THE USE OF CEFTIOFUR SODIUM IN THE EXTENSION AND COOLED STORAGE OF EQUINE SEMEN: ITS EFFECTS ON MOTION CHARACTERISTICS, pH, OSMOLALITY, AND BACTERIAL GROWTH

BY

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THESIS

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ABSTRACT

During semen collection in the stallion, contamination of the ejaculate occurs secondarily to the normal microflora present on the external genitalia. These bacteria and their metabolites can negatively affect spermatozoal motility and viability during cooled storage. Therefore, the addition of antibiotics to the semen extender is warranted to reduce bacterial load and prevent further growth. Naxcel® (ceftiofur sodium, Pfizer Inc., New York, NY) is a third generation cephalosporin antibiotic with high antibacterial activity and a broad resistance to β-lactamases. The purpose of the current study was to evaluate varying levels of ceftiofur sodium added to a skim milk-glucose based semen extender in comparison to control (antibiotic-free extender) and other commonly utilized antibiotics on spermatozoal motion characteristics, semen osmolality and pH, and bacterial growth during cooled storage for 48 hours.

Semen was collected and pH measurement, osmolality measurement, and aerobic culture were undertaken for each ejaculate (total of thirteen from three stallions). Each ejaculate was then divided and extended in a skim milk-glucose based semen extender (Har-Vet™ Semen Extender, Har-Vet™, Spring Valley, WI) without antibiotic (control) or with one of seven different antibiotic groups: ceftiofur sodium at 250µg/ml (CEFT250); ceftiofur sodium at 500µg/ml (CEFT500); ceftiofur sodium at 1,000µg/ml (CEFT1000); ceftiofur sodium at 2,500µg/ml (CEFT2500); combination of amikacin sulfate and potassium penicillin G at 1,000µg/ml and 1,000IU/ml, respectively (AMKPCN); gentamicin sulfate at 1,000µg/ml (GENT); and ticarcillin disodium at 1,000µg/ml (TICAR). Extended semen was cooled and stored in a semen-transport container (Equitainer™, Hamilton Research Inc, South Hamilton, MA) at approximately 5°C. Motility measures using computer-assisted semen analysis, pH measurements, and osmolality measurements were performed at 0, 24, and 48 hours after

collection. Aerobic culture was performed for the eight extended groups after 24 and 48 hours of storage. Motion characteristics measured included: total motility (MOT), progressive motility (PM), curvilinear distance (DCL), average path distance (DAP), straight line distance (DSL), curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity (LIN), straightness (STR), wobble (WOB), beat cross frequency (BCF), and amplitude of lateral head displacement (ALH).

Ceftiofur sodium had a dose-dependent effect on DCL, VCL, and ALH, with higher concentrations (1,000µg/ml and 2,500µg/ml) yielding increased values in comparison to controls (F test, $P<0.05$; post-test comparison, $P<0.0071$). There was also a significant effect of extender on STR (F test, $P<0.05$) with higher concentrations of ceftiofur sodium tending to decrease values in comparison to controls ($P<0.028$). The changes in motion characteristics are consistent with a trend toward spermatozoal hyperactivation. Stepwise, multiple regression analysis revealed pH was the strongest indicator for the increased motion characteristics, but it was only a minor predictor. There was no significant effect of extender noted for MOT, PM, DAP, DSL, VAP, VSL, LIN, WOB, and BCF. There was an effect of time on MOT and PM with parameters decreasing significantly over time (F test, $P<0.05$). Time also had an effect on DAP, DSL, VAP, VSL, LIN, STR, and WOB (F test, $P<0.05$) with most values decreased significantly after 48 hours of storage.

There was a significant effect of extender group on pH (F test, $P<0.05$). Overall, pH was significantly decreased for the CEFT2500, AMKPCN, and TICAR groups and significantly increased for the GENT group in comparison to control. Time also had a significant effect on pH, with values increasing during storage. Neither extender group nor time had a significant effect on osmolality.

The most frequent isolates from the ejaculates were *Corynebacterium* spp., coagulase-negative *Staphylococcus* spp., and *Pseudomonas* spp. with each stallion tending to grow a consistent "normal flora". All treatment groups grew bacteria at some point during the experiment except the amikacin/penicillin group after 48 hours of storage. *Corynebacterium* spp. was the most common isolate in the control groups at both 24 hours and 48 hours of storage. The AMKPCN extender group provided the most effective antimicrobial control with growth noted in two of the thirteen collections. The CEFT250 and TICAR extender groups provided the least effective antimicrobial control with growth noted in ten and eight of the thirteen collections, respectively. The CEFT1000 extender group provided acceptable antimicrobial control with growth noted in five of the thirteen collections.

The most significant finding of the current study was the dose-dependent relationship between ceftiofur sodium and increases in motion characteristics associated with hyperactivation of spermatozoa. While pH was the strongest predictor for these changes of the measured variables, it was only a minor indicator. Furthermore, hyperactivation of spermatozoa is associated with an increase in intracellular pH and a decrease in solution pH was noted with high concentrations of ceftiofur in comparison to control in the current study. Further study is indicated for the evaluation of ceftiofur sodium in the extension and cooled storage of stallion semen and its relationship to hyperactivation of spermatozoa.

Keywords: Spermatozoa, equine, antibiotics, semen extender, motion characteristics, bacteriology, pH, osmolality

To my family

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CHAPTER 01

INTRODUCTION

The stallion has a variable, normal microflora present in the distal urethra and on the surface of the penis and prepuce (Bowen et al., 1982; Burns et al., 1975; Clément et al., 1995; Hoyumpa et al., 1992; Kenney et al., 1975; Rideout et al., 1982; Varner et al., 1998), which contaminates the ejaculate during semen collection despite proper collection technique (Clément et al., 1995; Samper and Tibary, 2006). The normal, fertile mare can respond appropriately to bacterial invasion at the time of insemination. However, in susceptible mares, bacteria can become established, provide an environment unsuitable to a developing embryo, and negatively impact fertility (Bennett 1986; Blanchard et al., 1987; King et al., 2006; Varner et al., 1998). Also, bacteria and their metabolites can negatively affect spermatozoal motility and viability, further decreasing fertility (Rideout et al., 1982; Aurich and Spergser, 2006). Due to these factors, the addition of antibiotics into the semen extender is warranted to reduce bacterial load and prevent further growth.

As both gram-positive and gram-negative bacteria can be present or potentially pathogenic in the stallion, a broad-spectrum antibiotic is indicated. Ceftiofur is a third generation cephalosporin antibiotic with high antibacterial activity and a broad resistance to β-lactamases (Prescott et al., 2000). Bermúdez et al (1995) reported that intra-uterine administration of Naxcel® (ceftiofur sodium, Pfizer Inc., New York, NY) offered effective control of bacteria, decreased inflammation, and improved subsequent fertility. These aspects show ceftiofur sodium could potentially offer another effective choice for antibiotic in semen extender, but further research is warranted.

To date, little research has been published regarding the potential use of this antibiotic in stallion semen extension. Varner et al (1998) reported that when used in a milk-glucose semen extender, ceftiofur sodium (1,000µg/ml) provided adequate control of bacterial growth and did not negatively impact computer-analyzed motion characteristics after 24 hours of storage at 5°C. It is not uncommon that semen is stored longer than 24 hours in the shipping container, given that equine semen can travel great distances prior to insemination or can be held for the purpose of timely breeding. In previous work evaluating antibiotics and their effects on motion characteristics, the investigators found that at 48 hours of storage at 5°C gentamicin (1,000-µg/mL) significantly reduced percent motility and progressive motility, while at 24 hours there were no significant detrimental effects noted (Jasko et al., 1993). Prolonged exposure (>24 hours) to ceftiofur sodium may have a detrimental effect on spermatozoa that was not seen in previous work evaluating 24 hours of storage.

This study evaluated the effects of different concentrations of Naxcel® (ceftiofur sodium) on spermatozoal motion characteristics and semen bacteriology up to 48 hours of storage. To the author's knowledge, no previous work has been published evaluating the minimum effective and the toxic concentrations of Naxcel® in stallion semen extenders.

The hypotheses to be tested include:

1. Ceftiofur sodium can safely be used as a broad-spectrum antibacterial agent in the extension and cooled storage of equine semen over a 48 hour period, without negatively impacting motion characteristics in comparison to other broad spectrum antibiotics (combination amikacin/potassium penicillin G, gentamicin, and ticarcillin).
2. Based on bacterial growth and motion characteristics, the minimum effective and toxic concentrations of ceftiofur sodium will be 1,000µg/ml and 2,500µg/ml, respectively.

CHAPTER 02

LITERATURE REVIEW

A. Semen Collection in the Stallion

1. History

Although largely undocumented, it is commonly reported that the history of artificial insemination began in the 1300's with Arabian horses via the aspiration of semen from a recently bred mare. However, it was not until the beginning of the twentieth century that documented reports of successful artificial insemination in horses using this technique first appeared (Heape, 1897). By the 1920's, an intravaginal sponge technique was developed where stallion semen was collected post-mating from a sponge that had been inserted into the vagina of the mare (Berliner, 1940; Love, 1992). However, the technique was associated with high spermatozoal loss and damage as well as an increased risk of venereal disease spread and alternate techniques of semen collection were sought. Rubber sperm collectors of various sorts were subsequently developed without much success (Berliner, 1940). The first documented development of an artificial vagina (AV) in the United States was by F.F. McKenzie at the University of Missouri for use in the boar (McKenzie, 1931). Interestingly, a Russian researcher, V. K. Milovanov, was also developing several AV models at the same time as McKenzie (Berliner, 1940). However, due to the cumbersome, heavy nature of these AVs, their widespread use did not initially occur in the stallion. The collection of semen for the purpose of artificial insemination (AI) was often performed via vaginal aspiration of semen from a recently bred mare. In the 1940's, the

development of lighter weight, more flexible AVs allowed for the continued growth of stallion semen collection for the purpose of AI (Berliner, 1940). This newer model was further improved upon by McKenzie and became known as the Missouri model AV that is still commonly utilized (Love, 1992). Shortly after this time, the importance of semen evaluation in the stallion and of semen characteristics was documented (Day, 1940). The importance of this evaluation and its potential link to stallion fertility continued to develop. In today's equine industry, stallion semen collection is frequently practiced for multiple purposes including: breeding soundness examination, cooled extension and storage, semen cryopreservation, and advanced reproductive techniques (e. g. spermatozoal sexing, in vitro fertilization).

2. Procedure

Most stallions can be trained for semen collection via artificial vagina (AV) with relative ease. The stallion is conditioned to extend the penis and attain erection with exposure to a mare in estrus, an ovarectomized mare treated with estrogens, or a breeding phantom in cases of routinely collected stallions (Samper, 2007). The penis, prepuce, and urethra harbor a normal bacterial flora and semen is often contaminated with some of these bacteria (Rideout et al., 1982; Hoyumpa et al., 1992; Clément et al., 1995). Washing the erect penis of the stallion with plain water is generally performed prior to collection. Research has shown that all forms of washing will alter the normal flora of the stallion penis, but water alone has the least altering effect on the normal flora and is therefore recommended over antiseptics or soaps (Bowen, 1982). Most frequently, the stallion is collected via diversion of the penis into a properly prepared AV while mounting a breeding phantom or a receptive mare, although ground collection is also utilized. A

stallion condom can also be utilized for the collection of a stallion that will not breed with an AV. Due to the marked contamination of the sample with debris and bacteria, this method of collection should only be utilized when absolutely necessary (Love, 1992; Blanchard et al., 2003).

The stallion ejaculates in a series of five to ten jets that is divided into three fractions (Loomis, 2006; McDonnell, 2006). The pre-sperm or first fraction contains no spermatozoa and is comprised mainly of fluid from the bulbourethral glands. The sperm-rich or second fraction contains the majority of the spermatozoa and originates from the tail of the epididymis. The gel or third fraction contains minimal spermatozoa and is comprised mainly of fluid from the seminal vesicles (Love, 1992; Loomis, 2006). Generally, the entire ejaculate is collected in one receptacle although the ejaculate can be fractionated with the use of an open-ended AV. Alternatively, the AV can be prepared with an in-line, micro mesh filter to allow for the collection of gel-free semen, although the filter can occasionally become clogged if the gel-fraction is particularly large (personal observation).

Ex copula ejaculation is also possible in the stallion. The principal pharmaceutical compounds reported for the induction of ex copula ejaculation are α-adrenergics (detomidine and xylazine) and other smooth muscle-active agents, such as imipramine (McDonnell and Love, 1991; Rowley, 1999; McDonnell, 2001). These drugs have been known to affect contractions of genital smooth muscle in the horse and have been associated with the occasional side effect of induced-ejaculation. Reported regimens include a variety of doses, schedules, routes of administration, combinations of agents, and pre-treatment procedures. In recent literature, the two most commonly utilized drugs are imipramine and xylazine. Imipramine is a tricyclic antidepressant agent that has three main pharmacological actions: blockage of the amine pump,

which causes an increase in neurotransmitter levels (principally serotonin, but also norepinephrine), sedation, and central and peripheral anticholinergic activity. For reproductive purposes, this drug has been used to lower the ejaculation threshold and to induce ejaculation ex copula (McDonnell et al., 1987). Xylazine is an α2-adrenergic agonist that causes sedation, analgesia, and muscle relaxation. For reproductive purposes, this drug has been used to pharmacologically induce ejaculation ex copula (Turner et al., 1995; Card et al., 1997; McDonnell 2001). A common protocol includes the administration of imipramine (1mg/lb, orally) one to two hours prior to the administration of xylazine. If ejaculation occurs, it is most commonly within five minutes of the xylazine administration and is collected in a plastic bag either tied around the back of the stallion to cover the penis continually or attached to a pole for "free-catch" at the time of ejaculation (McDonnell, 2006). Overall, reported rates of ejaculation are approximately 30–75% of attempts (McDonnell, 2001). Ejaculate characteristics and fertility are similar to samples obtained from collection with an AV (McDonnell, 1991; Card et al., 1997).

3. Semen Handling Post-Collection

Collection of stallion semen via AV interrupts the natural "closed-loop" system association with natural or live-cover breeding. A primary consideration with this type of breeding system is to minimize harm to the spermatozoa when handling and preparing semen (Loomis, 2006). During the collection process, it is important to ensure that the AV has been properly prepared and that all surfaces the semen comes into contact with are non-spermacidal. After collection, the sample should be immediately transported to the laboratory while protecting

it from light exposure, cold shock, excessive heat, and/or physical trauma (Love and Varner, 1998; Blanchard et al., 2003). Once in the laboratory, all things in contact with the semen should be clean, non-toxic, and pre-warmed to approximately 37°C. The temperature of the semen should be maintained at 35°C to 37°C at all times. Prior to initial evaluation, the ejaculate should be filtered for removal of the gel fraction (if it was not done during the collection process) as well as for any dirt or debris that may be contaminating the semen (Love and Varner, 1998).

B. Routine Semen Analysis in the Stallion

1. The Relationship of Routine Semen Analysis to Fertility

Semen evaluation as a component of the breeding soundness examination was developed with the intent of predicting future fertility. Historically, the basis of the examination relies on the measurement of sperm motility (raw and extended), longevity of motility, morphological features, spermatozoal numbers, and the detection of evidence of inflammation (Pickett, 1993; Love, 2002). Unfortunately, attempts to consistently and strongly relate fertility to specific seminal parameters in the stallion are of limited capacity (Amann, 1989; Jasko, 1992; Magistrini et al., 1996; Love et al., 2000; Meyers, 2002). Some stallions that fall within normal parameters are sub-fertile, while stallions with abnormal parameters are normo-fertile, which makes predicting future fertility problematic.

The poor correlation of a single assay to fertility is likely due to the fact that a single parameter involves only one of the numerous attributes that a spermatozoon must possess to attain fertility, a relative few number of spermatozoa are evaluated when considering the entire ejaculate, and many studies consist of a small number of stallions (Amann and Graham, 1993; Graham, 2001; Amann, 2005). Attempts at basing prediction of fertility on single laboratory parameters should be abandoned, and evaluation of a large conglomerate of measures should be incorporated into the breeding soundness exam of the stallion. When several tests are used in combination and multiple samples are evaluated, the ability to predict future fertility is much improved (Love and Varner, 1998; Graham, 2001; Meyers, 2002; Colenbrander et al., 2003; Love et al., 2003). The standard breeding soundness exam should be utilized as a means of

selecting out obviously unsatisfactory breeding prospects versus the identification of sub-fertility.

2. Physical Characteristics of the Ejaculate

2.1 Appearance

In general, fresh stallion semen should have an opaque, white to grayish-white, somewhat watery appearance (Jasko, 1992; Graham, 1996; Love and Varner, 1998). The appearance allows a rough estimate of concentration. Semen of extremely low concentration yields the appearance of transparent, watery fluid consistent with pear-juice. Semen of extremely high concentration yields the appearance of white, creamy fluid consistent with whole milk. Color changes to the ejaculate can give an indication of the presence of abnormal cells or contamination. Semen contaminated with urine has a yellow hue while semen contaminated with blood/red blood cells has a pink-tinged to red hue. Semen grossly contaminated with white blood cells/purulent material can attain a thick green to yellow and/or flocculent appearance. The average volume of the ejaculate is 65mL with a range of 30 to 300mL considered to be within normal limits (Lopate et al., 2003).

2.2 Concentration

The concentration of the sperm-rich fraction of the ejaculate is required for calculation of total spermatozoa numbers. Concentration is dependent on the amount of testicular tissue

present, with one gram of testicular tissue producing roughly 19 million spermatozoa per day during breeding season (Rodriguez-Martinez, 1992). Several other factors will also affect the concentration of an individual ejaculate. Seasonal effects on sperm production have been well documented in the stallion with sperm output significantly decreased during the non-breeding season (Pickett et al., 1970; Thompson et al., 1977). Recent breeding activity will also affect the concentrations of an individual ejaculate. This is due to the presence and amount of extragonadal sperm reserves. This reserve, primarily present in the tail of the epididymis, can amount to three to four times the stallion's daily sperm output (Love, 2007). A sexually rested stallion will therefore typically ejaculate a highly concentrated sample due to the presence of this store. Sperm production, and frequently semen concentration increases with stallion age. This is secondary to an increase in the total length of the seminiferous tubules (Johnson and Neaves, 1981; Estrada and Samper, 2007). Consequently, stallion spermatozoa concentration varies widely. Measurements are commonly reported to range from 75 to 800 million spermatozoa per milliliter (Lopate et al., 2003) with means ranging from 100 to 200 million spermatozoa per milliliter (Dowsett and Pattie, 1982; Rousset et al., 1987).

While multiple methods of determining spermatozoal concentration have been described for man (Mahmoud et al., 1997), the most frequently used methods for the stallion include the hematocytometer and spectrophotometry (Brinsko et al., 2011). The hematocytometer provides a direct measurement that is highly accurate when performed properly. It has previously been used as the standard to determine the accuracy of other systems. However, proper technique is imperative as dilution and loading errors occur easily and significantly affect counts (Rigby et al., 2001). For this reason, multiple measurements of each sample are recommended to increase accuracy. Benefits of this system are low cost, and accuracy is not affected by sample

discoloration. Spectrophotometry provides an indirect estimate of spermatozoal concentrations via measurements of optical density. This is the most commonly utilized method of concentration determination due to its efficiency and ease of use. Rigby et al. (2001) found a high correlation between hemocytometer measurements and four commercially available photometric systems. However, there was a tendency for greater disparity between the two methods when spermatozoal concentrations were high (>300million/ml) or low (<100million/ml). This effect has been previously noted (Varner et al., 1991). A significant disadvantage of these systems includes inaccurate estimates due to the presence of contaminants within the sample (semen extenders, cellular debris, blood, urine, purulent material, and/or premature germ cells) or due to an improperly calibrated machine (Brinsko et al., 2011).

Alternative measures of spermatozoal concentration include computer-assisted semen analysis (CASA) and flow cytometry. Overall, CASA systems are infrequently used for measurements of spermatozoal concentration. This is not only due to the cost of the system, but there are also reported inaccuracies. This is secondary to the physics associated with loading the glass slide chambers utilized with the system causing an area of high spermatozoal concentration at the meniscus, which decreases the concentration at the area of measurement (Kuster, 2005; Douglas-Hamilton et al., 2005). Flow cytometry is a method of cell counting that entails the suspension of cells within a stream of fluid and passage before an electronic detection device. Cells are detected via fluorescent stain and the particular stain used determines which aspect of the individual cell is fluoresced. Recently, a commercially available flow cytometry unit has been validated for use in the stallion (Morrell et al., 2010). This device counts cells via fluorescence emission of DNA after staining with a fluorescent probe. This method of determining spermatozoal concentration is currently the most accurate with samples of either

very high or low concentration (Brinsko et al., 2011). However, it is not a widely used method due to expense and also likely due to lack of familiarity.

2.3 pH and Osmotic Pressure

Normal stallion semen is slightly basic in nature. Reported pH values typically range from 7.2 – 8.0 (Davis and Cole, 1939; Blanchard et al. 2003), while the optimal value for maximizing spermatozoal viability is considered to fall between 7.4 and 7.9 (Griggers et al., 2001). However, values have been reported outside of this range in normal stallions (Limone et al., 2002). The gel-fraction of the ejaculate has a higher pH than gel-free semen, which could account for slight differences in values depending on whether the measurement occurs prior to or after ejaculate filtration (Pickett et al., 1976). Both excessively acidic and excessively basic conditions are generally equally detrimental in altering motility and viability of the spermatozoon. However, it has been shown with human spermatozoa that the immobilization effects of acidic conditions are reversible, but excessive alkalinization will irreversibly harm spermatozoa (Makler A et al., 1981).

The most commonly reported normal osmotic pressure range of normal stallions is approximately 290-320 mOsm (Pickett et al., 1976; Griggers et al., 2001; Lopate et al., 2003). A large variation in values between stallions as well as between ejaculates from the same stallion is frequently encountered. Spermatozoa can tolerate approximately 100 mOsm variation prior to the occurrence of significant detrimental effects to motility and viability (Meyers, 2005). Great increases in osmotic pressure can occur with detrimental effects secondary to contamination of the ejaculate from debris, lubricants, urine, and/or purulent material (Duoos et al., 2002; Lopate

et al., 2003; Estrada and Samper, 2007). The most frequent cause of excessive alteration in spermatozoal pH and osmotic pressure are secondary to urospermia and/or pyospermia.

3 Spermatozoal Characteristics

3.1 Morphology

Assessment of spermatozoal morphology is an integral part of routine semen analysis in all species. Several methods of morphology evaluation are available and include: phase-contrast microscopy, differential interference-contrast microscopy, light microscopy with differential staining, and scanning and transmission electron microscopy. Scanning electron microscopy provides an ultra-detailed three-dimensional view of the entire spermatozoon, but is uncommonly used due to expense and lack of availability in most clinical settings. Transmission electron microscopy has also been described to more specifically evaluate certain tail abnormalities and acrosomal defects by providing a detailed cross-sectional view of the spermatozoon ultrastructure (Pesch et al., 2006). Again, this method of assessing spermatozoal morphology has limited use within the clinical setting. The use of differential stains for visualization is considered less superior to contrast microscopy secondary to increased artifactual changes that can be interpreted as morphologic abnormalities (Love and Varner, 1998; Blanchard et al., 2003). The most common stain reported for initial morphology evaluation is modified Wright-Giemsa stain (Diff-Quik), which provides a simple, rapid method of evaluating both nuclear and cytoplasmic components. Other commonly utilized stains include eosin-nigrosin and toluidine blue, which both provide measures of vitality. Contrast microscopy

evaluation of morphology requires fixation of the cells (via buffered formol-saline or buffered glutaraldehyde solution) prior to evaluation to decrease artifactual changes prior to interpretation (Blanchard et al, 2003). At least 100 spermatozoa (preferably 200) should be evaluated and recorded.

Abnormalities can be characterized by different classification systems that theoretically allow one to predict their effect on fertility. Three different classification systems are commonly used. The first system is primary, secondary, and tertiary abnormalities, which divides abnormalities on the basis of the location and timing of their appearance in relation to development. Primary abnormalities occur during spermatogenesis (within the testes proper) and include abnormally shaped heads, abnormally formed midpieces, and proximal cytoplasmic droplets. Secondary abnormalities occur during maturation of the spermatozoa (within the excurrent ducts) and include detached heads, bent midpieces, and distal cytoplasmic droplets. Tertiary abnormalities are considered to occur in vitro due to improper handling and can include looped, coiled, or bent tails and detached heads (Dowsett et al., 1984; Blanchard et al., 2003; Card, 2005). The second classification system describes abnormalities as major or minor and is based on their impact to fertility. Major abnormalities (such as proximal droplets, congenital acrosomal defects, and pyriform shaped heads) are thought to have a greater impact on fertility while minor abnormalities (such as distal droplets and simple bent tails) are thought to have little consequence on fertility (Card, 2005). The third classification is compensable and non-compensable abnormalities, which are divided on the basis of their ability to overcome infertility with increased insemination dose. Stallions that require a higher insemination dose to attain maximal fertility have compensable deficits and those that are sub-fertile regardless of insemination dose have non-compensable deficits (Saacke et al., 2000). Examples of

compensable defects include acrosomal and tail defects and examples of non-compensable defects include those of the head that affect the DNA integrity. While it is clearly understood that not all defects directly impact fertility, there are conflicting reports of which ones do and the mechanism by which they do so. Therefore, use of the classification systems can be helpful in identifying potential sub-fertile stallions, but evaluating individual deficits as a whole allows one to follow the prevalence of the abnormalities and reveals more specific information without assuming the origin of the abnormalities.

The average stallion has approximately 50% morphologically normal spermatozoa (Card, 2005). It is important to note that certain morphological characteristics are considered abnormal in most species, but are considered normal in the stallion. These include: abaxial implantation of the tail, the presence of a smaller acrosome, and an asymmetrically shaped head (Love, 2002; Pesch et al., 2006). As mentioned previously, improper handling and poor collection technique can result in artifactual changes in spermatozoa morphology. Reportedly, the most common morphological abnormalities in the stallion are: abnormal head, detached head, abnormal/broken neck, abnormal midpiece, proximal droplet, distal droplet, and coiled or kinked tails (Estrada and Samper, 2007).

The exact percentage of abnormal cells and the types of deficits that negatively impact fertility are not known. Varying correlations between abnormal morphology and decreased fertility have been reported (Bielanski and Kaczmarski, 1979; Voss et al., 1981; Jasko et al., 1990; Graham, 1996; Parlevliet and Colenbrander, 1999). In particular, abnormalities involving the head and midpiece have shown a more negative impact on fertility than those involving the tail end piece (Jasko et al., 1990; Love et al., 2000; Clément et al., 2001). In general the guidelines for concern in the stallions are: >10% premature germ cells; >30% head and/or

midpiece defects; >25% proximal droplets; and <30% morphologically normal spermatozoa (Card, 2005). It is assumed that abnormal spermatozoa do not exert a direct negative influence on normal spermatozoa. Therefore, the percent morphologically normal spermatozoa are more important than the percent morphologically abnormal spermatozoa (Love and Varner, 1998; Blanchard et al., 2003).

3.2 Motion Characteristics

Spermatozoal motion characteristics are very important parameters in the breeding soundness examination of the stallion. Initial motion characteristics of raw semen should be evaluated within five minutes of collection, and the use of a stage warmer to prevent cold shock will minimize artifactual decreases in motility. This allows the practitioner to evaluate future drops in motility and to speculate possible causes. Evaluation of raw semen is important as it allows for the detection of significant decreases in total motility with the addition of diluents (extender). However for the assessment of individual motility patterns, the stallion ejaculate is generally too concentrated for proper evaluation and should be extended in an appropriate concentration (25 to 50 million spermatozoa per milliliter). This enhances accuracy as motility is frequently over-estimated with samples of too high concentration (Estrada and Samper, 2007). In this method, a 5-10 µL drop of extended semen is placed on a pre-warmed microscope slide and covered with a coverslip. The center of the sample should be evaluated as motility at the edges declines more rapidly secondary to drying and exposure to air (Estrada and Sampler, 2007). Phase-contrast microscopy is ideal for evaluation, although bright-field microscopy can also be utilized. When measuring values subjectively, three to five fields should be evaluated

and the percentages averaged to yield results. Accuracy of measurements will increase if two individuals make approximations.

The most frequently evaluated subjective motion characteristics are total motility, progressive motility, and velocity. Progressive motility is considered to be of greater importance than total motility as progressive motility is essential for fertility (Pickett, 1993; Brinsko et al., 2011). A progressively motile spermatozoon is one that moves across the field in a reasonably rapid fashion, and with each back and forth lash of the tail, the head rotates 360°. At least 50% of the spermatozoa should be progressively motile in a normal stallion (Lopate et al., 2003). Spermatozoal velocity can also be measured in a subjective fashion. The spermatozoa are measured on an arbitrary scale of 0 to 4, immotile to rapidly motile respectively (Love and Varner, 1998). The extension of the ejaculate will generally alter the spermatozoal motion characteristics, generally by increasing velocity measures (Varner, 2008).

Computer-Assistant Semen Analysis (CASA)

With the use of a single, subjective estimation of percent motility, the 95% confidence interval is projected to be ±30-60% (Amann, 1987). Accordingly, several different methods have been developed to evaluate motility and other motion characteristics in an objective fashion. As spermatozoa appear to alter their motion characteristics as they move, objective analysis allows for the detailed examination of these changes. These methods include time-lapse photomicrography, frame-by-frame playback videomicrography, spectrophotometry, and computerized analysis. Computer-assisted semen analysis (CASA) is the most utilized of these options within the research laboratory setting.

Computer-assisted semen analysis allows for the objective measurement of motion characteristics taken from tracks of large numbers of spermatozoa. The first validated system for computer-analysis was developed over 30 years ago (Liu and Warme, 1977), and these systems have become more commercially available in recent years. The technology is based on the capturing of successive microscopic images, which are then digitalized. The motile spermatozoa in the image are subsequently identified in the successive images allowing for the establishment of their trajectories. The trajectories are then mathematically processed to allow for their definition into a numerical form. This yields a series of parameters that define the exact movement of each individual spermatozoon recorded. This objective analysis also allows for a more specific characterization of spermatozoa other the simple percent motility and percent progressive motility.

While the use of CASA in the evaluation of motion characteristics increases the objectivity of the analysis, there are limitations associated with the technology (Quintero-Moreno, 2003). First, there is an assumption that there is a normal distribution of all the variables measured among the spermatozoa population when frequently there is a high degree of asymmetry in the observations. Second, the large variety of separate parameters (generally twelve to twenty) makes it difficult to explain the overall movement of the spermatozoa accurately. Finally, ideal conditions under which the sperm cells are evaluated must be maintained (free from debris and at the appropriate concentration, fluid depth, and temperature) to prevent the generation of erroneous data (Amann, 1987). The benefit of objective measurement should outweigh the limitations. It is important to note that various features of these systems can be customized making comparisons in motion characteristics between laboratories extremely difficult (Amann and Katz, 2004). The most common movement

characteristics defined by CASA systems include: the amplitude of lateral head displacement, beat cross frequency, straightness, wobble, linearity, and multiple measures of velocity and distance traveled. Unfortunately, a uniform nomenclature is not utilized by the various systems available, which can further complicate interpretation.

With CASA, various measures of velocity are taken and reported in micrometers per second (µm/sec). These include curvilinear velocity, straight line velocity, and average path velocity. Curvilinear velocity (VCL) is the velocity of the total distance between each spermatozoa track along its actual curvilinear path. Straight line velocity (VSL) is the velocity between the beginning and end of the spermatozoa track, or a straight line between the spermatozoa's first detected position and its last detected position. Average path velocity (VAP) is the velocity of the spermatozoa along the smoothed, average path the spermatozoa takes during the measurement period. While the relevance of these measurements has not been clearly established in the stallion, they have been shown to correlate with certain requirements of fertility in other species (Olds-Clarke, 1996; Robayo et al., 2008; Gillan, 2008). Distance parameters measured via CASA include curvilinear distance traveled (DCL), straight line distance traveled (DSL), and average path distance traveled (DAP). These measurements are reported in micrometers (µm). Beat cross frequency (BCF), measured in hertz (Hz), is the number of lateral oscillatory movements of the sperm head per second around the cell's mean path. The amplitude of lateral head displacement (ALH) is the mean width of the head oscillation and is measured in micrometers (µm). The importance of these parameters in association with fertility has not been extensively evaluated, but hyperactivation of stallion spermatozoa has been described by changes in selected motion characteristics including increases in VCL and ALH (Rathi et al., 2001; McPartlin et al., 2009).

Various values are calculated from the measurements taken by CASA. These include linearity (LIN), straightness (STR), and wobble (WOB). Linearity is the ratio of the straight line velocity compared to the overall, actual path velocity the spermatozoa travel (VSL divided by VCL). Straightness is the ratio of the straight line velocity compared to the smoothed, average path the spermatozoa travel (VSL divided by VAP). Wobble is the ratio of the smoothed, average path compared to the overall, actual path velocity the spermatozoa travel (VAP divided by VCL). All of the calculated measurements are expressed as a percentage. Decreases in linearity and straightness have been utilized to describe hyperactivation of stallion spermatozoa and its association with capacitation (Rathi et al., 2001; McPartlin et al., 2009). Otherwise, these calculated parameters have not been extensively evaluated in the stallion.

Motion Characteristics and Fertility

Motion characteristics are not highly correlated with fertility in the stallion even with the use of computer assisted semen analysis (Voss et al., 1981; Samper et al., 1991; Jasko et al., 1992; Graham, 1996; Colenbrander et al., 2003). One could speculate that this is due to the fact that the environment in which we measure motion characteristics is vastly different from the environment of the mare's reproductive tract. Alternatively, it has been suggested that this lack of correlation may be due to improper use of the available data. Quintero-Moreno et al. (2003) utilized CASA to differentiate spermatozoa into four subpopulations based on specific motion characteristics. In this study, the majority of the motile spermatozoa in ejaculates of known high fertility were in a subpopulation with high progressive motility and low linearity. Furthermore, all the motile spermatozoa in ejaculates of known high fertility with a total sperm count of greater than or equal to 20 billion spermatozoa were in the subpopulation with high progressive

motility and low linearity. The study concluded this category of sperm potentially represents the spermatozoa with the highest fertilizing potential. Subjective evaluation of motion characteristics are also not well correlated with fertility (Dowsett and Pattie, 1982; Graham, 2001; Meyers, 2002). However, significant decreases in progressive motility are generally though to result in decreased fertility, as Colenbrander et al. (2003) reported compromised fertility when levels of progressive motility drop below 40%.

Longevity of Motility

Longevity of motility of raw and extended samples should be evaluated as well as initial motion characteristics. Raw semen stored at room temperature should maintain at least 10% progressive motility for 6 to 8 hours and extended semen should maintain the same for 24 hours provided samples are shielded from light (Kenney et al., 1971; Brinsko et al., 2011). Extended samples stored at 4-6°C should maintain motility similar to or only slightly less than original values for 24 hours. Evaluating total and progressive motility after cooled-storage is generally thought to be of more value in assessing a potential breeding prospect as this is the same manner in which semen is transported in artificial insemination programs.

C. Advanced Spermatozoal Analysis in the Stallion

1. Plasma Membrane Integrity

The plasma membrane surrounds the entire spermatozoon and is integral to cellular function and vitality. Consequently, there are multiple testing procedures utilized to evaluate its integrity and are generally referred to as sperm viability tests. The use of extracellular dyes is the most common assay as it is relatively easy and inexpensive. Common extracellular stains include: eosin-nigrosin and eosin-aniline-blue (Dott and Foster, 1972). The presence of an intact plasma membrane prevents the uptake of the eosin stain and differences between membrane-intact versus non-intact can be detected via light microscopy with the help of "background" stains (nigrosin or aniline blue) to provide contrast for live cells. When performed correctly, the use of extracellular stains can provide information concerning the plasma membrane as accurate as that obtained with the use of some fluorescent stains (Merkies et al., 2000).

The hypo-osmotic swell (HOS) test can also be utilized to evaluate plasma membrane functionality. When an intact sperm cell is placed into a hypo-osmotic solution, fluid is transported into the cell via active ionic mechanisms in the cell membrane causing it to swell (Caiza del la Cueva et al., 1997). This swelling separates the membrane away from the tail fibers causing a distinctive kink or bend in the tail of the intact spermatozoa. Tails of spermatozoa with damaged plasma membranes will remain straight and can be easily identified via light microscopy. The HOS test has not been significantly correlated with percent pregnancy rate, but it has shown a tendency to correlate with increased services per pregnancy, and horses with less

than 40% swelling scores can be considered of potentially questionable fertility (Neild et al., 2000).

More recently other methods of evaluating plasma membrane integrity with the use of supravital staining and fluorescent labeling have been developed. While a large array exists, commonly reported fluorescent stains indicating spermatozoal viability are propidium iodide (PI) and SYBR®-14. PI binds to DNA, but cannot access the nucleus of a cell with an intact plasma membrane. Therefore, cells that fluoresce red with PI staining are non-viable cells. Conversely, SYBR®-14 by itself is non-fluorescing and readily crosses the plasma membrane. The SYBR®-14 is converted to a fluorescent molecule within the cellular cytoplasm. An intact plasma membrane is required to retain the stain and viable cells therefore fluoresce a green color. Frequently, these two stains are utilized in combination to identify membrane-intact cells, membrane-damaged cells, and moribund cells via fluorescent microscopy or flow cytometry (Garner and Johnson, 1995).

As sperm viability is highly correlated with motility in the stallion (Love et al., 2003), the use of viability assays is not often indicated. They are also infrequently utilized in the clinical setting due to lack of availability and expense. However, these tests could be useful in identifying a small population of sub-fertile stallions that may not have been identified with other more conventional methods of evaluation.

2. Mitochondrial Integrity

The midpiece of the spermatozoon contains mitochondria, which is important for the conversion of glucose into ATP. Although the importance of mitochondrial ATP has been

challenged, it likely maintains a key role in sustenance of spermatozoal function (Varner, 2008). Clinically, this has been shown to translate into a relationship between mitochondrial function and motility (Papaioannou et al., 1997; Love et al., 2003). The use of fluorescent stains have been described to evaluate mitochondrial function, as only actively respiring mitochondria will take up stain and subsequently fluoresce. The most commonly utilized stain is the lipophilic, cationic compound JC-1, as it allows for the differentiation of low versus high functioning mitochondria (Gravance et al., 2000). Mitochondria with low membrane potential uptake low levels of dye and fluoresce green while those with high membrane potential uptake high levels and fluoresce orange. Comparing the ratio of high functioning cells vs. low functioning cells allows for characterization of the spermatozoal population and the assessment of overall sperm cell "health". To date, there is little available information concerning the correlation of mitochondrial function and stallion fertility and the assay is infrequently done in the clinical setting.

3. Sperm Chromatin Analysis

The nucleus of the spermatozoon contains highly condensed DNA and associated proteins, together known as chromatin. Several methods of chromatin analysis have been reported (Linfor and Meyers, 2002; Baumber et al., 2003; Makhlouf and Niederberger, 2006; López-Fernández et al., 2007), but the sperm chromatin structure analysis, or SCSA, is the most common in the stallion. SCSA was first introduced as an analysis of mammalian fertility thirty years ago (Evenson et al., 1980), and has since been correlated with fertility in the stallion. The assay is performed with the use of flow cytometry and provides a measurement of the structural

integrity of spermatozoal chromatin via the acridine orange stain that fluoresces green when bound to double stranded DNA and fluoresces red when bound to single stranded DNA. Prior to analysis, the spermatozoa are exposed to acid denaturing conditions and the subsequent level of chromatin denaturation is determined. Increased susceptibility to chromatin denaturation is significantly correlated with decreased fertility in the stallion (Evenson et al., 1995; Kenny et al., 1995; Love and Kenney, 1998; Love, 2005). As spermatozoa are transcriptionally inactive prior to spermatozoon-oocyte fusion, the assay provides an opportunity to discover a high population of abnormal cells that appear normal with conventional analysis (Varner, 2008). Due to the cost of equipment and the expertise required, this technique is not frequently utilized during routine examination. However, as the test requires frozen semen, samples can easily be transported to appropriate laboratories for testing making analysis possible for stallions with unexplained sub-fertility.

4. Evaluation of Spermatozoal Readiness for Fertilization

4.1 Capacitation

Capacitation is a poorly defined series of architectural and biochemical changes that a spermatozoon must undergo to allow binding to the zona pellucida, performance of the acrosome reaction, and ultimately fertilization of an oocyte (Yanagimachi, 1994; Visconti et al., 1998; Neild et al., 2005). For the stallion, it is difficult to definitively conclude much concerning these exact processes as there has been limited study. As significant species differences are known to exist, it is also difficult to extrapolate (Varner and Johnson, 2007). However, the most

commonly reported changes in mammals include an influx of extracellular calcium secondary to plasma membrane glycocalyx removal and subsequent protein tyrosine phosphorylation, an increase in cyclic AMP, an increase in intracellular bicarbonate and pH, a loss of membrane cholesterol, changes in membrane phospholipid content, and exposure of membrane progesterone receptors (Hunter and Rodriguez-Martinez, 2004; Brewis et al., 2005).

Tests to evaluated capacitation status are typically undertaken to determine if capacitation changes have occurred prematurely. Tests can also evaluate if capacitation can occur in vitro, but as in vitro capacitation of stallion spermatozoa has proven difficult and inconsistent this is not commonly done for routine semen analysis. Evaluations of capacitation status is rarely recommended in the clinical setting as there is limited data to determine what normal levels are in various circumstances (fresh spermatozoa, cooled-stored spermatozoa, or post-thaw frozen spermatozoa). Therefore, when undertaken, the side by side evaluation of a stallion with known fertility is recommended when attempting to utilize capacitation status as a measure of fertility.

Reported tests of capacitation include the evaluation of tyrosine phosphorylation, calcium metabolism, and membrane cholesterol/phospholipid changes (Sieme, 2009). Tyrosine phosphorylation of flagella proteins is a significant event associated with capacitation in the stallion. Measurements of tyrosine phosphorylated proteins via immunoblotting combined with anti-phosphotyrosine antibody have been described as a measure of capacitation status (Pommer et al., 2002b), but are not commonly performed. The significant increase in intra-cellular calcium that occurs with capacitation can also be used as a measure of capacitation status. Fluoresced chlortetracycline has been utilized as a method to evaluate the amount of free calcium within the spermatozoa as a measure of capacitation (Varner et al., 1987). The assay allows for the simultaneous evaluation of the acrosome reaction, but is technically challenging and time

consuming. Consequently, other markers of free intracellular calcium have been described (Landim-Alvarenga et al., 2004). These markers can be utilized with flow cytometry, allowing for rapid evaluation of a large population of spermatozoa. Significant changes within the plasma membrane are known to occur with capacitation including phospholipid reorganization and cholesterol loss (Gadella et al., 2001; Brinsko et al., 2007). The decrease in phospholipid density that occurs with early capacitation can be detected with a hydrophobic dye, Merocyanine-540 and subsequent flow cytometry analysis (Rathi et al., 2001). Alternatively, the use of fluorescent-labeled binding proteins and incorporation of fluorescent phospholipids has been described, but has not been widely studied (Gadella et al., 1999).

4.2 Hyperactivated Motility of Spermatozoa

Capacitation is frequently reported to be associated with spermatozoal hyperactivation (Ho and Suarez, 2001; Sieme, 2009). Hyperactivation of spermatozoa is required for navigation through the oviductal mucus, penetration of the zona pellucida, and penetration of the cumulus oophorus (Suarez and Ho, 2003). While hyperactivated motility is independent of capacitation (Rathi et al., 2001; McPartlin et al., 2009), the two processes share similar requirements including the presence of calcium and bicarbonate, and the activation of cAMP synthesis (Visconti et al., 1999; Ho and Suarez, 2001). Hyperactivated spermatozoa have been subjectively described as having a high-amplitude, asymmetric, whip-lash effect of the flagella leading to circulatory or non-progressive motility (Suarez and Ho, 2003). Objective measurements of the exact motility pattern determining hyperactivation are species specific and have not been determined for the stallion. In recent reports, increases in the amplitude of lateral

head displacement (ALH), increases in curvilinear velocity (VCL), and decreases in straightness (STR) as measured with computer-assisted semen analysis systems have all been attributed to hyperactivated motility although specific values or percent increases have not been validated (Rathi et al., 2001; McPartlin et al., 2009; Sieme, 2009). Ideally, spermatozoa should be evaluated under non-capacitating and capacitating conditions and subsequent motion characteristic comparisons should determine the exact classification of hyperactivation for a particular laboratory. It is also important to note that most CASA system measurements are based on spermatozoal head movements and not flagellar movements, which is the principle area of change with hyperactivate motility (Suarez and Ho, 2003).

4.3 Zona Binding

Only capacitated sperm can bind to the zona pellucida, which in turn induces the acrosome reaction. The acrosome reaction allows the spermatozoon to penetrate the zona and enter the perivitelline space. The zona binding assay is rarely performed within the clinical setting due to the level of expertise required and the lack of easily obtainable equine oocytes. Although non-equine oocytes have been used (Choi et al., 2003), the resulting increased variability makes interpretation of decreased binding difficult. Due to high inter-zona variability, a hemi-zona assay is recommended where an equine oocyte is bisected and binding of the test stallion's spermatozoa is compared to that of a stallion of known high fertility to increase test accuracy. The oocyte and fluorescent stained-spermatozoa are incubated for a defined period followed by washing to remove unbound sperm cells. The numbers of adherent spermatozoa are then counted. It is important to remember that for zona binding to take place, sufficient

capacitation changes must have occurred. Therefore, the test does not differentiate between non-capacitated spermatozoa that do not bind or capacitated sperm with an inherent binding defect. However, stallions of unexplained sub-fertility have been shown to have decreased zona binding (Fazeli et al., 1995; Pantke et al., 1995). Unfortunately, due to high cost as well as other factors mentioned previously, this assay is not routinely utilized.

4.4 The Acrosome Reaction

The acrosome is the membrane-bound organelle covering the anterior aspect of the spermatozoon head. The binding of the spermatozoon to the zona pellucida triggers a series of biochemical changes that result in fusion of the outer acrosomal membrane and the plasma membrane (Gadella et al., 2001). This fusion or vesiculation releases hydrolytic enzymes allowing penetration of the zona and entrance into the perivitelline space. The acrosome reaction is considered an end-product of capacitation and several assays to test it have been described. Similar to assays for capacitation, testing can be performed to evaluate the percentage of acrosome-reacted spermatozoa or to evaluate the ability of a spermatozoal population to undergo the acrosome reaction. Fluorescent markers or dyes have been described in the stallion to assess the acrosome status of a spermatozoal population (Cheng et al., 1996; Arruda et al., 2002). This method of assessment has the benefit of classifying three types of spermatozoa: acrosome-intact (fluorescence of the acrosome noted), acrosome-reacting (patchy fluorescence noted over the acrosome), and acrosome-reacted (no fluorescence noted or only in the equitorial segment). Electron microscopy is infrequently utilized to evaluate the acrosomal status in stallions, but is

considered the gold standard (Varner et al., 2000; Pesch et al., 2006). However, due to expense, lack of availability and inefficiency, it is rarely performed.

Several methods of inducing the acrosome reaction in the stallion have been described, most frequently with the use of progesterone or the calcium ionophore A23187 (Christensen et al., 1996; Cheng et al., 1998; Landim-Alvarenga et al., 2001). Following capacitation, the release of a glycocalyx matrix from the spermatozoal plasma membrane allows for exposure of progesterone receptors. The binding of these receptors has been shown to induce the acrosome reaction (Cheng et al., 1998), which has been correlated with fertility in the stallion (Meyers et al., 1995). The Acrosomal Responsiveness Assay (ARA) utilizes the calcium ionophore A23187, a strong inducer of the acrosome reaction via the influx of intracellular calcium. A benefit of this assay is that prior capacitation of the spermatozoa is not required for the reaction to occur allowing for more specific information for low reaction rate spermatozoal populations. The ARA has been correlated to fertility in the stallion, with over 80% of spermatozoa from fertile stallions undergoing the reaction and less than 20% of spermatozoa from sub-fertile stallions undergoing the reaction (Varner et al., 2001).

4.5 Oocyte Binding and Penetration

After penetration of the zona and the occurrence of the acrosome reaction, the spermatozoa must bind to and ultimately penetrate the oocyte plasma membrane. In vitro assays have been developed to evaluate this step of fertilization. Due to availability, hamster oocytes are routinely utilized for this assay as they are non-selective in their binding properties, although equine oocytes are preferred (Landim-Alvarenga et al., 2001). An association with fertility has

been reported for this assay (Wilhelm et al., 1996), although the nonselective properties of these oocytes can allow for significant binding by sub-fertile spermatozoa. Overall, there is limited data available correlating the usefulness of both the zona binding and oocyte penetration assays with stallion fertility. Currently, these tests are not readily available in the clinical setting.

D. Semen Extension and Cooled Storage in the Stallion

1. Semen Extenders

Countless formulas have been studied for the extension and cooled storage of stallion semen. While variable in actual ingredients, they all attempt to address several basic requirements. These include providing a proper combination of nutrients and mineral elements, buffering against changes in pH and osmotic pressure, stabilizing enzyme systems and protecting the integrity of the plasma membrane, and providing antibiotic coverage for bacterial control (Pickett and Amann, 1987). The best mechanisms to provide most of these requirements are not known, which is understandable as many attributes of spermatozoon biology and biochemistry are not known.

Most commercially available extenders utilize a sugar, commonly glucose, as a spermatozoal nutrient, which also serves as a buffering agent. As the spermatozoa utilize the energy source (whether from an external or internal source), extra-cellular by-products are produced including lactic acid and peroxides. Therefore, the extender must also provide a mechanism to buffer the resulting changes in pH as well as simply diluting the waste products via the addition of volume. While other buffers are typically added, the sugar also serves to buffer the semen. This stabilization of pH and osmotic pressure is extremely important to optimize viability under in vitro storage conditions. The pH and osmolarity of commercially available equine semen extenders typically range from 6.8 to 7.4 and 250 to 400 mOsm/L (Katila, 1997). It has been reported that extender pH values between 6.6 and 7.2 may optimize sperm motility (Wendt et al., 2002), but there is some discrepancy within the literature as

Griggers et al. (2001) reported an optimal pH of 7.7. The importance of this point is questionable as several studies have shown no significant differences in fertility with moderate variations in pH of extended semen (Bogart and Mayer, 1950; Province et al., 1984; Francl et al., 1987). The presence of egg yolk, milk, glycerol, bovine serum albumin, polyvinyl alcohol, and lipoproteins within the semen extender have all been evaluated for their ability to provide protection against temperature related damages ("cold shock") likely via the stabilization of enzyme systems and protecting the integrity of the plasma membrane (Watson et al., 1987; Katila, 1997).

Historically, egg yolk-based and milk-based extenders have been evaluated for use in the stallion, although milk-based extenders are utilized more frequently in the stallion. As milk is a biologic product, it has many components – some beneficial and some potentially detrimental to spermatozoa. Although skim-milk based extenders are commonly used with success, individual components of milk have been evaluated to determine which substances maintain better spermatozoal viability (Batellier et al., 1997). In particular, native phosphocaseinate or casein formulated extenders are available commercially and commonly utilized (LeFrapper et al., 2010). Most recently, a soybean lecithin based extender has been evaluated in the stallion as an alternative nutrient. Aurich et al. (2007) found similar spermatozoal motion characteristics with the extender in comparison to caseinate-based extenders, but overall spermatozoal viability was decreased on days 0 and 4. Further study is required to determine the potential for non-animal product-based semen extenders in the stallion.

The seminal plasma, secreted from the epididymides and the accessory sex glands during ejaculation, contains a complex mix of organic and inorganic components. It is an important mediator of the chemical functions of the ejaculate and serves to provide protection and nutrients

to the spermatozoa within the female reproductive tract. The role of seminal plasma in the extension and cooled storage of equine semen is somewhat complicated, as both beneficial and deleterious effects have been reported (Jasko et al., 1991; Kareskoski and Katila, 2008). However, there is a general recommendation within the literature that its presence at 5-10% within the final extended semen volume is best (Pruitt et al., 1993; Todd et al., 2001), although individual stallion differences exist (Brinsko et al., 2000a).

Raw semen should be extended appropriately within 15-20 minutes of collection to maximize spermatozoal viability (Samper, 2009). Final spermatozoal concentrations generally should be 25-50 million cells per ml for best preservation of viability for storage longer than six hours (Varner et al., 1987), although a few studies have shown no deleterious effects with slightly higher spermatozoal concentrations (Jasko et al., 1991; Dawson et al., 2000). However, it is important to note that the ejaculate must be diluted to a semen:extender ratio of at least 1:4, otherwise the resulting seminal plasma concentration is too high, which is detrimental to sperm viability (Todd et al., 2001). Therefore, excessively dilute ejaculates may require further processing to increase spermatozoal concentration prior to extension for optimal viability. Centrifugation (300-500 x g for 10-15 minutes) should be performed for ejaculates with spermatozoal concentrations of $\leq$100 million spermatozoa/ml (Love et al., 2005). Afterwards, most of the seminal plasma is gently aspirated and the sperm pellet is re-suspended in an appropriate extender. For inseminations performed shortly after collection (within six hours), extending semen at a ratio of 1:1 or 1:2 to extender and maintaining at room temperature is appropriate to maintain viability and control bacterial growth (Samper, 2009).

2. Cooled Storage of Extended Stallion Semen

Appropriate handling of semen should continue even after extension as spermatozoa remain vulnerable to damage. Several studies have evaluated optimal storage temperatures for equine semen. It is difficult to extrapolate all the information into blanket recommendations for all stallions due to significant stallion variation (Francl et al., 1987). However, general consensus of the literatures shows storage of extended semen at or around 5°C to be ideal in reducing bacterial growth and decreasing the metabolic processes of the spermatozoa while optimizing viability (Squires et al., 1988; Varner et al., 1988; Varner et al., 1989; Moran et al., 1992). This is particularly accurate for semen stored for greater than 6-12 hours.

A slow cooling rate for extended stallion semen is imperative to prevent temperature related damages resulting in reduced spermatozoal viability. The optimal rate is typically reported at -0.3°C per minute (Douglas-Hamilton et al., 1984; Province et al., 1985; Varner et al., 1988), although a wider range of cooling rates may be acceptable (Brinsko et al., 2000b). However, it has been shown that stallion spermatozoa can actually be cooled rapidly from 37°C to 20°C, followed by a more critical period of slow cooling (≤0.1°C per minute) until a final temperature of 5°C (Kayser et al., 1992). Further study has shown the most critical period of cooling to be from 19°C to 8°C (Moran et al., 1992). Although programmable coolers are available, the use of passive cooling devices is much more common in the clinical setting. Several systems are commercially available, many of which consist of a specialized Styrofoam cooler with ice packs placed near the packaged, extended semen. While these systems are quite efficient in cooling semen at an appropriate rate, many do not maintain a temperature of 5-8°C for longer than 24-36 hours (Samper, 2009). The Equitainer™ (Hamilton Research Inc, South

Hamilton, MA) is the most commonly utilized re-usable stallion semen shipper in the United States and has been shown to maintain its temperature and cooling rates better in comparison to Styrofoam systems (Katila et al., 1997; Brinsko et al., 2000b).

3. Role of Antibiotics in Stallion Semen Extension

3.1 Bacteriology of Stallion Semen

Research has shown the stallion has a variable, normal microflora present in the distal urethra and on the surface of the penis and prepuce (Burns et al., 1975; Kenney et al., 1975; Bowen et al., 1982; Rideout et al., 1982; Hoyumpa et al., 1992; Clément et al., 1995; Varner et al., 1998). The majority of bacteria are nonpathogenic, but some are capable of causing infection within the bred mare. Both gram-positive and gram-negative bacteria can be present and potentially pathogenic. The most commonly reported isolates are *Bacillus* spp., *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp., *Staphylococcus aureus*, *Streptococcus zooepidemicus*, and *Streptococcus equisimilis* (Sigler and Kirocofe, 1988; Corona et al., 2006; Samper and Tibary, 2006). Disruption of the normal flora can easily lead to opportunistic pathogen over-growth, including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Streptococcus zooepidemicus*. Frequently, stallions harboring pathogenic bacteria within their genital tract do not show clinical signs of disease (Hughes and Loy, 1975; Blanchard et al., 1987).

During semen collection, the ejaculate is often contaminated with some of these bacteria despite proper collection technique (Clément et al., 1995; Samper and Tibary, 2006). There are conflicting reports on open-ended vs. closed artificial vagina (AV) use and effects on bacterial

contamination of the ejaculate. Clément et al. (1995) determined contamination was significantly less with open-ended collection while Corona et al. (2006) found the opposite with ejaculates obtained via a closed AV containing on average $1.1x10^4$ CFU/ml less bacteria. While there is significant stallion variation in bacterial numbers within the ejaculate, the mean bacterial population of raw semen was found to be 573,000 ± 374,000 organisms/ml (Burns et al., 1975), or between 10^4 to 10^6 CFU/ml (Clément et al., 1995; Corona et al., 2006).

The normal, fertile mare can respond appropriately to bacterial invasion at the time of insemination. However, in susceptible mares, bacteria can become established, provide an environment unsuitable to a developing embryo, and negatively impact fertility (Bennett, 1986; Blanchard et al., 1987; Varner et al., 1998; King et al., 2006). Elimination of pathogens from semen is not practical and is likely not possible, which warrants the addition of antibiotics into the semen extender.

3.2 The Use of Antibiotics

Bacterial Control

As many bacterial metabolites suppress spermatozoal motility and viability (Rideout et al., 1982; Aurich and Spergser, 2006) and as bacteria present in stallion semen can establish infection in susceptible mares, the addition of appropriate antibiotic to the semen extender is a requirement. The addition of an antibiotic typically does not eliminate the bacterial load. It effectively controls further growth of bacteria, but frequently small amounts of bacterial contamination are still present after cooled-storage of extended semen. In fact, Varner et al. (1998) found persistence of bacterial growth with the use of several different antibiotics

(amikacin, gentamicin, streptomycin, potassium penicillin, sodium penicillin, ticarcillin, and polymixin B) added to a skim milk glucose-based extender after 24 hours of storage. However, several studies have shown antibiotics added to the semen extender to be effective in preventing bacterial over-growth and allowing for a significant, rapid reduction in bacterial count numbers (Burns et al., 1975; Kenney et al., 1975; Vaillancourt et al., 1993; Dietz et al., 2007). However, more recently the bacterial load of extended semen has been shown to be significant. A study evaluating the prevalence of bacterial contamination in cool-shipped stallion semen found a 65.9% contamination rate with average bacterial load of $6.9x10^3$CFU/ml (Althouse et al., 2010). Unfortunately, as the semen was from a variety of collection facilities, there was no note of which antibiotics were utilized or how the semen was processed prior to shipping.

Effect on Spermatozoa

There are many reports of the effect of antibiotics on spermatozoal characteristics (primarily motion characteristics). Although there are few studies evaluating the effect of antibiotic on stallion fertility, there are several studies showing increased fertility when breeding with extended semen (with antibiotic added) versus breeding with raw semen (Kenney et al., 1975; Woods et al., 1990). Currently, the most commonly reported antibiotics in current literature include: polymixin B sulfate, crystalline penicillin, gentamicin sulfate, amikacin sulfate, ticarcillin, and timentin.

Positive correlations between the presence of antibiotic and spermatozoal motion characteristics have been infrequently reported. Macedo et al. (2005) found an increase in total and progressive motility with the addition of amikacin at 2,000µg/ml to a skim milk-glucose extender in comparison to gentamicin at the same concentration. Interestingly, although not

statistically significant due to small numbers, single cycle pregnancy rates were greater for the amikacin group versus the gentamicin group (8/12 pregnant versus 4/12 pregnant, respectively). Varner et al. (1998) also found a positive correlation between amikacin combined with potassium penicillin (1,000µg/ml and 1,000IU/ml, respectively) on selected spermatozoal velocity characteristics after 24 hours of storage at 5°C in comparison to control (no antibiotic). Interestingly, Arriola and Foote (1982) determined no benefit of amikacin (50-1,000µg/ml) on spermatozoal motility during storage at 37°C for 10 hours, but did note decreased motility with concentrations of 10,000µg/ml.

There are conflicting reports in the literature regarding the effects of gentamicin on stallion spermatozoa. Several studies have shown the antibiotic to have detrimental effects on selected motion characteristics (Squires and McGlothlin, 1980; Jasko et al., 1993; Aurich and Spergser, 2007) while others have shown no detrimental effect (Back et al., 1975; Squires et al., 1981; Varner et al., 1998; LeFrapper et al., 2010). The conflicting results are likely due to differences in the base semen extender utilized that could affect the antibiotic's interaction with spermatozoa, the use of non-reagent-grade antibiotic allowing for different preservatives (Squires and McGlothlin, 1980), and inherent stallion differences. As most of these factors can play a role in the evaluation of any antibiotic-semen extender population, it is the general consensus that semen evaluations should include extension and storage in several different combinations due to the significant differences seen in individual stallions.

E. Cephalosporin Antibiotics

Cephalosporins are a group of broad-spectrum antibiotics derived from the fungus *Cephalosporium acremonium*. They contain β-lactam and dihydrothiazine rings and are relatively resistant to β-lactamases (Ahrens and Martin, 2008). They are bactericidal as a group, working by inhibition of mucopeptide synthesis in the bacterial cell wall. The group is divided into four sub-classifications known as generations. While these generations maintain some connectivity based on their spectrum of activity, they are also grouped upon the timing of each drug's development. First generation cephalosporins are effective against many Gram-positive bacteria. Second generation cephalosporins have a similar spectrum of activity, but are also effective against some Gram-negative bacteria. Third generation cephalosporins have a wider spectrum of activity against Gram-negative organisms, but have a narrower range of activity against Gram-positive bacteria than the first and second generations. More recently fourth generation cephalosporins have been developed and have the widest range of activity against Gram-positive and Gram-negative bacteria (Hornish and Kotarski, 2002).

Ceftiofur Sodium

Ceftiofur sodium is a third generation cephalosporin utilized in equine practice most commonly for respiratory infections (Folz et al., 1992). It has been shown to have a broad spectrum of activity against common equine isolates (Salmon et al., 1996), although more recently resistance to *Escherichia coli* and *Klebsiella pneumoniae* has been reported (Vo et al., 2007). It is important to note that the resistant isolates were resistant to multiple drugs and only 7 of 1347 isolates were resistant to ceftiofur. The drug is labeled for intramuscular use although

successful intravenous and subcutaneous use has been reported (Slovis et al., 2006). After administration, ceftiofur is hydrolyzed to desfuroylceftiofuracetamide, which has increased metabolic activity and a longer half-life (Cervantes et al., 1993; Witte et al., 2010). Its use for treatment of endometritis in the mare has also been evaluated. There are two conflicting reports within the literature concerning the level of ceftiofur derivatives within the endometrium of healthy mares after systemic injection. Jonker (1997) found detectable levels of ceftiofur in the endometrium after a single systemic treatment. However, Cerventes et al. (1993) had previously found no evidence of ceftiofur derivatives within the endometrium of healthy mares after five consecutive systemic treatments. Recently, Witte et al. (2010) repeated Jonker's results as ceftiofur derivate levels were found within the endometrium after single systemic treatment. The discrepancy of the Cerventes et al. study was likely due to different assays utilized.

The Use of Cephalosporins in Stallion Semen Extension

There is limited data available concerning the use of cephalosporins in the extension and cooled storage of semen. Miraglia et al. (2003) reported the use of ceftiofur sodium (Excenel® – Upjohn) for the control of leptospires in egg yolk citrate extender in three Holstein Friesian bulls and found no effect on progressive motility after 24 hours of storage. The exact antibiotic utilized in this study is confusing as Excenel® is ceftiofur hydrochloride and not "ceftiofur sodium", although the study was performed in Brazil where there may be different antibiotic formulations available. Cephalothin (a first-generation cephalosporin) and ceftazidime (a third-generation cephalosporin) were evaluated by de Jong et al. (2005) for their ability to control identified bacteria from the prepuce, urethra, and semen of the flying fox (*Pteropus* spp.), but their effects on spermatozoal survival and motion characteristics were not evaluated. Ahmad and

Foote (1986) evaluated the effects of varying concentrations (200-2,000µg/ml) of cephapirin (a first-generation cephalosporin) and ceforanide (a second-generation cephalosporin) to bull semen prior to cryopreservation and found no effects on total motility. Additionally, this study found no significant effect on fertility (as measured by 56 day no return to service rate) when cephapirin (500µg/ml) was added to the control extender. Gadea (2003) reported successful use of ceftiofur in the extension of boar semen, but noted that no conclusive results of its effects on motility and fertility were available.

To the author's knowledge, there has only been one published report of the effects of ceftiofur sodium on motility of equine sperm. The study (Varner et al. 1997) evaluated the effects of ceftiofur at 1,000µg/ml in a skim milk-glucose semen extender in comparison to control (no antibiotic) and the addition of several antibiotics including combination amikacin sulfate/potassium penicillin G (1,000µg/ml and 1,000IU/ml, respectively). Three gel-free ejaculates from each of three stallions were utilized. Selected motion characteristics were evaluated (including curvilinear velocity) after 24 hours of storage at approximately 5°C and no significant differences between groups were detected.

Parlevliet et al. (2006) evaluated the effects of cefquinome (a fourth-generation cephalosporin) at 990µg/ml added to a skim-milk based extender on stallion spermatozoal total motility and progressive motility and found no significance in comparison to gentamicin at time zero, after 24 hours of storage, and after 48 hours of storage. However, curvilinear velocity and average path velocity were significantly lower after 24 hours of storage, but not after 48 hours of storage leading the authors to conclude the difference was likely not clinically significant. The amplitude of lateral head displacement was found to be significantly lower after 24 and 48 hours

of storage in the cefquinome treatment group, which the authors concluded was likely not significant for potential fertility of spermatozoa.

CHAPTER 03

MATERIALS AND METHODS

Animals

Two Standardbred stallions and one Quarter Horse stallion, ranging in age from 14 to 17 years and located in central Illinois, were enrolled in the study. One Standardbred stallion (Stallion A) was maintained in a 12 x 12 box stall with continuous access to a small dry paddock and fed sufficient quantities of grass and alfalfa hay to maintain good body condition. The other Standardbred stallion (Stallion B) was maintained in a dry paddock with access to a shed, and was fed sufficient quantities of a locally formulated pelleted, complete feed for horses to maintain good body condition. The Quarter Horse stallion (Stallion C) was maintained in a 12 x 12 box stall with daily paddock exercise, and fed sufficient quantities of grass and alfalfa hay to maintain good body condition. Stallions A and B were collected three times weekly as part of the University of Illinois Horse Farm breeding program. Stallion C was collected intermittently as part of the University of Illinois teaching program. The study was performed in the late spring of 2007 and the early spring of 2008.

Experimental Design

Stallions were collected in the morning with the use of a breeding phantom after teasing with a mare in estrus. Ejaculates were collected using an artificial vagina (Missouri-model, Har-Vet™, Spring Valley, WI) prepared with an in-line, micro mesh filter (AV filters, Har-Vet-™, Spring Valley, WI) to allow for the collection of gel-free semen. Three ejaculates from Stallion B and five ejaculates each from Stallion A and Stallion C (for a total of thirteen ejaculates) were

obtained. Ejaculates were transported to the laboratory (within ten minutes of collection) at ambient temperature (average of 70°F).

The volume of each ejaculate was measured via graduated centrifuge tube (Fisherbrand Higher-Speed Easy Reader Plastic Centrifuge Tubes, Fisher Scientific Inc., Pittsburgh, PA) and the spermatozoa concentration determined using a spectrophotometer (Animal Reproduction Systems model 534B, Chino, CA). At ambient temperature, each ejaculate was extended within 15 minutes of collection to 25 x 10^6 spermatozoa/ml (Varner et al, 1987 and Web et al, 1993) in a total of eight different extender groups. For the first five groups, a commercially available skim milk-glucose based semen extender without antibiotic[1] was utilized with no antibiotic added (control) or with one of four different concentrations of ceftiofur sodium[2]: 250µg/ml (CEFT250), 500µg/ml (CEFT500), 1,000µg/ml (CEFT1000), and 2,500µg/ml (CEFT2500). For the last three extender groups, similarly commercially available semen extenders with varying antibiotics were utilized: combination amikacin sulfate and potassium penicillin G[3] at 1,000µg/ml and 1,000IU/ml, respectively (AMKPCN); gentamicin sulfate[4] at 1,000µg/ml (GENT); and ticarcillin disodium[5] at 1,000µg/ml (TICAR). A skim milk-glucose based semen extender was chosen due to its availability without added antibiotic and its widespread use in equine artificial insemination.

Two different sets of aliquots (20ml) of the extended semen groups were placed in plastic bags (Whirlpak®, Nasco, Fort Atkinson, WI) with the air extruded. One set was stored in a

[1] Har-Vet™ Semen Extender without Antibiotics, HarVet™, Spring Valley, WI
[2] Har-Vet™ Semen Extender without Antibiotics, HarVet™, Spring Valley, WI and Naxcel®, Pharmacia & Upjohn, Kalamazoo, MI
[3] Har-Vet™ Semen Extender with Amikacin and Potassium Penicillin, HarVet™, Spring Valley, WI
[4] Har-Vet™ Semen Extender with Gentamicin Sulfate, HarVet™, Spring Valley, WI
[5] Har-Vet™ Semen Extender with Ticarcillin, HarVet™, Spring Valley, WI

semen-transport container (Equitainer™, Hamilton Research Inc, South Hamilton, MA) for 24 hours around 5°C and the second group was stored in an identical container for 48 hours around 5° C (Varner et al, 1989). The temperature of the containers was continuously monitored and noted at 24 and 48 hours of storage using a digital, traceable thermometer (Fisher-Scientific #15-077-17B, Pittsburgh, PA). Osmotic pressure, pH, and spermatozoal motion characteristics were all measured for the eight extended groups immediately after placement in the test extender (time zero), after 24 hours of storage, and after 48 hours of storage. Aerobic bacterial culture was also undertaken for the eight extended groups after 24 and 48 hours of storage.

Analytical Methods

The osmolality of each ejaculate was measured using a laboratory freezing point osmometer (5010 Osmette III™, Precision Systems Inc., Natick, MA). The pH of each ejaculate was measured with a benchtop pH meter (Accumet AB15, Fisher Scientific, Hanover Park, IL) at ambient temperature (70°F). A 2-mL sample of the ejaculate was submitted to the University of Illinois Veterinary Diagnostic Laboratory for aerobic culture of bacterial isolates. After 24 hours of storage, the eight extended groups were plated individually onto blood agar, phenylethyl alcohol agar, and MacConkey agar and incubated at 37°C for 48 hours. This procedure was repeated after 48 hours of storage. Growth characteristics were evaluated after 24 and 48 hours of incubation and bacterial colonies were submitted to the University of Illinois Veterinary Diagnostic Laboratory for semi-quantitative identification.

Motion characteristics were evaluated using a commercially available computerized spermatozoal-motility analyzer (Sperm Vision® CASA, Minitube®, Verona, WI) validated for equine use (Sieme, 2009; Sepp Weigert, personal communication). At the time of analysis,

approximately 2ml of each extender group was transferred to a sterile test tube (BC Falcon Round-Bottom disposable Polypropylene Tubes, Fisher Scientific Inc., Pittsburgh, PA) and warmed at 37°C for 10-15 minutes. Pre-warmed four-chambered glass Leja slides (Minitube of America, Verona, WI) were utilized for evaluation on a heated (37°C) microscope stage. Each sample was gently mixed prior to evaluation. Five random fields were analyzed for each extender group and measurements averaged with a frame rate of 58/second. Motion characteristics evaluated included: total motility (MOT), progressive motility (PM), curvilinear distance (DCL), average path distance (DAP), straight line distance (DSL), curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity (LIN), straightness (STR), wobble (WOB), beat cross frequency (BCF), and amplitude of lateral head displacement (ALH). Total motility was defined as percent spermatozoa displaying any motion while PM was percent spermatozoa displaying forward motion. Curvilinear distance (DCL), DAP, and DSL were the distances (μm) the sperm head traveled along its actual path, its averaged trajectory, and along a straight line from its first position to its last position, respectively. Curvilinear velocity (VCL), VAP, and VSL were the time average velocities (μm/sec) of the sperm head along its actual path, its averaged trajectory, and along a straight line from its first position to its last position. Linearity (LIN) was the percent linearity of the curvilinear trajectory and calculated as VSL/VCL. Straightness (STR) was the percent straightness of the average path and calculated as VSL/VAP. Wobble (WOB) was the degree of oscillation of the actual sperm-head trajectory about its average path and was calculated as a percent, VAP/VCL. BCF was the time average rate (Hertz) that the actual sperm trajectory crossed the average path trajectory. Finally, ALH was the maximum amplitude of variation (μm) of the actual sperm head trajectory about its average trajectory.

Statistical Analysis

Data are expressed as least squares mean ± SE and $P<0.05$ was taken as significant. Mixed models analysis of variance (PROC MIXED) with a compound symmetry covariance structure was used to determine if there was a main effect of extender (8 levels), time (3 levels), and the interaction between extender and time, on pH, osmolality, and motion characteristics, using the repeated command and Friedman's test. The primary motion characteristics of interest were VCL and PM as they are the most frequently measured characteristics for routine breeding soundness examinations and both parameters are essential for normal fertility (Pickett, 1993; Colenbrander et al, 2003). When indicated by a significant F test, Bonferonni adjusted post-test comparisons were performed, with $P<0.0071$ indicating a significant difference for extender and $P<0.025$ indicating a significant difference for time. A stepwise, multiple regression model including stallion, time of measurement, and extender was utilized to evaluate possible predictors of significant spermatozoal motion characteristics. Regression coefficients were considered to be significant at $P<0.01$. A statistical software program (SAS 9.1, SAS Inc, Cary, NC) was used for all analyses.

CHAPTER 04

RESULTS

A. Gel-Free Ejaculate Characteristics and Storage Temperatures

Gel-free ejaculate characteristics are presented in Table 1. Mean volume was 70ml with a range of 12 to 210ml. Mean concentration was $379x10^6$ spermatozoa per ml with a range of $111x10^6$ to $673x10^6$ spermatozoa per ml. Average osmolality and pH were 304mOsm/kg (range 289 to 337mOsm/kg) and 7.24 (range 6.99 to 7.40), respectively. Internal temperature readings of the two semen transport containers containing the extended semen groups are reported in Appendix A. Mean temperature was 7.9°C (range 6.7 to 9.5°C) after 24 hours of storage and was 8.4°C (range 7.6 to 9.0°C) after 48 hours of storage.

B. Effect of Extender on Motion Characteristics, pH, and Osmolality at Time Zero, after 24 Hours of Storage, and after 48 Hours of Storage

Summary

Least squares mean ± standard error spermatozoal motion characteristic measurements at time zero, after 24 hours of storage, and after 48 hours of storage with statistical significance noted are presented in Appendix B. Least squares mean ± standard error osmolality and pH values with statistical significance noted at time zero, after 24 hours of storage, and after 48 hours of storage are presented in Appendix C. Differences in all variables were detected via differences of least squares mean testing with significance set at $P<0.05$.

Characteristics at Time Zero

As expected, there were no significant differences in spermatozoal motion characteristics at time zero in treatment groups versus the control group with no antibiotic added. There was a significant decrease in pH for the combination amikacin/penicillin and the ticarcillin groups compared to control ($P<0.0001$ and $P=0.0001$, respectively). There were no significant differences in extender group osmolality at time zero.

Characteristics after Twenty-Four Hours of Storage

The highest concentration of ceftiofur (2,500µg/ml) produced significant differences in several spermatozoal motion characteristics versus control after 24 hours of storage. Curvilinear distance (DCL), curvilinear velocity (VCL), and the amplitude of lateral head displacement (ALH) were significantly greater than control ($P=0.0037$, 0.0037, and 0.0005, respectively).

Similar to time zero, combination of amikacin/penicillin and ticarcillin significantly decreased pH compared to control (P=0.0002 and P<0.0001, respectively). Differing from measurements at time zero, gentamicin significantly increased pH versus control (P=0.0004). Again, there were no significant differences in extender group osmolality after 24 hours of storage.

Characteristics after Forty-Eight Hours of Storage

The differences in spermatozoal motion characteristics after 48 hours of storage were similar to differences noted after 24 hours of storage. High concentrations of ceftiofur (2,500µg/ml) significantly increased DCL, VCL, and ALH compared to control (P=0.0013, 0.0012, and 0.0004, respectively). However, at this time, DCL and VCL were also significantly greater with ceftiofur at 1,000µg/ml versus control (P=0.0059 and 0.0055, respectively).

Changes to pH and osmolality after 48 hours of storage were similar to changes seen after 24 hours of storage. The pH was significantly less in the presence of combination amikacin/penicillin and ticarcillin, and significantly increased in the presence of gentamicin versus the control extender (P=0.0024, P<0.0001, and P<0.0001, respectively). There were no significant differences in extender group osmolality after 48 hours of storage.

C. Main Effect of Extender, Time, and the Interaction between Extender and Time

Total Motility

There was not a significant effect of extender (F test, P=0.18) on total motility. However, overall total motility did significantly decrease over time (F test, P=0.0001). After 48 hours of storage, total motility for all the groups (antibiotic and control) was significantly less than the 24 hour values and the time zero values (Appendix B). Ceftiofur at 500µg/ml, 1,000µg/ml, and 2,500µg/ml prevented significant decreases in total motility between time zero and 24 hours of storage. There was no interaction between extender and time on total motility (F test, P=0.83).

Progressive Motility

There was not a significant effect of extender (F test, P=0.15) on progressive motility. However, overall progressive motility significantly decreased over time (F test, P=0.0002). After 48 hours of storage, progressive motility for all groups (antibiotic and control) was significantly decreased from the 24 hour values and the time zero values (Appendix B). Similar to total motility measurements, ceftiofur at 500µg/ml, 1,000µg/ml, and 2,500µg/ml all prevented significant decreases in motility between measurements at time zero and measurements after 24 hours of storage. However, unlike total motility, a similar effect was seen within the ticarcillin group. There was no interaction between extender and time on progressive motility (F test, P 0.80).

Curvilinear Distance (DCL)

There was a significant effect of extender (F test, P=0.0095) on DCL. Ceftiofur had a dose-dependent effect on DCL (Figure 1). In comparison to control, DCL was increased at

ceftiofur concentrations of 2,500µg/ml (P=0.0020) and 1,000µg/ml (P=0.0066). Ceftiofur concentrations of 500µg/ml tended (P=0.046) to increase VCL, whereas a ceftiofur concentration of 250µg/ml had no effect on VCL (P=0.37). Combination amikacin/penicillin, gentamicin, and ticarcillin had no effect on DCL versus control (P=0.21, 0.70, and 0.39, respectively). Time did not have a significant effect on DCL (F test, P=0.20) and there was no interaction between extender and time on DCL (F test, P=0.42).

Average Path Distance (DAP) and Straight Line Distance (DSL)

There was not a significant effect of extender (F test, P=0.10) on either DAP or DSL. Overall, time had a significant effect on both DAP and DSL (F test, P=0.034) with values significantly decreased after 48 hours of storage in comparison to values after 24 hours of storage (P=0.014). Within the extender groups (Appendix B), DAP and DSL were significantly decreased after 48 hours of storage compared to measurements at time zero for the control group only (P= 0.0077). Otherwise, no significant changes were noted within the individual extender groups. There was no interaction between extender and time on DAP and DSL (F test, P=0.60).

Curvilinear Velocity (VCL)

There was a significant effect of extender (F test, P=0.0090) on VCL. Similar to DCL, ceftiofur had a dose-dependent effect on VCL (Figure 2), with values being increased at concentrations of 2,500µg/ml (P=0.0016) and 1,000µg/ml (P=0.0057). Ceftiofur concentrations of 500µg/ml tended (P=0.042) to increase VCL, whereas concentrations of 250µg/ml had no effect on VCL (P=0.32). Combination amikacin/penicillin, gentamicin, and ticarcillin had no

effect on VCL (P=0.18, 0.81, 0.40, respectively). Time did not have an effect on VCL (F test, P=0.19), and there was no interaction between extender and time on VCL (F test, P=0.42).

Average Path Velocity (VAP)

There was not a significant effect of extender (F test, P=0.087) on VAP. However, time had a significant effect on VAP (F test, P=0.043) with overall values decreasing after 48 hours of storage in comparison to values after 24 hours of storage (P=0.017). Within the extender groups (Appendix B), this change was only noted in the control with measurements after 48 hours of storage significantly decreased from measurements at time zero (P= 0.013). There was no interaction between extender and time on VAP (F test, P=0.71).

Straight Line Velocity (VSL)

There was not a significant effect of extender (F test, P=0.75) on VSL, but there was a significant change in overall VSL values over time (F test, P=0.011). VSL values after 24 hours of storage were higher than values at time zero (P=0.018), then values decreased between 24 and 48 hours of storage (P=0.0046). Within extender groups (Appendix B), the increase between time zero and 24 hours of storage was noted with the ceftiofur 1,000µg/ml and 2,500µg/ml groups (P=0.0078 and 0.011, respectively), and the decrease between 24 hours and 48 hours was noted within the control group, the ceftiofur 1,000µg/ml group, and the combination amikacin/penicillin group (P=0.0092, 0.020, and 0.014, respectively). There was also a significant decrease in VSL values noted in the control group after 48 hours of storage compared to values at time zero (P=0.011). There was no interaction between extender and time on VSL (F test, P=0.54).

Linearity (LIN)

There was not a significant effect of extender (F test, P=0.077) on LIN. However, there was a significant change in LIN measurements over time (F test, P=0.0097) with values decreasing significantly between 24 and 48 hours of storage (P=0.0038). Within extender groups (Appendix B), this decrease was noted in the combination amikacin/penicillin group and the gentamicin group (P=0.012 and 0.0062, respectively). An increase in LIN was also noted after 24 hours of storage in the combination amikacin/penicillin group compared to time zero values (P=0.017). There was no interaction between extender and time on LIN (F test, P=0.93).

Straightness (STR)

There was a significant effect of extender (F test, P=0.034) on STR. Ceftiofur tended to have a dose-dependent effect (Figure 3), with STR tending to be decreased at ceftiofur concentrations of 2,500µg/ml (P=0.012) and 1,000µg/ml (P=0.027). Neither ceftiofur concentrations of 500µg/ml (P=0.051) nor 250µg/ml (P=0.50) had an effect on STR. Combination amikacin/penicillin, gentamicin, and ticarcillin had no effect on STR (P=0.72, 0.69, and 0.84, respectively).

There was a significant effect of time on STR (F test, P=0.016) with STR values significantly increased after 24 hours of storage versus values at time zero (P=0.011). Values then significantly decreased between 24 hours storage and 48 hours of storage (P=0.0099). Within extender groups (Appendix B), the only significant change in STR values was an increase noted after 24 hours of storage in combination amikacin/penicillin group (P=0.019). There was no interaction between extender and time on STR (F test, P=0.96).

Wobble (WOB)

There was not a significant effect of extender (F test, P=0.49) on WOB. However, there was a significant change in WOB over time (F test, P=0.0064) with values significantly decreased after 48 hours of storage in comparison to values at both time zero (P=0.0058) and after 24 hours of storage (P=0.0033) values. Within extender groups (Appendix B), this change was consistent for the gentamicin group (P=0.0092 and P=0.021). A decrease in WOB values between 24 and 48 hours of storage was noted in the ceftiofur 1,000µg/ml group and the combination amikacin/penicillin group (P=0.011 and 0.013, respectively). Finally, a decrease in WOB measurements between time zero and 48 hours of storage was noted with the ceftiofur 2,500µg/ml group (P=0.018). There was no interaction between extender and time on WOB (F test, P=0.97).

Beat Cross Frequency (BCF)

There was not a significant effect of extender (F test, P=0.63) on BCF. There was also not a significant effect of time on BCF (F test, P=0.053). There was no interaction between extender and time on BCF (F test, P=1.0).

Amplitude of Lateral Head Displacement (ALH)

There was a significant effect of extender (F test, P=0.0011) on ALH. Ceftiofur had a dose-dependent effect on ALH (Figure 4), with ALH values increased at concentrations of 2,500µg/ml (P=0.003) and 1,000µg/ml (P=0.0034). Concentrations of 500µg/ml tended (P=0.041) to increase ALH values, whereas a ceftiofur concentration of 250µg/ml had no effect

on ALH values (P=0.25). Combination amikacin/penicillin, gentamicin, and ticarcillin had no effect on ALH (P=0.20, 0.34, and 0.28, respectively).

Time did not have significant effect on ALH (F test, P=0.61), and there was no interaction between extender and time on ALH (F test, P = 0.45).

pH

Overall, there was a significant effect of extender (F test, P<0.0001) on pH (Figure 5). A significant decrease in pH was noted for the ceftiofur 2500µg/ml group (P=0.0046), the combination of amikacin/penicillin group (P<0.0001) and the ticarcillin group (P<0.0001). Finally, gentamicin significantly increased the pH in comparison to control (P<0.0001).

Time had an effect on pH (F test, P<0.0001), with pH increasing significantly over time. Within all the extender groups (Appendix C), pH values were significantly increased after both 24 hours and 48 hours of storage compared to values at time zero (P≤0.0006 for all comparisons). The pH significantly increased between 24 and 48 hours of storage in the ceftiofur 250µg/ml group, the combination amikacin/penicillin group, and the gentamicin group (P=0.024, 0.0062, and <0.0001, respectively). There was also a significant interaction between extender and time on pH (F test, P=0.014).

Osmolality

As expected, there was not a significant effect of extender (F test, P=0.10) on osmolality. Time also did not have a significant effect on osmolality (F test, P=0.36) and there was no interaction between extender and time on osmolality (F test, P=0.98).

D. Individual Stallions: Main Effect of Extender, Time, and the Interaction between Extender and Time on Curvilinear Distance, Curvilinear Velocity, the Amplitude of Lateral Head Displacement and pH

Stallion A

Consistent with the overall analysis, there was a significant effect of extender on DCL, VCL, and ALH for stallion A (F test, P<0.0001 for all), with a dose-dependent effect of ceftiofur seen (Figures 6, 7, and 8, respectively). All three parameters were increased with ceftiofur concentrations of 2,500µg/ml and 1,000µg/ml after 24 hours and after 48 hours of storage versus control at identical time points. Ceftiofur at 500µg/ml significantly increased DCL, VCL, and ALH versus control after 24 hours of storage. After 48 hours of storage, ceftiofur at 500µg/ml increased ALH in comparison to control, but only tended to increase DCL and VCL. Ceftiofur at 250µg/ml did not significantly increase any of the three measurements versus control at any time. For the combination amikacin/penicillin group, VCL was significantly increased versus control after 24 hours, but not after 48 hours of storage while ALH was significantly increased compared to control for both storage times. Gentamicin and ticarcillin had no effects on DCL, VCL, or ALH at any point of measurement.

Time also had a significant effect on DCL (F test, P=0.0047), VCL (F test, P=0.0043), and ALH (F test, P=0.0001) for stallion A (Figures 6, 7, and 8, respectively). Overall, there was an increase in these motion characteristics between time zero and 24 hours of storage plus an increase in ALH noted between time zero and 48 hours of storage. Within the extender groups, higher concentrations of ceftiofur (2,500 and 1,000µg/ml) yielded increased DCL, VCL, and ALH measurements after 24 hours of storage compared to time zero. The increase in ALH was

maintained in these groups after 48 hours of storage (compared to time zero values). There was no interaction between extender and time on DCL, VCL, or ALH (F test, P=0.19, 0.17, and 0.17, respectively).

There was also a significant effect of extender (F test, P<0.0001) on pH for stallion A (Figure 9). At time zero, the pH of the combination amikacin/penicillin group was significantly decreased in comparison to control (P<0.0001). After 24 hours of storage, combination amikacin/penicillin continued to decrease pH (P=0.0012) while gentamicin significantly increased pH in comparison to control at this time (P=0.0002). After 48 hours of storage, gentamicin continued to increase pH in comparison to control (P<0.0001). There was no significant effect of ceftiofur at any of the four concentrations on pH versus control at any time of measurement. Time had an effect on pH for stallion A (F test, P<0.0001) with values increasing significantly at each measurement within each extender group including control. There was also a significant interaction between extender and time on pH (F test, P<0.0001).

Stallion B

A significant effect of extender on DCL, VCL, or ALH was not present for stallion B (F test, P=0.88, 0.85, and 0.46, respectively). However, there was a significant overall effect of time (F test, P<0.0001). DCL and VCL measurements decreased significantly after both 24 and 48 hours of storage. ALH measurements were significantly decreased after 24 and 48 hours of storage compared to time zero. Within the extender groups, changes in DCL and VCL were similar (Figures 6 and 7, respectively). Values were significantly decreased from time zero after both storage times for the control group, the ticarcillin group, and the groups with lower concentrations of ceftiofur (250µg/ml and 500µg/ml). The addition of gentamicin significantly

decreased these measurements after 24 hours of storage; however this effect was lost after 48 hours of storage. Interestingly, for combination amikacin/penicillin and for higher concentration of ceftiofur (1,000µg/ml and 2,500µg/ml), the decreases in DCL and VCL values were delayed with no significance difference between values at time zero and after 24 hours of storage. Within the extender groups, ALH after 24 hours of storage was decreased from time zero in all of the extender groups and was also decreased from time zero after 48 hours of storage in all but the gentamicin group (Figure 8). There was no interaction between extender and time on DCL, VCL, or ALH (F test, P=0.30, 0.32, and 0.14, respectively).

There was a significant effect of extender (F test, P<0.0001) on pH for stallion B (Figure 9). At time zero, the pH of the combination amikacin/penicillin group and ticarcillin group was significantly decreased verses control (P<0.0001 and =0.0022, respectively). This decrease in comparison to control was also seen after both 24 and 48 hours of storage for the combination amikacin/penicillin group (P=0.0012, 0.0029, respectively). Gentamicin and all concentrations of ceftiofur had no significant effects on pH versus control at any time of measurement. Time also had an effect on pH for stallion B (F test, P<0.0001) with values increased significantly in comparison to time zero after both 24 and 48 hours of storage for all groups including control. There was no significant interaction between extender and time on pH (F test, P=0.30).

Stallion C

For stallion C, there was not a significant effect of extender on VCL (F test, P=0.051), but there was a significant effect of extender on DCL and ALH (F test, P=0.046 and 0.0001, respectively). Within the extender groups, there were no direct comparisons with the control group of significance for DCL. For ALH, only the addition of ceftiofur at 2,500µg/ml increased

values in comparison to control after 48 hours of storage. There was a significant effect of time on DCL, VCL, and ALH measurements (F test, P=0.0001, 0.0002, and <0.0001, respectively) with measurements at 24 and 48 hours of storage significantly greater than measurements at time zero. Within the extender groups, DCL and ALH were increased compared to time zero within the ceftiofur 2,500µg/ml and 1,000µg/ml groups after 24 hours and 48 hours of storage (Figures 6 and 8, respectively). For VCL, the increase was present in the ceftiofur 2,500µg/ml group for both times (compared to time zero) and after 24 hours of storage for the ceftiofur 1,000µg/ml group. VCL only tended to be increased within this treatment group after 48 hours of storage (Figure 7). Ceftiofur concentrations of 500µg/ml increased all three parameters after 48 hours of storage compared to time zero. There was no interaction between extender and time on either DCL or VCL (F test, P=0.58 and 0.62, respectively). There was a significant interaction between extender and time on ALH (F test, P=0.046).

Similar to the other two stallions, there was a significant effect of extender (F test, P<0.0001) on pH for stallion C (Figure 9). Ceftiofur had no significant effect on pH versus control at any time point (time zero, 24 hours of storage, and 48 hours of storage). At time zero, only combination amikacin/penicillin significantly decreased pH verses control (P=0.0046). After 24 hours of storage, only ticarcillin significantly affected pH, causing a decrease in comparison to control (P=0.0003). After 48 hours of storage, ticarcillin continued to significantly decreased pH versus control while gentamicin significantly increased pH verses control (both P=0.0005). Again, similar to the other two stallions, time also had an effect on pH for stallion C (F test, P<0.0001) with overall values increasing significantly at each measurement. Within the extender groups, increased pH values were noted after 24 and 48 hours of storage compared to time zero in the control group, the combination amikacin/penicillin

group, and the gentamicin group. The significant rise in pH in comparison to time zero was delayed for the three lower concentrations of ceftiofur (250µg/ml, 500µg/ml, and 1,000µg/ml) and the ticarcillin group until after 48 hours of storage. Only ceftiofur at the highest concentration (2,500µg/ml) yielded no significant increase in pH over time, although the value was near significance after 48 hours of storage in comparison to time zero (P=0.025). There was also a significant interaction between extender and time on pH (F test, P=0.046).

E. Multivariable Regression Analysis of Curvilinear Distance, Curvilinear Velocity, and the Amplitude of Lateral Head Displacement

A stepwise, multiple regression analysis including stallion and time of measurement revealed pH was the strongest predictor of curvilinear distance (DCL), curvilinear velocity (VCL), and the amplitude of lateral head displacement (ALH). While all predictors were minor, pH was the strongest for all three motion characteristics at 3.4% for DCL and VCL and 4.0% for ALH. Other predictors included were the CEFT2500 group (1.2%) and the TICAR group (1.0%) for DCL; the CEFT2500 group (1.3%), the TICAR group (1.1%), and the CEFT1000 group (0.8%) for VCL; and the CEFT2500 group (2.5%) and the TICAR group (1.4%) for ALH.

F. Bacterial Growth

Bacterial growth is summarized in Appendix D. The most common isolate from raw semen was *Corynebacterium* spp. as it was grown from each ejaculate. Other most common isolates were coagulase-negative *Staphylococcus* spp. and *Pseudomonas* spp. Each stallion tended to have a consistent "normal flora" of growth from their ejaculates.

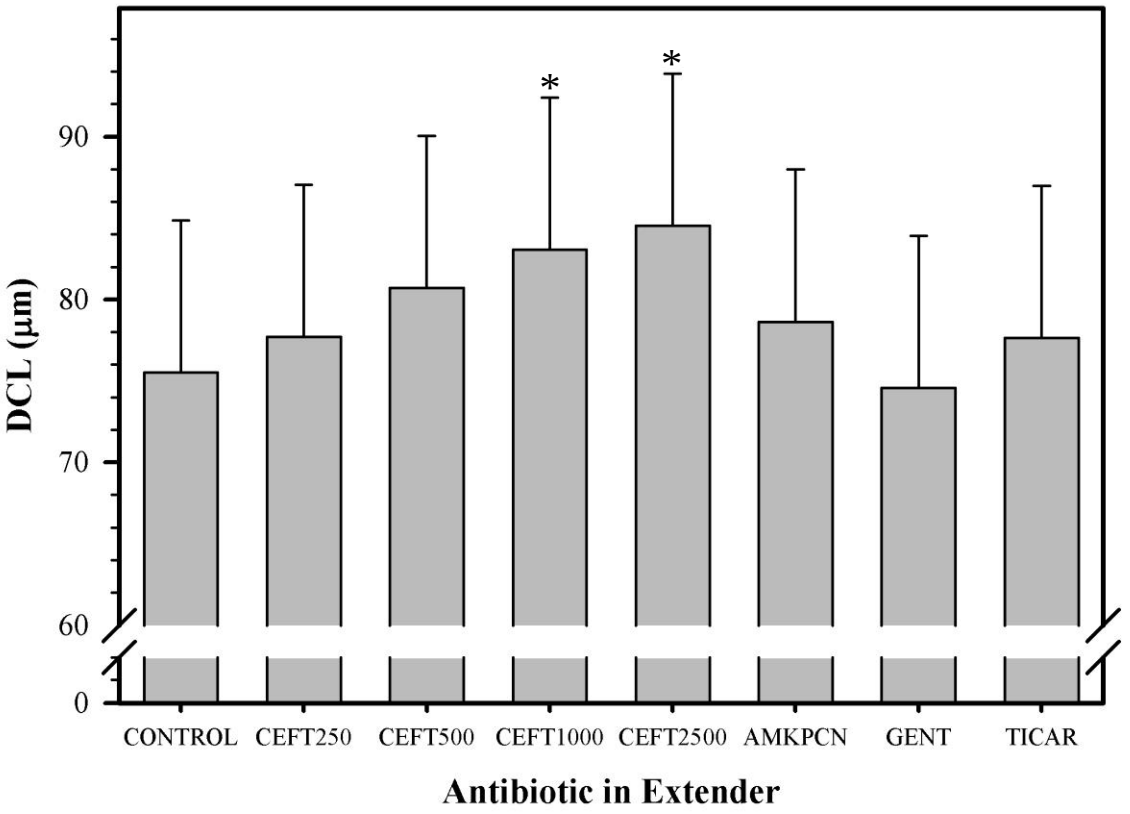
All treatment groups grew bacteria at some point during the experiment except the amikacin/penicillin group after 48 hours of storage. *Corynebacterium* spp. remained the most common isolate in the control groups at both 24 hours and 48 hours of storage. Growth within the treatment groups at both 24 and 48 hours of storage was limited to few colonies or less with the exception of very light growth within the ticarcillin group after 24 hours of storage (collection 10) and within the ceftiofur 2,500µg/ml group after 48 hours of storage (collection 10). Of the treatment groups, control of bacterial isolates was greatest for the combination amikacin/penicillin group (11/13 collections; 85%), the ceftiofur 2,500µg/ml group (10/13 collections; 77%), and the gentamicin group (10/13 collections; 77%). The treatment groups with poorest bacterial control were the ceftiofur 250µg/ml group (3/13 collections; 23%) and the ticarcillin group (5/13 collections; 38%) while the control group provided no bacterial control (0/13 collections).

Table 1. Characteristics of gel-free ejaculates from three stallions measured approximately ten minutes after collection using an artificial vagina (Missouri-model, Har-Vet™, Spring Valley, WI) prepared with an in-line, micro mesh filter (AV filters, Har-Vet™, Spring Valley, WI).

Stallion[1]	Ejaculate	Volume (ml)	Concentration (spermatozoa per ml)	pH	Osmolality (mOsm/kg)
A	**1**	45	417×10^6	6.99	303
	2	40	302×10^6	7.19	337
	3	80	302×10^6	7.27	313
	4	65	322×10^6	7.23	317
	5	70	378×10^6	7.05	314
B	**6**	160	138×10^6	7.39	293
	7	210	111×10^6	7.38	297
	8	130	120×10^6	7.24	297
C	**9**	15	673×10^6	7.40	300
	10	16	589×10^6	7.24	289
	11	24	611×10^6	7.25	293
	12	37	387×10^6	7.16	292
	13	12	575×10^6	7.37	309

[1]- Stallion A: 17-year-old Standardbred; Stallion B: 14-year-old Standardbred; Stallion C: 15-year-old Quarter Horse

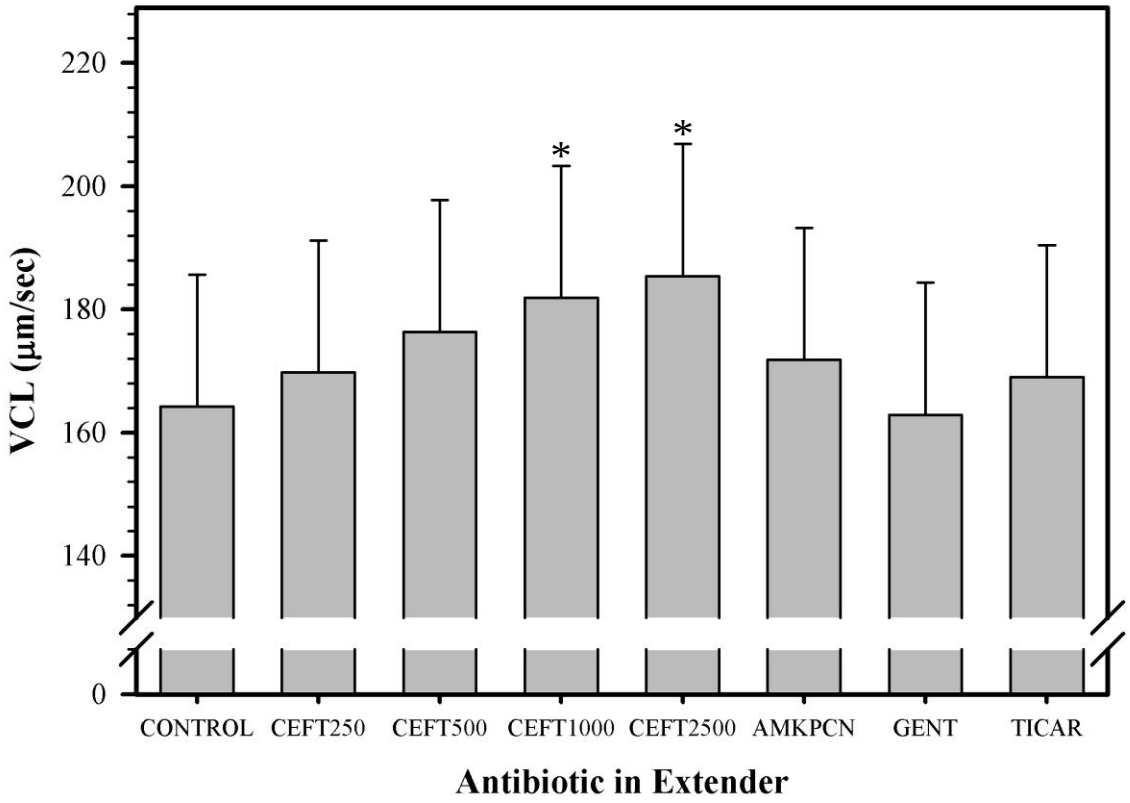
Figure 1. Bar plot of overall effect of various antibiotics in skim milk-glucose based semen extender on least squares mean (±SE) equine spermatozoal curvilinear distance (DCL) analyzed by computer-assisted semen analysis (Sperm Vision® CASA, Minitube®, Verona, WI). Analysis of repeated measures at time zero, after 24 hours of storage, and after 48 hours of storage.



CONTROL: no antibiotic added; CEFT250: ceftiofur sodium at 250µg/ml; CEFT500: ceftiofur sodium at 500µg/ml; CEFT1000: ceftiofur sodium at 1,000µg/ml; CEFT2500: ceftiofur sodium at 2,500µg/ml; AMKPCN: a combination of amikacin sulfate and potassium penicillin G at 1,000µg/ml and 1,000µg/ml, respectively GENT: gentamicin sulfate at 1,000µg/ml; TICAR: ticarcillin disodium at 1,000µg/ml.

*Significantly different than control. F test, $P \leq 0.05$. Bonferonni adjusted post-test comparison, $P \leq 0.0071$

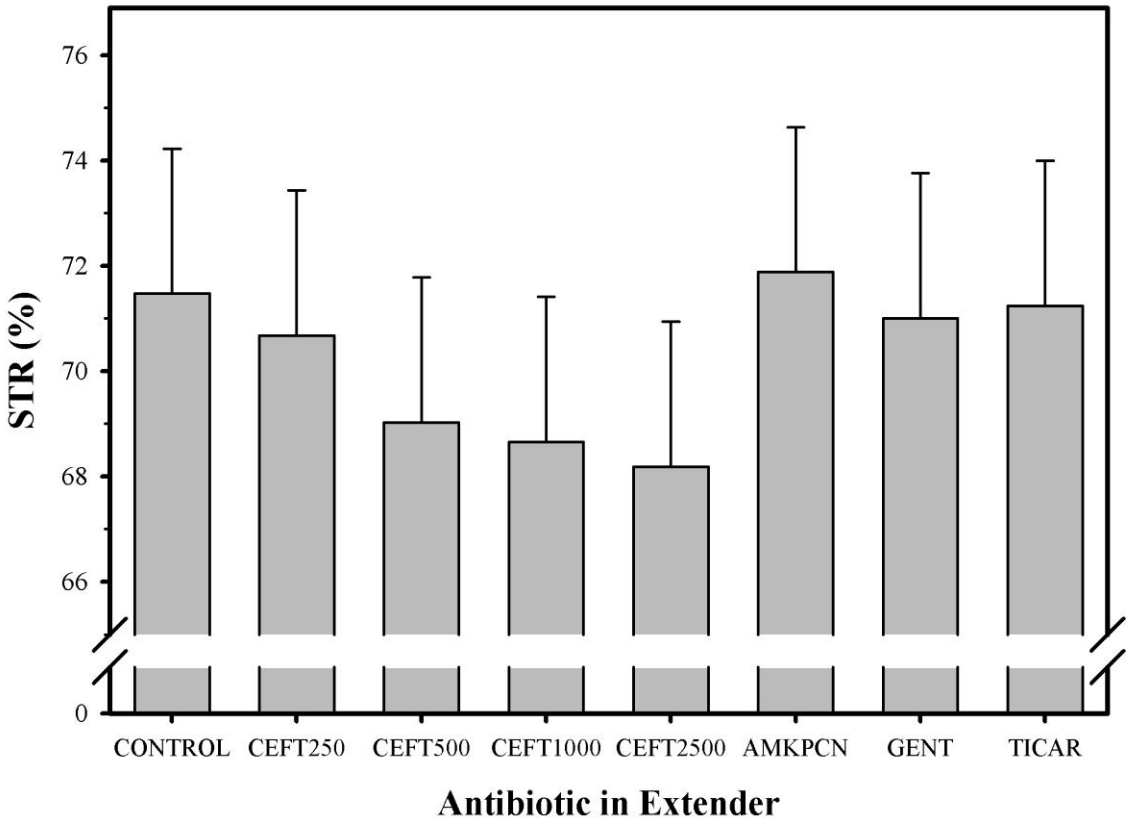
Figure 2. Bar plot of overall effect of various antibiotics in skim milk-glucose based semen extender on least squares mean (±SE) equine spermatozoal curvilinear velocity (VCL) analyzed by computer-assisted semen analysis (Sperm Vision® CASA, Minitube®, Verona, WI). Analysis of repeated measures at time zero, after 24 hours of storage, and after 48 hours of storage.



CONTROL: no antibiotic added; CEFT250: ceftiofur sodium at 250µg/ml; CEFT500: ceftiofur sodium at 500µg/ml; CEFT1000: ceftiofur sodium at 1,000µg/ml; CEFT2500: ceftiofur sodium at 2,500µg/ml; AMKPCN: a combination of amikacin sulfate and potassium penicillin G at 1,000µg/ml and 1,000µg/ml, respectively GENT: gentamicin sulfate at 1,000µg/ml; TICAR: ticarcillin disodium at 1,000µg/ml.

* Significantly different than control. F test, P≤0.05. Bonferonni adjusted post-test comparison, P≤0.0071

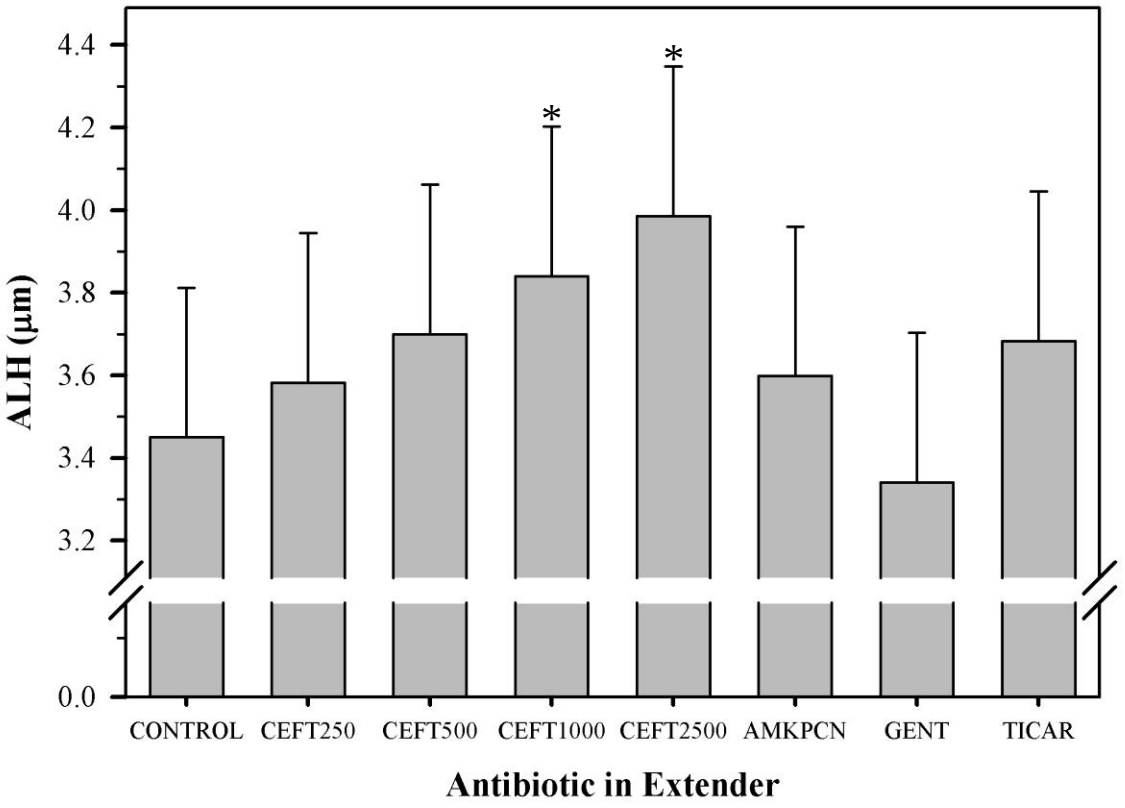
Figure 3. Bar plot of overall effect of various antibiotics in skim milk-glucose based semen extender on least squares mean (±SE) equine spermatozoal straightness (STR) analyzed by computer-assisted semen analysis (Sperm Vision® CASA, Minitube®, Verona, WI). Analysis of repeated measures at time zero, after 24 hours of storage, and after 48 hours of storage.



CONTROL: no antibiotic added; CEFT250: ceftiofur sodium at 250µg/ml; CEFT500: ceftiofur sodium at 500µg/ml; CEFT1000: ceftiofur sodium at 1,000µg/ml; CEFT2500: ceftiofur sodium at 2,500µg/ml; AMKPCN: a combination of amikacin sulfate and potassium penicillin G at 1,000µg/ml and 1,000µg/ml, respectively GENT: gentamicin sulfate at 1,000µg/ml; TICAR: ticarcillin disodium at 1,000µg/ml.

F test, $P \leq 0.05$. Bonferonni adjusted post-test comparison, $P \leq 0.0071$yielded no significance between groups.

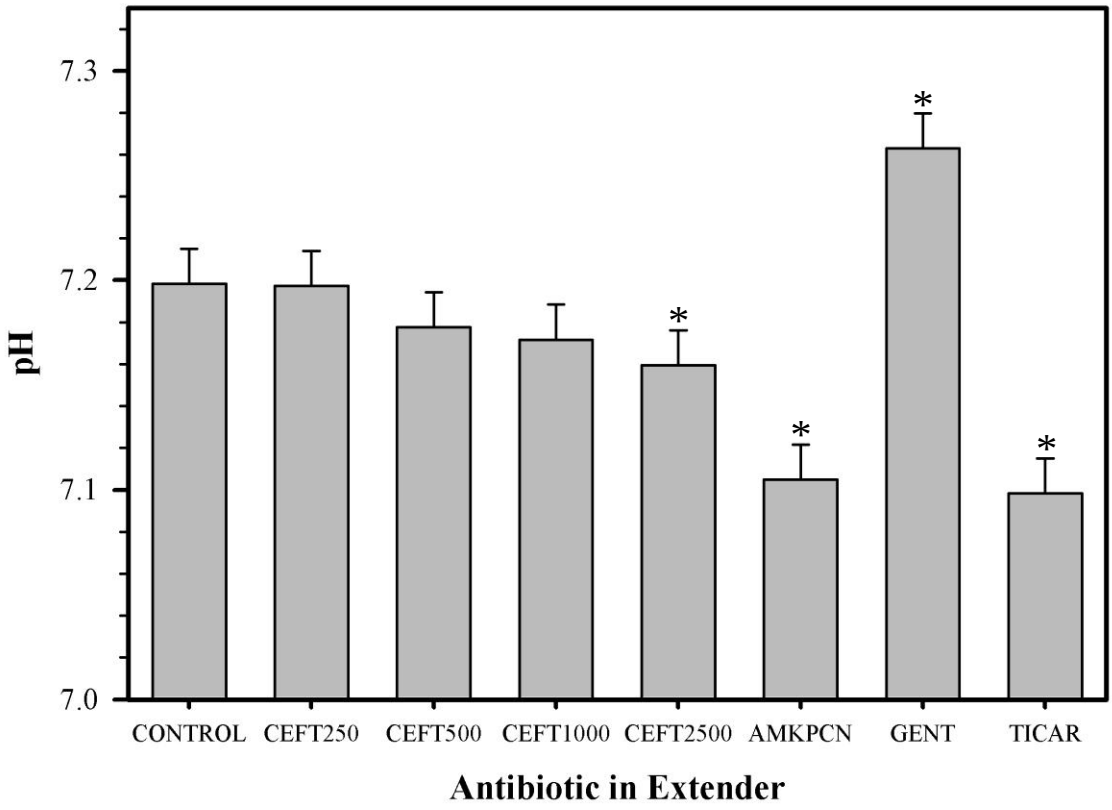
Figure 4. Bar plot of effect of various antibiotics in skim milk-glucose based semen extender on least squares mean (±SE) equine spermatozoal amplitude of lateral head displacement (ALH) analyzed by computer-assisted semen analysis (Sperm Vision® CASA, Minitube®, Verona, WI). Analysis of repeated measures at time zero, after 24 hours of storage, and after 48 hours of storage.



CONTROL: no antibiotic added; CEFT250: ceftiofur sodium at 250µg/ml; CEFT500: ceftiofur sodium at 500µg/ml; CEFT1000: ceftiofur sodium at 1,000µg/ml; CEFT2500: ceftiofur sodium at 2,500µg/ml; AMKPCN: a combination of amikacin sulfate and potassium penicillin G at 1,000µg/ml and 1,000µg/ml, respectively GENT: gentamicin sulfate at 1,000µg/ml; TICAR: ticarcillin disodium at 1,000µg/ml.

* Significantly different than control. F test, P≤0.05. Bonferonni adjusted post-test comparison, P≤0.0071

Figure 5. Bar plot of overall effect of various antibiotics in skim milk-glucose based semen extender on least squares mean (±SE) equine semen pH. Analysis of repeated measures at time zero, after 24 hours of storage, and after 48 hours of storage.



CONTROL: no antibiotic added; CEFT250: ceftiofur sodium at 250µg/ml; CEFT500: ceftiofur sodium at 500µg/ml; CEFT1000: ceftiofur sodium at 1,000µg/ml; CEFT2500: ceftiofur sodium at 2,500µg/ml; AMKPCN: a combination of amikacin sulfate and potassium penicillin G at 1,000µg/ml and 1,000µg/ml, respectively GENT: gentamicin sulfate at 1,000µg/ml; TICAR: ticarcillin disodium at 1,000µg/ml.

* Significantly different than control. F test, $P \leq 0.05$. Bonferonni adjusted post-test comparison, $P \leq 0.0071$

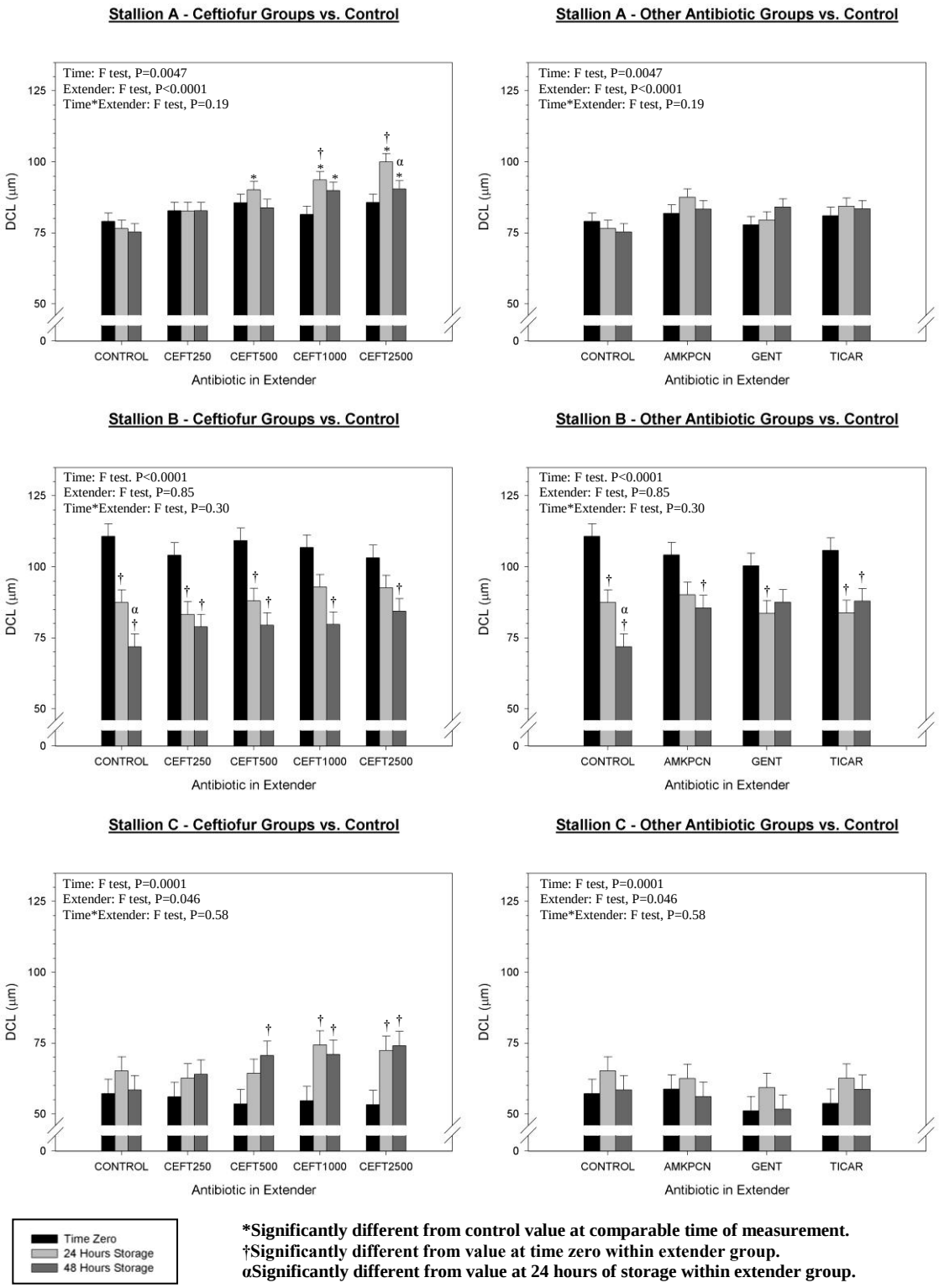


Figure 6. Bar plots of effect of ceftiofur sodium at various concentrations (250µg/ml – CEFT250, 500µg/ml – CEFT500, 1,000µg/ml – CEFT1000, and 2,500µg/ml – CEFT2500) and amikacin sulfate with potassium penicillin G (1,000µg/ml each - AMKPCN), gentamicin sulfate (1,000µg/ml - GENT), and ticarcillin disodium (1,000µg/ml - TICAR) versus no antibiotic (CONT) in skim milk-glucose based semen extender on LSMean (±SE) spermatozoal curvilinear distance (DCL) at different time points (time zero, after 24 hours of storage, and after 48 hours of storage) analyzed by CASA (Sperm Vision® CASA, Minitube®, Verona, WI) for three stallions (Stallion A – 17-year-old Standardbred, Stallion B – 14-year-old Standardbred, and Stallion C – 15-year-old Quarter Horse).

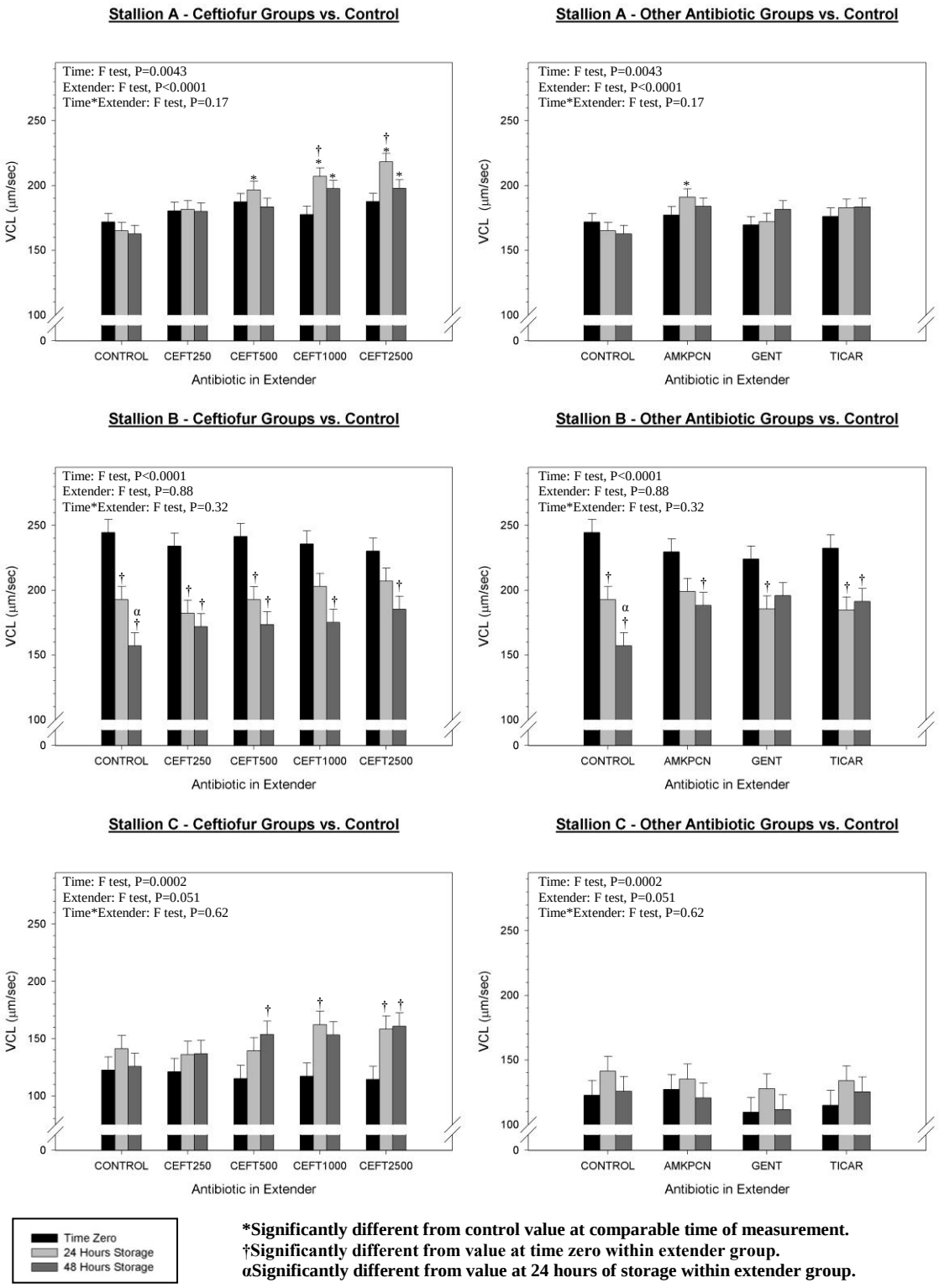


Figure 7. Bar plots of effect of ceftiofur sodium at various concentrations (250µg/ml – CEFT250, 500µg/ml – CEFT500, 1,000µg/ml – CEFT1000, and 2,500µg/ml – CEFT2500) and amikacin sulfate with potassium penicillin G (1,000µg/ml each - AMKPCN), gentamicin sulfate (1,000µg/ml - GENT), and ticarcillin disodium (1,000µg/ml - TICAR) versus no antibiotic (CONT) in skim milk-glucose based semen extender on LSMean (±SE) spermatozoal curvilinear velocity (VCL) at different time points (time zero, after 24 hours of storage, and after 48 hours of storage) analyzed by CASA (Sperm Vision® CASA, Minitube®, Verona, WI) for three stallions (Stallion A – 17-year-old Standardbred, Stallion B – 14-year-old Standardbred, and Stallion C – 15-year-old Quarter Horse).

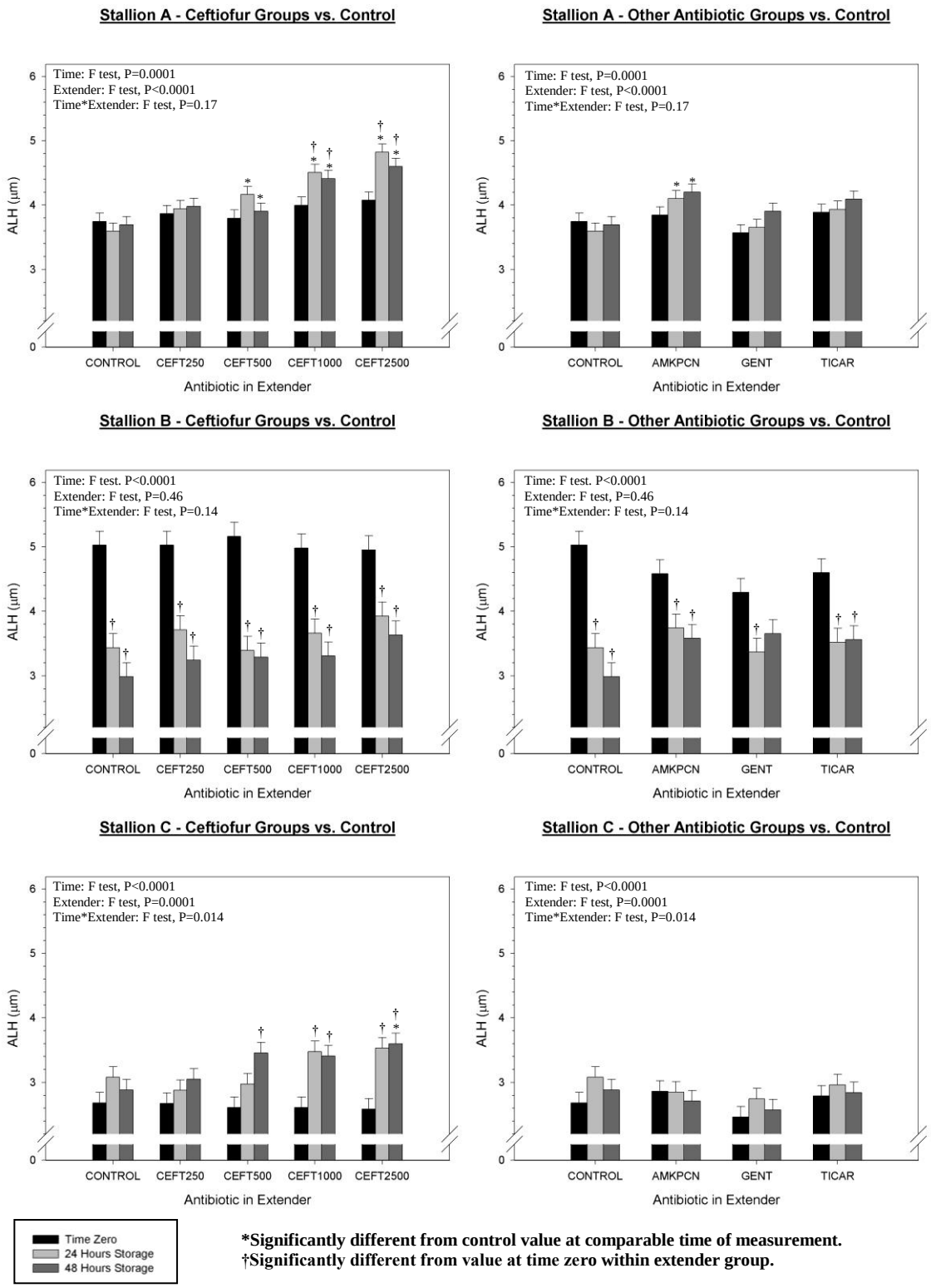


Figure 8. Bar plots of effect of ceftiofur sodium at various concentrations (250µg/ml – CEFT250, 500µg/ml – CEFT500, 1,000µg/ml – CEFT1000, and 2,500µg/ml – CEFT2500) and amikacin sulfate with potassium penicillin G (1,000µg/ml each - AMKPCN), gentamicin sulfate (1,000µg/ml - GENT), and ticarcillin disodium (1,000µg/ml - TICAR) versus no antibiotic (CONT) in skim milk-glucose based semen extender on LSMean (±SE) amplitude of lateral head displacement (ALH) at different time points (time zero, after 24 hours of storage, and after 48 hours of storage) analyzed by CASA (Sperm Vision® CASA, Minitube®, Verona, WI) for three stallions (Stallion A – 17-year-old Standardbred, Stallion B – 14-year-old Standardbred, and Stallion C – 15-year-old Quarter Horse).

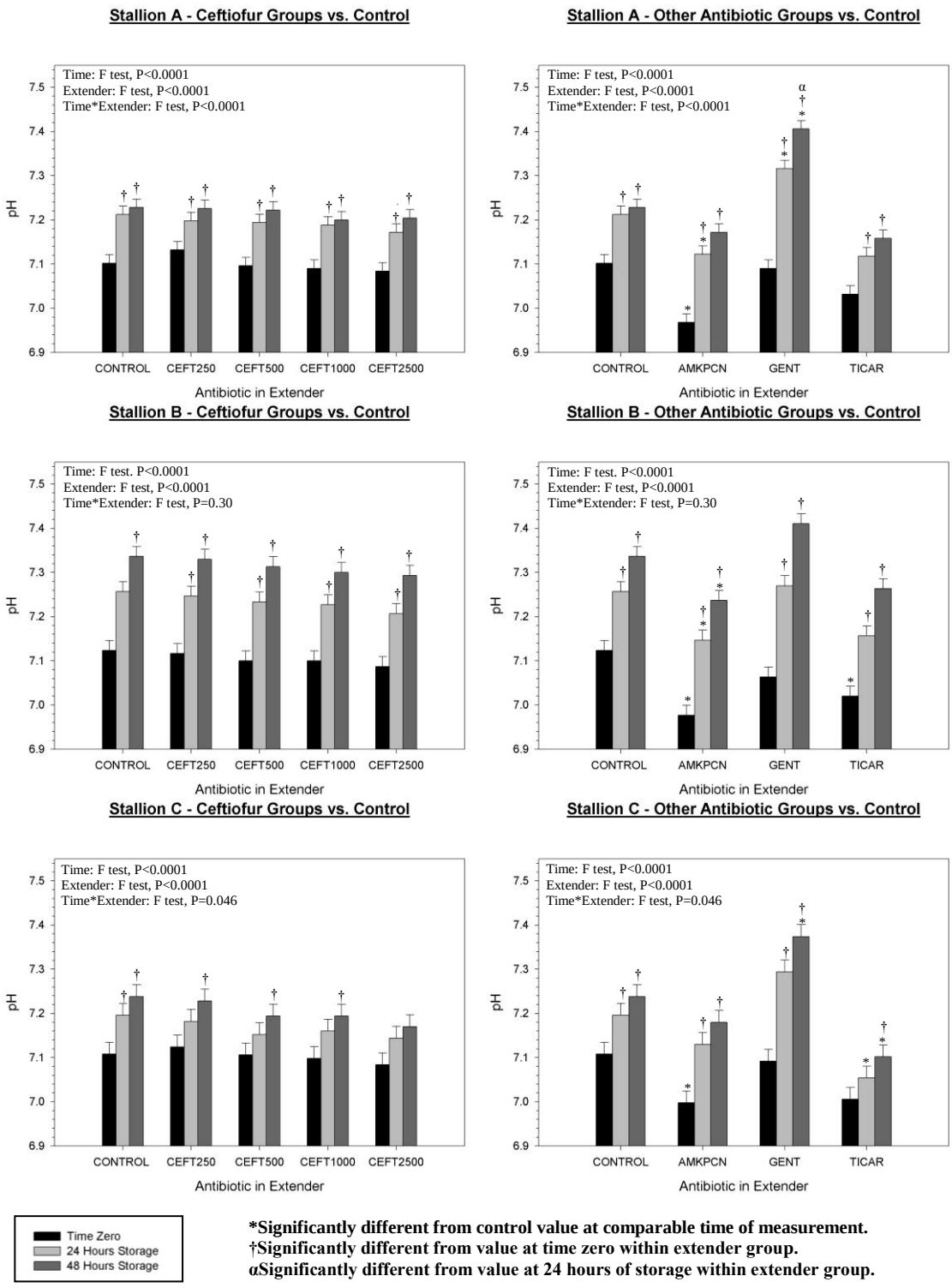


Figure 9. Bar plots of effect of ceftiofur sodium at various concentrations (250µg/ml – CEFT250, 500µg/ml – CEFT500, 1,000µg/ml – CEFT1000, and 2,500µg/ml – CEFT2500) and amikacin sulfate with potassium penicillin G (1,000µg/ml each - AMKPCN), gentamicin sulfate (1,000µg/ml - GENT), and ticarcillin disodium (1,000µg/ml - TICAR) versus no antibiotic (CONT) in skim milk-glucose based semen extender on LSMean (±SE) pH at different time points (time zero, after 24 hours of storage, and after 48 hours of storage) for three stallions (Stallion A – 17-year-old Standardbred, Stallion B – 14-year-old Standardbred, and Stallion C – 15-year-old Quarter Horse).

CHAPTER 05

DISCUSSION

A. Spermatozoal Motion Characteristics

Effects of Antibiotic in Semen Extender

The major finding of the present study was the dose-dependent relationship of ceftiofur sodium to increases in spermatozoal curvilinear distance (DCL), curvilinear velocity (VCL), and the amplitude of lateral head displacement (ALH). To the author's knowledge, this is the first report associating increasing concentrations of an antibiotic to an increase in selected spermatozoal motion characteristics in a dose-dependent fashion. Evaluation of increasing concentrations of antibiotic added to stallion semen extenders has been evaluated previously, but only for antibiotics other than ceftiofur. Jasko et al. (1993) evaluated selected spermatozoal motion characteristics (including curvilinear velocity) for several antibiotics (including amikacin sulfate, ticarcillin disodium, and gentamicin sulfate) added to a skim milk-glucose based semen extender at two concentrations (1,000µg/ml and 2,000µg/ml) after storage at 5°C for 48 hours. The two concentrations showed no significant increases in spermatozoal motion characteristics in comparison to control. Arriola and Foote (1982) evaluated total spermatozoal motility with increasing concentrations of amikacin sulfate in an egg yolk-tris semen extender. Total motility was not significantly different for concentrations of 50, 100, 250, 500, or 1,000µg/ml in comparison to positive controls (penicillin and penicillin-streptomycin) after storage for 10 hours at 37°C. However, amikacin sulfate concentration of 10,000µg/ml was detrimental to overall motility. Back et al. (1975) evaluated increasing concentrations (250µg/ml to 2500µg/ml) of

gentamicin sulfate and sodium penicillin G (as well as other antibiotics no longer commonly used in stud practice) in an egg yolk-tris semen extender. The researchers found the levels of gentamicin or penicillin had no significant effects on overall motility during storage at 5°C.

Positive correlations between the presence of certain antibiotics in the semen extender and improved spermatozoal motion characteristics have been infrequently reported. Macedo et al. (2005) found an increase in selected motion characteristics with the addition of amikacin at 2,000µg/ml to a skim milk-glucose based semen extender. An increase in total motility and progressive motility was noted in comparison to their control (gentamicin at 2,000µg/ml in the same base extender). This effect was not noted within the current study, possibly due to the decreased concentrations of these antibiotics used (1,000µg/ml versus 2,000µg/ml) and use of a combination of penicillin with the amikacin. Also, the lack of negative control (extender without antibiotic added) in their study prevents the evaluation of the direct effect of antibiotic. Varner et al. (1998) found the addition of combination amikacin sulfate/potassium penicillin G increased selected motion characteristics (including VCL, VAP, and VSL) in comparison to control after storage at 5°C for 24 hours in a skim milk-glucose based semen extender. This finding is inconsistent with the present study, which found no significant difference between control extender and the addition of combination amikacin sulfate/potassium penicillin G. This discrepancy is likely due to the small size of both studies (three ejaculates each from three stallions were utilized in the Varner et al. study) and the high variability of spermatozoal characteristics between stallions.

Gentamicin has been shown to have a detrimental effect on selected motion characteristics in some studies (Squires and McGlothlin 1980; Jasko et al. 1993; Macedo et al. 2005; Aurich and Spergser 2007). Although similar to the present study, other research (Back et

al., 1975; Squires et al., 1981; Varner et al., 1998; LeFrapper et al., 2010) showed no detrimental effect on selected spermatozoal motion characteristics associated with the use of gentamicin. These conflicting results could be associated with differences in semen extender used that could affect the antibiotic's interaction with spermatozoa, the use of non-reagent-grade antibiotic allowing for the presence of preservatives (Squires and McGlothlin, 1980), and inherent differences in the spermatozoal populations of the stallions studied. This later finding was noted by Back et al. (1975) and reported that higher concentrations of gentamicin were detrimental to spermatozoa in "a few" stallions.

Use of Cephalosporins in Semen Extension

Reports of the use of cephalosporins in the extension and cooled storage of stallion semen are scarce. Parlevliet et al. (2006) evaluated the effects of cefquinome (a fourth-generation cephalosporin) at 990µg/ml added to a skim milk based extender on spermatozoal total motility and progressive motility in the stallion and found no significance in comparison to gentamicin at time zero, after 24 hours or after 48 hours of storage. However, velocity measurements (VCL and VAP) were significantly lower after 24 hours of storage, but not after 48 hours of storage. The authors concluded the difference was likely not clinically significant. ALH was found to be significantly lower after 24 and 48 hours of storage in the cefquinome treatment group. These findings are opposite to those in the current study, which may be due to the utilization of a different cephalosporin antibiotic (ceftiofur, a third-generation cephalosporin) at a different concentration.

To the author's knowledge, there has only been one published report of the effects of ceftiofur sodium on motility of extended equine spermatozoa during cooled storage. The study

(Varner et al. 1998) evaluated the effects of ceftiofur at 1,000µg/ml in a skim milk-glucose based semen extender in comparison to control (no antibiotic) and the addition of several antibiotics. Selected motion characteristics were evaluated (including curvilinear velocity) after 24 hours of storage at approximately 5°C and no significant differences were detected. This differs from the findings reported in the current study. This is potentially due to the evaluation of spermatozoal motion characteristics after 48 hours of storage, and the increased concentration of ceftiofur sodium evaluated (2,500µg/ml). In fact in the current study, ceftiofur sodium at concentrations of 1,000µg/ml did increase selected spermatozoal motion characteristics, but only after 48 hours of storage (in the Varner et al. study, the same concentration was stored for only 24 hours).

Hyperactivation and Relationship to Capacitation

Spermatozoal capacitation is frequently reported to be associated with hyperactivation (Ho and Suarez, 2001; Sieme, 2009). While hyperactivated motility is independent of capacitation (Rathi et al., 2001; McPartlin et al., 2009), it does share similar requirements including the presence of calcium and bicarbonate, and the activation of cAMP synthesis (Visconti et al., 1999; Ho and Suarez, 2001).

The exact motility pattern to determine hyperactivation is species specific and has not been determined definitively for the stallion. The characterization is further complicated by differences in computer-assisted semen analysis systems. However, increases in the amplitude of lateral head displacement (ALH), increases in curvilinear velocity (VCL), and decreases in straightness (STR) have all been attributed to hyperactivated motility (Rathi et al., 2001; McPartlin et al., 2009; Sieme, 2009). Values specific to the SpermVision® system as reported by Minitube® for classification of hyperactive motility include VCL greater than 80µm/sec, LIN

less than 65%, and ALH greater than 6.5µm (Sepp Weigert, personal communication). For this study, these criteria were not met, but the significant increases in ALH and VCL seen with higher levels of ceftiofur are consistent with a trend toward hyperactivated motility. The increase in DCL is also consistent with hyperactivated spermatozoa, as the length of the actual path could easily increase the more the spermatozoa move in a non-linear pattern. However, the author is unaware of any reports correlating hyperactivation to DCL. Also consistent with hyperactivation of spermatozoa was the tendency for STR to be decreased when exposed to the higher concentrations of ceftiofur in this study.

The specific cause of this trend toward hyperactivated motility cannot be elucidated from the current study. Multivariate regression analysis showed that the strongest predictor of these increased spermatozoal motion characteristics was pH. Keller and Campbell (1972) evaluated pH changes in association with different antibiotics in a lactose-buttermilk extender and found a positive correlation between a decrease in pH and an increase in rapid sperm motion. This finding is not consistent with subsequent studies that noted an increase in intracellular bicarbonate with a subsequent increase in intracellular pH occurs during capacitation and hyperactivated motility (Boatman and Robbins, 1991; Garcia and Meizel, 1999; Rathi et al., 2001). In fact, Ho et al. (2002), found that an alkaline environment was required for the production of hyperactivated motility in bull spermatozoa. Furthermore, Wendt et al. (2002) found no difference in VCL or ALH when evaluating spermatozoa exposed to significant variation in the pH of a skim milk-glucose-sucrose based extender, but there was a significant decrease in VSL, STR, and LIN. The authors concluded this was consistent with hyperactivated motility possibly secondary to increases in intracellular pH. However, it is important to note that Visconti et al. (1999) showed increases in extracellular or intracellular pH did not induce the

acrosome reaction (an end product of capacitation) in hamster spermatozoa in the presence of bicarbonate-free media. These results suggest that the presence of bicarbonate is more important than the resulting increase in intracellular pH for the induction of capacitation changes and hyperactivation of spermatozoa.

Due to these previous results, it is difficult to explain the trend toward hyperactivated motility with increasing concentrations of ceftiofur sodium in association with pH changes in the current study. If increased pH and subsequent increases in intracellular pH were resulting in the trend toward hyperactivated motility, the trend should have been noted within all of the extender groups as there was an increase in pH measurements with increased storage in all groups. However, only the higher concentrations of ceftiofur resulted in hyperactivation within the current study. This leads to the conclusion that pH is contributing only minor influences on the increases in DCL, VCL, and ALH, which is consistent with the multivariate regression analysis results. Furthermore, if increased pH yielded the increases noted in DCL, VCL, ALH, the addition of gentamicin should have resulted in the strongest trend toward hyperactivated motility secondary to highest pH. In fact, the opposite occurred with DCL, VCL, and ALH values being the lowest in the GENT extender group.

Therefore, a separate component specific to ceftiofur sodium is leading to the increases in motion characteristics noted within the current study. It has been shown that the intracellular pH of capacitated human spermatozoa is decreased following incubation in Na^+-deficient media (Garcia and Meizel, 1999). It is possible that the presence of sodium is interfering with Na^+/H^+ exchanger within the spermatozoal membrane leading to increased intracellular bicarbonate although Hernández-González et al. (2006) found the addition of Na^+ to mouse spermatozoa does not increase internal pH. With the current data, it is not possible to determine that

biochemical changes occurring within the spermatozoa are leading to the motion characteristic changes noted.

Pommer et al. (2002a) evaluated differences in acrosomal reactions of stallion spermatozoa after incubation in either a skim milk based semen extender (without antibiotic) or in a Tyrode's medium containing albumin, lactate, and pyruvate. While there were no detected changes in baseline acrosomal status after 3 hours of incubation at 37°C, the authors did report an increased acrosomal reaction rate (induced with the calcium ionophore A23187) with the skim milk based extender. The authors concluded that the skim milk based extender appeared to facilitate capacitation allowing for higher rates of acrosome reaction after incubation with the ionophore. This conclusion is inconsistent with previous reports that prior capacitation is not required for A23187 to induce the acrosome reaction as the ionophore can overwhelm mitochondrial and plasma membrane pumps allowing for fee entry of calcium (Simpson et al., 1987; Varner et al., 2001). Regardless, the study shows a distinct increase in capacitation rate with incubation in skim milk extender. It is difficult to compare these results to that of the current study as only skim milk based extender was utilized. However, the use of skim milk-glucose based extender in the current study with the addition of high concentrations of ceftiofur seems to have lead to changes (hyperactivated motility) consistent with early capacitation changes.

The present study should be repeated with higher ceftiofur concentrations to determine if they can yield true hyperactivated motility of spermatozoa and to evaluate if the current results are repeatable with a different stallion group. If this occurs, measuring the capacitation status of these spermatozoa is indicated. Interestingly, Parlevliet et al. (2006) evaluated the acrosomal status of spermatozoa exposed to cefquinome (a fourth-generation cephalosporin) at 990µg/ml

and found significantly more acrosome intact cells after 48 hours of storage in comparison to spermatozoa exposed to gentamicin. This is the opposite of what would be expected from the current study if early capacitation is occurring, as the acrosome reaction is considered to be an end-point of capacitation (Sieme, 2009). As mentioned previously, this study also revealed the opposite effect of the cephalosporin antibiotic on both VCL and ALH, possibly due to differences between a fourth-generation and third-generation cephalosporins. If early capacitation was occurring in the current study, the use of ceftiofur sodium in the extension of cooled-shipped equine semen would be contraindicated as the longevity of these sperm cells would be significantly compromised resulting in decreased viability and fertility (Pommer et al., 2002a; Varner and Johnson, 2007). This conclusion prevents the recommendation of the use of ceftiofur sodium as a broad-spectrum antibacterial agent in the extension and cooled-storage of equine semen until further research is performed.

As both capacitation and spermatozoal hyperactivation are required for successful in vitro fertilization (IVF) in the horse (McPartlin et al., 2009), further study of the effects of ceftiofur on stallion spermatozoal capacitation and hyperactivation is recommended for a possible role in equine IVF. Holyoak et al. (1998) evaluated the effects of various levels of ceftiofur sodium on the development of bovine oocytes/embryos during oocyte maturation, oocyte fertilization, and embryo culture. They found no significant difference in embryo development with the addition of ceftiofur (10µg to 200µg) to the in vitro fertilization medium. The authors concluded that short-term exposure of ceftiofur sodium did not affect the fertilizing capacity of bovine spermatozoa. Unfortunately, there was no further study of the spermatozoa to note any potential differences in capacitation status and only one bull was utilized for the experiment. Therefore, it is difficult to compare these results to those of the current study, but one can conclude that the

presence of ceftiofur within the in vitro fertilization medium at the very least did not prevent capacitation from occurring. If the addition of ceftiofur sodium at high concentrations proves to increase hyperactivated motility and potentially lead to capacitation, it could be evaluated for use in IVF in the equine.

Effect of Time on Spermatozoal Motion Characteristics, pH, and Osmolality

The decrease in total and progressive motility over time seen in the current study is consistent with previous reports (Back et al., 1975; LeFrappe et al., 2010). Although it is important to note that there is a considerable variation between stallions in the maintenance of these motion characteristics, which is consistent with the current results. Within the current study, the higher concentrations of ceftiofur (500, 1,000, and 2,500µg/ml) prevented a decrease in total and progressive motility until after 48 hours of storage.

For DAP, DSL, VAP, VSL, LIN, STR, and WOB, it is difficult to draw conclusion from noted changes over time within the current study due to the paucity of information in the literature correlating their importance to semen analysis in the stallion. Some parameters changed significantly only between 24 and 48 hours of storage (DAP, DSL, VAP, and LIN). Whereas VSL and STR increased after 24 hours of storage, only to decrease after 48 hours of storage and WOB was relatively stable at time zero and 24 hour measurements, but significantly decreased after 48 hours of storage. With no consistent pattern elucidated, a conclusion concerning the meaning of these measurements is not possible.

While time did not have an effect on osmolality, it did result in increased pH. This increase was consistent for all extender groups although greater increases in pH were noted with the addition of gentamicin, which is interesting as gentamicin is particularly acidic in nature

(Pickett and Amann, 1987). The increase is likely due to extensive buffering of the base extender by the manufacturer prior to the addition of the gentamicin. The increase in pH noted is most likely due to the additive effects of spermatozoal and bacterial metabolites with prolonged storage although one might expect overall pH to decrease secondary to the production of acidic by-products (such as lactic acid).

Individual Stallion Effects

Stallions were evaluated separately for each of these motion characteristics to ensure the effect was not based on a single animal. Only stallion A followed the exact same statistical significance as the overall analysis for the effect extender on all three parameters. The effect of extender was not present for Stallion B for any of the three parameters. However, only three ejaculates were analyzed from this stallion in comparison to five each from the other two stallions, which may have contributed to the lack of effect noted. For stallion C, there was a significant effect of extender for DCL and ALH. While the significance was not as strong as for stallion A, there was a definite increase in these parameters in relation to higher concentrations of ceftiofur.

The effect of time for DCL, VCL, and ALH was significant for all three stallions, while the overall analysis found no significant effect. This difference can be explained by a significant increase in values for stallions A and C and a significant decrease in values for stallion B, which cancelled any significant effect for the overall analysis. The increases noted for stallions A and C were only detected with the higher concentrations of ceftiofur while the decreases noted for stallion B were detected with all the extender groups. For stallion B, it is worth noting that only the higher concentrations of ceftiofur and combination amikacin/penicillin were able to prevent a

significant decrease in DCL and VCL within the first 24 hours of storage, possibly indicating a protective effect on these motion characteristics. In conclusion, while stallion A contributed significantly to the overall results of the current study, the dose-dependent relationship of ceftiofur to increases in the three motion characteristics was confirmed in some fashion for each stallion.

Although there were subtle differences, individual stallion pH was similar to the overall analysis with both extender and time significant for all three stallions.

B. Ejaculate Characteristics

The gel-free ejaculate volumes and concentrations for this study are within previously reported ranges (Clay et al., 1987; Ionata et al., 1991; Sieme, 2009). The pH values for the gel-free ejaculates are also within reported ranges (Griggers et al., 2001; Lopate et al., 2003). Osmolality values for the gel-free ejaculates are within reported ranges (Lopate et al., 2003).

C. Bacterial Growth

The bacterial colonization of the ejaculates in the present study is consistent with previous reports. Interestingly, the most commonly isolated pathogen, *Corynebacterium* spp., in the current study was also the most frequently isolated pathogen in a recently reported study. Althouse et al. (2010) evaluated the bacterial contaminants present in cooled-shipped equine semen from a variety of sources and found *Corynebacterium* spp. and *Bacillus* spp. most frequently. The authors noted that the vast majority of bacteria isolated in their study appeared to be environmental contaminants. Of the most common isolates in the current study, *Corynebacterium* spp. and *Pseudomonas* spp. can be considered environmental contaminants (Hirsh and Biberstein, 2004; Hirsh, 2004), although *Pseudomonas* spp. is also considered to be a contributor to the normal flora of stallions (Corona et al., 2006; Samper and Tibary, 2006). It is likely that presence of *Corynebacterium* spp. in the current study represents environmental contamination that occurred during semen processing and excessive transporting of the semen and culture plates between the collection area and various laboratory areas.

The continued presence of pathogens within the extended semen is consistent with previous reports (Varner et al., 1998; Althouse et al., 2010). As antimicrobials within extended semen are generally thought to work during the cooling process and not during cooled storage itself (Aurich and Spergser, 2006), the persistent presence of bacteria noted in the current study is not unexpected. The addition of combination amikacin sulfate and potassium penicillin G (1000µg/ml and 1000IU/ml, respectively) provided the most effective antimicrobial control (85%), followed by gentamicin and ceftiofur at 2500µg/ml (both 77% control). These results are consistent with findings reported by Varner et al. (1998), which reported high bacterial control rates for amikacin sulfate and potassium penicillin G, and gentamicin. The lowest concentration

ceftiofur sodium group (250µg/ml) provided the least effective antimicrobial control (23%), which is expected. Ticarcillin provided the second least effective antimicrobial control (38%). This level of moderate to poor control is similar to levels reported by Varner et al. (1998). Ceftiofur sodium at 1,000µg/ml provided acceptable antimicrobial control at 62%. As there is limited evidence within the literature concerning what acceptable levels of bacterial contamination are, it is difficult to definitively conclude this to be the minimum effective concentration of ceftiofur in the current study. However, it is likely this level would provide acceptable control as growth was limited to few colonies or less and it is not likely that the limited bacteria would lead to direct decreases in fertility or increased post-breeding infectious endometritis rates.

The potential interaction between bacterial growth and antibiotic on spermatozoal characteristics was not evaluated in the current study. However, as the majority of growth after storage was limited to a few colonies or less, it is unlikely that the presence of bacteria significantly affected motion characteristics although this cannot be definitively determined.

CHAPTER 06

CONCLUSIONS

The present study allowed us to conclude:

- The presence of ceftiofur sodium in a skim milk-glucose based semen extender increases overall stallion spermatozoal DCL, VCL, and ALH, and tends to decrease STR versus control in a dose-dependent fashion during cooled storage. The motion characteristic changes indicate a trend toward spermatozoal hyperactivation, possibly due to early capacitation like changes. Of the measured variables, pH is the strongest indicator for the increased motion characteristics, although it is only a minor predictor.
- Spermatozoal motion characteristics are variable between individual stallions.
- Total motility and progressive motility of stallion spermatozoa decrease over time with cooled storage in skim milk-glucose based semen extender.
- The pH of semen extended in a skim-milk-glucose based semen extender increases with cooled storage regardless of antibiotic presence. The presence of ceftiofur sodium at high concentrations (2,500µg/ml), combination amikacin sulfate/potassium penicillin G (1,000µg/ml and 1,000IU/ml, respectively), and ticarcillin disodium (1,000µg/ml) significantly decrease extended semen pH in comparison to control. The presence of gentamicin sulfate (1,000µg/ml) significantly increases extended semen pH in comparison to control.
- The osmolality of semen extended in skim-milk-glucose based semen extender does not significantly changed with cooled storage.

- Stallion ejaculates are contaminated with bacteria consistent with normal flora and environmental contaminants during the collection and processing of semen. The most common isolates can be considered environmental contaminants from the collection procedure and subsequent extension process. This bacterial growth can be controlled, but not eliminated with the addition of various antibiotics to the semen extender.
- The minimum effective concentration of ceftiofur sodium for the control of bacterial growth is suggested to be 1,000µg/ml, which limits growth in the extended semen to few colonies or less. This level of bacterial growth would not likely lead to direct decreases in spermatozoal viability or increased post-breeding infectious endometritis.

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Appendix A. Internal temperature in °C of semen-transport containers containing extended semen from thirteen ejaculates from three stallions taken at twenty-four hours of storage (container #1) or at twenty-four and forty-eight hours of storage (container #2) recorded by a digital, traceable thermometer (Fisher-Scientific #15-077-17B, Pittsburgh, PA).

Ejaculate No.[1]	Storage Container No.	Temp. 24 hours	Temp. 48 hours[2]
1	1	9.5	n/a
	2	7.5	8.2
2	1	8.2	n/a
	2	7.7	8.2
3	1	8.4	n/a
	2	8.7	8.7
4	1	7.7	n/a
	2	8.4	9.0
5	1	8.3	n/a
	2	7.4	8.1
6	1	7.7	n/a
	2	7.9	8.7
7	1	8.1	n/a
	2	7.3	8.0
8	1	6.7	n/a
	2	7.8	8.7
9	1	7.9	n/a
	2	8.1	8.8
10	1	8.1	n/a
	2	7.6	8.6
11	1	8.1	n/a
	2	7.1	7.6
12	1	8.2	n/a
	2	8.1	8.8
13	1	8.2	n/a
	2	7.4	7.9

[1] – Ejaculates 1-5 were Stallion A, collections 6-8 were stallion B, and collections 9-13 were stallion C

[2] – n/a = not available

Appendix B. Effect of various antibiotics in milk-glucose based semen extender and time of storage on least squares mean (±SE) equine spermatozoal motion characteristics analyzed by computer-assisted semen analysis (Sperm Vision® CASA, Minitube®, Verona, WI) at the time of extension (time zero) and after storage in a semen-transport container at approximately 5°C for 24 and 48 hours.

		Treatment[1]									Repeated measures analysis of variance		
Variable[2] & Time[3]		CON	CEFT 250	CEFT 500	CEFT 1000	CEFT 2500	AMK PCN	GENT	TICAR	SE	Trt	Time	Trt*Time
MOT	0	77.3	78.0	78.0	76.5	75.8	76.5	78.5	78.2	5.6	0.2	0.0001	0.8
	24	66.2†	67.1†	69.4	69.7	67.9	63.5†	65.0†	68.7†				
	48	50.6$^{\dagger\alpha}$	55.1$^{\dagger\alpha}$	57.7$^{\dagger\alpha}$	57.8$^{\dagger\alpha}$	55.7$^{\dagger\alpha}$	47.1$^{\dagger\alpha}$	50.6$^{\dagger\alpha}$	53.5$^{\dagger\alpha}$				
PM	0	71.32	69.2	70.4	68.0	65.8	66.8	68.6	69.7	5.7	0.2	0.0002	0.8
	24	58.5†	58.1†	60.9	61.4	59.6	56.0†	55.1†	60.2				
	48	41.3$^{\dagger\alpha}$	45.9$^{\dagger\alpha}$	47.6$^{\dagger\alpha}$	48.1$^{\dagger\alpha}$	46.2$^{\dagger\alpha}$	36.9$^{\dagger\alpha}$	38.2$^{\dagger\alpha}$	44.6$^{\dagger\alpha}$				
DCL	0	79.9	79.4	80.7	79.0	79.2	80.1	74.7	78.2	9.6	0.01	0.2	0.4
	24	76.6	77.1	81.7	88.0	89.6*	80.5	74.6	77.8				
	48	70.0	76.6	79.7	82.3*	84.8*	75.4	74.4	76.9				
DAP	0	39.8	39.1	39.6	38.7	38.8	40.0	38.3	38.5	4.1	0.1	0.03	0.6
	24	38.2	38.5	41.1	44.0	43.6	40.8	37.6	39.1				
	48	33.2†	37.0	38.4	38.7	39.7	36.2	35.8	36.8				
DSL	0	39.8	39.1	39.6	38.7	38.8	40.0	38.3	38.5	4.1	0.1	0.03	0.6
	24	38.2	38.5	41.1	44.0	43.6	40.8	37.6	39.1				
	48	33.2†	37.0	38.4	38.7	39.7	36.2	35.8	36.8				
VCL	0	174.2	174.5	176.6	172.3	173.8	174.6	163.6	170.2	22.1	0.009	0.2	0.4
	24	166.9	168.8	178.2	193.5	197.2*	176.0	162.7	169.4				
	48	151.7	166.0	174.3	179.9*	185.3*	165.0	162.5	167.4				
VAP	0	86.9	79.4	86.6	84.6	85.3	86.3	83.8	83.9	9.3	0.09	0.04	0.7
	24	83.4	84.3	89.8	96.9	96.2	89.4	82.1	85.8				
	48	72.1†	80.1	84.1	84.7	87.1	79.3	78.4	80.1				
VSL	0	61.8	61.1	58.1	56.8	56.7	59.8	59.7	58.1	6.6	0.7	0.01	0.5
	24	62.1	61.3	64.8	68.3†	67.6†	67.3	61.1	63.3				
	48	50.9$^{\dagger\alpha}$	56.4	57.9	58.4$^{\alpha}$	59.3	56.9$^{\alpha}$	54.1	56.9				
LIN	0	36.1	35.6	33.6	33.4	33.3	34.6	36.3	34.4	2.9	0.08	0.01	0.9
	24	36.7	36.1	36.2	35.1	34.2	38.7†	37.6	37.5				
	48	33.4	33.7	32.7	32.2	31.7	34.4$^{\alpha}$	32.8$^{\alpha}$	33.7				
STR	0	71.3	70.1	67.7	67.6	67.1	69.9	71.0	69.4	3.0	0.03	0.02	1.0
	24	73.4	72.1	71.3	69.8	69.8	74.8†	73.8	74.0				
	48	69.8	69.8	68.1	68.5	67.6	70.9	68.2	70.3				
WOB	0	49.9	50.1	49.1	49.1	49.4	49.4	50.7	49.1	2.0	0.5	0.006	1.0
	24	49.7	49.8	50.2	49.9	48.7	50.9	50.4	50.1				
	48	47.4	48.1	47.9	47.0$^{\alpha}$	46.7†	48.1$^{\alpha}$	47.7$^{\dagger\alpha}$	47.7				
BCF	0	38.6	38.6	38.5	37.5	37.8	38.3	39.3	37.7	2.2	0.6	0.05	1.0
	24	39.7	38.9	40.4	39.7	38.6	40.5	40.8	39.6				
	48	37.7	38.4	37.8	38.3	37.7	38.2	39.0	38.5				
ALH	0	3.7	3.7	3.8	3.7	3.8	3.7	3.4	3.7	0.4	0.001	0.6	0.4
	24	3.4	3.5	3.6	4.0	4.2*	3.6	3.3	3.5				
	48	3.3	3.5	3.7	3.8	4.0*	3.5	3.4	3.5				

[1] CON: control, no antibiotic added; CEFT250: ceftiofur sodium at 250µg/ml; CEFT500: ceftiofur sodium at 500µg/ml; CEFT1000: ceftiofur sodium at 1,000µg/ml; CEFT2500: ceftiofur sodium at 2,500µg/ml; AMKPCN: a combination of amikacin sulfate and potassium penicillin G at 1,000µg/ml and 1,000µg/ml, respectively GENT: gentamicin sulfate at 1,000µg/ml; TICAR: ticarcillin disodium at 1,000µg/ml.

[2] MOT: total motility (%); PM: progressive motility (%); DCL: curvilinear distance (µm); DAP: average path distance (µm); DSL: straight line distance (µm); VCL: curvilinear velocity (µm/sec); VAP: average path velocity (µm/sec); VSL: straight line velocity (µm/sec); LIN: linearity (%); STR: straightness (%); WOB: wobble (%); BCF: beat cross frequency (Hz); ALH: amplitude of lateral head displacement (µm).

[3] 0: time zero; 24: 24 hours of storage; 48: 48 hours of storage

[*] **Significantly different from control value within row and variable (P<0.0071).**

[†] **Significantly different from value at time zero within column and variable (P<0.025).**

[α] **Significantly different from value at 24 hours of storage within column and variable (P<0.025).**

Appendix C. Effect of various antibiotics in milk-glucose based semen extender and time of storage on least squares mean (±SE) pH and osmolality at the time of extension (time zero) and after storage in a semen-transport container at approximately 5°C for 24 and 48 hours.

		Treatment[1]									Repeated measures analysis of variance		
Variable[2] & Time[3]		CON	CEFT 250	CEFT 500	CEFT 1000	CEFT 2500	AMK PCN	GENT	TICAR	SE	Trt	Time	Trt*Time
pH	0	7.11	7.13	7.11	7.10	7.09	6.99*	7.09	7.02*	0.020	< [4]	< [4]	< [4]
	24	7.22†	7.21†	7.19†	7.19†	7.17†	7.14*†	7.30*†	7.11*†				
	48	7.26†	7.26†α	7.24†	7.23†	7.22†	7.19*†α	7.40*†α	7.17*†				
OSM	0	339.6	334.8	337.5	340.4	337.0	329.5	334.8	341.0	3.8	0.10	0.36	0.98
	24	338.5	339.4	341.3	340.1	340.8	334.7	338.4	345.9				
	48	335.2	340.1	341.1	339.3	334.3	330.4	341.5	341.6				

[1] CON: control, no antibiotic added; CEFT250: ceftiofur sodium at 250µg/ml; CEFT500: ceftiofur sodium at 500µg/ml; CEFT1000: ceftiofur sodium at 1,000µg/ml; CEFT2500: ceftiofur sodium at 2,500µg/ml; AMKPCN: a combination of amikacin sulfate and potassium penicillin G at 1,000µg/ml and 1,000µg/ml, respectively GENT: gentamicin sulfate at 1,000µg/ml; TICAR: ticarcillin disodium at 1,000µg/ml.

[2] OSM: osmolality (mOsm/kg)

[3] 0: time zero; 24: 24 hours of storage; 48: 48 hours of storage

[4] P value <0.0001

*Significantly different from control value within row and variable (P<0.0071).

†Significantly different from value at time zero within column and variable (P<0.025).

αSignificantly different from value at 24 hours of storage within column and variable (P<0.025).

Appendix D. Antibiotic control of bacterial growth in equine semen: a semi-quantitative comparison of isolates in the ejaculate with isolates present in extended semen after 24 and 48 hours of storage at approximately 5°C in a milk-glucose based semen extender with various antibiotics added.

Col. No.[1]	Storage Time and Specimen[2]	Isolate	Growth Level
1	Ejaculate	*Corynebacterium* sp.	Moderate
		Acinetobacter lwoffi	Rare
		Coagulase Negative *Staphylococcus* sp.	V. light
		Klebsiella pneumonia	1 colony
		Alpha *Streptococcus* sp.	1 colony
	24 hours storage – No antibiotic	*Corynebacterium* sp.	Moderate
		Acinetobacter lwoffi	1 colony
		Bacillus sp.	1 colony
		Corynebacterium sp. (different colony type)	Few
		Gram-negative bacteria (closest ID is *Roseomonas fauriae*)	1 colony
	48 hours storage – No antibiotic	*Corynebacterium* sp.	Moderate
		Corynebacterium sp. (different colony type)	1 colony
	48 hours storage – Ceftiofur sodium 1,000µg/ml	*Streptomyces* sp.	1 colony
	48 hours storage –Ceftiofur sodium 2,500µg/ml	*Corynebacterium* sp.	Rare
		Bacillus sp.	1 colony
		Streptomyces sp.	1 colony
2	Ejaculate	*Klebsiella pneumonia*	Few
		Corynebacterium sp.	Moderate
		Coag. Neg. *Staphylococococcus* sp.	Rare
		Acinetobacter lwoffi	Rare
		Corynebacterium sp. (different colony type)	Rare
		Bacillus sp.	Rare
	24 hours – No antibiotic	*Corynebacterium* sp.	Light
		Corynebacterium sp. (different colony type)	Few
		Bacillus sp.	1 colony
	24 hours storage – Gentamicin sulfate 1,000µg/ml	*Streptomyces* sp.	1 colony
	24 hours storage – Ticarcillin disodium 1,000µg/ml	Gram neg. rod – closest ID *Brevundimonas vesicularis*	Rare
		Corynebacterium sp.	Few
	48 hours storage – No antibiotic	*Corynebacterium* sp.	Moderate
		Acinetobacter lwoffi	Moderate
		Klebsiella pneumonia	Rare
3	Ejaculate	*Corynebacterium urealyticum*	Moderate
		Corynebacterium sp.	Few
		Coag. Neg. *Staphylococcus* sp.	Rare
		Enterococcus sp.	1 colony
	24 hours storage – No antibiotic	*Corynebacterium urealyticum*	Light
		Corynebacterium sp.	Light
		Coag. Neg. *Staphylococcus* sp.	1 colony
	24 hours storage – Ceftiofur sodium 500µg/ml	*Bacillus* sp.	1 colony
		Pseudomonas sp.	1 colony
	24 hours – Ceftiofur sodium 1,000µg/ml	*Streptomyces* sp.	1 colony

[1] – Collections 1-5 were Stallion A, collections 6-8 were stallion B, and collections 9-13 were stallion C

Appendix D (cont.)

Col. No.[1]	Storage Time and Specimen[2]	Isolate	Growth Level
4	Ejaculate	*Corynebacterium* sp.	Moderate
		Corynebacterium sp. (different colony type)	V. light
		Coag. Neg. *Staphylococcus* sp.	Few
		Acinetobacter lwoffi	Rare
		Strep. Group D non-entero.	Rare
	24 hours storage – No antibiotic	*Corynebacterium* sp.	Moderate
		Coag. Neg. *Staphylococcus* sp.	Rare
		Corynebacterium sp. (different colony type)	Few
	24 hours storage – Ceftiofur sodium 250µg/ml	*Bacillus* sp.	1 colony
	48 hours storage – No antibiotic	*Corynebacterium* sp.	Moderate
		Strep. Group D non-entero.	1 colony
		Coag. Neg. *Staphylococcus* sp.	Rare
		Corynebacterium sp. (different colony type)	1 colony
		Chryseomonas luteola (CDC VE1)	1 colony
5	Ejaculate	*Corynebacterium* sp.	Moderate
		Coag. Neg. *Staphylococcus* sp.	Rare
		Corynebacterium sp. (different colony type)	V. light
		Streptomyces sp.	1 colony
		Acinetobacter sp.	1 colony
	24 hours storage – No antibiotic	*Corynebacterium* sp.	Light
		Corynebacterium sp. (different colony type)	Rare
	24 hours storage – Ceftiofur sodium 250µg/ml	*Ralstonia eutropha*	1 colony
	48 hours storage – No antibiotic	*Corynebacterium* sp.	Moderate
		Corynebacterium sp. (different colony type)	Few
		Corynebacterium sp. (different colony type)	Rare
6	Ejaculate	*Corynebacterium* sp.	Moderate
		Corynebacterium sp. (different colony type)	Light
		Coag. Neg. *Staphylococcus* sp.	V. light
		Coagulase Positive Staph Group	Rare
	24 hours storage – No antibiotic	*Corynebacterium* sp.	Light
		Corynebacterium sp. (different colony type)	Light
		Corynebacterium sp. (different colony type)	V. light
		Coagulase Positive Staph Group	1 colony
	24 hours storage – Ceftiofur sodium 250µg/ml	*Corynebacterium* sp.	Rare
		Bacillus sp.	1 colony
	24 hours storage – Ceftiofur sodium 500µg/ml	*Corynebacterium* sp.	Rare
	24 hours storage – Ceftiofur sodium 1,000µg/ml	*Corynebacterium* sp.	Few
		Streptomyces sp.	Rare
		Pantoea agglomerans	1 colony
	24 hours storage – Gentamicin sulfate 1,000µg/ml	*Corynebacterium* sp. (different colony type)	1 colony
	24 hours storage – Ticarcillin disodium 1,000µg/ml	*Corynebacterium* sp.	Rare
		Corynebacterium sp. (different colony type)	Rare
		Streptomyces sp.	1 colony
	24 hours storage – Amikacin sulfate 1,000µg/ml and potassium penicillin G 1,000µg/ml	*Corynebacterium* sp. (different colony type)	1 colony
	48 hours storage – No antibiotic	*Corynebacterium* sp.	Light
		Corynebacterium sp. (different colony type)	Rare
		Streptomyces sp.	1 colony

[1] – Collections 1-5 were Stallion A, collections 6-8 were stallion B, and collections 9-13 were stallion C

Appendix D (cont.)

Col. No.[1]	Storage Time and Specimen[2]	Isolate	Growth Level
7	Ejaculate	Gram-positive rod – *Cellumonas turbata*	Moderate
		Corynebacterium sp.	Light
		Strep. Group D non-entero.	Few
		Coag. Neg. *Staphylococcus* sp. (assorted colony types)	V. light
		Acinetobacter radoresistans	1 colony
		Enterococcus sp.	1 colony
		Corynebacterium sp. (different colony type)	V. light
	24 hours storage – No antibiotic	*Corynebacterium* sp.	V. light
		Coag. Neg. *Staphylococcus* sp. (assorted colony types)	V. light
		Strep. Group D non-entero.	1 colony
		Corynebacterium sp. (different colony type)	Rare
		Acinetobacter radioresistans	1 colony
	24 hours storage – Ceftiofur sodium 250µg/ml	Strep. Group D non-entero.	1 colony
		Coag. Neg. *Staphylococcus* sp.	1 colony
		Corynebacterium sp.	1 colony
	24 hours storage – Ceftiofur sodium 500µg/ml	*Corynebacterium* sp.	Rare
		Bacillus sp.	1 colony
		Alpha Strep spp. (not *Enterococcus* sp.)	1 colony
	24 hours storage – Ceftiofur sodium 1,000µg/ml	Coag. Neg. *Staphylococcus* sp.	1 colony
		Strep. Group D non-entero.	1 colony
	24 hours storage – Ceftiofur sodium 2,500µg/ml	Coag. Neg. *Staphylococcus* sp.	1 colony
	24 hours storage – Ticarcillin disodium 1,000µg/ml	Coag. Neg. *Staphylococcus* sp.	Few
		Enterococcus sp.	1 colony
	48 hours storage – No antibiotic	*Corynebacterium* sp.	Light
		Corynebacterium sp. (different colony type)	Light
		Coag. Neg. *Staphylococcus* sp. (assorted colony types)	Light
		Strep. Group D non-entero.	Light
		Acinetobacter radioresistans	Rare
		Pseudomonas fluorescens	1 colony
	48 hours storage – Ceftiofur sodium 250µg/ml	Coag. Neg *Staphylococcus* sp. (assorted colony types)	Rare
		Enterococcus sp.	1 colony
	48 hours storage – Ceftiofur sodium 500µg/ml	*Actinomyces* sp.	Rare
		Corynebacterium sp.	1 colony
	48 hours storage – Ceftiofur sodium 1,000µg/ml	Coag. Neg. *Staphylococcus* sp.	1 colony
	48 hours storage – Ceftiofur sodium 2,500µg/ml	*Streptomyces* sp.	1 colony
	48 hours storage – Ticarcillin disodium 1,000µg/ml	Coag. Neg. *Staphylococcus* sp.	1 colony
		Corynebacterium sp.	Rare
		Enterococcus sp.	1 colony

[1] – Collections 1-5 were Stallion A, collections 6-8 were stallion B, and collections 9-13 were stallion C

Appendix D (cont.)

Col. No.[1]	Storage Time and Specimen[2]	Isolate	Growth Level
8	Ejaculate	Gram-positive rod (*Cellulosimicrobium cellulans*)	Moderate
		Coag. Neg. *Staphylococcus* sp.	Light
		Corynebacterium sp.	Rare
		Coag. Neg. *Staphylococcus* sp. (different colony type)	Rare
		Strep. Group D non-entero.	1 colony
	24 hours storage – No antibiotic	Gram-positive rod (*Cellulosimicrobium cellulans*)	Light
		Coag. Neg. *Staphylococcus* sp. (assorted colony types)	Light
		Corynebacterium sp.	Rare
		Strep. Group D non-entero.	Rare
		Acinetobacter sp.	1 colony
	24 hours storage – Cefiofur sodium 250µg/ml	Coag. Neg. *Staphylococcus* sp. (2 different colony types)	Rare
		Strep. Group D non-entero.	1 colony
		Streptomyces sp.	1 colony
		Corynebacterium sp.	1 colony
	24 hours storage – Ceftiofur sodium 500µg/ml	Coag. Neg. *Staphylococcus* sp. (2 different colony types)	Rare
	24 hours storage – Ticarcillin disodium 1,000µg/ml	Coag. Neg. *Staphylococcus* sp. (2 different colony types)	Rare
	24 hours storage – Amikacin sulfate 1,000µg/ml and potassium penicillin G 1,000µg/ml	Fungi sp.	1 colony
	48 hours storage – No antibiotic	Gram-positive rod (*Cellulosimicrobium cellulans*)	Light
		Enterococcus sp.	Light
		Coag. Neg. *Staphylococcus* sp. (assorted colony types)	Light
		Corynebacterium sp.	Rare
		Strep. Group D non-entero.	1 colony
		Acinetobacter sp.	1 colony
	48 hours storage – Ceftiofur sodium 250µg/ml	*Corynebacterium* sp.	1 colony
	48 hours storage – Ceftiofur sodium 500µg/ml	Coag. Neg. *Staphylococcus* sp.	1 colony
	48 hours storage – Ticarcillin disodium 1,000µg/ml	*Corynebacterium* sp.	1 colony
9	Ejaculate	*Corynebacterium* sp. (mixed morphologies present)	Moderate
		Pseudomonas alcaligenes	Moderate
	24 hours storage – No antibiotic	*Corynebacterium* sp. (mixed colony morphologies present)	V. light
		Pseudomonas alcaligenes	V. few
	24 hours storage – Ticarcillin disodium 1,000µg/ml	*Corynebacterium* sp. (mixed colony morphologies present)	V. few
	48 hours storage – No antibiotic	*Corynebacterium* sp. (mixed colony morphologies present)	Light
		Pseudomonas alcaligenes	Few
	48 hours storage – Ceftiofur sodium 250µg/ml	*Pseudomonas alcaligenes*	1 colony
	48 hours storage – Ticarcillin disodium 1,000µg/ml	*Corynebacterium* sp. (mixed colony morphologies present)	Few
		Actinomyces sp.	Few

[1] – Collections 1-5 were Stallion A, collections 6-8 were stallion B, and collections 9-13 were stallion C

Appendix D (cont.)

Col. No.[1]	Storage Time and Specimen[2]	Isolate	Growth Level
10	Ejaculate	*Corynebacterium* sp. (mixed colony morphologies present)	Moderate
		Pseudomonas alcaligenes	Moderate
	24 hours storage – No antibiotic	*Corynebacterium* sp. (mixed colony morphologies present)	Light
		Pseudomonas alcaligenes	Light
	24 hours storage – Ceftiofur sodium 250µg/ml	*Corynebacterium* sp. (mixed colony morphologies present)	Rare
		Pseudomonas alcaligenes	V. rare
	24 hours storage – Ceftiofur sodium 500µg/ml	*Pseudomonas alcaligenes*	V. rare
	24 hours storage – Ticarcillin disodium 1,000µg/ml	*Corynebacterium* sp. (mixed colony morphologies present)	V. light
		Bacillus sp.	V. light
	48 hours storage – No antibiotic	*Corynebacterium* sp. (mixed colony morphologies present)	Light
		Pseudomonas alcaligenes	Light
	48 hours storage – Ceftiofur sodium 250µg/ml	*Pseudomonas alcaligenes*	V. rare
		Corynebacterium sp. (mixed colony morphologies present)	Rare
	48 hours storage – Ceftiofur sodium 500µg/ml	*Pseudomonas alcaligenes*	V. rare
	48 hours storage – Ceftiofur sodium 1,000µg/ml	*Pseudomonas alcaligenes*	Rare
		Corynebacterium sp.	1 colony
	48 hours storage – Ceftiofur sodium 2,500µg/ml	*Corynebacterium* sp. (mixed colony morphologies present)	V. light
11	Ejaculate	*Corynebacterium* sp. (mixed colony morphologies present)	Moderate
		Pseudomonas alcaligenes	Few
		Non-hemolytic *Staphylococcus* sp.	1 colony
	24 hours storage – No antibiotic	*Corynebacterium* sp. (mixed colony morphologies present)	Few
	24 hours storage – Ceftiofur sodium 250µg/ml	*Corynebacterium* sp. (mixed colony morphologies present)	Rare
	48 hours storage – No antibiotic	*Corynebacterium* sp. (mixed colony morphologies present)	V. few
	48 hours storage – Ceftiofur sodium 250µg/ml	*Corynebacterium* sp.	2 colonies
12	Ejaculate	*Corynebacterium* sp. (mixed colony morphologies present)	Light
		Pseudomonas alcaligenes	Few
	24 hours storage – No antibiotic	*Corynebacterium* sp. (multiple colony morphologies present)	V. light
		Pseudomonas alcaligenes	V. rare
	24 hours storage – Ceftiofur sodium 250µg/ml	*Corynebacterium* sp.	1 colony
	24 hours storage – Ticarcillin disodium 1,000µg/ml	*Corynebacterium* sp. (multiple colony morphologies present)	Rare
	48 hours storage – No antibiotic	Coag. Neg. *Staphylococcus* sp.	1 colony
		Corynebacterium sp. (multiple colony morphologies present)	Few
		Bacillus sp.	1 colony
	48 hours storage – Ceftiofur sodium 250µg/ml	*Pseudomonadaceae*	1 colony
		Corynebacterium sp.	1 colony
	48 hours storage – Ceftiofur sodium 500µg/ml	*Corynebacterium* sp.	1 colony
	48 hours storage – Ticarcillin disodium 1,000µg/ml	*Corynebacterium* sp. (multiple colony morphologies present)	V. few

[1] – Collections 1-5 were Stallion A, collections 6-8 were stallion B, and collections 9-13 were stallion C

Appendix D (cont.)

Col. No.[1]	Storage Time and Specimen[2]	Isolate	Growth Level
13	Ejaculate	*Corynebacterium* sp. (mixed colony morphologies present)	V. light
		Coag. Neg. *Staphylococcus* sp.	Rare
	24 hours storage – No antibiotic	*Corynebacterium* sp. (multiple colony morphologies present)	V. light
		Coag. Neg. *Staphylococcus* sp.	1 colony
		Strep. Group D non-entero.	1 colony
	48 hours storage – No antibiotic	*Corynebacterium* sp. (multiple colony morphologies present)	V. light
		Non-hemolytic *Staphylococcus* sp.	Few
	48 hours storage – Ceftiofur sodium 250µg/ml	*Corynebacterium* sp.	2 colony
	48 hours storage – Ceftiofur sodium 500µg/ml	*Corynebacterium* sp.	1 colonies
	48 hours storage – Gentamicin sulfate 1,000µg/ml	Coag. Neg. *Staphylococcus* sp.	1 colony
		Corynebacterium sp. (multiple colony morphologies present)	V. few
	48 hours storage – Ticarcillin disodium 1,000µg/ml	*Pantoea agglomerans*	V. rare
		Corynebacterium sp. (multiple colony morphologies present)	Few

[1] – Collections 1-5 were Stallion A, collections 6-8 were stallion B, and collections 9-13 were stallion C