

ABSTRACT

CROWELL, SARA SHUTE. Evaluating Temperature Effects and Extension Cooling Rates on Boar Semen Quality. (Under the Direction of Dr. William L. Flowers.)

Experiment one examined the effects of temperature variations between semen and extender on eleven different estimates of semen quality. Extender type, short or long term, and time interactions on the quality of semen were analyzed. Eight mature boars (n=8) were collected twice and extended with either Beltsville Thawing Solution (BTS) or Androhep Plus. Nine variations on temperature between semen and extender resulting from the combinations between 35°C, 37°C or 39°C were examined. Analyses were performed immediately after extension and at 24, 48 and 96 h after collection. All data was analyzed using SAS and the proc GLM procedure.

Analysis showed that although a four degree Celsius difference occurred between semen and extender for two of the samples, sample treatment was not significant ($p>.05$) for 8 of the 11 estimates of semen quality. Three estimates of mobility significantly affected ($p<.05$) the two samples with the coolest temperatures of semen, S35/E35 and S35/E37, however the 35°C semen with a four degree difference from the temperature of the extender was not affected.

Analysis also showed that Androhep Plus was able to better maintain semen quality at hour 96 as compared to BTS ($p>.05$). However, BTS at hour 48 had higher values than Androhep for semen quality parameters, which is the time in which BTS would be used in a commercial setting. Although BTS and Androhep Plus differed for many of the estimates, values did not fall below rates that have been shown to affect fertility, suggesting that

biologically the two extenders would not differ. There were no significant effects of extender on sample treatments ($p>.05$).

The findings of this study negate the rule that semen and extender must be within 1°C of each other before they are mixed together, if between 35°C and 39°C. This could result in time and labor savings during semen processing.

Experiment two examined the effects of three rates of cooling on freshly ejaculated semen on eight different estimates of semen quality. Extender type, short or long term and time interactions on the quality of semen were analyzed. Three mature boars ($n=3$) were tested three times each. Samples were extended with either Androhep Plus, a five day extender, or BTS, a three day extender. After extension, samples were cooled at a (1) slow rate, allowed to slowly reach room temperature before being placed in a 17-18°C semen storage unit; (2) a medium rate, immediately place in a 17-18°C semen storage unit; or a (3) fast rate, placed in an incubator set at 12°C for an hour before being placed in a 17-18°C semen storage unit. Analyses were performed on the neat semen at hour 0 and on cooled samples at 4, 24, 48 and 96 hours after extension. All data was analyzed using SAS and the proc GLM procedure.

The rate of cooling did significantly differ from one another ($p<.05$). For all estimates of semen quality, the medium cooling rate and slow cooling rates did not differ from one another ($p>.05$). Quality estimate for the fast rate of cooling was significantly lower from the medium and slow rates of cooling for motility and mobility estimates. Although the fast rate of cooling had reduced quality of semen, values did not fall below rates that have been shown to affect fertility, suggesting that physiologically the rates of

cooling will not differ. Rate of cooling was not significantly affected by extender or time ($p < .05$).

Extender and time influenced almost all estimates of semen quality. Values for Androhep Plus were consistently higher than BTS for all estimates of semen quality as time progressed ($p < .05$). Androhep Plus is a long term extender and contains ingredients better suited for the maintenance of semen quality. Although Androhep Plus and BTS significantly differed, neither saw values drop below what has been shown to affect fertility. This suggests that whether storing semen for a short period or a long period it will not result in decreased fertility for the chosen rate of cooling.

The results of these two experiments suggest that boar semen is able to withstand greater temperature fluctuations than previously thought. The first experiment showed that semen and extender do not have to be within 1°C of each other, a goal not easily obtainable in a commercial setting, resulting in saved time and effort. The second experiment showed that there does not have to be a holding time before storing semen, which can save time and money during semen processing.

Evaluating Temperature Effects and Extension Cooling Rates on Boar Semen Quality

by
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BIOGRAPHY

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**EVALUATING TEMPERATURE EFFECTS AND EXTENSION COOLING RATES
ON BOAR SEMEN QUALITY**

LITERATURE REVIEW

Introduction

Today in commercial swine production in N.C., ninety-nine percent of sows are bred via artificial insemination. This means 1.18 million of the 1.2 million sows in the state are bred artificially. With an average of two doses of semen used per mating and sows having an average of 2.3 litters per year, this results in a minimum of 7 million doses used in North Carolina a year. Estimates for the non-genetic costs for producing an insemination dose vary between \$2.00 and \$5.00. Based on these figures, the economic investment in producing insemination doses is between \$14 and \$35 million in N.C. Consequently, evaluation of current practices and development of new effective semen handling and processing techniques are critical for profitable and efficient production of pork. The main objectives of the research contained within this thesis are to examine how changes in temperature during semen processing after collection affect semen viability and longevity during storage. As a result, a thorough review of spermatogenesis followed by an examination of current industry practices for preparation of semen for fresh or frozen storage is in order.

Spermatogenesis

Spermatogonial stem cells originate from the primordial germ cell, which in turn derives from epiblast cells (Lawson and Pederson, 1992). When the primordial germ cells arrive in the genital ridges the differentiating Sertoli cells enclose them and seminiferous

cords are formed. (de Rooij, 1998). These cells colonize and become gonocytes, which are morphologically different from primordial germ cells (Capel, 2000). After formation, the gonocytes proliferate and then stay arrested in the G0/G1 phase. The interactions of Sertoli and germ cells result in the formation of the seminiferous cords, allowing for the normal development of spermatogenesis and inhibition of meiosis.

Spermatogenesis occurs in the epithelium of the seminiferous tubule and consists of three functionally and morphologically distinct phases. It is important to note that no other cell type will undergo such an extreme morphological change with a simultaneous genetic recombination and reduction in chromosomal ploidy (Hecht, 1998). The first stage, called spermatocytogenesis or the spermatogonial stage is proliferative where diploid spermatogonia will undergo 8-9 mitotic divisions. There are four classes of spermatogonia: differentiated type A spermatogonia, undifferentiated type A spermatogonia, Intermediate spermatogonia and type B spermatogonia. Differentiated type A spermatogonia can be subclassified into A₁, A₂, A₃, and A₄ as can undifferentiated type A spermatogonia, which are subdivided into A_{single} (A_s), A_{paired} (A_p) and A_{aligned} (A_{al}).

Type A spermatogonia are large cells with ovoid nuclei characterized by homogeneous and dusk-like chromatin (Courot, 1970). The undifferentiated type A spermatogonia exist at the beginning of spermatogenesis and differ depending on their topographical arrangement on the basement membrane (de Rooij 1998). A_s spermatogonia have two fates: they can become two new A_s daughter cells (self-renewing division) and replenish the stem cell pool; or they can stay connected via an intercellular bridge and become A_{pr} spermatogonia. The intercellular bridge occurs when telophase is incomplete,

leaving an open area of cytoplasmic continuity that allows for the sharing of gene products to aid in the synchronization of development (Weber and Russell, 1987; Lee, 1995). A_{pr} spermatogonia are destined to follow the differentiation path and divide further to form chains of 4, 8 or 16 A_{al} spermatogonia. The next generations to form from the A_{al} spermatogonia are differentiated A_1 spermatogonia. The differentiating spermatogonia go from subsequent divisions to become A_2 , A_3 , A_4 , Intermediate and B spermatogonia, with B spermatogonia becoming committed to further differentiation producing preleptotene, or resting diploid primary spermatocytes (de Rooij 1998). This process in boars is similar to spermatogenesis in the mouse (Frankenhuis 1982).

The second stage of spermatogenesis in the boar is the spermatocytary stage, which is meiotic. This stage involves primary and secondary spermatocytes and results in one primary spermatocyte becoming four haploid spermatids (Mann, 1964). The preleptotene primary spermatocytes are the last germ cells to undergo DNA synthesis and subsequently enter prophase I of meiosis I (Felig and Lawrence, 2001). The first meiotic division will have one primary spermatocyte yield two primary spermatocytes. Prophase I of meiosis I is the longest in duration and includes five stages: preleptotene, leptotene, zygotene, pachytene and diplotene. These stages can be recognized via unique changes in nuclear chromatin. The leptotene stage has chromosomes arranged in thin strands. A thickening and pairing off of the homologous chromosomes characterizes the zygotene phase. This stage usually is said to have ended when the mitotic division of the previous generation of spermatocytes has occurred. Pachytene begins with the completion of pairing which results in the crossing over between non-sister chromatids. Crossing over results in the redistribution of hereditary

material in the four chromatids (Roosen-Runge, 1977).

The diplotene phase has paired chromosomes that pull away from each other in preparation of diakinesis, a phase where the chromosomes shorten and broaden with the four chromosomes clearly visible. However, in this phase the sister chromatids remain attached. Franca et al. (2005) also noted that in the primary spermatocytes there was a large increase in size from pre-leptotene to diplotene. The increase is also followed by a decrease on average from ~500 to 10 μm^3 in cell size during the last stage of spermatogenesis (Russell and Franca 1995; Franca et al., 2005).

Prophase I is followed by metaphase I, anaphase I and telophase I. These occur rapidly. During metaphase I, paired chromosomes arrange at the metaphase, or equatorial plate. The homologous chromosomes move to opposite poles of the cell where they will be distributed to the resulting secondary spermatocytes. Secondary spermatocytes have a short interphase, prophase, metaphase, anaphase and telophase and are exclusively in phase 4 of the seminiferous epithelial cycle. This second maturation division ends with the division of the centromeres from the sister chromatids resulting in four spherical spermatids with a haploid set of chromosomes.

The last stage of spermatogenesis is spermiogenesis, where complete differentiation of round spermatids into elongated spermatozoa is achieved. During this stage the spermatozoa undergo a variety of species-specific morphological, metabolic and biochemical modifications, such as nuclear condensation, development and formation of the acrosome, formation of the fibrous sheath, and development and loss of organelles and a portion of the cytoplasm as a cytoplasmic droplet (Franca et al., 2005). This last stage is divided into the

Golgi phase, cap phase, acrosomal phase, and maturation phase. The Golgi phase is characterized by spherical nuclei and the formation of proacrosomal vesicles within the Golgi apparatus. These preacrosomal granules fuse to form a proacrosome. This phase also contains the early stages of tail development. Centrioles move in opposition to the position of adherence of the acrosomal granule to the nuclear envelope. The proximal centriole that migrates closest to the nucleus serves as the attachment point for the tail to the head, with the distal centriole serving as the central point for the developing axoneme.

During the next phase, the cap phase, the acrosomal granule begins to spread over the surface of the nucleus, forming a double-layered membrane sac that closely adheres to the nuclear membrane and will cover two-thirds of the anterior nucleus (Hafez, 2000). The distal centriole begins to develop further into the outgrowing flagellum and consists of two central tubules surrounded by nine pairs of additional tubules. The acrosomal phase is characterized early on by the elongation of the cell body and nucleus, the contents of which will condense helping to reshape the nucleus into a flattened elongated structure. This in turn causes the explosion of the cytoplasm to the developing tail (Mann, 1964). The manchette also forms within the cytoplasm when microtubules associate to form this temporary cylindrical sheath. Within the sheath is where a chromatoid body condenses around the axoneme to form the ring-like structure known as the annulus, which will move posteriorly along the tail (Hafez, 2000). The spermatid rotates in the direction of the spot on the basement membrane in which the acrosome is pointed and then rotates, oriented towards the nucleus of the Sertoli cell with the tail towards the lumen. Mitochondria aggregate around the proximal part of the flagellum, close to the axoneme to form the middle piece (Junqueira, 2005).

The final transformations of the spermatids occur during the maturation phase. Within the nucleus, transitional proteins are replaced with protamines, which fill the nucleus. In addition, a fibrous sheath and the underlying nine course fibers are formed around the axoneme. The fibrous sheath covers the axoneme from the neck to the beginning of the end piece, while the annulus migrates to the point where it separates the middle and principal pieces (Hafez, 2000). The final modification includes the formation of a post nuclear cap, after which the spermatids are ready for spermiation.

Each cycle takes 8.6-9 days to complete and boars will have 4.5 cycles lasting a total of 38.7-40 days (Sharpe, 1994). The germ cells in boars are arranged in specific segmental associations called stages where there is only one stage per tubular cross-section (Franca and Russell, 1998). It has been shown that boars have eight of these stages. Franca and Russell (1998) were able to compare the frequencies of these stages utilizing criteria developed for laboratory animals that include the time when the acrosome is developed in newly formed spermatids and is based on changes in the morphological characteristics of the nucleus of the spermatids. They have divided the spermatogenic process into three phases in relation to meiosis. The first phase is called the premeiotic phase, which occurs after spermiation and prior to the metaphase of meiosis I. This phase has two generations of spermatocytes and one generation of spermatids and lasts 28.3-31.4 days. The second phase is called the meiotic phase, which occurs from meiosis I through meiosis II, is short comprising only 11.7-12.1 days. The last stage is a post meiotic phase that occurs after the completion of meiosis until spermiation. This phase is characterized by two generations of spermatids and one generation of spermatocytes that lasts 56.5-60 days (Franca and Russell, 1998). It has

been found that the premeiotic phase occurs in the basal compartment of the seminiferous epithelium and the meiotic and postmeiotic phases occur in the luminal compartment (Eddy et al., 1996).

Spermatozoa Maturation

The final stages of spermatozoa maturation occur after spermiation when the spermatozoa leave the testis and enter into the epididymal tubule. These final maturation steps are not under the genomic control of the germ cells and are essential to the quality of the spermatozoa (Dacheux et al., 2003). It is believed that these final maturation steps require sequential interactions with the medium surrounding the spermatozoan during its transit through the epididymis (Dacheux et al., 2005). The epididymis is roughly 50-100 m in the boar and contains three distinct regions known as the caput, the corpus and the cauda (Franca et al., 2005). Boar epididymi are also unique in the fact that numerous arterioles and capillaries penetrate into the subepithelial region (Dacheux et al., 1987; Stoffel and Friess, 1994).

Spermatozoa traverse the entire epididymis in 9-11 days with time spent in the caput and corpus being between 5.4 and 7 days and time in the cauda being 6.4 days (Mann, 1993; Franca et al., 2005). The primary function of the epididymis is for spermatozoa maturation, transport, storage and maintenance of viability in a specialized microenvironment. As the spermatozoa move through the epididymis they will have modifications in patterns of movement, changes in the metabolic pattern and the structural condition of the specific

organelles of the tail, changes in the nuclear chromatin and modification in the acrosomal shape. In addition there will be progressive loss of water and an increase of specific gravity, migration through the midpiece and then detachment of the cytoplasmic droplet and modifications of the plasma membrane that determine variations in their biophysical qualities (Briz et al., 1995). As spermatozoa progress through the duct, their movement will decrease and conversely spermatozoa agglutination will increase.

To help achieve these changes the epididymis is lined with epithelium that contains a variety of specialized cells giving character to the environments of each section. It should be noted that the epithelium is the thickest in the proximal caput and the thinnest in the cauda. Principal cells function in protein secretion and absorption; however, it is the apical tight junctions between these cells that create the blood epididymal barrier (Dacheux et al., 2005). There have been 125 proteins discovered in the epididymal regions in the boar with secretions in the caput, corpus and cauda being 1%, 83% and 16%, respectively (Syntin, 1996). Clear cells function in endocytosis. Along with narrow cells, clear cells have secretory activities and are both are thought to be responsible for the acidification of the lumen fluid (Rodriguez-Martinez, 1990; Franca et al., 2005). Finally, the basal cells are responsible for immune defense, phagocytosis and secretion of antioxidant proteins. These cells are all present in a segment specific manner to give rise to the morphological, biochemical and physiological changes as the spermatozoa navigate the epididymis.

When spermatozoa enter the caput from the testes they are immature and do not have any fertilizing ability. Spermatozoa in the caput are characterized by a proximal cytoplasmic droplet and have a circular, non- unidirectional swimming pattern (Hafez, 2000). It is

thought that the spermatozoa in the caput lack fertility due this pattern of movement. Next, the spermatozoa will enter the corpus and have an estimated 10-50% fertilizing ability. Here the spermatozoa achieve osmotic resistance and are characterized with having a distal cytoplasmic droplet. Towards of the middle of the corpus the spermatozoa will develop rapid forward motility patterns. Sperm movement through the first two segments occurs via continual peristaltic contractions of smooth muscle cells lining the epididymal duct (Amann et al., 1983).

The majority of maturation occurs in the caput and corpus, therefore when the spermatozoa enter the cauda they have the most fertilizing ability of 90-100% (Dacheux and Paquigaon, 1980). The dominant pattern of movement that occurs is a unidirectional rapid forward motility that enhances the fertilizing ability of these spermatozoa. In addition, the spermatozoa will maintain their osmotic resistance gained in the corpus and it is here where there is the highest percent of mature spermatozoa (Dacheux et al., 2005). Some of the modifications such as the movement and loss of the cytoplasmic droplet and changes in the specific gravity of the spermatozoon are unknown as to their impact on fertilizing ability (Hafez, 2000). The cauda is also the major site of spermatozoa storage.

Insemination and Maturation in the Female Reproductive Tract

Spermatozoa are moved out of the cauda during ejaculation when the smooth muscle cells of the epididymal lining are stimulated (Amann et al. 1983). Upon leaving the boar, the spermatozoa will enter into the female tract where spermatozoa maturation is not halted at

ejaculation, but continues (Mann, 1964). Boar spermatozoa are ejaculated in a suspension of seminal plasma composed of cauda luminal contents and secretions of the accessory sex glands, including the bulbourethral glands, prostate and seminal vesicles. The seminal plasma influences the physiology of the spermatozoa by increasing the motility at ejaculation and membrane stability (Tamuli and Watson, 1994). Rodríguez-Martínez et al. (2005) described the ejaculate as being composed of three series of fractions. The first fraction is called pre-sperm and is composed of secretions from the urethral and bulbourethral glands and prostate. The second is called the sperm-rich fraction and is characterized by containing the majority of spermatozoa. Fluid from the seminal vesicles and prostate is also present, along with the epididymal fluid. The initial portion of this fraction contains most of the spermatozoa and after this the initial portion concentrations of spermatozoa decrease progressively.

The final fraction is known as the post-sperm rich where the number of spermatozoa has almost disappeared, a stark contrast to the previous fraction. The post-sperm rich fraction initially contains increased secretions from the seminal vesicles, prostate and the bulbourethral glands. It is in this fraction where the floccula from the bulbourethral glands coagulates the seminal plasma that is known as the “gel-fraction.” Rodríguez-Martínez et al. (2005) stated that this aids in retaining the ejaculate in utero to minimize retrograde flow through the cervix.

The boar ejaculates 200-250 mL suspension of spermatozoa with the average number of spermatozoa being greater than 3.0×10^9 . With such a large amount of ejaculate, even though sows are mated with an intracervical insemination, the semen is distributed to the

uterine lumen and is therefore said to be intrauterine (Langendijk et al., 2005). Between 70 and 90% of spermatozoa are eliminated from the uterine lumen (Lavon and Boursnell, 1975) via phagocytosis and vaginal reflux (Rozeboom et al., 2000). In what is known as the rapid-phase, the remaining spermatozoa are directed towards the uterotubal junction via myometrial contractions (Rodríguez-Martínez et al., 2005). These contractions are at a maximum during estrus and under endocrine regulation via increased estrogen and progesterone (Langendijk et al., 2005). The spermatozoa gather in the caudal isthmus in the uterotubal junction of the oviduct where they are immersed in tubal fluid. This gathering is known as the functional sperm reservoir (Tienthai et al., 2004).

The functional sperm reservoir contains 10^5 to 10^8 stored spermatozoa maximum; however, that number slowly decreases (Rodríguez-Martínez, 2001). The function of the sperm reservoir is to maintain fertilizing capacity and spermatozoa viability (Mburu et al., 1997; Rodríguez-Martínez et al., 2001). Mburu et al. (1996; 1997) was able to discern two subpopulations spermatozoa in the sperm reservoir. The first subpopulation is found in groups inside the spaces formed by the folded endosalpinx. This group has the head oriented towards the epithelial lining with straight or slightly curved tails. The other subpopulation is found in the central lumen. Many in this group are in direct contact with the apical surface of the epithelium. Natural mating occurs when the female displays estrous behavior no more than 30 hours before ovulation. The reservoir will release a steady flow of restricted numbers of spermatozoa towards the ampullary-isthmic junction, the site of fertilization, thereby ensuring that an oocyte will be fertilized when ovulated. The sperm reservoir is free of polymorphonuclear leukocytes (Rodríguez-Martínez et al., 1990).

The sperm reservoir is the site of capacitation. Cheng and Austin (1951) identified capacitation as a crucial pre-fertilization event in the reproductive tract. Capacitation is a gradual process and is the last event spermatozoa need to undertake in order to be fully functional for fertilization (Yanagimachi et al., 1994). Capacitated sperm must be able to be released from the sperm reservoir; penetrate the cumulus layers of an oocyte; and bind to the zona pelucida (Rodriguez-Martinez, 2001). A number of bound proteins, acquired from the cauda epididymis and seminal plasma, are removed from the acrosomal region. Cholesterol is then extracted from the plasma membrane via the female luminal fluids. Lipid scrambling occurs which results in enhanced membrane fluidity. The transformation of the plasma membrane is seen as the earliest sign of capacitation (Rodriguez-Martinez et al., 2005).

Bicarbonate seems to be an important factor in the reorganization of the plasma membrane. In fact it is bicarbonate (HCO_3^-), along with calcium (Tardif et al., 2003) that initiates the initial destabilization and subsequent lipid scrambling in the plasma membrane (Gadella and Harrison, 2002). Other indicators of capacitation include the uptake of extracellular calcium and shifts in motility patterns. The spermatozoa are released from the sperm reservoir in a progressive and continual manner allowing for low numbers, which reduces the chance of polyspermy and increases the availability of capacitated spermatozoa during the lengthy time interval of deposition and female ovulation (Rodriguez-Martinez et al., 2005).

Artificial Insemination with Fresh Semen

In 1678, Leeuwenhoek and his assistant were able to first view spermatozoa under a primitive microscope to which they called their specimens “animalcules” (Foote, 2002). It was more than 100 years later in 1784 when Spallanzani successfully inseminated a dog and almost 250 years later when Ivanow completed an insemination in swine (Foote, 2002). The work of Ivanow was taken over by Milovanov in the 1930s. Milovanov proposed using glucose-sulphate and glucose-tartrate as early diluents (Johnson et al., 2000). Milovanov inseminated sows on the second day of estrus with 100-150 mL of fresh one-to-three diluted semen. The inseminations resulted in a conception rate of 70% (cited by Polge, 1956). In addition, it was found that boars were easily trained to mount immobile benches called dummies (Milovanov, 1938; Polge, 1956). Artificial vaginas that applied pressure to the glans penis were subsequently developed as a collection aid (McKenzie, 1931). During this time, the recommended storage temperature for semen was 7 to 12°C. Ito et al. (1948) recommended that semen be stored at 15 to 20°C. Polge (1956) modified a yolk-phosphate, yolk-citrate and milk extender commonly used for preservation of bovine spermatozoa for use with cooled boar semen.

During these early studies, it also became apparent that with a reduction in temperature there was also a reduction in the surviving populations of spermatozoa. Polge (1956) called this phenomenon “cold shock.” Cold shock was found to occur when freshly ejaculated boar spermatozoa were cooled quickly from body temperature to temperatures below 15°C resulting in a loss of viability (Johnson et al., 2000). Semen was able to acquire

resistance to cold shock when held at or above 15°C for several hours (Pursel et al., 1973).

The concept of cold shock has been studied in depth and many reasons for its occurrence have been proposed. It is possible that it is related to the lipid composition of the membrane bilayer, which may affect fluidity (Watson, 1996).

De Leeuw et al. (1990) found that as the temperature is lowered, phospholipids in the membrane have restricted lateral movement, which can result in a transition from a fluid state to a gel phase. Membrane lipids have different transition temperatures, therefore when phase separations occur, proteins become irreversibly clustered. De Leeuw et al. (1990) concluded that the membrane composition of cells is strongly related to how a cell membrane responds to low temperatures. The boar spermatozoan membrane has a low ratio of cholesterol/phospholipids and in addition has a lower percentage of phosphatidylcholine with a higher percentage of phosphatidylethanolamine and sphingomyelin, as compared to cold-resistant bull spermatozoa (Johnson et al., 2000).

Watson and Plummer (1985) were the first group to suggest that the sensitivity to cold shock may be internally caused by a particular composition in cholesterol and phospholipids. De Leeuw et al. (1990) found that cholesterol is distributed asymmetrically with a higher percentage located in the outer monolayer of the membrane. This group also hypothesized that this cholesterol imbalance renders the inner monolayer susceptible to cold shock. The membrane function changes when chilled and this, in turn causes increased leakage of cations and enzymes (increased permeability); changes in the lateral motion through channels; and reductions in diffusion controlled membrane transport and enzyme activity. It was later hypothesized that the increased membrane permeability coupled with

phase shifts, allows the flow of calcium ions and capacitation (Watson, 1996).

Consequently, premature capacitation events could result in a sub-fertile population of spermatozoa undergoing rapid cooling after ejaculation.

There are several factors that can influence the ability of spermatozoa to withstand the detrimental effects of rapid cooling such as composition of extenders, rate of cooling and holding time after extension. Pursel et al. (1972) stated that spermatozoa acquired resistance to cold shock during incubation at 5°C. Pursel and Johnson (1974) also concluded that the ability to develop resistance to cold damage is an inherent property of spermatozoa. It is recommended that when semen are stored below 15°C protocols should include a holding time at or above this temperature to decrease the effects of cold shock (Eriksson et al., 2001). Optimal holding times vary but range from 2 hours to 8 hours (Eriksson et al., 2001).

Rate of cooling is another factor that can be manipulated when extending boar semen. When sperm are rapidly cooled, there is a loss of membrane integrity and cell function in the range of 0 to 20°C (Watson, 1995). Early protocols suggest cooling raw semen to room temperature over a period of an hour before further cooling. Cooling usually occurred at a rate of less than 5° an hour because sperm motility was reduced in storage if cooled at faster rates (Dzuik, 1958). Semen is typically processed using variations of this protocol. It is either diluted and cooled to room temperature over two hours before it is placed into semen storage units (Bamba and Cran, 1985), or it is placed immediately into an incubator maintained at 15 to 17°C (Kuster and Althouse, 1999).

A final consideration is the semen extender. When spermatozoa are ejaculated, they are diluted in seminal plasma composed of fluids from the accessory sex glands. Seminal

plasma provides for nutrition, osmotic protection, and regulation of motility, capacitation and binding for spermatozoa. In addition, the seminal plasma also affects the female tract by stimulating uterine contractions and modulating the immune response (Johnson et al., 2000). These actions affect spermatozoa indirectly. Extenders reduce the metabolic activity and preserve the spermatozoa until fertilization (Johnson et al., 2000). One of the original extenders was a yolk-phosphate, yolk-citrate and milk extender developed by Polge (1956). Extenders have become more complex as more has been discovered about how boar spermatozoa respond to dilution. A major factor to overcome is the concept of the dilution effect proposed by Harrison et al. (1982). Harrison et al. (1982) suggested that there is a loss of cell viability due to cell injury, when semen is diluted presumably due to the reduction of protective agents normally found in seminal plasma.

The main purpose of extending semen is to maintain the viability of spermatozoa during storage prior to insemination. In order to accomplish this, semen extenders must be able to do the following: (1) retard bacterial growth; (2) maintain a pH between 6.8 and 7.2; (3) stabilize the plasma membrane; (4) maintain the function of the $\text{Na}^+ - \text{K}^+$ membrane pump; and (5) regulate the osmotic pressure to which spermatozoa are exposed (Johnson et al., 2000). The most widely used extender, Beltsville-TS (BTS), was originally developed for thawing boar spermatozoa by Pursel and Johnson (1975). However, the extender is able to maintain intracellular concentrations of potassium due to the near absence of the ion in the extender (Johnson et al., 1988; Johnson et al., 2000). With adequate extenders, semen can be routinely preserved for 3 to 5 days allowing artificial insemination protocols to become one of the most important management tools (Bailey et al., 2008).

Three different types of extender are commercially available. These include the short-term extenders, long-term extenders and ultra-long term extenders. Short-term extenders, such as BTS, are used up to three days. Long-term extenders such as Androhep are used up to five days. Ultra-long term extenders are used for storing samples over five days, and up to eight days. Long-term extenders typically have a much more complex chemical formula and a higher cost than do the short-term extenders. Most extenders are formulated to enhance preservation capabilities by providing nutrients for energy in the form of glucose. BTS contains ethylenediamine-tetra-acetic acid (EDTA), a chelating agent discovered in the 1960's that blocks the action of calcium as a mediator of sperm capacitation and the acrosome reaction by limiting its movement across the plasma membrane (Gadea, 2003, Johnson et al., 2000). BTS is also characterized by containing a low concentration of potassium and is believed to play a role in maintaining the intracellular concentration of this ion at physiological levels during storage (Johnson et al., 2000). Androhep contains EDTA but also contains HEPES and Bovine Serum Albumin (BSA) (Gadea, 2003). HEPES is a zwitterionic organic buffer that captures heavy metals and controls pH over a wide range, and is not temperature dependent (Johnson et al. 2000). Controlling of the pH is important because the spermatozoa's glycolytic metabolism leads to a reduced intracellular pH, which suppresses the cell metabolism. Buffers like sodium citrate and sodium bicarbonate are present in both BTS and Androhep, which also help to control pH levels (Gadea, 2003). BSA is thought to compensate for the dilution effect on seminal plasma proteins. It is known to stimulate motility in a reversible manner (Harrison et al.,

1982, Waberski et al., 1989). BSA is also a potent inhibitor of lipid peroxidation in sperm, a damage that reduced sperm motility (Alvarez et al., 1995).

Antibiotics are added to semen extenders routinely because extenders contain glucose and are stored at 15-16°C, conditions that promote the growth of Gram negative bacteria. Aminoglycosides such as gentamicin, neomycin and kanamycin are generally used to combat bacterial growth (Gadea, 2003). Limited information is available on X-cell, due to commercial interests, however it is known to contain BSA and gentamicin sulfate. An improved form of X-cell was introduced in 2004 called Tri-Xcell, which contains three antibiotics: gentamicin sulfate, sodium amoxicillin and tylosin, which provide broad spectrum control of bacterial growth. With bacteria being spermicidal, it is thought that with adequate antibiotics the semen will be able to withstand longer storage times (Althouse et al., 2000).

Finally, it is important to note that other factors independent of spermatozoa's ability to survive the effects of cooling are associated with fertility differences among boars and their ejaculates. Age and ejaculation frequency can account for much of the variability in semen characteristics (cited by Swiestra and Dyck, 1976). In general, young boars and those maintained under frequent collection frequencies tend to have reduced fertility compared with their older and less frequently used counterparts (Swiestra and Dyck, 1976). In addition, even when age and collection frequency are constant litter size can still vary among boars even when the same insemination dose is used (Flowers, 2002). Reasons for these differences are not known but could be related to how spermatozoa from different boars interact with the sow's reproductive tract after fertilization.

Once semen has been properly extended and stored, it is inseminated into sows. The most common way that this is achieved is via transcervical insemination of sows. Sows typically are inseminated twice with 3×10^9 spermatozoa per dose (Knox et al., 2008). Artificial insemination is achieved using a catheter of which several types are available ranging from a modified boar glans penis to a spongy plug. In order for a successful insemination to occur, the semen needs to be in the sow's tract when the ova become available to discourage aging, thus rendering the ova unviable (Bathgate, 2004). An optimal insemination regimen is insemination 12-18 hours after the standing estrus with a second dose 18-24 hours later (Almond et al., 1998). However, previous research clearly shows that insemination strategies are often herd specific (Flowers and Esbenshade, 1993). This is mainly due to differences in the timing of ovulation among and within herds (Flowers and Esbenshade, 1993; Flowers, 1998).

Cryopreservation

Cryopreservation offers several advantages over use of fresh semen because it (1) allows global genetic selection, (2) enables the preservation of top genetic lines, (3) and can offer extra time to complete health safeguards prior to insemination (Eriksson and Rodriguez-Martinez, 2000).

Despite the wide spread use of cryopreservation in the cattle industry, the swine industry has been slow to adopt this technology. In 2000, the use of frozen-thawed semen on a worldwide basis was reported to be only 1% (Wagner and Thibier, 2000). The main reason

frozen-thawed semen has not been routinely adopted is due to the reduced fertility compared with fresh semen. Farrowing rates with fresh semen are greater than 90%, while those associated with frozen-thawed semen are between 40% to 70% (Eriksson and Rodriguez-Martinez, 2000; Eriksson et al., 2002; Bolarin et al., 2006). In addition, frozen-thawed semen has more technical obstacles to overcome than fresh semen. There is large individual boar variation in the success of freezing. The procedure is expensive, cumbersome and uses a high semen dosage of $5-6 \times 10^9$ (Eriksson and Rodriguez-Martinez, 2000; Saravia, 2008). Lastly, frozen-thawed spermatozoa have a lower survival rate with a shorter life span *in vitro* leading to a reduction in the window of optimal fertility for artificial insemination (Larsson and Einarsson, 1977).

Moreover, problems associated with cold shock are magnified with frozen-thawed semen compared with cooled liquid semen. As mentioned earlier, there is a significant amount of variation among different boars and among ejaculates from the same boar. These internal factors probably have a genetic basis and it might be possible to select for boars that “freeze” well. External factors such as the concentration of the cryoprotective agents and rates of dilution and cooling can be altered to achieve improved results (Johnson et al., 2000).

Advances in freezing semen began in 1949 when Polge serendipitously discovered that glycerol was an effective cryoprotectant with the successful freezing and thawing of rooster semen (Foote, 2002). In 1956, Polge was then able to freeze boar semen for the first time using high glycerol content in glass ampules (Polge, 1956; Eriksson and Rodriguez-Martinez, 2000). More progress was made in 1970 when he was able to use frozen-thawed semen to achieve a pregnancy via oviductal insemination (Polge et al., 1970). A year later,

three groups of scientists published successful fertility with the use of frozen-thawed semen via transcervical insemination (Crabo and Einarsson 1971, Graham et al 1971, Pursel and Johnson 1971).

Two methods are routinely used today for freezing boar semen. Pursel and Johnson (1975) developed the first method which was largely adapted from Nagase and Niwa's success in freezing bull semen (Nagase and Niwa, 1963). This method, also called the Beltsville method, holds the sperm-rich fraction of the ejaculate for two hours in seminal plasma. After this holding time has expired, the sample is centrifuged, cooled for three hours and is pelleted on dry ice. The pellets range in size from .15 to .20 mL. The other method used commercially is the Westendorf method (Westendorf et al., 1975). In this method, the semen is diluted 1:2, with diluent, cooled and then frozen over liquid nitrogen vapor in 5-6 mL "maxistraws". Recently there have been some successes with freezing highly concentrated spermatozoa in small volumes (Saravia et al., 2005). However, a major breakthrough in commercial application has not yet occurred.

With two commercially available procedures, the rate of use is still 1% worldwide. Freezing semen still has a myriad of challenges to overcome before surpassing liquid stored semen as the superior storage mechanism. Difficulties freezing may lie with a concept known as the two-factor theory, which was proposed by Mazur et al. (1972). The two-factor theory states that cell damage, or even cell death, can result as a consequence of either exposure to high concentrations of solutes when the freezing rate is too low, or the formation of intracellular ice when the freezing rate is too high. These events are said to be reversed when thawing, therefore if the freezing rate is low, the thawing rate should be as well.

Glycerol is used as the primary cryoprotectant; however, boar spermatozoa are sensitive to high concentrations. Therefore, boar spermatozoa should be frozen rapidly with low levels of glycerol, according to the two-factor theory (Mazur, 1977). Using .5 mL plastic straws, Fiser and Fairfull (1990) looked at the combined effect of glycerol concentrations of 0-10% and cooling rates from 1 to 1,500°C/min on boar spermatozoa with regards to motility and acrosomal integrity. Their results showed a C-shape survival curve where optimum cooling velocities shifted to higher values with decreasing glycerol concentration. Fiser and Fairfull (1990) concluded that the optimum balance was freezing in 3% glycerol with a cooling rate of 30°C/min to achieve optimum cryosurvival.

Cooling velocity is also an important factor to consider. Johnson et al. (2000) stated that when a sperm suspension is cooled below the freezing point of the solution, it will continue supercooling until intracellular ice crystal form. When the temperature continues to decrease the differential osmotic pressure increases and the cellular water diffuses out and freezes. The rate of water diffusing out of the cell depends on the following factors: (1) the cell volume: (2) volume to surface ratio and (3) the permeability of the cell membrane to water and glycerol at specific temperatures. The rate of exosmosis can be controlled by the rate of freezing and the concentration of glycerol. When frozen rapidly the intracellular water freezes before leaving the cell. In contrast, if frozen slowly, water is able to leave the cell without formation of internal ice and thus avoiding supercooling. Applying Mazur's (1970) theory to the cooling velocity, it can be seen that damage to the cell will occur with dehydration at suboptimum rates and intracellular ice formation, thus freezing rate will typically appease the two extremes.

While the optimum cryofactors have been recognized, studies have shown that the ability of sperm to survive freeze thawing varies from boar to boar indicating a genetic predisposition for freezability (Thurston et al., 2002). In fact, it has been stated that the boar is the most important factor for explaining the variability in sperm cryosurvival among ejaculates (Roca et al., 2006). This phenomenon has been seen in the bull as well, resulting in bulls being genetically selected for their freezability (Hahn et al., 1969). For the boar, differences in specific DNA sequences have been identified between boars in which post-thaw sperm quality was classified as poor or good (Thurston et al., 2002). Further giving credence to a genetic component, one study suggested that the variability between animals could be due to their breed. Roca et al. (2006) found that Landrace and Pietrain boars had the greatest percentage of post-thaw sperm motility and viability. Yet it was noted that there is likely to be great variability among ejaculates within the breed itself. This study also suggested that standard semen measurements in fresh ejaculates and sperm quality assessments before freezing were of limited value in explaining post-thaw sperm quality. Furthermore, significant differences in post-thaw sperm motility exist between ejaculates of boars in different age ranges (Joyal et al., 1986). Therefore, freezability may only exist during a certain time during the productive life of boars.

The last factor affecting the survival of spermatozoa post-thaw is the rate of thawing. Using the accepted cooling and thawing rates, acceptable post-thaw sperm survival rates (>50%) are possible with a high proportion of boars (Großfeld et al., 2008). The accepted thawing rate is 1,200°C/min in .5 mL straws (Fiser et al., 1993), which can be described as quick. The two commercially used methods of freezing use pellets frozen on

dry ice or “maxistraws” frozen with liquid nitrogen. Pellets benefit from a faster thawing rate, which prevents the formation of small ice crystals. In addition, pellets are small and have a cryobiologically suitable shape with large surface-to-volume ratio, however labeling of pellets is difficult and there can be risks of cross contamination with storing and thawing. The small 0.25 and 0.5 mL straws also have a cryobiological-suitable shape, but 10-20 straws need to be thawed to achieve an insemination dose. On the contrary, the “maxistraw” is 5 mL and contains one insemination dose, but has a small surface-to-volume ratio. This limits the optimal thawing and freezing throughout the sample (Weitze et al., 1987). It has been seen that there is a 4-5 fold lowering of the thawing rate, roughly between -20 and 0°C, in the center of the “maxistraw” (Eriksson and Rodriguez-Martinez, 1999). To combat this effect, flat PET-plastic packages (FlatPack) have been found to improve results and may be replacements for the “maxistraws” in the Westendorf method. The FlatPack showed improved sperm motility due to a rapid thawing throughout the sample (Eriksson and Rodriguez-Martinez, 1999). The improved sperm motility correlates with fertility and therefore may improve results.

The concept of holding time is of more importance for frozen than fresh semen. Boar spermatozoa can acquire resistance to cold shock if incubated at low extension rates, for 1-5 hours at ambient temperatures (Pursel et al., 1972; Pursel et al., 1973). It is because of these findings that nearly all freezing protocols include a holding time at or above 15°C. Others have found that if the holding time is increased to 16 hours or 24 hours the proportion of spermatozoa surviving cold shock increases dramatically (Weber, 1989; Tamuli and Watson,

1994). In addition to a higher proportion surviving, a longer holding time produced benefits to post-thaw sperm viability (Kotzias-Banderia, 1997) and *in vivo* fertility (Simmet, 1993).

Frozen-thawed spermatozoa go through many changes during the freezing-thawing process. During freezing, the lipid composition and organization of the membrane changes during rapid cooling (Buhr et al., 1994). This allows for increased permeability causing frozen thawed spermatozoa to go through changes similar to capacitation (Watson, 1996). The capacitation-like changes cause the semen to have a shorter survival time in the female tract. The structure of spermatozoa is unique, and unlike other cells they have a compartmentalized structure resulting in each section having a specific requirement for freezing and thawing (Hammerstedt et al., 1990). There are different parameters that can be used to monitor the number of spermatozoa that survive post-thaw. These parameters include motility, viability and acrosomal status; however even these *in vitro* parameters seem to have low correlations with *in vivo* fertility (Flowers, 1997).

Another parameter that has been looked at is Chlortetracycline staining. This particular staining pattern has shown researchers that frozen-thawed spermatozoa undergo accelerated capacitation-like process. These effects may affect the ability to establish sperm reservoirs and account for the shortened lifespan of these cells (2-8 hours). In addition, it also indicates that the freezing thaw-process results in a more homogenous cell population, which may be functionally compromised (Curry, 2000). The freezing process affecting a particular population of cryo-sensitive cells, thus selecting a population of sub-fertile cryo-resistant cells, may explain the gain of homogenous cell populations. Another explanation is the appearance of a homogenous population caused by freeze-thaw masking subtle

differences (Curry, 2000). These two reasons arose with the examination of post-thaw spermatozoa with the technique of partitioning in aqueous two-phase systems to detect subtle differences in surface properties, which demonstrated that the heterogeneity is diminished after freeze-thawing (Ollero et al., 1998).

The affects of a homogeneous population can be counteracted by inseminating closer to the site of fertilization (Polge et al., 1970) or time of ovulation (Waberski et al., 1994). While oviductal insemination is not practical in a commercial setting, deep intra-uterine insemination is showing promising results. Roca at al., (2003) used deep intrauterine inseminations on 111 hormonally treated sows with 1×10^9 frozen-thawed semen and achieved farrowing rates of 77.6% and litter sizes of 9.3 ± 0.4 . Other studies achieved similar results leading to the conclusion that fertility can be optimized with frozen-thawed semen and a deep intrauterine insemination with an insemination dose of 1×10^9 spermatozoa, within 4-8 hours before ovulation (Großfeld et al., 2008)

In summary, both fresh and frozen boar semen are subject to cold shock after collection and during extension. The effects of factors such as cooling rate, holding time, and semen extenders with different cryoprotectants have been studied for frozen semen, but not for fresh semen. Because the majority of sows in N.C. and the U.S. are bred with fresh semen, it seems appropriate to determine whether these factors have the same detrimental effects on fresh as frozen semen.

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**EFFECT OF TEMPERATURE VARIATION BETWEEN SEMEN AND EXTENDER
ON BOAR SEMEN QUALITY**

Introduction

A common management practice within the commercial swine industry is to make sure that semen and the extender are within 1°C of each other before they are mixed together (Almond et al., 1998). The rationale behind this recommendation is that cold shock will be minimal if temperature differences between semen and extender are small. Cold shock typically occurs when the fresh ejaculate is cooled too quickly and results in a loss of viability (Johnson et al., 2000). However, this guideline is often not practical from a commercial standpoint for several reasons. First, semen from boars at collection can vary from 35°C to 40°C, which makes it difficult to pre-warm the extender to a temperature that is within 1°C of all ejaculates collected. Second, ejaculates from several different boars are often pooled to make insemination doses (Knox et al., 2008). Hence, in order to adhere to the 1°C rule, semen would most likely have to be either warmed or cooled prior to pooling. Finally, empirical data generated from a systematic investigation that supports this recommendation is lacking. If the temperature difference between the freshly collected semen and the semen extender could be relaxed without compromising fertility, then this would result in significant time and labor savings when processing semen. This study aims to determine the effect of temperature differences between semen and extender on spermatozoa during storage in vitro prior to insemination.

Materials and Methods

Experimental Animals

Eight mature boars (26 ± 2 months of age; 314 ± 10 kg) were used in this study. The boars were the offspring of Yorkshire x Large White x Landrace sows bred to Duroc x Hampshire x Spot boars. 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 during the summer months was provided by 18, 5000 cfm stirring fans evenly spaced throughout the barn and a thermostatically controlled dripper system. Boars had ad libitum access to water via water nipples and received 2.7 kg of a corn and soybean meal based diet formulated to meet all the nutritional requirements of adult breeding boars (NRC, 1998). Prior to the onset of the study, boars were maintained on a weekly collection schedule. All procedures performed on boars were approved by the N.C.S.U. Institutional Animal Care and Use Committee (06-036-A).

Experimental Design

Each boar was collected twice between January and June 2008. One ejaculate from each boar was extended with a 3-day extender, Beltsville Thawing solution (BTS; Minitube of America, Verona, WI), and the other was extended with a 5-day extender, Androhep Plus (Minitube of America, Verona, WI). After collection, each ejaculate was transported to the Swine Reproduction laboratory at North Carolina State University, approximately 7 miles from the Swine Educational Unit. Once in the laboratory, nine aliquots of the neat semen

were placed in 15 mL tubes (Fisher Scientific, St. Louis, MO) and three were incubated at 35°C, 37°C, or 39°C for 30 minutes. After the aliquots of neat semen had reached its respective incubation temperature, these were extended with semen extender maintained at the same three temperatures. This resulted in nine experimental treatments (Table 1). In three of the treatments, the neat semen and semen extender were at the same temperature of either 35°C, 37°C, or 39°C when mixed. These were considered to be the control treatments. In four of the treatments, the neat semen was two degrees either cooler or warmer than the extender when mixed. Temperature differences for these treatments were between 35 and 37°C or between 37 and 39°C. Finally, in two of the treatments, the difference between the semen extender and neat semen when mixed was 4°C, 35°C versus 39°C. After extension, all treatments were allowed to cool to room temperature, placed in an environmentally controlled semen storage unit (Minitube of America, Verona, WI), and maintained at 17°C for the duration of the study.

For each ejaculate, estimates of semen quality were obtained on the neat semen prior to extension; immediately after extension (time 0); and at 24, 48, and 96 h after collection. At 0, 48, and 96 h the proportion of spermatozoa with normal acrosome morphology; the proportion of spermatozoa with normal head and tail morphology (including the presence of proximal, distal and translocated cytoplasmic droplets); the proportion of motile spermatozoa; proportion of spermatozoa exhibiting progressive forward motility; the average straight-line velocity (VSL) of motile spermatozoa; the average curvilinear velocity of motile spermatozoa (VCL); the average straight-line distance traveled by motile spermatozoa (DSL); the average curvilinear distance traveled by motile spermatozoa (DCL); the acrosin

activity; and the capacitation status of spermatozoa in the ejaculate were assessed. At 24 hours post extension, only the motility and morphology analyses mentioned previously were performed (Figure 1).

Table 1. Formation of experimental treatments by combining neat semen and semen extender maintained at various temperatures.

Sample Treatments		
	Semen	Extender
1	35	35
2	35	37
3	35	39
4	37	35
5	37	37
6	37	39
7	39	35
8	39	37
9	39	39

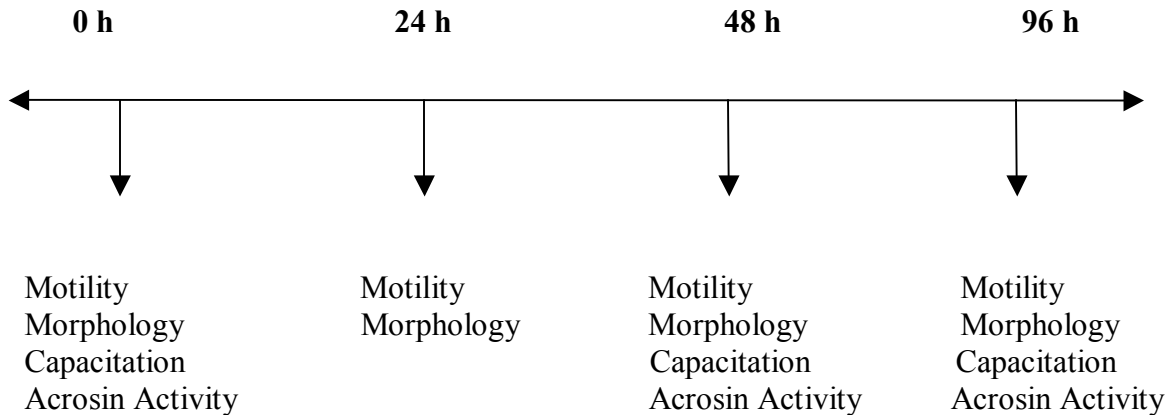


Figure 1. Representative time line showing the timing of semen quality estimates relative to collection and extension (Time 0).

Semen Collection

Each boar was collected by the double-gloved hand technique (Almond et al., 1998). The collection pen was 2.43 x 3.65 m and the collection dummy (Minitube of America, Inc., Verona, WI) was 0.3 m wide and 1.21 m long. Collections were completed using powder-free polyvinyl gloves (IMV America, Eden Prairie, MN). Each boar was housed in stalls separated from the collection pen by a 1 m alley. The semen was collected into a sterile plastic semen collection bag (Minitube of America, Verona, WI), which was placed inside a 1 -qt thermos covered with a milk filter (IMV America, Eden Prairie, MN). The collection cup was kept at 37°C. The same technician collected all boars during the study and allowed the boar to terminate the collection. Boars were allowed to dismount and remount during the collection.

After collection, the milk filter and rubber band were removed and the collection cup was weighed (Fisher Scientific, St. Louis, MO). From this, the pre-collection weight was

subtracted. Using the assumption that 1 mL semen weighs 1 g the semen volume was then recorded along with the boar's identification number. A spectrophotometer (SpermaCue, Minitub of America, Verona, WI) was used to determine the concentration. This was also recorded and then the ejaculates were placed into an insulated bag (Minitube of America, Verona, WI) and transported to the Swine Reproduction Laboratory located on the campus of North Carolina State University.

Treatment Preparation

As mentioned previously, nine different treatments were prepared from each ejaculate. Each treatment dose contained 3 billion total spermatozoa in 60 mL. This is representative of the standard insemination dose used in the industry. Treatment doses were prepared by first adding the appropriate aliquot of neat semen to a 50 mL conical tube (Port City Diagnostics, Inc., Wilmington, NC) via a 10 mL serological pipette (Fisher Scientific, Atlanta, GA). To avoid shocking the spermatozoa, extender was slowly dripped from the pipette down the sides of the conical tube until a total volume of 60 mL was achieved.

Evaluations of Semen Quality

Percentage of Normal Acrosomes

One mL of semen was placed into a test tube (12 x 75 mm; Port City Diagnostics, Inc., Wilmington, NC) and mixed with 100 μ L of 10% formalin. Five μ L was placed on a glass microscope slide (Fisher Scientific, Atlanta, GA) and covered with an 18 x 18 mm plastic coverslip (Fisher Scientific, Atlanta, GA). A phase contrast microscope (Zeiss,

Berlin, Germany) was used to evaluate 100 spermatozoa with normal tails under oil immersion at 40x. Spermatozoa with a normal shape and smooth surface of acrosomal ridges were classified as normal, while spermatozoa with misshaped, detached or loose acrosomes were recorded as abnormal (Briz et al., 1995; Pursel et al., 1972).

Percentage with Normal Morphology

Assessments of normal head and tail morphology were performed on the same subsamples that were used for evaluation of normal acrosome morphology. Two hundred sperm cells were evaluated from at least five different fields. Spermatozoa with normal heads and tails were recorded. Spermatozoa with a coiled, broken, or detached tails were recorded as having an abnormal tail. Spermatozoa displaying a cytoplasmic droplet distal to the head were recorded as having a distal droplet. Spermatozoa displaying a cytoplasmic droplet proximal to the head were recorded as having a proximal droplet. Spermatozoa with an abnormal head shape were recorded as abnormal head. Lastly, spermatozoa displaying tails that were looped around so that they crossed by the head were recorded as translocated.

Percentage of Motile Spermatozoa

One mL of semen was placed into a test tube (12x 75 mm; Port City Diagnostics, Inc., Wilmington, NC) and allowed to equilibrate for 30 minutes at 37°C. Leja slides (Minitube of America, Verona, WI) were prewarmed on a slide warmer (Minitube of America, Verona, WI) for 30 minutes prior to analysis. Each sample was mixed before 3 μ L were pipetted into one chamber on the Leja slide. Slides were analyzed using a computer assisted semen analysis system (SpermVision®, Minitube of America, Verona, WI) connected to a florescent microscope (Olympus BX41, Washington, DC). Five fields were

chosen at random and the average motility, average progressive motility average curvilinear distance (DCL), average straight-line distance (DSL), the average curvilinear velocity (VCL), and the average straight-line velocity (VSL) were recorded. This procedure was repeated so that a total of 10 fields and approximately 500 spermatozoa were evaluated.

Percentage of Capacitated Spermatozoa

The chlorotetracycline technique (CTC), which was originally described by Fraser and Herod (1990) and modified by Popwell (1999), was used to determine the percentage of capacitated spermatozoa in each sample. Before preparing the samples, .87% saline, DABCO stock solution, DABCO working solution, NaCl/Tris buffer, CTC solution and paraformaldehyde were prepared. The 12.5% (w/v) paraformaldehyde in a 0.5 M-Tris buffer was prepared with 12.5g paraformaldehyde, 7.88 Tris-HCL and 100 mLs of distilled water. Under a hood, the solution was mixed over low heat until it became a solution. If needed the pH was adjusted to 7.4. This solution was made once at the beginning of the study. The .22M DABCO stock solution was made when needed and consisted of 2.46g of 1,4-diazabicyclo (2,2,2)-octane (DABCO; Sigma Chemical Company, St. Louis, MO) and 100 mL of PBS. The .87% saline was made by adding 8.7g of NaCl to 1 liter of sterile water. The solution was then sterilized by autoclaving. The NaCl/Tris buffer and DABCO working solutions were prepared weekly. The NaCl/Tris buffer consisted of 1.8975g of NaCl and .6055g Tris (Sigma Chemical Company, St. Louis, MO) mixed with 250 mL of sterilized water. The DABCO working solution was made with 1 mL DABCO stock solution into a 15 mL conical tube (Port City Diagnostics, Inc., Wilmington, NC) and then adding 9 mL of glycerol. Inverting the tube until the contents were thoroughly incorporated sufficiently

mixed the combination. The CTC solution was prepared the day of analysis and was mixed using a 100 mL bottle (Pyrex, Germany) covered with aluminum foil to avoid light. The solution consisted of .019g of CTC (Sigma Chemical Company, St. Louis, MO), .039g of cysteine (Sigma Chemical Company, St. Louis, MO), and 50 mL of the NaCl/Tris buffer. The solution was refrigerated until needed for the assay.

For the assay, 1 mL was placed into a 15 mL conical tube (Port City Diagnostics, Inc., Wilmington, NC) and centrifuged (Angle Centrifuge; Hamilton Bell Co, Inc., Montvale, NJ) at 2000 rpm for five minutes. After removing from the centrifuge, the seminal plasma was removed and discarded and 1 mL of the .87% saline was added and mixed. Ethanol-cleaned glass microscope slides were placed on a 37°C slide warmer (Fisher scientific, Atlanta, GA). The CTC slides were prepared with the lights off. On each slide, 2.5µL of saline/sperm was pipetted onto a warmed slide. Next, 10 µL of the CTC solution was added and mixed with a gel loading pipette tip (Fisher Scientific, Atlanta, GA), which helped to minimize damage to the spermatozoa. Once mixed, a tiny bead of paraformaldehyde dropped from a gel loading pipette tip (Fisher Scientific, Atlanta, GA), was added and mixed which functioned to fix the CTC pattern. A single drop from a 0.5 mL disposable pipette (Fisher Scientific, Atlanta, GA) of the DABCO working solution was added and mixed to prevent fading of fluorescence. A 24 x 50 mm coverslip (Port City Diagnostics, Inc., Wilmington, NC) was placed onto the slide. The prepared slides were stored in a slide box (Fisher Scientific, Atlanta, GA) lined with aluminum foil and damp lab bench paper. The slide box was kept closed while other slides were being prepared.

Slides were analyzed using a VANOX microscope (Olympus Optical Co, Ltd., Japan) under a blue-violet illumination (excitation at 400-440 emission at 470 nm). A random sample of 100 cells was counted per slide. Spermatozoa were classified as either capacitated, uncapacitated, acrosome reacted or abnormal according to patterns described by Fraser and Herod (1990). Capacitated spermatozoa displayed a fluorescence pattern in the pre-acrosomal region of the head, but none in the post-acrosomal region of the head. Uncapacitated spermatozoa displayed fluorescence over the entire head. Acrosome reacted spermatozoa were characterized with having little to no fluorescence over the entire head except from a bright band in the equatorial segment of the head. Lastly, any sperm displaying fluorescence patterns, unlike the ones aforementioned or unusual heads were counted as abnormal.

Acrosin Activity

To determine the acrosin activity of the spermatozoa gelatin slides using a procedure originally describe by Penn et al. (1972) and slightly modified by Popwell and Flowers (2003). To begin the procedure, glass microscope slides (Fisher Scientific, Atlanta, GA) are cleaned with 70-80% ethanol by dipping into a 50 mL conical tube (Port City Diagnostics, Inc., Wilmington, NC) containing ethanol. Slides were wiped clean and dried with a Kimwipe (Kimberly Clark Professional, Roswell, GA) and placed on a slide warmer (Fisher Scientific, Atlanta, GA) set at 37°C . For identification, all slides are marked with a “G” in the upper left corner of each slide. The 3.5% gelatin solution was prepared by placing 100 mL deionized water into a 250 mL bottle (Pyrex, Germany) with a magnetic stir bar. The bottle was placed on a hot plate (Model PC-520, Corning) set to stir and on low heat. Three

and one-half grams of 3.5% gelatin (Fisher Scientific, Atlanta, GA) and 30 μ L of (.03%) Tween 80 (Fisher Scientific, Atlanta, GA) were added. The mixture was stirred until it became clear. Ten μ L of the 3.5% gelatin was placed onto the slide by starting at the top of the labeled slide and slowly pipetting in a line down to the bottom of the slide. A glass spreader was used to spread the gelatin slowly and evenly across the slide. The prepared slides were dried for 2-3 minutes and stored in a slide box in the refrigerator. They were removed and allowed to warm up to room temperature before performing analysis.

One mL of each semen sample was placed in a 12x75mm plastic test tube (Port City Diagnostics, Inc., Wilmington, NC) and placed in a heated stage set to 37°C (Minitube of America, Inc., Verona, WI) for 30 minutes. Samples were mixed and 10 μ L was pipetted on the slide near the labeled end in a line from top to bottom. A glass spreader was used to spread the sample evenly along the slide with a similar technique as that used to spread the gelatin. Slides were air dried for five minutes and then placed on a slide warmer (Fisher Scientific, Atlanta, GA) for 15 minutes. Slides were then placed in a CO₂ incubator (NAPCO Model 6100; NAPCO Scientific Company, Tualatin, OR) with 100% humidity at 37°C for 1.5 hours. After the incubation, slides were immediately stained with a toluidine blue stain. The stain was prepared by mixing 0.03 g of toluidine blue in a 250 mL glass beaker with 100 mL of borate buffer with a pH of 10. After stirring, the stain was transferred to a Coplin staining jar. The slides were immersed in the stain for 15 seconds. Excess stain was blotted with paper towels and each slide was rinsed three times in deionized water. Slides were air dried vertically until analysis.

The analysis was performed using a phase contrast microscope (Zeiss, West Germany) under 25X magnification. A glowing “halo” around the head of the sperm was considered indicative of acrosin activity. Sperm heads that lacked a “halo” were classified as not having acrosin activity. The magnification was reduced to 10X when the identification of digestion halos was difficult at 25X. A random sample of 100 sperm cells was counted per slide.

Statistical Analysis

The effects of extender (Androhep Plus and BTS); time (0, 24, 48, 96 hours); and treatment on semen quality parameters were analyzed with analysis of variance procedures for repeated measures (Gil and Hafs, 1971) using the general linear models procedure (GLM) of SAS (SAS, 2002, Version 9.1). Main effects of extender, treatment and extender by sample interaction were tested using boar nested within sample and extender as the error term. Effects of time and its interactions were tested using the residual error. No significant three-way interactions were observed. When significant two-way interactions between time and extender, time and treatment, or treatment and extender were observed they were analyzed further. When a significant interaction between time and extender was present, the change over time within each extender was determined. When a significant effect of time was present, differences among means were evaluated by examining probability values (PDIFF) with standard error (STDERR) for all possible combinations of least-squares estimates of marginal means (LSMEANS). Statistical significance was determined by resulting p-values with a p-value less than or equal to 0.05. A trend or tendency was noted if

the p-value was greater than 0.05, but less than 0.1. A complete list of SAS analysis program codes is located in Appendix A.

Results

Main effects of extender, treatment, time, and their interactions on 11 dependent variables associated with estimates of semen quality are shown in table 2.

Morphology

Time ($p < .0001$) and extender ($p < .0001$) significantly influenced the proportion of spermatozoa with normal, intact acrosomes (Table 2 and Figure 2). Androhep Plus had more spermatozoa with normal acrosomes (96.43 ± 0.2) as compared with BTS (95.50 ± 0.2). The proportion of normal acrosomes steadily declined ($p < .0001$) from time 0 to the 96 hour after collection. A significant interaction ($p = .0006$) between extender and time was observed for the proportion of normal acrosomes (Table 2 and Figure 2). Acrosome morphologies were similar at the 0 and 24 hours ($p > .05$). However, at 48 ($p = .0330$) and 96 hours ($< .0001$), BTS had fewer normal acrosomes than Androhep Plus ($p < .05$). Treatment had no effect ($p = .5670$) (Figure 3). The model showed no significance between extenders for the proportion of spermatozoa with normal head and tail morphology ($P = 1.0000$). There was a significant difference observed between the extenders for the percentage of spermatozoa with normal head and tail morphology ($p = .0097$). Androhep Plus ($90.73 + 0.6$) maintained a higher percentage of spermatozoa with normal morphology compared with BTS ($88.34 + 0.6$; Figure 4). When individual characteristics were examined, the proportion of spermatozoa

with abnormal tails and heads, proximal droplets and translocated tails were not different ($p > .05$). No interactions were observed to be significant (Table 1).

Motility

The proportion of motile spermatozoa were influenced by extender ($p < .0001$) and time ($p < .0001$) (Table 1). There was also an interaction between the extender and time ($p < .0001$). Semen extended with BTS had higher motility than Androhep Plus (74.18 ± 1.0 versus 60.95 ± 1.0 ; Figure 7). Motility also decreased over time ($p < .0001$) Mean values were (76.90 ± 1.4) at 0 h and (61.84 ± 1.4) by 96 h (Figure 7). The interaction between extender and time was one of magnitude. Overall, the proportion of motile spermatozoa decreased over time in both extenders. However, the decrease was greater in Androhep Plus compared with that in BTS (Figure 7). No other interactions were shown to be significant. Sample treatment had no effect ($p = .4730$) (Table 2 and Figure 8). The average motility of individual boars appeared to be influenced by extender (Table 3). Semen extended with BTS had higher motility than Androhep Plus for boars 111, 151, 240, 12505, 13909 and 158 (Table 3). Semen extended with Androhep Plus had higher motility than BTS for boars 112 and 156 (84.61 ± 0.6 versus 78.16 ± 0.6 ; 73.30 ± 2.0 versus 72.33 ± 2.0 ; Table 3).

The average progressive motility was also significantly affected by extender ($p < .0001$) and over time ($p < .0001$). Androhep Plus is significantly lower ($p < .0001$) than BTS with a means of 36.65 ± 1.1 and 49.85 ± 1.1 , respectively. Over time, the progressive motility decreased. There is a strong tendency ($p = .0555$) between the 24th and 48th hour with a significant difference between the 0 and 24th hour ($p < .0001$) and the 48th and 96th hours ($p < .0001$). There were significant interactions between the effects of extender and time

($p < .0001$) (Figure 9). Over time, both extenders followed the same trend with a sharp decline from the 0 hour to the 24th hour, with both extenders gradually increasing until the 96th hour. As time continued BTS had greater progressive motility than Androhep Plus with significant differences occurring at the 0 ($p < .0001$) and 24th hours ($p < .0001$). Treatment of samples had no effect ($p = .3642$) (Figure 10). Interactions between time and sample treatment ($p = .9996$) and extender and sample treatment ($p = .3483$) had no effect (Table 2).

Mobility

The average straight-line distance traveled were influenced by extender ($p < .0001$), treatment ($p = .0060$) and time ($p = .0084$) (Table 2). There were also interactions between extender and time ($p = .0009$). Semen extended with BTS had higher straight-line distances traveled than Androhep Plus (19.27 ± 0.2 versus 17.03 ± 0.2). The average straight-line distance traveled also decreased over time ($p = .0084$). Mean values were (18.97 ± 0.3) at 0 h and (17.71 ± 0.3) by 96 h. The interaction between extender and time was significant ($p < .0001$). Treatments extended with BTS decreased over time, while treatments extended with Androhep Plus decreased until h 48, where it increased to become no different than BTS at h 96 ($p = .3123$) (Figure 11). When samples were examined, samples that had the coolest semen at 35°C traveled less and significantly differed ($p < .05$) from the two samples which had the highest straight-line distance values which were S35/E39, S37/E37 and S39/E35 (Figure 12).

The average curvilinear distance traveled as influenced by extender ($p < .0001$) and treatment ($p = .0083$) (Table 2). Semen extended with BTS had higher average curvilinear distance than samples extended in Androhep Plus (44.82 ± 0.4 versus $42.35 \pm$

0.4). There were also interactions between extender and time ($p < .0001$). Treatments extended with BTS decreased over time, while treatments extended with Androhep Plus, initially less than BTS, increased to become higher than BTS at h 96 ($p = .0058$) (Figure 13). When samples were examined, samples that had the coolest semen at 35°C traveled less and significantly differed ($p < .05$) from the two samples which had the highest curvilinear distance values which were S37/E37 and S39/E35 (Figure 14).

The average straight-line velocities were influenced by extender ($p < .0001$), time ($p = .0012$) and treatment ($p = .0262$) (Table 2). There were also interactions between extender and time ($p = .0027$). Semen extended with BTS had higher straight-line velocities than Androhep Plus (41.94 ± 0.4 versus 37.57 ± 0.4). The average straight-line velocity traveled also decreased over time ($p = .0012$). Mean values were (41.66 ± 0.6) at 0 h and (38.65 ± 0.6) by 96 h. The interaction between extender and time was significant ($p < .0001$). Treatments extended with BTS decreased over time, while treatments extended with Androhep Plus increased at the h 24, decreased until h 48, and then increased to become no different than BTS at h 96 ($p = .7255$) (Figure 15). No other interactions were observed to be significant. When samples were examined, the two samples that had the coolest semen at 35°C and lowest temperature variations between extender traveled slower and significantly differed ($p < .05$) from the two samples which had the highest straight-line velocity values which were S35/E39, S37/E37 and S39/E39 (Figure 16). Sample S39/E35, with a four degree variation between semen and extender demonstrated a tendency to be different from S35/E37 ($p = .0669$), as did sample S35/E35 from S37/E39 ($p = .0722$).

There was a significant differences observed between extender as time progressed for the average curvilinear velocity ($p < .0001$) (Figure 17). Treatments extended with BTS decreased over time, while treatments extended with Androhep Plus increase at the h 24, decrease until h 48 and are no different than BTS at these times of analysis ($p > .05$). At h 96 samples extended in Androhep Plus increase to levels above those extended in BTS having means of 97.72 ± 2.0 and 90.78 ± 1.8 , respectively ($p = .0105$). There were no differences among treatments for average curvilinear velocity ($p = .2161$) (Figure 18).

Capacitation

There were no differences between the proportion of spermatozoa that were acrosome reacted among treatments ($p = .9919$). Time ($p = .0261$) significantly influenced the population of spermatozoa undergoing the acrosome reaction (Table 2). BTS has more spermatozoa displaying fluorescence patterns indicating the occurrence of an acrosome reaction ($26.68 \pm 1.8\%$) as compare to Androhep Plus ($20.49 \pm 1.8\%$). A significant interaction ($p = .0001$) between extender and time was observed for the proportion of acrosome reacted spermatozoa (Figure 19). Acrosome reacted spermatozoa were significantly different at hour 0 ($< .0001$) with samples extended in BTS having the greater percentage of reacted spermatozoa. Samples extended in BTS continue to decline as time progresses, as compared to Androhep Plus which continued to increase to have a tendency to differ with the greater percentage of acrosome reacted spermatozoa at hour 96 ($p = .0645$).

There were no differences observed between effects ($p > .05$) (Table 2). A difference was observed between extender over time on the number of spermatozoa displaying fluorescence indicating a capacitation reaction ($p = .0002$). Androhep Plus had a higher

number of cells that capacitated as compared to BTS at h 0, (53.66 ± 4.0 verses 27.29 ± 4.5) ($p < .0001$) (Figure 21). However, samples extended with Androhep Plus continue to decrease until h 96, when an apparent increase occurred. Although initially lower than Androhep Plus, capacitation increased at h 24, where it leveled off and Androhep Plus and BTS were no longer significantly different ($p > .05$) (Figure 21). There was no difference between the number of capacitated spermatozoa among treatments ($p = .9197$) (Figure 22). No other significant interactions were observed.

A significant interaction was observed between extender and time ($p = .0115$) (Figure 23). Uncapacitated and abnormal cells were similar at the 0 hour ($p > .05$) however samples extended in BTS decline at the 24th hour and remain steady until the 96th hour, but is significantly lower than Androhep Plus at the time points 24 and 48 ($p < .02$). However, at hour 96 Androhep declines to a proportion less than BTS, but is not significantly different. There was no differences observed between spermatozoa possessing abnormal or uncapacitated patterns of fluorescence among treatments ($p = .7537$) (Figure 24). Extender ($p = .0387$) and time ($p = .0820$) influenced the proportion of abnormal and uncapacitated spermatozoa (Table 2). Androhep Plus had the greater proportion of uncapacitated/abnormal spermatozoa (39.83 ± 1.3) as compared to BTS (34.83 ± 1.4). The proportion of uncapacitated and abnormal acrosomes is similar at the 0 and 24 hours ($p > .05$), but increases significantly at hour 48 ($p < .05$) before declining to the lowest proportion at the 96th hour. No other interactions were observed to be significant.

Discussion

The objective of this study was to determine the effect of temperature differences between semen and extender on spermatozoa during storage *in vitro* prior to insemination. It was found that there was no main effect for temperature variations up to four degrees Celsius, nearly ten degrees Fahrenheit, on the overall quality of semen. These results indicate that there is no need to follow the 1°C rule if semen and extender are between 35 and 39° C. Many of the estimates of semen quality demonstrate differences between the effects of extender and time, and their subsequent interaction. It should be noted that the present study tested temperatures between 35 and 39° C, and if extending at cooler temperatures, semen may be affected adversely.

Currently there exists a myriad of procedures to test the quality of semen. Flowers (2002) describes two major categories: (1) tests that determine a proportion of spermatozoa that have characteristics thought to be involved with fertilization and (2) tests that quantify the ability of an individual spermatozoa to express these advantageous characteristics. Evaluating normal morphology falls into the first category that indicates spermatozoan viability and fertility. It has been demonstrated that fertility is not affected when primary abnormalities as with head and tail do not exceed ten percent. In addition, when secondary abnormalities, such as proximal and distal droplets, do not exceed 20 percent fertility is not affected (Flowers, 1996). Another study found that the percentage of spermatozoa with normal morphology positively correlated to litter size (Xu et al., 1998). The present study showed observed that samples extended with Androhep Plus had a greater proportion of spermatozoa with normal morphology. This may suggest that Androhep Plus is able to

provide a better thermotolerance than BTS, thus preserving semen quality. Flowers (1997) found no differences in fertilizing capabilities between ejaculates that had >70% normal sperm morphology. None of the samples reached this benchmark, and although there were observed differences there is no physiological relevance.

The present study also looked at the proportion of intact acrosomes. The acrosome reaction is a prerequisite for zona pellucida penetration. It has been seen that acrosome-intact boar spermatozoa initiate binding to the zona pellucida, suggesting that semen comprised of spermatozoa without intact acrosomes may lead to reduced fertility (Fazeli et al., 1997). In addition, previous studies have reported improvements in farrowing rates as the proportion of spermatozoa exhibiting progressive motility or having normal acrosomes in an ejaculate increases (Flowers, 1997 as cited in Popwell et al. 1999). The present study observed no differences between sample treatments suggesting that farrowing rate, an important measure of fertility, would not be affected by the variations in temperature. Significant differences between extenders were observed ($p=.0002$). Androhep Plus had a higher percentage of spermatozoa with normal acrosomes, 96.43 ± 0.2 , as compared to BTS, 95.50 ± 0.2 . In addition, an interaction between extender and time presented itself. Androhep Plus and BTS are not significantly different at hour 0 and 24, however are significantly different at the 48th and 96th hour, $p=.0330$ and $p<.0001$, ending with Androhep Plus having the greater proportion of normal acrosomes (Figure 2). Flowers (1997) found no differences in fertilizing capabilities between ejaculates that had greater than seventy percent normal acrosome morphologies. The present study did not observe percentages of above thirty percent abnormal, which indicates that although the extenders differ, biologically these

negative aspects would not cause a reduction in fertility. With BTS being a short-term extender and Androhep Plus being considered a long-term extender, it can be expected that when the averages are pooled, samples extended in Androhep Plus will have higher proportions as compared to BTS.

Motility and mobility are also measurable tests that can be examined using computer assisted semen analysis software (CASA) and are useful in determining fertility in boars (Holt et al., 1997). CASA allows the quantification of the degree of individual spermatozoa to exhibit a given characteristic, which are also involved in fertilization. The study did not find motility or progressive motility to be significantly associated with fertility, and these are thought to be more associated with sperm viability, than fertility. When motility is greater than sixty percent, there is no clear relationship between *in vitro* and *in vivo* as estimates of fertility (Flowers, 1997). Motility can also indicate an active metabolism and integrity of the membrane (Johnson *et al.* 2000). When average motility and average progressive motility are examined in the present study, there are significant differences over time, between extender, and the interactions between time and extender. Over time, average motility and average progressive motility do decrease because of resource depletion and loss of viability. Average motility and average progressive motility were not influenced by the temperature variations of semen and extender. Boar spermatozoa are more susceptible to circular patterns of motion, which has been described as an indicator of cold shock (Eriksson et al., 2001). The average progressive motility in this study was not found to be influenced by temperature variations suggesting that the present population did not fall

subject to cold shock, despite the four degree Celsius temperature differences in two of the sample treatments.

When both extenders are looked at against one another over time, BTS has greater motility and progressive motility at all time points, with both extenders having a steady increase of motility and progressive motility from the 24th hour to the 96th hour. As a long-term (5-7 day) extender, Androhep Plus may contain Bovine Serum Albumin (BSA), which not only protects against cold, but also stimulates motility in a reverse manner, initially retarding motility in order to preserve the viability of the spermatozoa (Gadea, 2003). This was observed in the present test with samples extended in Androhep Plus initially displaying a decrease in motility before continuing to increase by the 5th day. Waberski et al. (1989) found that BSA stimulated the motility of spermatozoa during a six-day storage test. BSA is also thought to overcome the fluidity of the membrane, which may hinder the trans-membrane movement of calcium to stave off capacitation (Buhr, 1990). BTS, originally developed as a thawing medium and adapted for refrigerated use later, uniquely contains potassium chloride which preserved the sodium potassium pump and avoid intracellular potassium depletion, which is related to a loss in sperm motility, which may explain the greater motility seen in this experiment. The initial difference between BTS and Androhep Plus may also be explained by the concentration of glucose present in the media. BTS contains 37.0 g/L of glucose, while Androhep Plus contains 26.0 g/L. The additional energy in the BTS may increase the initial motility of the spermatozoa, resulting in the greater motility observed for the first three days. BTS is commercially used before three days, so this initial increase in motility may be advantageous for its use.

The average motility of individual boars appeared to be individually affected by extender choice as well (Table 3). The large standard errors seen with Boar 240 suggest there is variation occurring between samples extended with Androhep Plus. These types of relationships are demonstrated between many of the boars in the study. Between-boar variation is widely seen when samples are cryopreserved but in these situations, immense stress is placed on the samples with the current processes for freezing and thawing semen. The current study suggests that subtle differences between boars may be present as well. Androhep Plus and BTS have different components that are advantageous in their own respect. For example, the semen from boar 240 may have benefited from the small amount of potassium present in the BTS allowing for the motility to be better preserved. Conversely, the HEPES thought to be in Androhep Plus may have benefited the semen from boar 112 (Johnson et al. 2000; Gadea, 2003).

The mobility estimates of straight-line distance and velocity and curvilinear distance were significantly different ($p < .009$). The mobility estimates were also the only estimates of semen quality that were influenced by temperature variations between sample and extender. The parameters were also impacted by extender at the various time points examined. The present study examined curvilinear velocity and distance, which takes into account deviations in spermatozoan head movement and straight-line velocity and distance, which is the distance between the beginning and end of the sperm tract. Holt et al. (1997) saw increased straight-line and curvilinear velocities equated to greater litter sizes indicating increased fertility. Individual samples treatment also saw significant differences between straight-line distance and velocity and curvilinear distance. The two samples that were lower

than the other samples were S35/E35 and S35/E37. This suggests that if samples begin too cool are within two degrees of one another that semen quality could be affected. However, currently there are no present studies that have examined the relationship between the measures of velocity and distance and *in vivo* measurements of fertility. Interestingly the S35/E39 that has a four-degree difference was not adversely affected. This defies the accepted rule, by having a better result than when the rule is followed. This is beneficial from a commercial standpoint where it may be difficult to keep semen and extender within such a narrow range with one another. In fact, a 4-degree difference allows for some “wobble” room in case samples begin to waver from one another. Samples could also be affected by various components thought to be in Androhep Plus. Androhep Plus is thought to contain BSA, which protects against the diluents effect and may be beneficial to samples with 35° and 37° semen.

When extender choice is examined for the straight-line distance and velocity and curvilinear distance, each saw Androhep Plus having increased values at the 96th hour, while BTS continued to steadily decline as time progressed. Androhep Plus may have had lower values than BTS because Androhep Plus is thought to initially reduce motility and then allowing it to rise. While this phenomenon was only observed when individual boars were looked at, it appears to have manifested itself in these mobility estimates allowing them to be initially reduced before they continue to rise until the 96th hour, the time where in a commercial setting, samples extended with Androhep would be used.

When capacitation was examined, both extenders initially saw increased proportions of uncapacitated spermatozoa that decreased by the 96th hour, however the amount of

capacitated spermatozoa had comparable numbers to that of the proportion of uncapacitated spermatozoa. When individual extenders were examined, Androhep Plus had the amount of uncapacitated spermatozoa increasing until the 48th hour, before falling to levels below BTS at the 96th hour. The proportion of capacitated spermatozoa extended with BTS is initially small, increases to levels above Androhep Plus at the 24th hour, and then remains constant. BTS does and Androhep Plus may contain ethylenediaminetetraacetic acid or EDTA, which is a chelating agent that blocks the action of calcium as a mediator of sperm capacitation and the acrosome reaction. With significant differences occurring only between the interaction of extender and time, this suggests that the mixing semen and extender at temperatures up to four degrees Celsius apart has no effect on cells under going early capacitation reactions.

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Table 2. Summary of Independent and Dependent Variables and their P-values

Dependent Variables	Independent Variables						
	Extender	Time	Sample	Extender * Time	Time * Sample	Extender * Sample	Extender * Time * Sample
% Normal Acrosomes	.0002	<.0001	.5670	.0006	.8607	.4149	.6215
% Normal Morphology	.0097	.9544	.9999	.9611	1.0000	1.0000	1.0000
Average Motility	<.0001	<.0001	.4730	<.0001	.9424	.3483	.4988
Average Progressive Motility	<.0001	<.0001	.3642	<.0001	.9996	.1884	.8597
DSL	<.0001	.0084	.0060	.0009	.9480	.4913	.9477
DCL	<.0001	.2597	.0083	<.0001	.4179	.6737	.8033
VSL	<.0001	.0012	.0262	.0027	.9868	.8400	.9983
VCL	.0687	.4229	.2092	<.0001	.8352	.9475	.9881
% Acrosome Reacted	.0261	.5031	.9970	.0001	1.0000	.9847	.9999
% Capacitated	.4771	.3813	.9586	.0002	.9881	.9119	.8952
% Abnormal/Un-capacitated	.0387	.0820	.9404	.0115	.9145	.7011	.7865

Table 3. Individual boar effects on extender for dependent variables average motility and average progressive motility, LSMEANS \pm S.E

Boar ID	Average Motility		Average Progressive Motility	
	Androhep	BTS	Androhep	BTS
111	69.7 \pm 1.3 ^a	78.33 \pm 1.3 ^b	54.43 \pm 1.9	57.55 \pm 1.9
112	84.61 \pm 0.6 ^a	78.16 \pm 0.6 ^b	68.9 \pm 0.9 ^a	64.21 \pm 0.9 ^b
151	64.26 \pm 1.4 ^a	74.64 \pm 1.3 ^b	32.49 \pm 1.4 ^a	50.86 \pm 1.4 ^b
156	73.3 \pm 2.0 ^a	72.33 \pm 2.0 ^a	60.08 \pm 2.3 ^a	49.72 \pm 2.3 ^b
240	79.62 \pm 1.1 ^a	83.63 \pm 1.1 ^b	60.51 \pm 1.5 ^a	59.39 \pm 1.5 ^a
12505	74.89 \pm 1.4 ^a	79.85 \pm 1.4 ^b	59.39 \pm 1.7 ^a	64.78 \pm 1.7 ^b
13909	52.18 \pm 1.4 ^a	75.00 \pm 1.4 ^b	31.88 \pm 1.3 ^a	48.26 \pm 1.3 ^b
158	27.39 \pm 2.7 ^a	69.26 \pm 2.9 ^b	13.94 \pm 2.4 ^a	41.86 \pm 2.6 ^b

^{a,b} Columns with differing superscript in a row indicate significant differences between extender for variables, $p < .05$

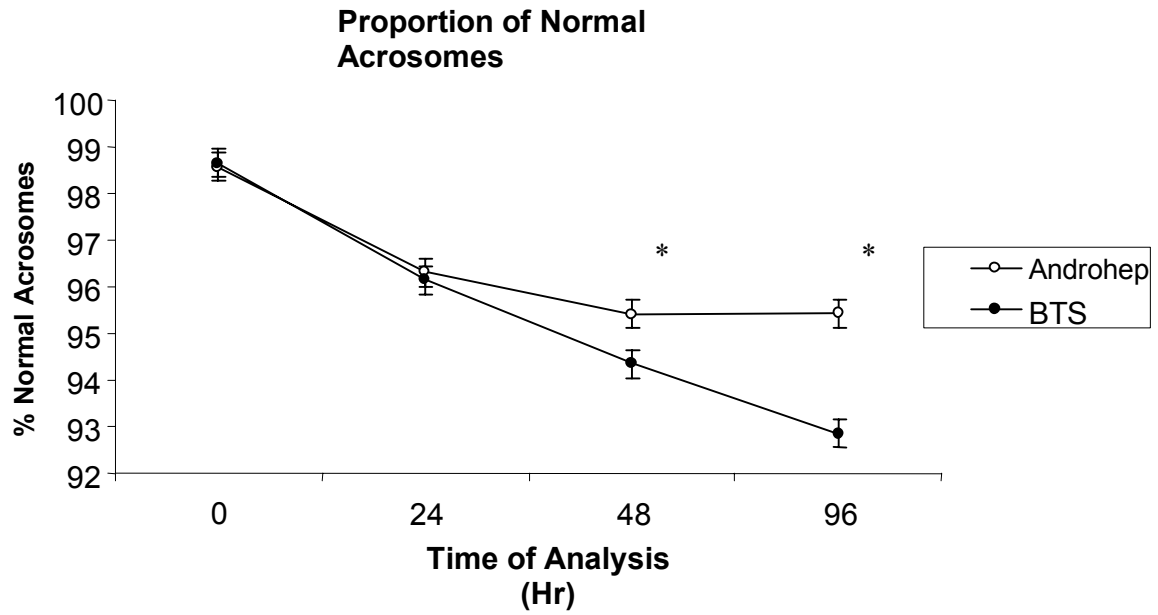


Figure 2. Mean proportions of normal acrosomes between extenders over time

* Data points with a star indicate significant differences between extender for variables,

$p < .05$

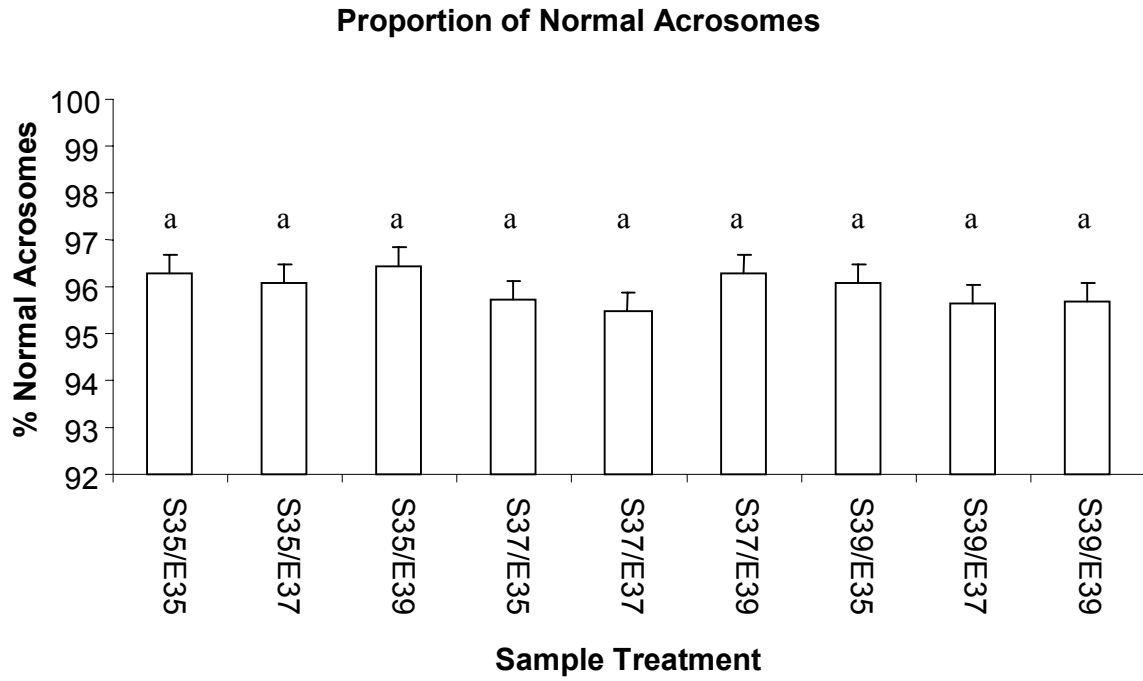


Figure 3. The Proportion of Normal Acrosomes between Treatments of Samples

^{a,b} Points with differing superscripts indicate significant differences between extender for variables, $p < .05$

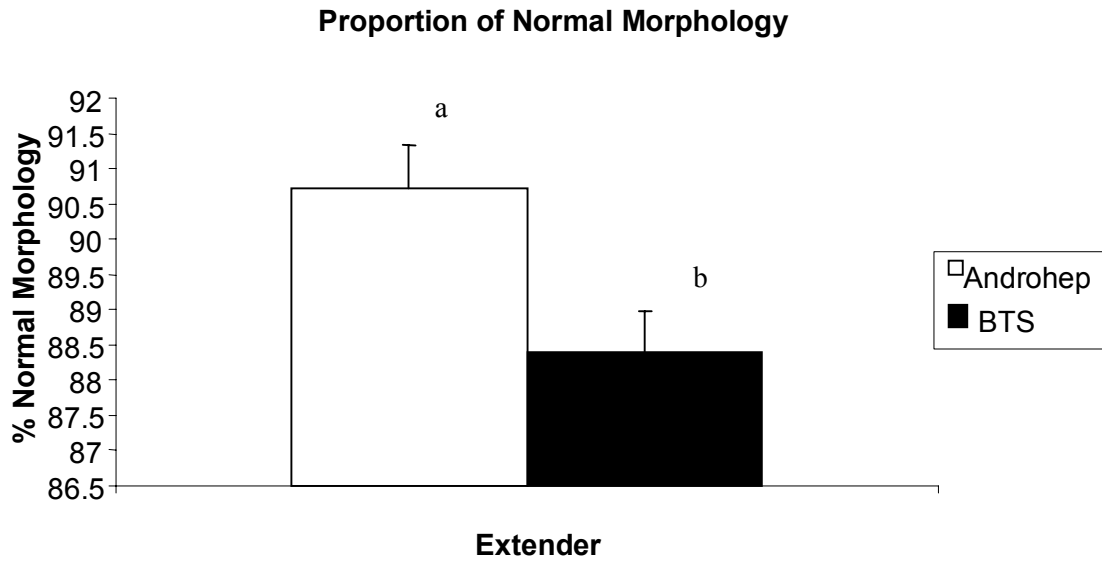


Figure 4. Mean Proportion of Total Number of Normal Morphology between Androhep Plus and BTS

^{a,b} Points with differing superscripts indicate significant differences between extender, $p < .05$

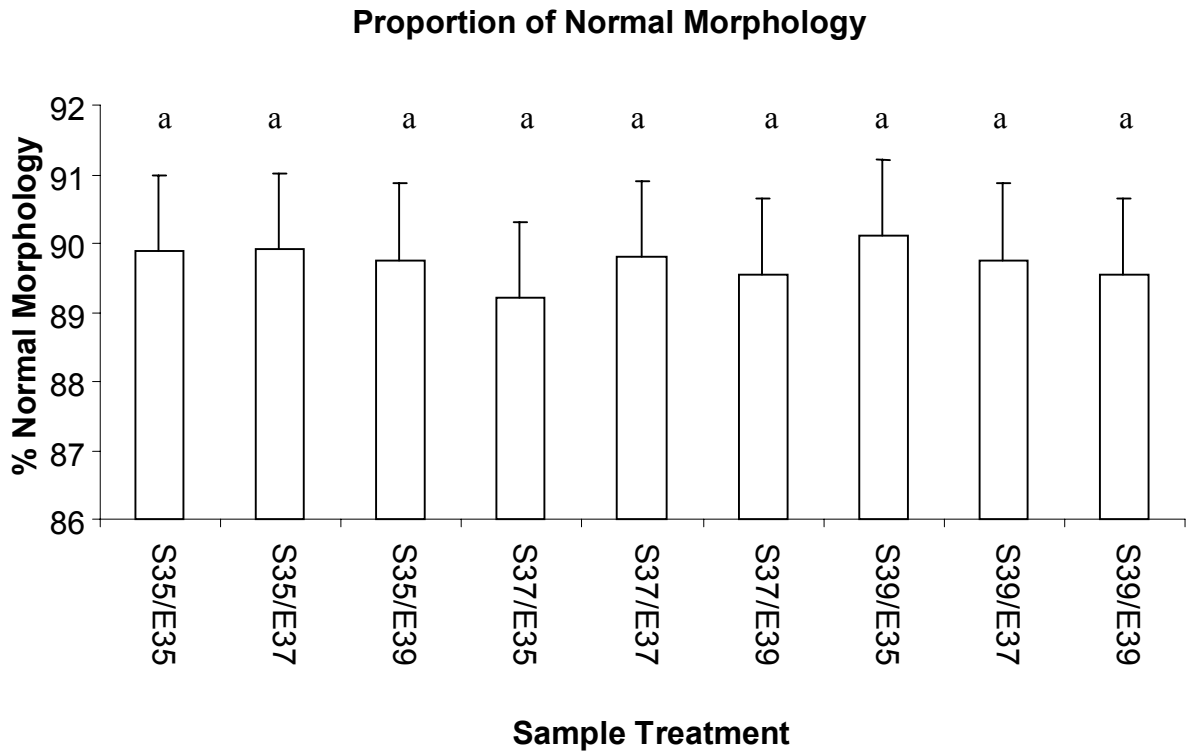


Figure 5. Mean Proportion of Total Number of Normal Morphology between Treatment of Samples

^{a,b} Points with differing superscripts indicate significant differences between extender, $p < .05$

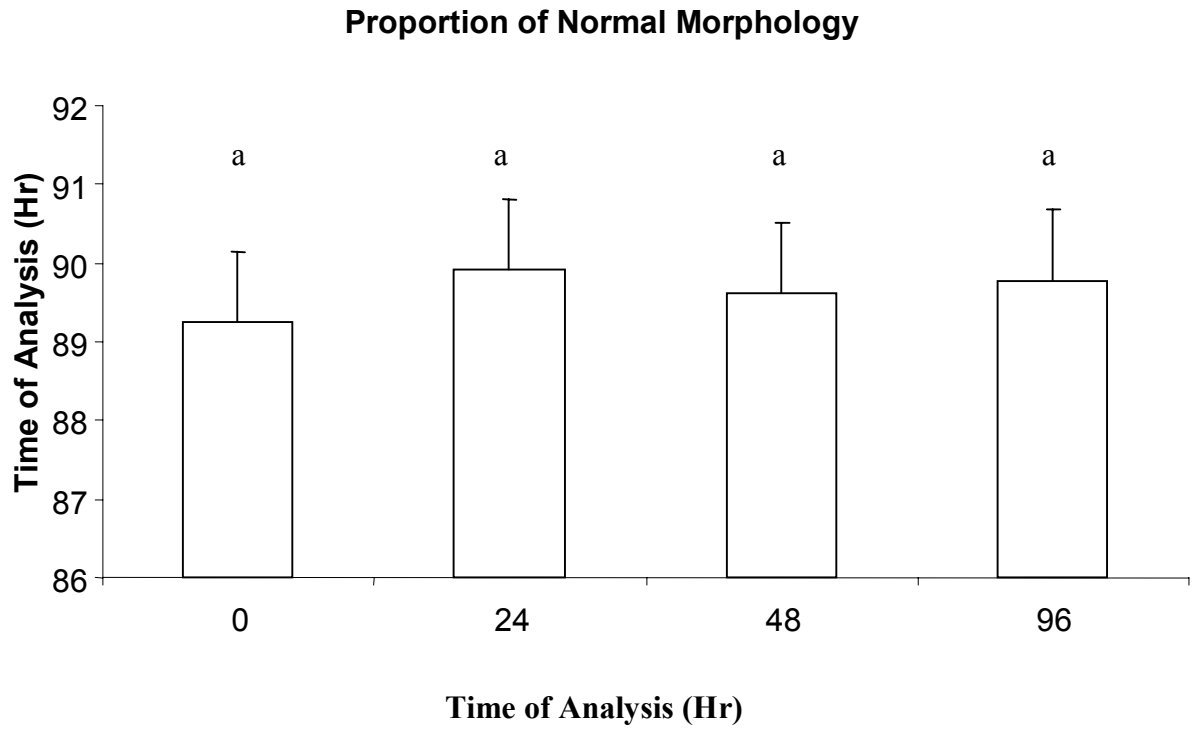


Figure 6. Mean Proportion of Total Number of Normal Morphology between Times of Analysis

^{a,b} Points with differing superscripts indicate significant differences between time of analysis, $p < .05$

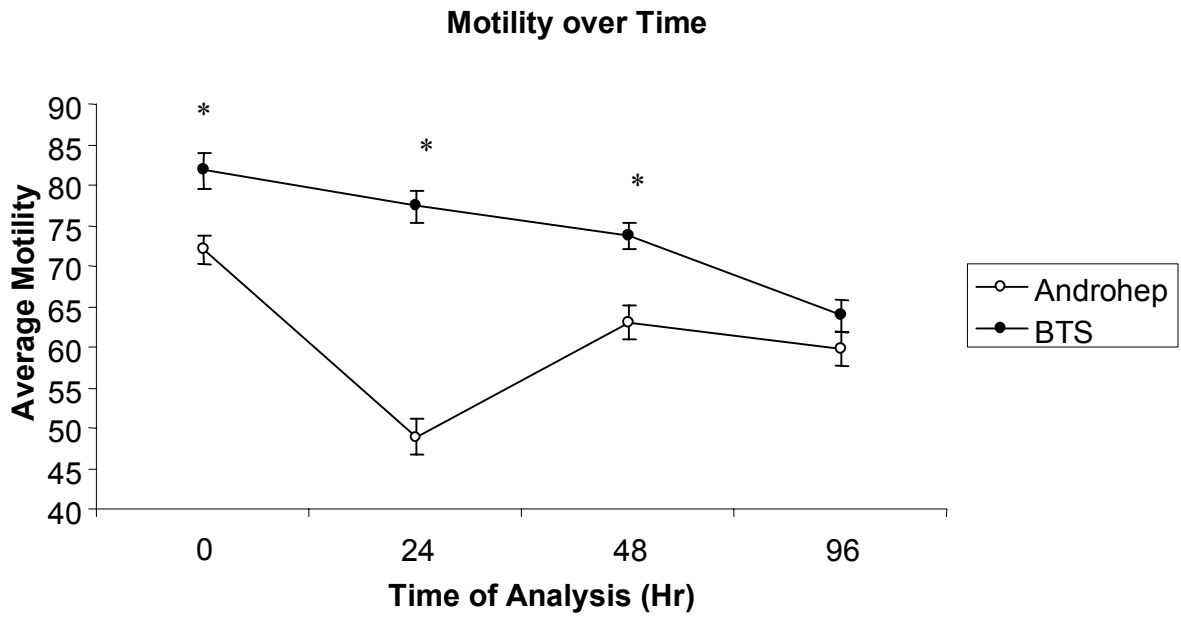


Figure 7. Average Motility between Extenders over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

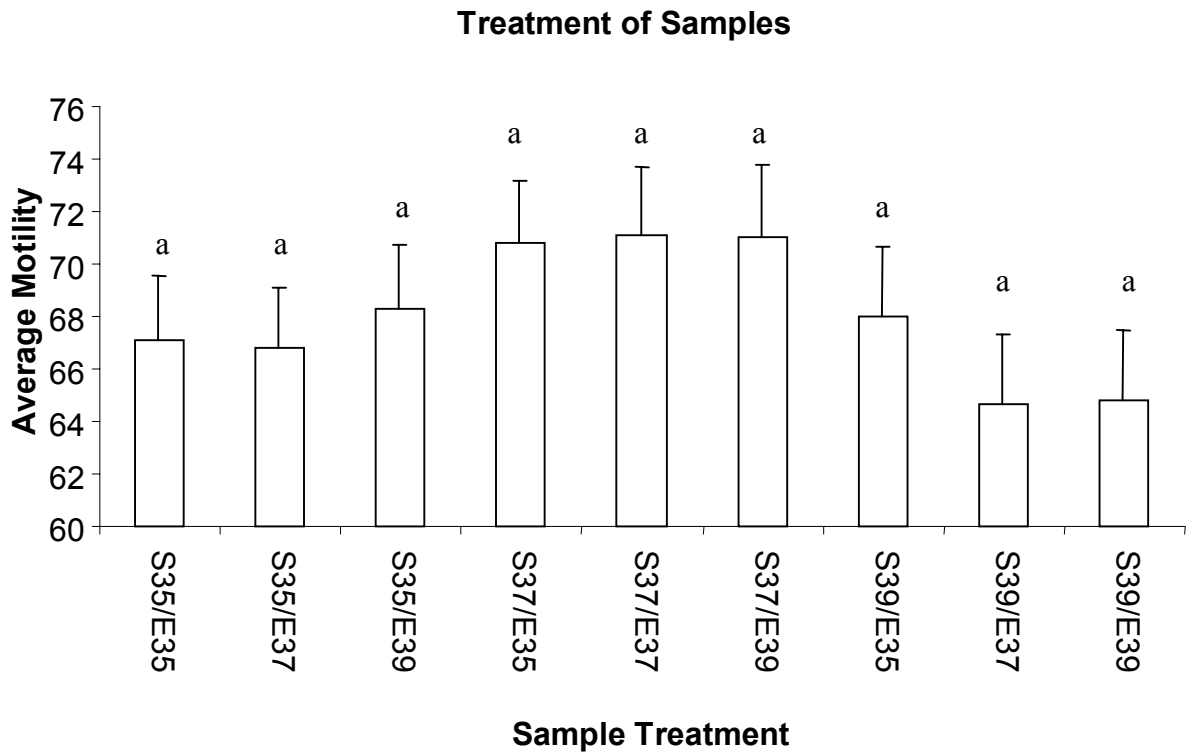


Figure 8. Average Motility for Treatment of Samples

^{a,b} Points with differing superscripts indicate significant differences between sample treatment, $p < .05$

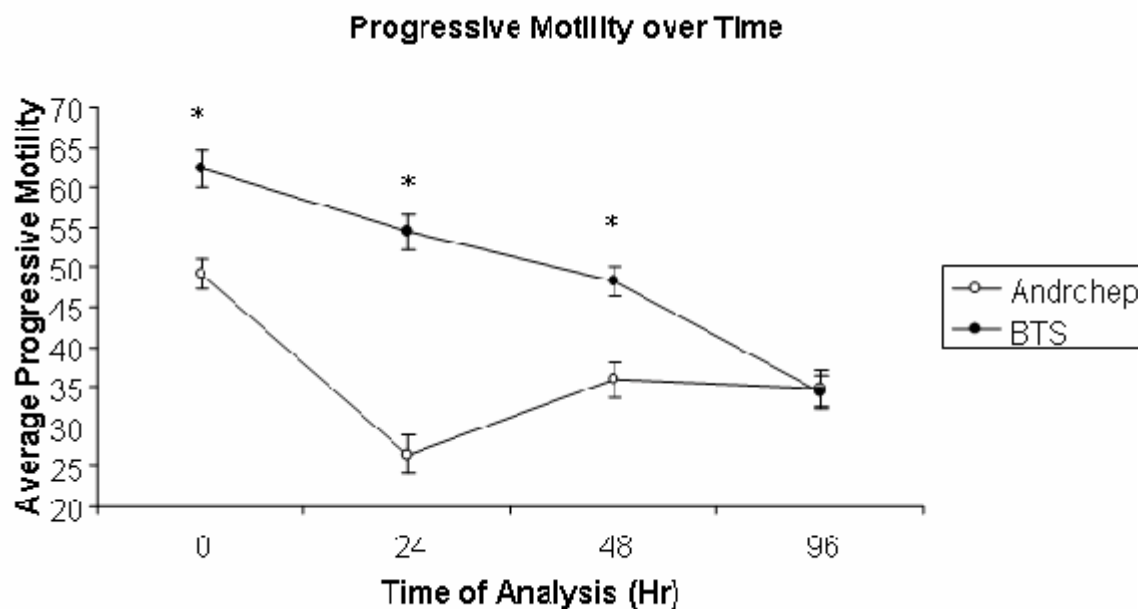


Figure 9. Average Progressive Motility between Extenders over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

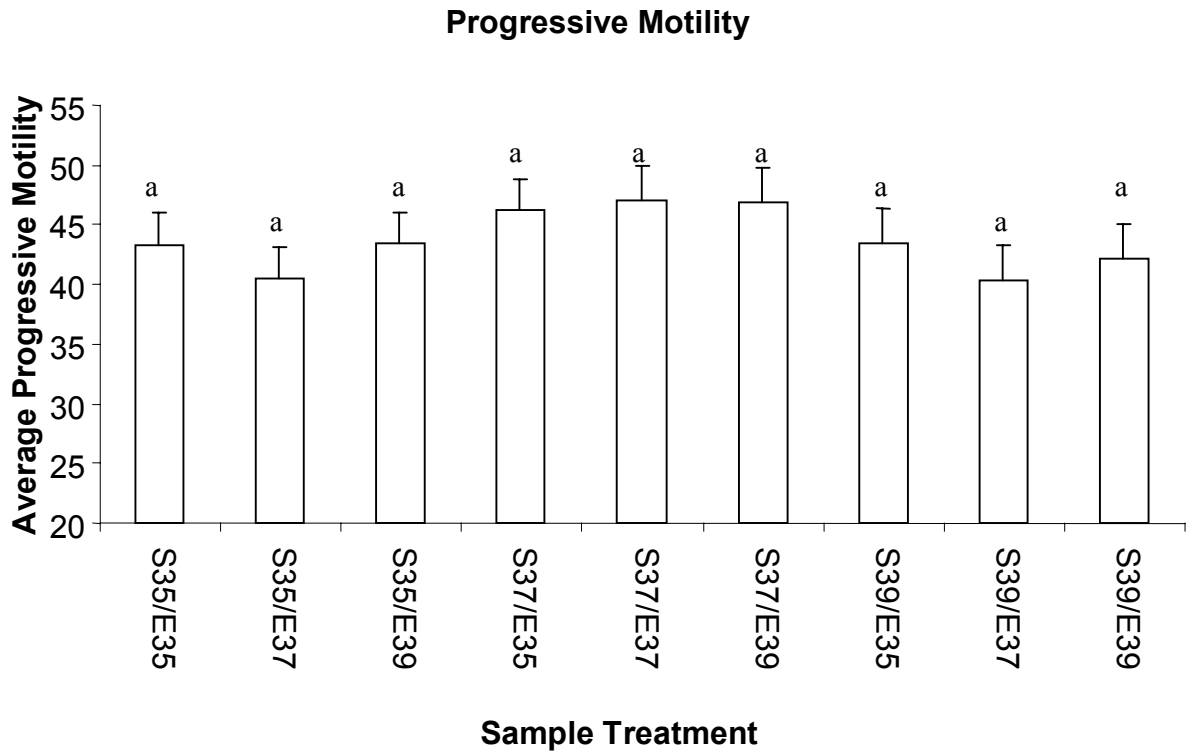


Figure 10. Average Progressive Motility for Treatment of Samples

^{a,b} Points with differing superscripts indicate significant differences between sample treatment, $p < .05$

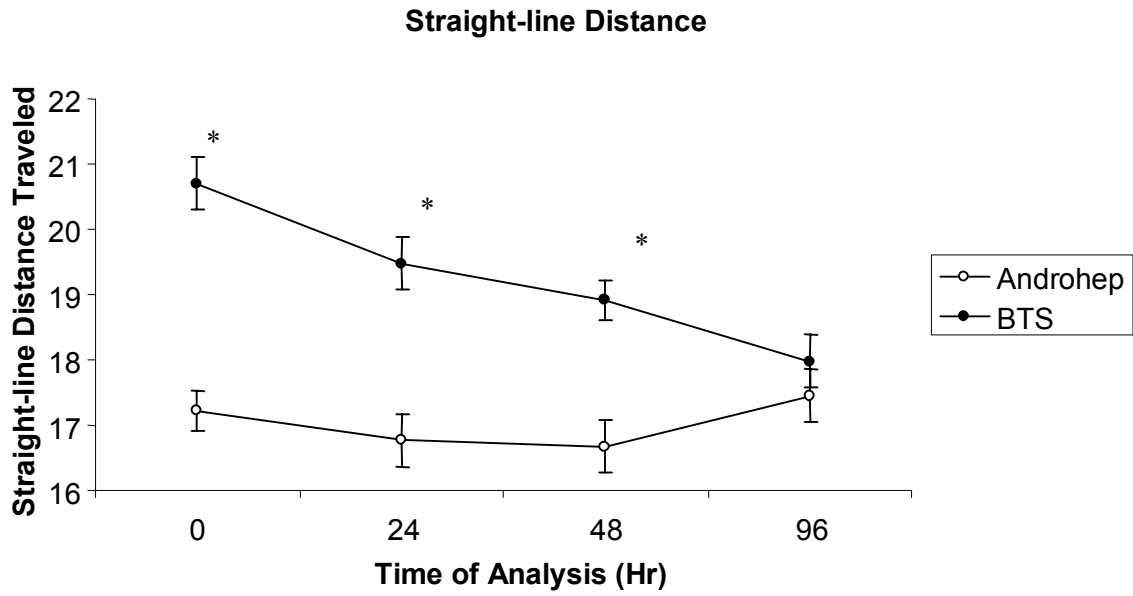


Figure 11. Mean Straight-line Distance Traveled between Extenders over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

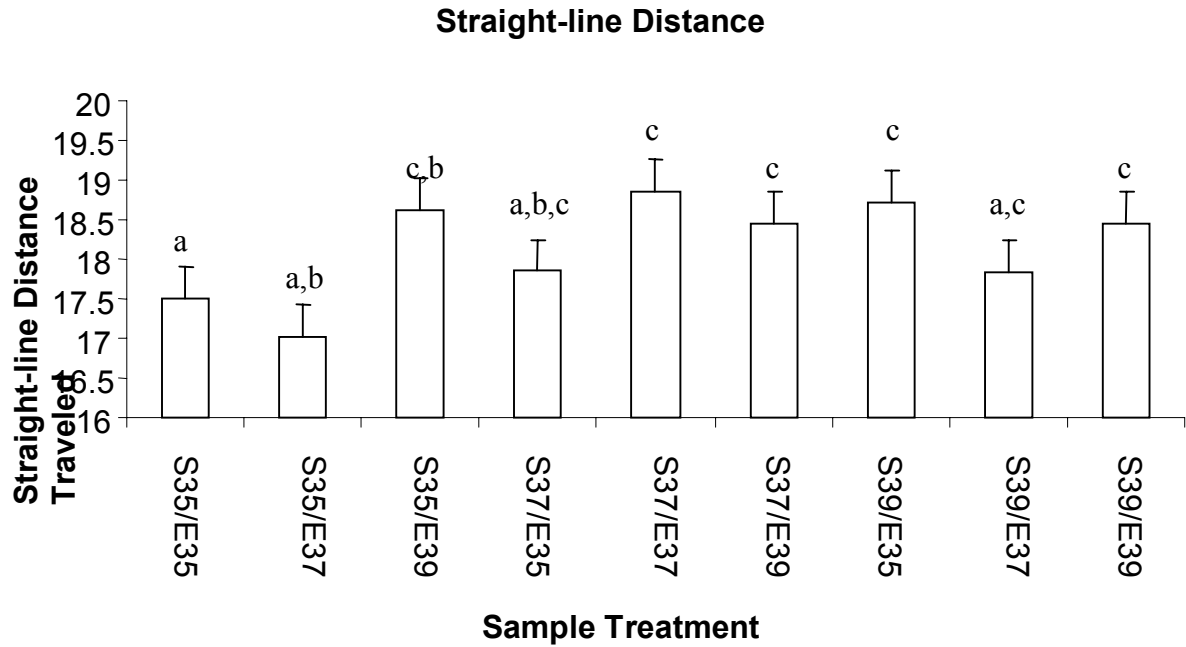


Figure 12. Mean Straight-line Distance Traveled between Treatment of Samples

^{a,b} Points with differing superscripts indicate significant differences between sample treatment, $p < .05$

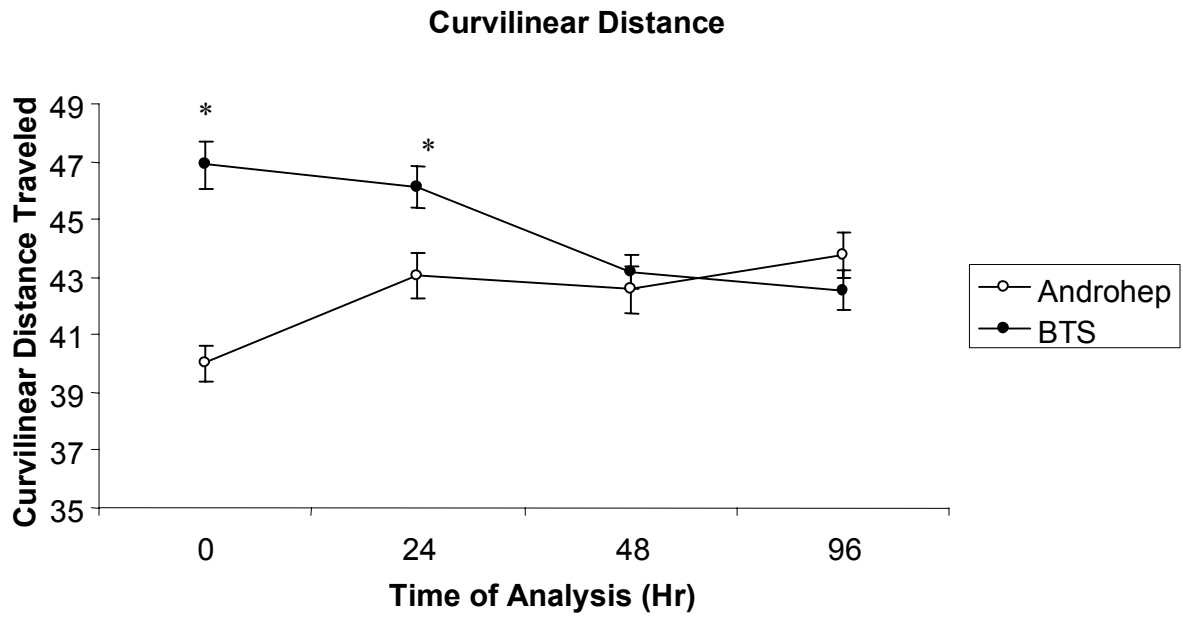


Figure 13. Mean Curvilinear Distance Traveled between Extenders over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

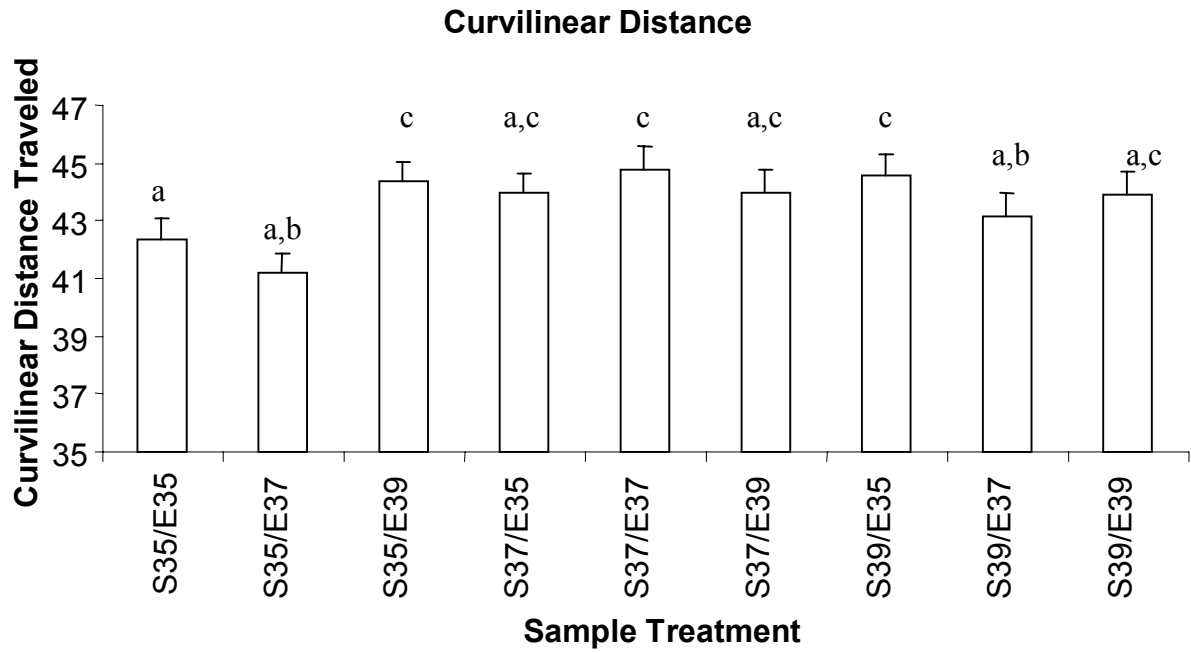


Figure 14. Mean Curvilinear Distance Traveled between Sample Treatments

^{a,b} Points with differing superscripts indicate significant differences between sample treatment, $p < .05$

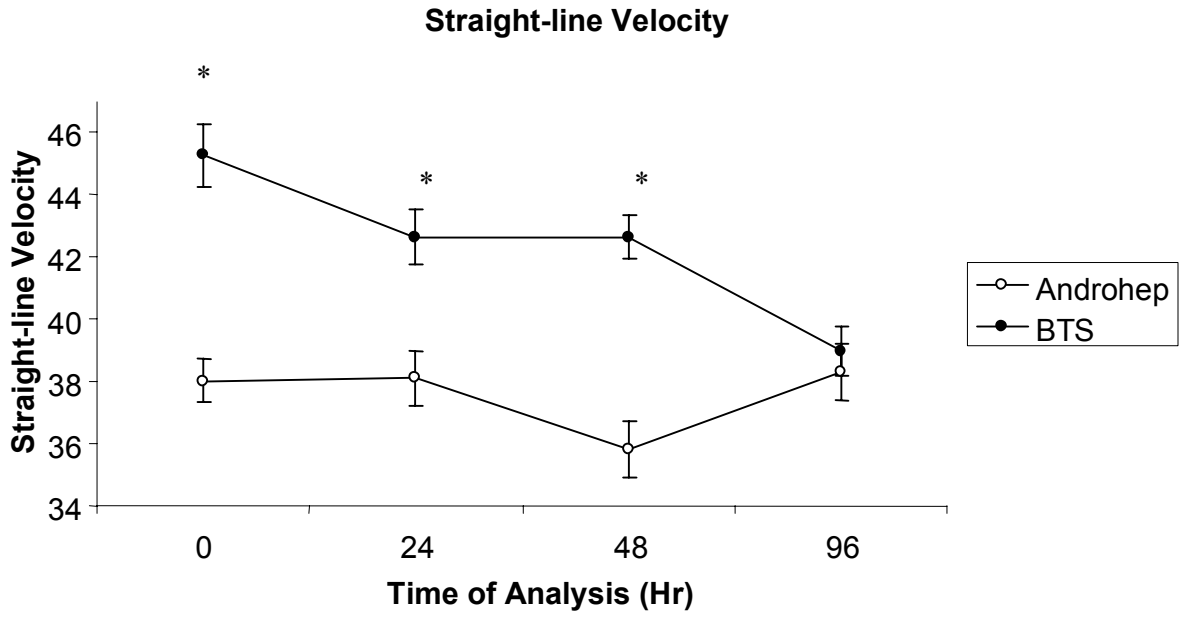


Figure 15. Mean Straight-line Velocity between Extenders over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

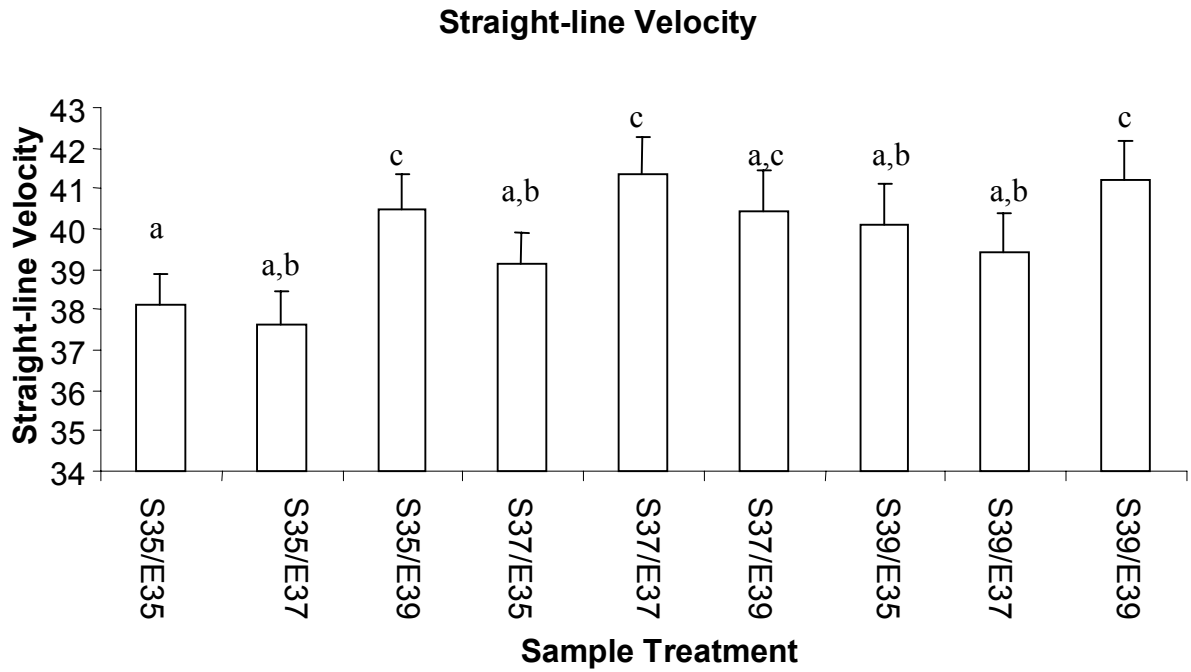


Figure 16. Mean Straight-line Velocity between Treatment of Sample

^{a,b} Points with differing superscripts indicate significant differences between sample treatment, $p < .05$

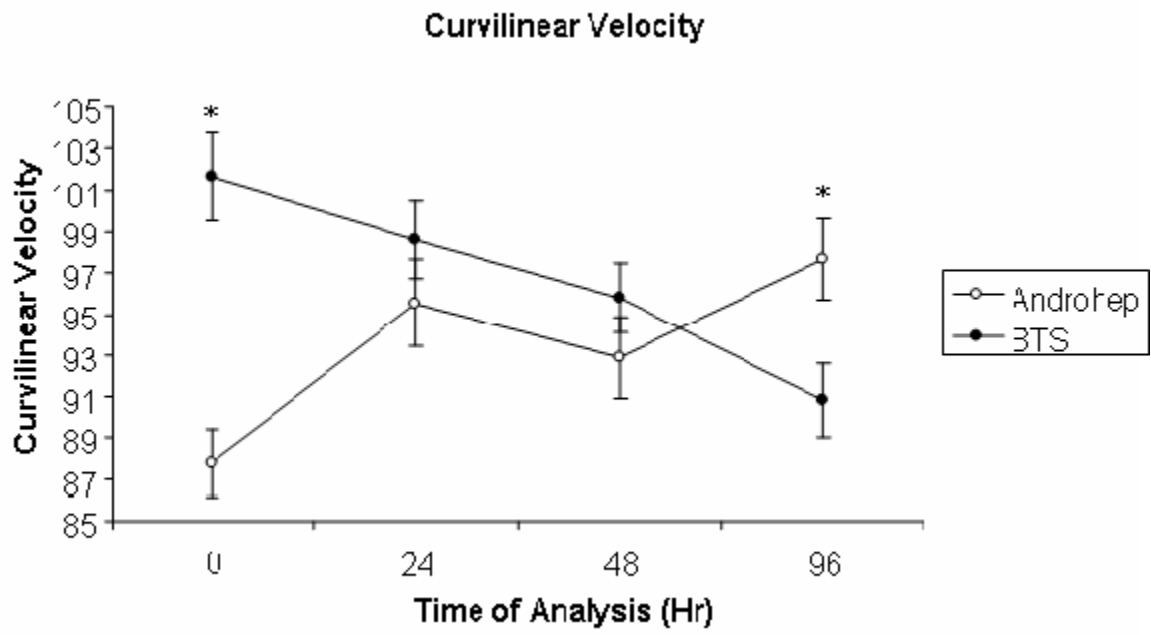


Figure 17. Mean Curvilinear Velocity between Extender over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

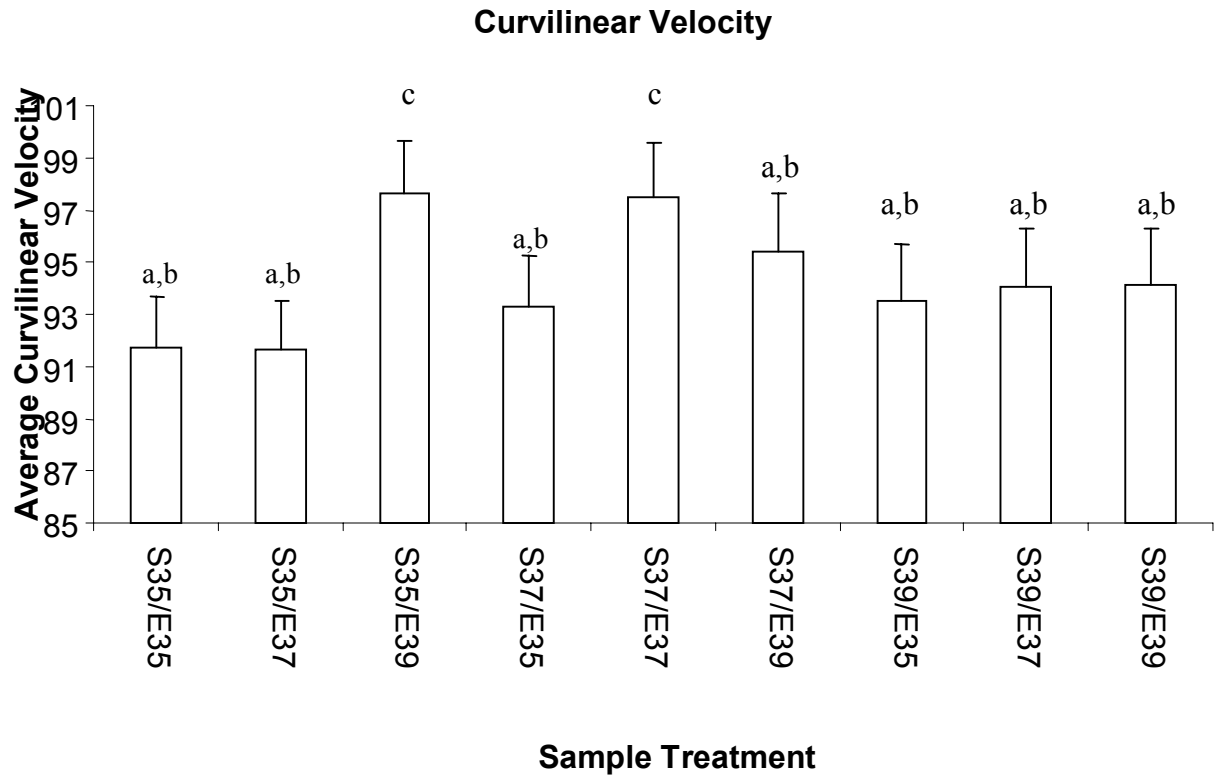


Figure 18. Mean Curvilinear Velocity between Treatment of Samples

^{a,b} Points with differing superscripts indicate significant differences between sample treatment, $p < .05$

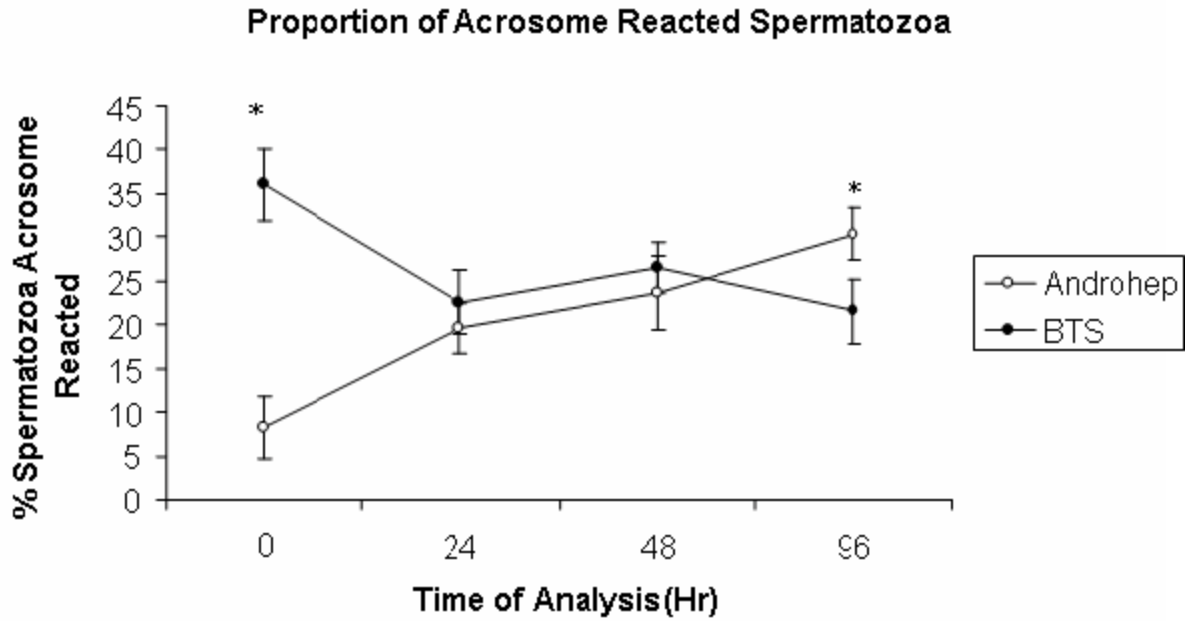


Figure 19. Proportion of Acrosome Reacted Spermatozoa between Extenders over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

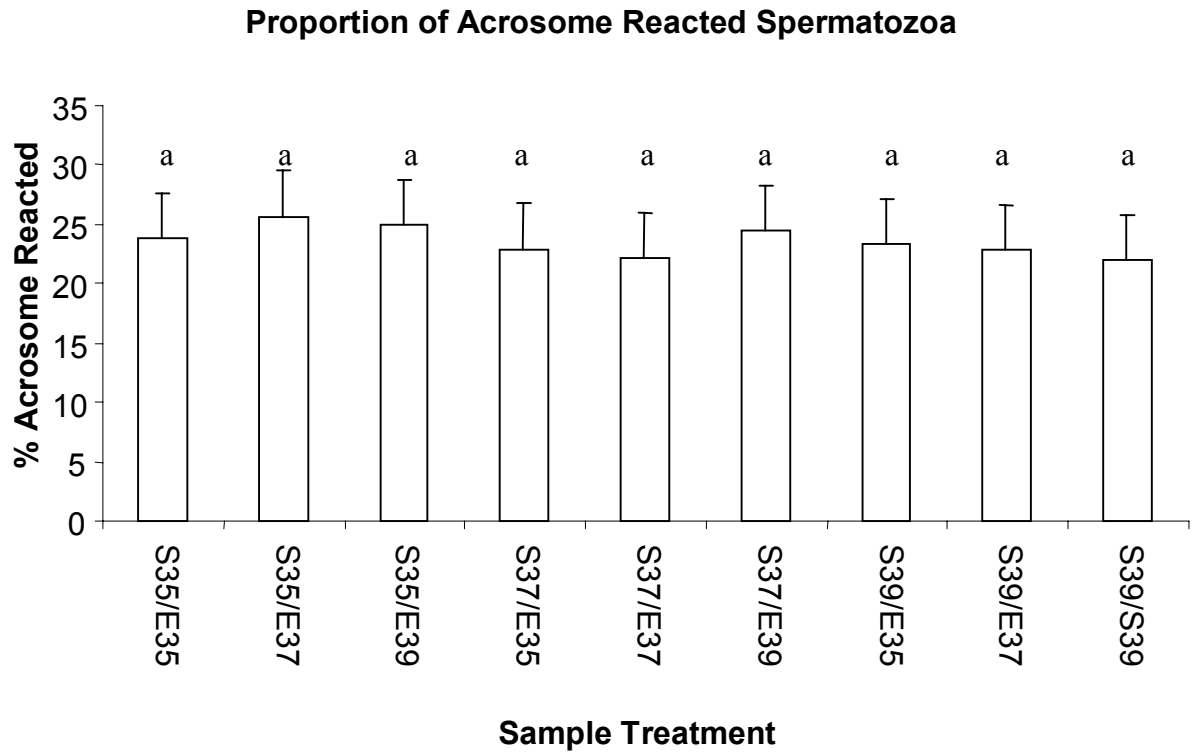


Figure 20. Proportion of Acrosome Reacted Spermatozoa between Sample Treatments

^{a,b} Sample Treatments lacking a common subscript differ significantly, $p < .05$

Proportion of Capacitated Spermatozoa

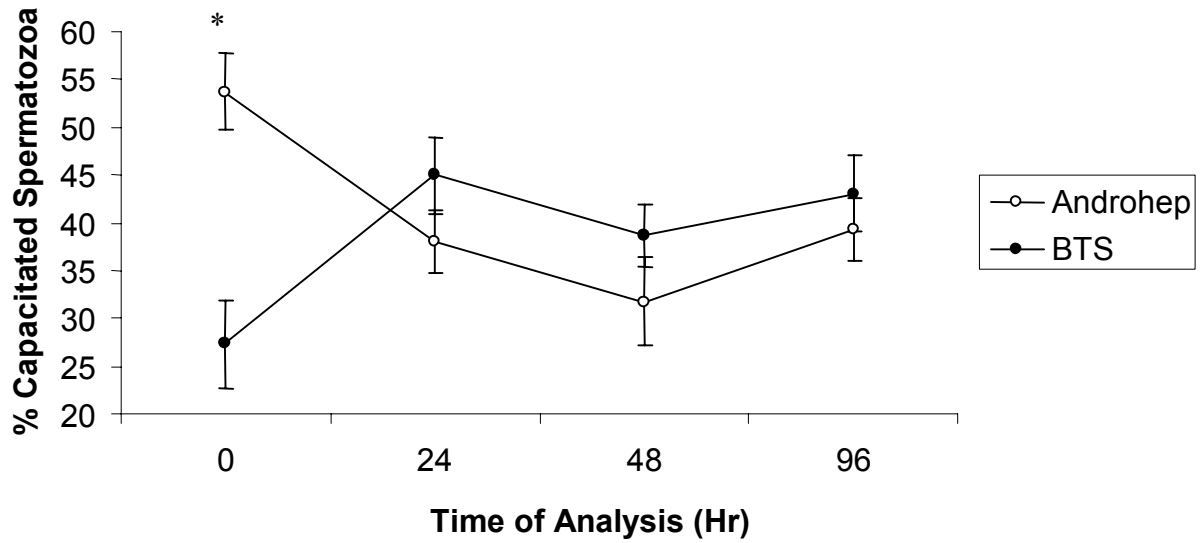


Figure 21. Proportion of Spermatozoa Demonstrating the Fluorescence Pattern Indicating Capacitation between Extenders over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

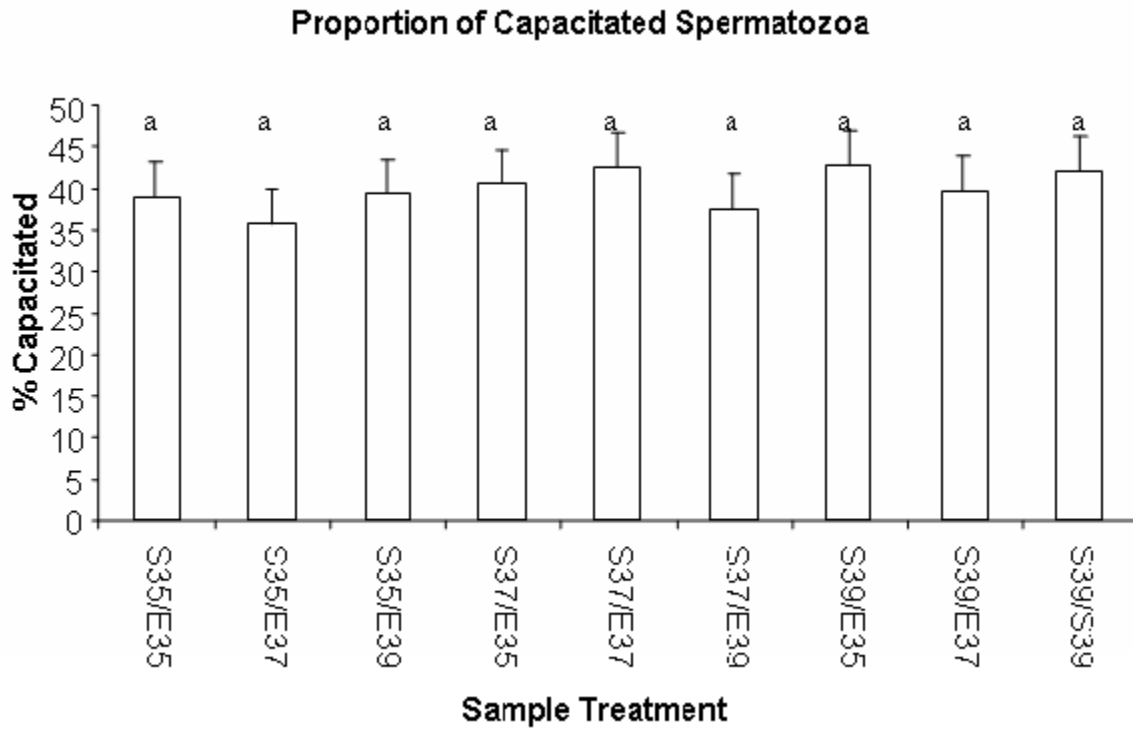


Figure 22. Proportion of Spermatozoa Demonstrating the Fluorescence Pattern Indicating Capacitation per Treatment Sample

^{a,b} Sample Treatments lacking a common subscript differ significantly, $p < .05$

Proportion of Spermatozoa with Abnormal and Uncapacitated Patterns

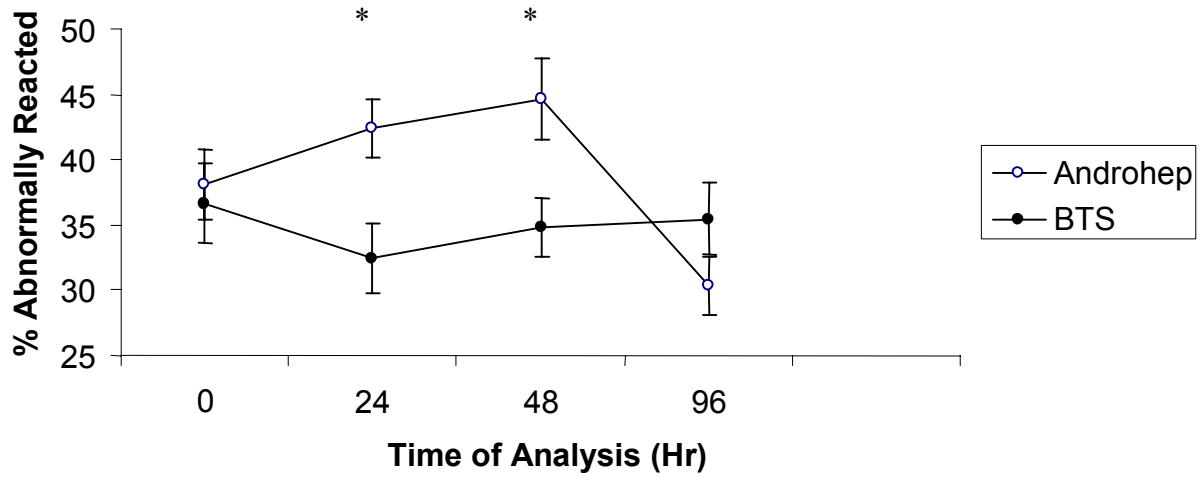


Figure 23. Proportion of Spermatozoa Demonstrating both Abnormal and Uncapacitated Patterns between Extenders over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

Proportion of Uncapacitated and Abnormal Spermatozoa

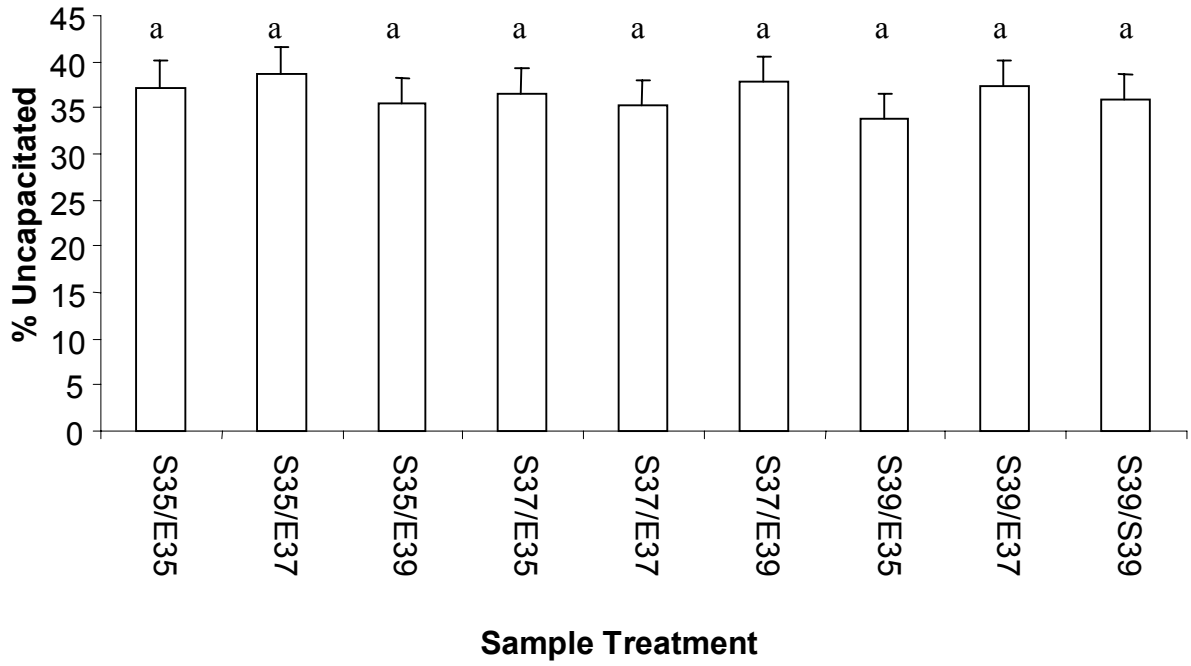


Figure 24. Proportion of Spermatozoa Demonstrating both Abnormal and Uncapacitated Patterns per Sample Treatment

^{a,b} Sample Treatments lacking a common subscript differ significantly, $p < .05$

**EFFECTS OF RATE OF COOLING ON THE OVERALL QUALITY OF BOAR
SEMEN**

Introduction

After collection and extension, insemination doses are typically between 35 and 39°C. In contrast, the recommended storage temperature for boar semen is between 15 and 18°C. Consequently, extended semen has to undergo a decrease of 20°C between extension and storage. The commercial swine industry currently uses two different approaches to accomplish this. Both were developed to avoid damage to the sperm cell otherwise described as “cold shock.” The first is considered as a “slow” cool and involves allowing semen to cool to room temperature around 25°C first before it is placed into a semen storage unit at 16 to 18°C. The proponents of this process believe that if the semen is allowed to equilibrate at room temperature before being placed into the semen storage unit, less damage is likely to occur. The other approach involves taking extended semen at temperatures above 35°C and placing it directly in a 16-18°C semen storage unit. The effects of cooling rate on semen quality have been well documented for frozen semen. However, the temperature ranges over which semen must be cooled are quite larger for frozen than fresh semen (Maxwell and Johnson, 1997). Consequently, it is also reasonable to speculate that cooling rates for fresh semen after extension may also influence its quality. These effects could be immediate as well as latent. This experiment aims to assess the effect cooling rate has after extension on the quality of fresh boar semen.

Materials and Methods

Experimental Animals

Three mature boars (26 ± 2 months of age; 314 ± 10 kg) were used in this study. The boars were the offspring of Yorkshire x Large White x Landrace sows bred to Duroc x Hampshire x Spot boars. 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 during the summer months was provided by 18, 5000 cfm stirring fans evenly spaced throughout the barn and a thermostatically controlled dripper system. Boars had ad libitum access to water via water nipples and received 2.7 kg of a corn and soybean meal based diet formulated to meet all the nutritional requirements of adult breeding boars (NRC, 1998). Prior to the onset of the study, boars were maintained on a weekly collection schedule. All procedures performed on boars were approved by the N.C.S.U. Institutional Animal Care and Use Committee (06-036-A).

Experimental Design

Each boar was collected three times between July and September 2008. Each ejaculate from each boar was extended with a 3-day extender, Beltsville Thawing solution (BTS; Minitube of America, Verona, WI), and with a 5-day extender, Androhep Plus (Minitube of America, Verona, WI). After collection, each ejaculate was transported to the Swine Reproduction laboratory at North Carolina State University, approximately 7 miles from the Swine Educational Unit. Once in the laboratory, the neat semen was placed into a water bath set at 37°C until extension. Twelve aliquots of the neat semen were placed in 80

mL tubes (Mini Tüb, Tiefenbach, Germany). Six of the aliquots will serve as vessels for temperature readings, and will be replicates of the other six treatments, which samples for analysis will be extracted from. Six of the aliquots of neat semen were extended with BTS, and the other six extended with Androhep Plus. Three rates of cooling were utilized: (1) a fast rate where samples were placed into a 12°C incubator (Ambi-Hi-Lo Chamber, Lab-line Instruments, Inc., Melrose Park, IL) for one hour before being placed into a semen storage unit set at 17°C, (2) a medium rate where after extension samples were immediately placed into the semen storage unit, and (3) a slow rate where after extension samples were allowed to sit at room temperature for two and a half hours before being placed into the semen storage unit. This resulted in six experimental treatments (Table 4). The samples with the medium rate of cooling were considered the control, being immediately cooled 19°C. The samples cooled at a fast rate are placed in an environment 25°C cooler than previously exposed. The samples with a slow rate of cooling are cooled in an environment 12°C cooler than previously exposed. After cooling was completed per treatment group, all treatments were placed in an environmentally controlled semen storage unit (Minitube of America, Verona, WI), and maintained at 17°C for the duration of the study. Samples were gently agitated daily to prevent sedimentation.

For each ejaculate, estimates of semen quality were obtained on the neat semen prior to extension; immediately after extension (time 0); and at 4, 24, 48, and 96 h after collection. At 0, 4, 24, 48, and 96 h the proportion of spermatozoa with normal acrosome morphology; the proportion of spermatozoa with normal head and tail morphology (including the presence of proximal, distal and translocated cytoplasmic droplets); the proportion of motile

spermatozoa; proportion of spermatozoa exhibiting progressive forward motility; the average straight-line velocity (VSL) of motile spermatozoa; the average curvilinear velocity of motile spermatozoa (VCL); the average straight-line distance traveled by motile spermatozoa (DSL); the average curvilinear distance traveled by motile spermatozoa (DCL); and the capacitation status of spermatozoa in the ejaculate were assessed (Figure 25). At hours 0, 1, 2, 3, 4, 24, 48, 72 and 96 temperature readings via a digital thermometer (Dual Chamber, Fisher Scientific, Atlanta, GA) were taken. Ambient temperatures with the samples cooled fast and slow were recorded with data loggers (2400 Baud Communications, Ertco, West Patterson, NJ), which recorded the temperature every 15 minutes for the first 24 hours.

Table 4. Formation of Experimental Treatments by Combining Neat Semen Extender with Rate of Cooling Maintained at Various Temperatures.

Sample Treatment		
	Extender	Cooling Rate
1	BTS	Fast
2	BTS	Medium
3	BTS	Slow
4	Androhep	Fast
5	Androhep	Medium
6	Androhep	Slow

0 Hour

4 Hour

24 Hour

48 Hour

72 Hour

96 Hour

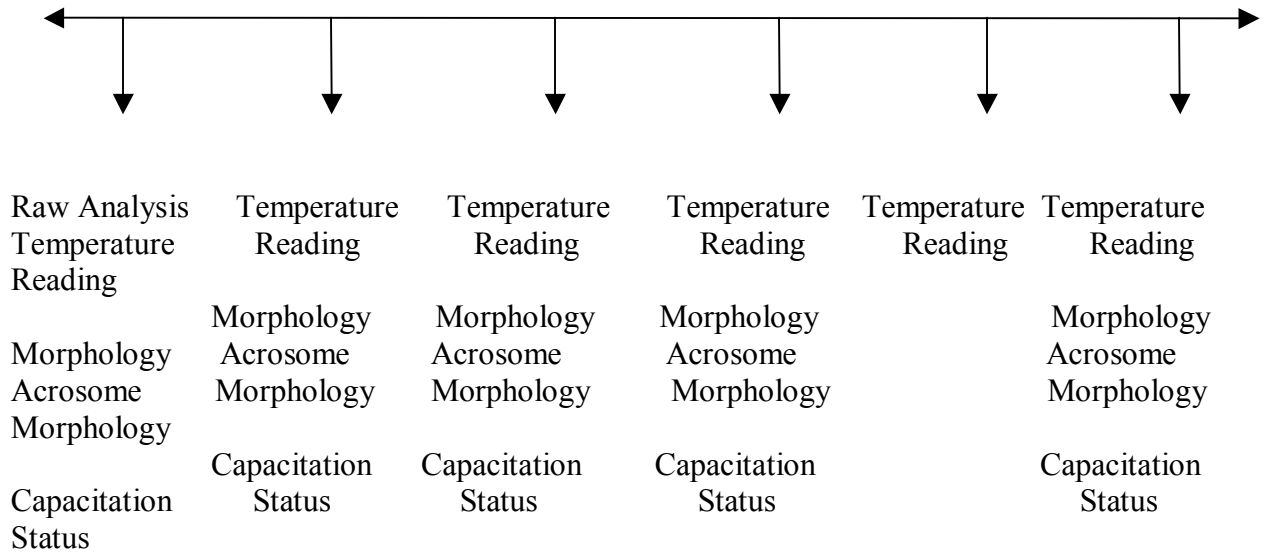


Figure 25. Representative time line showing the timing of semen quality estimates relative to collection and extension (Hour 0)

Semen Collection

Each boar was collected by the double-gloved hand technique (Almond et al., 1998). The collection pen was 2.43 x 3.65 m and the collection dummy (Minitube of America, Inc., Verona, WI) was 0.3 m wide and 1.21 m long. Collections were completed using powder-free polyvinyl gloves (IMV America, Eden Prairie, MN). Each boar was housed in stalls separated from the collection pen by a 1 m alley. The semen was collected into a sterile plastic semen collection bag (Minitube of America, Verona, WI), which was placed inside a 1 -qt thermos covered with a milk filter (IMV America, Eden Prairie, MN). The collection cup was kept at 37°C. The same technician collected all boars during the study and allowed the boar to terminate the collection. Boars were allowed to dismount and remount during the collection.

After collection, the milk filter and rubber band were removed and the collection cup

was weighed (Fisher Scientific, St. Louis, MO). From this, the pre-collection weight was subtracted. Using the assumption that 1 mL semen weighs 1 g the semen volume was then recorded along with the boar's identification number. A spectrophotometer (SpermaCue, Minitube of America, Verona, WI) was used to determine the concentration. This was also recorded and then the ejaculates were placed into an insulated bag (Minitube of America, Verona, WI) and transported to the Swine Reproduction Laboratory located on the campus of North Carolina State University.

Treatment Preparation

As mentioned previously, twelve different treatments were prepared from each ejaculate. Each treatment dose contained 3 billion total spermatozoa in 60 mL. This is representative of the standard insemination dose used in the industry. Treatment doses were prepared by first adding the appropriate aliquot of neat semen to a 50 mL conical tube (Port City Diagnostics, Inc., Wilmington, NC) via a 10 mL serological pipette (Fisher Scientific, Atlanta, GA). To avoid shocking the spermatozoa, extender was slowly dripped from the pipette down the sides of the conical tube until a total volume of 60 mL was achieved.

Evaluations of Semen Quality

Percentage of Normal Acrosomes

One mL of semen was placed into a test tube (12 x 75 mm; Port City Diagnostics, Inc., Wilmington, NC) and mixed with 100 μ L of 10% formalin. Five μ L was placed on a glass microscope slide (Fisher Scientific, Atlanta, GA) and covered with an 18 x 18 mm

plastic coverslip (Fisher Scientific, Atlanta, GA). A phase contrast microscope (Zeiss, Berlin, Germany) was used to evaluate 100 spermatozoa with normal tails under oil immersion at 40x. Spermatozoa with a normal shape and smooth surface of acrosomal ridges were classified as normal, while spermatozoa with misshaped, detached or loose acrosomes were recorded as abnormal (Briz et al., 1995; Pursel et al., 1972).

Percentage with Normal Morphology

Assessments of normal head and tail morphology were performed on the same subsamples that were used for evaluation of normal acrosome morphology. Two hundred sperm cells were evaluated from at least five different fields. Spermatozoa with normal heads and tails were recorded. Spermatozoa with a coiled, broken, or detached tails were recorded as having an abnormal tail. Spermatozoa displaying a cytoplasmic droplet distal to the head were recorded as having a distal droplet. Spermatozoa displaying a cytoplasmic droplet proximal to the head were recorded as having a proximal droplet. Spermatozoa with an abnormal head shape were recorded as abnormal head. Lastly, spermatozoa displaying tails that were looped around so that they crossed by the head were recorded as translocated.

Percentage of Motile Spermatozoa

One mL of semen was placed into a test tube (12x 75 mm; Port City Diagnostics, Inc., Wilmington, NC) and allowed to equilibrate for 30 minutes at 37°C. Leja slides (Minitube of America, Verona, WI) were prewarmed on a slide warmer (Minitube of America, Verona, WI) for 30 minutes prior to analysis. Each sample was mixed before 3 μ L were pipetted into one chamber on the Leja slide. Slides were analyzed using a computer assisted semen analysis system (SpermVision®, Minitube of America, Verona, WI)

connected to a florescent microscope (Olympus BX41, Washington, DC). Five fields were chosen at random and the average motility, average progressive motility average curvilinear distance (DCL), average straight-line distance (DSL), the average curvilinear velocity (VCL), and the average straight-line velocity (VSL) were recorded. This procedure was repeated so that a total of 10 fields and approximately 500 spermatozoa were evaluated.

Percentage of Capacitated Spermatozoa

The chlorotetracycline technique (CTC), which was originally described by Fraser and Herod (1990) and modified by Popwell (1999), was used to determine the percentage of capacitated spermatozoa in each sample. Before preparing the samples, .87% saline, DABCO stock solution, DABCO working solution, NaCl/Tris buffer, CTC solution and paraformaldehyde were prepared. The 12.5% (w/v) paraformaldehyde in a 0.5 M-Tris buffer was prepared with 12.5g paraformaldehyde, 7.88 Tris-HCL and 100 mLs of distilled water. Under a hood, the solution was mixed over low heat until it became a solution. If needed the pH was adjusted to 7.4. This solution was made once at the beginning of the study. The .22M DABCO stock solution was made when needed and consisted of 2.46g of 1,4-diazabicyclo (2,2,2)-octane (DABCO; Sigma Chemical Company, St. Louis, MO) and 100 mL of PBS. The .87% saline was made by adding 8.7g of NaCl to 1 liter of sterile water. The solution was then sterilized by autoclaving. The NaCl/Tris buffer and DABCO working solutions were prepared weekly. The NaCl/Tris buffer consisted of 1.8975g of NaCl and .6055g Tris (Sigma Chemical Company, St. Louis, MO) mixed with 250 mL of sterilized water. The DABCO working solution was made with 1 mL DABCO stock solution into a 15 mL conical tube (Port City Diagnostics, Inc., Wilmington, NC) and then adding 9 mL of

glycerol. Inverting the tube until the contents were thoroughly incorporated sufficiently mixed the combination. The CTC solution was prepared the day of analysis and was mixed using a 100 mL bottle (Pyrex, Germany) covered with aluminum foil to avoid light. The solution consisted of .019g of CTC (Sigma Chemical Company, St. Louis, MO), .039g of cysteine (Sigma Chemical Company, St. Louis, MO), and 50 mL of the NaCl/Tris buffer. The solution was refrigerated until needed for the assay.

For the assay, 1 mL was placed into a 15 mL conical tube (Port City Diagnostics, Inc., Wilmington, NC) and centrifuged (Angle Centrifuge; Hamilton Bell Co, Inc., Montvale, Nj) at 2000 rpm for five minutes. After removing from the centrifuge, the seminal plasma was removed and discarded and 1 mL of the .87% saline was added and mixed. Ethanol-cleaned glass microscope slides were placed on a 37°C slide warmer (Fisher scientific, Atlanta, GA). The CTC slides were prepared with the lights off. On each slide, 2.5µL of saline/sperm was pipetted onto a warmed slide. Next, 10 µL of the CTC solution was added and mixed with a gel loading pipette tip (Fisher Scientific, Atlanta, GA), which helped to minimize damage to the spermatozoa. Once mixed, a tiny bead of paraformaldehyde dropped from a gel loading pipette tip (Fisher Scientific, Atlanta, GA), was added and mixed which functioned to fix the CTC pattern. A single drop from a 0.5 mL disposable pipette (Fisher Scientific, Atlanta, GA) of the DABCO working solution was added and mixed to prevent fading of fluorescence. A 24 x 50 mm coverslip (Port City Diagnostics, Inc., Wilmington, NC) was placed onto the slide. The prepared slides were stored in a slide box (Fisher Scientific, Atlanta, GA) lined with aluminum foil and damp lab bench paper. The slide box was kept closed while other slides were being prepared.

Slides were analyzed using a VANOX microscope (Olympus Optical Co, Ltd., Japan) under a blue-violet illumination (excitation at 400-440 emission at 470 nm). A random sample of 100 cells was counted per slide. Spermatozoa were classified as either capacitated, uncapacitated, acrosome reacted or abnormal according to patterns described by Fraser and Herod (1990). Capacitated spermatozoa displayed a fluorescence pattern in the pre-acrosomal region of the head, but none in the post-acrosomal region of the head. Uncapacitated spermatozoa displayed fluorescence over the entire head. Acrosome reacted spermatozoa were characterized with having little to no fluorescence over the entire head except from a bright band in the equatorial segment of the head. Lastly, any sperm displaying fluorescence patterns, unlike the ones aforementioned or unusual heads were counted as abnormal.

Statistical Analysis

The effects of extender (Androhep Plus and BTS); time (0, 4, 24,48, 96 hours); and sample (Neat Semen, Fast, Medium, Slow) on semen quality parameters were analyzed with analysis of variance procedures for repeated measures (Gill and Hafs, 1971) using the general linear models procedure (GLM) of SAS (SAS, 2002, Version 9.1). Main effects of extender and sample, and extender by sample interaction were tested using boar nested within sample and extender, Boar (sample x extender) as the error term. Effects of time, time by extender, time by sample and time by extender by sample were tested using the residual error. No significant three-way interactions were observed. When significant two-way interactions between time and extender, time and sample or sample and extender were observed they

were analyzed. When a significant interaction between time and extender was present, the effect over time for each extender was determined by examining probability values (PDIFP) with standard error (STDERR) for all possible combinations of least-squares estimates of marginal means (LSMEANS). This procedure was consistent when significant interactions were observed with the interaction of time with the samples and the interaction of extender on the various samples.

Statistical significance is determined by resulting p-values with a p-value less than or equal to 0.05. A trend or tendency was noted if the p-value was greater than 0.05, but less than 0.1. A complete list of SAS analysis program codes is located in Appendix B.

Results

Main effects of extender, treatment, time, and their interactions on 8 dependent variables associated with estimates of semen quality are shown in table 5.

Morphology

Extender ($p=.0001$) and time ($p<.0001$) significantly influence the proportion of spermatozoa with normal, intact acrosomes (Table 4 and Figures 26 and 28). Androhep Plus had more spermatozoa with normal, intact acrosomes, (96.38 ± 0.2) as compared with BTS (95.40 ± 0.6). The proportion of normal acrosomes steadily declined ($p<.0001$) from time 0 to the 96th hour after extension (Figure 26). A significant interaction ($p=.0029$) was observed for the proportion of normal acrosomes (Table 5). Treatments extended with Androhep Plus had fewer abnormal acrosomes, as compared to BTS, from time 0 to the 96th hour after extension (Figure 26). When the various rates of cooling were examined, there was a tendency ($p=.0614$) for treatments to differ. The samples that were cooled fast had a

tendency to differ from samples cooled at a medium rate ($p=.0666$) and significantly differed from samples cooled at a slow rate ($p=.0237$). Samples cooled at a medium rate did not differ than those cooled at a slow rate ($p=.6650$). Samples cooled at a medium and slow rate were had higher percentages of acrosomes ($96.67 \pm 0.3\%$, $96.98 \pm 0.3\%$) than those cooled at a fast rate ($95.59 \pm 0.3\%$) (Table 8). When different extenders were examined per sample, Androhep Plus had significantly higher percentages of acrosomes for each rate of cooling ($p<.05$) (Figure 27).

The percentage of spermatozoa with normal morphology did not significantly differ for the effects and their subsequent interactions ($p=.4995$) (Table 5). Cooling Rate did not affect the percentage of spermatozoa with normal morphology (Table 8 and Figures 29 and 30).

Motility

Extender ($p=.0091$), time ($p<.0001$) and rate of cooling ($p<.0001$) significantly influenced the average motility. Androhep Plus had higher motility values (81.14 ± 2.1) as compared with BTS (78.63 ± 2.1). When the influence of extender is looked at for each cooling rate, Androhep Plus has higher motility as compared to BTS for each rate of cooling. Androhep Plus (76.44 ± 1.1) is significantly higher than BTS (72.20 ± 1.1) for samples cooled at a fast rate ($p<.05$). Androhep Plus (82.98 ± 1.1) is significantly higher than BTS (79.47 ± 1.1) for samples cooled at a slow rate ($p<.05$) (Table 6 and Figure 32). Average motility was lowest when the neat semen was analyzed at hour 0. However once samples were extended and cooled to, the analysis at hour 4 saw the average motility increased before steadily decreasing until hour 96 ($p<.05$).

There was a significant difference observed between samples $p < .0001$ (Table 6 and Figure 33). The motility observed for the neat semen did not differ significantly from the treatment samples ($p > .05$). The samples that were cooled at a fast rate significantly differed from those cooled at a medium rate ($p < .0001$) and from those cooled at a slow rate ($p < .0001$), however samples cooled at a medium rate did not differ from samples cooled at a slow rate ($p = .2273$). No interactions were observed to be significant (Table 4 and Figure 32).

Extender ($p < .0001$), time ($p < .0001$) and rate of cooling ($p < .0001$) significantly influenced the average progressive motility. Androhep Plus had higher progressive motility values (67.03 ± 0.8) as compared with BTS (61.63 ± 0.8). Progressive motility was lowest when the neat semen was analyzed at hour 0. However once samples were extended and cooled to, the analysis at hour 4 saw the average progressive motility increased before steadily decreasing until hour 96 ($p < .05$).

There was a significant difference observed between samples $p < .0001$ (Table 4 and Figure 37). The motility observed for the neat semen did not differ significantly from the treatment samples ($p > .05$). The samples that were cooled at a fast rate significantly differed from those cooled at a medium rate ($p < .0001$) and from those cooled at a slow rate ($p < .0001$), however samples cooled at a medium rate did not differ from samples cooled at a slow rate ($p = .9849$). Extender influenced the progressive motility for individual cooling rates. Androhep Plus was significantly higher than BTS for all rates of cooling (Table 6). No interactions were observed to be significant (Table 4 and Figure 35).

Mobility

Extender ($p < .0001$) and cooling rate ($p < .0001$) significantly influenced the straight-line distance traveled (Table 4). Samples extended in Androhep Plus traveled further (23.63 ± 0.3) as compared to BTS (21.93 ± 0.3). There is a tendency for samples to differ over time ($p = .0602$). After being cooled and analyzed at hour 4, the straight-line distance steadily declined over time. There is a tendency observed for the interaction between extender and time ($p = .0806$) (Figure 41). Androhep Plus and BTS are comparable when the neat semen is analyzed at hour 0 ($p > .05$). At hour four both Androhep Plus and BTS demonstrate increased straight-line distance traveled, with Androhep Plus traveling further than BTS ($p = .0012$). After the peak, samples extended in Androhep Plus continue to decline until the 96 hour. Samples extended in BTS are significantly lower than Androhep Plus at hour 24 ($p = .0001$), and have a tendency to differ at the 48th hour ($p = .0902$). At hour 96, samples extended in BTS increase to become comparable to Androhep ($p = .7133$). The various rates of cooling for straight-line distance traveled are significantly different ($p < .0001$) (Table 7). Samples cooled at a fast rate have lower straight-line distance traveled (22.16 ± 0.4) when compared to samples cooled at a medium rate (23.21 ± 0.4) ($p = .0202$) and samples cooled at a slow rate (23.17 ± 0.4) ($p = .0304$). No other interactions were observed to be significant.

Extender ($p = .0001$), time ($p = .0198$) and cooling rate ($p = .0004$) influenced the straight-line velocity (Table 4). Samples extended in Androhep Plus traveled faster (52.74 ± 0.6) as compared to BTS (48.66 ± 0.6). Straight-line velocity steadily declined ($p = .0198$) from the 0 to the 48th hour, where it increased slightly at the 96th hour (Figure 47). A significant interaction ($p = .0469$) between extender and time was observed for the average

straight-line velocity (Table 4 and Figure 47). Straight-line velocities were similar at the 0 hour between extenders, and samples extended in both BTS and Androhep Plus increased at hour 4. BTS was slower than Androhep Plus at hour 4, 24 and 48 ($p=.0015$, $p<.0001$ and $p=.0035$). However at the 96th hour, Androhep Plus continues to decline, while BTS increases to become comparable to Androhep Plus ($p=.9596$). When the rates of cooling were examined, the neat semen did not significantly differ from the rates of cooling ($p>.05$). Samples that were cooled at a fast rate had a significantly slower straight-line velocity (121.70 ± 1.9) as compared to samples cooled at a medium rate (128.53 ± 1.9) ($p=.0103$) and slow rates (127.64 ± 1.9) ($p=.0256$) (Figure 48). When Androhep Plus and BTS are compared for each cooling rate, Androhep Plus is significantly faster than BTS for all rates of cooling (Table 6 and Figure 46). No other interactions were observed to be significant.

Time ($p<.0001$) significantly influenced the curvilinear velocity (Table 4). Curvilinear velocity steadily declined from hour 0 (139.71 ± 3.6) until hour 96 (121.19 ± 2.2) (Figure 44). Extender ($p=.0581$) and cooling rate ($p=.0587$) had a tendency to differ for curvilinear velocities. Androhep Plus (128.82 ± 1.5) had faster curvilinear velocity than BTS (123.09 ± 1.5). There was a significant interaction between extender and time ($p=.0002$) (Figure 44). BTS and Androhep Plus are comparable at hour 0 ($p>.05$), however Androhep Plus is significantly higher than BTS at hours 4 ($p<.0001$) and 24 ($p=.0062$). At hour 48 Androhep Plus and BTS are not significantly different ($p=.2992$). At hour 96, BTS increases above Androhep Plus, as Androhep Plus continues to decline ($p=.0266$). Samples cooled at a fast rate were significantly lower (121.70 ± 1.9) than samples cooled at a medium rate (128.53 ± 1.9) ($p=.0103$) and a slow rate (127.64 ± 1.9) ($p=.0256$) (Table 7) (Figure 45).

BTS and Androhep Plus also influenced the curvilinear velocity for the various rates of cooling ($p=.0317$) (Figure 46). For samples that were cooler at a fast rate, BTS had a higher curvilinear velocity than Androhep Plus ($p>.05$) (Table 6). Samples cooled at medium and slow rates had higher curvilinear velocities when extended with Androhep Plus as compared to BTS ($p<.05$) (Table 6). No other significant interactions were observed (Table 4).

Time ($p=.0001$) significantly influence the curvilinear distance traveled (Table 4). The curvilinear distance traveled is comparable at the 0 (59.93 ± 1.5) and 4th hours ($p>.05$), and then declines steadily until the 96th hour (56.55 ± 0.8). The analysis of the neat semen significantly differs from at the 48th ($p=.0191$) and 96th hours ($p=.0455$) with a tendency to differ at the 24th hour ($p=.0776$). There is a weak tendency for Androhep Plus and BTS to differ ($p=.0965$). Androhep Plus has a higher curvilinear distance traveled (58.95 ± 0.6) as compared to BTS (56.84 ± 0.6). The interaction between extender and time is significant ($p=.0006$) (Figure 38). Cooling rates are not significantly different ($p=.2234$) (Table 7) (Figure 39). No other significant interactions were observed (Table 4).

Discussion

The objectives of this study were to determine the rates of cooling on fresh boar semen. It was found that semen quality is not adversely affected if freshly extended ejaculates are placed directly into a semen storage unit set at 17°C, or if they are allowed to slowly reach room temperature around 25°C for two and a half hours before being placed into a semen storage unit at 16-18°C. Samples were able to withstand a 20°C drop in temperature immediately after extension without affect the quality of the sample.

Boar spermatozoa are very susceptible to cold shock, a phenomena occurring when the fresh ejaculate is cooled quickly to temperatures below 15°C resulting in a loss of viability (Johnson et al., 2000). Scientists have suggested that irreversible damage caused by cold shock occurs when boar semen is exposed to temperatures below 15°C (Althouse et al., 1998). When the overall rate of cooling is examined for each of the parameters, the samples that were cooled in a fast manner had significant decreased motility and progressive motility when compared to the samples allowed to reach room temperature (slow) and those that were immediately placed into a semen storage tank (medium). Progressive motility of spermatozoa is an indicator of both impaired metabolism and intactness of membranes (Johnson et al., 2000). Interestingly the two accepted methodologies of cooling extended boar semen did not differ from one another, suggesting that both procedures are similar and will not cause detrimental effects to the spermatozoa. This notion negates the accepted notion of needed a holding time in order to avoid cold shock (Eriksson et al., 2001). This demonstrates that spermatozoa are able to withstand a temperature decrease of 20°C without affecting semen quality. Motility and progressive motility decrease at each analysis time for each of the cooling rates, with the fast rate of cooling being significantly lower than the medium and slow rates of cooling. Although the samples that were cooled at a fast rate differed significantly than samples cooled at slower rates, the values for motility did not fall below 60%. Flowers (1997) found no differences in fertilizing capacities when motility is greater than 60%. It can be speculated that spermatozoa are able to withstand decreases of 25° C in temperature without affecting semen quality.

A study using boar ejaculates (n=9) found that when samples are extended in

Androhep and stored in a 12°C for 60 hours, no difference is observed in farrowing rate, total offspring boar, or number boar alive with samples stored at the recommended 17°C temperature for the same amount of time (Althouse et al., 1998). These authors concluded that acceptable fertility could be obtained with Androhep extended boar semen exposed to temperatures as low as 12°C without compromising fertility. The present study had a group that was placed into a 12°C cooler for one hour before being placed into the semen storage unit at 17°C. When Androhep Plus is examined over time, there are no significant differences between average motility and average progressive motility for each analysis time when examined against one another.

Extender influenced a majority of the parameters for semen quality. Androhep Plus is a long-term extender and is commercially available for storing extended samples for up to five days post collection. BTS is a short-term extender and is commercially available for storing extended samples for up to 3 days post collection. Androhep Plus also contains ingredients that promote a higher quality of semen for longer. Because Androhep Plus is a five-day extender, this sufficiently explains why for a majority of the semen quality parameters, Androhep Plus is superior to that of BTS.

For all samples, Androhep Plus was able to maintain semen quality over time better than BTS. Both extenders were able to keep motility greater than 70% over 96 hours, with both extenders being comparable at the 96th hour. With motility greater than 70%, this suggests there are no latent effects due storage time on overall semen quality. While BTS is marketed as a 3-day short-term extender, it was originally formulated for use in thawing after cryopreservation and uniquely contains a small amount of potassium, which preserves the

sodium potassium pump (Ambrogi et al., 2006). In addition, the depletion of intracellular potassium can reduce sperm motility (Alvarez and Storey, 1982). It is possible that the lack of intracellular potassium depletion is allowing for the sustained motility, even at the 96th hour, 48 hours past the recommended usage time. Another study that looked at the long-term storage of samples in Androhep Plus and BTS found that Androhep Plus maintained better motility and progressive motility over 12 days of CASA analysis (Dubé et al., 2003). These authors suggest that it is the bovine serum albumin (BSA) thought to be in the Androhep Plus that is able to inhibit the lipid peroxidation in the sperm, a damage which reduces sperm motility.

Androhep Plus was also able to maintain significantly higher proportions of acrosomes with respect to time. Androhep Plus was also beneficial to the curvilinear distance and velocities as time progressed until the 96th hour where sperm viability was higher in the BTS extender. Androhep Plus may contain HEPES, which is known to control pH by capturing heavy metals because it is a zwitterionic organic buffer (Crabo et al. 1972). Factors such as pH are important to spermatozoa function and can change during storage. It appears that Androhep was able to preserve viability in this manner. It is possible that a more accurate assessment of the 96th hour phenomena of BTS surpassing Androhep Plus in viability could be examined with an analysis at the 120th hour or longer.

There were no significant differences observed for the proportion of spermatozoa with normal morphology. Cytoplasmic droplets represent the most frequent morphological alteration observed and it has been shown to have a negative correlation between the proportion of distal droplets and fertility (Waberski, 1994). In the present study, there was

no significance observed for the main effects when the proportion of distal droplets was examined ($p=.9441$). All parameters for semen quality, with the exception of morphology, showed significant decreases over time. This decrease in viability can resemble the natural aging process, which is usually determined by the conditions and length of *in vitro* storage (Ambrogi et al., 2006).

Trends were observed between sample and extender for average motility, and curvilinear distance and velocities. Androhep and BTS were not significantly different for the fast cooled samples, and both extenders were less than extenders for the medium and slow cooled samples. For the slow and medium cooled samples, Androhep is significantly higher than BTS for all three parameters suggesting that the extender is able to better maintain viability for the two widely accepted ways of cooling semen.

It appears that the two accepted ways of cooling extended boar semen do not differ from one another. When extending samples, viability will be compromised when freshly extended boar spermatozoa is placed into 12°C cooler, as shown in previous studies. The findings also support the fact that Androhep Plus would better preserve sperm fertilizing capacity than samples extended in BTS, however both extenders would result in good artificial inseminations results.

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Table 5. Summary of Independent and Dependent Variables and their P-values

Dependent Variables	Independent Variables						
	Extender	Time	Sample	Extender * Time	Time * Sample	Extender * Sample	Extender * Time * Sample
% Normal Acrosomes	<.0001	<.0001	.0614	.0029	.9584	.3022	.8578
% Normal Morphology	.7272	.8808	.4505	.7199	.9049	.9472	.7658
Average Motility	.0091	<.0001	<.0001	.5336	.9258	.1619	.9010
Average Progressive Motility	<.0001	<.0001	<.0001	.1047	.6088	.3546	.9840
DSL	<.0001	.0602	<.0001	.0806	.9865	.2861	.9672
DCL	.0965	.0001	.2234	.0006	.7945	.1084	.9560
VSL	.0001	.0198	.0004	.0469	.8029	.2927	.9997
VCL	.0581	<.0001	.0587	.0002	.9975	.0317	.7804

Table 6. Extender comparisons for extension protocols for various dependent variables, LSMEAN \pm S.E.

	Neat Semen		Fast		Medium		Slow	
	Androhep	BTS	Androhep	BTS	Androhep	BTS	Androhep	BTS
% Normal Acrosomes	99.60 \pm .4 ^a	98.8 \pm .4 ^a	97.25 \pm .4 ^a	95.13 \pm .4 ^b	97.70 \pm .4 ^a	95.95 \pm .4 ^b	97.50 \pm .4 ^a	96.45 \pm .4 ^a
% Normal Morphology	87.11 \pm 2.7 ^a	86.78 \pm 2.7 ^a	88.84 \pm .9 ^a	88.19 \pm .9 ^a	88.47 \pm .8 ^a	88.56 \pm .8 ^a	88.99 \pm .9 ^a	88.78 \pm .9 ^a
Average Motility	84.72 \pm 1.7 ^a	83.20 \pm 1.7 ^a	76.44 \pm 1.1 ^a	72.70 \pm 1.1 ^b	80.20 \pm 1.1 ^a	79.71 \pm 1.1 ^a	82.98 \pm 1.1 ^a	79.47 \pm 1.1 ^b
Average Progressive Motility	68.72 \pm 2.9 ^a	65.88 \pm 2.9 ^a	64.68 \pm 1.3 ^a	57.16 \pm 1.3 ^b	68.56 \pm 1.4 ^a	64.55 \pm 1.4 ^b	69.72 \pm 1.3 ^a	63.70 \pm 1.3 ^b
Curvilinear Velocity	138.98 \pm 5.5 ^a	139.53 \pm 5.5 ^a	120.92 \pm 3.1 ^a	122.96 \pm 3.2 ^a	133.04 \pm 2.8 ^a	123.10 \pm 2.8 ^b	133.26 \pm 2.6 ^a	123.48 \pm 2.6 ^b
Straight-line Velocity	50.44 \pm 2.5 ^a	48.44 \pm 2.5 ^a	50.55 \pm 1.1 ^a	47.00 \pm 1.1 ^b	56.46 \pm 1.5 ^a	48.92 \pm 1.4 ^b	53.13 \pm 1.1 ^a	50.33 \pm 1.1 ^a
Curvilinear Distance	62.64 \pm 2.3 ^a	63.01 \pm 2.3 ^a	56.59 \pm 1.1 ^a	56.77 \pm 1.1 ^a	60.91 \pm 1.2 ^a	56.29 \pm 1.2 ^b	60.24 \pm 1.0 ^a	57.02 \pm 1.0 ^b
Straight-line Distance	23.02 \pm 1.1 ^a	22.08 \pm 1.0 ^a	23.05 \pm 0.5 ^a	21.62 \pm 0.5 ^b	25.15 \pm 0.6 ^a	22.04 \pm .06 ^b	23.92 \pm 0.5 ^a	22.55 \pm 0.5 ^b

^{a,b} superscripts that are different between extender indicate a significant different, $p < .05$

Table 7. Differences between times for each rate of cooling with LSMEANS \pm S.E.

	Fast				Medium				Slow			
	4	48	96		4	24	48	96	4	24	48	96
% Normal Acrosomes	97.70 \pm .6 ^a	96.8 \pm .6 ^{a,b}	95.9 \pm .6 ^b	94.5 \pm .6 ^c	98.3 \pm .5 ^a	97.1 \pm .5 ^{a,b}	96.3 \pm .5 ^{b,c}	94.2 \pm .5 ^c	98.5 \pm .5 ^a	97.4 \pm .5 ^{a,b}	96.4 \pm .5 ^c	95.5 \pm .5 ^c
% Normal Morphology	89.36 \pm 1.3 ^a	89.5 \pm 1.3 ^a	88.2 \pm 1.4 ^a	86.8 \pm 1.3 ^b	89.2 \pm 1.1 ^a	89.2 \pm 1.1 ^a	86.2 \pm 1.3 ^b	87.3 \pm 1.1 ^{b,c}	88.3 \pm 1.3 ^a	89.1 \pm 1.3 ^a	89.2 \pm 1.5 ^a	88.9 \pm 1.3 ^a
Average Motility	77.41 \pm 1.6 ^a	75.3 \pm 1.5 ^a	71.6 \pm 1.6 ^b	73.5 \pm 1.6 ^{a,b}	82.5 \pm 1.4 ^a	80.9 \pm 1.4 ^{a,b}	78.4 \pm 1.5 ^{b,c}	77.3 \pm 1.5 ^c	83.7 \pm 1.2 ^a	82.8 \pm 1.2 ^{a,b}	79.0 \pm 1.3 ^c	78.7 \pm 1.3 ^{c,d}
Average Progressive Motility	63.77 \pm 1.9 ^a	62.0 \pm 1.9 ^a	58.6 \pm 2.0 ^a	58.9 \pm 2.0 ^a	72.2 \pm 1.8 ^a	66.3 \pm 1.8 ^b	64.5 \pm 1.9 ^{b,c}	62.3 \pm 1.9 ^{b,c}	69.7 \pm 1.8 ^a	69.4 \pm 1.8 ^{a,b}	64.6 \pm 2.0 ^{a,c}	62.2 \pm 2.0 ^c
Curvilinear Velocity	130.73 \pm 4.4 ^a	120. \pm 4.3 ^{a,b}	118. \pm 4.5 ^{a,b}	117. \pm 4.5 ^{b,c}	140. \pm 3.5 ^a	126. \pm 3.5 ^{b,c}	124. \pm 3.7 ^{b,c}	122. \pm 3.8 ^{b,c}	138. \pm 3.6 ^a	125. \pm 3.6 ^{b,c}	124. \pm 3.8 ^{b,c}	123. \pm 3.8 ^{b,c}
Straight-line Velocity	52.07 \pm 1.6 ^a	48.6 \pm 1.6 ^{a,b}	48.0 \pm 1.7 ^{b,c}	46.5 \pm 1.7 ^{b,c}	55.4 \pm 1.6 ^a	51.1 \pm 1.6 ^{a,b}	48.5 \pm 1.7 ^{b,c}	52.4 \pm 1.7 ^{b,c}	54.9 \pm 1.5 ^a	51.8 \pm 1.5 ^{a,b}	48.9 \pm 1.6 ^{b,c}	50.5 \pm 1.6 ^{b,c}
Curvilinear Distance	58.89 \pm 1.6 ^a	56.3 \pm 1.5 ^a	55.6 \pm 1.6 ^a	55.6 \pm 1.6 ^a	61.9 \pm 1.6 ^a	57.3 \pm 1.6 ^a	56.7 \pm 1.7 ^a	58.0 \pm 1.7 ^b	62.4 \pm 1.3 ^a	58.3 \pm 1.3 ^{b,c}	56.4 \pm 1.4 ^{b,c}	56.1 \pm 1.4 ^{b,c}
Straight-line Distance	23.58 \pm .7 ^a	22.1 \pm .7 ^a	22.1 \pm .7 ^a	20.7 \pm .7 ^b	25.0 \pm .7 ^a	23.1 \pm .7 ^{a,b}	22.4 \pm .7 ^{b,c}	22.0 \pm .7 ^{b,c}	24.8 \pm .7 ^a	23.1 \pm .6 ^{a,b}	22.6 \pm .7 ^{b,c}	21.9 \pm .7 ^{b,c}

^{a,b} are significantly different at $p < .05$ between rows for individual extension cooling treatment

Table 8. Extension Protocols with various dependent variables with LSMEANS \pm S.E.

	Fast	Medium	Slow
% Normal Acrosomes	95.59 \pm .3 ^a	96.67 \pm .3 ^b	96.98 \pm .3 ^b
% Normal Morphology	88.93 \pm .6 ^a	89.02 \pm .6 ^a	89.41 \pm .6 ^a
Average Motility	74.44 \pm .7 ^a	79.92 \pm .7 ^b	81.20 \pm .7 ^b
Average Progressive Motility	60.76 \pm 1.0 ^a	66.53 \pm 1.0 ^b	66.68 \pm 1.0 ^b
Curvilinear Velocity	121.84 \pm 2.0 ^a	128.81 \pm 2.0 ^b	128.25 \pm 2.0 ^b
Straight-line Velocity	48.85 \pm .8 ^a	51.99 \pm .8 ^b	51.64 \pm .8 ^b
Curvilinear Distance	56.37 \pm .7 ^a	57.32 \pm .7 ^a	58.31 \pm .7 ^a
Straight-line Distance	22.16 \pm .4 ^a	23.21 \pm .4 ^b	23.17 \pm .3 ^b

^{a,b} significant differences are present with differing superscripts between each column per dependent variables

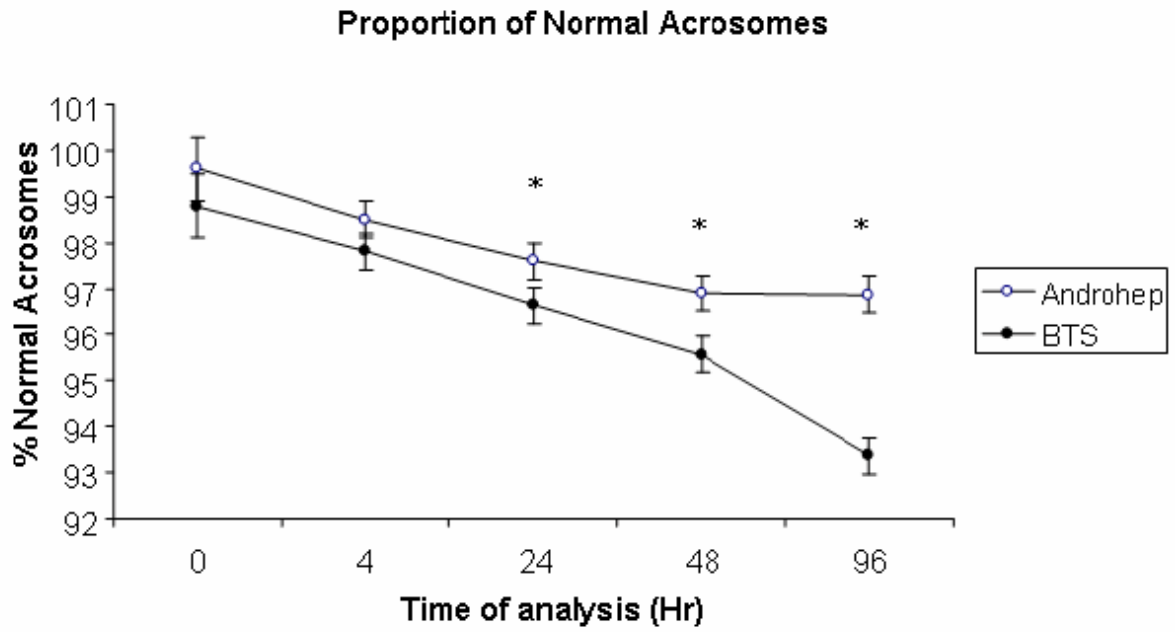


Figure 26. The Proportion of Normal Acrosomes between Extenders over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

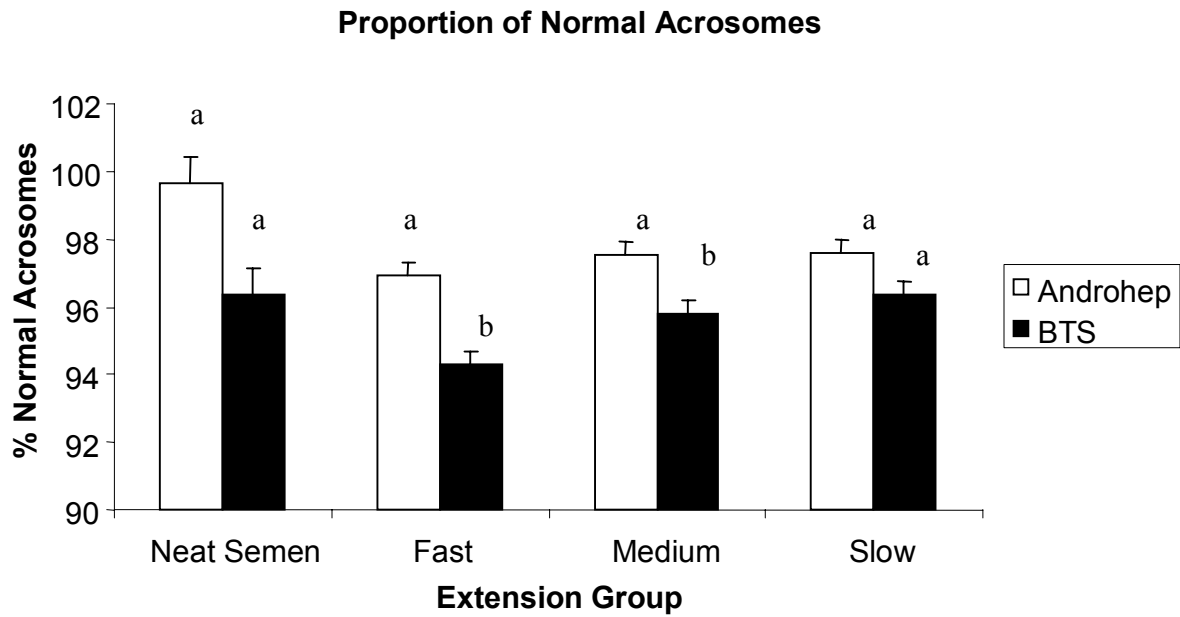


Figure 27. Proportion of Normal Acrosomes between Extender for various Rates of Cooling

^{a,b} Extenders lacking a common superscript indicate a significant difference, $p < .05$

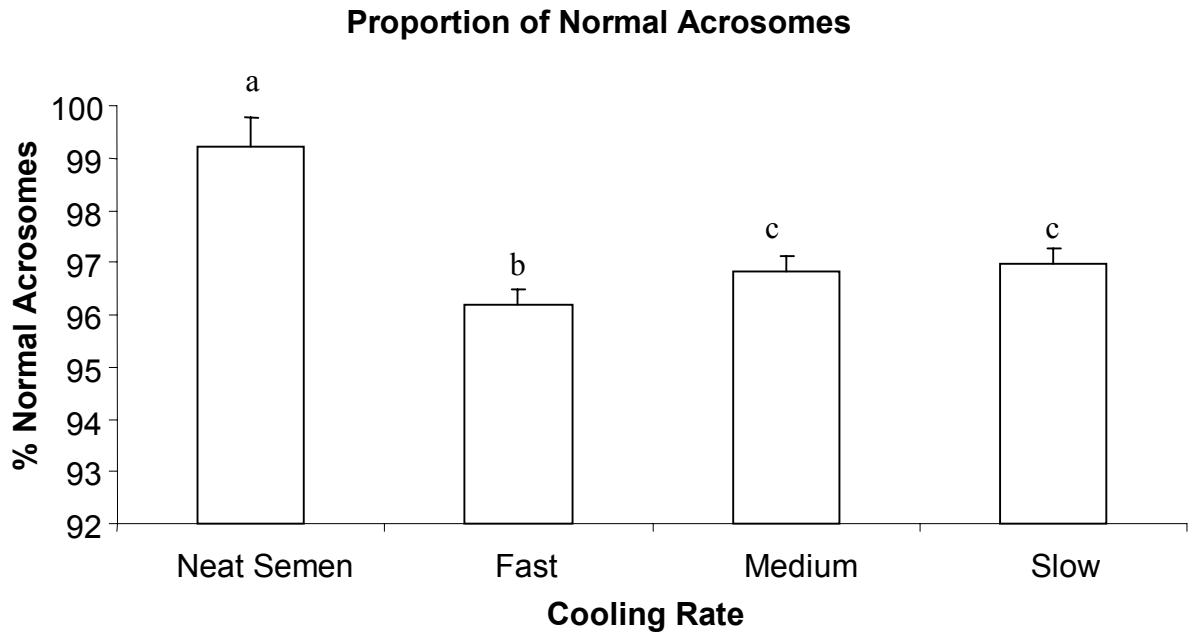


Figure 28. Proportion of Normal Acrosomes for Rate of Cooling

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

Proportion of Normal Morphology

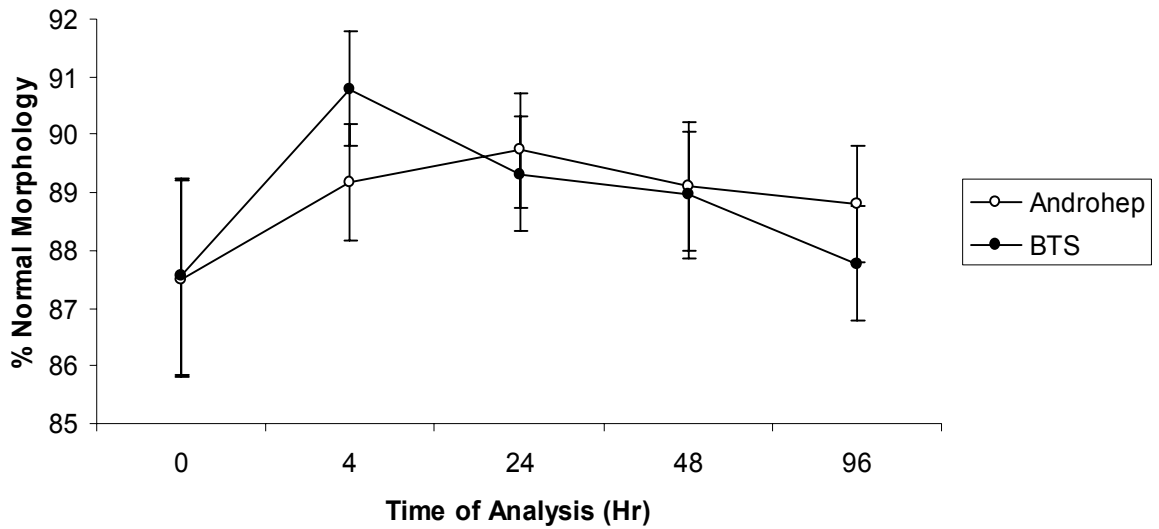


Figure 29. Proportion of Spermatozoa Expressing Normal Morphology over Time between Two Extenders

* Data points with a star indicate significant differences between extender for variables, $p < .05$

Proportion of Normal Morphology

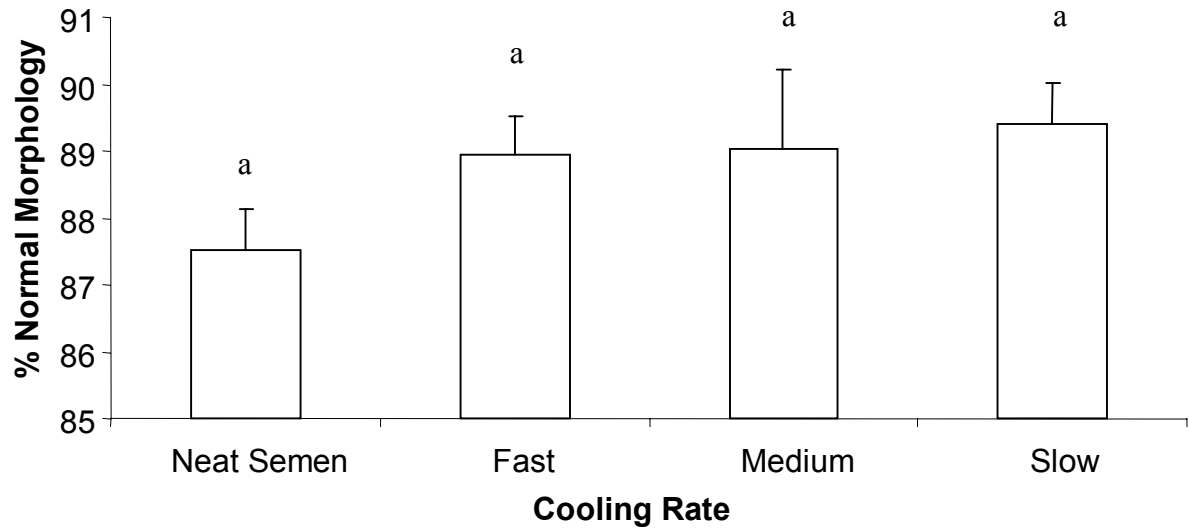


Figure 30. Proportion of Spermatozoa with Normal Morphology between Different Rates of Cooling

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

Proportion of Normal Morphology

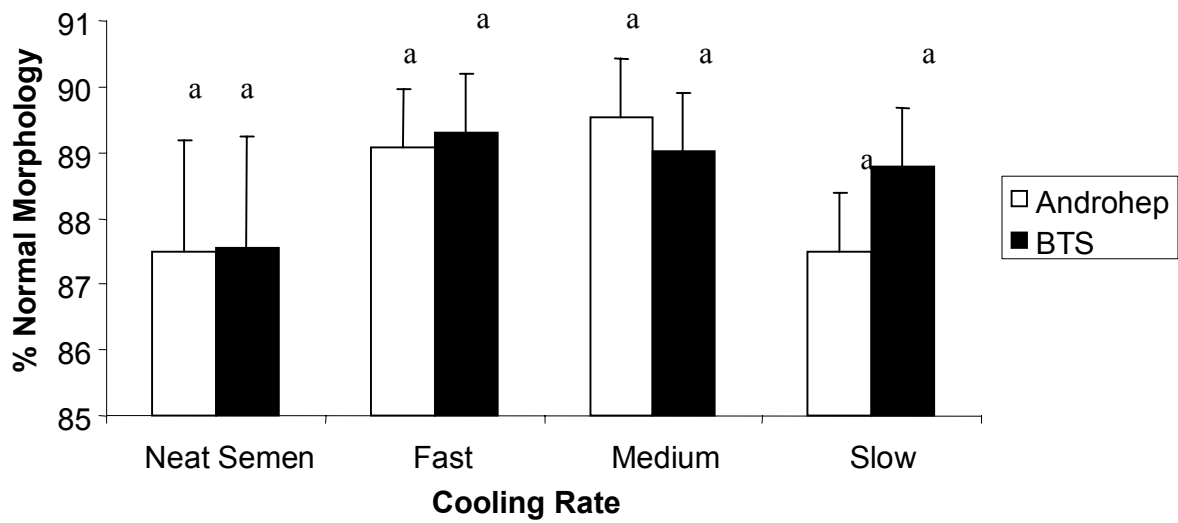


Figure 31. Proportion of Normal Spermatozoa between Extenders for Different Rates of Cooling

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

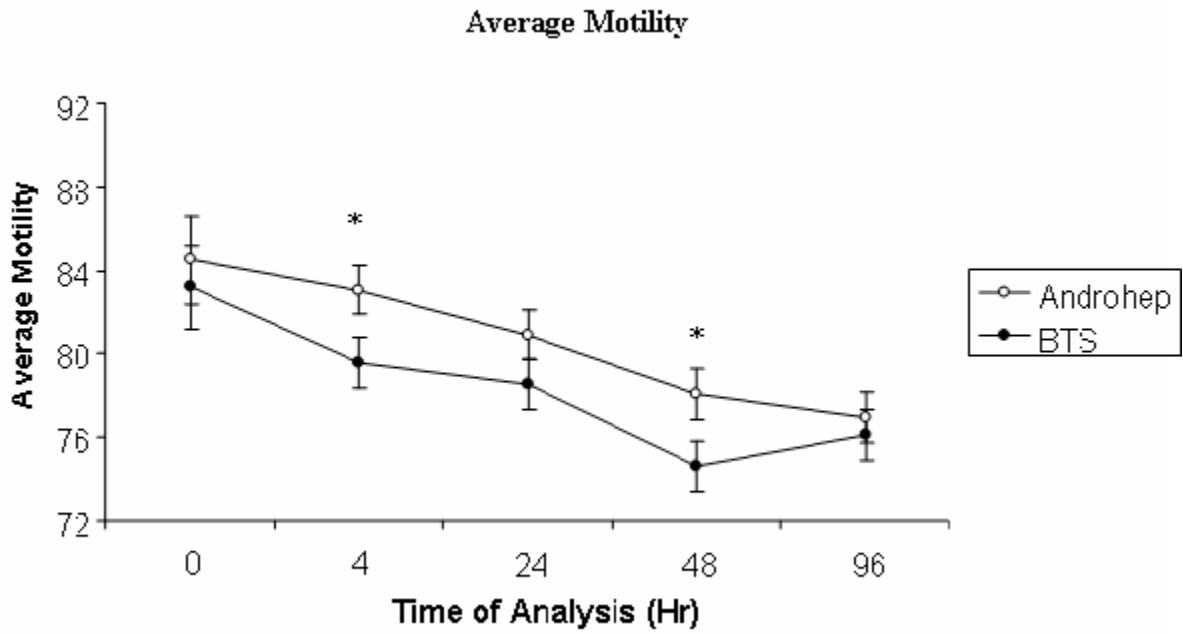


Figure 32. Average Motility Expressed over Time between Extenders

* Data points with a star indicate significant differences between extender for variables, $p < .05$

Average Motility

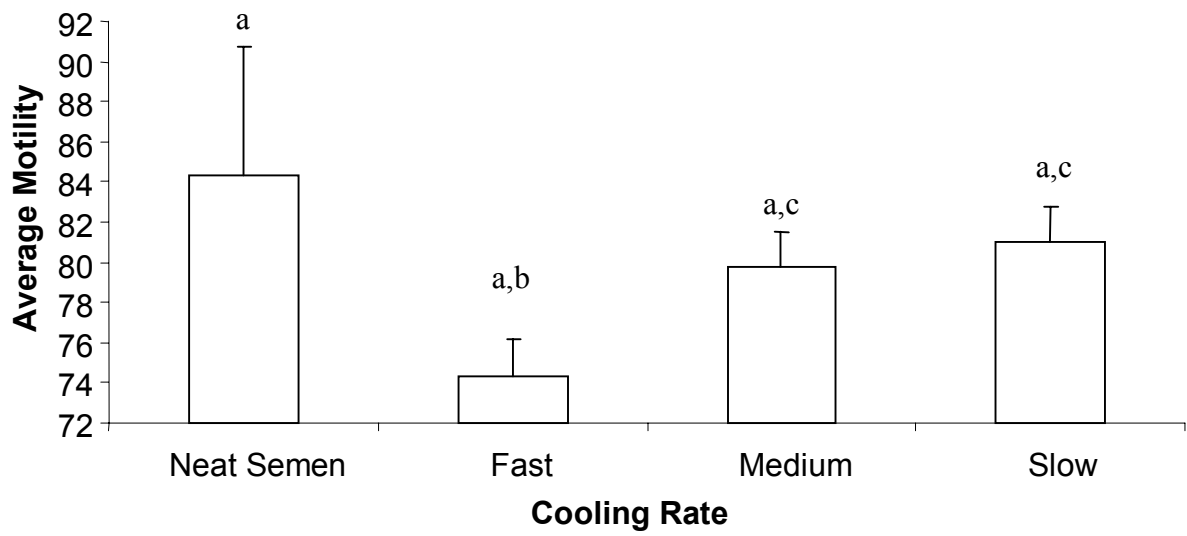


Figure 33. Average Motility for Different Rates of Cooling between Extenders

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

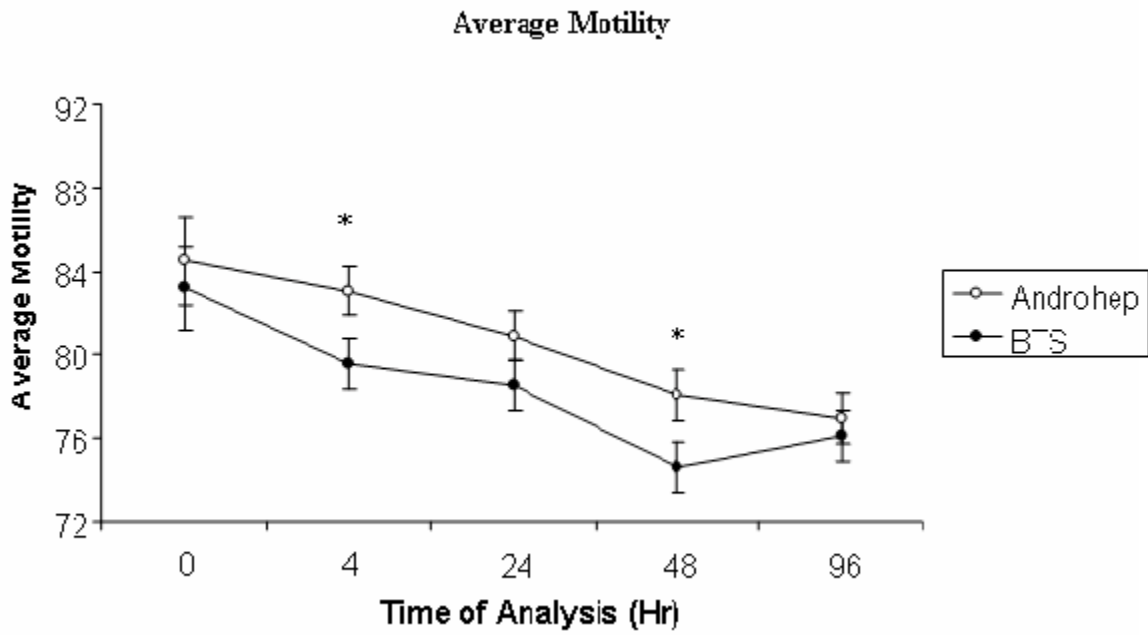


Figure 34. Average Motility between Extenders for the Different Rates of Cooling

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

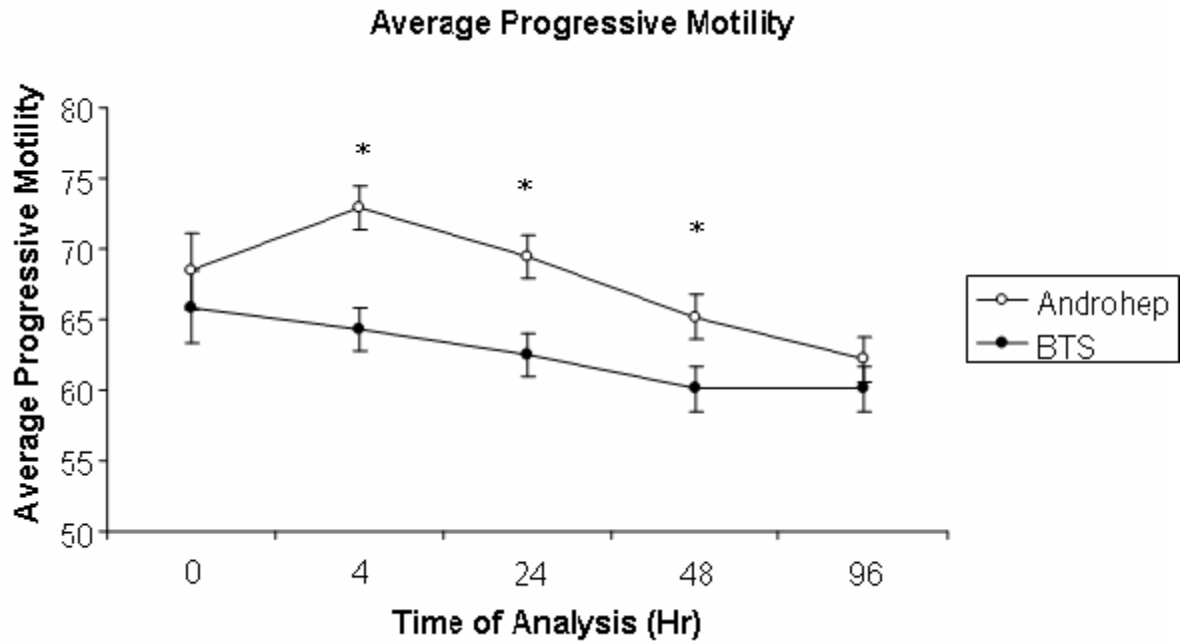


Figure 35. Average Progressive Motility between Extenders over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

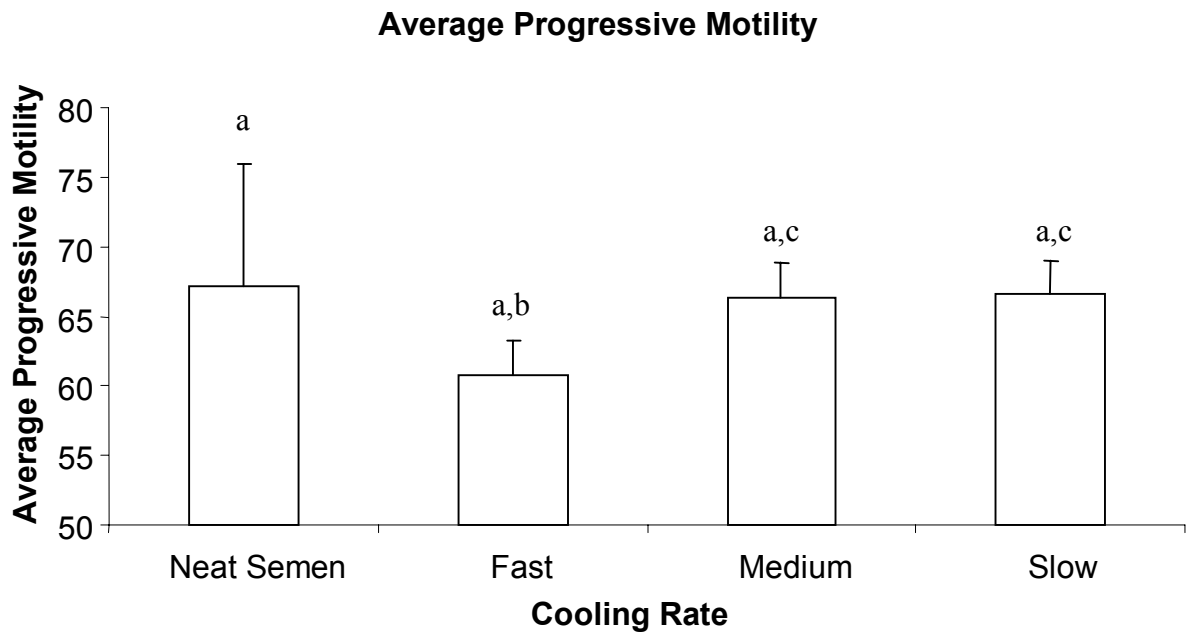


Figure 36. Average Progressive Motility for Cooling Rates

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

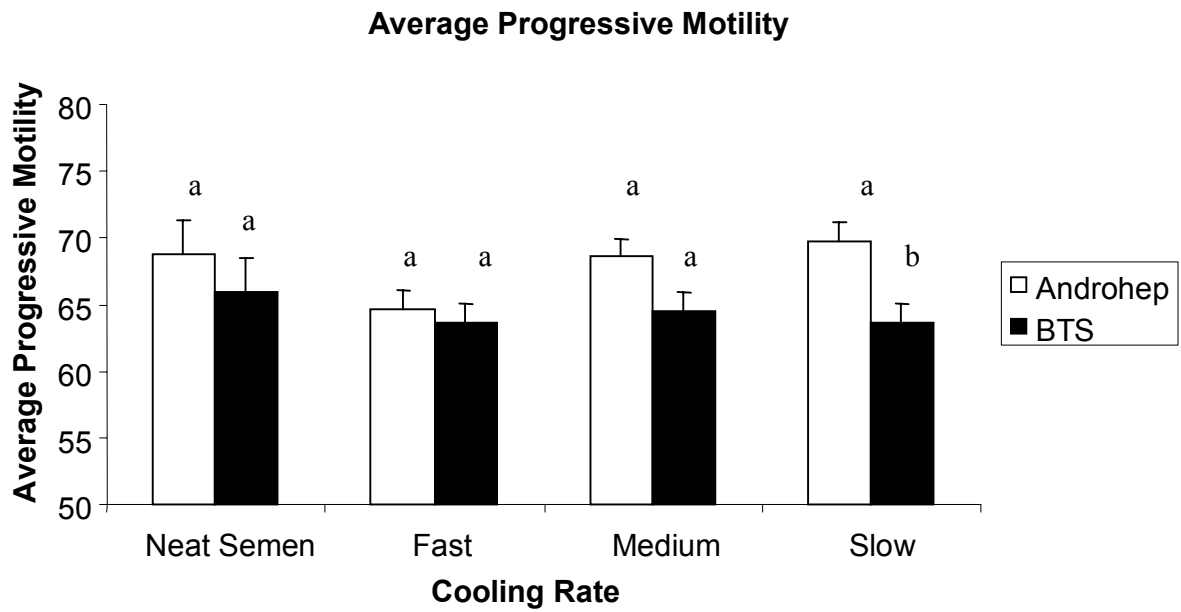


Figure 37. Average Progressive Motility between Extenders for Different Cooling Rates

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

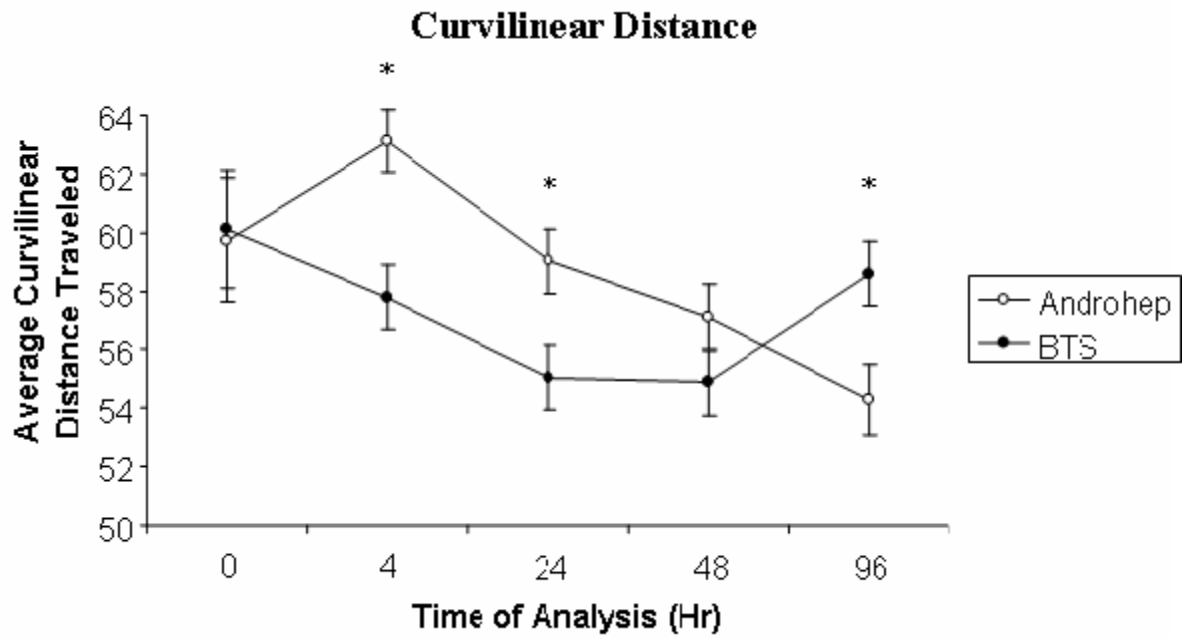


Figure 38. Average Curvilinear Distance Traveled between Extenders over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

Curvilinear Distance

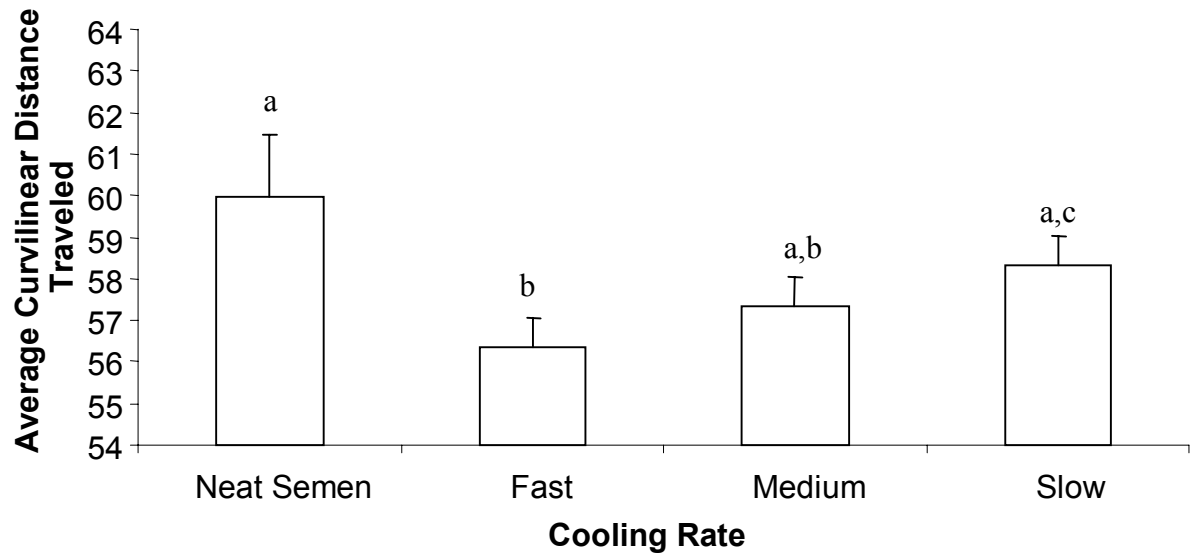


Figure 39. Average Curvilinear Distance Traveled between Different Rates of Cooling

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

Curvilinear Distance

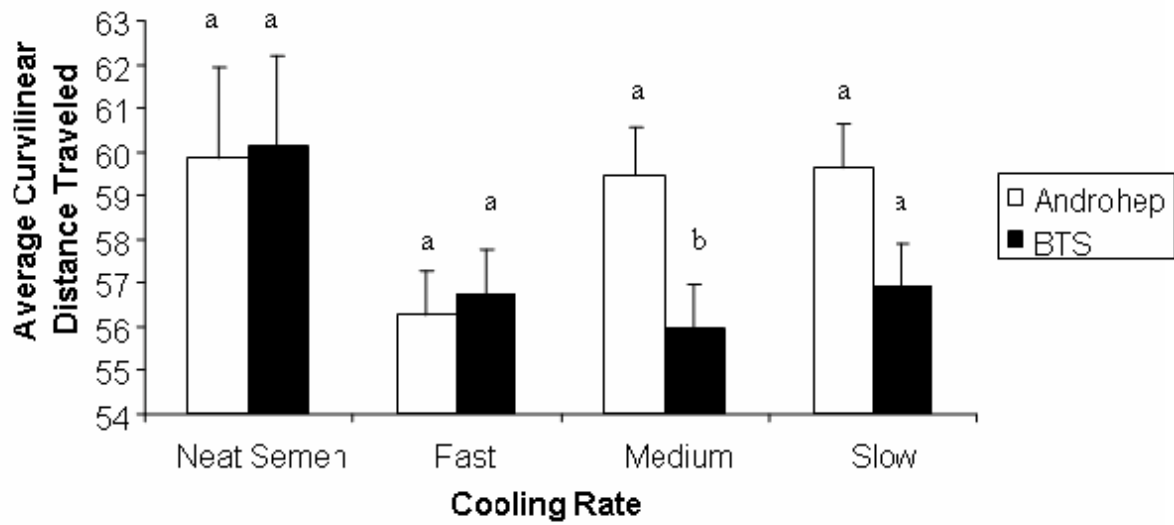


Figure 40. Average Curvilinear Distance between Extenders for Different Rates of Cooling

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

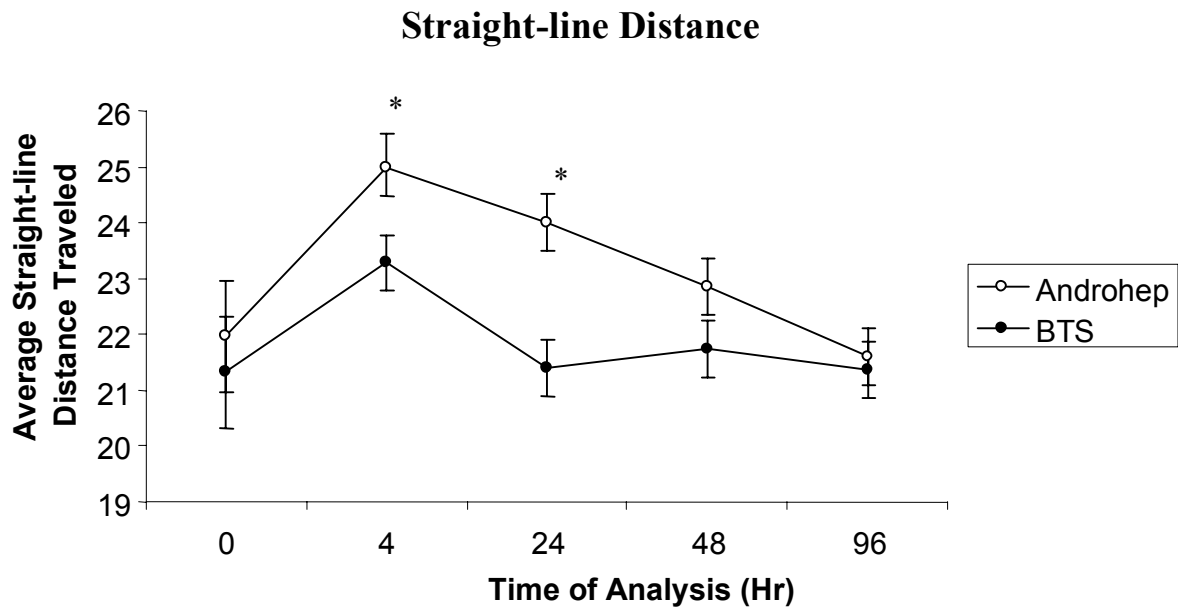


Figure 41. Average Straight-line Distance Traveled between Extenders over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

Straight-line Distance

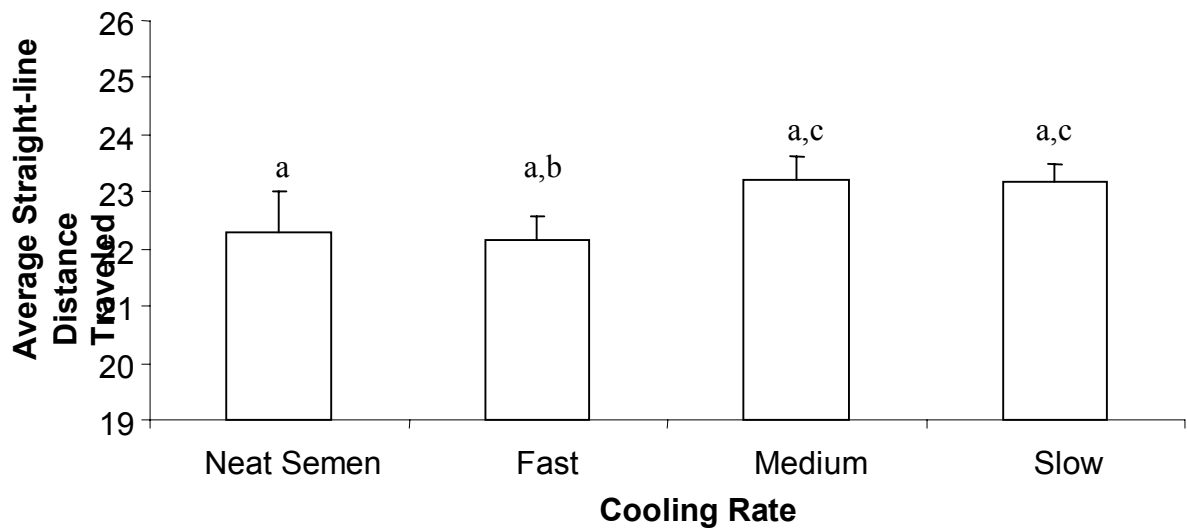


Figure 42. Average Straight-line Distance Traveled for Different Rates of Cooling

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

Straight-line Distance

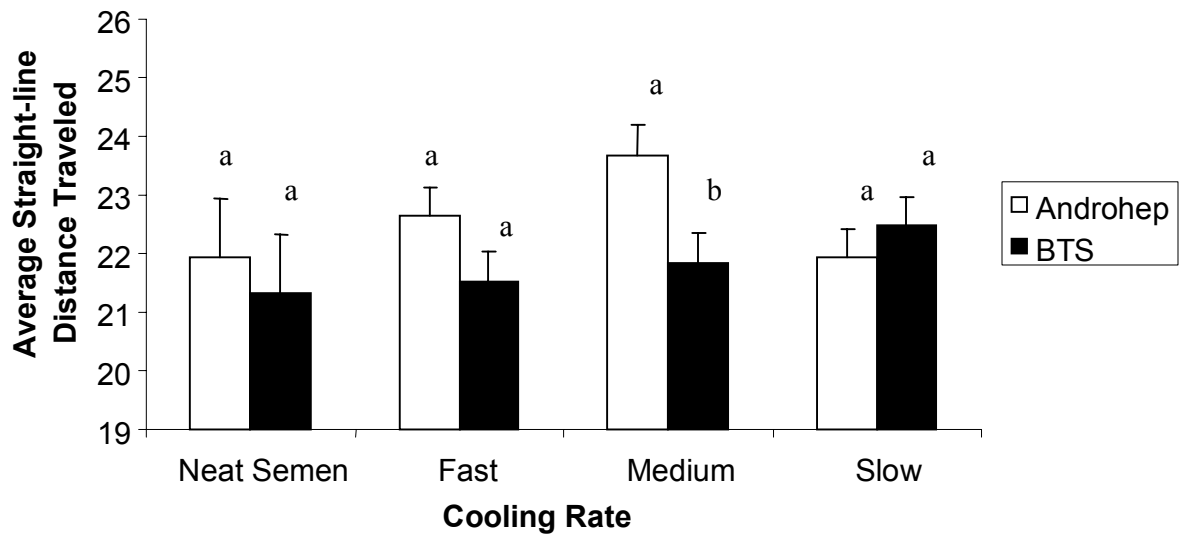


Figure 43. Average Straight-line Distance Traveled between Extenders for Different Rates of Cooling

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

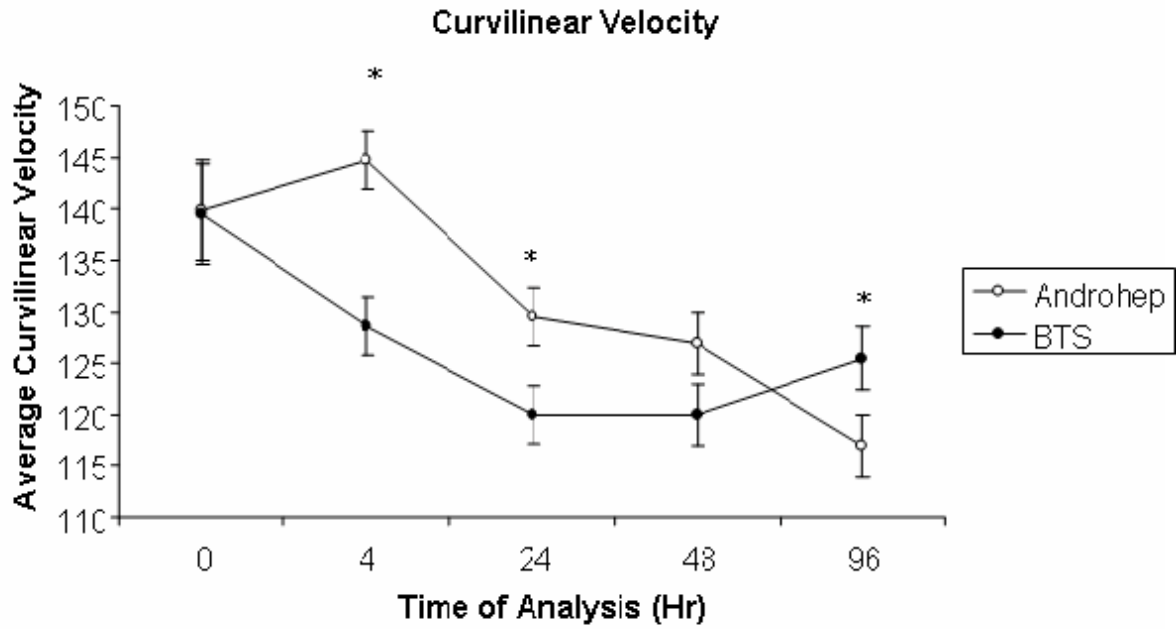


Figure 44. Average Curvilinear Velocity between Extenders over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

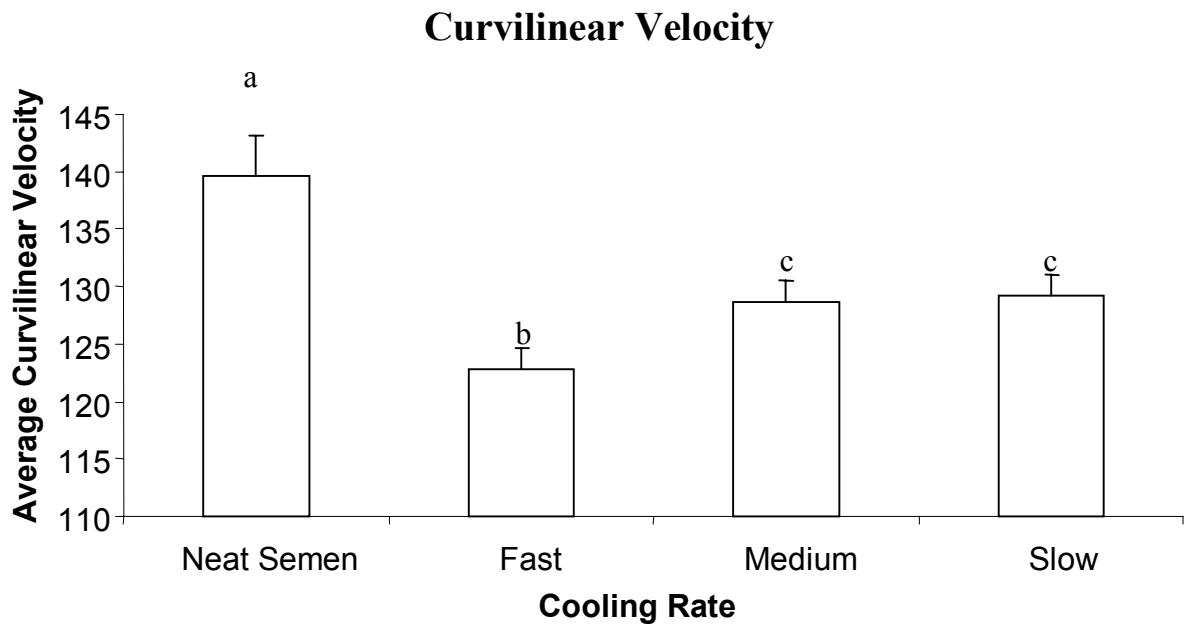


Figure 45. Average Curvilinear velocity for Different Rates of Cooling

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

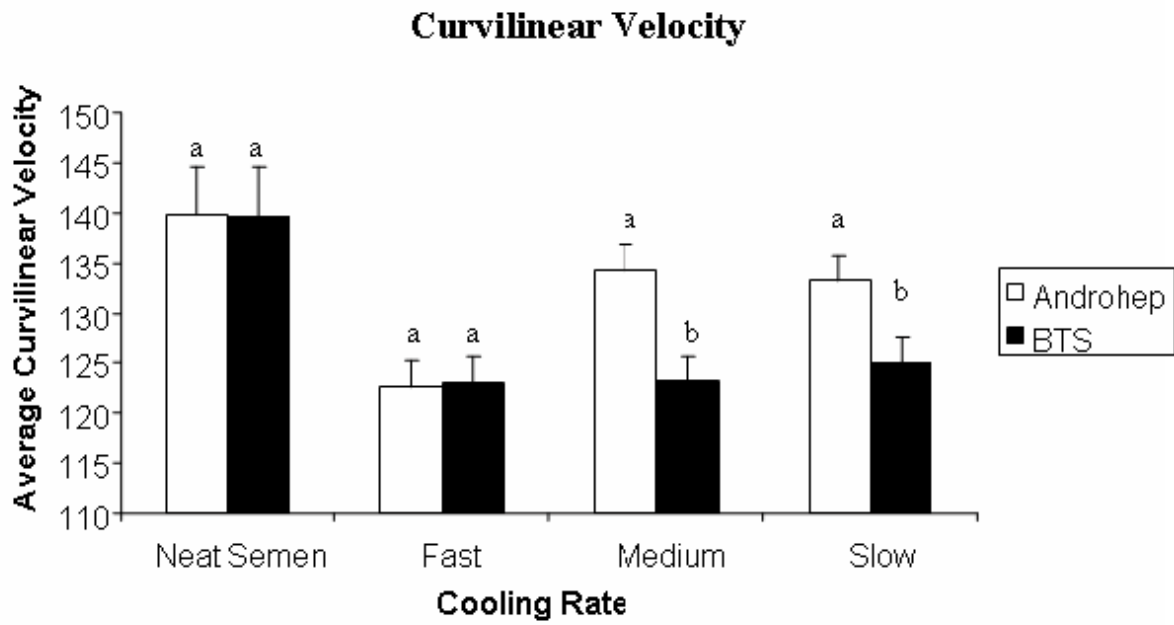


Figure 46. Average Curvilinear Velocity between Extenders for Different Rates of Cooling

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

Straight-line Velocity

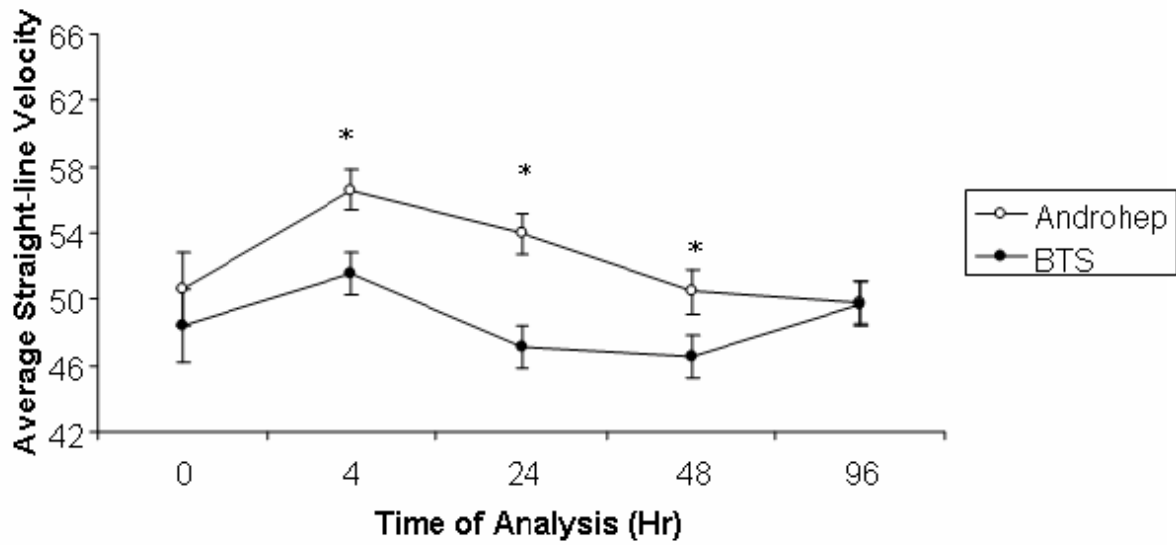


Figure 47. Average Straight-line Velocity between Extenders over Time

* Data points with a star indicate significant differences between extender for variables, $p < .05$

Straight-line Velocity

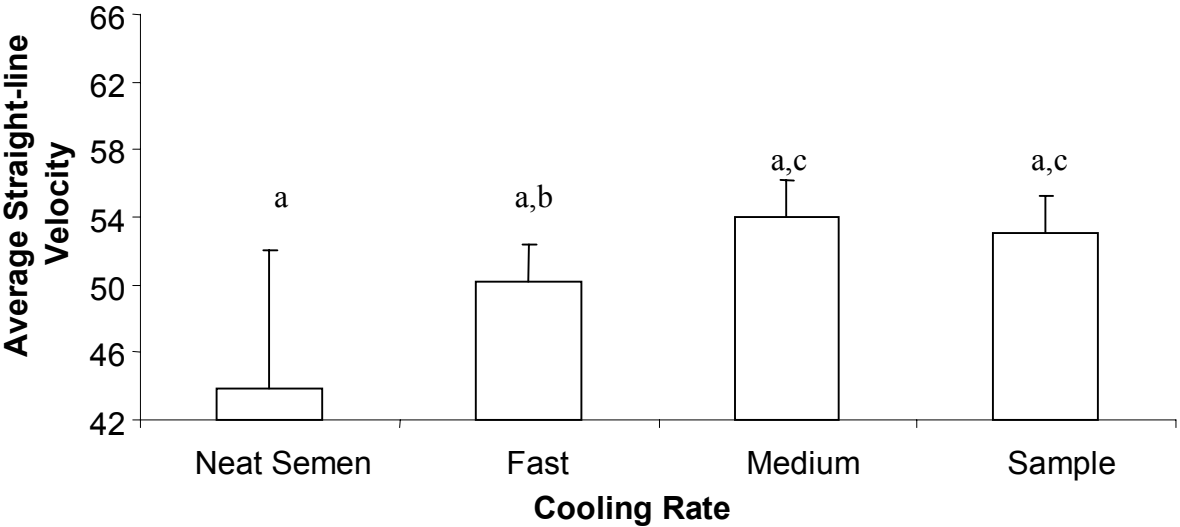


Figure 48. Average Straight-line Velocity between Different Rates of Cooling

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

Straight-line Velocity

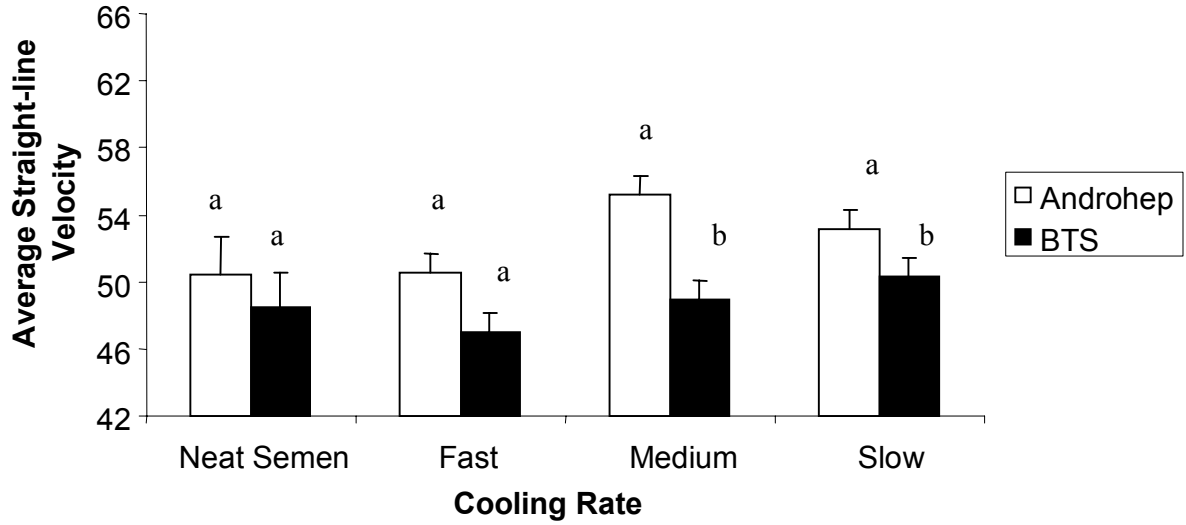


Figure 49. Straight-line Velocity between Extenders for Different Rates of Cooling

^{a,b} Extension Rates lacking a common superscript indicate a significant difference, $p < .05$

Data Logger Reading

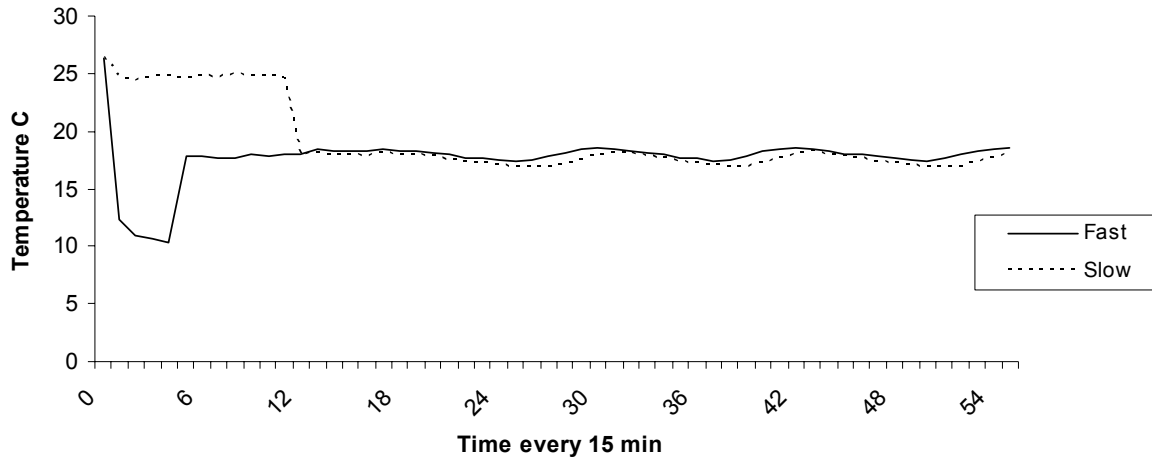


Figure 50. Representative Graph of Data Logger Output for Boar 113 during Week 1

CONCLUDING DISCUSSION

The results of the first experiment demonstrated that spermatozoa can withstand up to four degree of temperature difference between extender and semen during the extension process without sacrificing semen quality. The results of the second experiment demonstrated that after semen has been extended there is no difference if the samples are immediately placed into a 17-18°C storage unit, or if they are allowed to slowly approach room temperature before storage. These two experiments demonstrate that fresh semen is less resistant to changes in temperature than previously thought. Extender composition has changed since many of the preliminary studies examining temperature effects were conducted. It can be speculated that the extender formulations commercially available today are able to better regulate temperature fluctuations and provide a more thermostable environment. Both of these findings have the potential to save money and time with semen processing.

APPENDICES

APPENDIX A

SAS Coding for Statistical Analysis For Experiment 1

Repeated Measures for Proportions of Normal Acrosomes

```
proc glm;
class EXT SAMPLE TIME;
model PERCENTAB = ext sample time ext*time sample*time ext*sample ext*sample*time;
test h = ext sample ext*sample e=boar(sample*ext);
LSMEANS ext sample time ext*time sample*time ext*sample ext*sample*time /pdiff
stderr;
run;
quit;
```

Repeated Measures for Proportions of Normal Morphology

```
proc glm;
class EXT SAMPLE TIME;
model PERNORM PERHEAD PERTAIL PERHEADTAIL PERDIST PERPROX
PERTRANS PERDISTPROX= ext sample time ext*time sample*time ext*sample
ext*sample*time;
test h = ext sample ext*sample e=boar(sample*ext);
LSMEANS sample time ext*time sample*time ext*sample ext*sample*time /pdiff stderr;
run;
quit;
```

Repeated Measures for Estimates of Sperm Quality

```
proc glm;
class EXT SAMPLE TIME;
model DCL DSL VCL VSL AVMOT AVPRGMOT= ext sample time ext*time sample*time
ext*sample ext*sample*time;
test h = ext sample ext*sample e=boar(sample*ext);
LSMEANS sample time ext*time sample*time ext*sample ext*sample*time /pdiff stderr;
run;
quit;
```

Repeated Measures for CTC Analysis

```
proc glm;
class EXT SAMPLE TIME;
model NUMCAP NUMACRO= ext sample time ext*time sample*time ext*sample
ext*sample*time;
test h = ext sample ext*sample e=boar(sample*ext);
LSMEANS sample time ext*time sample*time ext*sample ext*sample*time /pdiff stderr;
run;
quit;
```

APPENDIX B

SAS Coding for Statistical Analysis For Experiment 2

Repeated Measures for Proportions of Normal Acrosomes

```
proc glm;
class EXT SAMPLE TIME;
model PERCENTAB = ext sample time ext*time sample*time ext*sample ext*sample*time;
test h = ext sample ext*sample e=boar(sample*ext);
LSMEANS ext sample time ext*time sample*time ext*sample ext*sample*time /pdiff
stderr;
run;
quit;
```

Repeated Measures for Proportions of Normal Morphology

```
proc glm;
class EXT SAMPLE TIME;
model PERNORM PERHEAD PERTAIL PERHEADTAIL PERDIST PERPROX
PERTRANS PERDISTPROX= ext sample time ext*time sample*time ext*sample
ext*sample*time;
test h = ext sample ext*sample e=boar(sample*ext);
LSMEANS sample time ext*time sample*time ext*sample ext*sample*time /pdiff stderr;
run;
quit;
```

Repeated Measures for Estimates of Sperm Quality

```
proc glm;
class EXT SAMPLE TIME;
model DCL DSL VCL VSL AVMOT AVPRGMOT= ext sample time ext*time sample*time
ext*sample ext*sample*time;
test h = ext sample ext*sample e=boar(sample*ext);
LSMEANS sample time ext*time sample*time ext*sample ext*sample*time /pdiff stderr;
run;
quit;
```