A Comparison of Equine Spermatozoa Viability Using the TrueBreed+, TrueBreed, and Standard Semen Collection Devices

by

Melissa Ann Sillivent, M.S., B.S.

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Approved

Samuel Prien, Ph.D.
Chair of the Committee

Bradley Johnson, Ph.D.

Sam Jackson, Ph.D.

Leslie Thompson, Ph.D.

Lindsey Penrose, Ph.D.

Jerry Black, DVM

Mark Sheridan, Ph.D.
Dean of the Graduate School

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ABSTRACT

Previous research has demonstrated that the Device for Improved Semen Collection (DISC- marketed under TrueBreed™) helped maintain semen parameters for extended periods in a human and equine model compared to semen collected in a traditional collection vessel. By design, the DISC (1) slows the cooling rate, (2) limits exposed surface areas, (3) provides nutrients, and (4) lessens or eliminates osmotic shock during processing, theoretically protecting the cells from collection induced damage. The system was recently redesigned to include fixed scavengers to harvest reactive oxygen species (ROS). The objective of this study was to determine if this physiological redesign of the DISC protects sperm cell function in the equine model, using a series of biochemical assays to assess cellular function.

Three semen samples were obtained from each of 10 stallions as part of a routine collection program. Each stallion was randomly collected one time in a standard container, the original equine TrueBreed, and the TrueBreed+ modified for antioxidant properties, using standard equine extension techniques. Once prepared, the samples were incubated at room temperature in the collection device, an evaluated holding temperature (20-23°C) to induce ROS. Sample aliquots were analyzed, and slides were prepared at 6, 9, 12, 24, 48, 72, and 96 hrs. Samples were assessed for standard motility parameters using a CASA instrument, and slides were prepared to assess morphology, acrosomes, mitochondrial function, and DNA fragmentation.

The slides were prepared using standard commercial assays and a Biotek Cytation 5 with appropriate fluorescent filtration. Data were analyzed by ANOVA with repeated measures. While the study was designed to increase ROS generation, results indicate that

stallion sperm cells collected in either the TrueBreed or TrueBreed+ maintained significantly better quality across all variables at all time points past six hours compared to the control ($P < 0.05$). This data suggests that more biochemically intact, physiologically active cells are found in samples collected and held in either the TrueBreed or TrueBreed+ devices. The results of this study continue to support previous studies supporting the role of the TrueBreed and TrueBreed+ systems in maintaining healthy sperm biochemistry and physiology. The present data demonstrate that the addition of ROS scavengers to the plastic matrix effectively protects sperm from DNA damage without adding anti-oxidant compounds to the media.

Furthermore, the study suggests the TrueBreed+ system effectively protects stallion sperm cells from damage to the mitochondria and acrosome membranes at the time of collection. Future experimentation is necessary to determine the optimal scavenger to prevent cell damage. Lessening sperm damage in the sperm collection process provides the potential for improved sperm quality and potentially higher equine pregnancy rates.

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CHAPTER I

INTRODUCTION

According to the Equine Business Association, the equine industry has a tremendous impact on the global economy, contributing over $300 billion annually and supplying more than 1.6 million full-time jobs (Alltech, 2021). This industry is primarily driven by equine competition in the horse racing, showing, and performance horse divisions. In this competitive market, owners are awarded prize money and prestige for superior performance. This has led horse owners to desire enhanced genetics continuously. These sought-after genetics can be attained through assisted reproductive technologies, such as artificial insemination. Artificial insemination of mares allows horse breeders access to various genes from numerous geographic locations, allowing them to breed to superior stallions to create offspring with desired traits. This is one of the many reasons artificial inseminations have become a standard practice in the equine industry (McKinnon & Voss, 1992).

The practice of collecting stallion semen and then inseminating mares with it is thought to have begun in the 1300s. According to legend, Arabian tribes are reported to have stolen semen from rival tribes' stallions to inseminate their mares, essentially trying to improve their own herd genetics (Bearden et al., 2004). However, the first scientific documentation of artificial insemination (AI) did not occur until the 1780s, when Spallazani, an Italian physiologist, would inseminate a female dog with a stud dog's semen, resulting in the birth of three puppies (Bearden et al., 2004). From this time forward, the collection of semen and artificial insemination practices have continued to improve. One early observation was that once sperm cells were excreted from the male's

body, they would only remain viable for a short duration of time (Palmer, 1984). This posed a problem: collected semen would have to be used almost immediately, or they were rendered useless. Therefore, females had to be receptive and present at the collection location. This led to research into methods for prolonging the life of sperm cells. (Hamilton & Waites, 1990)

Studies of sperm cell physiology found that these cells were highly susceptible to many environmental factors that would decrease their viability. These factors included temperature shifts, exposed surface area, light, pH shifts, osmatic pressures, and free radicals in the media (Prien & Johnson, 2006). To minimize these issues, semen extenders were invented as an additive for collected semen. They typically either were milk or egg yolk-based. These extenders prolong the life of sperm cells by providing the cells protection against pH shifts by adding buffers, energy sources in the form of glucose and fructose, proteins, antibodies, and various other nutrients designed to prolong their life (England, 2005).

Along with the development of extenders, better devices for the actual semen collection process were also invented. These devices included the artificial vagina (AV) and were meant to enhance the stimulation of the male to produce a superior sample. The AV was first used in 1914 by Giuseppe Amantea for collecting dogs. Since then, a variety of AV models have been developed for several species, including the stallion (Taylor & Field, 2001). Missouri and Colorado are the most common two AVs used for stallion semen collection today. Both models simulate a mare's vaginal vault with the same temperature, pressure, and feel present in natural copulation. A collection vessel (bottle) is attached at the distal end of the AV, which will collect the semen after

ejaculation (McKinnon & Voss, 1992). The industry standard for this device is typically a baby bottle with a liner and a gel filter inside. This method leaves the excreted semen in the bottle exposed to the environment and unprotected at the time of collection (Johnson & Prien, 2003). Recognizing issues these exposures might cause semen collection, this lab developed a new collection device to be placed at the end of the AV. This device is called TrueBreed. The equine TrueBreed was adapted to be the same size and shape as a baby bottle to minimize disruption in the stallion collection process with existing AVs. The TrueBreed provided an optimal environment for sperm cells during semen collection by reducing light exposure, minimizing surface area, regulating temperature, providing buffering agents to reduce shifts in pH, and providing nutrients. These qualities increased the viability and longevity of collected stallion semen (Langdon, 2007).

Previous studies demonstrated dramatic improvement in the longevity of semen samples, resulting in higher pregnancy rates. Data suggested this results from better maintenance of cell biochemistry, including membrane structure and mitochondrial function. However, it is unclear if the current TrueBreed design offered protection from reactive oxygen species (ROS), known to build up the longer a sample stays in storage. ROS buildup has been associated with several detrimental effects, including damage to the cell's DNA. Recognizing this limitation, TrueBreed was redesigned with plastics, specially designed to scavenge ROS from the media environment. In theory, this redesign, referred to as TrueBreed+, will provide sperm cells with all the protection of the original TrueBreed and add antioxidants in the lining of the device, reduce free radical exposure to the sperm cells and minimize DNA fragmentation, thus increasing the

likelihood of a viable conception.

There is currently a significant concern in the horse industry that DNA damage in sperm may lead to decreased pregnancies or loss. If the TrueBreed+ limits ROS build-up at the time of collection, it may prevent DNA fragmentation and improve intrauterine fertilization outcomes. The current study represents the first trial of the new device and its effectiveness in maintaining ROS at physiologically acceptable levels, allowing for increased cell longevity and decreased cell damage.

CHAPTER II

LITERATURE REVIEW

Spermatogenesis

Male fertility requires the production of mature gametes, otherwise known as sperm cells, in large numbers by the testis. This complex process is known as spermatogenesis. It can be broken down into three major steps, (1) the multiplication of spermatogonia by the process of mitosis, (2) meiosis, the reduction of chromosome number from diploid to haploid, and (3) commences during the prophase of the first meiotic division. As single spermatogonia will lead to the production of 64 spermatozoa cells (De Kretser et al., 1998).

Spermatogonia develop into spermatozoa in the seminiferous tubules of the testis. In the interstitial space, Leydig cells produce testosterone and Sertoli cells, which lie within the seminiferous tubules that aid in spermatogenesis. Sertoli cells, also called *nurse cells*, secrete fluids containing proteins necessary for the germ cells to grow and mature into spermatozoa (Bardin et al., 1988). Sertoli cells produce lactate which will supply energy to the spermatozoa cells. After spermatozoa are released from the seminiferous tubules, lactate is converted to pyruvate by spermatid mitochondria (Grootegoed & Boer, 1987).

Spermatozoa are released from seminiferous tubules into the rete testes and then pushed to the epididymis. Throughout this transport, they are surrounded by fluid that contains proteins, energy substrates, such as glucose, lactate, and a variety of other substances (Grootegoed & Boer, 1987). Upon leaving the testis, the spermatozoa will enter the epididymis. When the sperm cells reach the epididymis, they are still immature

and immotile. In the cauda or tail of the epididymis, the sperm are concentrated and stored in a highly favorable environment. The epididymal cells secrete low pH fluid and have a high potassium-to-sodium ratio (Bardin et al., 1988). These conditions allow the sperm to be stored and remain viable for an extended time. While in the cauda epididymis, spermatozoa cells gain the ability to be motile and fully mature until the final stage of capacitation occurs in the female tract.

During ejaculation, the sperm cells in the cauda epididymis are pushed into the vas deferens, urethra, then out of the body. Along this pathway, the sperm cells are mixed with fluid from the accessory sex glands, called seminal plasma (Bardin et al., 1988). The accessory sex glands include the seminal vesicles, prostate, and bulbourethral glands (Cooper, 1979; Setchell, 1977; Thompson et al., 1980). The secretions of these glands contain buffers, nutrients, and a variety of other organic and inorganic substances. These secretions aid the sperm cells for survival in the female vaginal vault (Little & Woods, 1987). The vaginal of the female tract, where semen will be deposited in most species, has a very high pH. Buffers such as phosphates and carbonate buffers are necessary to protect against the female tract's pH shifts. Other pH shifts, such as lactic acid, can result from sperm cell metabolic waste. The organic and inorganic ions such as sodium ($Na2^{+}$), potassium (K^{+}), and calcium ($Ca2^{+}$) are necessary to initiate sperm motility and fertilizing capability. Other nutrients, such as fructose and sorbitol, are utilized by the sperm to meet energy requirements (Johnson & Prien, 2000).

Freshly ejaculated stallion spermatozoa, like spermatozoa from all mammals, cannot fertilize oocytes without undergoing further modification. One major modification involves changes in the spermatozoa plasma membrane, which facilitates the fusion of

the plasma membrane with the outer acrosomal membrane during the acrosome reaction (McKinnon & Voss, 1992). Spermatozoa motion is modified, and spermatozoa change from moving progressively forward to a non-progressive motion in which the spermatozoa exhibit whiplike tail motions causing the head of the sperm cell to move in a star-like or figure-eight pattern; this is referred to as hyperactivated motility or hyperactivation. The process that induces these modifications is called capacitation(McKinnon & Voss, 1992). Spermatozoa undergoing aging or trauma after ejaculation will cause spontaneous changes to spermatozoa plasma membrane and metabolism that render the spermatozoa infertile; these are similar to what is seen during capacitation (McKinnon & Voss, 1992). Some of these processes are enhanced in the female reproductive tract.

The head of the spermatozoa includes the nucleus with its nuclear envelope, the acrosome, post chromosomal lamina, and plasma membrane (see Figure 2.1). Each of these steps represents a key element in the spermatogenic process. Defects in any of these stages can result in the failure of the entire process and lead to the production of defective spermatozoa and the reduction or absence of functional sperm production.

Sperm Cell Structure

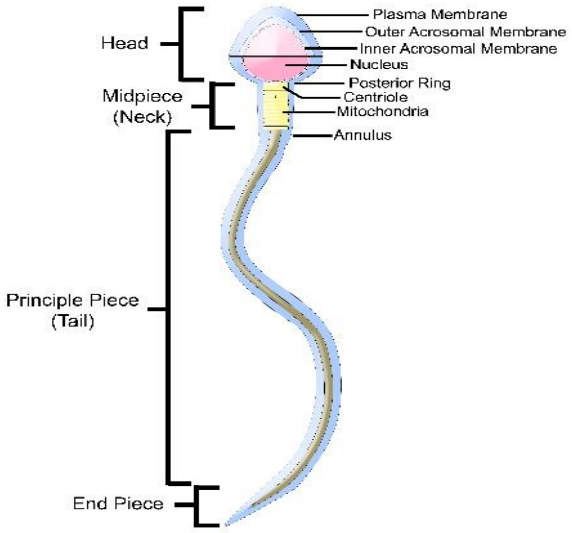

Figure 2.1

Sperm Cell Structure

The shape of the head is determined primarily by the shape of its nucleus. The head and the nucleus are broad and relatively flat for stallion spermatozoa. The nucleus contains the DNA.

The nucleus is enclosed by a double-layer nuclear envelope that contains few pores. The rostral portion of the nucleus is overlain by the acrosome, a specialized vesicle formed from the double-layered membrane; the acrosome contains glycolipids and enzymes. Hyaluronidase, proacrosin/acrosin, and lipases are the primary enzymes. In the stallion spermatozoa, the acrosome is slightly thickened but sharply tapered at the rostral end (McKinnon & Voss, 1992).

After capacitation, which takes place in the female and is the final step in sperm cell maturation, the sperm cell is ready to fertilize an oocyte. Once the sperm cell binds to the zona pellucida surrounding the egg's membrane, pores are formed between the

acrosome membrane and the plasma membrane allowing the acrosome components to be released. Among the acrosome components released are enzymes, such as protease, that degrade the zona pellucida surrounding the oocyte's plasma membrane.

Degradation of the zona pellucida makes it possible for the head of the spermatozoa to pass through and fuse with the vitelline membrane of the oocyte. This fusion prevents other spermatozoa from penetrating the zona pellucida for fertilization. Once the egg is fertilized, no other sperm cells will be able to enter the oocyte regardless of acrosome status (McKinnon & Voss, 1992).

The midpiece extends from the caudal end of the neck, distal through the annulus. It is characterized by numerous mitochondria arranged circumferentially, end to end, in a continuous double spiral (McKinnon & Voss, 1992). Mitochondria are necessary for energy conversion to ATP and contain enzymes and cofactors for producing adenosine triphosphate (ATP) via cellular respiration. The majority of ATP produced by the mitochondria is used for cell motility. As ATP is converted, this causes contractile activity of the tail motion (England, 2005).

The sperm tail is a thin, elongated structure that makes up about 80% of the entire length of the sperm (De Jonge & Barratt, 2017). While the tail may appear to be one long continuous structure, it is divided into several parts, including the connecting piece, which connects the flagellum to the sperm head. Some sources consider the midpiece as part of the tail: the principal piece or axial filament and the end piece. The principal piece and the end piece of the flagella help generate the waveform, which via contraction and

relaxation of actin and myosin, allows for movement (Durairajanayagam, 2015;

Yanagimachi, 2011).

Sperm Cell Metabolism

It is important to understand sperm cell metabolism to improve the efficiency of equine-assisted reproductive technology. When using techniques such as artificial insemination, it is critical to understand how the metabolism of sperm cells operates to retain the cell's functionality during collection, storage, and insemination. The metabolic pathways sperm cells use to sustain energy can differ between mammals (Swegen et al., 2016).

Glycolysis

In a purely biological sense, glycolysis is a metabolic pathway that utilizes enzymes to break down six-carbon sugars, such as glucose, to form pyruvate, ATP, and NADH/H+. In spermatozoa, the enzymes needed to carry out glycolysis are associated with the fiber sheath of the principal piece of the tail.

The role of glycolysis in driving ATP production for sperm motility has been well researched due to its relative importance in human and laboratory species. Large polar molecules such as glucose cannot diffuse across membranes, so their transport is facilitated by membrane-bound proteins called glucose transporters (GLUTs). Glucose transporters are categorized according to their relative ability to transport hexoses (glucose, mannitol, and fructose), amino sugars, or vitamins (Evans et al., 1990). Since the discovery of the glucose transporter GLUT1, many additional GLUTs have been characterized in spermatozoa, including spermatozoa of the stallion (Pickett et al., 1976). Glucose transporters are localized to the tail and acrosome, suggesting that glycolytic processes are involved in generating energy for the membrane modifications required for

capacitation and the acrosome reaction. In glycolytic spermatozoa, the distribution of GLUTs changes along with the capacitation status of the cell (between non-capacitated and capacitated states) to provide energy at the sites requiring membrane modifications or hyperactivation of motility (Brisko et al., 2010). In contrast, the distribution of GLUTs on non-glycolytic spermatozoa, such as stallion spermatozoa, does not change with the capacitation status of the cell, indicating glycolysis is not required to support ATP production for motility, capacitation, or the acrosome reaction in these species.

Oxidative Phosphorylation

Oxidative phosphorylation (OXPHOS), in contrast to glycolysis, occurs in the mitochondria located in the midpiece of the sperm cell. Oxidative phosphorylation OXPHOS is a significantly more efficient method for ATP production. It has the advantage of rapidly producing high levels of ATP and facilitating higher sperm motility, than glycolysis. However, due to rapidly producing high ATP levels, reactive oxygen species (ROS) are also rapidly produced as a by-product. Thus, mitochondria are the main site of ROS production. High levels of ROS, can be viewed as a sign of healthy spermatozoa in the sense that the sperm cell metabolism is highly functioning and positively correlated with stallion fertility. However, spermatozoa continually exposed to high levels of ROS exhaust their antioxidant reserves and ultimately deteriorate as they engage the intrinsic apoptosis pathway to cell death (Gibb & Aitken, 2016).

Stallion Sperm Metabolism

Glycolysis and OXPHOS are the two ways in which stallions' spermatozoa produce ATP. These cells can switch glucose utilization mechanisms during times of cellular stress when the energy demand is high, but oxygen availability is low. The

necessary ability of stallion spermatozoa to switch between different substrates to generate energy allows them to supply their high demand for ATP even in high-stress environments such as low oxygen availability. Stallion spermatozoa are often stored in low-oxygen and high-glucose environments in vitro (Swegen et al., 2016).

Despite the well-characterized presence of GLUTs on equine sperm, it has become very evident that stallion spermatozoa differ from that of other well-studied mammalian species, in that their energy demands are met not by glycolytic pathways but by using OXPHOS (Agarwal, et al., 2020; Plaza Davila et al., 2015) and in the presence of mitochondrial inhibitors, they suffer a rapid loss of velocity and a dramatic decline in ATP content. This dependence results in a nonconventional relationship between ROS production and fertility in the stallion (Myers, 2009; Plaza Davila et al., 2015), with the source of ROS being the mitochondrial electron transport chain, in which about 1–3% of O_2 reduced in the mitochondria during OXPHOS forms superoxide (Hernández-Avíles et al., 2020).

There is a long-standing theory that it is the nonviable or poor-quality spermatozoa that generate the most ROS, but in reality, rapidly metabolizing spermatozoa from highly fertile stallions exhibit higher levels of OXPHOS activity, following *in vitro* storage before artificial insemination (AI) present with elevated levels of ROS generation and lipid peroxidation. Thus, while human clinical data steadily report negative correlations between male fertility and sperm oxidative stress (Carter et al., 1995), a recent study has revealed a contradictory inverse relationship between fertility and the percentage of live cells *without* oxidative damage in the stallion (Plaza Davila et al., 2015). In addition, more fertile ejaculates (those which resulted in a pregnancy

following insemination) had lower vitality and a higher percentage of cells displaying ROS-induced damage following *in vitro* storage than ejaculates that did not result in a pregnancy (Plaza Davila et al., 2015).

From these results, it was hypothesized that during *in vitro* storage, spermatozoa from the more fertile stallions (assumed to be more metabolically active) were becoming exhausted more rapidly, such that, by the time that the assays were performed in the laboratory, these cells had suffered an accelerated demise due to the accumulation of metabolic by-products, such as ROS and cytotoxic lipid aldehydes in a "live fast, die young" paradigm. Another interesting observation was that the greater efficiency of OXPHOS mediated ATP production by equine spermatozoa supported a higher velocity, with stallion spermatozoa being around 60% faster than human spermatozoa. Ultimately, high ROS production by stallion spermatozoa appears to be a physiologically normal scenario brought about by superoxide leakage from the mitochondrial electron transport chain during OXPHOS (Agarwal et al., 2020) with a positive relationship between mitochondrial ROS production and sperm velocity, leading to increased rates of lipid peroxidation (Plaza Davila et al., 2015) and, following prolonged storage, a loss of motility and vitality (Dart et al., 1994). This phenomenon introduces a number of implications for the *in vitro* storage of stallion spermatozoa, since the prolonged generation of ROS in the absence of extracellular free radical and lipid aldehyde scavengers will lead to irreversible oxidative damage impairing DNA integrity and sperm functionality (Gibb & Aitken, 2016).

Collection of Stallion Semen

Artificial Vagina

Due to having a Musculo vascular penis type, the stallion does not respond favorably to electroejaculation. Therefore, an artificial vagina (AV) is essential to collect high-quality semen. Several models of AVs and their modifications have been described for collecting stallion semen (McKinnon & Voss, 1992). Two of the most used AV types are the Colorado and Missouri AV. Each has distinct attributes and peculiarities, so AV selection is based on specific requirements and stallion preference, but all AV types rely on the same basic principles. Most AVs have a closed collection system, although some are open-ended, and semen must be caught. There is a rigid outer tube and a soft inner liner (this may be plastic or latex); the space between these is filled with warm water (England, 2005). The Missouri model AV comprises a double-walled rubber lining containing a permanently sealed water chamber and a leather carrying case. The Colorado model AV consists of two independent rubber liners and a heavy plastic case covered by a leather collar (Brisko et al., 2010). The Japanese model AV, uncommon in the USA, comprises a small, rigid aluminum case and a single rubber liner, so it is lightweight and easy to maneuver.

Condom

A condom is a poor alternative to an AV for semen collection but may be the only viable option if the stallion will not breed an AV or if an AV is unavailable. The quality of semen collected in a condom is inferior to that obtained with an AV because of the marked contamination of the sample with bacteria and debris from the exterior of the penis (Blanchard et al., 2003). Stallions most reluctant to breed an AV are those that have

never bred before and those accustomed to breeding mares by natural service. All potential collection materials need to be tested for biocompatibility.

Live

For standard semen collection protocols with the stallion mounted on a mare, either gonad intact or ovariectomized mares. If a mare is intact, she should be in estrus and readily allow the stallion to mount (McKinnon & Voss, 1992). The mare should be physically restrained before the stallion is allowed to mount. Leg hobbles and a twitch on the muzzle can be applied to the mare (Blanchard et al., 2003). Mount mares should have their rump and vaginal area washed to prevent the spread of microorganisms (Evans et al., 1990).

Phantom

Stallions with good libido can be easily trained to mount a phantom, particularly during the physiological breeding season. A phantom has a barrel shape and a sturdy stand centrally placed upright, where the height can be adjusted. It is padded to be nonabrasive and should be installed in an area that is free of obstructions and has enough space around it for a mare to stand abjectly if need be (England, 2005). The collection room floor should be comprised of a sturdy non-slick surface. There are several advantages to having stallions trained to a phantom. Phantom usage greatly decreases the likelihood of stallion injury during semen collection and mare injury resulting from the vicious biting of some stallions. The size, shape, and composition of breeding phantoms are quite diverse, ranging from padded hot water tanks to sophisticated structures with mounted AVs and hydraulic controls for adjusting the height. A stallion can be trained by placing a mare alongside the phantom (Brisko et al., 2010). The AV holder should stand

on the same side of the mount as the stallion handler and allow the stallion to mount. Immediately, the erect penis should be deflected to the side of the mount and introduced to the AV; this may be difficult because of the stallion's thrusting (England, 2005). The AV can be pushed along the side of the mount to help the collector support and steady the AV during the collection process.

Manual Stimulation

To train the stallion for ground collection, the stallion is teased to erection, and the penis is washed and dried. (Brisko et al., 2010). Some stallions trained to ejaculate using an AV can also be collected via manual stimulation while standing on the ground. The AV is pushed towards the base of the stallion's penis to encourage thrusting. Stallions usually ejaculate after 5 to 10 thrusts. If the stallion does not ejaculate after the first attempt, the procedure is repeated until successful ejaculation is achieved (Genetti, 2013).

Chemical Ejaculation

Semen may be collected from stallions with severe physical disabilities by a procedure known as pharmacology-induced ex copula ejaculation or, more commonly, chemical ejaculation. The protocol involves oral administration of imipramine followed two hours later by intravenous administration of xylazine. Passive emission of semen usually occurs one to three minutes after administration of xylazine or, less commonly, 15–25 minutes after xylazine administration as the sedation effect is wearing off (Brisko et al., 2010). A thin plastic mitt hangs over the stallion's penis and is used to collect the semen as he stands on the ground.

Semen Collection Devices

A Missouri or Colorado AV can be used to collect a stallion. On either of these devices, a pooling device (bottle) must be attached to the end of the AV, where the semen will be collected once the stallion's ejaculation is complete. In general, a bottle with a similar shape to that of a baby bottle is commonly used due to the large ejaculate volume from stallions. Often a blue cone is placed over this bottle to help insulate the collection bottle helping to reduce thermal shock. Within this bottle, there is a liner made of polypropylene plastic and within this liner is a filter to collect the gel fraction of the ejaculate. The gel filter is typically made of nylon, and most of the semen will easily pass through this filter while it catches the gel fraction. (McKinnon & Voss, 1992). It is important to remove this gel fraction, so it does not contaminate the semen sample. The filter will be discarded along with the gel (Genetti, 2013). All materials with direct contact with the seman should be tested for biocompatibility before use and collection procedures.

Methods of Evaluating Semen

Gross Evaluation

The gel-free semen is transferred into a warm graduated cylinder and initially evaluated for color, consistency, and volume. The normal ejaculate is pale white, similar to skimmed milk in appearance. Normally, the sample is free from contamination with blood or urine, which will cause discoloration. Clarity or consistency of the sample can often be used as an initial assessment of sperm concentration. The volume of the gel-free sample should also be measured. Ejaculate volume is normally 60-70 ml, but this may vary between 30-300 ml depending on the stallion's size and the year's season. The

volume of the gel fraction may also vary (England, 2005). However, it is seldom important in determining fertility; it is used in calculating the total sperm number in the ejaculate (Brisko et al., 2010).

Concentration

An accurate determination of sperm concentration is a critical parameter in calculating the total number of sperm cells in the ejaculate (Brisko et al., 2010). The concentration of spermatozoa per ml in the ejaculate may be manually counted using a hemocytometer, Makler counting chamber, or. Another alternative is using an electronic device known as a computer-assisted semen analysis (CASA Machine, IVOS II). The total sperm number in ejaculates obtained from mature stallions typically ranges from four to $12x10^9$ but may exceed 15 to $20x10^9$ in sexually rested stallions. Total sperm output per ejaculate is subject to seasonal variations but also is affected by numerous other factors that include age, testicular size, spermatogenic efficiency, size of extragonadal spermatozoa reserves, and various forms of the reproductive disease (Brisko et al., 2012).

Motility

It is important to assess spermatozoa motility. This should include total spermatozoa motility (percentage of spermatozoa exhibiting motility of any form), progressive spermatozoa motility (percentage of spermatozoa that exhibit rapid, linear movement), and spermatozoa velocity (manually on an arbitrary scale of 0, immotile, to 4, rapidly motile). The normal percentage of motile sperm is 40 to 75% and is estimated to the nearest five percent (Pickett et al., 1976). To standardize the spermatozoa motility for testing protocol, all semen samples should be diluted with an extender before

analysis. It is preferred to dilute sperm to a standard concentration of $25x10^6$ sperm/ml for manual motility assessment (Blanchard et al., 2003).

Morphology

At least 100 cells should be evaluated for evidence of morphological defects. The most common deficiencies observed in stallion spermatozoa are the abnormal head, detached head, abnormal/broken neck, abnormal midpiece, proximal droplet, distal droplet, coiled tail, and kinked tail. Morphology is the percentage of spermatozoa conforming to the normal shape for stallion semen. Studies have reported that fertility in stallions is positively correlated with the percentage of morphologically normal sperm and inversely correlated with the percentage of sperm with abnormal heads, proximal droplets, and abnormal midpieces (Varner, 2009). The percentage of abnormal sperm in an ejaculate can range from 10% to 40%. A standard bright field microscope can be used to examine air-dried semen smears with appropriate stains. Specific stains for sperm morphology include eosin and 19 nigrosine.

Seminal pH

The pH of gel-free semen should be determined using a properly calibrated pH meter, preferably within one hour after semen collection. Measurements obtained using pH paper are less precise than those derived with a meter, so this method of pH determination should be used only as a last resort (Brisko et al., 2010). The normal pH of equine semen ranges from 7.2 to 7.7. The pH of normal semen can be affected by season, frequency of ejaculation, sperm concentration, and time between collection frequency. Inflammation of the reproductive tract or contamination of the ejaculate with soap or urine can lead to an abnormally high pH (Varner, 2009).

Mitochondria

It can be argued that mitochondria are the most important organelle in sperm cells, after the nucleus, as they perform several critical functions. Mitochondrial DNA (mtDNA) copy number has been utilized to measure sperm quality in several species, including mice, dogs, and humans. It has been suggested as a potential biomarker of fertility in stallion sperm. The mitochondria of the spermatozoa control numerous functions and are considered to be hallmarks of sperm functionality (Amaral et al., 2013). In addition to their role as an ATP source via OXPHOS, other functions regulating the lifespan of spermatozoa have attracted major research attention to these organelles (Pena et al., 2009). Important cellular functions in the spermatozoa are redox-regulated; the production of ROS is an early event during the series of the modifications that occur during capacitation. However, alteration in the redox homeostasis of the cell leads to sperm senescence and, finally, death; in humans, it has been reported that the mitochondria of defective sperm are the major source of ROS originating from electron leakage in the electron transport chain (ETC) (Koppers et al., 2009). This has also been assumed to be true for horses, as reviewed in, and recent data supports this hypothesis (Plaza Davila et al., 2015).

Acrosome

The acrosome is derived from the Golgi complex of the spermatid. The acrosome is between the plasma membrane in the anterior head region and the nuclear envelope. It has its own set of membranes: the inner acrosomal membrane bordering the nuclear envelope and the outer acrosomal membrane, which is overlaid by the plasma membrane and fuses with that structure during acrosomal exocytosis, or the acrosome reaction. The

acrosome consists of a protein matrix core and contains numerous hydrolytic and glycolytic enzymes, which are important for fertilization. Although not described for stallion sperm specifically, the most well-known acrosomal enzymes in other species are proacrosin-acrosin, hyaluronidase, β-galactosidase, various proteinases, neuraminidases, esterases, arylsulfatase, and phospholipases A and C, as well as numerous phosphatases and regulatory enzymes and proteins. It is likely that stallion sperm have many of these enzyme systems (Meyers, 2009).

When the acrosome is absent, the sperm usually has a spherical shape, appearing round on the stained smear. A rare clinical condition called globozoospermia is present when most of the sperm are round without acrosomes. These sperm cannot penetrate the ovum but can be fertile if injected into the ovum using ICSI. Several gene mutations have been identified in globozoospermia especially deletions of DPY19L2 on chromosome 12, which plays an active role in acrosome formation and sperm elongation (Coutton et al., 2015; Elinati et al., 2012; Koscinski et al., 2011; Zhu et al., 2015).

In recent years, assessing acrosome intactness and function has become necessary when other sperm quality tests, such as sperm motility, morphology, or DNA integrity, cannot explain the causes of reduced fertility. The use of certain stains for assessment of acrosome intactness under field conditions has been reported; however, in the authors' opinion, their capacity to detect true acrosome reaction or acrosomal damage is limited, particularly when cooled or frozen semen is analyzed. The use of fluorescent stains for acrosomal assessment is superior to conventional light microscopy, but this requires access to expensive equipment such as fluorescent microscopes or flow cytometers. Using fluorescent techniques, several combinations of dyes have been proposed to

simultaneously evaluate the percentage of sperm with intact plasma and acrosome membranes (Hernández-Avíles et al., 2020).

DNA

Sperm DNA integrity is crucial for normal fertilization and essential to healthy offspring's future development. The spermatozoon undergoes extensive molecular remodeling of its nucleus during later phases of spermatogenesis, imparting compaction and protecting the genetic content. Testicular and post-testicular mechanisms are involved in the etiology of sperm DNA fragmentation (SDF), which affects both natural and assisted reproduction. Several clinical and environmental factors are known to impact sperm DNA integrity negatively. An increasing number of reports emphasize the direct relationship between sperm DNA damage and male infertility. Currently, several assays are available to assess sperm DNA damage; however, professional organizations do not recommend routine SDF assessment in clinical practice. Sperm DNA fragmentation testing significantly improved fertility outcomes (Agarwal et al., 2020). More recent data suggests that even intact DNA may undergo abnormal epigenetics signaling during this period, resulting in abnormal fetal development.

Instrumentation

Makler

The Makler counting chamber is used for sperm count and motility estimation. The Makler counting chamber allows the rapid and direct sperm count of an undiluted preheated sample. This chamber is only 10 microns deep, allowing for horizontal movement of spermatozoa in one focal plane while examined under a microscope. The chamber has a grid that is made up of 10 x 10 squares. The concentration can be

determined by counting three rows of 10, which will be averaged to indicate the number of spermatozoa per ml. Statistical evaluation of the results revealed high precision, accuracy, and reliability of sperm counts compared with the hemocytometric method. Easy performance, rapid sperm counts, and improvement of motility estimation make this chamber a useful tool where sperm analysis is carried out (Seaman et al., 1996).

Hemocytometer

Spermatozoa concentration of the gel-free semen can be determined using a standard clinical hemocytometer. On a hemocytometer, nine large squares are present with additional cross-hatched dividing lines within the center and central squares. When put under a microscope the number of spermatozoa within one of the nine large squares is counted, and this number is multiplied by one million to provide the number of spermatozoa per ml (Brisko et al., 2010). Advantages of using hemocytometer counting to determine spermatozoa concentration include the following: it is a direct method for counting spermatozoa visually identified under a microscope; discoloration of the sample does not affect the count's accuracy, and equipment expense is minor (Brisko et al., 2010).

CASA

Computer Assisted Semen Analysis (CASA) is a phrase for automatic or semi-automatic semen analysis techniques. Most systems are based on image analysis, but alternative methods exist, such as tracking cell movement on a digitizing tablet. Computer-assisted techniques are often used for assessing sperm concentration and mobility characteristics, such as velocity and linear velocity. Some CASA machines are based on image analysis and, using new techniques, can do a full analysis in a few

seconds. With some techniques, sperm concentration and motility measurements are as reliable as current manual methods. A CASA can determine many sperm attributes such as basic counts and motilities or detailed analysis of sperm motion. It can provide results for motile, progressively motile, and static sperm, including actual number counted, sample total, concentrations, and percentages. Additional results include velocities' averages, distribution bar charts, motion characteristics, and morphometry (Hamilton Thorne, 2020). The systems use fixed slides on devices with known volumes to provide reproducible counts. Chambers may vary between CASA systems.

Flow Cytometry

Flow cytometry is a procedure that is used to assess individual sperm cells. Samples that have cells of interest are stained with a particular dye, also called probes, that fluoresces under a specific color under a particular circumstance. For the dye to fluoresce within a cell, the cells must be passed by a laser beam, which excites the dye as it flows past. The fluorescent color is transmitted to electrical signals processed through a multichannel analyzer to determine the amount of specific fluorescence emitted per cell. Due to the recent development of fluorescent probes, flow cytometry is now capable of analyzing a number of sperm characteristics like viability, capacitation, acrosomal integrity, membrane permeability, membrane integrity, mitochondrial status, DNA integrity, de-condensation of DNA and differences between gametes based on sex (Maxwell & Johnson, 1997). Flow cytometry is useful for detecting problems within the cell or cell damage. It can also assess many cells quickly compared to more routine sperm cell assessment practices.

Cytation 5

The BioTeck Cytation 5 is a multichannel cell imaging platform capable of automated cell counting from fluorescently labeled nuclear images. This type of imaging has a range of applications for cell-based assays that cannot be performed on a standard plate reader (Yoonseok et al., 2017). Information on cell morphology, localization of signal, cell count, and more is obtained with Cytation 5 imaging mode. Plate reading modes include absorbance, fluorescence, luminescence, and advanced read modes. Imaging modes include fluorescence, phase contrast, HC brightfield, brightfield, and color brightfield. Cytation 5 automates many traditionally manual microscopy tasks, from slide scanning to time-lapse live-cell assays, from low to high magnification. This machine has a 6-objective turret, 1.25x to 60x, 20+ colors available, and a wide field view camera. Full automation includes automated stage, autofocus, and automated turret. Live-cell imaging has temperature and gas (CO_2) control for time-lapse live-cell imaging. This automation allows sperm cell imaging to be done in a shorter time with greater accuracy (Agilent, 2022).

Cytation 5 was used for the fluorescent analysis of sperm cell mitochondrial function, acrosomal integrity, and DNA fragmentation analysis.

CHAPTER III

MATERIALS AND METHODS

Redesign of the TrueBreed Collection Device

Previous research from this laboratory has demonstrated that human, swine, bovine, equine and canine semen quality, both physiological and biochemically, can be improved via a simple redesign of the semen collection cup, which helped maintain semen parameters for much longer periods of time in the equine model compared to semen collected in a tradition collection vessel. However, the original design was not specifically designed to help reduce DNA fragmentation. Recently the system underwent a redesign incorporating a proprietary method to harvest free oxygen radicals, an identified cause of DNA fragmentation. The purpose of this study was to determine the effects, of the redesign of the Device for Improved Semen Collection (DISC – trade name TrueBreed+) on all cellular functions of stallion semen. By design, the DISC: (1) slows the cooling rate, (2) limits exposed surface areas, (3) provides nutrients, and (4) lessens or eliminates osmotic shock during processing, potentially protecting the cells from collection induced damage. The system was recently redesigned to include fixed scavengers to harvest reactive oxygen species (ROS). The objective of the current study was to verify these findings in the equine model using a series of physiological and biochemical assays to assess cellular function.

Study Design

This experiment was designed to compare the traditional method of collecting stallion semen into a standard plastic baby bottle versus collection into the TrueBreed and the TrueBreed+ devices. Samples from 10 mature, reproductively sound, Quarter horse

stallions were collected and examined across multiple parameters to determine if sperm cells had increased viability in the TrueBreed+ device. Furthermore, this study specifically aimed to determine if stallion samples collected in the TrueBreed+ device had significantly reduced DNA fragmentation compared to the original TrueBreed and the standard collection device.

Collection and Extension Process

A commercial stallion operation in Guthrie, TX, the 6666's Ranch, provided stallion semen samples for this study. The 6666's ranch and its staff collected 10 mature Quarter horse stallions in their traditional protocol manner as part of their routine collection procedures. Semen donated for use in these studies would have otherwise been collected and discarded as no differences were required in animal handling and no study personnel actively engaged with the animals. The study was exempt from IACUC Oversight. However, the Texas Tech University Health Sciences Center IACUC and IVET (home institution of PI) were made aware of this study. Stallions were trained by the 6666's ranch to mount a dummy and were collected with a Missouri artificial vagina. Figure 3.1 shows an example of the Missouri artificial vagina, a soft walled artificial vagina (AV design controlling temperature and pressure on studs penis. Each stallion was collected once per day, every other day, for three collections from each stallion. Through randomization, each stallion was either collected with the standard device attached to the AV, the TrueBreed attached to the AV, or the TrueBreed+ attached to the AV. Stallions were collected in the same manner regardless of the bottle attached to the AV, using standard safety protocols. At the time of collection, information was recorded by the 6666's reproductive clinic. This information included the animal's name, age, type of

AV, number of mounts, the sample volume collected, and concentration and motility of spermatozoa were recorded using a CASA machine (Hamilton Thorne, 2020). Once these recordings were complete, semen was extended at a 1:1 ratio with INRA 96 extender (produced by IMV Technologies) directly into the collection device. Lids were then placed on collection devices and held at room temperature until pickup. Samples were transported at room temperature from the 6666's ranch to the Texas Tech University Health Sciences Center reproductive lab.

Figure 3.1

Missouri Artificial Vagina

Semen Parameters

Semen samples remained in the original collection device throughout the evaluation. Samples were held at room temperature and were evaluated at 6, 9, 12, 24, 48, 72, and 96 hours; until 0% motility was reached. This unusually high holding temperature increased cell metabolism and ROS production. To evaluate semen at these time frames, a small amount (.5 mL) of semen was removed and used for the specific protocol to analyze that sample at that time frame. Selected semen parameters were

recorded at each time interval for all samples collected in the standard device, true breed, and true breed plus. Parameters evaluated included: volume, concentration, motility, and progressive motility, as well as a variety of motility parameters provided by a CASA (Hamilton Thorne, 2020), including path velocity, progressive velocity, track speed, elongation, lateral displacement, beat cross frequency, straightness, and linearity. Other evaluations included morphology, acrosome integrity, mitochondrial function, and DNA fragmentation. All measurements were taken to evaluate the viability of semen samples collected and maintained in each device and how individual stallion's semen performed. This data also compared the viability of each sample from each device at each individual time frame.

Volume

The initial total volume of each stallion semen sample was recorded immediately after collection. It included the sperm cell-rich fraction of the ejaculate, and the volume of the gel fraction was removed from the sample, was not included. The volume was not recorded at any other time frame because the sample volume should have remained the same except for a small aliquot (.5mL) which was removed at each timeframe from each sample for each parameter evaluation.

CASA Analysis

Computer-assisted semen analysis (Hamilton Thorne, 2020) measured several sperm cell viability parameters. The CASA machine was used for each sample throughout the experiment. At each time frame, every sample was gently mixed, and a small aliquot was pulled from the center of the sample. This aliquot was placed on an 8-micron fixed slide and placed in the CASA machine. Each sample identification was

entered into the CASA machine before analysis. Computer-assisted semen analysis was used to determine spermatozoa parameters. Parameters that were assessed included: smooth path velocity (VAP), straight-line velocity (VSL), track velocity (VCL), the amplitude of lateral head (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN), elongation, size, motile percent, and progressively motile percent. After a short delay to let specimen space over the slide, it was analyzed at 20x magnification. The CASA parameters were as follows: 30 frames acquired, a frame rate of 60Hz, minimum contrast of 80, the minimum cell size of five pixels, VAP cutoff of 50 um, progressive minimum VAP cutoff of 20um/s, VSL cutoff of 1µm/s, cell intensity of 120, and magnification of 1.58. A minimum of five fields or 1000 sperm were analyzed for each sample.

Morphology

Morphology is the percentage of spermatozoa conforming to the normal shape for stallion semen. The most common defects observed in stallion spermatozoa are the abnormal head, detached head, abnormal/broken neck, abnormal midpiece, proximal droplet, distal droplet, coiled tail, and kinked tail. Studies have reported that fertility in stallions is positively correlated with the percentage of morphologically normal sperm and inversely correlated with the percentage of sperm with abnormal heads, proximal droplets, and abnormal midpieces (Varner, 2009). The percentage of abnormal sperm in an ejaculate can range from 10% to 40%. For evidence of morphological defects, a minimum of 100 cells were evaluated at each time frame from each stallion. Slides were prepared using a standard Hematoxylin-Eosin stain technique followed by a bright-field examination of 100 cells using a 100x oil immersion lens and noting head, mid-piece, and

tail defects. A smear of 4 µL from the native sample was placed on a slide and allowed to dry in a slide box. Morphology was recorded at 6, 9, 12, 24, 48, and 72 hours until zero percent motility was reached. The 96-hour time point was dropped due to no significant motility in any treatment at this time point. Morphology analysis was performed on all stallions at each time point from both treatments and control to verify that morphology did not change due to the collection device (see Figure 3.2).

Figure 3.2

Normal Equine Spermatozoa Morphology

Acrosome Reaction

Acrosome determinations were made using the chlortetracycline technique of chlortetracycline fluorescence assay (Lee et al., 1987). The assay using samples fixed in glutaraldehyde, an aliquot was prepared at each time point to evaluate the percentage of spermatozoa that had undergone the acrosome reaction as an indicator of cells that had begun the process of capacitating (Brisko et al., 2010). Slides were prepared by mixing a four microliter drop from the sample and four microliters of 12.5% Glutaraldehyde, smeared on a slide and allowed to dry. The dried slides were stained in a dark room at

room temperature until all slides were collected. Once stained, slides were stored. Dried slides were then stained with a chlortetracycline stain, using the technique of Keel and Webster. The slides were examined using the Biotek Cytation 5 machine, equipped with fluorescence. The prepared slides were examined using a 520μm excitation filter and a 570μm barrier filter. A minimum of 100 cells per slide from each sample were evaluated using the Biotek Cytation 5. The intact acrosome (see Figure 3.3) cap appeared a fluorescent green, with normal morphology, while the acrosome reacted cells (see Figure 3.4) had lost their acrosome cap and appeared faded and not of normal shape.

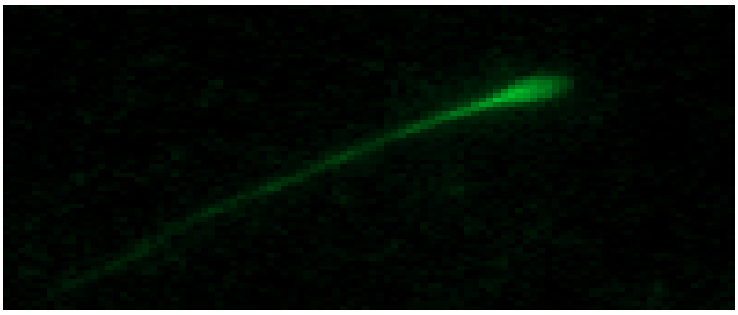

Figure 3.3

Acrosome Intact

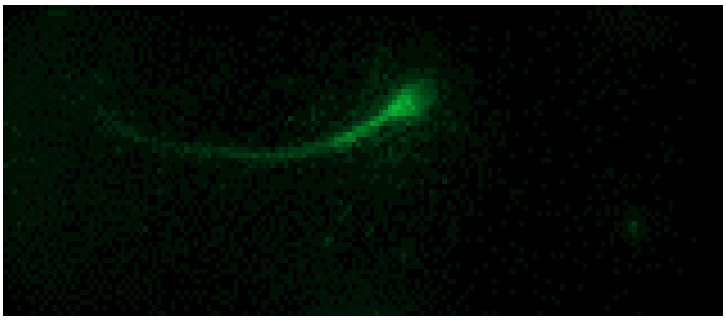

Figure 3.4

Acrosome Reacted

Mitochondria

In fluorescent microscopic evaluation, the functionality of mitochondrion is detected by organelle-specific dyes. These dyes exploit the membrane-polarizing attribute of mitochondria to differentiate metabolically active from functionally dead spermatozoa. MitoTracker Red CMXRos is a red-fluorescent dye that stains mitochondria in live cells, and its accumulation is dependent upon membrane potential. MitoTracker® Red CMH2XRos (MT-R; M-7513) and JC-1 (T-3168) were purchased from Molecular Probes. The dye is well-retained after aldehyde fixation. The stain was made by adding .5 ml PBS to a vial of Mitotracker Red that was stored at five degrees C. In a bullet tube, a sample aliquot of 50µL was mixed with 50µL of the Mitotracker solution and incubated for 15 minutes. The sample was then centrifuged for five minutes at 500g. The supernatant was removed, and 100µL of four percent Formalin was mixed in with the sample. A 10µL suspension of the sample was smeared on slides shieled from light. Once all slides were collected, the slide box was wrapped in parafilm and refrigerated. Sperm suspensions were mounted on a microscope slide and observed using the Biotek Cytation 5, at a minimum of 100 cells in at least four different fields were counted per sample. Sperm were categorized according to the patterns of staining in the midpiece. Functioning mitochondria (see Figure 3.5) would fluoresce a bright red, and low or nonfunctioning mitochondria (see Figure 3.6) would appear faded or with black gaps, demonstrating inactive mitochondria.

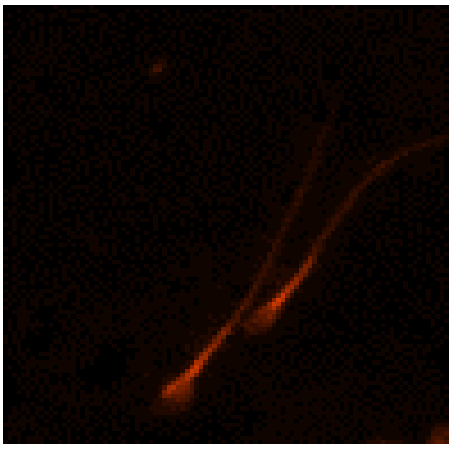

Figure 3.5

Functioning Mitochondria

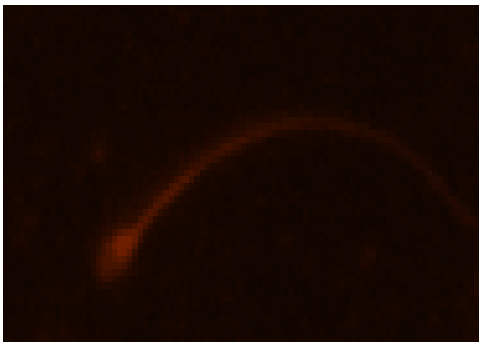

Figure 3.6

Non-Functioning Mitochondria

DNA Fragmentation

DNA fragmentation is the separation or breaking of DNA strands into pieces. Spontaneous or accidental DNA fragmentation is fragmentation that gradually accumulates in a cell. DNA fragmentation was assessed using Halosperm kits from

Spectrum Technologies. Halosperm is based on a controlled DNA denaturation process to facilitate the removal of the proteins contained in each spermatozoon. Normal spermatozoa create halos formed by loops of DNA at the head of the sperm, which are not present in those with damaged DNA. Testing preparation began with warming an agarose solution to 37° C in an Eppendorf tube, transferring 50µl of the sperm sample, and mixing gently. A drop of the suspension was placed in the well of the slide with a cover slip, the sample was refrigerated at 4° C for five minutes to solidify the agarose. The slide was removed, flooded with Lysis solution, and then incubated for five minutes. The sample was flushed with distilled water for five minutes. The next slide was flooded with 70% alcohol and incubated for two minutes. After the incubation slide was washed with 100% alcohol for two minutes. The slide was dyed and place in refrigerator until the stain was applied. DNA was stained using premixed DAPI (diamidino-2-phenylindole) prolong gold antifade mount from Thermo fisher scientific (Invitrogen). DAPI stain is excited by UV light at 360nm when bound to DNA, with an emission maximum at 460nm, and is detected using a DAPI traditional filter. SYTOX Deep Red stain is excited by a red light at 660nm when bound to DNA, with an emission maximum at 682nm, and is detected using a Cy5/deep red traditional filter. To prep slides for stain, they were placed in 1x PBS solution for 10 minutes, dried, and a DAPI stain was dropped on samples. Samples using the Biotek Cytation 5 and a minimum of 100 sperm cells per slide were analyzed.

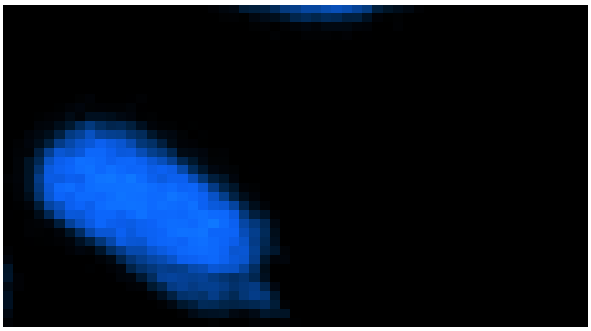

Figure 3.7

Intact DNA

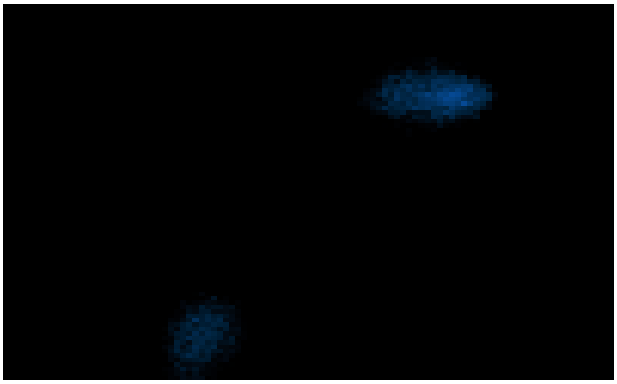

Figure 3.8

Fragmented DNA

Data Analysis

All data were analyzed using the statistical program for Social Sciences SPSS Version 25. Data was parametric. All data were normalized to 100% from the six-hour time. Comparisons between stallions, treatments, and time for CASA data, morphology, mitochondria function, acrosome integrity, and intact DNA were made using a General Linear Model. A Multivariant ANOVA was performed with repeated measures for time when significant at that level ($p < .05$). The Tukeys HSD test was used to determine the relationship between time, device, and time/device interaction variables.

Preliminary data analysis revealed that over time there was a significant difference for all variables, as expected. It was also observed that there was a substantial difference between devices in the traditional collection method and the two DISC devices. Significant differences were observed between devices for CASA data, including motility, rapid cell, path velocity elongation, and beat cross frequency. Significant differences between devices were also observed for all types of morphological defects, mitochondria, acrosomal integrity, and DNA. A significant difference was also detected for the device/time interaction for beat cross frequency and DNA.

At the nine-hour time point and beyond, samples collected in the traditional device significantly decreased in all semen parameters to 96 hours. There was no significant difference between the DISC and the DISC+ across time as expected. Except for DNA fragmentation at the 48-hour time, samples collected in the only samples collected in the DISC+ had significantly less DNA fragmentation than those collected in the traditional device or the original DISC.

CHAPTER IV

RESULTS

CASA Data

As expected, there were a significant difference in time for all Computer Assisted Semen Analysis (CASA) parameters ($p < .05$). There was also a substantial difference for all CASA variables between devices ($p < .05$). There was only a significant difference in the time-by-device interaction for one CASA parameter, beat cross frequency.

Concentration

Figure 4.1 demonstrates sample cell concentration over time in equine semen samples stored at high temperatures to induce cellular metabolism. As expected, there was no difference in concentration for time, device, or time-by-device interaction ($p < .05$). The graph shows changes in the cell concentration by volume over time, but not between devices. As expected, there was no significant difference over time, no difference between devices, and no device X time interaction significant difference. The device should not have impacted the concentration of samples.

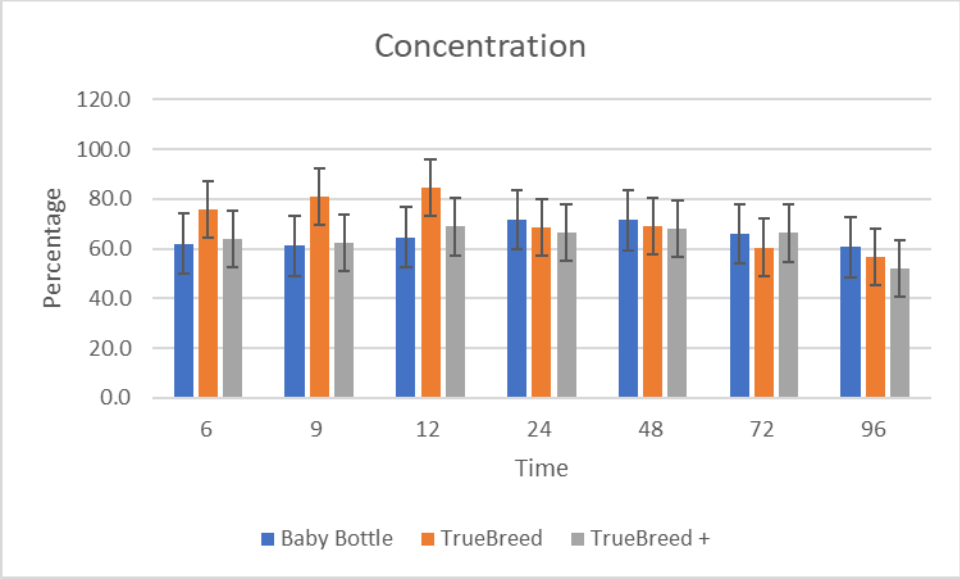

Figure 4.1

Concentration by Treatment Over Time demonstrating no significant differences. Bars represent standard error of means.

Motility

As expected, motility decreased over time regardless of device ($p < .001$). However, there was an independent treatment effect ($p < .03$). No significant interaction between time and device type. Means suggest an early benefit to the TrueBreed+ device followed by a rapid decline in all treatments, possibly due to the unfavorable holding conditions used to induce reactive oxygen species development. Data was normalized. Figure 4.2 demonstrates cell motility over time in equine semen samples stored at high temperatures to induce cellular metabolism. Data indicated a significant difference over time ($p < .001$), there was also a difference between devices ($p < .03$) and no device X time interaction significant difference. Means suggest slower cooling rate, limiting exposed surface areas, providing nutrients, and lessening or eliminating osmotic shock gave benefit to samples help in the TrueBreed and TrueBreed+ devices up to12 hours.

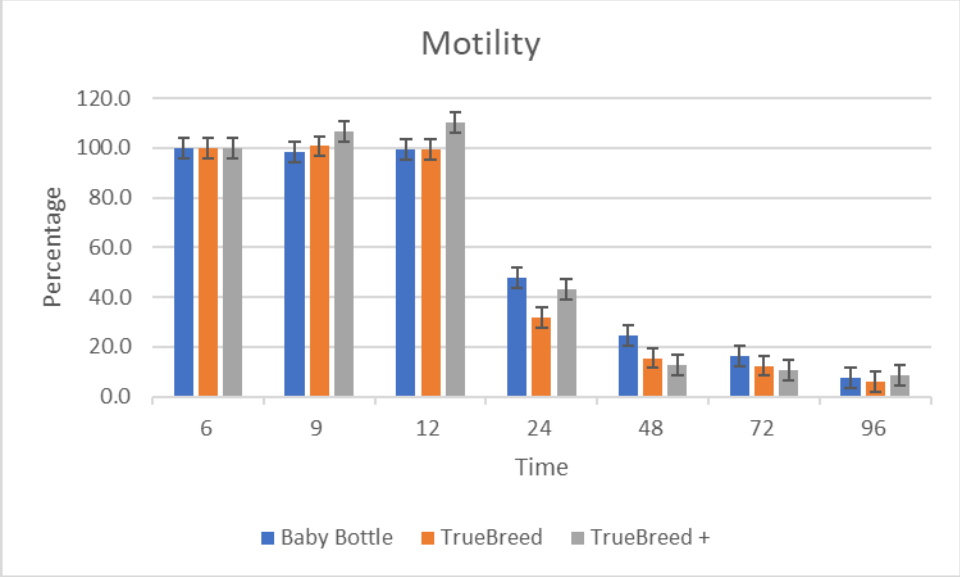

Figure 4.2

Motility by Treatment Over Time suggesting the *TrueBreed+ to be the superior device until 24-hours. Bars represent standard error of means.*

Rapid Cell

As expected, there was a difference in Rapid cell over time as it decreased regardless of treatment ($p < .001$). However, there was also an independent treatment effect ($p < .03$). There was also no difference in the interaction between time and treatment. Means again suggest an early benefit to the TrueBreed+ device followed by a rapid decline in all treatments, after 12 hours. Data was normalized. Figure 4.3 demonstrates rapid cell over time in equine semen samples stored at high temperatures to induce cellular metabolism. Data indicated only a significant difference over time ($p < .001$), there was also a difference between devices ($p < .025$) and no device X time interaction significant difference. Means suggest a slower cooling rate, limiting exposed surface areas, providing nutrients and reducing or eliminating osmotic shock gave benefit

to samples help in the TrueBreed and TrueBreed+ devices for up to 12 hours.

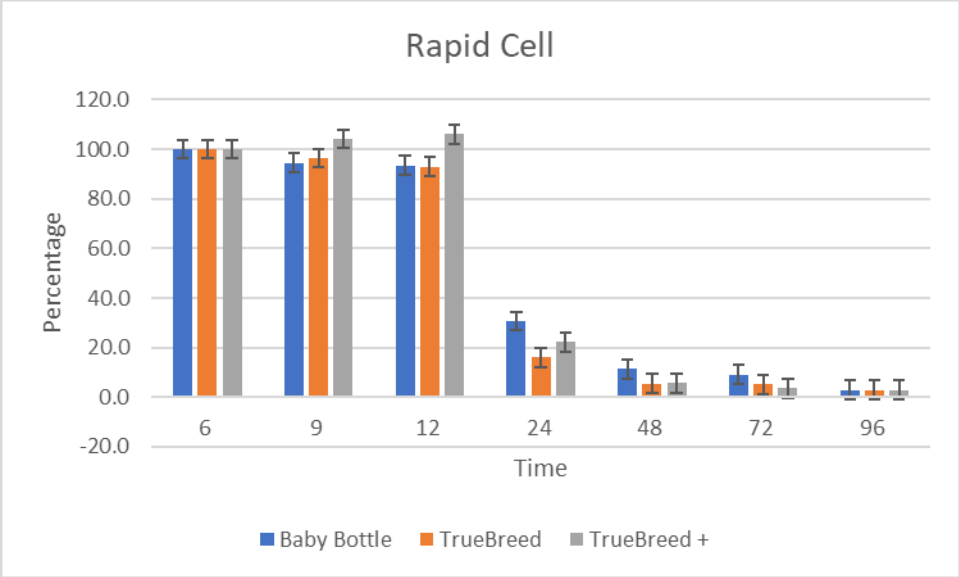

Figure 4.3

Rapid Cell by Treatment Over Time suggesting the TrueBreed+ device cells had higher rate of travel up to 24 hours. Bars represent standard error of means.

Path Velocity

As expected, there was a difference in path velocity over time as it decreased regardless of treatment ($p < .001$). However, there was also an independent treatment effect among devices ($p < .031$). There was also no interaction difference between time and path velocity treatment. Means again suggest an early benefit to the TrueBreed device followed by a rapid decline in all treatments, after 12 hours. Then at 72 hours, suggest a more favorable path velocity in the TrueBreed +. There was also a significant difference in path velocity between the standard device and the TrueBreed+. However, there was no difference in path velocity between the standard and the TrueBreed or between the TrueBreed and the TrueBreed+. Figure 4.4 demonstrates path velocity over time in equine semen samples stored at high temperatures to induce cellular metabolism. Data

indicated only a significant difference over time ($p < .001$), there was also a difference between devices ($p < .031$) and no device X time interaction significant difference. Means suggest slower cooling rate, limiting exposed surface areas, providing nutrients, and reducing or eliminating osmotic shock gave a slight early benefit to samples in the TrueBreed device, followed by a rapid decline of progression in all treatments, after 12 hours.

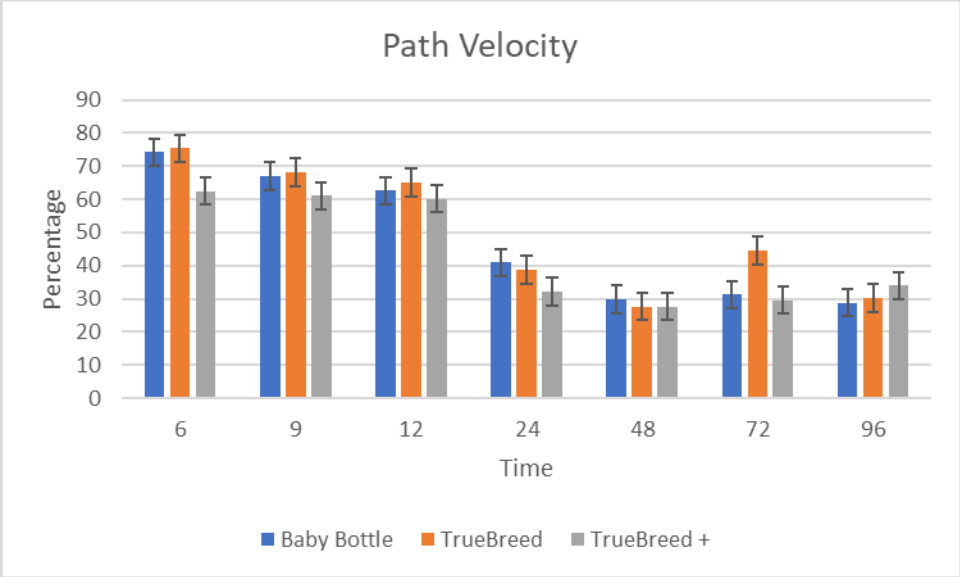

Figure 4.4

Path Velocity by Treatment Over Time, suggesting the *Truebreed device had higher velocity up until 12 hours with a spike at 72 hours. Bars represent standard error of means.*

Progression

As expected, there was a difference in progression over time as it decreased regardless of treatment ($p < .001$). However, there was no independent treatment effect among devices ($p < .05$). There was also no interaction between time and treatment for progression. Means again suggest a slight early benefit to samples in the TrueBreed

device followed by a rapid decline of progression in all treatments, after 12 hours. Figure 4.5 demonstrates progression over time in equine semen samples stored at high temperatures to induce cellular metabolism. Data indicated only a significant difference over time ($p < .001$ no between devices and no device X time interaction significant difference. Means suggest a slower cooling rate, limiting exposed surface areas, providing nutrients, and reducing or eliminating osmotic shock did not significantly affect cell progression.

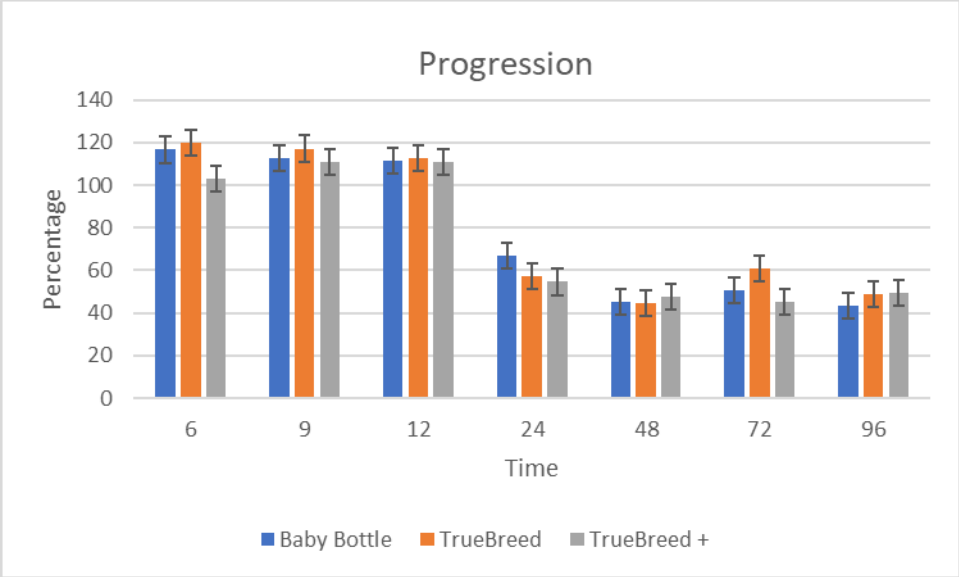

Figure 4.5

Progression by Treatment Over Time, no significant difference between devices Bars represent standard error or means.

Track Speed

There was a difference in track speed over time as it decreased regardless of treatment ($p < 0.001$). However, there was no independent treatment effect among

devices ($p < 0.05$). There was also no significant interaction between time and treatment for track speed. Means again suggests no benefit to samples in the TrueBreed or TrueBreed+ device over time, with all samples declining after the 12-hour time point. Figure 4.6 demonstrates track speed over time in equine semen samples stored at high temperatures to induce cellular metabolism. Data indicated only a significant difference over time ($p < .001$) between devices and no significant difference between device X time interaction. Means suggest a slower cooling rate, limiting exposed surface areas, providing nutrients, and reducing or eliminating osmotic shock did not significantly protect cells held in the TrueBreed or TrueBreed+ devices after 12 hours.

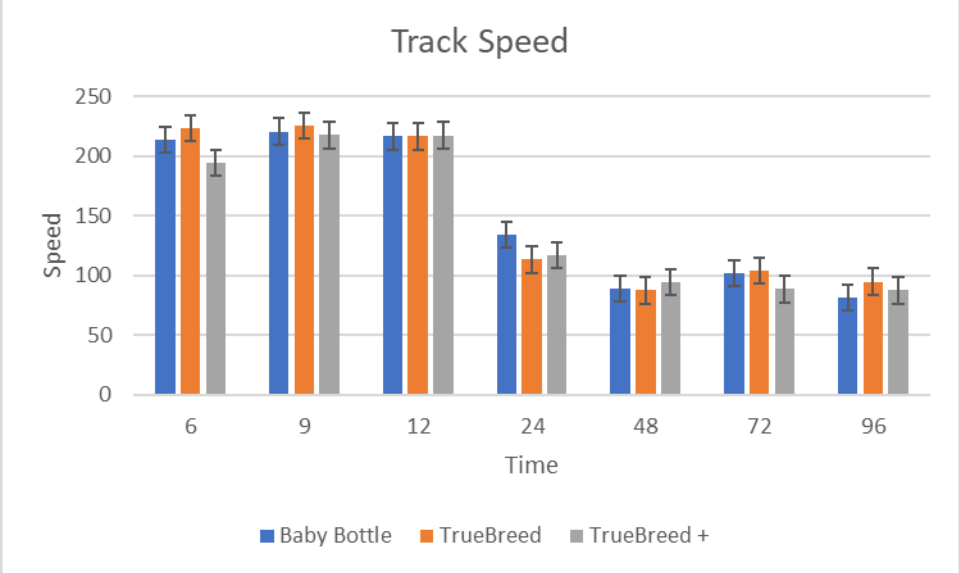

Figure 4.6

Track Speed by Treatment Over Time, demonstrating no difference between devices. Bars represent standard error of means.

Elongation

There was once again a difference in elongation over time as it decreased regardless of treatment ($p < .001$). There was also a difference in independent treatment effect among devices ($p < .05$). There was no difference in the interaction between time and treatment for elongation. Means again suggest a slight advantage for the sample collected and held in the TrueBreed+ after 12 hours. Means also suggest a strong benefit to samples collected and held in the TrueBreed device 24 hours and beyond. Figure 4.7 demonstrates elongation over time in equine semen samples stored at high temperatures to induce cellular metabolism. Data indicated a significant difference over time ($p < .007$), as well as differences between devices ($p < .05$), but there was no device X time interaction significant difference. Means suggest a slower cooling rate, limiting exposed surface areas, providing nutrients, and lessening or eliminating osmotic shock during processing; protecting the cells held in the TrueBreed or TrueBreed+ devices.

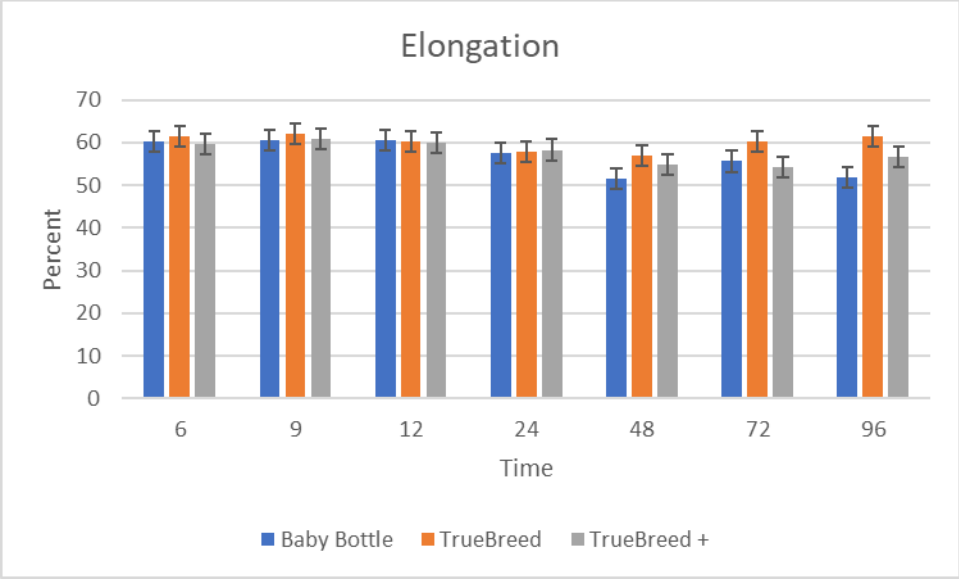

Figure 4.7

Elongation by Treatment Over Time. Bars represent standard error of means.

Lateral Displacement

There was no difference in lateral displacement over time ($p <$ .05). There was also no significant difference in lateral displacement among devices ($p <$ .05). There was also no difference detected in the device by time interaction for lateral displacement. Means suggest an advantage to the TrueBreed+ after 12 hours, with samples collected and held in the TrueBreed and standard device more rapidly declined up to 72 hours. Figure 4.8 demonstrates lateral displacement over time in equine semen samples stored at high temperatures to induce cellular metabolism. Data indicated no significant difference over time between the device or device X time interaction significance.

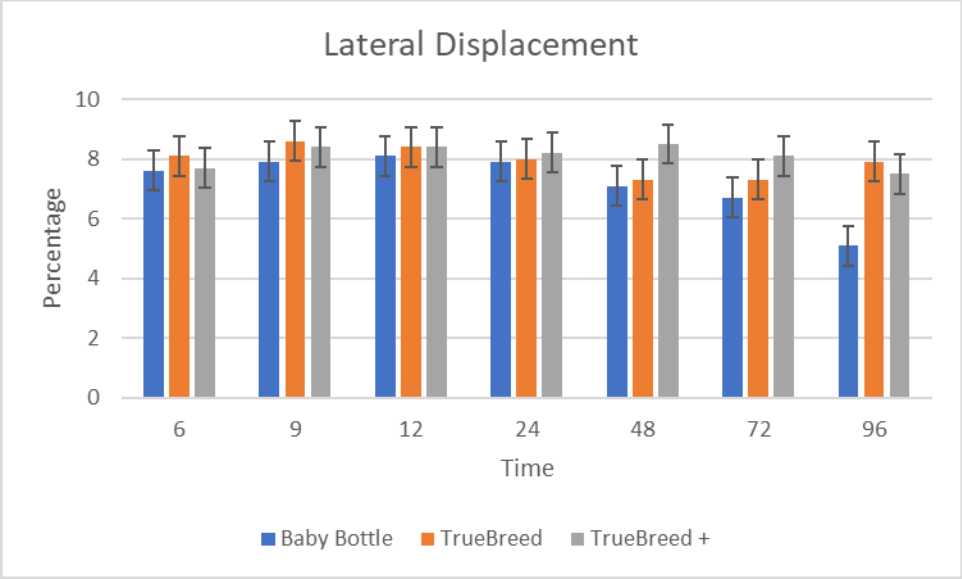

Figure 4.8

Lateral Displacement by Treatment Over Time, no significant difference over time or between devices. Bars represent standard error of means.

Beat Cross Frequency

As expected, there was a significant difference over time for beat cross frequency as it decreases over time ($p <$ .001). There is no difference in beat cross frequency between devices ($p <$ .05). However, there is a significant difference between time and device interaction for beat cross frequency (p <.03). Means suggest that past 24 hours samples collected and held in the TrueBreed and TrueBreed+ were superior, and at 72 hours and beyond the samples in the TrueBreed+ had a more favorable beat cross frequency. At nine hours, there was a significant difference between the standard device and the TrueBreed, and almost a difference between the TrueBreed+ and the Standard. There was no significant difference between the TrueBreed and the TrueBreed+ for Beat cross frequency. Figure 4.9 demonstrates beat cross frequency over time in equine semen samples stored at high temperatures to induce cellular metabolism. Data indicated a

significant difference over time ($p < .001$), no differences between devices, but there was a device X time interaction significant difference ($p < .031$). Means suggest a slower cooling rate, limiting exposed surface areas, providing nutrients, and lessening or eliminating osmotic shock during processing; protecting the cells held in the TrueBreed or TrueBreed+ devices, thus maintaining reduced beat cross frequency up to 72 hours.

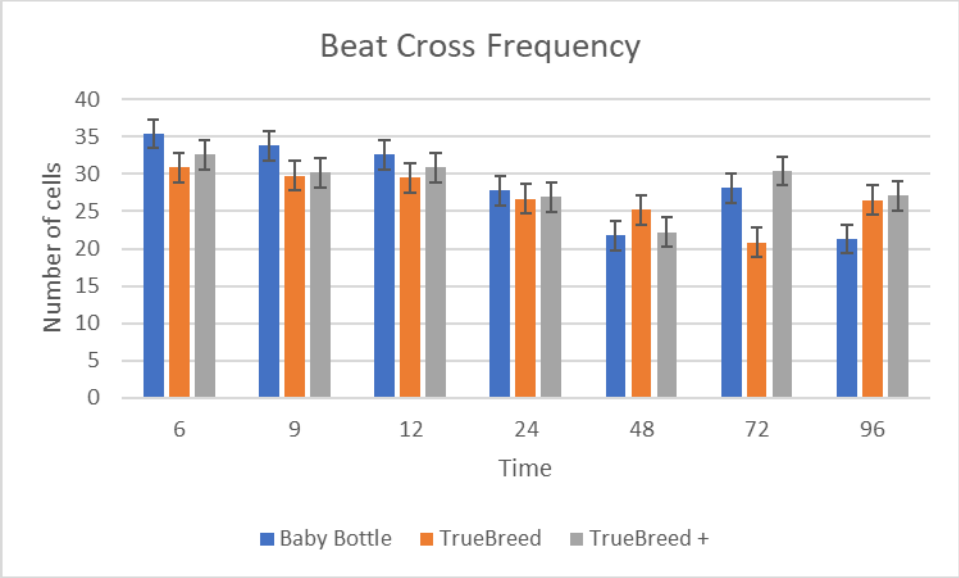

Figure 4.9

Demonstrating no significant difference between the TrueBreed and the Truebreed+ for beat cross frequency, Truebreed + became the superior device after the 48-hours. Bars represent standard error of means.

Straightness

There was no difference in straightness over time ($p < .05$). There was also no significant difference in the straightness of the device ($p < .05$). There were also no significant differences between the interaction of time by device for straightness ($p < .05$). Means suggest a slight advantage in linearity in the TrueBreed and the TrueBreed+

after 24 hours. Figure 4.10 demonstrates straightness over time in equine semen samples stored at high temperatures to induce cellular metabolism. Data indicated no significant damage over time, no differences between devices, and no device X time interaction. Means suggest no advantage in straightness over time in cells held in another TrueBreed device.

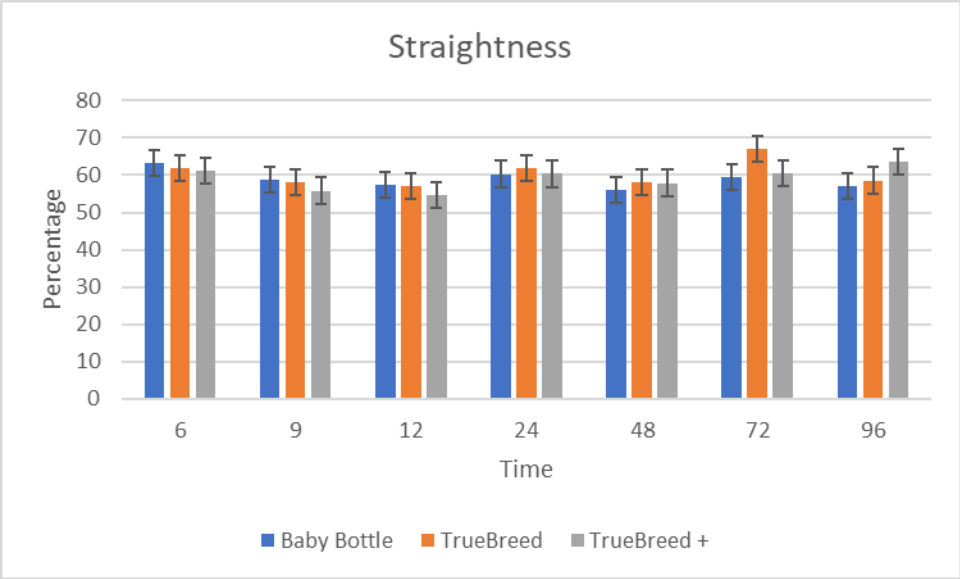

Figure 4.10

Straightness by Treatment Over Time, no significant difference over time or between devices. Bars represent standard error of means.

Linearity

There was no difference in linearity over time ($p <$ .05). There was also no significant difference in linearity for the device ($p <$ .05). There were also no significant differences between the interaction of time by device for linearity ($p <$ .05). This is expected as the results correspond to straightness. Means suggest a benefit for samples

collected in the TrueBreed+ after 12 hours and up to 72 hours. Figure 4.11 demonstrates linearity over time in equine semen samples stored at high temperatures to induce cellular metabolism. Data indicated no significant damage over time, no differences between devices, and no device X time interaction. Means suggest no advantage in linearity over time in cells held in another TrueBreed device.

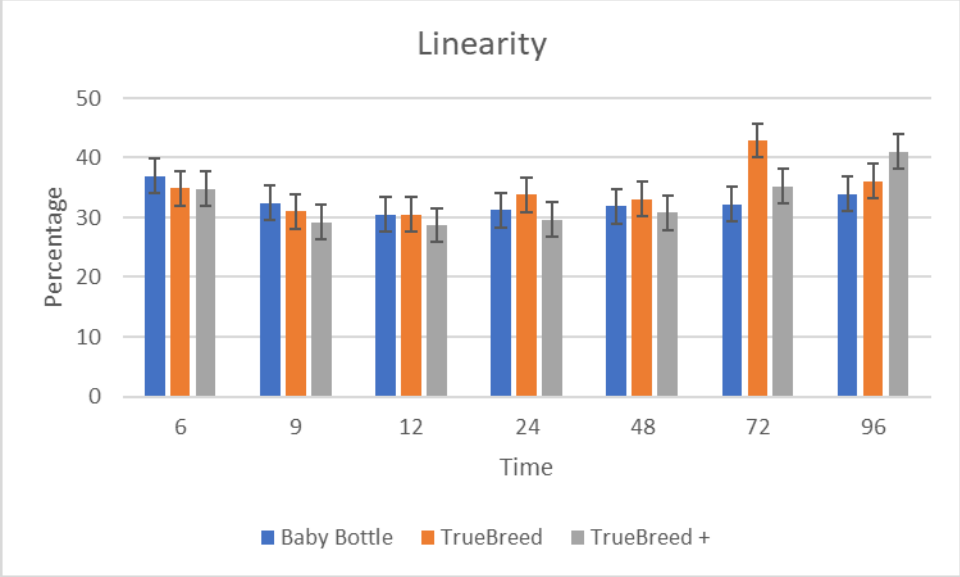

Figure 4.11

Linearity by Treatment Over Time, no significant difference over time or between devices. Bars represent standard error of means.

Morphology

Overall normal morphology had no significant difference over time ($p < .05$). There was a significant difference in devices for morphology. This was the only difference observed in overall morphology ($p < .03$). There was no difference in the time-by-device interaction for overall morphology ($p < .05$). However, trends in the means highly suggest favorable conditions for samples collected in the TrueBreed device over

time. Also, the TrueBreed+ demonstrates a significant advantage over the stander device over time. At the 48-hour time, overall morphology was significantly different between the TrueBreed and the standard device. However, for overall normal morphology, there was no significant difference between the standard device and the TrueBreed+ or between the TrueBreed and the TrueBreed+. Figure 4.12 demonstrates overall normal morphological over time in equine semen samples stored at high temperatures to induce cellular damage. Data indicated no significant damage over time and differences between devices ($p <$ .003). There was no device X time interaction. Means suggest a slower cooling rate, limiting exposed surface areas, providing nutrients, and lessening or eliminating osmotic shock during processing; protecting the cells held in the TrueBreed or TrueBreed+ devices, thus maintaining better overall morphology.

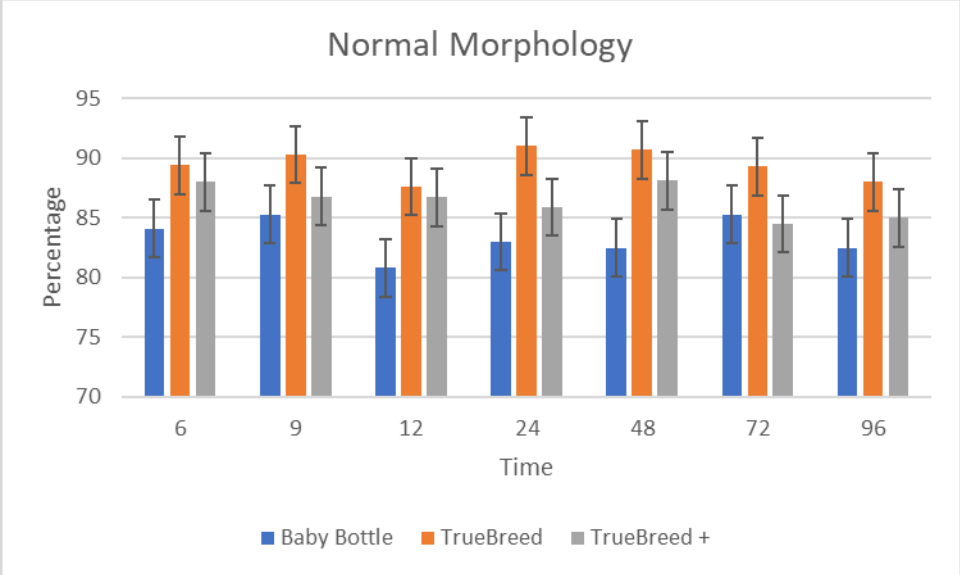

Figure 4.12

Overall Morphology by Treatment Over Time, demonstrating a greater number of cells with normal Morphology in TrueBreed and Truebreed+ at all time points. Bars represent standard error of means.

Head Morphology Defects

Overall morphological head defects had no significant difference over time ($p <$ .05). There was a significant difference in devices for morphological head defects depending on what device was used to collect and store the sample ($p < .005$). There was no significant difference between time and deice interaction for morphological head defects ($p < .05$). Means suggest that head defects significantly increased in the standard device after 72 hours. Before that, head defects seemed to be minimized in samples collected in the TrueBreed and TrueBreed+ devices. Means also suggest that samples collected and held in the TrueBreed+ had fewer defects than the other two devices. This also suggests that samples were collected and held. At the nine-hour time, there was a significant difference in head defects between the standard device and the TrueBreed and between the standard device and the TrueBreed+. However, there was no significant difference in head defects between the TrueBreed and the TrueBreed+. Figure 4.13 presents morphological head defects over time in equine semen samples stored at high temperatures to induce cellular damage. Data indicated no significant damage with time and differences between devices with cells in the standard baby bottle having greater tail defects than either of the Truebreed devices ($p <$ .005). There was no device X time interaction. Means suggest a slower cooling rate, limiting exposed surface areas, providing nutrients, and lessening or eliminating osmotic shock during processing; protecting the cells in the TrueBreed and TrueBreed+.

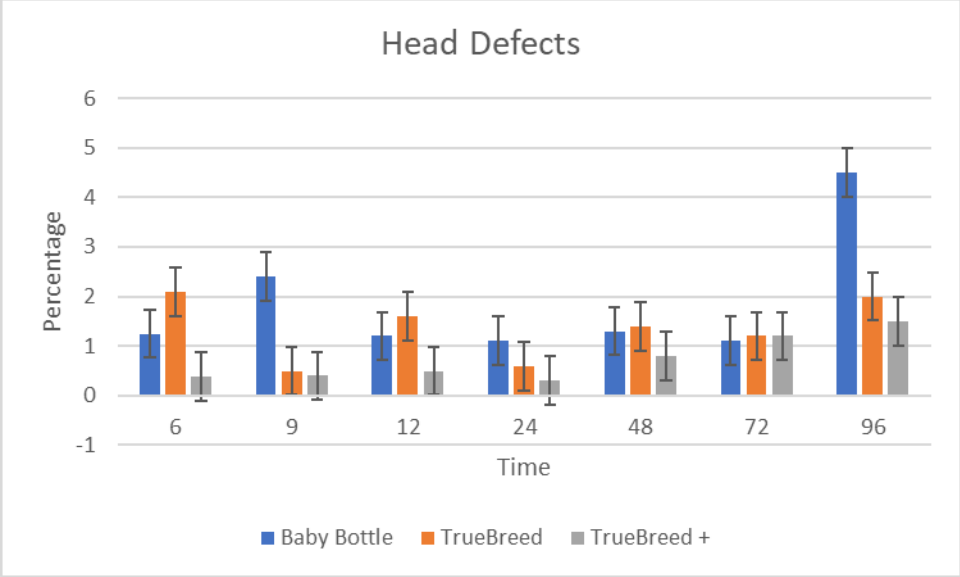

Figure 4.13

Morphological Head Defects for the Device Over Time, head Defects lower in TrueBreed+, highest in standard device at 96 hours. Bars represent standard error of means.

Midpiece Morphological Defects

Overall morphological midpiece defects had no significant difference over time ($p < .05$). There was a substantial difference in devices for morphological midpiece defects depending on what device was used to collect and store the sample ($p < .01$). There was no significant difference between time and device interaction for morphological midpiece defects ($p < .05$). Means appear to demonstrate that samples collected in the TrueBreed and the TrueBreed+ had substantially less damage to the midpiece over time compared to the standard device., with the TrueBreed+ being superior until the 96-hour time point. Figure 4.14 shows morphological midpiece defects over time in equine semen samples stored at high temperatures to induce cellular damage. Data indicated no significant damage with time and differences between devices with cells in the standard baby bottle

having grater tail defects at every time point compared to cells held in either of the Truebreed devices ($p <$ .011). There was no device X time interaction. Means suggest a slower cooling rate, limiting exposed surface areas, providing nutrients, and lessening or eliminating osmotic shock during processing; protecting the cells membrane in the TrueBreed and TrueBreed+.

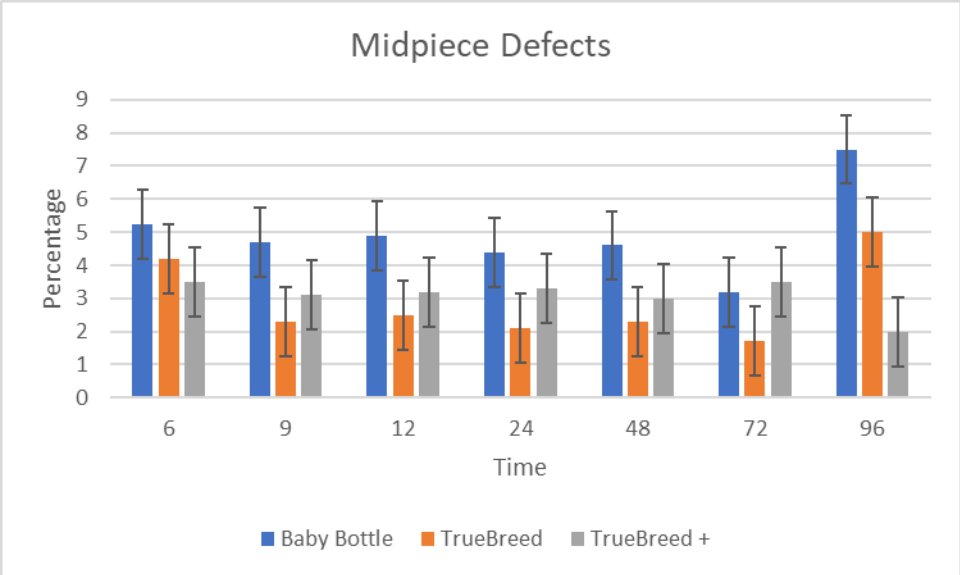

Figure 4.14

Morphological Midpiece Defects for the Device Over Time, demonstrating midpiece defects were consistently higher in samples collected in the standard device. Bars represent standard error of means.

Tail Morphological Defects

Overall morphological tail defects had no significant difference over time ($p <$ .05). There was a substantial difference in devices for morphological tail defects depending on what device was used to collect and hold the sample ($p <$ .041). There was no significant difference between time and device interaction for morphological tail

defects ($p <$ .05). Means demonstrate that samples collected in either the TrueBreed or TrueBreed+ device had fewer defects than the standard device over time. Figure 4.15 presents morphological tail defects over time in equine semen samples stored at high temperatures to induce cellular damage. Data indicated no significant damage with time and differences between devices with cells in the standard baby bottle having greater tail defects than either of the Truebreed devices ($p <$ .042). There was no device X time interaction. Means suggest a slower cooling rate, limiting exposed surface areas, providing nutrients and lessening or eliminating osmotic shock during processing; protected the cells in the TrueBreed and TrueBreed+, up to after the 72-hour time point.

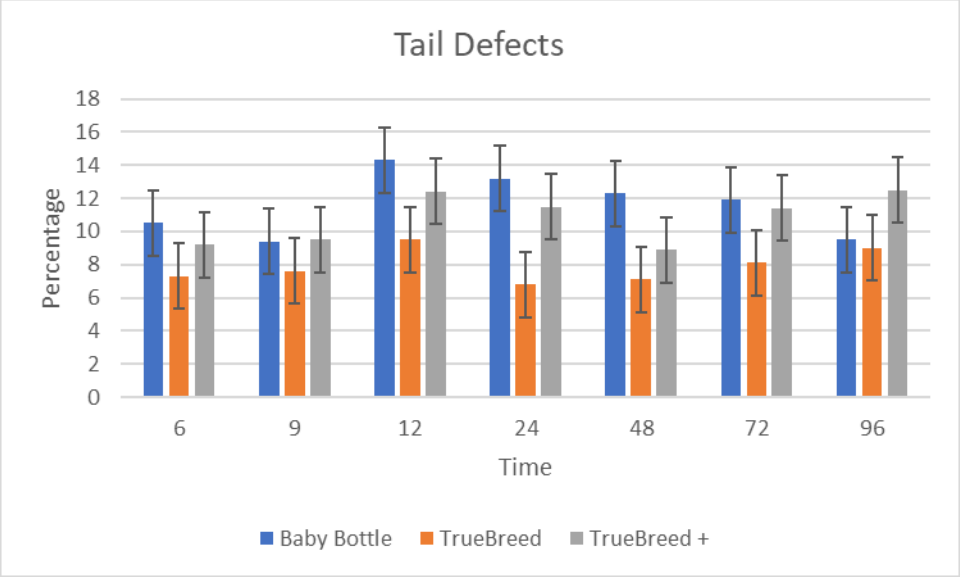

Figure 4.15

Tail Defects for the Device Over Time, Tail defects were consistently higher in samples in the standard device until 96 hours. Bars represent standard error of means.

Mitochondria Function

As expected, there was a significant difference over time for mitochondria

function ($p < .001$). There was also a significant difference in mitochondrial function between samples collected and held in different devices ($p < .001$). There was no significant difference in the interaction of the time be the device for mitochondrial function ($p < .05$). Means suggest that samples collected in the TrueBreed and TrueBreed+ had superior mitochondrial function compared to the standard device. Means also suggest that samples collected in the TrueBreed+ were superior to the TrueBreed at 48 hours and beyond, with superior performance at 96 hours. At the 24-hour time point, there was a significant difference between the standard device and the TrueBreed and TrueBreed+ devices. There was no difference in mitochondrial function between the TrueBreed and the TrueBreed+ devices. Figure 4.16 shows functioning mitochondria over time in equine semen samples stored at high temperatures to induce metabolic rate. Data indicated an increase in damage with time ($p < .001$) and differences between devices, with the standard baby bottle having significantly fewer cells with functioning mitochondria than either of the Truebreed Device ($p < .001$). There was no device X time interaction. Means suggest a slower cooling rate, limiting exposed surface areas, providing nutrients, and lessening or eliminating osmotic shock during processing; protecting the cells in the TrueBreed and TrueBreed+, delaying acrosome reaction.

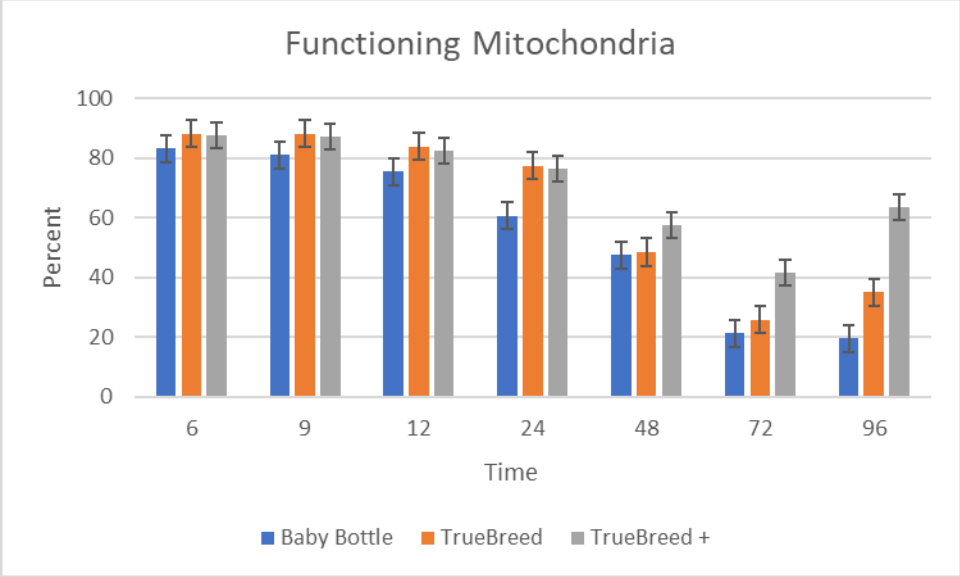

Figure 4.16

Mitochondrial Function in the Device Over Time, demonstrating a greater number of functional mitochondria in TrueBreed and TrueBreed+, + being superior after 24 hours. Bars represent standard error of means.

Acrosomal Integrity

As expected, acrosomal integrity is significantly different over time ($p < .001$). There was also a significant difference in acrosomal activity by the device ($p < .001$). There was no significant difference in acrosomal activity for the interaction of time by the device ($p > .05$). Means demonstrate that samples collected in the TrueBreed and TrueBreed+ were superior to samples collected in the standard device. After six hours, samples collected in the TrueBreed+ were superior to TrueBreed until 72 hours, when the TrueBreed samples had the advantage. There was a difference in acrosomal integrity at 12 hours between the standard device and the TrueBreed and the TrueBreed+. There was no difference in acrosomal integrity between the TrueBreed and the TrueBreed+ at 12 hours. However, at the 24-hour time point, there is only a difference between the standard

device and the TrueBreed+, not between the standard device or the TrueBreed or between the TrueBreed or the TrueBreed+. Figure 4.17 shows intact acrosomes over time in the equine sample stored at high temperatures to induce metabolism. Data indicated an increase in damage with time ($p < .001$) and differences between devices, with the standard baby bottle having significantly fewer cells with intact DNA than either of the Truebreed Devices ($p < .001$). There was no device X time interaction ($p < .029$). Means suggest a slower cooling rate, limiting exposed surface areas, providing nutrients, and lessening or eliminating osmotic shock during processing; protecting the cells in the TrueBreed and TrueBreed+, delaying acrosome reaction.

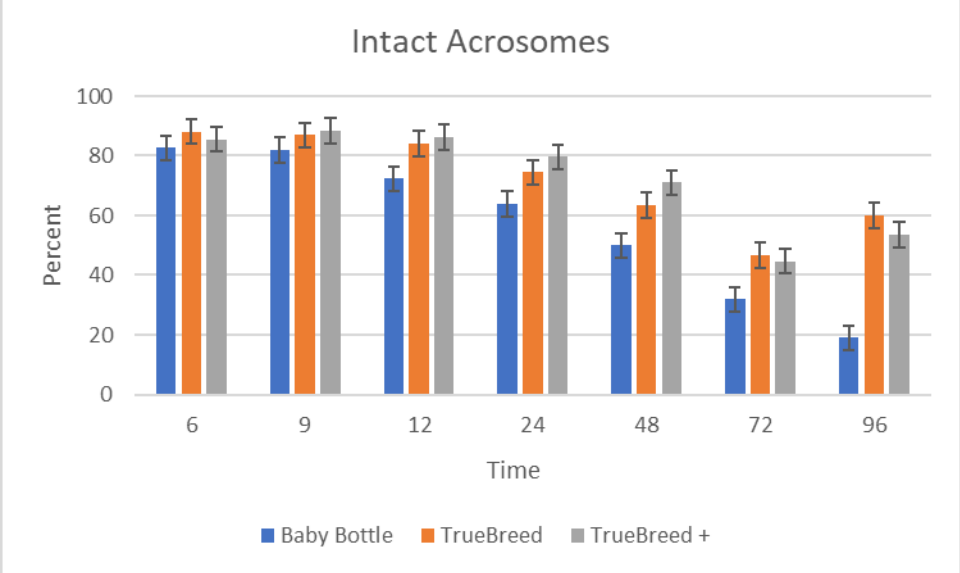

Figure 4.17

Intact Acrosomes by the Device Over Time, demonstrating more intact Acrosomes in TrueBreed devices, + superior until 72 hours. Bars represent standard error of means.

DNA

As expected, there was a significant difference over time when evaluating DNA

fragmentation ($p < .001$). DNA fragmentation was also significantly different between different devices ($p < .001$). There was also a significant difference in device interaction over time for DNA fragmentation. Samples collected in the TrueBreed or the TrueBreed+ had far less fragmented DNA than those collected in the standard device. Means would also suggest that samples collected in the TrueBreed+ consistently had fewer fragmented DNA than those collected in the TrueBreed device. At the 12-hour time, there is a significant difference in DNA fragmentation between the standard device and the TrueBreed and the TrueBreed+ device and no difference between the TrueBreed and the TrueBreed+ device. However, at the 48-hour time point, there is only a difference between the standard device and the TrueBreed+. At the 72-hour time, there is again a difference in DNA fragmentation between the standard device and the TrueBreed and the TrueBreed+ device and no difference between the TrueBreed and the TrueBreed+ device. Figure 4.18 shows the presence of DNA fragmentation over time in equine samples stored at high temperatures to induce reactive oxygen species compared across different collection vessels with differing antioxidant potentials. Data indicated an increase in damage with time ($p < .001$) and differences among devices, with the standard baby bottle having significantly fewer cells with intact DNA than either of the Truebreed Device ($p < .001$). There was also a device X time interaction ($p < .029$).

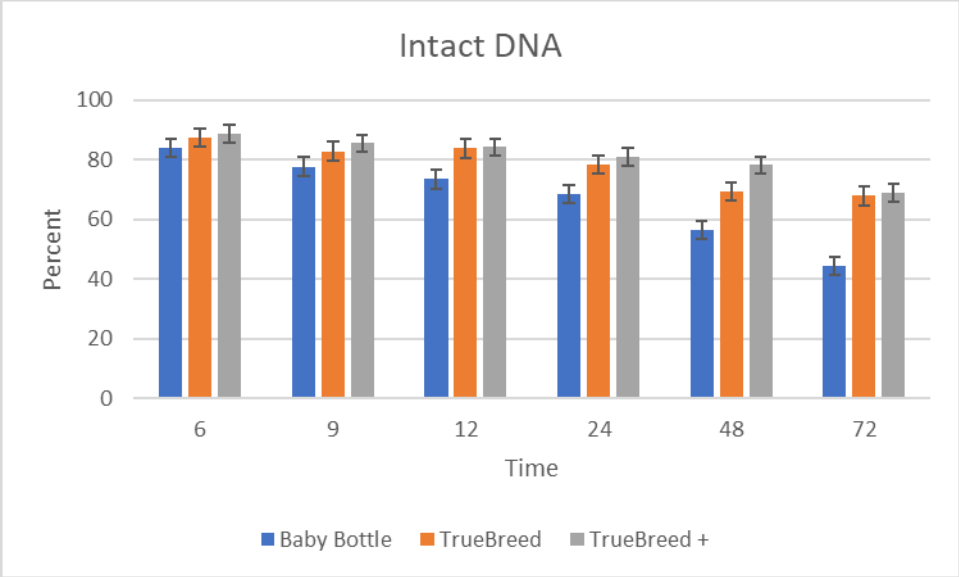

Figure 4.18

DNA Intact by Device Over Time, demonstrating DNA intact more frequently in TB and +, + superior at every time point. Bars represent standard error of means.

CHAPTER V

DISCUSSION AND CONCLUSIONS

CASA Data

Computer Assisted Semen Analysis (CASA) techniques assess sperm concentration and mobility characteristics, such as velocity and linear velocity. The CASA machine can detect many sperm cell attributes, primarily those associated with motility. While CASA measurements are vital to determining stallion semen quality, they are not a complete picture of sperm cell viability. Results demonstrate that a device difference was discovered in multiple CASA variables. This would suggest that the attributes of both the TrueBreed and the TrueBreed+ improved samples stored and collected in these devices. These attributes include providing a physiologically stable environment for sperm function by controlling the temperature, surface area to volume ratio, pH, and osmotic stress. The TrueBreed and the TrueBreed+ regulate temperature by providing an insulated container that funnels the semen sample to a center well to control the surface area to volume ratio. These devices were designed to be used with a small amount of media, which provides additional protection. The buffers in the media protect the semen from pH shifts and allow for osmoregulation before additional media is added in the laboratory.

The first CASA measurement assessed was concentration. The total number of cells measures a sample's concentration at the time of collection. This is a set number. This number should not fluctuate over time, and the device should have no bearing on this number. Concentration does not account for live or dead cells; it is simply an overall cell count. This measurement was important to determine whether the sample was

adequate to work with and the implication that higher concentration samples could have on other study parameters. As expected, there was no significant difference in concentration over time or device, and no time-by-device interaction since the cell number in the device should remain stable throughout the experiment. As the means indicate, although not significant, there are some differences in the overall concentrations in devices over time. This most likely has to do with how the aliquot was pulled from the sample. Over time spermatozoa cells will settle towards the bottom of the device, and although every attempt was made to have a consistent aliquot, completely identical pulls are not possible.

The second and perhaps most important CASA parameter was motility. Motility is defined by the moving cells to the total concentration. Motility alone does not explain the movement of the cells but how many are moving, and cell movement is correlated to live cells. This is an important parameter as cells must be alive to potentially fertilize an oocyte. The percentage of motile cells is indicative of the quality of the sample. As the results show, this is an expected result of motility decrease over time. As sperm cells die, motility should decrease regardless of the device over time, and motility should decrease. The results indicate this is a sign of ($p < .001$). Although there was no difference in interaction for the device over time, there was a significant difference in the device ($p < .03$). The data for motility had to be normalized since motility numbers per stallion varied.

Once the data was normalized, it demonstrated that the sample collected in both the TrueBreed and the TrueBreed+ had better motility over time. This is indicative of the condition of the improved devices versus the standard. Motility had a rapid drop off after

the12 hour time point, which is to be expected as the cells begin to die. At this point, cells held in the True breed+ had higher motility until 24 hours. This potentially due to the attributes of the TrueBreed+, especially in terms of the added fixed scavengers in the plastic that harvest reactive oxidative species. Therefore, if the reactive oxygen species (ROS) decreases, this should lead to a more stable environment for spermatozoa cells, thus improving overall motility over time, especially in comparison to the standard device. It can be assumed that the original TrueBreed had higher motility than the standard device due to the benefits of the plastic and the device itself, allowing less membrane damage to cells.

The next parameter that CASA assessed was the rapid cell. Rapid cell describes the speed of motile cells by determining their rate of travel in seconds per frame. Every species has a standard, so this measurement compares using the equine standard. The rapid cell can only be measured in motile cells, so once again, there would be a decrease over time in the overall rapid cell, as the cells naturally begin to die. Although there was no interaction between time and treatment, there was a device difference ($p < .025$). The TrueBreed+ device showed superior rapid cell count, up until 24 hours. This measurement is highly correlated with overall motility. Therefore, the components of the TrueBreed+ device appear to be beneficial to spermatozoa cells up until the 24-hour time point. The standard device showed the lowest rapid cell count. The performance of individual stallion spermatozoa can explain this.

Path velocity is the speed of the direction a sperm cell is traveling in a particular direction. While often seen as a positive indicator, this also could be indicative of early capacitation if the sperm cell is traveling too fast or if it is traveling at a normal rate. This

could also be indicative of healthy cellular function. Unfortunately, Path velocity does not distinguish between these two components; rather, it just indicates which were moving at a higher velocity. Once again, over time, we would expect to see a significant difference in path velocity as the cell begins to expire ($p < .001$). While once again, there was no difference in the interaction between time and treatment for path velocity. There was a device difference ($p < .031$). Figure 4.4 suggests a rapid decline in impact velocity once again after 12 hours. This is a continuing trend of all CASA parameters indicative of motility. Means suggest that the TrueBreed device had higher path velocity up until 12 hours, with a spike at 72 hours. I believe that this is indicative of cells traveling at a normal pace. Means also suggest that samples in the standard device had the highest path velocity at 24 and 48 hours, which could be a sign of capacitation earlier than samples in the TrueBreed or TrueBreed+ devices. Once again, the similar components of the TrueBreed and the TrueBreed+ led to overall and proved path velocity compared to the standard device. The TrueBreed and the TrueBreed+ support better overall motility and perhaps delayed capacitation leading to healthier sperm over time.

The CASA machine also measured progression. Progression essentially measures how straight the cell travels, which indicates the cells' fertilization capability and normal cell morphology and movement. Progression is also an indicator of overall motility. Once again, we see a difference over time as expected ($p < .001$). Trends continue to follow the same as overall motility, whereas cells have a continual drop-off after the 12-hour time point. Progression data also suggests that samples held in either the TrueBreed or the TrueBreed+ had better overall progression at the longer time points than samples

collected and stored in the standard device. There was no difference in the interaction between device and time, and there was no significant difference among devices.

Track speed measures curve linear velocity. Unlike progression, which measures the straightness, track speed measures the curve's linear velocity. This could indicate that a cell is moving in a circular direction versus a straighter one. Curvilinear could be seen as a negative parameter of sperm cell quality. Curve linear tracks significantly decrease over time, indicating overall motility ($p < .001$). However, there were no differences in track speed between device and time interaction, nor did we see any difference in track speed among devices. While one may have predicted greater curve linear velocity in samples collected with the standard device, the data did not indicate this. One conclusion that can be drawn from this data is that samples collected in either the TrueBreed or TrueBreed+ did not have increased curve linear, meaning that these devices did not have negatively impact samples for curve linearity.

Elongation is a measurement of the sperm cell head and path velocity. Data demonstrated a significant difference over time expected ($p <. 007$). There was also a device difference for elongation ($p < .05$). Means suggest a slight advantage for samples collected in the TrueBreed+ after 12 hours and the TrueBreed catching up to the TrueBreed+ at 24 hours. Throughout all of these CASA parameters, we continue to see that the original components of the original TrueBreed tend to positively influence all parameters, and the additives in the plus act as a supplement.

Lateral displacement once again is based on motile cells only. This measurement has to do with the head oscillation as the sperm cell swims, which is why the cell must be motile to be measured. The amplitude of the lateral head essentially means the maximum

displacement or distance moved by the cell from its equilibrium. So essentially, this is measuring how far the head swings from its original position. The data revealed no significant difference over time, device, or time-by-device interaction difference.

Beat cross frequency (BCF) measures the rate at which something is repeated. In the case of sperm cell motility, this has to do with how many cells are crossing a particular path within a given time. Beat cross frequency can only measure motile cells and can be a positive indicator for overall spermatozoa health. The data for beat cross frequency showed significance for overall time ($p < .001$), no significance between devices, but BCF had a device and time interaction significance ($p < .031$). While data only gives a significant difference between the standard and the TrueBreed devices ($p < .043$), the TrueBreed+ was very close to the TrueBreed at the nine-hour time point ($p < .06$). Both modified devices outperformed the standard device. Data demonstrated no significant difference between the TrueBreed and the TrueBreed+ for beat cross frequency. Again, I believe this is due to the repeated components found in the original TrueBreed also being expressed in the TrueBreed+. Means also suggests that the TrueBreed+ became the superior device after the 48-hour time point. This could be due to the reactive oxidative species added to this plastic.

I would consider deviation from a straight line, straightness, or linearity to be a negative indicator. Straightness measures the departure of the cell's path from a straight line, whereas linearity measures the departure of the cell's track from the straight line. These two measurements are extremely similar, and both are indicative of motility. I would also expect that straightness and linearity would be highly correlated. As the data suggests, there was no difference in straightness or linearity over time. There was also no

significant difference in linearity or straightness between devices. And there were no significant differences between the interaction of time by device for either linearity or straightness.

Morphology

The morphological number has to do with the percentage of spermatozoa that conform to the shape as accepted as normal. Sperm cells must display normal head shape, midpiece, and tail shape to be classified as morphologically normal. Morphology is most affected during spermatogenesis, which takes place in the seminiferous tubules of the testes and is complete upon entering into the cauda epididymis. Therefore, once the cells are ejaculated, they may or may not have normal morphology, depending on how they developed. This was an important indicator to the study to ensure that the sperm cells had acceptable morphology to apply to other parameters. Results indicated that there was no significant difference in morphology over time. I believe this is expected as most morphological changes would have occurred before the collection. However, that is not to say that some morphological defects cannot happen after ejaculation. And I think we begin to see this as a significant difference in overall morphology between devices ($p <$ .004). There were two very important components to assessing morphology in this study period, the first being when the morphological significance began to occur and the second being what was the morphological defect. The second of these two is answered in subsequent paragraphs. After 48 hours, overall morphology begins to taper off as the samples age. There is also a significant difference at this time between the TrueBreed and the TrueBreed+. However, looking at Figure 4.12 means also suggests that TrueBreed+ was superior to the standard device. This may be attributed to many factors, primarily the

aging spermatozoa cells. Primarily, as cells begin to age, we begin to see shock protein set off, which can cause transformation of the cell membrane leading to the formation, particularly when it comes to the tail as the cell begins to expire. We also see swelling of the head, and we also notice swelling of the midpiece as the mitochondria begin to cease function. I believe that both the TrueBreed and the TrueBreed+ prolonged these events from occurring in comparison to samples that were held in the standard device.

It was important to look at the different morphological defects starting with the head. Data suggests significant head morphological defects among devices ($p < .005$). Means suggest that head defects became significantly more prominent in the standard device, especially after 72 hours. Head defects are also minimized in samples collected in either the TrueBreed or the TrueBreed+ devices. This may be due to their protective capabilities, which have proven to lessen and eliminate osmotic shock during storage. This decrease in osmotic shock potentially protects cells from induced damage.

The second type of morphological defect that was analyzed was the midpiece. Primarily when the midpiece of a sperm cell is discussed, it is often correlated to mitochondrial function. There was an observed difference in midpiece morphology between devices ($p < .011$). Data suggests that the device collected in either the TrueBreed or the TrueBreed+ had substantially less damage to the midpiece over time when compared to the standard device. Midpiece defects were consistently higher in samples collected in the standard device. Samples collected in either the TrueBreed or TrueBreed+ had greater motility and mitochondrial function over time. As a result, I believe this is what we are seeing in reduced midpiece defects over time. Figure 4.14

shows a continuous increase in midpiece defects as time continues. These trends are expected to carry through with mitochondrial function and motility.

The last morphological defect examined was the tail. There was a significant difference in tail defects between devices ($p < .042$). As seen in Figure 4.15, the means suggest that tail defects were consistently higher in samples collected and held in the standard device versus the TrueBreed or TrueBreed+. As sperm cells begin to get stressed, they have changes to their cellular membrane, particularly in their tails. This is commonly displayed as a coiled tail. Tail defects in both TrueBreed devices were minimized until 96 hours. Once again, I think this is indicative of overall spermatozoa health, which can be attributed to the components of the device.

Mitochondria

The mitochondria of a sperm cell are found in the midpiece. Without functional mitochondria, the cell would have no motility. Mitochondria are responsible for creating energy in the form of Adenosine triphosphate (ATP), allowing the sperm cell flagellum to move. Mitochondrial function is critical for motility and, therefore, crucial for fertilization. Samples with cells of higher functioning mitochondria will have greater motility which is highly correlated to fertilization. However, samples with extremely high motility and functioning mitochondria can also experience cellular death rapidly due to the byproducts produced through oxidative respiration. This is where the TrueBreed and the TrueBreed+ play an imperative role. Both devices create a more physiological stable environment allowing for overall improved sperm function and mitochondrial function. As expected, there was a significant difference in mitochondrial function over time ($p < .001$). As the cells do not maintain the same viability over time and begin to perish, the

mitochondrial function will cease. While there was no significant difference in the interaction of time by device for mitochondrial function, there was a significant difference between devices ($p < .001$). Figure 4.16 displays that samples collected in the TrueBreed and TrueBreed+ were superior to those collected in the standard device. A likely reason the TrueBreed+ became superior to the TrueBreed at 48 hours was the fixed scavengers in the plastic that helped reduce ROS, which in turn prolonged the life of the cells. I believe this data suggests that samples collected in the TrueBreed or the TrueBreed+ had an advantage in mitochondrial function over the standard device. Seeing that there is a difference between the plus and the original, it can be assumed that the fixed scavengers in the redesign increased overall mitochondrial health.

Acrosome

The acrosome is a critical component of the sperm cell. If the acrosome becomes compromised, it can affect the fertilization capability of the sperm cell. The acrosomal cap contains numerous hydrolytic and glycolytic enzymes, which are critical for penetrating the oocyte. If the acrosome reacts before being in the vicinity of the egg, fertilization will not be possible for this sperm cell. Therefore, assessing acrosomal integrity is important to determining overall sperm cell quality. Once sperm cells become stressed, they release shock proteins, leading to capacitation and acrosome reaction. Therefore, the longer a sperm cell is in a healthy environment with reduced stress which could be caused by temperature, surface area, pH, or osmotic changes, the better likelihood the sperm cell will have of maintaining an intact acrosome until the time of fertilization. Results demonstrate that overall, acrosomal integrity decreased over time as expected ($p < .001$). While once again, there was no interaction between device and time

for acrosomal integrity, there was a significant difference between devices alone ($p <$ .001). Data suggest that samples collected in either the TrueBreed or the TrueBreed+ were at an acrosomal advantage compared to those collected in the standard device. At six hours, the TrueBreed+ was superior to the TrueBreed up until 72 hours. Differences begin to level off again at 72 hours due to all of the acrosomes slowly reacting, which is a natural process for the cells. It is critical to acknowledge that within the first six hours, the TrueBreed+ outperformed the original TrueBreed. While many components of the TrueBreed and the TrueBreed+ devices are similar, better acrosomal integrity in the TrueBreed+ could be due to the fixed scavengers in the plastic that was not present in the original TrueBreed. This could indicate that reducing ROS in the sample leads to increased acrosomal integrity, eliminating shock and maintaining a more homeostatic environment.

DNA Fragmentation

Sperm cells rely on ATP for energy. Sperm cells use glycolysis and primarily oxidative phosphorylation to produce ATP. As ATP is produced by these two pathways, byproducts such as NADH and FADH 2, along with ROS, are unavoidable during oxygen metabolism. Reactive oxygen species are molecules with unpaired electrons that have high reactivity and search for molecules with which to pair. Reactive oxygen species can react with molecules of the sperm cell structure and cause damage to the cell. These ROS include superoxide anion (O_2^-) hydroxy radical (OH^-) along with many others (du Plessis, 2015).

The goal of creating the TrueBreed+ was to reduce DNA fragmentation by adding fixed scavengers in the plastic to reduce ROS in the sample. Fixing the scavenger

molecule within the container, the scavenger molecules are not free to enter the cell. When scavengers are in media, they rapidly absorb most of the free-oxygen species at first introduction. By having the scavengers within the plastic wall, they will create a constant but limited absorption point and an equilibrium within the semen sample. The sample should remain biochemically stable to prevent a rapid rise or fall in free-oxygen species. More intact membranes mean a longer functional cell life. Fewer reactive species could also mean less DNA damage. DNA fragmentation is the separation or breaking of DNA strands into pieces. When DNA fragmentation occurs, this will compromise the fertilization of an oocyte, thus leading to decreased conceptions if the nuclear membrane becomes compromised. The DNA will fragment and therefore is no longer intact, leading to potential genetic defects or no conception.

The TrueBreed+ with the fixed scavengers was designed to reduce DNA fragmentation. As indicated by the results, there was a significant difference in DNA fragmentation over time ($p < .001$) between devices ($p < .001$), and there was a significant interaction of the device over time ($p < .029$). It is assumed that natural DNA fragmentation will occur as the cells begin to age and become more stressed. However, at the 12-hour time point, there is significantly less DNA fragmentation in both the TrueBreed and the TrueBreed+ samples versus the samples collected in the standard device. At 48 hours, there is an observation where there is only a significant difference between the true breed plus and the standard device. Then, at 72 hours, we once again see a significant difference between samples and the standard device and those collected in either the TrueBreed or the TrueBreed+. While both the TrueBreed and the TrueBreed+ maintain a more physiological stable environment, thus reducing overall stress and

maintaining optimal pH, the TrueBreed+ with the fixed scavengers reduced DNA fragmentation to a greater degree, up to 48 hours. This is significant because stallion semen should be bred within 48 hours of collection. Since TrueBreed+ performed the best at this point, this should lead to greater conception rates. As the cells begin to age and become more damaged, we see the TrueBreed level with the TrueBreed+.

Conclusions

Previous research has determined that semen samples collected in the original TrueBreed device demonstrated superior semen quality over time. This study further supports this previous research; stallion semen samples collected and held in either the TrueBreed or the TrueBreed+ device improved quality in almost all semen quality parameters. This study also determined that samples collected in the TrueBreed+ upheld the integrity of the original TrueBreed device. Furthermore, this study aimed to determine if the redesign of the TrueBreed would harvest reactive oxygen species, thus reducing DNA fragmentation. Results suggest that DNA fragmentation was reduced in samples collected and held in the TrueBreed+ device. However, it should be noted that DNA fragmentation was also reduced in the original TrueBreed device compared to the standard device. While both devices significantly reduced DNA fragmentation compared to the standard device, it is the TrueBreed+ that surpassed the original TrueBreed at the 48-hour time point. Therefore, it can be concluded that both devices reduce DNA fragmentation, with the TrueBreed+ having a slight advantage at particular time points. While the original TrueBreed did not have the fixed scavengers in the plastics, its other protective qualities, such as a reduction in surface area exposure, increase in temperature regulation, added nutrients, and reduction of osmotic shock, not only protect the cells

from shock and induced damage but also protect the nuclear membrane and reduce DNA fragmentation, thus producing more intact DNA over a prolonged time.

A significant role not only in maintaining the viability and quality of the sperm but also in ensuring fertilization is the pH of the seminal fluid (Zhou et al., 2015). Upon reflection of this study, a few improvements could be made in the future. One improvement would be to test for pH, as pH shifts in semen occur as sperm cells continue to metabolize and produce byproducts that alter pH making it more acidic. Testing pH changes in every device at every time point could be helpful to determine if the fixed scavengers of the TrueBreed+ help reduce pH drops

A second improvement would be how the samples were collected from each stallion. The 6666's ranch graciously donated collections before breeding season. These collections are what the industry considers *clean out* collections. Typically, the first stallion collection of the year is regarded as a poor sample since the stallion has not been cleaned out for many months. Stallions were collected in one of the three devices based on randomization. However, upon the first collection from each stallion, regardless of the device samples were collected in, were subpar in comparison to subsequent collections. Therefore, the study could be improved if each stallion was cleaned out three times before using samples. This would have put all collections and devices on a more even playing field.

In conclusion, modification of the standard stallion collection device improved semen parameters over extended periods. The results also suggest that collecting and holding stallion semen in a device provides the sample with a more physiologically optimal environment by reducing surface area, regulating temperature, providing

nutrients, and reducing shock, increasing the viability of the sperm cells, thus improving the chance of fertilizing an oocyte. Furthermore, the modification of the TrueBreed to the TrueBreed+ that adds fixed scavengers appears to reduce DNA fragmentation in cells collected and held in this device.

The capability of improving semen quality parameters in stallions would have several implications for evaluating semen and artificial insemination. First, the environment provided by TrueBreed+ would allow a technician to provide a more accurate analysis of the stallion semen by reducing damage during collection and processing. Reduction in damage invitro would give a more precise assessment of stallion sperm in vivo. Second, stallions with low quality or even sub-fertile samples might now have acceptable quality for artificial insemination and give cells a better survival rate for cryopreservation, which may not have been possible before. Finally, samples collected in the TrueBreed+, which reduced DNA fragmentation, should not only increase conception rates but should, in theory, result in increased overall full-term gestations leading to an increase in offspring from the stallion. Further studies, including evaluating pregnancy rates in mares, should take place to confirm these observations.

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APPENDIX A

STANDARD DEVICE

APPENDIX B

TRUEBREED

APPENDIX C

TRUEEBREED+

APPENDIX D

PATENTS APPROVAL

UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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DICKINSON WRIGHT PLLC - WASHINGTON, DC
1825 EYE ST., NW
SUITE 900
WASHINGTON, DC 20006

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Patents Stakeholder Experience (OPSE), Stakeholder Support Division (SSD) at (571)-272-4200.

INVENTOR(s) (Please see PAIR WEB site http://pair.uspto.gov for additional inventors):

Samuel D. Prien, Shallowater, TX;
Lindsay L. Penrose, Lubbock, TX;
Diana M. Peninger, Flower Mound, TX;

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Texas Tech University System, Lubbock, TX;
RSI Technology Group, LLC, Dallas, TX;

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