ABSTRACT

TURNER, FRANCES BYRD. Evaluating Relationships Between Various Estimates of Semen Quality Based on Fertility Assessments of Boars (Under the Direction of Dr. William L. Flowers).

In commercial swine production, boars are an integral part of profitability. Since 99% of breeding in the swine industry uses artificial insemination, a boar can breed between 10,000 and 15,000 sows over his lifetime. Current measurements of semen quality used in the industry have not been studied in terms of their association with boar fertility. The objective of this study was to determine whether and to what degree common assessments of semen quality in boars are related to their fertility.

Boars (n = 12) that were between two and two and a half years of age were used. Heterospermic insemations and paternity testing of the piglets were used to group these boars into High, Medium, and Low fertility groups. Average number of pigs sired per litter by boars in the High (n=3), Medium (n=6), and Low (n=3) groups were 71.2 $\pm$ 0.7%, 51.2 $\pm$ 1.5%, and 25.5 $\pm$ 4.6%, respectively. The assumption was made that there was a positive relationship between the proportion of piglets sired in a litter and boar fertility. Both analysis of variance and multiple regression procedures were used to determine relationships between fertility and 76 different estimates of semen quality. Thirteen dealt with various anatomical and functional aspects of spermatozoa, while 63 were associated with proteins in seminal plasma.

Proportions of motile spermatozoa ($p < 0.075$); spermatozoa exhibiting progressive forward motility ($p < 0.075$); spermatozoa with normal acrosome morphology ($p < 0.05$); and spermatozoa undergoing an acrosome reaction ($p < 0.05$) and the average straight line distance traveled by motile spermatozoa ($p < 0.05$) exhibited a positive relationship with

fertility. Boars in the High group were higher than their counterparts in the Low group and boars in the Medium group being intermediate between the two. The average straight line velocity of motile spermatozoa was higher in the High and Medium boars compared with the Low boars.

Concentrations of three known and 60 unknown seminal plasma proteins were examined in ejaculates from High, Medium, and Low fertility boars. Of the three known proteins, HSP-70, TNF-α, and TGF-β, only HSP-70 had a positive, linear association ($p < 0.05$) with boar fertility. Of the 60 seminal plasma proteins that were identified via two dimensional gel electrophoresis, only 10 had either positive or negative relationships with boar fertility. Two – a 26 kD/7.2 pI protein and a 28 kD/6.6 pI protein – exhibited a positive linear ($p < 0.05$) with fertility; and three – a 19 kD/9.2 pI protein, a 35 kD/6.3 pI protein, and an 186 kD/8.7 pI protein – had negative associations ($p < 0.05$) with fertility. For the remaining five proteins, 46 kD/6.8 pI ($p < 0.10$) and 59 kD/5.2 pI proteins were greater in High boars compared with Medium and Low boars. In contrast, concentrations of 19 kD/8.5 pI, 20 kD/6.2 pI, 40 kD/5.0 pI, and 111 kD/8.7 pI proteins were higher ($p < 0.075$) in Low than Medium and High boars.

Results from the multiple regression analyses showed that three variables accounted for 87.6% of the variation in the average number of pigs sired per litter. These were concentrations of the 28 kD/6.6 pI protein (partial $R2 = 0.6619$; $p < 0.0001$); proportion of acrosome-reacted spermatozoa (partial $R2 = 0.1549$; $p = 0.0002$); and concentrations of the 17 kD/3.8 pI protein (partial $R2 = 0.0590$; $p = 0.0090$). In conclusion, two seminal plasma proteins and the proportion of spermatozoa that are acrosome-reacted per ejaculate hold the most promise for assessing boar fertility.

Evaluating Relationships between Various Estimates of Semen Quality Based on Fertility Assessments of Boars

by
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BIOGRAPHY

Frances Turner was born on February 4th, 1984 in Charlotte, North Carolina to Tim and Sarah Turner. She has two older sisters Virginia and Rachel and one younger sister Lila. She attended Charlotte Latin School in Charlotte, North Carolina for kindergarten through high school. She graduated from Charlotte Latin School in 2002.

After graduation from high school Frances came to Raleigh to attend North Carolina State University. She Graduated with her Bachelors of Science in Animal Science in May 2006. In August of 2006 she began to pursue her Masters of Science in Animal Science at North Carolina State University.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

EVALUATING RELATIONSHIPS BETWEEN VARIOUS ESTIMATES OF SEMEN QUALITY BASED ON FERTILITY ASSESMENTS OF BOARS

LITERATURE REVIEW

Introduction

Today in a commercial production, ninety-nine percent of sows are bred with artificial insemination. Due to this trend semen from one boar can be used to inseminate between 10,000 and 15,000 sows in his lifetime. Because of this, the fertility of a single boar can influence the efficiency of the entire herd. For this reason being able to decipher a highly fertile from a low fertility boar is valuable to the breeding herd. Semen is evaluated based on many different quality assessments. These assessments are used to decipher whether or not an ejaculate should be extended and used for insemination or discarded. These quality assessments would become more valuable to the producer if the relationships between these various estimates of semen quality and boar fertility were known. The objective of this project is to decipher which quality assessments of semen are good indicators of fertility.

In order to determine this, it is important to first understand how fertilization occurs. First, there must be the formation of oocytes and spermatozoa. Then, male gametes must then be able to properly travel through the female reproductive tract, undergo capacitation and the acrosome reaction in order to properly fuse with the female gametes. Consequently, it seems prudent to review these processes in order to understand exactly what is required of spermatozoa during fertilization.

Next, there have been a number of factors that have been implicated as predictors of the semen quality. For example, there are many tests that assess motility and morphology of

the spermatozoa. Other sperm quality assays have been developed that are linked to what spermatozoa have to do once inside the female reproductive tract. Finally, there is good evidence that non-spermatozoal components of semen, such as seminal plasma proteins, may be integral in assessing the fertility of the ejaculate. Obviously, a thorough review of this body of work is important for fully understanding the results of the current study.

Oocyte

"The major function of the female gonad is the differentiation and release of the mature oocyte for fertilization and successful propagation of the species" (McGee and Hsueh, 2000). Consequently, oocyte quality as defined as the potential for the oocyte to develop into viable offspring is central to the reproductive process (Hunter, 2000). Oocyte quality, in turn, is influenced by a number of factors that direct folliculogenesis because the follicle houses the oocyte during most of its development. Oogenesis is initiated by the female germ cells during prenatal development shortly after the germ cells under go one proliferation of oogonial division. This typically occurs just prior to oocyte formation within the primordial follicle. Germ cells begin meiotic divisions along the junction between the cortical and medullary regions of the ovary. Oogonia are converted into primary oocytes when they come into contact with cells from the ovary that are of mesonephric origin. The relationship between the germ and somatic cells is crucial for their subsequent development (Greenwald and Roy, 1994).

The formation of various cytoplasmic components and rearrangement and reduction of chromosomes are parts of the maturation process that are linked and it is crucial that both

follicular cells and oogonia mature in a synchronous manner. In pigs, germ cells start to mitotically divide beginning at the onset of pregnancy and have been seen to continue mitotic division until 7 days after birth. The total number of germ cells reaches a peak of approximately 1,100,000 germ cells at 50 days after mating. This represents an active period of mitotic activity because fetal ovaries at 20 days post mating typically only have 5000 germ cells. Once a sufficient level of germ cells is reached, mitosis ceases and meiosis begins. This transition has been observed as early as 40 days after mating. During meiosis germ cells are transformed into oogonia which are arrested at prophase stage. This process is usually complete by 35 days after birth. The distinguishing characteristic of oogonia is the presence of a single layer of granulosa cells. At this point of development they are housed within primordial follicles of which there are about 500,000 present in gilts when they are born (Hunter, 2000).

Oocyte and follicular growth seem to occur in two separate phases (Hunter, 2000). In the first phase of growth, the follicle and oocyte grow together in a linear fashion until the oocyte is close to its maximum size. At this time the second phase of growth begins during which the follicle develops granulosa cells, theca cells, and follicular fluid (Greenwald and Roy, 1994). An individual follicle consists of an oocyte surrounded by inner layers of granulosa cells and outer layers of thecal cells (McGee and Hsueh, 2000). In the first phase of growth oogonia enter Meiosis I and go through the G1 and S phases, and then arrest in the G2 phase. After this oocytes continue to grow for the sole purpose of making mRNA for later use (Parrish et al., 1992). Follicles are simultaneously going through changes as well. Primordial follicles change into primary follicles in which the oocytes become surrounded by

a unilaminar layer of cubodial grannulosa cells. Oocytes seem to control the transition of pregranulosa cells of primordial follicles into the cuboidal cells of primary follicles. It has also been shown that follicle stimulating hormone (FSH) plays a major role in early follicle cell division and early oocyte growth. A primary follicle becomes a secondary follicle by a series of mitotic divisions in the granuloa cell layer. Secondary follicles contain multiple layers of granulosa cells (Greenwald and Roy, 1994).

At the beginning of the secondary stage, follicles of most mammalian species start to develop thecal cells, but in pigs this process occurs later during development when follicles are approximately 206-287 micrometers in diameter and just about ready to form antra. The transition of secondary follicles into tertiary follicles involves the formation of antral cavities. The development of a primordial follicle into an antral follicle takes about 84 days and ends with a follicle that is about 400 micrometers. After the follicles reach this stage, they will either under go atresia or enter the preovulatory stage of development (McGee and Hsueh, 2000). If the latter occurs, 19 days later the follicle will reach a size of about 3 mm in diameter. The hormonal control of follicles in the antral stage primarily relies on FSH (Greenwald and Roy, 1994).

Follicles that are chosen to ovulate undergo a process which has three parts: recruitment; selection; and dominance. This process begins when follicles are in the antral stage. Recruitment refers to the process whereby follicles form an antrum and continue to increase in size. After follicles are recruited they start to produce small amounts of estradiol (Senger, 1997). During recruitment oocytes complete their growth; form zona pellucidae; and acquire the ability to resume meiosis (McGee and Hsueh, 2000). The zona pellucida is a

thick mucoprotein that is translucent and surrounds the oocyte (Senger, 1997).

Selection occurs next. During follicular selection the previously recruited follicles have one of two fates: they either will become atretic or will continue to develop. Follicles that are chosen to continue developing start to produce more estradiol and become dominant follicles. Dominant follicles produce large amounts of estradiol and inhibin. Inhibin is a hormone that inhibits the release of FSH from the anterior pituitary. This helps in arresting the growth of the non-dominant follicles as they become atretic. The blood supply to dominant follicles is also increased and, as a result, they are exposed to more gonadotropins. In contrast, the blood supply to non-dominant follicles is decreased, reducing the amount of gonadotropin delivery available to them. Dominant follicles ovulate. In polytocous species like pigs, more than one follicle is chosen to be dominant (Senger, 1997). At this point in the process, the follicles are in the preovulatory stage of development during which meiosis is resumed. Resumption of meiotic activity is caused by a surge of LH which also causes ovulation. The hormone responsible for keeping the oocyte from maturing during most of oogenesis is oocyte maturation inhibitor (OMI). It is produced by the granulosa cells in the follicle, highlighting the important relationship between the oocyte and follicle. It has been shown that OMI is only present in antral follicles, (Tsafriri, 1984). One theory is that LH reverses the effects of OMI. It has been shown that testosterone and dihydrotestosterone decrease OMI and allow the oocyte to mature. This could occur by at least three different mechanisms. Firstly, LH can be a direct antagonist of OMI. In other words, high levels of LH can block the suppressive actions of OMI. Secondly, LH could stimulate theca cells to produce testosterone, which in turn inhibits OMI. Lastly, LH causes lutenization of granulosa

cells which may also cause a decrease in OMI. Apparently, the latter process does occur as the follicle matures (Channing et al., 1982).

In addition to the removal of mechanisms that block maturation, pig oocytes also require positive stimulation during their final development. Active transcription and translation are required in order for chromatin condensation and germinal vesicle breakdown to occur. These events are thought to be regulated by cytostolic kinases, of which the principal kinase is maturation promoting factor (MPF). This protein triggers a series of reactions including nuclear membrane breakdown, chromosome condensation, and spindle formation. Oocytes progress through meiosis until they reach Metaphase II where meiosis undergoes its second arrest. Meiosis II is only completed after fertilization has occurred (Hunter, 2000).

As mentioned earlier, the relationship between the oocyte and somatic cells is fundamental to its maturation and complete development. Follicular cells are required during oocyte maturation in a pig. They play important roles in maturation such as regulating meiotic arrest and resumption, and providing nutrients. The cumulus cells also play a role in maintaining communication between the follicle and oocyte by an anatomical link known as intracellular coupling. There is evidence that follicular cells secrete substances that play an important role in cytoplasmic maturation of the oocyte (Hunter, 2000). Cytoplasmic maturation must occur for an oocyte to be able to complete development. A mature oocyte, by definition must be able to resume meiosis, successfully undergo fertilization, and sustain development of an embryo. Some examples of the changes that occur in the cytoplasm are mRNA transcription, protein translation, post-translation modifications of proteins, and ultra

structural changes. One of the proteins that are produced in the cytoplasm is MPF (Krisher, 2004). Follicular cells have another effect on cytoplasmic development - only oocytes surrounded by a follicle have the ability for male pronucleus formation. There is evidence that direct cell-to-oocyte contact must occur for the oocyte to fully mature (Mauro et al., 1988). Oocytes that are larger and from more mature follicles have been proven in pigs to produce better quality oocytes, at least for in vitro fertilization. The quality of the oocyte is determined by whether it can be fertilized by a single spermatozoa and form a male pronucleus (Hunter, 2000). In summary, Krisher (2004) states, "Pig oocytes clearly depend on the presence of follicle cells to generate specific cellular signals that coordinate oocyte growth and maturation".

The ovulatory process encompasses a series of biochemical and morphological changes that aid in the release of a mature oocyte and the transformation of the Graffian follicle into a corpus luteum (Channing et al., 1978). The process begins when the follicle reaches the preovulatory stage, where a LH surge causes a cascade of events which lead to ovulation (Senger, 1997). Ovulation can be defined as an "inflammatory-like process that occurs during the early stages of luteinization" (Espey and Lipner, 1994). When a mature ovarian follicle is stimulated by gonadotropins, most importantly LH, the initial cellular response is for the theca interna and the granulosa layers to luteinize. The follicle then becomes hyperemic, or having localized elevated blood flow, within a few hours of the surge (Espey and Lipner, 1994). This hyperemia is caused by prostaglandin E_2 and histamines, which also cause the theca interna to have increased vascular permeability. This raises the level of hydrostatic pressure around the follicle, which is important later when the follicle

ruptures. The LH surge also causes the theca cells to switch from producing testosterone to producing progesterone before ovulation occurs. Progesterone, in turn, is essential for ovulation because it stimulates the synthesis of collagenase which causes the breakdown of collagen in the basement membrane by the theca interna. This enzyme is also important because it breaks down the collagen of the connective tissue holding the oocyte to the ovary called the tunica albuginea. Simultaneously, the follicular fluid volume is building, and these two processes are creating a stigma, the region at apex of the follicle which begins to protrude. Another event associated with the hormonal cascade after the LH surge is that the ovary starts to produce prostaglandin F_2 alpha which causes contraction of the myoid component of the ovary. Myoid is smooth muscle whose contraction causes the stigma to protrude further from the circumference of the ovary. Another function of PGF_2 alpha is to release enzymes from lysosomes in granulosa cells that break down connective tissue. When all of the processes are combined there is a rupture of the follicle and the mature oocyte is released. After the release, the remaining follicle is transformed into a corpus luteum by prostaglandin E_2 to support embryonic development via the production of progesterone (Senger, 1997).

Sperm Production

The production of sperm occurs at puberty and continues throughout life (Courot et al., 1970). The process begins with spermatogenesis which is a mitotic and meiotic process that occurs in the seminiferous tubules of the testis (Johnson, 1995). Spermatogenesis occurs in stages which, in turn, are defined by cellular associations between the developing

spermatogonia and the seminiferous epithelium. The rate at which a cohort of spermatozoa progress through these well-defined stages of development is referred to as "cycle of seminiferous epithelium" (Swierstra, 1968). It has been shown that boars have 8 segmental stages (Sharpe, 1994). A layer of cells all in the same stage of development has been termed as a generation (Courot et al., 1970).

At puberty the final stages of spermatogenesis are initiated by an increase in the secretion of FSH and the Leydig cells are stimulated to produce testosterone. While the Leydig cells are present at birth, the total number of Sertoli cells is not fixed until approximately four months before puberty and is directly related to the amount of sperm production in adults (Franca et al., 2005). In response to FSH, sertoli cells undergo considerable changes in structure and function (Sharpe, 1994). One of the changes in the sertoli cell that is coordinated with the first wave of proliferation is the formation of tight junctions with the adjacent cells creating compartments. This is also known as the blood-testis barrier (Sharpe, 1994.).

Spermatogenesis has three major stages which are characterized by the function and morphology of the developing spermatozoa. The first stage involves mitotic divisions which result in approximately ten differentiations or replications in the boar (Franca et al., 2005). This stage involves mostly proliferation and is called the spermatogonial phase. In this stage, only spermatagonia are present. Spermatagonia can be defined as unspecialized diploid germ cells that are able to carry out mitotic divisions. They have three main roles in spermatogenesis. The first role is to initiate spermatogenesis. A second role is to increase the germ cell population via mitosis. Their third role is to assist with the regulation of the germ cell number (Rooij and Russell, 2000).

There are four classes of spermatagonia in pigs: undifferentiated spermatagonia type A; differentiated spermatagonia type A; intermediate spermatagonia; and spermatgonia type B. The spermatagongenic process takes approximately 8.6-9.0 days (Franca et al., 2005) and ends with the formation of a primary spermatocyte.

The next phase of spermatogenesis is the spermatocyte stage. In this stage the primary spermatocyte goes through first and secondary stages, where they are still diploid cells, and then they become spermatids which have a haploid nucleus as a result of having undergone meiosis (Courot et al., 1970). After spermatocytogenesis the spermatids are spherical (Johnson, 1995). This is when they enter spermiogenesis which is when differentiation occurs. In this step the spherical spermatid becomes a mature spermatid (Franca et al., 2005). The process of going from a round spermatid to a spermatozoon is called spermiogenesis. During this period of development, the spermatid elongates and transcription is terminated (Toshimori, 2003). Spermatozoa at the end of spermiogenesis have a compact head with nuclear compact inactive DNA. On one side of the outside of the head is the acrosome which is located on the anterior surface. On the other side is the tail with mitochondria (Sharpe, 1994). This step is where most of the germ cell loss occurs. Only two to three of ten spermatozoa are produced from each differentiated type A spermatagonia. In the elongation process 15% of the germ cells are lost. The entire spermiogenic process takes about 40 days after which the spermatids enter the epididymis (Franca et al., 2005). At this point the spermatozoa are not fertile or motile and require post-gonadal modifications in the epididymis to acquire these traits (Dacheux et al., 2005).

The epididymal duct in boars is between 50 and 100 meters in length, and is separated

into different sections. These sections are the initial segment, caput, corpus and cauda (Franca et al., 2005). Once the spermatids enter the epididymis they are no longer under the genomic control of the germ cells so in order to complete the maturation process interactions occur with the epididymal fluid (Dacheux et al., 2005). The epididymis has two main mechanisms that aid in the maturation: absorption and secretion (Dacheux et al., 2005). In order for the spermatid to become motile and fertile, including the ability to recognize the zona pellucida and undergo the acrosome reaction, it must undergo modifications as it passes through the epididymis, such as nuclear condensation, formation of acrosomal membranes, perinuclear theca development, creation of a fibrous sheath, cytoplasmic droplet formation, and modifications to the plasma membrane (Franca et al., 2005).

The epididymis consists of polarized epithelium which contains basal and principal cells. These two types of cells create a complex environment for the spermatozoa. The cells in the epididymis have many important functions such as protein secretion and absorption which are performed by the principal cells and endocytosis, secretory activity, immune defence, phagocytosis, and production of antioxidants which are performed by both cell types (Franca et al., 2005). During their passage through the epididymis proteins are incorporated into the membranes of the developing spermatozoa (Toshimori, 2003). These proteins come from a variety of different sources. Some are secreted by the rete testis whose proteins mainly reside in the proximal part of the epididymis. Others are produced by the caput and caudal portions of the epididymis. In addition, still other proteins are the result of proteolysis of pre-existing proteins. The epididymal enzymes modify proteins produced by other portions of the epididymis or by the spermatozoa, themselves. In the epididymus, 125

proteins from 187 different locations have been identified (Dacheux et al., 2005). The passage of spematazoa through the epididymis takes approximately 10 days: 3 days in the caput; 2 days in the corpus; and 4 to 9 days in the cauda until they are ejaculated (Briz et al., 1995).

Six main modifications of spermatozoa that occur during their passage through the epididymis are changes in the pattern of movement; alterations in the metabolic pattern and structural conditions of the specific organelle's tail; modifications to the nuclear chromatin and adjustments of the acrosomal shape; progressive loss of water and change in specific gravity; detachment of the cytoplasmic droplet after it travels through the midpiece; and lastly modifications to the plasma membrane (Briz et al., 1995). Dacheux (2005) describes spermatozoa's passage through the epididymis by stating that "ultimately it is coordinated activities of secretion and endocytosis of various substances by the epithelial cells along the duct that influence the final maturation of sperm as well as their concentration, protection and storage."

Once spermatozoa finish their transit through the epididymi they possess a flat, oval shaped head attached to a long tail which is also referred to as a flagellum. The cell surface is covered by a plasma membrane. The head has two major regions which consist of the acrosomal cap and the post-acrosomal region. The nucleus occupies the majority of the space in the head and is separated from the acrosome by perinuclear theca which surrounds the entire nucleus. The acrosome is separated into two regions: the acrosomal cap which is the anterior part and the equatorial segment which is the posterior acrosome. The flagellum is attached to the head at the neck, which consists of the distal-most part of the nucleus, the

basal plate, the capitulum, the proximal centriole, and segmented columns. The flagellum is separated into three different parts: the middle piece; principal piece; and the end piece (Toshimori, 2003).

The ejaculate that a boar produces is a suspension of spermatozoa in a fluid medium called seminal plasma (Mann, 1964). Seminal plasma is produced in the accessory sex organs. These consist of the vas deferens, seminal vesicles, ejaculatory duct, bulbourethral glands and the prostate. Seminal plasma is an extracellular fluid which provides a medium and a vehicle for spermatozoa. It is a mixture of secretions from the accessory sex glands and contains citric acid, prostatic phosphatase, fructose, phosphorylcholine, inositol, glycerlphosphorylcholine, and particulate bodies such as granules, globules, and other elements. The boar can produce up to 250 ml of whole semen in one ejaculation, and in boars most of the ejaculate is seminal plasma. The density of spermatozoa can be from 300,000 to 25,000 cells per microliter. Compared with semen from other mammals, boar semen is unique in that it contains high concentrations of ergothioneine, citric acid and inositol and has low amounts of fructose. Boar ejaculates are also unique in that they are released in definitive fractions with distinct fluid a gel fraction. Seminal plasma comes from all of the accessory sex glands with 15-20% from the seminal vesicle, 2-5% from the epididymis, 10-25% from the bulbourethral gland, and the rest coming from the urethral gland (Mann, 1964). The urethral gland seems to refer to the part of the urethra where the semen gathers prior to ejaculation.

Sperm Transport; Sperm Capacitation; and the Acrosome Reaction

In swine, artificial insemination is the most common way to breed sows. A volume between 80 and 300 ml of semen is deposited in the sow with an A.I. catheter. The site of deposition in the sow is the cervix and the large volume of semen helps to push the spermatozoa into the uterine horns and avoid getting caught in the cervical folds (Langendijk et al., 2005). Spermatozoa travel through the uterine horns to the oviduct where a sperm reservoir forms. They are assisted by uterine contractions. Uterine contractions can be caused by multiple stimuli. Studies have proven that the presence of a boar can release oxytocin which stimulates contractions (Langendijik et al., 2005). Also it has been shown that prostaglandins are released that increase contractions when there is estrogen in the inseminating fluid (Langendijik et al., 2005). Studies in which either oxytocin or estrogens were added to insemination doses resulted in improved fertilization rates. Seminal plasma also aids in the movement of spermatozoa to the oviduct and the infusion of seminal plasma prior to insemination increased the number of normal embryos 3 to 4 days later. At least a portion of this stimulatory effect was due to an increase in the transport of spermatozoa as increased numbers were present in the oviduct in 1 to 6 hours after administration of seminal plasma (Langendijik et al., 2005). Thus, it is clear that there are several components of the medium in which spermatozoa are traveling that enable them to travel from the cervix where insemination occurs to the oviduct.

In the pig, insemination occurs prior to ovulation, so this creates the need for a storage place to ensure that there is still competent sperm available to fertilize eggs. This storage site is called the sperm reservoir and is located in the furrows of the utero-tubual junction (UTJ) and the segment of the isthmus adjacent to the uterus (Mburu et al., 1997).

After the sperm enter this site they begin to undergo changes that enable them to properly fertilize eggs. These changes collectively are called capacitation. Capacitation was originally discovered by Austin. In 1952, he concluded that sperm must spend some time in the reproductive tract before they are capable of penetrating eggs (Chang, 1984). After this initial definition of capacitation it was found that there were two processes that are necessary for sperm to undergo before they are capable of penetrating eggs. These two processes are capacitation and the acrosome reaction. It is now thought that capacitation is the preparation of the sperm so that the acrosome reaction can occur (Chang, 1984). Currently, more is known about capacitation than the acrosome reaction. Tienthai (2004) states that "capacitation modifies the architecture of the intrinsically stable plasma membrane by removal of absorbed epididymal and seminal plasma proteins, as well as the promotion of lipid disorder with consequent protein relocation". Even though what happens to spermatozoa during capacitation is reasonably understood, where it actually occurs is a debatable issue. It was initially thought that the sperm was capacitated in the sperm reservoir (Fazeli et al., 1999), but recently some researchers have begun to hypothesize that capacitation may occur in the oviduct after the sperm has exited the sperm reservoir (Hunter, 2004). It is generally accepted though, that only a few sperm leave the sperm reservoir at any given time (Yanagimachi, 1994). This occurs because once capacitation has occurred and the plasma membrane is no longer stable, cell death occurs if the sperm is not in the immediate vicinity of an oocyte (Rodriguez-Martinez, 2007). The combination of cell death after capacitation and the length of time between insemination and ovulation necessitate this slow release from the sperm reservoir. The release of spermatozoa from the sperm reservoir

is facilitated by the fact that there are two subpopulations of sperm within the UTJ. There are spermatozoa bound to the epithelial lining and those that are unbound (Mburu et al., 1997). Sperm that bind to the epithelial lining are uncapacitated. When they bind to the epithelia the bond is quite strong and one from which sperm are not easily detached (Fazeli et al., 1999). It has been found that the binding to the epithelial wall helps the sperm keep their intracellular calcium levels low, and by so doing, they protect themselves against premature capacitation and hyper-activation (Hunter, 2004).

Other factors also regulate capacitation. There is evidence that ovulation affects the number, distribution and membrane integrity of spermatozoa within the sperm reservoir. Interactions among spermatozoa, the oviductal epithelium and the ovary via the occurrence of ovulation are complex. However, there are three main things that must occur. The first is timing. Capacitation should coincide with peri-ovulation, if fertilization is going to occur in an efficient manner. Secondly, there is the imperative need for some way to store spermatozoa within the oviduct because of the lengthy period of between insemination and ovulation. If sperm were capacitated as soon as they entered the female reproductive tract, then the instability of the plasma membrane would be detrimental to their survival during storage. Lastly, ovarian control mechanisms associated with ovulation are the main regulators of capacitation (Hunter, 2004). It is now thought by some researchers that the sperm reservoir helps to block sperm from capacitating until the appropriate time (Tienthai, 2004). Seminal plasma has also been shown to inhibit capacitation (Harrison, 1996).

The actual changes that spermatozoa undergo to become capacitated are complicated, but culminate in changes of ion flux, membrane fusogenicity, and the exposure of receptors

that allow sperm to bind to the zona pellucida (Harrison, 1996). Biochemically, capacitation occurs when bicarbonate and calcium act synergistically. Signaling pathways involved in capacitation also are complicated. The first thing that occurs is an efflux of calcium which causes a rise in intracellular calcium levels. This also causes bicarbonate to activate adenylate cyclase which in turn produces cyclic AMP which, in turn, triggers protein kinase A which indirectly phosphorylates proteins on tyrosine. This phosphorylation is dependent on actin polymerization and the membrane bound phospholipase C (Breitbart, 2003). In summary, Bart and Visconti (2006) state that "initiation of capacitation lipid rearrangement in the sperm plasma membrane, ion fluxes resulting in alteration of the sperm membrane potential and an increase in tyrosine phosphorylation of proteins involved in induction of hyperactivation and the acrosome reaction" .

The mammalian oocyte is surrounded by a thick layer of glycoproteins, referred to as the zona pellucida, which in turn is surrounded by a layer of cumulous cells. Sperm must be able to pass through all of these in order to fuse with the plasma membranes of ova. In order to accomplish this, spermatozoa must be able to undergo acrosome reactions and periods of hyperactive motility. These functions are described as "expression of vigorous flexible flagellar movement" (Harrison, 1996). The acrosome is a cap-like structure on the anterior of the head of the spermatozoa. It is a membrane-bound sac that can be separated into two parts. The cap is anterior and the equatorial segment is posterior. The cap contains hydrolyzing enzymes while the equatorial segment is thought to be enzymatically empty (Yanagimachi, 1994). It has been suggested that the acrosomal cap performs the same functions as a lysome (Yanagimachi, 1994). It is also thought that the acrosome is highly

organized, in which the carbohydrates in the matrix may play a role to orchestrate the proper release of acrosome components. The acrosome serves two purposes: to provide a place for sperm to penetrate through the membrane, and to fuse sperm with the oocyte's plasma membrane (Yanagimachi, 1994).

The acrosome reaction is defined as a release of the hydrolytic enzymes stored in the cap which weaken the zona pellucida by lysing components so that the sperm can penetrate and allow the equatorial region to fuse with the egg plasma membrane (Harrison, 1996). In pigs, the outer acrosomal membrane creates many intra-acrosomal vesicles and which release the acrosomal contents. Of the sperm that are inseminated, very few reach the actual fertilization site where the acrosome reaction occurs. Those that do reach the site of fertilization and have been capacitated experience a rapid increase of cyclic AMP and protein kinases A and C when they bind to the zona pellucidae of ova (Yanagimachi, 1994). Protein kinase C opens calcium channels in the plasma membrane, while protein kinase A stimulates calcium channels in the acrosomal membrane, which causes calcium levels in the cytosol to increase. This increase in calcium, in turn, activates f-actin which causes fusion of the two membranes allowing enzyme release and sperm penetration (Bretbart, 2003). There are many obstacles spermatozoa must overcome in order to be successful in fertilizing eggs. The initial meeting of the spermatozoon and egg is at the "oocyte cumulus complex" (Talbot, 1985). The cumulus layer is a composition of cells and a matrix. The matrix is made up of a polymerized hyaluronic acid that is conjugated with glycoproteins (Yanagimachi, 1994). This cumulus layer develops when meiosis is resumed just prior to ovulation. In order for sperm to be able to penetrate through this cumulus layer, which is very important because the

sperm to egg ratio at the point of fertilization is 1:1, the spermatozoon must be capacitated. If the spermatozoon has not fully capacitated when it comes in contact with the cumulus layer it can still attach but will not be able to penetrate (Yanagimachi, 1994). This may be a mechanism for preventing unviable sperm from fertilizing eggs. It has been hypothesized that the hyperactivated motility and hyaluronidase both aid the sperm in its passage through the cumulus complex (Primakoff and Myles, 2002). It was initially thought that when the spermatozoon passed through the cumulus layer acrosome reaction started to occur because the hyaluronidase that breaks down the hyaluronic acid within the matrix is housed within the acrosome (Anderson, 1991). It is now known that the cumulus layer can be penetrated by spermatozoa that have not undergone an acrosome reaction. The reason for this could be because hyaluronidase, along with other enzymes such as acrosin betagalactosidase and arylsulfatase, are located on the sperm membrane. It is possible that due to location, they could be absorbed during spermatogenesis or leak out of the acrosome before the full reaction occurs (Yanagimachi, 1994). If either of these situations occurred, then penetration could occur prior to the completion of the acrosome reaction.

The role of the cumulus cells has been a subject of much debate. Some people think that the cumulus cells are useless, but others think they serve a purpose in the ultimate fertilization of the egg (Yanagimachi, 1994). It has been discovered in pigs that the cumulus layer is critical for fertilization *in vitro* (Yanagimachi, 1994). It has been suggested that the cumulus matrix reduces individual variation in male fertility and that there are parts of the matrix that stimulate sperm motility. The following sequence of events has been proposed as an explanation for this speculation. The cumulus layer sensitizes the spermatozoon to the

zona pellucida. This effectively creates a complex that offers a larger target for the spermatozoon to aim for. Once a spermatozoon enters the cumulus cells, it cannot leave. From the oocyte's perspective this accomplishes several things. It prolongs the life of the oocyte by slowing zona hardening; weeds out spermatozoa that are weak swimmers; and stops the zona from rotating while the sperm is attempting to penetrate (Yanagimachi, 1994). After the sperm has overcome the obstacle of the oocyte cumulus complex it must navigate its next obstacle - the zona pellucida.

The zona pellucida is a matrix of sulfated glycoprotein. In pigs there are 5 different kinds of zona pellucida proteins (Barros et al., 1996). Studies have shown that the zona pellucida must go through a maturation process to be able to bind the spermatozoa (Rath et al., 2005). The primary protein that binds to a receptor on the sperm plasma membrane is ZP3 (Yanagimachi, 1994). It has been hypothesized that the receptor on the sperm plasma membrane is a 95-KD tyrosine kinase protein (Barros et al., 1996). The sperm receptor doesn't actually bind to the ZP3 but to an oligosaccharide chain that is connected to the ZP3. The composition of this chain is still unknown, but four critical monosaccharides have been found (Talbot et al., 2003). Once binding occurs there are two possible cascades of events that occur to initiate the acrosome reaction. One possibility is that when spermatozoa bind GalT is stimulated which activates GTP-binding protein and phospholipase C. This, in turn, raises the level of calcium (Primakoff and Myles, 2002). Another possibility is that spermatozoa bind to the same receptor but instead of an increase in GalT calcium enters through type T channels (Primakoff and Myles, 2002). Regardless of which hypothesis is correct, the rise of cytoplasmic calcium causes the exocytosis of the acrosomal enzymes from the cap (Primakoff and Myles, 2002).

The acrosome reaction is explained by Wasserman et al. (2005) as: "The acrosome reaction is characterized by multiple fusions of the plasma membrane at the anterior region of the sperm head with outer acrosomal membrane just underlying the plasma membrane". The acrosomal reaction has several morphological effects on the spermatozoon which are directly related to its ability to bind ova. First, the contents of the vesicles are expelled, leading to an acrosomal ghost, which is the non-soluble components that remain adhered to the vesicle after the contents have been released. In addition, the acrosomal cap is lost. This exposes the receptors for subsequent binding events (Talbot, 1985). This adhesion to the zona pellucida seems to be achieved with proacrosin/acrosin. Proacrosin is the zymogen for acrosin which autoactivates into acrosin which is a hydrosoluble basic glycoprotein with proteolytic activity. It has two functions. The first is to act as a sperm binding protein and the second is to enable the spermatozoon to penetrate the zona. The binding caused by acrosin is thought to involve ZP2 from the zona pellucida. It has also been proven in boars that the ZP proteins aid in the degradation of the zona after binding (Barros et al., 1996). Once the plasma membrane of the spermatozoon has attached to the zona pellucida, the penetration process begins. There are two hypotheses that have been proposed as to how the spermatozoon accomplishes this. One asserts that the passage is purely mechanical and the removal of the acrosome cap creates a sharp edge which allows the motility of the spermatozoon to push through the zona (Yanagimchi, 1994). The second hypothesis assumes that penetration is purely the function of enzymatic action. In other words, the acrosin hydrolyzes the glycoprotein of the zona (Yanagimchi, 1994). It is important to recognize that acrosin does not destroy the gross macroscopic structures of the zona or the sperm binding sites (Yanagimchi, 1994).

After the penetration, the next step that must occur is the fusion of the egg's plasma membrane and the spermatozoon's plasma membrane. There are many hypotheses of how this happens. The most popular one involves ADAM family proteins specifically fertilin, integrin and cyritestin. Binding is thought to occur by interactions between these proteins. However, recent knockout tests have shown that sperm and egg membranes can fuse without any of these proteins being functional (Wassarman et al., 2005). Because of this two other proteins have been studied to explain how membrane fusion occurs. They are the GPI and CD9 proteins, both of which are also located on the egg surface. Based on the study of Primakoff and Myles (2002), CD9 currently is viewed as the leading candidate for the putative binding protein during fertilization. It is a member of the tetraspanin superfamily that aids in membrane fusion in other cellular systems. There is evidence that it is the long extracellular loop that aids in fusion (Wassarman et al., 2005).

Once the fusion of the egg and sperm plasma membranes has occurred, the oocyte starts to take precautions to ensure its survival. Collectively, this is referred to as oocyte activation. Oocyte Activation is defined as meiosis resumption, pronuclear formation and DNA replication. The oocyte is arrested at meiosis II after ovulation, so if fertilization is to progress normally, then egg must come out of arrest. The exact mechanism for this is unknown (Alberio et al., 2001). It is thought that calcium, MPF, and stabilizing factors play a role in this process. It has been found that calcium is an absolute requirement for normal oocyte activation and embryonic development (Perry et al., 2000). When the sperm and egg membrane bind, the influx of calcium begins at the initial point of contact and proceeds with a wave-like motion (Alberio et al., 2001). Calcium has two key targets: cyclin B1 and

securin (Jones, 2005). It is thought that cyclin B1 helps to maintain the calcium oscillations (Albiro et al., 2001). Securin allows seperase to release. Seperase is the structural component that holds together sister chromatids (Jones, 2005). The first calcium increase in mammals occurs one to three minutes after the spermatozoal and oocyte membranes fuse (Swann et al., 2006).

There are three main hypotheses that have been proposed to explain how intracellular calcium is elevated during fertilization: the conduit hypothesis; receptor hypothesis; and the fusion hypothesis (Malcuit et al., 2006). The conduit hypothesis was one of the earliest hypotheses. It has also been called the calcium bomb hypothesis (Sutovsky at al., 2003). This hypothesis suggests that the acrosomal reaction starts the rapid inflow of calcium and then when the sperm and egg fuse, calcium is steadily pumped through channels. In essence, the initial build up of calcium builds starts the fertilization wave (Alberio et al., 2001). This model was soon proven not to be plausible because there was no indication of elevations in intracellular calcium concentrations where the sperm penetrates the egg (Alberio et al., 2001).

The next hypothesis was the receptor hypothesis. This hypothesis argues that calcium influx is controlled by a receptor on the sperm plasma membrane and its coupling to a second membrane bound receptor (Sutovsky et al., 2003). These receptors involve a G-protein and a PTK phoshorylation cascade. This hypothesis is puzzling because there has been no receptor found on the oocyte plasma membrane (Jones, 2005). Also, additional studies have found that the G-proteins are not crucial for activation (Perry et al., 2000).

This brought about the development of a third possibility which attempts to explain

calcium influx via the involvement of cytostolic sperm components (Perry et al., 2000). These cytosolic components are referred to as sperm-borne oocyte activating factors (SOAF) (Perry et al., 2000). One problem with this hypothesis is that spermatozoa contain no cytosolic material that can induce ovulation on its own (Perry et al., 2000). This observation has lead to the belief that there might be two types of SOAF. Studies have found that SOAF-I can not work alone, perhaps because it is heat labile (Perry et al., 2000). It is thought that the second SOAF- II is stable and is part of the sperm head sub-membrane (Perry et al., 2000). Both SOAF-I and SOAF-II are needed for oocyte activation. After SOAF-I and II form a complex, a signal cascade that involves an IPS receptor is initiated (Perry et al., 2000). The perinuclear theca (PT) are also thought to play a role in this process. The PT dissolves after membranes fuse. This allows it to release signaling molecules (Sutovsky et al., 2003). An interesting observation is that in order for the sperm nucleus to remodel, it needs factors that are made in the egg cytoplasm. What makes this interesting is that at the point of remodeling the PT has already dissolved into the cytoplasm, so some of the necessary factors may have originally come from the PT (Sutovsky et al., 2003). It is also possible that the sperm-borne factors that initiate the calcium influx and the resumption of meiosis may be stored on the PT. Proteins have been found in the PT membranes that have SOAF activity. A protein that has been identified to have SOAF properties is PT32 as (Sutovsky et al., 2003). Recently, it has been shown that PLC (zeta) is important and triggers the calcium oscillation that leads to activation (Suvosky et al., 2003). Phospholipase C (zeta) is a protein and it measures about 70 kDa. It has been shown that PLC (zeta) can cause calcium oscillations when injected into the egg (Swann et al., 2006). There are quite a few different

theories on what causes activation to occur, but one thing is known, that the spermatozoa play a key role in the initiation (Malcuit et al., 2006).

Another important component of oocyte activation is the block to polyspermy. The main elements of this are the cortical granules. A cortical granule is a membrane-bound organelle (Hoodbhoy and Talbot, 1994), which contains sulfated mucopolysaccharides, hyaline, serine protease and peroxidase (Yanagimachi, 1994). The cortical granules are made in the Golgi apparatus and originate in the oocyte as it begins to mature (Hoodbhoy and Talbot, 1994). The depolarization of the membrane from the calcium influx at membrane fusion causes the cortical granules to exocytose (Yanagimachi, 1994). It is thought that this exocytosis begins less than two minutes after membrane fusion (Yanagimachi, 1994). They release their contents into the perivitelline space (PVS) (Sun, 2003).

How the cortical granules exocytose is not yet known. Some think that the reaction is mediated by the inositol phosphate cascade (Sun, 2003). Another possibility is that cortical granule exocytosis is triggered by calcium dependent pathways. Finally, protein kinase C could be responsible for exocytosis (Tsaadon, 2006). The exocytosis causes the zona pellucida to harden and repel the other spermatozoon that approach (Yanagimachi, 1994). After the cortical granules exocytose they form an envelope around the PVS. The PVS contains a matrix of hyaluronan, which inhibits membrane fusion, and expands when the envelope forms. Calcium is essential for the dispersion of the cortical granules and the formation of an envelope. The envelope within the PVS is filled with a substance that inhibits membrane fusion. This is commonly referred to as the second block to polyspermy

(Talbot and Dandekar, 2003). Pigs are unique in the fact that they have another built-in safe guard against polyspermy. Their cytoplasm can remove accessory sperm (Sun, 2003). The combination of the zona pellucida hardening and the envelope polyspermy is blocked allowing embryonic development.

Techniques used to Estimate Semen Quality and Boar Fertility

The main objective of the current research contained within this thesis is to explore new ways to improve the accuracy of estimating semen quality and boar fertility. Over the years, many different aspects of semen from boars have been evaluated in hopes of finding a single measure that is precise, accurate, and cost effective. To date, there does not appear to a single test that fulfills all of these criteria. However, when the results of several different tests are used, some general relationships emerge. Consequently, a brief summary of these tests and their relationship with semen quality is in order.

Visual Estimates

Characteristics of the ejaculate have been used for years to approximate the fertility of boars. In order to get an accurate evaluation of the boar's fertility it is thought that at least three ejaculates that have been taken two to three days apart to give the boar time to recuperate should be evaluated (Gardner, 1991). There are many components that can be a part of evaluating the sample. The most common first step is to visually look at the quality of the semen. The sample should be free from trash and clumps and should be slightly cloudy. If the sample is too clear it can suggest that the number of sperm cells is low in the ejaculate (Gardner, 1991 and ESHRE, 2002).

Semen Volume and Concentration of Spermatozoa

Semen volume, total number of spermatozoa, and the concentration of spermatozoa have all been used to estimate semen quality. The volume is measured by pouring the sample into a graduated cylinder or by simply weighing the ejaculate. The concentration of spermatozoa can be determined with a hemocytometer, Burker counting chamber, spectrophotometer, or cell counting machines. The total sperm number is then found by multiplying the concentration by the sperm volume.

It was originally thought that more sperm volume indicated a more fertile boar. Gardner (1991) presents a table of seminal characteristics which displays the average amounts for domestic animals. In this table it shows that boars have an average volume of 255 ml per ejaculate, a concentration of 200 million spermatozoa per milliliter, and a total number of spermatozoa of 45 billion under a collection regimen of 3 times per week. Since these tests turned out to be poor estimates of semen quality and fertility, other aspects of spermatocytes and their accompanying fluid in the ejaculate have been examined as possible measures of evaluating semen quality and predicting boar fertility (Perez-Llano, 2001).

Normal Morphogy

Spermatozoa are divided into three morphological regions - the head, midpiece, and tail. Spermatozoa with abnormalities in any of these regions are considered to be subfertile. Consequently, the percentages or proportions of morphologically abnormal spermatozoa collectively are considered better estimates of semen quality than any single abnormality. In

other words, the critical observation is that a spermatozoon is abnormal and not the specific type of abnormality that it has. It is commonly accepted in boars that ejaculates with more than 30 to 40% morphogically abnormal spermatozoa have reduced fertility (Flowers, 1997). However, there have been numerous cases in which ejaculates have nearly 100% normal morphology, yet their fertility is low (Flowers, 1997). As a result, morphology is currently viewed as more of a qualitative than quantitative measure of semen quality and boar fertility.

Motility of Spermatozoa

Sperm motility was originally assessed visually with a light microscope (Gardener, 1991. ESHRE, 2002. Schilling and Vengust, 1987. Sancho et al., 2004. Ciereszko et al., 2000. Hoffman et al., 1999. Strzezek et al., 2000. Borg et al., 1993). The relationship between semen quality and boar fertility has been studied in detail (Flowers, 1997; Xu and Foxcroft, 1999). The general consensus of these studies is that ejaculates that contain less than 60% motile spermatozoa are less fertile compared with those that have a higher proportion of motile spermatozoa. Consequently, like normal morphology, the proportion of motile spermatozoa is considered to be a good qualititative, but poor quantitative measure of fertility and semen quality.

Recent advancements in visual imagery have lead to the development and use of computer-assisted sperm analysis (CASA) systems. With CASA, information about the type of movement that spermatozoa possess can be monitored such as the average path velocity, curvilinear and straight line velocities, amplitude of lateral sperm head displacement, and beat cross frequency (Aitken, 2006; Gardner, 1991). Because spermatozoa are stimulated to

initiate hyperactivity when they are in close proximity to ova, it has been suggested that the type of movement spermatozoa display is a better estimate of their potential for fertilization than simply evaluating the number that are motile. At this time, CASA is mainly used in human fertility testing and there is no evidence that the type of motility of spermatozoa in boars is correlated with their fertility (Verstegen, 2002).

Acrosome and Plasma Membrane Integrity

Osmotic resistance tests have used to examine the plasma membrane which has been shown to be critical during fertilization in vivo. The rationale behind this test is that a functional plasma membrane will allow fluids to pass into and out of the cytoplasm of spermatozoa. Consequently, if spermatozoa are placed in a hypoosmotic solution and the plasma membrane is functional, then water will move across the plasma membrane into the cytoplasm . The end result of this process is that spermatozoa with a functional plasma membrance will appear to have "swollen heads" when viewed microscopically (Perez-Llano et al., 2001). The original osmotic resistance test was referred to as HOST, the hypoosmotic swelling test, and recently has been improved with the introduction of the short HOST (sHOST) (Perez-Llano et al., 2001). One study has shown that a sHOST rate lower than 60% will lower farrowing rates (Perez-Llano et al., 2001). The main criticism of the hyperosmotic swelling test is that it simply measures whether spermatozoa are alive or dead – something that can be done without subjecting them to osmotic stress.

Flow Cytometry

Another test that has been used to improve the evaluation of male fertility is flow

cytometry. Flow cytometry is an improvement upon the past practices of fluorochromes and fluorescent probes. The problems with these past tests were that only a small number of the spermatozoa could be evaluated so it gave a poor picture of the whole sample. Flow cytometry, as described by Gillan et al., (2005), "is a system for making measurements on single cells as they travel in suspension one by one past a sensing point". This test has enabled the evaluation of acrosomal integrity, sperm viability, capacitation status and membrane fluidity at the same time. There are a few drawbacks to the use of flow cytometry. The equipment needed is very large and expensive (Gillan et al., 2005). In addition, most of the traits that flow cytomotry can measure are ones that have been shown to have poor quantitative relationships with fertility such as morphology, sperm viability, etc. Consequently, measurement of a larger proportion of spermatozoa with an inaccurate test does little to improve its precision.

Acrosin Activity of Spermatozoa

This assay was first used when testing for human fertility because low levels of acrosin activity have been associated with sub-fertility or infertility. It was shown that motile sperm with an intact plasma membrane were still infertile if they had low levels of acrosin (Kennedy et al., 1989). The initial assays that were being used to evaluate acrosin activity were very time-consuming and measured the amount of acrosin and proacrosin, which is the zymogen. An assay that cannot distinguish between the amount of acrosin and proacrosin in the acrosome of spermatozoa is problematic because if proacrosin was not converted to acrosin during the assay, then it would not be detected with this test and the activity that is

detected may not be representative of what is available to the spermatozoa. To remedy this problem, a simple assay was created that uses amidase activity as a measure of acrosin activity. Amidase is a good measure because it has been shown that its activity is only caused by sperm acrosin activity (Glogowski et al., 1998; Kennedy et al., 1989). Another benefit to the amidase test was its simplicity.

This assay has only three steps. First, the sample must be washed and spun down to separate the spermatozoa from the seminal plasma (Kennedy et al., 1989). This step is done because acrosin activity is suppressed by inhibitors that are in the seminal plasma (Glogowski et al., 1998). The next step is to suspend the sample in detergents, and the last step is to evaluate the color change with a spectrophotometer (Kennedy et al., 1989). This assay for humans was adapted with a few changes to become an efficient assay of acrosin activity for boars (Glogowski, 1998). These changes consisted of decreasing the amount of spermatozoa per assay and extra washing of the sperm to remove the seminal plasma (Glogowski, 1998). Despite these modifications, acrosin activity is still viewed as a qualitative rather than a quantitative test for fertility in domesticated farm animals.

Sperm Binding Tests

There are two popular assays that look at the binding of the sperm to the zona pellucida. These two assays are the hemizona assay and the zona pellucida binding test (Oehinger et al., 2006). Other assays that look at the actual gamete interaction are the sperm penetration assay and the sperm binding with zona free hamster oocytes (Oehinger et al., 2006; Berger et al., 1996, 1989, 1988; Hammitt et al., 1989). The sperm penetration assay

was found to be a poor assay in term of fertility this is because sperm can penetrate without undergoing acrosome excytosis then the equatorial segment would not be able to fuse with the oocyte. For this reason the hamster assay became more popular (Oehinger et al., 2006; Hammitt et al., 1989). This assay is possible because hamster oocytes share a lot of the same sperm plasma membrane proteins with porcine oocytes (Berger et al., 1996). Since the hamster oocyte is free of the zona pellucida, the fusion of the sperm with the oocyte plasma membrane can be examined with *in vitro* fertilization (Berger et al., 1996). This assay was tested for accuracy using heterospermic mating. When this process was done they compared infertile boars with fertile boars. Infertile boars were used because the binding zona free hamster egg assay does not test for any other parameters that are routinely used to measure fertility (Berger et al., 1989, 1988). After the matings were conducted it was found that the fertile boars sired the main percentage of piglets 83% (Berger et al., 1989) and 93% (Berger et al., 1988) that were born. This demonstrated that the zona free hamster egg assay is useful in assessing the quality of semen (Berger et al., 1988).

Heterospermic matings is a variation in testing fertility. The spermatozoa from two boars are in competition to fertilize the oocytes. The piglets from the litter are then DNA-fingerprinted to determine which boar is the father. Then the boars can be ranked by fertility. One problem with heterospermic matings is that it is a relative test that can only rank the boars compared to each other but cannot determine the boar's fertility in terms of farrowing rates or litter size (Collins et al., 2008).

A competitive sperm binding assay which was basically an in vitro variation of heterospermic matings was used to see if one boar's spermatozoa was more fertile than

another. This assay uses two flourometric dyes that are lipophilic to label spermatozoa from different boars. Labeled spermatozoa are then combined in a droplet and allowed to bind with the oocytes in a competitive manner. This assay allows for boars to be compared against one another (Miller, 1998). This test has the same disadvantage of heterospermic matings. It is a relative test that can only rank boars against each other, but is not be able to provide any fertility information in terms of farrowing rates and litter size (Collins et al., 2008).

Motility and Fertility

Motility has been looked at as an indicator of fertility. Fertility of the boars was assessed through analysis of farrowing rates and litter size. Popwell et al., (2004) found a positive relationship between litter size and progressive motility. This relationship was only present when changes in motility were examined within individual boars and not between ejaculates from different boars. In contrast, Gadea et al. (2004) found that motility was significantly correlated with farrowing rate when ejaculates from different boars were evaluated.

Sperm Chromatin Structure

The assays that have been discussed so far have dealt with the sperm's interaction with the oocyte. The next group of assays looks at the actual composition of spermatozoa. They are collectively called sperm chromatin assays. Sperm with damaged chromatin are infertile because even though the spermatozoa can fertilize the egg and progress through the early stages of development, the resulting embryos cannot maintain their growth to term

(Evenson et al., 2002). The original tests used to look at the chromatin structure were alanine blue and CMA_3. These are both stains with negative aspects that led to a search for different tests. The problem with the alanine blue stain was that not only did it stain the lysine-rich histones, but it also would highlight the abnormal epididymal problems. The problem with CMA_3 was that there was no way to determine a threshold for fertility (Evenson et al., 2002). These problems led to the development of assays that examined DNA integrity. These assays are the single-cell gel electrophoresis assay (COMET), terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL), in situ nick translation (NT), sperm chromatin structure assay (SCSA), and the acridine orange test (AOT) (Evenson et al., 2002).

All of these assays have advantages as well as disadvantages, but the SCSA is viewed as the most informative test (Ballachey et al., 1988; Evenson et al., 1993, 2002). This assay looks at the denaturation of DNA in response to an acidic environment (Ballachy et al., 1988; Evenson et al., 1993, 2002). This test seems to have no correlation to any of the other tests that are normally performed to assess fertility (Evenson et al., 1993). This particular assay has been used in beef cattle and swine and now is beginning to be used in humans. In boars, it was used in conjunction with heterospermic matings. It was found to be highly correlated to the number of piglets sired and, when compared to more conventional tests, it was found to be a better predictor of fertility (Ballachy et al., 1998; Evenson et al., 1993). The only problem with this particular assay is that it requires flow cytometry which, as mentioned previously, requires a significant investment in equipment. The AOT was developed to eliminate the need for the equipment. It uses the same stain that SCSA uses, but spermatozoa are examined under the microscope. Evaluation of sperm chromatin structure by this method

increases the variation observed among boars, so there are, as yet, unresolved questions concerning whether it actually induces DNA damage in spermatozoa instead of just measuring it. Nevertheless, it is still the most popular way of testing sperm chromatin structure (Evenson et al., 2002).

Measurement of Non-Sperm Components in Seminal Plasma

Measurement of various non-sperm components in seminal plasma has also been used to estimate the fertility of boars. Boars are unique in that they have a lot of estrogen in their semen. It is thought that the estrogen comes from a few different areas in the male reproductive tract. The majority is believed to originate from the leydig cells, but significant amounts also come from the accessory sex glands (Claus et al., 1987). Estrogens in the semen are thought to cause an increase in uterine contractions and myometrial activity. It also is hypothesized that the estrogen leads to an increase in PGF_2-α, which influences ovulation (Raeside and Christie, 1997; Claus et al., 1987). An interesting finding in boars is that the amount of estrogen present in semen is higher than the amount that is found in the boar's blood plasma (Claus et al., 1987). Since the boars' levels are so high, the thought of testing for estrogens as in other species, including testing in stallions, has been undertaken. The seminal plasma of stallions has been tested for the concentrations of estrogen, estrogen sulfate, estradiol-17β, testosterone, androstenedione, dehydroepiandrosterone, and 5α-dihydrotestosterone (Hoffman et al., 1999). In this study, they were looking for an effect of seasonality on sperm production. Seasonal changes were observed in all the hormones studied except estradiol-17β and androstenedione.

Another hormone that has been linked to fertility and semen quality is IGF-I. In beef cattle, IGF-I has been found in seminal plasma and its receptor is present on spermatozoa. The rationale behind why semen contains IGF-I is that it has a positive effect on the motility of spermatozoa and, thus, might be associated with fertility (Henricks et al., 1998). To date, there are no definitive studies that have shown a cause and effect relationship between IGF-I and male fertility in domesticated livestock species.

Seminal Plasma Proteins

Seminal Plasma is important to the success of the spermatozoa to fertilize the oocyte. It has been found that if 10-12% of seminal plasma is included in an A.I. dose in swine, it has beneficial effects on spermatozoa (Strzezek et al., 2005). In the bull, boar, and stallion, seminal plasma is rich in proteins which have been shown to have many functions, one of which may be predicting fertility.

The first species in which seminal plasma proteins were shown to be related to fertility was cattle. In bulls, there is a family of proteins that are referred to as the bovine seminal plasma (BSP) proteins. These are also referred to as heparin binding proteins (HBP) and have molecular weights ranging from 14- 31 kiloDaltons. These proteins are thought to bind to the plasma membrane of spermatozoa at the time of ejaculation and cause molecular changes that allow them to capacitate (Nauc and Manjunath, 2000). It is thought that this group of proteins is very important in sperm storage in the oviduct, especially in preventing early capacitation (Nauc and Manjunath, 2000; Asadpour et al., 2007). There are three main proteins that are associated with BSP. These proteins are PDC-109, which is also referred to

as BSP- A1/A2; BSP-A3; and BSP-30KDa. The most abundant protein in seminal plasma is PDC-109. These proteins are involved in the fertilization process. Their main contributions are to increase the time that spermatozoa are capable of being motile and to facilitate binding of spermatozoa to the epithelium of the oviduct to create the sperm reservoir (Asadpour et al., 2007; Gwathmey et al., 2006).

Four additional proteins have been linked to the fertility of bulls. These four particular proteins were discovered by comparing the seminal plasma of bulls of with high and low calving rates. Killian et al. (1993) found that two of the protein markers were consistently present in bulls with above average fertility. These proteins had molecular weights and isoelectric points of 26kDa, pI6.2 and 55kDa, pI4.5, respectively. There were also two proteins associated with the lower fertility bulls and these had molecular weights and isoelectric points of 16kDa, pI4.1 and 16kDa, pI 6.7, respectively. It is important to note that all the bulls used had normal semen parameters (Killian et al., 1993). Subsequent studies found that the two fertility proteins were 3.5 times higher in high fertility bulls compared with their average or lower fertility counterparts (Gerena et al., 1998).

The identity of the two fertility proteins is known. The 55kDa protein is Osteopontin. Osteopontin is a highly acidic phosphorylated glycoprotein. It is thought to have 14kDa, 45kDa, and 55kDa isoforms. The reason that 14kDa and 45kDa proteins were not identified as fertility markers is because they probably are cleaved from the original 55kDa protein (Cancel et al., 1997; Nauc and Manjunath, 2000). The 26kDa protein has been identified as lipocalin-type prostalaglandin D synthase (Gerena et al., 1998; Nauc and Manjunath, 2000).

These proteins were measured in semen from buffalo bulls (Jobim, 2004). The

26kDa protein was positively correlated with progressive motility and the 55kDa protein was correlated with sperm viability (Jobim, 2004). These proteins seem to play key roles in predicting fertility in buffaloes and more research is being conducted to see if the proteins are useful for predicting the freezability of semen (Asadpour, 2007).

Two of the four proteins discussed previously have been linked to fertility in boars. It was found that the 26kDa, pI 6.2 and 55kDa, pI 4.5 proteins were positively correlated with in vitro fertility (Flowers, 1998). This finding prompted more studies that found high concentration of these two proteins in the semen of boars with high farrowing rates and increased number of pigs born alive (Strzezek, 2005). In a study using heterospermic inseminations where all the boars had similar parameters of semen, boars with increased concentrations of the two proteins sired 90% of the piglets (Flowers, 1998).

Other proteins that have been found in the seminal plasma of boars are spermadhesion proteins. The spermadhesion proteins make up the bulk of the proteins in the seminal plasma. These proteins bind to the sperm during ejaculation similar to the BSP proteins in cattle. These proteins are multi-functional in terms of their ability to bind to ligands. The binding of these proteins forms a coat which is thought to aid the spermatozoa in forming the sperm reservoir and facilitate sperm capacitation, oocyte recognition and sperm-oocyte binding (Jonakova et al., 2007; Strzezek, 2005). Seven different sperm adhesion proteins have been identified and all except for one show heparin binding, which is similar to BSP proteins. These proteins are thought to play crucial roles in the longevity of sperm motility. These spermadhesion proteins have also been found in equine and bovine (Strzezek, 2005).

The spermadhesion proteins found in boars and bulls have also been identified in horses through radioimmunoassay and western blotting. It was discovered that these seminal plasma proteins were homologues (Menard et al., 2003). Jobim et al. (2000) found that two proteins had quantitative differences between their high and low fertility stallions. In this particular study, they reported the identity of the proteins as spots and provided ranges of 25-30kDa, pI 7.5-7.7 and 20-25kDa, pI8.5-8.7. The low fertility stallions' seminal plasma contained the 25-30kDA, pI7.5-7.7 protein, while the higher fertility stallions contained the 20-25kDa, pI8.5-8.7 protein. In another study conducted by Brandon (1999), four proteins were found that correlated with fertility. Only one of these four indicated higher-than-normal fertility and this protein had a molecular weight of 72kDa and an isoelectric point of 5.6. The other three proteins (15kDa, pI6.0; 18kDa, pI4.3; and 16kDa, pI6.5) were associated with lower-than-average fertility. When the 72kDa protein was examined with a western blot, it was found that it was homologous to the 55kDa protein found in bulls and boars.

The tests designed to assess the quality of ejaculates quality assessment tests need to be quick, accurate and inexpensive if they are going to be used commercially (Gadea, 2004). Based on a current review of the literature, motility, morphology, capatitation status, arosome assays and seminal plasma characteristics all appear to have potential for determining a boar's fertility.

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INTRODUCTION

Today in commercial swine production, ninety-nine percent of sows are bred with artificial insemination. Due to this trend one boar can breed between 10,000 and 15,000 sows in his lifetime. Because of this, the fertility of a single boar can influence the efficiency of the entire herd. For this reason being able to decipher a highly fertile from a low fertility boar is valuable to the breeding herd. Semen is evaluated based on many different quality assessments. These assessments are used to decipher whether or not an ejaculate should be extended and used for insemination or discarded. These quality assessments would become more valuable to the producer if the relationships between these various estimates of semen quality and boar fertility were known. The objective of this study was to decipher which quality assessments of semen are good indicators of fertility.

MATERIALS AND METHODS

Experimental Design

The relative fertility of twelve boars was estimated via the use of heterospermic inseminations and paternity testing (n=5 or 6 per boar). The assumption was made that the proportion of piglets that a boar sired in a litter had a direct, positive relationship with his fertility. Based on the results of the paternity testing, boars were placed into one of three fertility groups: High (n=3); Medium (n=6); and Low (n=3). Ejaculates from each boar (n= 10 per boar) were evaluated for selected estimates of semen quality. These included the following: proportion of motile spermatozoa; proportion of spermatozoa exhibiting progressive forward motility; average straight line velocity (VSL) of motile spermatozoa;

average curvilinear velocity of motile spermatozoa (VCL); average straight line distance traveled by motile spermatozoa (DSL); average curvilinear distance traveled by motile spermatozoa(DCL); the proportion of spermatozoa with normal head and tail morphology; the proportion of spermatozoa that were uncapacitated, capacitating, and acrosome reacted; the proportion of spermatozoa with normal acrosome morphology; the proportion of spermatozoa with acrosin activity; and concentrations of the 70 kD heat shock protein; tumor necrosis factor; transforming growth factor-β, and 60 seminal plasma proteins. Analyses of variance and multiple regression analyses were conducted to determine statistical relationships among boar fertility groups and semen quality estimates.

Experimental Animals

Twelve mature boars that were 29 $\pm$ 3.1 months old and weighed 215 $\pm$ 5.7 kg were used in this study. These boars were the offspring of Yorkshire x Landrace x Large White dams and Hampshire x Duroc x Pietran sires and were expected to exhibit 100% heterosis in their reproductive traits as adults. Of the 12 boars used in the study, six were born in October, 2003 and the remaining six were born in March, 2004. There were 3 pairs of full siblings in the group born in October and 2 pairs of full siblings in the group born in March. Four of the boars in the October-born group were half siblings with boars born in March.

All boars were housed in crates that were 1.1 m wide by 3 m long and located in a curtain-sided breeding and gestation facility with underslat ventilation. Supplemental cooling was provided by overhead fans and a mister system that was set to be fully operational when the temperature in the barn reached 26^{o}C. Boars had ad libitum access to

water via nipple type waterers and received 3 kg of a 15% protein, corn and soybean based diet that was balanced to meet maintenance requirement for mature boars (N.R.C., 2005).

Sixty eight mature crossbred sows (Yorkshire x Landrace x Large White) with an average parity of 3.5 $\pm$ 1.1 were also used in the study. After breeding, sows were housed in gestation stalls that were 1.0 m wide and 3.0 m long. Gestation stalls were located in a curtain-sided building with an underslat ventilation system which included 18 stirring fans (5000 cfm per fan) situated in 3 rows of 6 fans each. A dripper system that was set to run 8 out of every 10 minutes as long as the ambient temperature was greater than 26^{o}C provided supplemental cooling for the sows during the summer months. Sows were fed between 2 and 4 kg of a 15% protein, corn and soybean based diet that was formulated to meet all nutrient requirements for pregnant sows expected to gain 30 kg during gestation (N.R.C., 2005). Individual feeding levels were adjusted weekly based on visual estimation of a sow's body condition. At 108 days of gestation, sows were moved into solid-walled farrowing rooms equipped with a side-wall baffle ventilation system. Evaporative cooling units served as the main air inlet when the ambient temperature reached 24^{o}C. Each room had 12 farrowing crates that were 1.5 m wide 4.0 m long. Sow movement within each crate was restricted to an area that was 1.0 m wide by 3.5 m long. Piglets had access to the entire crate. Sows were fed according to appetite throughout lactation. Peak daily lactational intake for all sows during the study averaged 8.3 $\pm$ 1.2 kg. The lactation diet was a corn-soybean meal-based diet formulated to contain 18% protein and adequate energy, vitamins and minerals for sows anticipated to wean litters in which piglets gained 300 g per day (N.R.C., 2005). The breeding phase of the study (heterospermic inseminations) began February 21, 2006 and

ended August 8, 2006. As a result, experimental sows farrowed between June 15, 2006 and December 10, 2006.

Six hundred seventy six, 1-day old piglets that resulted from mating different combinations of the 12 boars to the 68 sows also were used. Skin samples were collected from each piglet at 1-day of age when they were ear notched for identification purposes. All experimental procedures performed on the boars, sows and baby piglets were approved by the North Carolina State University Institutional Animal Care and Use Committee and were consistent with policies and procedures described for swine in the Guide for the Use and Care of Animals in Agricultural Research (F.A.A.S., 1998).

Semen Collection

Each boar was collected once a week by a double- gloved hand technique (Almond et al., 1998) during exposure to a collection dummy. Powder-free, polyvinyl gloves were used (IMV America, Eden Prairie, MN). The collection dummy (Minitube of America, Verona, WI) was 1.5 m long and adjusted to a height of 0.67 m high during the collection periods. The collection vessel consisted of an insulated, 1-quart thermos that was maintained at 37^{o}C prior to collection. A plastic, 1-quart collection bag (IMV America, Eden Prairie, MN) was placed down inside the thermos. The mouth of the thermos was covered with a milk filter (IMV America, Eden Prairie, MN) and secured in place with a rubber band. The entire ejaculate from the boar was collected. Boars were allowed to dismount and remount during collection. The collection was terminated when boars would no longer mount. All boars were collected by the same, experienced technician during the study.

Immediately after collection, the volume and concentration of spermatozoa were determined and the ejaculate placed into a 37^{o}C water bath until all the boars were collected. Ejaculates were weighed on a programmable scale (Fisher Scientific, St. Louis, Mo.) in order to estimate volume. The assumption was made that l mL of semen weighed 1 g. A self-calibrating spectrophotometer (SpermaCue® , Minitube of America, Verona, WI) was used to determine concentration. A 5 mL sample of each ejaculate was placed in a portable incubator at 37^{o}C and transported back to the laboratory for subsequent analyses. The remainder of each ejaculate was processed for heterospermic inseminations. The laboratory was located in Polk Hall on the campus of North Carolina State University, Raleigh, NC, which was located approximately 10 minutes from the research farm. Once in the laboratory, the temperature of each sample was recorded and the samples were placed directly into a preheated water bath at 37^{o}C.

Semen Preparation for Heterospermic Matings and Inseminations

Heterospermic insemination consisted of 3 billion total spermatozoa each from two different boars for a total of 6 billion spermatozoa in 80 mL of Androhep Plus® semen extender (Minitube of America, Verona, WI). Semen from the two different boars was combined in the following manner. Each ejaculate was extended 1:1 by adding 3 equal parts of extender slowly over a 30 min period. After waiting for 30 min to allow each ejaculate to equilibrate with the extender, the partially extended ejaculates were combined in such a way that there were 3 billion total spermatozoa from each boar. All extensions were carried out at 37oC.

As mentioned earlier, there were two contemporary groups of boars based on their birth dates: a group of 6 born in October, 2003 and a group born in March, 2004. Collectively, there were 5 pairs of full siblings and 4 boars that were half siblings. Consequently, because of the difficulty in using paternity testing to distinguish piglets sired by siblings, heterospermic insemination pairings were arranged such that fertility evaluations were conducted in such a way that siblings and half-siblings were not compared against one another. This resulted in semen from each of the 12 boars being used to breed either 5 or 6 sows.

Sows were checked for estrus by daily exposure to mature boars (36 $\pm$ 2 months and 257 $\pm$ 5 kg). Boars were confined to an area in the alley way in front of the crates in which weaned sows were housed. With the boar providing face-to-face interactions with the sows, two breeding technicians applied back pressure to groups of four to five sows. Sows exhibiting a standing reflex were inseminated using Golden Pig® insemination catheters (IMV America, Eden Prairie, MN). Insemination doses were packaged in insemination cochettes (IMV America, Eden Prairie, MN) and were used within 72 hours of collection. Sows were bred once each day of estrus by one of two experienced technicians during the study.

Paternity Testing

Procedures used to determine the paternity of the piglets born were identical those described by Collins et al. (2008). For boars, DNA was extracted from semen obtained at during collection using a Puregene® Purification kit for 60 to 100 million spermatozoa

according to the manufacturer's instructions (Gentra Systems, Minneapolis, MN). For sows and male piglets, DNA was extracted from skin (tail samples) shortly after farrowing using Puregene® Purification kit for 5 to 10 mg of mouse tail tissue according to the manufacturer's instructions (Genta Systems, Minneapolis, MN).

Oligonucleotide primers for paternity identification were selected based on normal variation inherent to the population of boars for DNA microsatellite markers from the X-Y chromosome map published by U.S.D.A. (www.marc.usda.gov/genome/swine/ChromosomeX-Y/). Primers are shown in Table 1 and were purchased from Qiagen (Valencia, CA).

Table 1. Oglionucleotide Primers used for Paternity Analyses

Primer	Sequence 5' to 3'	Length (base pairs)	Mol. Wt.
sw1325F	CATTCATTTTTGTTCTTCTGCC	22	6608.44
sw1325R	CACCACTTCGTCTTCACTTCC	21	6228.11
S0022F	CTGGGCAGCTCTATAATATC	20	6092.05
S0022R	TTACCCTTTCTACTCTGTGC	20	5984.99
SW259F	CCTTTCATGCTGTATTTAACCC	22	6611.41
SW259R	CAGAGAACAGAAGTTGGGGG	20	6280.14
SW290F	CTTCAGTGTAGTCCAAGTGGC	21	6437.26
SW290R	GATGTTTTGCTGATAGGAAGGG	22	6885.89
SW2456F	GAGCAACCTTGAGCTGGAAC	20	6151.04
SW2456R	AATGTGATTGATGCTGTGAAGC	22	6829.56
SW2137F	CATTGACACAGTCCCCTGG	19	5748.78
SW2137R	GGTAAATACACAAACCTCCACG	22	6681.41
S0117F	CAGGAAGCTGAAGCTGAGATTTGA	24	7465.95
S0117R	CTGTCAGGGAGCTAGTTTCACTCA	24	7343.86

For each of the microsatellite markers, PCR reactions were performed in volumes of 20 ul consisting of 0.2 uM of each primer (forward and reverse), 0.5 U of Taq DNA Polymerase, 100 ng DNA, 200 uM dNTPs and PCR buffers supplied by the manufacturer.

Mineral oil was placed on top of the reaction mixtures and the 0.5 mL reaction tubes were placed in a thermocycler. All reagents were purchased from Qiagen(Valencia, CA) unless otherwise indicated. Amplification reactions were conducted according to the following program: 2 minutes at 94oC; 40 cycles of denaturing (10 sec at 93oC), annealing (20 sec at 58oC), and extension (20 sec at 72oC) followed by a final extension phase for 5 min at 72oC. For each reaction, a control sample in which distilled water was added to the mixture instead of DNA and processed via the amplification cycle described previously. Single and double-stranded DNA fragments from PCR amplication reactions were purified using QIAquick spin columns according to the instructions supplied by the manufacturer (Qiagen). Alleles were identified via gel electrophoresis (40 V/cm for 2 h) with 8% denaturing acrylamide gels using a sequencing gel apparati.

A subset of samples (30 of the 63 sows) were sent to GeneSeek, Inc. (Lincoln, NE) as a means of quality control. GeneSeek, inc. examined the genetic heterogeneity of 22 different alleles in their paternity testing procedures. Paternity results reported by GeneSeek, Inc. were in agreement with those obtained by the procedures discussed previously in 99.2% of the piglets evaluated.

Semen Quality Evaluations

Motility and Mobility

The proportion of motile spermatozoa; proportion of spermatozoa exhibiting progressive forward motility; average straight line velocity (VSL) of motile spermatozoa;

average curvilinear velocity of motile spermatozoa (VCL); average straight line distance traveled by motile spermatozoa (DSL); and average curvilinear distance traveled by motile spermatozoa (DCL) were determined with a computer assisted semen analysis system (SpermVision®, Minitube of America, Verona, WI). In preparation for analyses one mL of neat semen from each ejaculate was extended in a 1:13 ratio with AndrohepPlus® extender. The extension process was started by adding the neat semen to 50ml conical tubes (Port City Diagnostic, Inc., Wilmington, NC) and then adding the extender in such a manner that it slowly dribbled down the inside of the conical tube. This was done to minimize the exposure of spermatozoa to temperature shock. A one mL sample of the extended semen was placed into a test tube (12 x 75mm; Port City Diagnostics, Wilmington, NC) and allowed to equilibrate in a 37^{o}C incubator (Fisher Isotemp Oven 200 series; Fisher Scientific, Atlanta, GA) for 30 minutes. After the equilibration period the sample of extended semen was removed and mixed thoroughly by gently rotating the conical tube until a uniform suspension of spermatozoa was achieved. Three ul was placed into a Leja slide (Minitube of America, Verona, WI), pre-warmed to 37^{o}C, and analyzed on an Olympus (BX41) equipped with a heated stage that was maintained at 37^{o}C. Twenty different fields (between 600 to 800 spermatozoa depending on the initial concentration of the ejaculate) were analyzed.

Proportion of Morphologically Normal Acrosomes

One mL of extended semen used for motility and mobility analyses was placed in a test tube (12 x 75mm; Port City Diagnostics, Wilmington, NC). One hundred ul of a 10% formalin solution was added to prevent deterioration of the spermatozoa. Each tube was

thoroughly mixed. Samples of approximately 10 ul were placed on an ethanol-cleaned, glass microscope slide (Fisher Scientific, Atlanta, GA). An 18x18mm glass cover slip (Fisher Scientific, Atlanta, GA) was then gently placed on top of the sample. The spermatozoa were allowed to settle by letting the slide sit for a couple of minutes. All microscopic analyses were conducted with a phase contrast microscope (Zeiss, Olympus B071, Berlin, West Germany). Spermatozoa were initially located using the 25x objective. Once located, a drop of immersion oil (Fisher Scientific, Atlanta, GA) was placed on the slide and the 100x oil immersion objective was used to visualize the acrosomes. A random sample of 100 spermatozoa was counted excluding spermatozoa with abnormal tails or heads. The acrosome classification scheme was based on the classification system of Pursel et al. (1972). In this classification system, a normal acrosome with a normal shape would have a smooth, unbroken surface. In contrast an abnormal acrosome would appear to have a broken and uneven surface. The proportion of spermatozoa with normal acrosomes was obtained by dividing the number of spermatozoa with normal acrosomes by the total number of spermatozoa evaluated.

Proportion of Spermatozoa with Acrosin Activity

A procedure developed by Penn et al. (1972) and modified by Popwell and Flowers (2003) was used to determine the number of spermatozoa with normal acrosin activity. Prior to analysis a gelatin solution was made by placing 100ml of distilled water, 3.5g of gelatin (Fisher Scientific, Atlanta, GA) and 30ul of Tween 80 (Fisher Scientific, Atlanta, GA) into a 250 mL glass beaker. The mixture was placed on hot plate to mix under low heat until the

solution became clear. Next 80% ethanol-cleaned glass slides (Fisher Scientific, Atlanta, GA) were placed on a 37oC slide warmer (Fisher Scientific, Atlanta, GA). After the slides were warmed for 30 min 10 ul of the gelatin solution was pipetted into a straight line along one end of the slide. A glass spreader was used to evenly spread the gelatin along the vertical length of the slide. The slides were allowed to air dry over night and then refrigerated until they were used. To prepare the toludine blue stain, 100 mL of borate buffer (pH=10) and 0.3g toludine blue were added to a glass beaker and stirred. Once the solution obtained a consistent color it is poured into a plastic jar for use at a later time.

Gelatin-coated slides were removed from the refrigerator and allowed to equilibrate to room temperature for at least 4 hours before the assay was performed. A one mL sample of the extended sample from each boar was placed into its own test tube (12 x 75mm; Port City Diagnostics, Wilmington, NC) and placed in an incubator (Fisher Isotemp Oven 200 series; Fisher Scientific, Atlanta, GA) at 37^{o}C for 40 minutes. After the incubation period 10 ul of the sample was pipetted across the end of the slides then using a glass spreader was spread evenly down the vertical length of the gelatin-coated slide. The gelatin slides were the placed on a slide warmer (Fisher Scientific, Atlanta, GA) at 37^{o}C for 15minutes. The slides were then placed in a CO_2 incubator (NAPCO Model 6100; NAPCO Scientific Company, Tualatin Oregon) set at 37oC and 100% humidity for 1.5 hours. After this incubation the slides were immediately stained with the toluidine blue solution by dipping them into the stain for a few seconds.

The end of each slide was blotted with a paper towel before it was rinsed in three separate beakers of distilled water. Then the slides were positioned vertically and allowed to dry.

The gelatin slides were then analyzed at 25x using a phase contrast microscope (Zeiss, West Germany). A random sample of 100 sperm cells was analyzed for acrosin activity. Acrosin activity was visually identified by a "halo" appearing around the head of the spermatozoa. Once released, acrosin will digest the protein matrix in the gelatin around the head of spermatozoa. This area around the head of the spermatozoa resembles a circle of light because it is devoid of gelatin and therefore does not stain with toluidine blue. If "halos" were present, then spermatozoa were classified as having acrosin activity. If there were no "halos", then spermatozoa were classified as not having acrosin activity. To calculate the percentage of spermatozoa expressing acrosin activity the number sperm with "halos" was divided by the total number of sperm cells evaluated.

Assessment of Capacitation Status of Spermatozoa

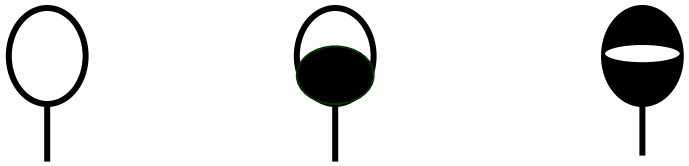
A chloratetracycline (CTC) fluorescence technique originally described by Fraser and Herod (1990) and modified by Popwell (1999) was used to determine the capacitation status of freshly ejaculated spermatozoa. The CTC solution was prepared fresh daily by adding 750 uM CTC (Sigma Chemical Company, St. Louis, MO) in a buffer of 5 mM cysteine (Sigma Chemical Company, St. Louis, MO)), 130 mM NaCl, and 20 mM Tris (Sigma Chemical Company, St. Louis, MO). The pH of the solution was adjusted to 7.8 via the addition of HCl or NaOH as appropriate. Briefly, 5 μl of the neat semen was added to 5 μl of the CTC solution on a clean warm slide. After 30 sec, 0.2 μl of 12.5% (w/v) paraformaldehyde in 0.5 M-Tris buffer (pH 7.4) was added and mixed. Finally, a drop of 0.22 M- 1,4-diaza-bicyclo (2,2,2)-octane (DABCO; Sigma Chemical Company, St. Louis, MO) in glycerol: PBS (9:1)

was added and mixed to retard fading of fluorescence. A coverslip was added to the slide and then compressed using a tissue to remove excess solution. Slides were kept in a light-shielded container in a moist environment until examination under a microscope with phase-contrast with epifluorescent optics (Make and Model of VanOx Scope). Spermatozoa were observed under blue-violet illumination (excitation at 400-440 nm, emission at 470 nm) and classified according to CTC staining patterns described by Fraser and Herod (1990). These were as follows:

Uncapacitated - uniform yellow fluorescence over the entire head;

Capacitating - fluorescence free band (clear) in the post- acrosomal region

Acrosome Reacted - dull fluorescence over the whole head except for a yellow band at the equatorial segment.



Uncapacitated Capacitated Acrosome Reacted

A total of 250 spermatozoa were evaluated from each ejaculate. The proportion of uncapacitated, capacitating, and acrosome-reacted spermatozoa were determined by dividing the appropriate counts by the total number of spermatozoa evaluated.

Two Dimensional Gel Electrophoresis

A 1.5 mL sample of whole semen was centrifuged (Fisher Model 235C Micro-Centrifuge, Fisher Scientific) at 12,000 rpm for eight minutes. Seminal plasma was then removed and placed into another 1.5 mL bullet tube and frozen at -20^{o}C for subsequent analysis. Two dimensional gel electrophoresis was performed on a minimum of four seminal plasma samples from each boar. All supplies were purchased from InVitrogen (Carlsbad, CA) unless otherwise specified. The basic reagents including the pre-cast gels were as follows:

Isoelectric Focusing (IEF) Gels

Product	Catalog #
Novex® pH 3-10 IEF gel 1.0 mm, 10 well	EC6655A
Novex® IEF Cathode Buffer pH 3-10 (10x)	LC5310
Novex® IEF Anode Buffer (50x)	LC5300
Colloidal Blue Staining Kit	LC6025
IEF pre-cast gels (20% TBE Gel/1.0 mm x 10 wells)	

Two dimensional (2D) Gels

Novex® 4-20% Tris-Glycine Gel 1.5 mm x 2D well	EC6029
Novex® Tris-Glycine SDS Running Buffer (10x)	LC2675
See Blue® Plus2 Pre-Stained Standard	LC5925
Gel-Dry™ Drying Solution (1x)	LC4025
2D pre-cast gels (4-20 % Tris-Gly gel / 1.5mm x 2D well)	

Seminal plasma proteins were first separated according to their isoelectric points on IEF gels. All reagents were prepared according to the manufacturer's instructions. One hundred ul of seminal plasma was mixed with an equal volume of sample buffer and loaded onto the gel. A programmable power source (Novex® PowerEase 500, Invitrogen, Carlsbad, CA) was used to stimulate migration of proteins in the IEF gel according to the following sequence: 100 volts for 1 h; 200 volts for 1 h; and 500 volts for 45min. Gels were removed; rinsed with distilled water; and allowed to stain overnight (~ 16 h).

The next step was to separate proteins with common isoelectric points according to their molecular weight (2D gel analysis). All reagents for the 2D gel analysis were prepared according to the manufacturer's instructions. The IEF gels were rinsed and lanes from each of the individual samples were removed and placed on top of a 2D gel. The 2D gels were subjected to 125 Volts for 1h and 45 min. Gels were then removed; rinsed four times in

distilled water; and stained for a minimum of 16 h. After staining, gels were dried for 24 h in the dark at room temperature.

Digital images were made of each gel (HP Scanjet 8200, Hewlett-Packard, San Jose, CA) and analyzed with the Image Quant TL program v2003 (Habersham Biosciences, Piscataway, NJ). Relative amounts of 60 seminal plasma proteins were recorded and expressed based on the total amount of protein in each seminal plasma sample. A sample of seminal plasma pooled from a single boar not involved in the study was analyzed with each run of the unknown samples as a means of determining the inter-assay (run) variation. All unknown samples were adjusted for differences among runs in this common pooled sample.

Seminal Plasma Concentrations of Heat Shock Protein (HSP) 70, Tumor Necrosis Factor(TNF)-α , and Transforming Growth Factor(TGF)-β

Concentrations of HSP70, TNF-α, and TGF-β in seminal plasma were determined with commercially available ELISA kits. Neat seminal plasma samples were subjected to centrifugation at 10,000 rpm for 10 min prior to analyses. One hundred ul from the supernatant was used for each assay. Instructions provided with the kits were followed for each assay with the exception that the original sample was not diluted (HSP70 – Stressgen, Ann Arbor, MI; TNF-α – Pierce Biotechnology, Rockford, IL; and TGF-β – Promega, Madison, WI).

Each kit was validated for porcine seminal plasma by examining parallelism and recovery. Each protein showed parallelism with the standard curve over a range of dilutions

between 1:2 and 1:16. Recovery of HSP70, TNF-α, and TGF-β in seminal plasma samples supplemented with 125 pg/ml to 250 pg/ml averaged 53%, 19%, and 45%, respectively.

Statistical Analyses

Two separate analyses were performed to examine relationships among fertility and various measures of semen quality. In the first analysis, boars were placed in fertility groups post priori based on the average number of piglets they sired from the heterospermic inseminations. This was done in two different ways. The first method was quantitative and based on the actual percentage of piglets in a litter that a boar sired. The three groups were as follows: High (n=3 boars; 71.2 $\pm$ 0.7%); Medium (n=6 boars; 51.2 $\pm$ 1.5%); and Low (n=3 boars; 25.5 $\pm$ 4.6%). The second method was qualitative and based on the average number of litters in which a boar sired the majority of the piglets. If two boars sired exactly the same number of piglets in a litter, then the entry was not recorded as a win or a loss. The three groups in this classification scheme were as follows: High (n=3; 78.3 $\pm$ 1.7%); Medium (n=6; 46.7 $\pm$ 2.1%); and Low (n=3; 8.3 $\pm$ 8.3%).

Because ejaculates were collected over time, a preliminary analysis was performed to determine whether semen quality estimates changed significantly over time during the experimental period. Analysis of variance for repeated measures (Kaps and Lamberson, 2004) using PROC MIXED in SAS® (Version 8.01, Cary, NC) was used. Boar was considered to be a random effect. The results from these analyses revealed no significant changes in time for any semen quality traits ($p > 0.25$). Due to the lack of time effects, averages for each semen quality variable were calculated for each boar. The average semen

quality estimates were treated as the dependent variables in the subsequent analyses.

Analyses of variance procedures (Kaps and Lamberson, 2004) using PROC MIXED in SAS® (Version 8.01, Cary, NC) were used to determine the effect of fertility group on the following dependent variables: proportion of motile spermatozoa; proporation of spermatozoa exhibiting progressive forward motility; average straight line velocity (VSL) of motile spermatozoa; average curvilinear velocity of motile spermatozoa (VCL); average straight line distance traveled by motile spermatozoa (DSL); average curvilinear distance traveled by motile spermatozoa(DCL); the proportion of spermatozoa with normal head and tail morphology; the proportion of spermatozoa that were uncapacitated, capacitating, and acrosome reacted; the proportion of spermatozoa with normal acrosome morphology; the proportion of spermatozoa with acrosin activity; and concentrations of the 70 kD heat shock protein; tumor necrosis factor; transforming growth factor-β, and 60 seminal plasma proteins. One analyses used the fertility groups established with the average number of piglets that each boar sired in a litter (quantitative), while a second analyses used fertility groups established with the average number of litters in which each boar sired the majority of the pigs (qualitative). When a significant effect of fertility group was present difference among fertility groups was determined using Student-Newman-Keuls multiple range test (Snedecor and Cochran, 1989).

In the second analyses, multiple regression techniques and analyses of colinearity (Snedecor and Cochran, 1989) were used to examine relationships among the proportion of piglets that a boar sired in a litter and semen quality estimates using PROC REG procedures of SAS® (Version 8.01, Cary, NC). In this analysis, the ejaculate was treated as the experimental unit and not the individual boar.

RESULTS

Boar Fertility Groups

Boars were divided post priori into 3 fertility groups based on either the average number of piglets sired per litter (quantitative) or the average number of litters in which they sired the majority, defined as 50.1% and greater, of the pigs in the litter (qualitative). Average number of pigs sired per litter by boars in the High (n=3), Medium (n=6), and Low (n=3) groups were 71.2 $\pm$ 0.7%, 51.2 $\pm$ 1.5%, and 25.5 $\pm$ 4.6%, respectively. Average number of litters in which the boar sired the majority of the pigs for High (n=3), Medium (n=6), and Low (n=3) boars were 78.3 $\pm$ 1.7%, 46.7 $\pm$ 2.1%, and 8.3 $\pm$ 8.3%. Based on this estimate of fertility, none of the boars in the High group had a perfect record, so to speak. Two of the boars had averages of 80% and one had an average of 75%. In contrast, two boars placed in the Low fertility group failed to produce a single litter in which they sired the majority of the pigs, while one boar produced only 1 litter out of 4 in which he sired the majority of the piglets.

Classification of boars based on their quantitative or qualitative assessments of fertility affected the group in which 4 boars were placed. There was one boar in the High group based on average number of pigs sired per litter that was ranked in the Medium group based on the average number of litters in which the boar sired the majority of the piglets. The opposite was also true for another boar. The same situation was present for 2 boars in the Medium and Low fertility groups.

Motility and Mobility

Using fertility groups based on the percent of piglets sired by each boar, the proportion of motile spermatozoa ($p = 0.07$) and the proportion of spermatozoa exhibiting progressive motility ($p = 0.06$) were significantly affected by fertility group (Table 2). The High fertility group was significantly higher ($p < 0.075$) than the Low fertility group but not from the Medium fertility group. The Medium fertility group was not different from the Low fertility groups. Results for DSL were similar to those for progressive motility (Table 2). High fertility boars were higher ($p < 0.05$) than Low fertility boars while Medium fertility boars were intermediate. For VSL, High and Medium boars were not different ($p > 0.05$) from each other, but both were greater ($p < 0.05$) than Low boars. There were no differences among fertility groups for DCL ($p = 0.11$) and VCL ($p = 0.16$; Table 2).

When fertility groups were established based solely on qualitative criteria, only overall motility ($p < 0.075$) and progressive motility ($p < 0.05$) were different among fertility groups (Table 3). Relationships were similar to those observed when quantitative criteria were used. The High fertility groups were superior to the Low fertility groups with the Medium fertility groups being intermediate. Estimates of mobility were not affected by fertility groups ($p > 0.10$; Table 3).

Acrosome Morphology, Acrosin Activity, and Capacitation Status

Effects of boar fertility on morphological and functional aspects related to acrosomes of spermatozoa are shown in Tables 4 and 5. When the average number of piglets sired per litter was used as the grouping criteria for fertility, only acrosome morphology ($p =$

0.01) and the proportion of spermatozoa undergoing the acrosome reaction ($p = 0.01$) were affected by fertility group (Table 4). High and Medium boars had a higher ($p < 0.05$) proportion of spermatozoa with normal acrosomes than Low boars. Ejaculates from boars with High fertility had more ($p < 0.05$) spermatozoa undergoing the acrosome reaction than their Low counterparts. Boars in the Medium group were intermediate and not different ($p > 0.05$) than the High or Low groups. There were no differences among fertility groups for the proportion of spermatozoa that were uncapacitated ($p = 0.20$) and capacitating ($p = 0.79$) or had an abnormal capacitation pattern ($p = 0.26$). Fertility group did not affect measures of acrosin activity in spermatozoa ($p = 0.11$).

The proportion of spermatozoa exhibiting an acrosome reaction ($p = 0.02$) and with normal acrosomes ($p = 0.07$) also were influenced by fertility group when boars were classified based on the proportion of litters in which they sired the majority of the piglets (Table 5). However, using this criterion, boars in the High group had more ($p < 0.05$) acrosome-reacted spermatozoa than those in either the Medium or Low groups. The proportion of spermatozoa with normal acrosomes were highest ($p < 0.075$) and lowest in the High and Low groups, respectively, and intermediate in the Medium group. Boars of Low fertility tended to have a higher ($p < 0.075$) proportion of spermatozoa with abnormal capacitation patterns than boars of Medium fertility. The proportion of uncapacitated ($p = 0.76$) and capacitating ($p = 0.06$) spermatozoa and estimates of the acrosin activity of spermatozoa ($p = 0.34$) were not influenced by the average number of litters in which boars sired the majority of the piglets.

Seminal Plasma Proteins

Concentrations of HSP-70, TNF-α, and TGF-β in High, Medium, and Low fertility boars are shown in Tables 6 and 7. Regardless of how fertility was classified, there was a linear increase ($p = 0.02$) in HSP-70 across the three fertility groups. Boars in the High group had higher ($p < 0.05$) concentrations than their counterparts in the Medium group which, in turn, had higher ($p < 0.05$) levels than boars in the Low group. No effect of fertility group was observed in concentrations of TNF-α ($p = 0.27$) or TGF-β ($p = 0.54$).

Sixty seminal plasma proteins were identified via two dimensional gel electrophoresis. Because the identities of the majority of these are unknown, they are identified with the combination of their molecular weight (kD) and isoelectric point (pI) in tables 8 to 20. The majority of these proteins were not affected ($p > 0.1$) by fertility groups based on the average number of piglets sired per litter (45/60; Tables 14-17) or the proportion of litters in which a boar sired the majority of the piglets (37/60; Tables18-20). Proteins that were affected by boar fertility are presented as those that were highly significant ($p < 0.05$; Tables 8 and 9); those that exhibited a strong tendency ($p = 0.051$ to $p = 0.075$; Tables 10 and 11); and those that exhibited a slight tendency ($p = 0.076$ to $p = 0.10$; Tables 12 and 13).

When the average number of pigs sired per litter was used as the fertility classification, there were eight (Table 8), five (Table 10), and two (Table 12) proteins that showed significant, strong, and slight associations with boar fertility. Of the eight that were significantly ($p < 0.05$) affected by boar fertility, two – a 26 kD/7.2 pI protein and a 28 kD/6.6 pI protein – exhibited a positive linear ($p < 0.05$) with fertility; and three – a 19

kD/9.2 pI protein, a 35 kD/6.3 pI protein, and an 186 kD/8.7 pI protein – had negative associations ($p < 0.05$) with fertility. There were two proteins, 17 kD/3.8 pI and 60 kD/4.2 pI, for which concentrations in Medium boars were higher ($p < 0.05$) than in the High and Low groups. Concentrations of a 103 kD protein with a pI of 6.3 were lower ($p < 0.05$) in the Medium fertility group compared with their counterparts in the High and Low groups.

For the remaining seven proteins that exhibited either a strong or slight tendency to be different among fertility groups, only 46 kD/6.8 pI ($p < 0.10$) and 59 kD/5.2 pI proteins were greater in High boars compared with Medium and Low boars. In contrast, concentrations of 19 kD/8.5 pI, 20 kD/6.2 pI, 40 kD/5.0 pI, and 111 kD/8.7 pI proteins were higher ($p < 0.075$) in Low than Medium and High boars. Finally, Medium boars had an 181 kD/5.7 pI protein in their ejaculates whereas this protein was absent from ejaculates of High and Low boars.

When boars were placed into fertility groups based on the proportion of litters in which they sired the majority of the piglets, there were 12 (Table 9), 8 (Table 11), and 3 (Table 13) proteins that showed significant, strong, and slight associations with boar fertility. Of the proteins that had significant associations with boar fertility, the 28 kD/6.6 pI protein, also identified in the previous analysis, was the only protein whose concentrations were higher ($p < 0.05$) in High and Medium than Low boars. In contrast, there were four proteins whose concentrations increased ($p < 0.05$) as fertility decreased. One of these, a 19 kD/9.0 pI protein, was identified earlier, while three were new. These proteins were as follows: 35 kD/6.3 pI; 187 kD/8.7 pI; and 187 kD/8.2 pI. Concentrations of the remaining proteins were either higher or lower in the Medium fertility group relative to the High and Low groups.

There were three proteins that exhibited tendencies to be positively associated with

boar fertility. A 26 kD/7.2 pI protein was higher ($p < 0.075$) in High and Medium boars compared with their Low counterparts, while a 17 kD/3.8 pI protein and a 59 kD/4.2 pI protein both showed a slight tendency ($p < 0.10$) to be reduced in seminal plasma from boars with low fertility. In contrast, two proteins, 19 kD/8.5 pI and 114 kD/7.6 pI, were lower ($p < 0.075$) in High and Medium fertility boars compared with their Low fertility counterparts. The other proteins identified as having strong and slight associations with fertility groups were either higher or lower in the Medium boars compared with High and Low boars.

Multiple Regression and Colinearity Analysis

Results from the multiple regression and colinearity analyses are shown in Table 21. Three variables accounted for 87.6% of the variation in the average number of pigs sired per litter and the proportion of litters in which a boar sired the majority of the piglets. These were concentrations of the 28 kD/6.6 pI protein (partial $R2 = 0.6619$; $p < 0.0001$); proportion of acrosome-reacted spermatozoa (partial $R2 = 0.1549$; $p = 0.0002$); and concentrations of the 17 kD/3.8 pI protein (partial $R2 = 0.0590$; $p = 0.0090$).

DISCUSSION

Currently, the most common methods used to analyze the quality of boar semen are based on morphological aspects of spermatozoa. The relationships between these assessments of fertility have been ambiguous (Gadea, 2004, Flowers, 1997, Holt, 1997, Xu,

1998, Popwell et al., 2003). Part of the reason for this is probably related to the fact that in all of these previous studies farrowing rates and litter size from homospermic matings were used to assess fertility. There are two potential problems with this approach. One is that any potential sow effects that were present would be confounded with boar fertility. If a boar by chance was bred to a group of sows with reduced fertility compared to his counterparts, then the farrowing rates and litter sizes he produced from these matings would be biased. Another problem with using homospermic matings for fertility assessment is that the reproductive physiology of the sow could limit identification of boars with exceptional fertility. Most modern genotypes of sows typically average between 11 and 13 pigs born alive. Technically, it would not be possible to identify boars consistently capable of producing 14 or more pigs born alive. In the present study heterospermic matings and paternity testing were used address the limitations associated with using homospermic inseminations. Heterospermic matings and then using paternity tests to decipher who sired the off spring is a relative measure of fertility. This can be used to rank the boars from highest to lowest fertility or alternatively place them into fertility groups.

In the present study, boars were assigned to High, Medium, or Low fertility groups in two different ways. One set of analyses used the mean percentage of piglets that the boar sired in the litter to assign fertility groupings. Average number of pigs sired per litter by boars in the High, Medium, and Low groups were 71.2 $\pm$ 0.7%, 51.2 $\pm$ 1.5%, and 25.5 $\pm$ 4.6%, respectively. This was viewed as a quantitative assessment of a boar's fertility. The second way that the boars were assigned to fertility groups was by looking at the number of litters in which they sired the majority of the piglets. Average number of litters in which the

boar sired the majority of the pigs for High, Medium, and Low boars were 78.3 ± 1.7%, 46.7 ± 2.1%, and 8.3 ± 8.3%, respectively. This was viewed as a qualitative assessment of a boar's fertility. In theory, two boars could be identical in terms of the average number of litters in which they sired the majority of the pigs, but have quite different average number of piglets sired per litter. Consequently, both estimates of fertility have merit when trying to decipher boar fertility.

The proportion of spermatozoa exhibiting motility and progressive forward motility showed a positive linear trend with boar fertility regardless of the criteria used to assess fertility. Interestingly, when fertility groups were based on the litters in which boars sired the majority of the pigs, these relationships were much stronger statistically. These results coincide with previous research by Gadea et al. (2004) and Popwell and Flowers (2004). These two studies used litter size and farrowing rate from homospermic matings to estimate the fertility of boars. Gadea et al. (2004) found that motility significantly correlated with farrowing rate and litter size. While Popwell and Flowers (2004) saw that progressive motility correlated linearly with litter size. Studies by Xu et al. (1998), Ruiz-Sanchez et al. (2006), Holt (1997) and Tardif (1999) found no relationships among motility or progressive motility and fertility. This could be because of the experimental procedure used for the evaluation of motility. In some of the studies listed visual assessment of motility with light microscopy was used to assess motility (Tardif, 1999), while others video-taped samples and conducted a visual assessment of motility of the video footage. We were able to use CASA which provides a more objective evaluation of motility with a greater degree of precision.

What is interesting about the results of our study is that it challenges the commonly

accepted belief that motility has weak associations with fertility at levels above 60% (Flowers, 1997). The proportions of motile spermatozoa were 82.4%, 72.1%, and 64.6% in the High, Medium, and Low fertility groups respectively. One explanation for this observation is that the use of heterospermic inseminations removed the limitations placed on fertility assessment by the reproductive physiology of the sow. With homospermic matings, it is possible that most boars with greater than 60% motility have sufficient numbers of spermatozoa to fertilize all the ova a sow is capable of carrying to term. With heterospermic matings, spermatozoa from boars actually compete to fertilize ova. The boar's whose spermatozoa fertilize the most ova would proportionately produce more pigs in each litter regardless of the limitations of the sow's uterine environment.

Even though motility and progressive motility were significantly associated with fertility, they were not important factors in explaining fertility differences among boars in the multiple regression analyses. This may be because after insemination the spermatozoa travel through the uterine horns and form the sperm reservoir in the oviduct. In the reservoir the sperm metabolism and motility decreases significantly until the sperm are slowly released from the reservoir just before fertilization. Since ovulation occurs after insemination in the pig. The spermatozoa come in contact with the ova long after the initial motility assessment is taken (Popwell and Flowers, 2004: Yanagimachi, 1994: Langendijik, 2005: Mburu et al., 1997). For this reason, the inclusive support of motility or progressive motility measured at collection may not be the most important factor associated with a process that occurs several hours later and after spermatozoa have been subjected to conditions that are known to alter its forward motion.

The assessments of mobility of the ejaculate using CASA analysis showed that straight-line velocity (VSL) and the straight-line distance that spermatozoa travel (DSL) were positively associated with fertility, while curvilinear velocity (VCL) and the curvilinear distance that spermatozoa travel (DCL) were not. A study by Holt (1997) found that the CASA parameters were useful in determining fertility in boars. They examined the ejaculates over a period of two hours to obtain the velocity measurements and found that these were the most useful in determining the fertility of boars based on litter sizes. The study by Holt (1997) was one of the first to look at CASA measurements of velocity as indicators of fertility. It is interesting to note that Holt (1997) did not find motility or progressive motility to be significantly associated with fertility.

The use of CASA to estimate relationships among motility traits and fertility is hindered by the same limitations as were previously discussed. The time interval between assessment and fertilization is long and the physiological status of the spermatozoa has changed dramatically. This was supported by the fact that DSL and VSL were not significant in terms of explaining variation among boars in fertility according to the multiple regression analyses. Our interpretation of the relationships between motility and fertility is that along with other measurements of sperm quality these could be helpful in determining fertility, but on their own they would not be good indicators.

Morphology of the sperm has been often evaluated as a sperm quality assessment. We found that spermatozoa that under went an acrosome reaction and had normal acrosome morphology had a positive linear relationship with fertility. It is physiologically rational that both of these traits would be positively related to fertility because in order for the

spermatozoa to undergo the acrosome reaction they would need to have normal acrosomes. This coincides with other studies that found the ability to under go the acrosome reaction was an indicator of fertility (Tardif, 1999). In contrast, it conflicts with others that found different morphological features that were poor indicators of fertility (Xu et al., 2000; Xu et al., 1998; Gadea, 2000). We did not find any other measures of morphology to be significantly associated with the fertility groups. This is contrary to other studies that found the proportion of spermatozoa with normal morphology to be significant in determining fertility (Xu et al., 2000; Xu et al., 1998; Gadea, 2000).

The reason for these discrepancies is not known. One difference could be related to the innate characteristics of the boars used. In our study, there was very little variation in normal morphology. In fact, most boars had less than 10% morphologically abnormal spermatozoa. In the studies from other groups, there was a greater range in morphologically normal spermatozoa with some boars having as high as 40% abnormalities. Therefore, it is possible that there was not enough variation in normal morphology among our boars for us to critically evaluate the impact of head and tail morphology on fertility. Nevertheless, biologically it makes senses that the acrosome would be important for fertilization of the egg. In order for the spermatozoa to be able to fertilize the egg it must pass through the cumulus cells and be able to bind to the zona pellucida (Harrison, 1996). Spermatozoa without equatorial regions are not capable of fertilizing ova so it is important that acrosome morphology be normal (Yanagimachi, 1994; Harrison, 1996; Moure et al., 1997; Langendijk, 2005). This speculation is supported by the observation that the percentage of spermatozoa that could undergo the acrosome reaction was the second most important trait in terms of

explaining variation among boars in fertility. Our interpretation is that this trait would be a good indicator in fertility.

In contrast to motility and morphology estimates, concentrations of seminal plasma proteins seemed to have the most potential as a prospective indicator of semen fertility. A total of 63 seminal plasma proteins were evaluated. Concentrations of three proteins, HSP-70, TNF-α, and TGF-β, were measured by ELISA, while relative amounts of 60 other proteins were determined with two-dimensional gel electrophoresis. There were 16 proteins identified that exhibited either positive or negative associations with boar fertility.

Of the three proteins analyzed with ELISA, HSP-70 was the only one that had a significant positive relationship with boar fertility. This is in agreement to some extent with a study done by Turba et al. (2007). They found that HSP- 70 concentrations were correlated with semen quality measurements known to be associated with fertility as previously reported by other scientists. However, it is important to note that in their study, no measures of fertility were actually made on the ejaculates from which they obtained their HSP-70 measurements. Therefore, our interpretation is that results from our study are definitive of an association between concentrations of HSP-70 and fertility, while those reported by Turba et al. (2007) are not.

Heat shock protein 70 is thought to protect the sperm from inverse environmental impacts and play an important role in thermotolerance (Huang et al., 2000; Spinaci et al., 2005). Previous studies found a positive correlation with HSP-70 and the quality of semen (Huang et al., 2000; Spinaci et al., 2005; Turba et al., 2007). In the aforementioned studies they looked at the amount of heat shock protein in the spermatozoa, while we looked at the

amount of heat shock protein in the seminal plasma. In human males, it is thought that the presence of HSP-70 antibodies in the seminal plasma will inhibit the fertilization process (Bohring and Kraus, 2003). This would explain why higher levels of heat shock protein would indicate that this ejaculate is more fertile. It is interesting to note that when heat shock protein is examined against all of the other qualities of an ejaculate with multiple regression analyses, it did not enter as a significant trait. This means that although there is a difference between fertility groups its ability as a unique test to determine fertility is low.

In contrast, Turba et al. (2007) also reported a positive correlation between fertility and TNF-α and we did not see any effect of boar fertility on TNF-α concentrations. The interest in TNF-α stems from the finding that in bull semen it was negatively correlated with other pro-inflammatory cytokines (Vera et al., 2003). High levels of anti-inflammatory cytokines in semen were interpreted as being protective of semen in this study. When TNF-α was measured in human semen there were no differences found between men that were fertile and men that were not (Camejo et al., 2001, Paradigms et al., 2003). Unfortunately, our results with regard to TNF-α do not provide much assistance in the elucidation of its role in semen fertility evaluations.

The other known protein that was examined was TGF-β. We also found no relationship between it and fertility. The reason for looking at TGF-β was that it is has been linked to embryonic survival in cattle via its actions during an immune response. When seminal plasma enters the system it causes the release of cytokines and it has been reported that TGF-β facilitates this process (Rhodes et al., 2006). Our results indicate that TGF-β was not an indicator in fertility in boars. It is interesting to note that despite its effects on cytokine

release addition of TGF-β to seminal plasma did not increase embryonic development in bulls (Rhodes et al., 2006).

Relative concentrations of seminal plasma proteins determined with two-dimensional gel electrophoresis showed the most promising and consistent relationships with boar fertility. There were 5 proteins that had positive relationships with boar fertility and 7 different ones that had negative relationships with boar fertility. In the latter case, concentrations of these proteins were low in high fertility boars and high in their low fertility counterparts.

The seven proteins with a negative association with boar fertility were as follows: MW 19.1, pI 9.02; MW 19.27, pI 8.5; MW 20.02, pI 6.19; MW 35.45, pI 6.63; MW 40.16, pI 5.01; MW 110.97, pI 8.68; and MW 187.07, pI 8.67. These proteins appear to be different from the ones that have been found to be indicators of low fertility in bulls and stallions. In bulls, the seminal plasma proteins that indicated below average fertility were 16kDa, pI4.1 and 16kDa, pI 6.7 (Killian, 1993). The low fertility stallions' seminal plasma contained proteins that were 15kDa, pI6.0; 18kDa, pI4.3; and 16kDa, pI6.5 (Brandon, 1999). At the present time, we have no explanation for the discrepancy in the results of our studies and those conducted in bulls and stallions. However, it is important to recognize that most proteins are initially produced as dimers and trimers and then are modified to their active form. Since most of the proteins we identified as having a negative correlation with fertility in boars were larger in MW than those reported in bulls and stallions, it is possible that in boars post-translational modifications occur at a later stage of the reproductive process compared with cattle and horses. If this speculation is true, then the proteins we found in boar

semen might be dimeric or trimeric forms of the same proteins identified in other species. Additional studies will need to be conducted to determine whether this speculation is true.

The seminal plasma proteins that exhibited the strongest relationship with fertility were as follows: MW 26.02, pI 7.21; MW 28.09, pI 6.63; MW 46.22 pI 6.81; MW 59.53, pI 5.19; and MW 103.4, pI 6.31. The 28 kD/6.6 pI and 59 kD/5.1 pI proteins are very close to proteins that have previously been reported to be associated positively with fertility in boars. These proteins were 26kDa, pI 6.2 and 55kDa, pI 4.5 (Flowers, 1999). A previous study found that these two proteins when in the seminal plasma of boar lead to higher farrowing rates and litter size (Strzezek, 2000). In another study, it was also found that boars with similar semen quality estimates based on motility and morphology differed significantly in fertility. Boars with high levels of these two proteins were more fertile than boars with lower concentrations (Flowers, 1998). Finally, proteins with similar molecular weights and isoelectric points have also been identified in the seminal plasma of bulls and linked to high fertility (Killian, 1993). Consequently, it appears that our results, at least based on these two proteins are in agreement with previous work reported in swine and cattle. In cattle these two proteins have been identified. The 26kDa, pI 6.2 is thought to be lipocalin-type Prostaglandin D synthase (Gerena et al., 1998). Osteopotin is 55Kda, pI 4.5 seminal plasma protein (Cancel et al., 1997). The most impressive result that we obtained was the observation that about 67% of the total variation in boar fertility observed in our study was due to concentrations of the 28 kD/6.6 pI protein. A single trait with such a great contribution to fertility differences would be an excellent candidate for development as a proactive fertility test.

Seminal plasma proteins that were found to correlate with high fertility in horses were

20-25kDa, pI 8.5-8.7 and 72kDa, pI 5.6. In our study, one of the proteins that indicated low fertility in boars was similar to the protein that indicated high fertility in stallions. Reasons for this apparent discrepancy are not known at the present time. However, since the identity of this protein is not known in either boars or stallions, it is possible that they might actually be different and simply have migrated to the same location of the gels. As mentioned earlier, perhaps in boar semen the proteins at this location are dimers or trimers of smaller proteins, whereas in stallions they are the actual active form of a larger protein.

The only protein that comes close to those observed as fertility proteins in stallions in our study is the 75 KD protein. It could be a similar protein that is in a different form in boars. In bulls, it has been demonstrated that the 55kd protein can be found in three different isoforms with three separate molecular weights. The only way to resolve these differences is to sequence the proteins from boars and other species.

The majority of the proteins found in seminal plasma are thought to be sperm adhesion proteins (Jonakova et al., 2007; Strzezek, 2000). It is thought that these proteins bind to the spermatozoa during or shortly after ejaculation. The proteins are thought to form a protein coat around the spermatozoa and aid in the formation of the sperm reservoir. This protein coat is also thought to play a role in sperm capacitation, oocyte recognition and sperm-oocyte binding (Jonakova et al, 2007; Strzezek, 2000). The proteins that are associated with higher fertility in boars could very well be sperm adhesion proteins. Their molelcular weights and isoelectric points are consistent with sperm adhesion proteins identified in boars and other species. If they in fact do turn out to be sperm adhesions proteins, then results from our study would be the first definitive evidence that their

concentrations in semen can be used to predict boar fertility.

In summary, based on all of the quality measurements of the ejaculate the best indicator of fertility would be to use concentrations of seminal plasma proteins, in particular, the protein that has a MW 28.09, and a pI 6.63. Concentrations of this protein alone were highly significant among boars with different fertility and compared with all the other parameters we examined explained two-thirds of the total variation in fertility among boars in our study.

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Table 2. Relationships among Boar Fertility based on the Average Number of Piglets Sired per Litter and Semen Quality Estimates for Motility and Mobility (means ± S.E.).

	Fertility Group		
Variable	High	Medium	Low
Proportion of motile spermatozoa (%)	82.4 ± 1.9[m]	73.1 ± 2.5[m,n]	64.6 ± 8.9[n]
Proportion of spermatozoa exhibiting progressive motility (%)	58.3 ± 2.6[m]	47.7 ± 2.7[m,n]	40.8 ± 7.9[n]
Average curvilinear distance traveled by motile spermatozoa (DCL, um)	52.5 ± 1.9	50.0 ± 2.1	55.4 ± 2.9
Average straight line distance traveled by motile spermatozoa (DSL, um)	21.3 ± 0.7[a]	19.9 ± 0.4[a,b]	18.1 ± 1.0[b]
Average curvilinear velocity of motile spermatozoa (VCL, um/s)	114.5 ± 4.3	109.5 ± 4.5	119.3 ± 6.6
Average straight line velocity of motile spermatozoa (VSL, um/s)	46.8 ± 1.7[a]	43.7 ± 1.0[a]	39.1 ± 2.2[b]

[a,b] means within the same row with different superscripts are different ($p < 0.05$).
[m,n] means within the same row with different superscripts are different ($p < 0.075$).

Table 3. Relationships among Boar Fertility based on the Average Number of Litters in which the Boar Sired the Majority of the Piglets and Semen Quality Estimates for Motility and Mobility (means ± S.E.).

	Fertility Group		
Variable	High	Medium	Low
Proportion of motile spermatozoa (%)	80.8 ± 1.2[a]	76.2 ± 2.9[a,b]	64.1 ± 6.6[b]
Proportion of spermatozoa exhibiting progressive motility (%)	57.4 ± 1.8[m]	49.8 ± 3.4[m,n]	40.6 ± 5.8[n]
Average curvilinear distance traveled by motile spermatozoa (DCL, um)	48.5 ± 2.1	51.3 ± 2.2	55.8 ± 2.2
Average straight line distance traveled by motile spermatozoa (DSL, um)	20.4 ± 0.4	20.5 ± 0.6	18.8 ± 0.9
Average curvilinear velocity of motile spermatozoa (VCL, um/s)	105.3 ± 4.7	112.0 ± 4.9	120.5 ± 5.0
Average straight line velocity of motile spermatozoa (VSL, um/s)	43.9 ± 0.9	45.2 ± 1.5	40.5 ± 1.9

[a,b] means within the same row with different superscripts are different ($p < 0.05$).
[m,n] means within the same row with different superscripts are different ($p < 0.075$).

Table 4. Relationships among Boar Fertility based on the Average Number of Piglets Sired per Litter and Semen Quality Estimates for Morphology (means ± S.E.).

	Fertility Group		
Variable	High	Medium	Low
Proportion of spermatozoa that are not showing capacitation (%)	0.3±0.11	0.81±0.14	0.66±0.23
Proportion of spermatozoa capacitating (%)	79.33±0.9	80.02±0.9	79.13±2.1
Proportion of spermatozoa exhibiting acrosome reaction (%)	5.65±0.5[a]	4.42±0.4[a,b]	3.18±0.3[b]
Proportion of spermatozoa with abnormal acrosome reaction (%)	14.95±0.4	14.63±0.5	17.09±2.2
Proportion of spermatozoa exhibiting acrosin activity (%)	93.15±0.4	94.62±0.5	92.45±1.4
Proportion of spermatozoa not exhibiting acrosin activity (%)	5.17±0.6	4.4±0.4	3.43±0.5
Proportion of spermatozoa with normal acrosome morphology (%)	91.87±0.5[a]	89.4±0.9[a]	79.06±5.5b

[a,b] means within the same row with different superscripts are different ($p < 0.05$).
[m,n] means within the same row with different superscripts are different ($p < 0.075$).

Table 5. Relationships among Boar Fertility based on the Average Number of Litters in which the Boar Sired the Majority of the Piglets and Semen Quality Estimates for Morphology (means ± S.E.).

	Fertility Group		
Variable	High	Medium	Low
Proportion of spermatozoa that are not showing capacitation (%)	0.53±.05	0.72±0.20	0.70±0.18
Proportion of spermatozoa capacitating (%)	78.60 ± 1.1^{m}	81.57 ± 0.4^{n}	78.24 ± 1.6^{m}
Proportion of spermatozoa exhibiting acrosome reaction (%)	5.76 ± 0.5^{b}	4.00 ± 0.3^{a}	3.93 ± 0.6^{a}
Proportion of spermatozoa with abnormal acrosome reaction (%)	15.43 ± 0.6^{m}	$13.82\pm0.3^{m,n}$	17.13 ± 1.6^{n}
Proportion of spermatozoa exhibiting acrosin activity (%)	93.96±0.7	94.62±0.5	92.78±1.1
Proportion of spermatozoa not exhibiting acrosin activity (%)	4.36±0.4	4.52±0.5	4.14±0.7
Proportion of spermatozoa with normal acrosome morphology (%)	92.51 ± 0.8^{m}	$88.58\pm0.8^{m,n}$	82.58 ± 4.5^{n}

^{a,b} means within the same row with different superscripts are different ($p < 0.05$).
^{m,n} means within the same row with different superscripts are different ($p < 0.075$).

Table 6. Relationships among Boar Fertility based on the Average Number of Piglets Sired per Litter and Concentrations of Known Seminal Plasma Proteins (means ± S.E.).

	Fertility Group		
Variable	High	Medium	Low
Heat Shock Protein 70 (ng/mg)	45.42±10.3[a]	36.03±3.3[a,b]	20.04±0.7[b]
Transforming Growth Factor-Beta (ng/mg)	0.47±0.22	0.58±0.05	0.74±0.23
Tumor Necrosis Factor-Alpha (ng/mg)	0.86±0.1	0.88±0.02	0.7±0.13

[a,b] means within the same row with different superscripts are different ($p < 0.05$).

Table 7. Relationships among Boar Fertility based on the Average Number of Litters in which the Boar Sired the Majority of the Piglets and Concentrations of Known Seminal Plasma Proteins (means ± S.E.).

	Fertility Group		
Variable	High	Medium	Low
Heat Shock Protein 70 (ng/mg)	50.60±7.9[a]	32.92±4.0[b]	20.04+0.7[b]
Transforming Growth Factor-Beta (ng/mg)	0.6±0.21	0.5±0.07	0.74±0.23
Tumor Necrosis Factor-Alpha (ng/mg)	0.94±0.08	0.83±0.04	0.70±0.13

[a,b] means within the same row with different superscripts are different ($p < 0.05$).

Table 8. Relationships among Boar Fertility based on the Average Number of Piglets Sired per Litter and Seminal Plasma Proteins (arbitrary units / ng of total protein ± S.E.). These proteins show significance at $p < 0.05$.

Code	MW (kd)	pI	High Fertility Group	Middle Fertility Group	Low Fertility Group
A1	19.1	9.02	238.23 ± 26.13^{a}	265.46 ± 11.37^{a}	403.37 ± 72.85^{b}
A11	17.52	3.84	0 ± 0^{a}	41.29 ± 7.62^{b}	0 ± 0^{a}
B1	26.02	7.21	61.75 ± 6.75^{a}	$45.72 \pm 12.66^{a,b}$	13.12 ± 8.30^{b}
B2	28.09	6.63	67.96 ± 3.42^{a}	33.64 ± 9.60^{b}	0 ± 0^{c}
C6	35.45	6.3	32.72 ± 10.81^{a}	$52.49 \pm 11.08^{a,b}$	100.39 ± 26.95^{b}
E7	59.53	4.17	$37.07 \pm 15.85^{a,b}$	72.58 ± 14.35^{a}	15.79 ± 9.99^{b}
G2	103.4	6.31	202.31 ± 11.84^{a}	160.54 ± 4.57^{b}	189.57 ± 4.58^{a}
I1	187.07	8.67	25.59 ± 8.01^{a}	37.1 ± 11.37^{a}	108.03 ± 34.17^{b}

^{a,b,c} means within the same row with different superscripts are different ($p < 0.05$).

Table 9. Relationships among Boar Fertility based on the Average Number of Litters in which the Boar Sired the Majority of the Piglets and Seminal Plasma Proteins (arbitrary units / ng of total protein ± S.E.). These proteins show significance at $p < 0.05$.

Code	MW (kd)	pI	High Fertility Group	Middle Fertility Group	Low Fertility Group
A1	19.1	9.02	263.16 ± 23.70^{a}	250.1 ± 14.03^{a}	403.37 ± 72.85^{b}
A6	20.02	6.19	284.52 ± 28.67^{a}	213.25 ± 5.10^{b}	$274.22 \pm 82.95^{a,b}$
B2	28.09	6.63	42.06 ± 13.38^{a}	49.18 ± 9.13^{a}	0 ± 0^{b}
C6	35.45	6.30	13.50 ± 8.54^{a}	64.02 ± 6.89^{b}	100.39 ± 26.59^{b}
E3	59.53	5.19	104.63 ± 33.51^{a}	146.11 ± 10.29^{b}	139.84 ± 13.29^{b}
F5	76.68	6.48	15.58 ± 9.85^{a}	112.13 ± 17.68^{b}	46.50 ± 29.41^{a}
F6	103.40	6.31	174.43 ± 9.31^{a}	$177.27 \pm 10.54^{a,b}$	189.57 ± 4.58^{a}
H4	115.34	6.86	107.23 ± 27.34^{a}	123.83 ± 8.69^{b}	$100.60 \pm 21.35^{a,b}$
I1	187.07	8.67	13.18 ± 8.33^{a}	44.55 ± 9.62^{a}	108.03 ± 34.17^{b}
I2	187.07	8.21	38.95 ± 12.71^{a}	37.10 ± 11.37^{a}	132.23 ± 42.54^{b}
I3	187.07	7.74	34.39 ± 21.75^{a}	94.68 ± 21.97^{b}	117.42 ± 37.17^{b}
I4	187.07	6.79	59.11 ± 37.39^{a}	107.69 ± 30.22^{b}	$91.66 \pm 38.99^{a,b}$

^{a,b} means within the same row with different superscripts are different ($p < 0.05$).

Table 10. Relationships among Boar Fertility based on the Average Number of Piglets Sired per Litter and Seminal Plasma Proteins (arbitrary units / ng of total protein ± S.E.). These proteins show significance at $p < 0.075$.

Code	MW (kd)	pI	High Fertility Group	Middle Fertility Group	Low Fertility Group
A2	19.27	8.5	255.09 ± 13.45^{m}	262.5 ± 5.47^{m}	385.04 ± 82.95^{n}
A6	20.02	6.19	199.18 ± 18.27^{m}	222.82 ± 7.21^{m}	274.22 ± 18.03^{n}
C7	40.16	5.01	0 ± 0^{m}	29.54 ± 8.14^{n}	36.77 ± 16.06^{n}
E5	59.53	5.19	168.81 ± 9.18^{m}	107.61 ± 19.18^{n}	139.84 ± 13.29^{n}
I7	181.3	5.66	0 ± 0^{m}	22.94 ± 9.62^{n}	0 ± 0^{m}

^{m,n} means within the same row with different superscripts are different ($p < 0.075$).

Table 11. Relationships among Boar Fertility based on the Average Number of Litters in which the Boar Sired the Majority of the Piglets and Seminal Plasma Proteins (arbitrary units / ng of total protein ± S.E.). These proteins show significance at $p < 0.075$.

Code	MW (kd)	pI	High Fertility Group	Middle Fertility Group	Low Fertility Group
A2	19.27	8.5	251.46 ± 13.84^{m}	264.68 ± 4.62^{m}	385.04 ± 82.95^{n}
B1	26.02	7.21	48.26 ± 15.27^{m}	53.82 ± 10.35^{m}	13.12 ± 8.30^{n}
C7	40.16	5.01	0 ± 0^{m}	29.54 ± 8.14^{n}	36.77 ± 16.06^{n}
E1	46.22	6.81	182.93 ± 18.41^{m}	132.01 ± 13.54^{n}	148.50 ± 22.52^{n}
E8	57.34	3.73	0 ± 0^{m}	9.44 ± 6.30^{n}	0 ± 0^{m}
H1	110.97	8.68	$89.40 \pm 7.64^{m,n}$	68.55 ± 14.24^{n}	124.42 ± 21.66^{m}
H3	113.78	7.61	164.68 ± 7.77^{m}	165.44 ± 14.07^{m}	$207.55+26.75^{n}$
I7	181.3	5.66	0 ± 0^{m}	22.94 ± 9.62^{n}	0 ± 0^{m}

^{m,n} means within the same row with different superscripts are different ($p < 0.075$).

Table 12. Relationships among Boar Fertility based on the Average Number of Piglets Sired per Litter and Seminal Plasma Proteins (arbitrary units / ng of total protein ± S.E.). These proteins show significance at $p < 0.10$.

Code	MW (kd)	pI	High Fertility Group	Middle Fertility Group	Low Fertility Group
D5	46.22	6.81	186.2 ± 17.94^{x}	130.04 ± 12.87^{y}	148.5 ± 22.52^{y}
H1	110.97	8.68	83.15 ± 5.09^{x}	72.29 ± 15.06^{x}	124.42 ± 21.66^{y}

^{x,y} means within the same row with different superscripts are different ($p < 0.10$).

Table 13. Relationships among Boar Fertility based on the Average Number of Litters in which the Boar Sired the Majority of the Piglets and Seminal Plasma Proteins (arbitrary units / ng of total protein ± S.E.). These proteins show significance at $p < 0.10$.

Code	MW (kd)	pI	High Fertility Group	Low Fertility Group	High Fertility Group
A11	17.52	3.84	20.52 ± 12.97^{x}	28.99 ± 8.37^{x}	0 ± 0^{y}
B3	28.09	6.63	19.21 ± 2.14^{x}	54.93 ± 9.49^{y}	25.43 ± 16.09^{x}
C3	30.74	7.96	117.46 ± 20.67^{x}	165.5 ± 16.60^{y}	116.61 ± 24.64^{x}
E7	59.53	4.17	$47.83 \pm 22.42^{x,y}$	66.13 ± 12.67^{x}	15.79 ± 9.99^{y}
I5	193.66	6.43	$59.11+37.39^{x}$	$64.20+21.75^{x}$	$57.23+36.19^{x}$

^{x,y} means within the same row with different superscripts are different ($p < 0.10$).

Table 14. Relationships among Boar Fertility based on the Average Number of Piglets Sired per Litter and Seminal Plasma Proteins with Molecular Weights from 20-31 (arbitrary units / ng of total protein ± S.E.).

Code	MW (kd)	pI	High Fertility Group	Middle Fertility Group	Low Fertility Group	P-Value
A3	20.82	7.88	214.53±63.17	290.63±7.03	363.76±96.90	0.23
A4	19.88	7.14	308.83±14.19	284.69±9.80	303.62±43.95	0.71
A5	20.25	6.66	299.64±31.25	261.95±17.58	289.28±32.78	0.53
A7	20.44	5.35	192.5±12.14	199.35±12.08	192.1±12.48	0.89
A8	19.88	4.77	145.61±14.48	155.71±13.39	164.79±16.58	0.71
A9	19.88	4.42	1.6±10.94	112.43±12.23	140.52±14.19	0.24
A10	18.75	4.17	38.18±12.12	68.93±14.08	37.72±13.14	0.19
B3	28.09	6.63	43.52±3.42	40.33±11.24	25.34±16.09	0.65
B4	26.42	5.01	64.45±14.05	94.11±17.44	107.58±51.89	0.62
C1	30.26	8.95	83.35±0.95	67.11±14.28	103.07±43.16	0.55
C2	30.74	8.39	169.23±27.68	104.45±24.38	104.99±34.25	0.24
C3	30.74	7.96	117.46±20.67	165.5±16.60	116.61±24.64	0.14

Table 15. Relationships among Boar Fertility based on the Average Number of Piglets Sired per Litter and Seminal Plasma Proteins with Molecular Weights from 32-57 (arbitrary units / ng of total protein ± S.E.).

Code	MW (kd)	pI	High Fertility Group	Middle Fertility Group	Low Fertility Group	P-Value
C4	32.09	7.54	71.59+28.81	82.24+15.07	93.5+34.42	0.84
C5	34.96	6.81	77.29±14.79	87.49±18.12	96.11±23.41	0.83
C8	41.71	4.43	0±0	12.98±5.34	7.3±4.62	0.17
D1	45.67	8.95	56.37±7.56	35.96±10.79	26.38±9.84	0.19
D2	45.29	8.39	122.38±8.80	94.8±5.61	115±19.04	0.17
D3	44.76	7.96	132.04±6.17	136.11±4.24	113.25±17.55	0.22
D4	45.76	7.21	161.37±9.82	147.55±9.45	164.94±16.14	0.51
D6	45.29	5.01	70.58±18.31	87.36±12.38	52.83±33.41	0.48
D7	45.29	4.43	0±0	28.38±12.09	23.71±15.00	0.25
E1	56.74	7.00	62.21+26.69	30.65+9.00	17.74+11.22	0.18
E2	56.74	6.5	72.16+22.94	63.86+13.52	62.09+27.21	0.94

Table 16. Relationships among Boar Fertility based on the Average Number of Piglets Sired per Litter and Seminal Plasma Proteins with Molecular Weights from 59-80 (arbitrary units / ng of total protein ± S.E.).

Code	MW (kd)	pI	High Fertility Group	Middle Fertility Group	Low Fertility Group	P-Value
E3	61.22	6.24	66.18+14.82	73.04+15.67	109.27+12.41	0.17
E4	59.53	5.57	127.85+19.39	79.49+17.92	149.59+45.61	0.18
E6	59.53	4.34	136.42+14.80	117.52+12.85	91.76+15.47	0.16
E8	57.34	3.73	0+0	9.44+9.44	0+0	0.30
F1	70.87	8.76	113.2±6.68	68.21±68.21	101.74±32.31	0.16
F2	73.73	8.21	168.27±16.35	127.21±9.44	146.22±25.14	0.20
F3	72.29	7.62	138.9±25.93	121.14±9.74	134.37±30.79	0.79
F4	76.68	7.03	99.53±27.00	76.91±4.15	122.3±27.97	0.22
F5	76.68	6.48	84.65±36.69	70.68±14.15	46.5±29.41	0.63
F6	75.20	6.16	105.71±21.72	91.21±19.84	140.11±7.45	0.21
F7	73.73	5.49	87.9±10.80	135.32±24.98	124.18±1.29	0.27

Table 17. Relationships among Boar Fertility based on the Average Number of Piglets Sired per Litter and Seminal Plasma Proteins with Molecular Weights from 100-200 (arbitrary units / ng of total protein ± S.E.).

Code	MW (kd)	pI	High Fertility Group	Middle Fertility Group	Low Fertility Group	P-Value
G1	103.4	6.49	93.58+27.63	81.48+10.30	110.83+23.58	0.55
G3	103.83	5.51	125.04±23.89	114.48±12.32	147.45±13.42	0.36
H2	113.78	8.03	196.58±5.09	175.44±6.64	197.78±12.40	0.11
H3	57.34	3.73	164.68±6.11	165.44±14.07	207.55±26.75	0.19
H4	57.34	3.73	107.23±7.77	123.83±8.69	100.6±21.35	0.61
H5	113.78	6.49	77.95±27.34	67.8±15.35	64.29±22.46	0.87
I2	187.07	8.21	38.95±12.71	94.68±21.97	132.23±42.54	0.11
I3	187.07	7.74	34.39±21.75	107.69±30.22	117.42±37.17	0.19
I4	187.07	6.79	59.11±37.39	64.2±21.75	91.66±38.99	0.76
I5	193.66	6.43	82.58±26.22	90.13±25.16	57.23±36.19	0.72
I6	187.07	6.12	0±0	72.87±22.43	48.64±30.76	0.11

Table 18. Relationships among Boar Fertility based on the Average Number of Litters in which the Boar Sired the Majority of the Piglets and Seminal Plasma Proteins with Molecular Weights from 15-45 (arbitrary units / ng of total protein ± S.E.).

Code	MW (kd)	pI	High Fertility Group	Middle Fertility Group	Low Fertility Group	P-Value
A3	20.82	7.88	227.95±65.68	282.58±7.66	363.73±96.90	0.30
A4	19.88	7.14	292.62±6.15	294.41±13.22	303.62±43.95	0.95
A5	20.25	6.66	284.52+28.67	271.02±20.31	289.28±32.78	0.87
A7	20.44	5.35	187.86±9.21	202.14±12.66	192.10±12.48	0.69
A8	19.88	4.77	146.15±14.81	155.38±13.30	164.79±16.58	0.73
A9	19.88	4.42	102.83±10.51	116.49±12.05	140.52±14.19	0.18
A10	18.75	4.17	46.8±15.88	63.76±13.81	37.72±13.14	0.43
B4	26.42	5.01	81.85±24.22	83.67±14.30	107.58±51.89	0.81
C1	30.26	8.95	86.75±1.23	65.07±13.97	103.07±43.16	0.49
C2	30.74	8.39	143.21±13.33	120.06±30.71	104.99±34.25	0.72
C4	32.09	7.54	50.55±17.19	94.86±18.12	93.50±34.42	0.36
C5	34.96	6.81	64±6.41	95.46±18.72	96.11±23.41	0.44
C8	41.71	4.43	0±0	12.98±5.34	7.30±4.62	0.17

Table 19. Relationships among Boar Fertility based on the Average Number of Litters in which the Boar Sired the Majority of the Piglets and Seminal Plasma Proteins with Molecular Weights from 45-60 (arbitrary units / ng of total protein ± S.E.).

Code	MW (kd)	pI	High Fertility Group	Middle Fertility Group	Low Fertility Group	P-Value
D1	45.67	8.95	53.7±5.87	37.56±11.42	26.38±9.84	0.27
D2	45.29	8.39	97.23±8.08	109.89±7.78	115.0±19.04	0.60
D3	44.76	7.96	130.18±5.00	137.23±4.58	113.25±17.55	0.20
D4	45.76	7.21	163.51±10.95	146.27±8.77	164.94±16.14	0.42
D5	46.22	6.81	182.93±18.41	132.01±13.54	148.50±22.52	0.14
D6	45.29	5.01	99.2±16.74	70.19±12.18	52.83±33.41	0.34
D7	45.29	4.43	32.27±20.72	8.73±3.56	23.71±15.00	0.37
E2	56.74	6.50	33.94±21.47	86.79±9.54	62.09±27.21	0.13
E4	59.53	5.57	64.94±20.67	117.23±16.96	149.59±45.61	0.15
E5	59.53	5.19	104.63±33.51	146.11±10.29	139.84±13.29	0.29
E6	59.53	4.34	129.71±11.65	121.55±14.31	91.76±15.47	0.23

Table 20. Relationships among Boar Fertility based on the Average Number of Litters in which the Boar Sired the Majority of the Piglets and Seminal Plasma Proteins with Molecular Weights from 70-200 (arbitrary units / ng of total protein ± S.E.).

Code	MW (kd)	pI	High Fertility Group	Middle Fertility Group	Low Fertility Group	P-Value
F1	70.87	8.76	95.95±5.86	78.56±12.91	101.74±32.31	0.63
F2	73.73	8.21	135.54±20.45	146.85±10.18	146.22±25.14	0.88
F3	72.29	7.62	100±17.24	144.48±12.27	134.37±30.79	0.27
F4	76.68	7.03	62.24±3.42	99.29±14.85	122.30±27.97	0.11
F7	73.73	5.49	117.28±22.62	117.20±24.26	124.18±1.29	0.97
G1	103.4	6.49	60.1±6.63	101.57±16.74	110.83±23.58	0.16
G2	103.4	6.31	174.43±9.32	177.27±10.54	189.57±4.58	0.57
G3	103.83	5.51	118.24±24.13	118.57±12.35	147.45±13.42	0.4
H2	113.78	8.03	186.67±10.84	181.39±5.90	197.78±12.40	0.44
H5	113.78	6.49	64.84±4.45	75.67±16.65	64.29±22.46	0.86
I6	187.07	6.12	0±0	72.87±22.43	48.64±30.76	0.10

Table 21. Results from Multiple Regression Analysis among Semen Quality Estimates and Boar Fertility based on the Average Number of Piglets Sired per Litter.

Variable	Partial R^2	Total R^2	F-value	P-value
28.09Kd/6.63pI	0.66	0.66	39.16	<0.0001
Proportion of spermatozoa exhibiting acrosome reaction (%)	0.15	0.82	16.06	0.0008
17.52Kd/3.84pI	0.06	0.88	8.56	0.009

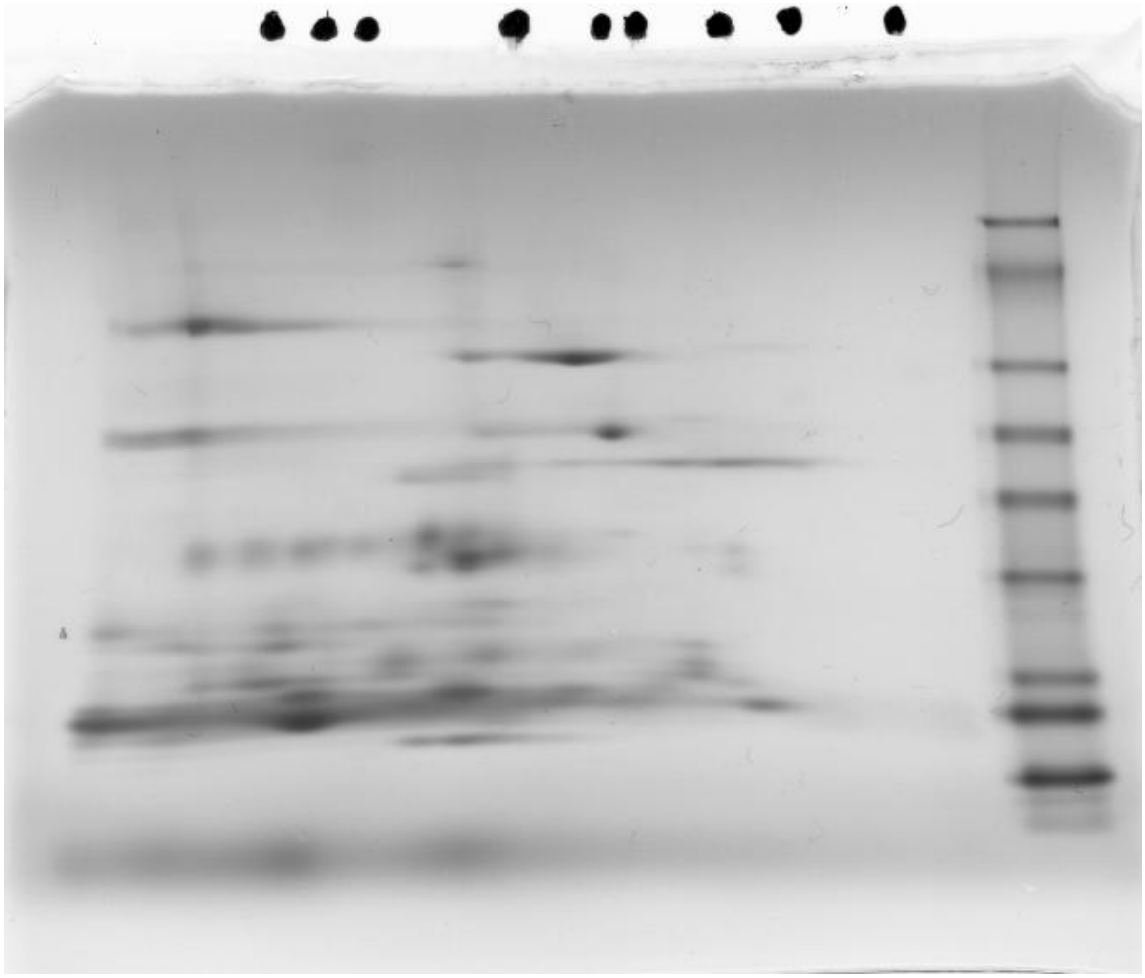

Figure 1. Representative Photomicrograph of a Two Dimensional Polyacrylamide Gel from a High Fertility Boar.

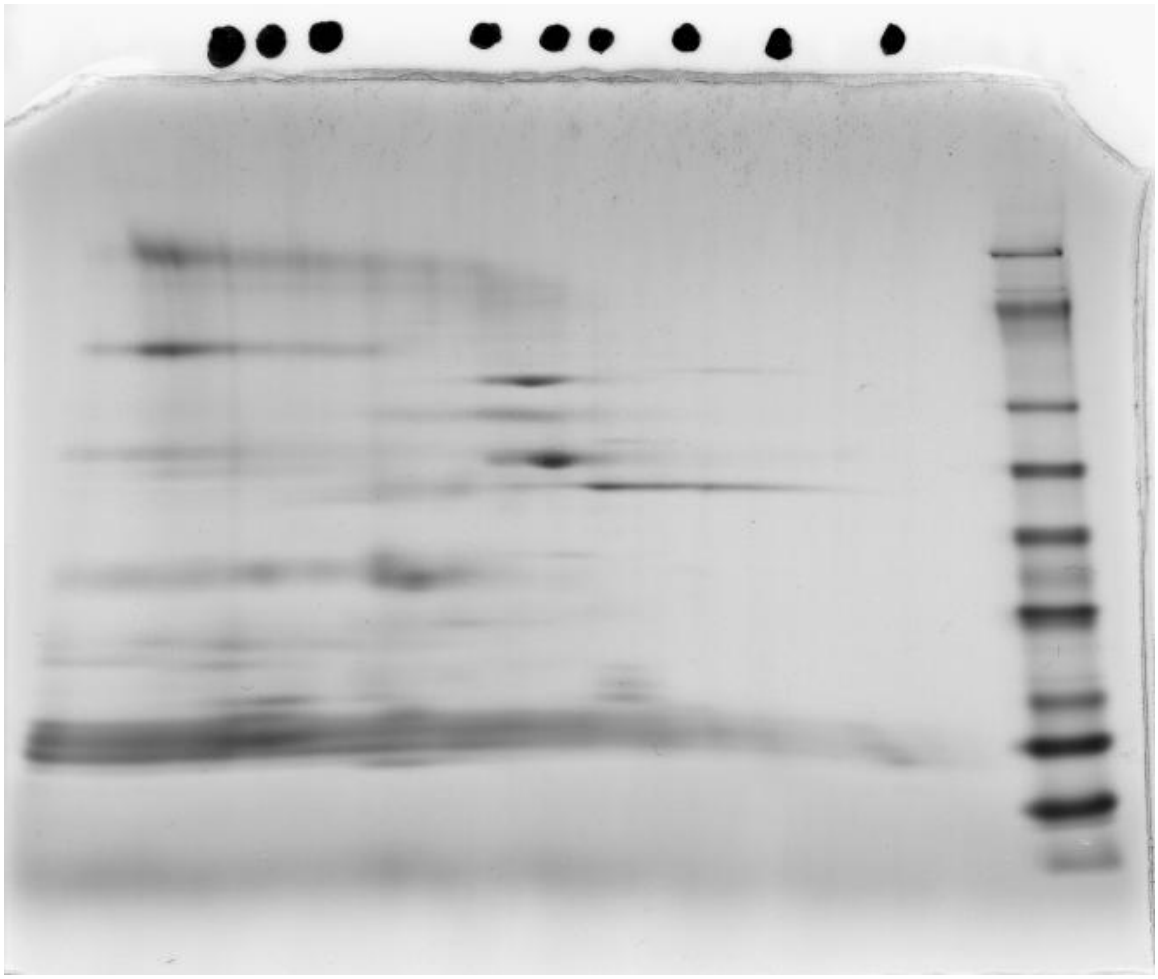

Figure 2. Representative Photomicrograph of a Two Dimensional Polyacrylamide Gel from a Medium Fertility Boar.

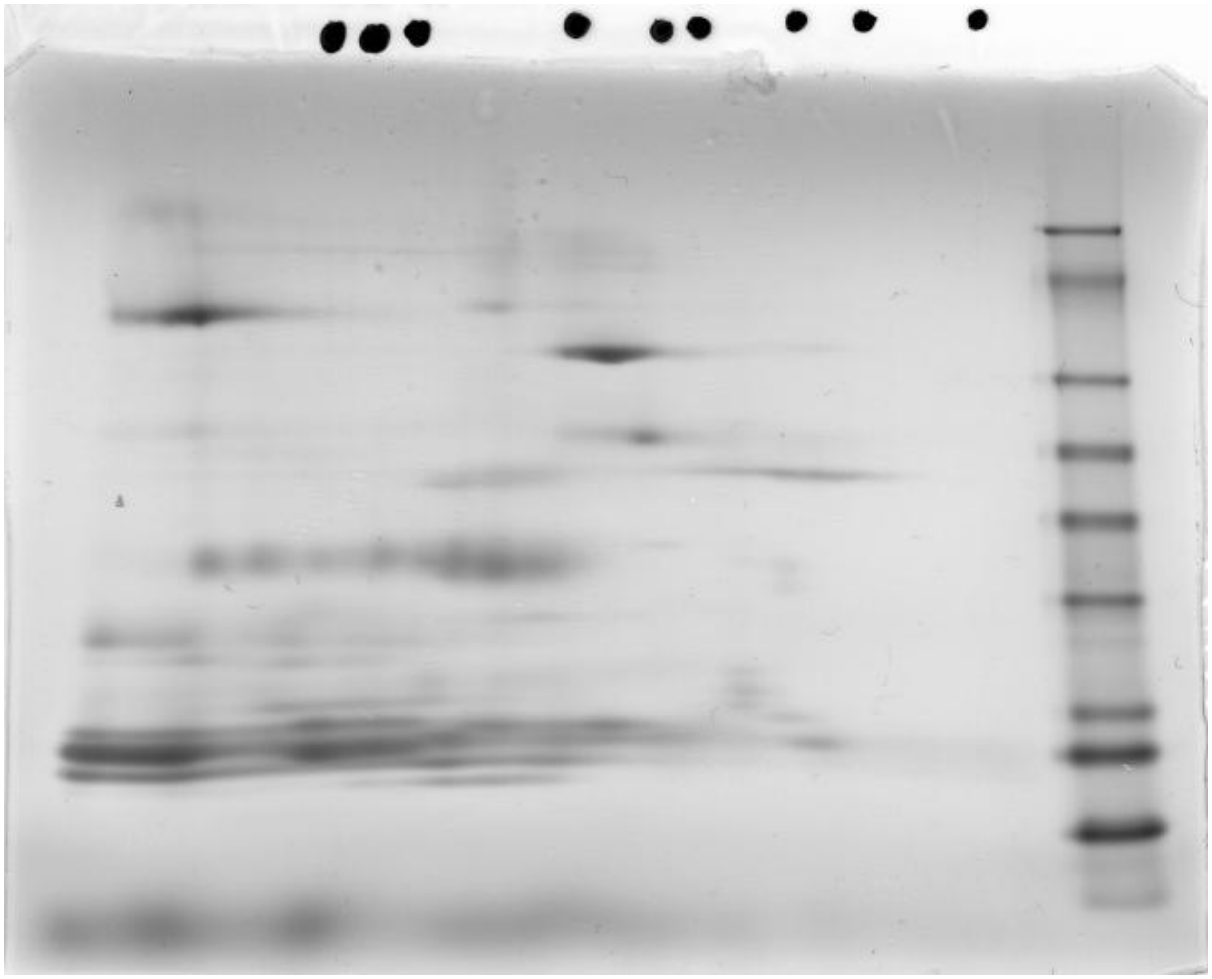

Figure 3. Representative Photomicrograph of a Two Dimensional Polyacrylamide Gel from a Low Fertility Boar.

APPENDICES

APPENDIX A

Protein Electrophoresis Procedure:

Solution Preparation:

1.) IEF Anode Working Buffer: Two 500ml bottles are prepared because about 550mls are used per run. 490mls of distilled water is measured in a graduated cylinder and added to the 500ml bottle. Then 10ml of Novex 50X IEF Anode Buffer (Invitrogen, Carlsbad, California) is measured with a serological pipette and added to the 500ml bottle. The bottle is then inverted several times to mix. A label with the date made, initials of the maker, and buffer name is placed on the bottle.

2.) IEF Cathode Working Buffer: One 500ml bottle of this buffer is made because only 175mls are used per run. Distilled water is measured to the 450ml mark in a graduated cylinder and is then added to the 500ml bottle. Next 50mls of Novex 10X IEF Cathode Buffer (Invitrogen, Carlsbad, California) is measured with a serological pipette and added to the 500ml bottle. The bottle is inverted multiple times to mix and then a label with the same information as above is added to the bottle.

3.) Fix Solution: Both solid ingredients are weighed out, 8.65 grams of Sulphosalicylic Acid and 28.65 grams of TCA, and put into a glass bottle. Next 250ml of distilled water is added to the bottle. The bottle is then inverted several times to mix and a label is added.

4.) Coomasie G-250 Stain: This is made using the ingredients that come in the Collodial Blue Staining Kit (Invitrogen, Carlsbad, California). This will be made in a glass bottle. Measure 116mls of distilled water in a graduated cylinder and pour into bottle. Next add 40mls of Stainer A, that can be measured with a serological pipette, 4mls of Stainer B, and 40mls of methanol. Then invert the bottle several times to mix.

IEF Gel Run:

1.) Remove samples for particular run from the freezer including the standard sample, which are four samples in total since we are using a 10 well gel and run the samples in duplicate.

2.) While the samples are thawing retrieve the cathode buffer from the refrigerator and pour about 250mls into a volumetric flask. Then degas the buffer by attaching the flask to the air hose and turning on the water. Degas the buffer for ten minutes.

3.) While degassing obtain four 0.5ml bullet tubes and label them with boar number and date of the sample they correspond to. Once sample has thawed mix sample by inverting it several times. Then pipette 100uls of sample into its corresponding bullet tube.

4.) Remove the IEF Sample Buffer (Invitrogen, Carlsbad, California) from the refrigerator and pipette 100uls into each sample tube. Then invert the sample a few times to mix.

5.) Set up the gel running apparatus. Remove an IEF gel from the refrigerator and document its gel number in the lab note book. Rinse the gel with distilled water and pat it dry with a paper towel. Trace the wells with a sharpie. Remove the comb from the top of the gel ant the tape from the bottom of the gel. Place the gel down in the slot at the front of the gel. Assemble the rest of the apparatus making sure that it is tight.

6.) Add enough cathode buffer to cover the bottom of the inner chamber that is created by the gel with a serological pipette. Check the chamber for leaks; if it is not leaking fill the rest of the chamber to the top of the wells. If there is a leak disassemble the apparatus rinse it off, pat dry, and start over.

7.) Fill the outer chamber with anode buffer by pouring it in the front. Fill until the buffer reaches the bottom of the wells on the gel.

8.) Pipette 5uls of IEF marker 3-10 (Invitrogen, Carlsbad, California) in the 5^{th} and 10^{th} well.

9.) Pipette 25uls of sample into the remaining wells. The samples should be placed in duplicate. For example the control sample would be placed into wells 1 and 2.

10.) Sketch the gel and the lab notebook and write what is in each well for reference.

11.) Place the lid on the gel running apparatus and place the plugs into there corresponding receptacles on the power source, red plugged into red and black plugged into black.

12.) Turn on the power source which is the Novex PowerEase 500 power supply. Then select IEF gel run and 1 gel. Check the pre-settings for correctness, 100 volts for 1 hour, 200 volts for 1hour and 500 volts for 45minutes. If correct push start.

13.) Once dye front reaches close to the bottom of the gel, turn off the power supply and disconnect the cords from it.

14.) Take lid off gel running apparatus. Disassemble apparatus and remove the gel. Pour out the buffers from the apparatus and rinse it with distilled water.

15.) Rinse gel with distilled water as well and pat it dry. Trace the dye front and standard lines with a sharpie.

16.) Fill a Pyrex dish with distilled water. Crack open the gel using the gel knife. Push the foot out of the casing and float the gel off the casing into the water in the Pyrex, be careful not to rip the gel. Discard the casing with no markings. Date the case with the markings and save it for later use.

17.) Place the gel on a square plastic cutting board. Move the gel with flimsy plastic squares to help deter ripping. Then cut of the fingers and foot of the gel. Then place the gel back into the distilled water.

18.) Let it rinse in distilled water for five minutes.

19.) Fill the Pyrex dish labeled fix with fix solution and move the gel into this dish and cover the dish with plastic wrap. Place the dish on the shaker at a low setting. Allow the gel to shake in the fix for 45 minutes to an hour.

20.) Transfer the gel back into the rinse dish and allow the gel to rinse for five minutes.

21.) Obtain the Pyrex labeled stain and pour the coomassie blue G-250 stain that was made previously into dish (made need to be inverted a few times to mix it thoroughly).

22.) Transfer gel into stain cover with plastic wrap and place on shaker at a low setting. Let stain over night.

23.) When the lines are distinguishable on the IEF gel, place the gel in distilled water in the rinsing Pyrex dish. Allow the gel to rinse while the 2-D apparatuses are assembled.

24.) Obtain four Novex 4-20% Tris-Glycine Gels, 1.5mm, 2D well (Invitrogen, Carlsbad, California) from the refrigerator. Record the gel numbers in the notebook and assign each gel to a sample. Then rinse the gels with distilled water and pat them dry with a paper towel. Then trace the sample well with a sharpie and remove the comb and the tape.

25.) Now take the gel out of the rinsing Pyrex and place it onto a clear plastic square to be cut. Use a comb from a 2D gel to approximate how long the pieces should be. Cut the first to lanes of the gel out and place them into the sample well of the appropriate gel. When placing in put the dark end farthest to the standard well and make sure there are no bubbles under the strip of IEF gel. Repeat this for all four samples.

Solutions for 2D Run:

1.) Tris-Glycine Running Buffer: Three 500ml bottles should be made for one run of the 2D gels. Place 450mls of distilled water measured by a graduated cylinder into each of three 500ml bottles. Then measure 50mls of Tris Glycine SDS Running Buffer 10X (Invitrogen, Carlsbad, California) with a serological pipette and place it the bottle, do this for all three. Then invert the bottles several times to mix the solution. Place a label on the bottom with the name, maker's initials and date.

2.) 2D Stain: This can be maid from the contents of the Collodial Blue Staining Kit (Invitrogen, Carlsbad, California). Make two bottles for one run. Place 220mls of distilled water measured by a graduated cylinder in to each of the two bottles. Use serological pipettes to add in the remaining, 80mls Stainer A, 20mls Stainer B, 80mls methanol into the each of the bottles. Invert both bottles several times to mix. Place a label on the bottle with name, maker's initials and date.

2D Gel Run:

1.) Insert the 2D gels into the gel running apparatus. Place two in each, one in front with the wells facing the back and one in the back with the wells facing toward the front.

2.) Fill the inner chamber with SDS running buffer, and check the chamber for leaks. Uses a transfer pipette to remove all the bubbles from the top of the inner chamber. Next fill the outer chamber with SDS running buffer to the bottom of the sample well and remove the bubbles from the top with a transfer pipette.

3.) Take the SeeBlue Plus2 Standard (Invitrogen, Carlsbad, California) out of the refrigerator and pipette 10uls into the standard well of each gel.

4.) Place the tops on the running apparatus and insert plugs into the corresponding color on the Novex PowerEase 500 power source.

5.) Turn on the power source and choose the Tris glycine gel option and change the gel number to four. Then check the setting of 125 volts for an hour and 45 minutes. Push start.

6.) Check the gels periodically to make sure that the gel front does not run off the end of the gel.

7.) Once the dye front has reached near the bottom turn off the power supply. Disconnect the plugs and remove the lid from the gel running apparatus.

8.) Remove the gels from the running apparatus and rinse them with distilled water. When rinsing the gel remove the IEF sample and dispose of it. Dry the gel with a paper towel and outline the standard and dye front with a sharpie. Do this to all four gels

9.) Crack the gel casing open using a gel knife, and float gel off into a Pyrex of distilled water. Save the part of the gel casing that has the sharpie markings on it.

10.) Then take the gel out of the water and place on the clear plastic square and cut of the foot and fingers, and then it needs to be cut to identify which gel it is as follows. For the first gel the bottom right corner is cut off, 2 would have both bottom corners cut, 3 would have the two bottom corners cut off plus the top right corner, and 4 would have all the corners trimmed.

11.) Rinse the gel in the water for five minutes

12.) Fill four Novex staining dishes labeled 1-4 with 200ml of stain each. Place each gel in its appropriate staining dish. Next place the staining dishes on the shaker at the

setting of 5.5. Make sure that none of the gels are stuck to the bottom of the staining dishes.

13.) Let shake over night or over a weekend. New stain should only take over night, but the second use of a stain may take two nights. The gels were checked occasionally to make sure they were not sticking to the bottom of the dish.

14.) When the gels have stained sufficiently, separate the basket part of the staining dish from the bottom dish. Place the basket on bench paper. Pour the stain out of the dish and refill the dish with water. Put the basket back into the dish that is now filled with water. Repeat process for all four staining dishes.

15.) Repeat in 2 hrs if water is blue.

16.) When lanes standout and the background is fairly clear they are done distaining.

APPENDIX B

ImageQuant TL Procedure

Molecular Weight Analysis:

1. Open the ImageQuant TL program on the computer

2. Select the 1D analysis when the start menu comes up. Then choose the gel that needs to be analyzed from those that were previously scanned in.

3. The program will then display four different windows. The image window size may need to be adjusted to allow the gel to fit in the whole window space.

4. Then the stepwise option is chosen by click on the hand icon in the top left of the screen.

5. When the edit options come up, the manual edit is chosen and the edit mode of create lanes is selected. Then the parameters are set to: # of tiers=1, # of lanes=12, lane % width=100.

6. The lanes are created by clicking on the top left corner of the gel above the dots for the pI and drag across the gel until the molecular weight markers are included.

7. Now that the lanes are on the gel the edit mode is changed to edit single lanes and the 11^{th} lane is deleted because it usually contains no proteins. Then select bend and select the 12^{th} lane that includes the molecular weight markers. Click on the bottom of the lane and bend it till the markers are all in the middle of the lane. Then click on move and adjust any lanes so that the proteins are fairly centered in the lane.

8. Then the lanes are accepted by clicking on accept. Then click next.

9. Select the background as Rubber Band. Click next.

10. The next step is band detection using the window to the right of the image window which is the pixel window. Lane 1 is selected by clicking on the top of the lane. Then on the pixel window click on the top of every peak. Make sure to decipher between back ground noise and peaks. Then continue this process for all the remaining lanes. If you wish to remove a band right click on it.

11. After all the proteins are marked. Click next.

12. The standard SeeBlue Plus 2 is selected and scroll down to ensure the correctness of the values. Then click on lane 12 which contains the molecular weight marker bands.

Then click on compute and check the data window at the bottom of the screen to make sure that molecular weights are showing in the other lanes.

13. Select the curve type as Cubic Spline Curve.

14. Click next.

15. There is no calibration need so next is clicked when this option appears.

16. When the normalize option appears select the first band in lane 12 then click the normalize button. The normalize volume should be 200ug. Click next. This ends the molecular weight analysis.

17. To print the gel the image window is selected then goes to edit and click on export to file. The gel is then saved in the Frances stuff folder with a name that is different from the scanned image. The image is opened from the file in Adobe Photoshop (San Jose, California) and cropped to just include the gel. Then the image is printed. After a successful print, the image is deleted from the file because it will be saved in the ImageQuant TL program.

18. Return to the ImageQuant screen and the data window is selected. Then export to excel is selected from the edit menu. The excel spread sheet is saved to the file Frances stuff, and rearranged for easy printing.

19. The gel and analysis is automatically saved to the file where the gel was originally selected from.

pI Analysis:

1. The ImageQuant TL program is selected.

2. The 1D gel analysis is clicked on when the menu screen comes up. Then the scanned gel that needs to be analyzed is chosen.

3. Four windows appear and the image window which is the one that contains the image of the gel is resized if needed by clicking on the right hand bottom corner and dragging to the appropriate size.

4. Then the stepwise option is chosen by click on the hand icon in the top left of the screen.

5. When the edit options come up, the manual edit is chosen and the edit mode of create lanes is selected. Then the parameters are set to: # of tiers=1, # of lanes=11, lane % width=100.

6. The lanes are created manually in the same manor as the molecular weight lanes. Accept the 11^{th} lane contains the pI dots.

7. The edit mode is then changed to edit single lane and the 10^{th} lane is deleted because it normally does not contain proteins. Then the bend option is selected. The 11^{th} lane is bent by clicking on the bottom middle dot and dragged till all the pI dots are in the middle of the lane. The move option is selected and the lanes are adjusted so that most of the proteins fall in the middle of the lanes.

8. Once all the edits have been made accept is clicked.

9. Next is clicked.

10. Rubber Band is selected as the back ground method. Next is clicked.

11. The next step is to detect bands. Using the printout from the gels molecular weight analysis the bands are marked on the pI as close to how they are on the molecular weight sheet. Accept for lane 11 which has the pI dots. A band is placed on each dot.

12. Once satisfied with the bands next is clicked.

13. The IEF3-10 Standard (Invitrogen, Carlsbad California) is selected and the values for the standards are checked and those the nine dots do not include are unchecked. Then the 11^{th} lane is selected and compute is pressed. The data window is checked to make sure that the other lanes are showing pI values.

14. The curve type is selected as the Cubic Spline Curve.

15. Next is clicked.

16. Calibration is not needed so next is clicked.

17. Normalization is not needed so next is clicked again.

18. Now the pI analysis is complete. The gel is printed by clicking on the image window and export to file is selected from the edit menu. Save the file as a different name the scan into the Frances stuff file and open it in Adobe Photoshop (San Jose, California). Crop the picture so that it is just the gel and print. The Image can now be deleted from the file.

19. The ImageQuant program is maximized and the data window is selected then export to excel is selected from the edit menu. It is then saved to the Frances stuff folder and is rearranged for easy printing.

20. ImageQuant TL automatically saves the analyzed gel to the original file that the scan was selected from.

Analysis of Protein Identification:

1. Find the gel that has the most proteins when analyzed with ImageQuant TL

2. Use this gel as a template and assign a letter and a number to every protein on the gel (see picture below).

3. Using the molecular weights assigned by the computer program label each protein with its molecular weight.

4. Find the corresponding protein on the pI picture of the gel.

5. Label the proteins on the template gel with the corresponding pI obtained from the pI gel.

6. Use this template to identify the proteins on all the other gels. If a protein which is on the template does not appear on the gel being analyzed, put a 0 down for that protein.

7. Record molecular weights and pIs for all of the proteins from the ImageQuant TL software.

8. Then record the volume, volume + background and normalized volume for each protein from the molecular weight analysis.

9. To obtain total protein sum all the normalized volumes for each protein on the gel of interest.

APPENDIX C

Transforming Growth Factor Beta ELISA Validation:

In order to validate the TGFB kit for seminal plasma parallelism and percent recoveries were conducted. For parallelism an acid treated and non-acid treated sample from two different boars (110, 150) were used. For each of these four samples serial dilutions of 1:1, 1:2, 1:4, 1:8, and 1:16 were made and placed in duplicate on the plate. To test for percent recovery 2 boars (110 and 150) samples were spike to 500 pg/ml, 250pg/ml and 125pg/ml. This was done by first diluting the stock concentration of standard to the appropriate concentration. For the 500pg/ml spike 0.7uls of standard was diluted in 140uls of sample buffer. To make the 250pg/ml 200uls of sample buffer was used with 0.7uls of stock standard. Lastly to make the 125pg/ml spike 0.7uls of stock standard was added to 560uls of sample buffer. Once all the desired concentration was made then 10uls of the spike concentration was added to 90uls of each sample. The samples were then placed on the plate in duplicate.

Manufacture's guidelines were used to run the assay and it was then read at 450nm absorbance. Parallelism was computed by multiplying the concentration found by the dilution and then plotting it on a graph with dilution on the x-axis and concentration on the y-axis this is compared to the standard curve that has been manipulated in the same fashion as the sample serial dilutions.

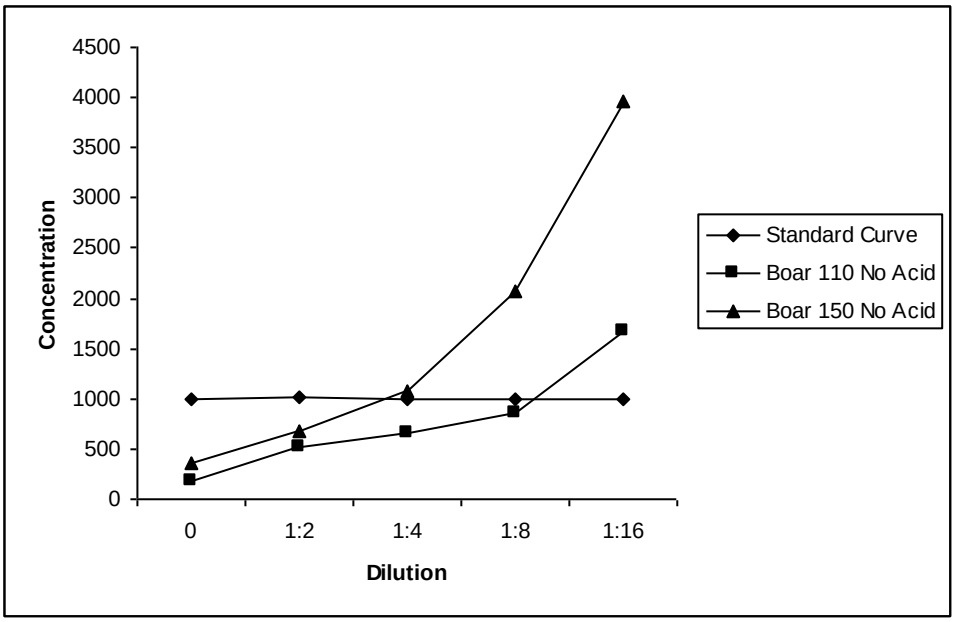

Figure 4. Transforming Growth Factor Beta Linear Dilution of Non-Acid Treated Samples

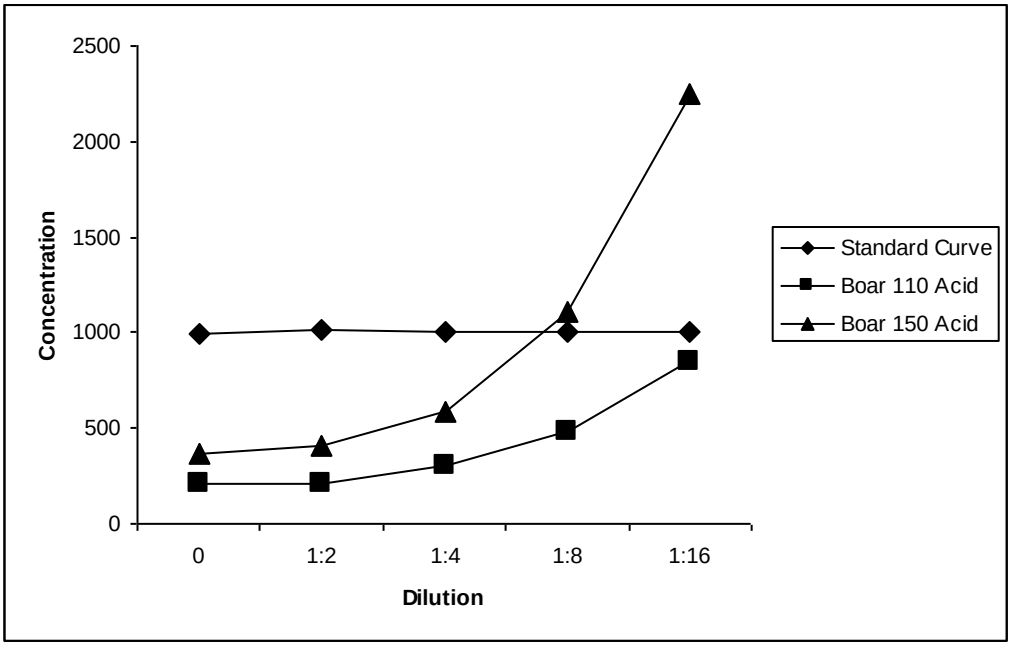

Figure 5. Transforming Growth Factor Beta Linear Dilution of Acid Treated Samples

The Percent Recoveries were determined by using the non dilute concentration of the sample and then determining expected values accounting for the spike. The actual concentration is divided by the spike. That number is multiplied by 100 to give the percent.

Table 22. Percent Recovery Calculations for Transforming Growth Factor Beta

Boar 110 Spike	Actual Concentration(acid)		average	expected	%recovery
ND	173.9	173.5	173.7		
500	206	164.4	185.2	673.7	27.48998
250	151.9	122.3	137.1	423.7	32.3578
125	127.7	115	121.35	298.7	40.62605

Boar 150 Spike	Actual Conc(acid)		avg	expected	%recovery
ND			358.5		
500	400.6	334.1	367.35	858.5	42.78975
250	367.9	350.6	359.25	608.5	59.03862
125	371.9	296	333.95	483.5	69.06929

Boar 110 Spike	Actual Conc(no acid)		avg	expected	%recovery
ND	191.2	211.4	201.3		
500	165.2	197.1	181.15	701.3	25.8306
250	163.9	182.5	173.2	451.3	38.37802
125	137.6	173.2	155.4	326.3	47.62489

Boar 150 Spike	Actual Conc(no acid)		avg	expected	%recovery
ND			367.5		
500	387.2	327	357.1	867.5	41.16427
250	308	295.6	301.8	617.5	48.87449
125	288.5	248.7	268.6	492.5	54.53807

Based on the acid treated sample showing better percent recovery and appearing more parallel; acid treated samples were used to perform the TGFB ELISA. The samples were run in triplicate on two separate plates. The intra-assay coefficient was found to 11.6 and the inter-assay coefficient was 8.9.

APPENDIX D

Tumor Necrosis Factor Alpha ELISA Validation:

In order to validate the porcine tumor necrosis factor alpha kit for seminal plasma parallelism and percent recoveries were conducted. For parallelism a sample from three different boars (110, 150, 115) were used. For each of these three samples serial dilutions of 1:1, 1:2, 1:4, 1:8, and 1:16 were made and placed in duplicate on the plate. To test for percent recovery each of the three samples were spike to 1000 pg/ml, 250pg/ml and 62.5pg/ml. This was done by first diluting the standard stock solution which has 4000pg/ml in 678ul to the appropriate concentration. For the 1000pg/ml spike 12.5uls of standard was diluted in 87.5ul of sample. To make the 250pg/ml 96.9uls of sample buffer was used with 3.1uls of stock standard. Lastly to make the 62.5pg/ml spike 0.8uls of stock standard was added to 99.2uls of sample buffer. The samples were then placed on the plate in duplicate.

Manufacture's guidelines were used to run the assay and it was then read at 450nm absorbance. Parallelism was computed by multiplying the concentration found by the dilution and then plotting it on a graph with dilution on the x-axis and concentration on the y-axis this is compared to the standard curve that has been manipulated in the same fashion as the sample serial dilutions.

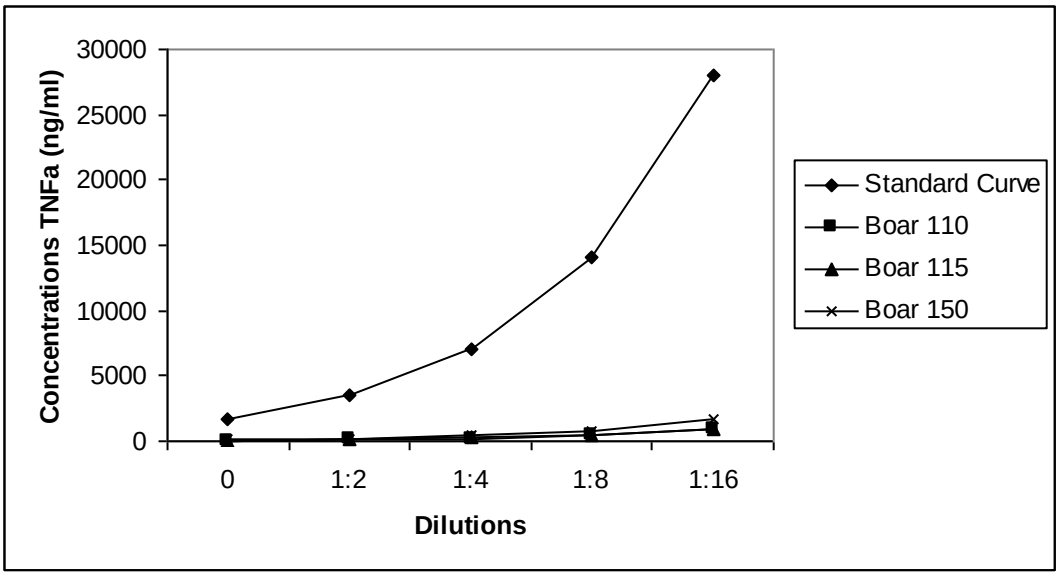

Figure 6. Linear Dilution for Tumor Necrosis Factor Alpha

The Percent Recoveries were determined by using the non dilute concentration of the sample and then determining expected values accounting for the spike. The actual concentration is divided by the spike. That number is multiplied by 100 to give the percent.

Table 23. Percent Recovery Calculations for Tumor Necrosis Factor Alpha

Boar 110 Spike	Actual Conc		avg	expected	%recovery
ND	59.07	52.9	55.985	55.985	
1000	91.7	111.8	101.75	1055.985	9.635554
250	71.62	51.53	61.575	305.985	20.12354
62.5	31.3	31.3	31.3	118.485	26.41685

Boar 150 Spike	Actual Conc		avg	expected	%recovery
ND	141.7	62.5	102.1	102.1	100
1000	176.1	188.3	182.2	1102.1	16.53208
250	90.79	74.36	82.575	352.1	23.45214
62.5	52.6	36.44	44.52	164.6	27.04739

Boar 115 Spike	Actual Conc		avg	expected	%recovery
ND	59.07	61.13	60.1	60.1	100
1000	119.1	148.3	133.7	1060.1	12.61202
250	52.9	58.39	55.645	310.1	17.94421
62.5	31.3	31.3	31.3	122.6	25.53018

The samples were run in triplicate on two separate plates. The intra-assay coefficient was found to 10.45 and the inter-assay coefficient was 9.84.

APPENDIX E

Heat Shock Protein 70 Validation:

In order to validate the heat shock protein 70 kit for seminal plasma parallelism and percent recoveries were conducted. For parallelism a sample from three a boar (4291) was used. For this sample serial dilutions of 1:1, 1:2, 1:4, 1:8, and 1:16 were made and placed in duplicate on the plate. To test for percent recovery a boar's (158) sample was spiked to 2 pg/ml, 5pg/ml, 10pg/ml and 20pg/ml. The samples were then placed on the plate in duplicate.

Manufacture's guidelines were used to run the assay and it was then read at 450nm absorbance. Parallelism was computed by multiplying the concentration found by the dilution and then plotting it on a graph with dilution on the x-axis and concentration on the y-axis this is compared to the standard curve that has been manipulated in the same fashion as the sample serial dilutions.

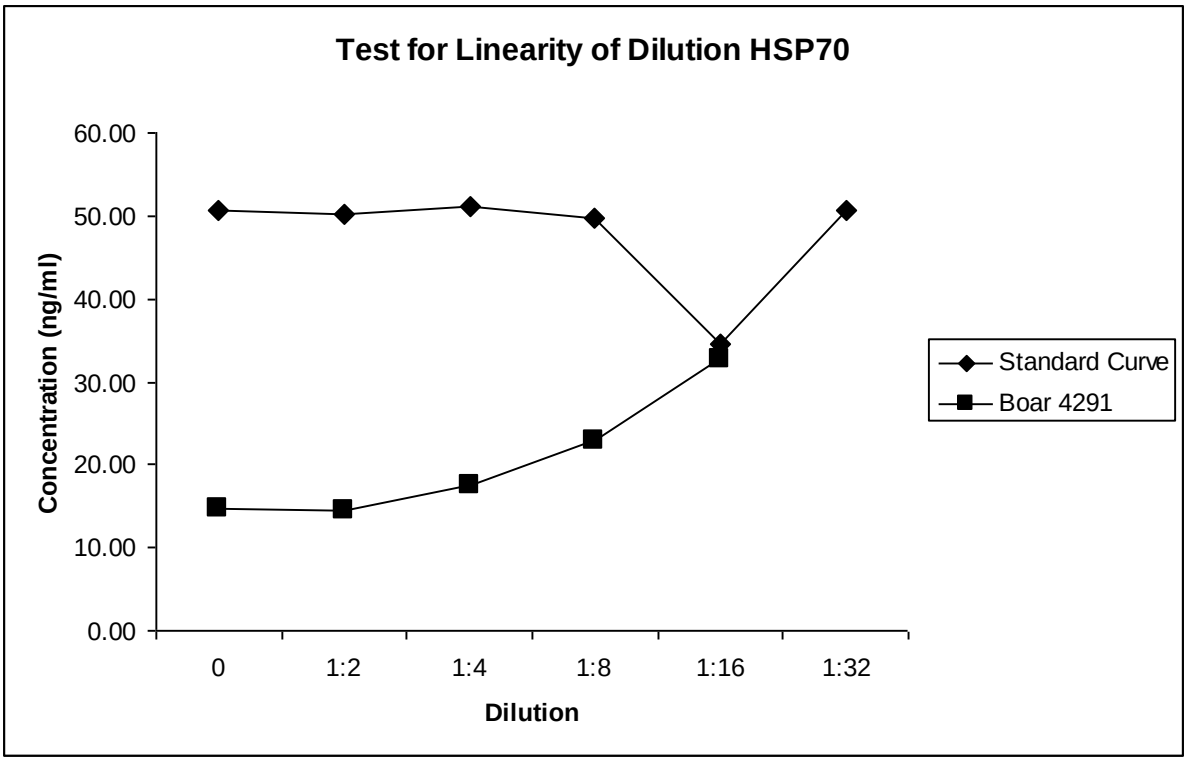

Figure 7. Linear Dilution for the Validation of Heat Shock Protein 70.

The Percent Recoveries were determined by using the non dilute concentration of the sample and then determining expected values accounting for the spike. The actual concentration is divided by the spike. That number is multiplied by 100 to give the percent.

Table 24. Percent Recoveries for the Validation of Heat Shock Protein 70.

Spike	**Boar 158**		
sample	**Expected**	**Observed**	**% Recovery**
2	2.78	0.98	35.2518
5	5.78	3.527	61.02076
10	10.78	6.573	60.97403
20	20.78	11.351	54.62464

The samples were run in triplicate on two separate plates. The intra-assay coefficient was found to 5.73 and the inter-assay coefficient was 0.34.

APPENDIX F

SAS programming used to obtain Semen Quality Means:

Log Statement:

```
74   dm 'output; clear; log; clear';
75   data SQT;
76   filename SEM 'K:\Thesis work\SEMEN QUALITY DATAM.prn';
77   infile SEM missover;
78   input BOAR 4-9  @12 DATE MMDDYY8. TYPE $ 23-27 MOTILITY 32-36
PROGMOT 40-44 DCL 48-52 DSL
78 ! 55-60 VCL 63-68
79   VSL 72-76 UNCAP 81-85 CAP 87-92 ARX 96-100 ABN 104-108 AACTIVITY 112-
116 NONACT 121-124
79 ! ACROMORPH 128-131;
80   *proc print;
81

NOTE: The infile SEM is:
      File Name=K:\Thesis work\SEMEN QUALITY DATAM.prn,
      RECFM=V,LRECL=256

NOTE: 113 records were read from the infile SEM.
      The minimum record length was 130.
      The maximum record length was 131.
NOTE: The data set WORK.SQT has 113 observations and 16 variables.
NOTE: DATA statement used (Total process time):
      real time          0.00 seconds
      cpu time           0.01 seconds


82   proc sort; by BOAR;

NOTE: There were 113 observations read from the data set WORK.SQT.
NOTE: The data set WORK.SQT has 113 observations and 16 variables.
NOTE: PROCEDURE SORT used (Total process time):
      real time          0.00 seconds
      cpu time           0.01 seconds


83   proc means data=SQT n mean maxdec=2; by BOAR;
84   var MOTILITY PROGMOT DCL DSL VCL VSL UNCAP CAP ARX ABN
AACTIVITY NONACT ACROMORPH;
85   run;
```

```
NOTE: There were 113 observations read from the data set WORK.SQT.

NOTE: PROCEDURE MEANS used (Total process time):
      real time          0.04 seconds
      cpu time           0.01 seconds


86
87   quit;
```

SAS programming used to obtain Seminal Plasma Protein Means:

Log Statement:

```
95   dm 'output; clear; log; clear';
96   proc means data=prmeans n min max mean var MAXDEC=2; by BOAR;
97   var HSP TGB TNF A1  A2  A3  A4  A5  A6  A7  A8  A9  A10 A11 B1  B2
98   B3  B4  C1  C2  C3  C4  C5  C6  C7  C8  D1  D2  D3  D4  D5  D6  D7
99   E1  E2  E3  E4  E5  E6  E7  E8  F1  F2  F3  F4  F5  F6  F7  G1  G2
100  G3  H1  H2  H3  H4  H5  I1  I2  I3  I4  I5  I6  I7;
101  run;

NOTE: There were 28 observations read from the data set WORK.PRMEANS.
NOTE: PROCEDURE MEANS used (Total process time):
      real time          0.14 seconds
      cpu time           0.11 seconds


102  proc sort; by BOAR;
103  run;

NOTE: Input data set is already sorted, no sorting done.
NOTE: PROCEDURE SORT used (Total process time):
      real time          0.01 seconds
      cpu time           0.00 seconds


104  quit;
```

SAS programming used to obtain Fertility Means:

Log Statement:

```
297  dm 'output; clear; log; clear';
298  data FDATA;
299  filename PAT 'K:\Thesis work\FERTILITYM.prn';
300  infile PAT missover;
301  input BOAR 4-9  DATE  16 TYPE 23 OPPONENT  28-32 SIRED 39-40 TOTAL 47-48
PERCENT 53-57 WINS
301!  65 LOSSES 73 TIES 81;

302  *proc print;

NOTE: The infile PAT is:
      File Name=K:\Thesis work\FERTILITYM.prn,
      RECFM=V,LRECL=256

NOTE: 60 records were read from the infile PAT.
      The minimum record length was 56.
      The maximum record length was 81.
NOTE: The data set WORK.FDATA has 60 observations and 10 variables.
NOTE: DATA statement used (Total process time):
      real time          0.01 seconds
      cpu time           0.02 seconds


303  proc sort; by boar;

NOTE: There were 60 observations read from the data set WORK.FDATA.
NOTE: The data set WORK.FDATA has 60 observations and 10 variables.
NOTE: PROCEDURE SORT used (Total process time):
      real time          0.00 seconds
      cpu time           0.01 seconds


304  proc means n mean maxdec=2; by boar;
305  var PERCENT;
306  RUN;
```

SAS programming used for Proc GLM Fertility Groups Percent Sired:

Log Statement:

```
1    data MEANS; set means;
2    /*if boar=09006 then FGROUP=1;
```

```
3    if boar=37904 then FGROUP=1;
4    if boar=37911 then FGROUP=1;
5    if boar=08509 then FGROUP=2;
6    if boar=08906 then FGROUP=2;
7    if boar=08910 then FGROUP=2;
8    if boar=09609 then FGROUP=2;
9    if boar=31209 then FGROUP=2;
10   if boar=37209 then FGROUP=2;
11   if boar=08511 then FGROUP=3;
12   if boar=37208 then FGROUP=3;
13   if Test=SAMPLE then delete;*/

NOTE: There were 24 observations read from the data set WORK.MEANS.
NOTE: The data set WORK.MEANS has 24 observations and 81 variables.
NOTE: DATA statement used (Total process time):
      real time          0.22 seconds
      cpu time           0.02 seconds


15   proc glm data=MEANS;
16   classes TRT;
17   model ProgMot DCL DSL VCL VSL UnCap Cap ARX ABN Aactivity   NonAct
18   AcroMorph HSP TGB TNF A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 B1 B2 B3 B4 C1
C2  C3  C4 C5
19   C6 C7 C8 D1 D2  D3  D4  D5  D6  D7 E1 E2 E3 E4 E5 E6 E7 E8 F1 F2 F3 F4 F5 F6 F7
G1 G2
20   G3 H1 H2 H3 H4  H5  I1  I2  I3  I4  I5  I6  I7=TRT;
21   run;

22   quit;

NOTE: PROCEDURE GLM used (Total process time):
      real time          0.86 seconds
      cpu time           0.20 seconds
```

SAS programming used for Proc GLM Fertility groups Percent Wins/ Matches:

Log Statement:

```
177  data MEANS; set means;
178  /*if boar=09006 then FGROUP=1;
179  if boar=37904 then FGROUP=1;
180  if boar=37911 then FGROUP=1;
```

```
181  if boar=08509 then FGROUP=2;
182  if boar=08906 then FGROUP=2;
183  if boar=08910 then FGROUP=2;
184  if boar=09609 then FGROUP=2;
185  if boar=31209 then FGROUP=2;
186  if boar=37209 then FGROUP=2;
187  if boar=08511 then FGROUP=3;
188  if boar=37208 then FGROUP=3;
189  if boar=37605 then FGROUP=3;
190  if Test=SAMPLE then delete;*/

NOTE: There were 24 observations read from the data set WORK.MEANS.
NOTE: The data set WORK.MEANS has 24 observations and 81 variables.
NOTE: DATA statement used (Total process time):
      real time          0.00 seconds

cpu time          0.01 seconds


191  proc glm data=MEANS;
192  classes TRT;
193  model  Motilty ProgMot DCL DSL VCL VSL UnCap Cap ARX ABN Aactivity
NonAct
194  AcroMorph HSP TGB TNF A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 B1 B2 B3 B4 C1
C2  C3  C4 C5
195  C6 C7 C8 D1 D2  D3  D4  D5  D6  D7 E1 E2 E3 E4 E5 E6 E7 E8 F1 F2 F3 F4 F5 F6
F7 G1 G2
196  G3 H1 H2 H3 H4  H5  I1  I2  I3  I4  I5  I6  I7=TRT;
197  run;

198  quit;

NOTE: PROCEDURE GLM used (Total process time):
      real time          1.63 seconds
      cpu time           1.54 seconds
```

SAS programming used for Proc GLM/SNK ALPHA=0.05 Percent Wins:

Log Statement:

```
69   data MEANST; set meansT;
70   /*if boar=09006 then FGROUP=1;
71   if boar=37904 then FGROUP=1;
```

```
72   if boar=37911 then FGROUP=1;
73   if boar=08509 then FGROUP=2;
74   if boar=08906 then FGROUP=2;
75   if boar=08910 then FGROUP=2;
76   if boar=09609 then FGROUP=2;
77   if boar=31209 then FGROUP=2;
78   if boar=37209 then FGROUP=2;
79   if boar=08511 then FGROUP=3;
80   if boar=37208 then FGROUP=3;
81   if boar=37605 then FGROUP=3;
82   if Test=SAMPLE then delete;*/

NOTE: There were 24 observations read from the data set WORK.MEANST.
NOTE: The data set WORK.MEANST has 24 observations and 81 variables.
NOTE: DATA statement used (Total process time):
      real time          0.00 seconds
      cpu time           0.01 seconds


83   proc glm data=MEANST;
84   classes TRT;
85   model DSL VSL ARX AcroMorph HSP A1 A6 A11 B1 B2 C6 E7 G2 I1=TRT;
86   means TRT/SNK;
87   run;

88   quit;

NOTE: PROCEDURE GLM used (Total process time):
      real time          0.39 seconds
      cpu time           0.28 seconds
```

SAS programming used for Proc GLM/SNK ALPHA=0.075 Percent Sired:

Log Statement:

```
109  data MEANST; set meansT;
110  /*if boar=09006 then FGROUP=1;
111  if boar=37904 then FGROUP=1;
112  if boar=37911 then FGROUP=1;
113  if boar=08509 then FGROUP=2;
114  if boar=08906 then FGROUP=2;
115  if boar=08910 then FGROUP=2;
116  if boar=09609 then FGROUP=2;
117  if boar=31209 then FGROUP=2;
```

```
118  if boar=37209 then FGROUP=2;
119  if boar=08511 then FGROUP=3;
120  if boar=37208 then FGROUP=3;
121  if boar=37605 then FGROUP=3;
122  if Test=SAMPLE then delete;*/

NOTE: There were 24 observations read from the data set WORK.MEANST.
NOTE: The data set WORK.MEANST has 24 observations and 81 variables.
NOTE: DATA statement used (Total process time):
      real time          0.01 seconds
      cpu time           0.01 seconds


123  proc glm data=MEANST;
124  classes TRT;
125  model MOTILTY PROGMOT A2 C7 E5 I7=TRT;
126  means TRT/SNK ALPHA=.075;
127  run;

128  quit;


NOTE: PROCEDURE GLM used (Total process time):
      real time          0.23 seconds
      cpu time           0.16 seconds
```

SAS programming used for Proc GLAM/SNK ALPHA=0.10 Percent Sired:

Log Statement:

```
129  data MEANST; set meansT;
130  /*if boar=09006 then FGROUP=1;
131  if boar=37904 then FGROUP=1;
132  if boar=37911 then FGROUP=1;
133  if boar=08509 then FGROUP=2;
134  if boar=08906 then FGROUP=2;
135  if boar=08910 then FGROUP=2;
136  if boar=09609 then FGROUP=2;
137  if boar=31209 then FGROUP=2;
138  if boar=37209 then FGROUP=2;
139  if boar=08511 then FGROUP=3;
140  if boar=37208 then FGROUP=3;
141  if boar=37605 then FGROUP=3;
142  if Test=SAMPLE then delete;*/
```

```
NOTE: There were 24 observations read from the data set WORK.MEANST.
NOTE: The data set WORK.MEANST has 24 observations and 81 variables.
NOTE: DATA statement used (Total process time):
      real time          0.00 seconds
      cpu time           0.01 seconds


143  proc glm data=MEANST;
144  classes TRT;
145  model D5 H1=TRT;
146  means TRT/SNK ALPHA=.1;
147  run;

148  quit;

NOTE: PROCEDURE GLM used (Total process time):
      real time          0.13 seconds
      cpu time           0.06 seconds
```

SAS programming used for Proc GLM/SNK ALPHA=0.05 Percent Wins/Total:

Log Statement:

```
490  data MEANSW; set meansW;
491  /*if boar=09006 then FGROUP=1;

492  if boar=37904 then FGROUP=1;
493  if boar=37911 then FGROUP=1;
494  if boar=08509 then FGROUP=2;
495  if boar=08906 then FGROUP=2;
496  if boar=08910 then FGROUP=2;
497  if boar=09609 then FGROUP=2;
498  if boar=31209 then FGROUP=2;
499  if boar=37209 then FGROUP=2;
500  if boar=08511 then FGROUP=3;
501  if boar=37208 then FGROUP=3;
502  if boar=37605 then FGROUP=3;
503  if Test=SAMPLE then delete;*/

NOTE: There were 24 observations read from the data set WORK.MEANSW.
NOTE: The data set WORK.MEANSW has 24 observations and 81 variables.
NOTE: DATA statement used (Total process time):
      real time          1.64 seconds
```

```
      cpu time          0.00 seconds


504  proc glm data=MEANSW;
505  classes TRT;
506  model MOTILTY ARX HSP A1 A6 B2 C6 E3 F5 F6 H4 I1 I2 I3 I4=TRT;
507  means TRT/SNK;
508  /*proc reg;
509  model PATERNT=MOTILTY PROGMOT DSL VSL ARX ACROMORPH HSP A1
A2 A6 A11 B1 B2 C6 C7 D5
510  E5 E7 G2 H1 I1 I7/SELECTION=STEPWISE;
511  run;
512  quit;
513
514  proc reg;
515  model PATERNT=MOTILTY PROGMOT DSL VSL ARX ACROMORPH HSP A1
A2 A6 A11 B1 B2 C6 C7 D5
516  E5 E7 G2 H1 I1 I7/SELECTION=MAXR;*/
517  /*PROC SORT; BY TRT;
518  PROC MEANS DATA=MEANST N MEAN STDERR MAX MIN MAXDEC=2;BY
TRT;
519  VAR motilty ProgMot DCL DSL VCL VSL UnCap Cap ARX ABN Aactivity NonAct
520  AcroMorph HSP TGB TNF A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 B1 B2 B3 B4 C1
C2  C3  C4 C5
521  C6 C7 C8 D1 D2  D3  D4  D5  D6  D7 E1 E2 E3 E4 E5 E6 E7 E8 F1 F2 F3 F4 F5 F6
F7 G1 G2
522  G3 H1 H2 H3 H4  H5  I1  I2  I3  I4  I5  I6  I7;*/

523  run;

524  quit;

NOTE: PROCEDURE GLM used (Total process time):
      real time          7.99 seconds
      cpu time           0.15 seconds
```

SAS programming used for Proc GLM/SNK ALPHA=0.075 Percent Wins/Total:

Log Statement:

```
315  data MEANSW; set meansW;
316  /*if boar=09006 then FGROUP=1;
317  if boar=37904 then FGROUP=1;
318  if boar=37911 then FGROUP=1;
```

```
319  if boar=08509 then FGROUP=2;
320  if boar=08906 then FGROUP=2;
321  if boar=08910 then FGROUP=2;
322  if boar=09609 then FGROUP=2;
323  if boar=31209 then FGROUP=2;
324  if boar=37209 then FGROUP=2;
325  if boar=08511 then FGROUP=3;
326  if boar=37208 then FGROUP=3;
327  if boar=37605 then FGROUP=3;
328  if Test=SAMPLE then delete;*/

NOTE: There were 24 observations read from the data set WORK.MEANSW.
NOTE: The data set WORK.MEANSW has 24 observations and 81 variables.
NOTE: DATA statement used (Total process time):
      real time          1.64 seconds
      cpu time           0.00 seconds


329  proc glm data=MEANSW;
330  classes TRT;
331  model PROGMOT CAP ABN ACROMORPH A2 B1 C7 E1 E8 H1 H3 I7=TRT;
332  means TRT/SNK ALPHA=.075;
333  /*proc reg;
334  model PATERNT=MOTILTY PROGMOT DSL VSL ARX ACROMORPH HSP A1
A2 A6 A11 B1 B2 C6 C7 D5
335  E5 E7 G2 H1 I1 I7/SELECTION=STEPWISE;
336  run;
337  quit;
338
339  proc reg;
340  model PATERNT=MOTILTY PROGMOT DSL VSL ARX ACROMORPH HSP A1
A2 A6 A11 B1 B2 C6 C7 D5
341  E5 E7 G2 H1 I1 I7/SELECTION=MAXR;*/
342  /*PROC SORT; BY TRT;
343  PROC MEANS DATA=MEANST N MEAN STDERR MAX MIN MAXDEC=2;BY
TRT;
344  VAR motilty ProgMot DCL DSL VCL VSL UnCap Cap ARX ABN Aactivity NonAct
345  AcroMorph HSP TGB TNF A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 B1 B2 B3 B4 C1
C2  C3  C4 C5
346  C6 C7 C8 D1 D2  D3  D4  D5  D6  D7 E1 E2 E3 E4 E5 E6 E7 E8 F1 F2 F3 F4 F5 F6
F7 G1 G2
347  G3 H1 H2 H3 H4  H5  I1  I2  I3  I4  I5  I6  I7;*/
348  run;
349  quit;
```

NOTE: PROCEDURE GLM used (Total process time):
real time 7.84 seconds
cpu time 0.12 seconds

SAS programming used for Proc GLM/SNK ALPHA=0.10 Percent Wins/Total:

Log Statement:

```
420  data MEANSW; set meansW;
421  /*if boar=09006 then FGROUP=1;
422  if boar=37904 then FGROUP=1;
423  if boar=37911 then FGROUP=1;
424  if boar=08509 then FGROUP=2;
425  if boar=08906 then FGROUP=2;
426  if boar=08910 then FGROUP=2;
427  if boar=09609 then FGROUP=2;
428  if boar=31209 then FGROUP=2;
429  if boar=37209 then FGROUP=2;
430  if boar=08511 then FGROUP=3;
431  if boar=37208 then FGROUP=3;
432  if boar=37605 then FGROUP=3;
433  if Test=SAMPLE then delete;*/
```

NOTE: There were 24 observations read from the data set WORK.MEANSW.
NOTE: The data set WORK.MEANSW has 24 observations and 81 variables.
NOTE: DATA statement used (Total process time):
real time 1.62 seconds
cpu time 0.00 seconds

```
434  proc glm data=MEANSW;
435  classes TRT;
436  model A11 C3 E7 I5=TRT;
437  means TRT/SNK ALPHA=.1;
438  /*proc reg;
439  model PATERNT=MOTILTY PROGMOT DSL VSL ARX ACROMORPH HSP A1
A2 A6 A11 B1 B2 C6 C7 D5
440  E5 E7 G2 H1 I1 I7/SELECTION=STEPWISE;
441  run;
442  quit;
443
444  proc reg;
445  model PATERNT=MOTILTY PROGMOT DSL VSL ARX ACROMORPH HSP A1
```

```
A2 A6 A11 B1 B2 C6 C7 D5
446  E5 E7 G2 H1 I1 I7/SELECTION=MAXR;*/
447  /*PROC SORT; BY TRT;
448  PROC MEANS DATA=MEANST N MEAN STDERR MAX MIN MAXDEC=2;BY
TRT;
449  VAR motilty ProgMot DCL DSL VCL VSL UnCap Cap ARX ABN Aactivity NonAct
450  AcroMorph HSP TGB TNF A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 B1 B2 B3 B4 C1
C2  C3  C4 C5
451  C6 C7 C8 D1 D2  D3  D4  D5  D6  D7 E1 E2 E3 E4 E5 E6 E7 E8 F1 F2 F3 F4 F5 F6
F7 G1 G2
452  G3 H1 H2 H3 H4  H5  I1  I2  I3  I4  I5  I6  I7;*/
453  run;

454  quit;

NOTE: PROCEDURE GLM used (Total process time):
      real time           6.99 seconds
      cpu time            0.06 seconds
```

SAS programming used for Multiple Regression:

Log Statement:

```
234  data MEANST; set meansT;
235  /*if boar=09006 then FGROUP=1;
236  if boar=37904 then FGROUP=1;
237  if boar=37911 then FGROUP=1;
238  if boar=08509 then FGROUP=2;
239  if boar=08906 then FGROUP=2;
240  if boar=08910 then FGROUP=2;
241  if boar=09609 then FGROUP=2;
242  if boar=31209 then FGROUP=2;
243  if boar=37209 then FGROUP=2;

244  if boar=08511 then FGROUP=3;
245  if boar=37208 then FGROUP=3;
246  if boar=37605 then FGROUP=3;
247  if Test=SAMPLE then delete;*/
248  /*proc glm data=MEANST;
249  classes TRT;
250  model D5 H1=TRT;
251  means TRT/SNK ALPHA=.1;*/

NOTE: There were 24 observations read from the data set WORK.MEANST.
```

```
NOTE: The data set WORK.MEANST has 24 observations and 81 variables.
NOTE: DATA statement used (Total process time):
      real time          0.01 seconds
      cpu time           0.02 seconds


252  proc reg;
253  model PATERNT=MOTILTY PROGMOT DSL VSL ARX ACROMORPH HSP A1
A2 A6 A11 B1 B2 C6 C7 D5
254  E5 E7 G2 H1 I1 I7/SELECTION=STEPWISE;
255  run;

256  quit;

NOTE: PROCEDURE REG used (Total process time):
      real time          0.10 seconds
      cpu time           0.04 seconds


257
258  proc reg;
259  model PATERNT=MOTILTY PROGMOT DSL VSL ARX ACROMORPH HSP A1
A2 A6 A11 B1 B2 C6 C7 D5
260  E5 E7 G2 H1 I1 I7/SELECTION=MAXR;
261  run;

262  quit;

NOTE: PROCEDURE REG used (Total process time):
      real time          0.14 seconds
      cpu time           0.06 seconds
```

SAS programming used for Proc GLM with out Outliers:

Log Statement:

```
24   data MEANS; set means;
25   /*if boar=09006 then FGROUP=1;
26   if boar=37904 then FGROUP=1;
27   if boar=37911 then FGROUP=1;
28   if boar=08509 then FGROUP=2;
29   if boar=08906 then FGROUP=2;
30   if boar=08910 then FGROUP=2;
```

```
31   if boar=09609 then FGROUP=2;
32   if boar=31209 then FGROUP=2;
33   if boar=37209 then FGROUP=2;
34   if boar=08511 then FGROUP=3;
35   if boar=37208 then FGROUP=3;
36   if boar=37605 then FGROUP=3;
37   if Test=SAMPLE then delete;*/
38   if motilty<60 then delete;

NOTE: There were 24 observations read from the data set WORK.MEANS.
NOTE: The data set WORK.MEANS has 22 observations and 81 variables.
NOTE: DATA statement used (Total process time):
      real time          0.05 seconds
      cpu time           0.01 seconds


39   proc glm data=MEANS;
40   classes TRT;
41   model motilty ProgMot DCL DSL VCL VSL UnCap Cap ARX ABN Aactivity NonAct
42   AcroMorph HSP TGB TNF A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 B1 B2 B3 B4 C1
C2  C3  C4 C5
43   C6 C7 C8 D1 D2  D3  D4  D5  D6  D7 E1 E2 E3 E4 E5 E6 E7 E8 F1 F2 F3 F4 F5 F6 F7
G1 G2
44   G3 H1 H2 H3 H4  H5  I1  I2  I3  I4  I5  I6  I7=TRT;
45   run;

46   quit;

NOTE: PROCEDURE GLM used (Total process time):
      real time          0.27 seconds
      cpu time           0.20 seconds
```

SAS programming used for Multiple Regression with out Outliers:

Log Statement:

```
180  data MEANS; set means;
181  /*if boar=09006 then FGROUP=1;
182  if boar=37904 then FGROUP=1;

183  if boar=37911 then FGROUP=1;
184  if boar=08509 then FGROUP=2;
185  if boar=08906 then FGROUP=2;
186  if boar=08910 then FGROUP=2;
```

```
187  if boar=09609 then FGROUP=2;
188  if boar=31209 then FGROUP=2;
189  if boar=37209 then FGROUP=2;
190  if boar=08511 then FGROUP=3;
191  if boar=37208 then FGROUP=3;
192  if boar=37605 then FGROUP=3;
193  if Test=SAMPLE then delete;*/
194  if motilty<60 then delete;
195  /*proc glm data=MEANS;
196  classes TRT;*/
197  */model motilty ProgMot DCL DSL VCL VSL UnCap Cap ARX ABN Aactivity
NonAct
198  AcroMorph HSP TGB TNF A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 B1 B2 B3 B4 C1
C2  C3  C4 C5
199  C6 C7 C8 D1 D2  D3  D4  D5  D6  D7 E1 E2 E3 E4 E5 E6 E7 E8 F1 F2 F3 F4 F5 F6
F7 G1 G2
200  G3 H1 H2 H3 H4  H5  I1  I2  I3  I4  I5  I6  I7=TRT*/;

NOTE: There were 22 observations read from the data set WORK.MEANS.
NOTE: The data set WORK.MEANS has 22 observations and 81 variables.
NOTE: DATA statement used (Total process time):
      real time          0.00 seconds
      cpu time           0.01 seconds


201  PROC REG;
202  model PATERNT=MOTILTY PROGMOT ARX ACROMORPH
203  DSL VSL HSP A6 A11 B2 B4 C1 C3 C7 D2 D3 D5 E5 E7 F2 G2 I5 I6
I7/SELECTION=STEPWISE;
204
205  run;

206  quit;

NOTE: PROCEDURE REG used (Total process time):
      real time          0.15 seconds
      cpu time           0.04 seconds


207  data MEANS; set means;

208  /*if boar=09006 then FGROUP=1;
209  if boar=37904 then FGROUP=1;
210  if boar=37911 then FGROUP=1;
```

```
211  if boar=08509 then FGROUP=2;
212  if boar=08906 then FGROUP=2;
213  if boar=08910 then FGROUP=2;
214  if boar=09609 then FGROUP=2;
215  if boar=31209 then FGROUP=2;
216  if boar=37209 then FGROUP=2;
217  if boar=08511 then FGROUP=3;
218  if boar=37208 then FGROUP=3;
219  if boar=37605 then FGROUP=3;
220  if Test=SAMPLE then delete;*/
221  if motilty<60 then delete;
222  /*proc glm data=MEANS;
223  classes TRT;*/
224  */model motilty ProgMot DCL DSL VCL VSL UnCap Cap ARX ABN Aactivity
NonAct
225  AcroMorph HSP TGB TNF A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 B1 B2 B3 B4 C1
C2  C3  C4 C5
226  C6 C7 C8 D1 D2  D3  D4  D5  D6  D7 E1 E2 E3 E4 E5 E6 E7 E8 F1 F2 F3 F4 F5 F6
F7 G1 G2
227  G3 H1 H2 H3 H4  H5  I1  I2  I3  I4  I5  I6  I7=TRT*/;

NOTE: There were 22 observations read from the data set WORK.MEANS.
NOTE: The data set WORK.MEANS has 22 observations and 81 variables.
NOTE: DATA statement used (Total process time):
      real time          0.00 seconds
      cpu time           0.01 seconds


228  PROC REG;
229  model PATERNT=MOTILTY PROGMOT ARX ACROMORPH
230  DSL VSL HSP A6 A11 B2 B4 C1 C3 C7 D2 D3 D5 E5 E7 F2 G2 I5 I6
I7/SELECTION=MAXR;
231
232  run;

233  quit;

NOTE: PROCEDURE REG used (Total process time):
      real time          0.14 seconds
      cpu time           0.06 seconds
```