Effect of Sperm Insemination Dose on Fertility and Use of *in vitro* Sperm Analyses to Explain Fertility Differences of Angus Sires in Brazilian Beef Cattle

A Thesis

Presented in Partial Fulfillment of the Requirements for the

Degree of Master of Science

with a

Major in Animal Science

in the

College of Graduate Studies

University of Idaho

by

Saulo Menegatti Zoca

Major Professor: Joseph C. Dalton, Ph.D.

Committee Members: Amin Ahmadzadeh, Ph.D.; Ahmed Tibary, DVM

Department Administrator: Amin Ahmadzadeh, Ph.D.

May 2018

Authorization to Submit Thesis

This thesis of Saulo Menegatti Zoca, submitted for the degree of Master of Science with a Major in Animal Science and titled "**Effect of Sperm Insemination Dose on Fertility and Use of *in vitro* Sperm Analyses to Explain Fertility Differences of Angus Sires in Brazilian Beef Cattle**," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor: _____Date_____

Joseph C. Dalton, Ph.D.

Committee members: _____Date_____

Amin Ahmadzadeh, PhD.

_____Date_____

Ahmed Tibary, DVM

Department _____Date_____

Administrator: Amin Ahmadzadeh, PhD.

ABSTRACT

The objectives of the first study were, primarily, to determine if beef bull fertility varied by number of sperm inseminated and, secondarily, to characterize the potential impact of random variation through the use of two identical sperm per dose treatments, which differed only by straw color. In the second (*in vitro*) study, the primary objective was to identify sperm characteristics associated with fertility differences between bulls from the field fertility trial. A secondary objective was to investigate the pattern and variability of *in vitro* sperm characteristics using principal component analysis. In the first study, although fertility differences between bulls were detected, fertility following timed artificial insemination with 10, 20, 20 or 40×10^6 sperm per dose resulted in a similar proportion of cows pregnant per timed artificial insemination (P/TAI) at first service in synchronized beef cattle. Although the overall P/TAI between the two control groups (20×10^6 sperm per dose) were not different, the numerical variation within bull ranged from 0.5 to 4.9 percentage points, providing evidence that variation in reproductive field trials should not be ignored. In the second study, morphologic evaluation, computer-assisted sperm analysis and flow cytometry were not able to explain the difference in field fertility between bulls. Principal component analysis simplified the complexity of data from computer-assisted sperm analysis and flow cytometry and allowed for the identification of key sperm characteristic variables. The use of principal component analysis should be investigated further, as it allows for the grouping and identification of key sperm characteristics, and provides a visual aspect to understanding variability.

ACKNOWLEDGEMENTS

I would like to thank my Major Professor, Dr. Joseph C. Dalton, for his support, time and dedication during my master's program. Dr. Dalton's help writing this thesis, conference abstracts and manuscripts, and to prepare seminar presentation slides was essential for my learning.

I would like to thank my committee members Dr. Amin Ahmadzadeh and Dr. Ahmed Tibary. I am grateful to have such wonderful committee members that supported and helped me during my master's program.

I would like to express my appreciation to Select Sires, Inc (Plain City, OH, USA) for their support and funding of my projects, as well as all their staff involved with preparation, logistics and equipment utilization. I would like to especially thank Dr. Cruppe, Mr. DeJarnette, Dr. Harstine, Dr. McDonald and Dr. Utt for their help during each of my projects. Also, I would like to thank Select Sires do Brasil (Porto Alegre, RS, Brazil) for facilitating the field fertility trial in Brazil.

Additionally, I would like to thank Dr. Vasconcelos (FMVZ – UNESP – BR) who was my advisor during my veterinary degree in UNESP and provided me the opportunity of coming to the USA for an internship with Dr. Dalton. Also, I would like to thank Dr. Vasconcelos and Dr. Cruppe for their support in the field fertility trial.

Finally, I would like to thank all the collaborating farms in Brazil and personnel involved in the fertility trial; without their help the project would not have been possible.

DEDICATION

I would like to dedicate this thesis to all the people who made it possible, including my family, advisor and friends. Primarily, I would like to thank and dedicate this thesis to my parents, my father Moises Zoca and my mother Regina A. Menegatti Zoca, and my siblings, Samuel Menegatti Zoca and Sara Menegatti Zoca, whose emotional and financial support were indispensable for me in pursuing my dream of receiving my Master of Science degree (in Animal Science) in the USA. Secondly, I would like to thank my advisor Dr. Joe Dalton for the scientific support, friendship and patience since my first time working with him in 2014. Finally, I would like to especially thank, Dr. Amin Ahmadzadeh, Anna Rodriguez, Jenn Spencer, Jim Vinyard (my roommate and also his dog Sully), Kim Davenport, Maggie Reynolds, McKenzie Corpron, Toni Colacchio and other fellow grad students, as well as faculty and staff from the University of Idaho Animal and Veterinary Science Department for the friendship and support. Also, all my Brazilian and international friends, from Moscow, ID and Pullman, WA, who had to listen to all my palpation stories.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABREVIATIONS

AC	Acre
acrosome	intact acrosome
AI	artificial insemination
ALH	amplitude of lateral head displacement
ANCa	Within sperm population with intact plasma membranes, sperm with intact acrosome and normal calcium influx
AO	acridine orange
BCF	beat cross frequency
BCS	body condition score
BR-State	Brazilian state
BSA	bovine serum albumin
CASA	computer-assisted sperm analysis
CWE	carcass-weight equivalent
d	days
D0	day zero
D7	day seven
D8	day eight

D9	day nine
D10	day ten
D11	day eleven
DFI	DNA fragmentation index
DIC	differential interference contrast
EB	estradiol benzoate
eCG	equine chorionic gonadotropin
ECP	estradiol cypionate
FC	flow cytometry
Fluo-3	fluo-3-acetomethoxy ester
FSH	follicle stimulating hormone
GDP	gross domestic product
GnRH	gonadotropin releasing hormone
GO	Goias
IVF	*in vitro* fertilization
LH	luteinizing hormone
LIN	linearity
MT	Mato Grosso

P/TAI	proportion of cows pregnant per timed-artificial insemination
PCA	principal component analysis
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PI	propidium iodide
PM	progressive motility
PNA	fluorescein isothiocyanate-conjugated peanut agglutinin-647
Prin1	principal component 1
Prin2	principal component 2
Prin3	principal component 3
SEM	standard error of the mean
SM	subjective motility
STR	straightness
TAI	timed-artificial insemination
TM	total motility
VA	intact plasma membrane with intact acrosome
VANCa	intact plasma membrane with intact acrosome and normal calcium influx
VAP	average path velocity

viable	intact plasma membrane on multiple stain analysis
viablePI	intact plasma membrane on dual stain analysis
VNCa	intact plasma membrane with normal calcium influx
VSL	straight line velocity
WOB	wobble

CHAPTER 1

"Literature review"

Introduction to the Brazilian beef cattle industry

The Brazilian gross domestic product (GDP) in 2015 was valued at approximately US$1.8 trillion (ABIEC, 2016). Livestock was valued at approximately US$122 billion, or approximately 30% of Brazil's total agribusiness GDP (6.8% of the total GDP; ABIEC, 2016).

According to the Brazilian Beef Exports Association (ABIEC, 2015) the cattle herd in Brazil totaled approximately 209 million animals with a stocking rate of 1.25 animals per hectare (0.51 animals per acre). The number of animals slaughtered for beef, and the dressed carcass weight has increased in almost linear fashion the past 19 years (206% and 225%, respectively; IBGE, 2015). Of 39.16 million animals slaughtered, Brazil produced 9.56-million-ton carcass-weight equivalent (CWE; IBGE, 2015). Approximately 20% of beef produced in Brazil was exported (ABIEC, 2016) and in 2017, Brazil and India ranked as the top beef exporters in the world (MICA, 2018). Five countries, Hong Kong, Egypt, Russia, China, and Venezuela received 57.4% of Brazilian beef exports in 2015 (ABIEC, 2016). Livestock-related exports generated US$5.9 billion in revenue in 2015 (ABIEC, 2016).

The majority of the Brazilian cattle herd is *Bos indicus*, with 80% of the beef cattle being Nelore (approximately 100 million animals; ABCN, 2018). The use of Nelore animals in Brazil started between 1868 and 1878 (OK State, 2018). The original Nelore animals were then known as Ongole, and were imported from India (OK State, 2018). The Nelore has since been incorporated in the Brazilian culture as stated by the Brazilian Association of Nelore Breeders: "The Brazilian Nelore, besides being considered as national patrimony, like

Carnival, soccer, caipirinha and barbecue, can be considered as a big victory of the Brazilian beef industry. Healthy and natural beef, exported to more than 146 countries with increasing demand by enlightened consumers around the world" (ABCN, 2018).

According to the Brazilian Association of Artificial Insemination (ASBIA, 2016), the total number of beef cattle semen doses sold in 2015 was 8.3 million, representing 65.9% of total doses sold in Brazil. Angus represented 47% of beef cattle semen doses sold, or approximately 3.9 million doses. Also, the Brazilian beef cattle industry imported approximately 1.7 million semen doses in 2015, with the majority being Angus semen from the United States (ASBIA, 2016).

It has been reported that *Bos taurus* beef exhibits greater marbling and fat thickness and is more tender than *Bos indicus* beef (Duarte et al., 2013; Martins et al., 2015; Rodrigues et al., 2017). Consequently, the use of crossbred Angus × Nelore cattle has resulted in greater tenderness compared to Nelore cattle (Lage et al., 2012). Due to the Nelore influence in the Brazilian beef cattle industry and its adaptability to Brazil, it would be almost impossible to use pure bred *Bos taurus* animals in most of the country. Accordingly, to satisfy the desire of consumers for greater tenderness and flavor (due to marbling), the use of crossbred animals, mainly, F1 Angus × Nelore has been incorporated in the Brazilian beef cattle industry, evidence of which can be recognized through the recent use of Angus semen.

Spermatogenesis

Male fertility plays an important role in the success of reproduction. The quality and quantity of inseminated sperm will be described throughout this thesis. However, to understand the importance of sperm to fertility it is necessary to understand spermatogenesis.

Spermatogenesis includes all cell transformation in the production of sperm. In bulls, it takes approximately 61 days for spermatogonia to be transformed into spermatozoa and for spermiation to occur (Senger, 2012). Spermatogenesis occurs within the seminiferous tubule in the testes. In the bull, normal spermatogenesis requires a temperature lower than body temperature (4-6^{o}C lower; Senger, 2012). To achieve the necessary temperature for normal spermatogenesis, the testes are contained within the scrotum, which contains many sweat glands (Senger, 2012). The tunica dartos muscle, which lies below the scrotal skin, also plays a role in thermoregulation, relaxing during periods of heat stress, thereby increasing the surface area of the scrotum (Senger, 2012). The pampiniform plexus allows for the exchange of heat from the testicular artery to veins, resulting in the cooling of arterial blood on the way to the testis (Senger, 2012).

The Leydig cells in the testis have receptors and are stimulated by luteinizing hormone (LH) which is released from the anterior pituitary after gonadotropin releasing hormone (GnRH) stimulation (Senger, 2012). Leydig cells produce progesterone (following LH receptor stimulation), which is converted to testosterone. The pulsatile nature of LH prevents Leydig cells from becoming refractory to LH stimulation, and consequently reduces the chance of impaired testosterone production. Testosterone concentration in the testis is 100-500 times higher than arterial blood entering the testes due to exchange of testosterone in the pampiniform plexus (Senger, 2012). A high concentration of testosterone in the testis is important for normal spermatogenesis, while a lower concentration in the peripheral blood avoids down-regulation of the gonadotropin releasing hormone (GnRH)/LH system (Senger, 2012).

The function of Sertoli cells is dependent on follicle stimulating hormone (FSH). As described by Senger (2012), the pulsatile nature of testosterone is thought to allow negative feedback on FSH to be removed, avoiding chronically high testosterone, which suppresses FSH. Sertoli cells convert testosterone to estradiol, however, the role of estradiol in the male is not clear. Undoubtedly, estradiol negatively feeds back on the hypothalamus and GnRH, to reduce LH and FSH. Sertoli cells also produce inhibin which acts to directly suppress FSH secretion from the anterior pituitary. Although the importance of the inhibin-FSH relationship in the male is not clear (Senger, 2012), it is thought inhibin suppresses spermatogenesis, as long-term immunization of bulls against inhibin during pre- and post-pubertal development resulted in increased sperm output in most treated bulls (Bame et al., 1999).

Spermatogenesis can be divided in three phases: spermatocytogenesis (proliferation), meiosis, and spermiogenesis (differentiation). During spermatocytogenesis, spermatogonia undergo mitotic divisions, generating B spermatogonia, which following another mitotic division become primary spermatocytes (Senger, 2012). The process continues indefinitely as a pool of stem cells is maintained from which A spermatogonia arise (Senger, 2012).

The meiotic phase begins with primary spermatocytes. Genetic diversity is ensured by meiosis as complete DNA replication and crossing over of DNA occurs, resulting in random assortment of different segments of each chromosome (Senger, 2012). After the first meiotic division, the primary spermatocyte becomes the secondary spermatocyte. The second meiotic division results in the formation of haploid spermatids (Senger, 2012).

The final phase of spermatogenesis is known as spermiogenesis. During spermiogenesis, spermatids are morphologically transformed into spermatozoa (Senger, 2012). In the end of this process, spermatozoa will be composed of a head and a tail. The head

includes the nucleus, acrosome, and post-nuclear cap, while the tail (flagellum) includes the midpiece (which contains the mitochondrial helix), principle piece and terminal piece (Senger, 2012).

The formation of the acrosome starts during the Golgi phase. As described by Senger (2012) small vesicles of the Golgi fuse resulting in proacrosomic granules. Continued vesicle fusion results in a large acrosomic vesicle that contains an acrosomic granule. Further development of the acrosome occurs during the cap phase, in which the acrosomic vesicle spreads over the nucleus (Senger, 2012). Then, during the acrosomal phase, the nucleus and cytoplasm elongate (Senger, 2012). As described by Senger (2012) the acrosome covers the anterior two-thirds of the nucleus. The acrosome has a key role in fertilization, as it contains numerous enzymes (acrosin, hyaluronidase, zona lysin, esterases, and hydrolases) required for attachment and penetration of the oocyte's zona pellucida (Senger, 2012). Finally, the maturation phase is where mitochondria are assembled around the flagellum, and where dense outer fibers and the fibrous sheath are produced. The entire sperm is covered with a plasma membrane, which is required for survival and function of sperm (Senger, 2012).

Chromatin (DNA) condensation in the nucleus of sperm is due to the replacement of histones by protamines, keratinoid proteins with high levels of disulfide cross-linking (Miller et al., 2010; Senger, 2012). During this process the DNA in the nucleus of sperm becomes compact and almost inert (Miller et al., 2010; Senger, 2012). The process of chromatin condensation is thought to protect DNA between spermiation and fertilization, and optimize nuclear shape to support appropriate sperm motility (Miller et al., 2010). After fertilization the DNA must be able to undergo decondensation in order to form the male pronucleus and the zygote (Senger, 2012).

Spermiation occurs with the release of fully differentiated sperm from Sertoli cells into the lumen of the seminiferous tubule (Senger, 2012). Final maturation and concentration of sperm occurs in the epididymis. Epididymal transit time (through the caput, corpus and cauda epididymis) in bulls is 14 days (Senger, 2012). Sperm undergo maturation during epididymal transit, including acquisition of progressive motility, final condensation of the nucleus, alteration of the plasma membrane, and migration of the cytoplasmic droplet from a proximal to a distal position (Barth and Oko, 1989). Absorption of seminiferous and rete fluid also occurs in the epididymis, concentrating the sperm in an environment with a slight oxygen deficiency to inhibit sperm metabolism (Barth and Oko, 1989). Sperm storage occurs in the cauda epididymis until ejaculation occurs (Senger, 2012). During epididymal transit sperm gradually gain fertilization capacity, as sperm in the caput epididymis are unable to fertilize ova, yet sperm from the distal corpus and cauda epididymis have the ability to fertilize, and can be used for in vitro fertilization (IVF) or artificial insemination (AI) (Barth and Oko, 1989).

Semen extension, semen cryopreservation, and artificial insemination

In 1940 Phillips and Lardy described the use of egg yolk as a semen extender to protect sperm from damage during cooling, allowing fresh semen to be preserved and used over a few days. The addition of antibiotics (Almquist et al., 1946; 1949) to semen resulted in a 10% increase in fertility, while Polge et al. (1949) and Polge and Rowson (1952) studied long-term cryopreservation of semen with glycerol as a cryoprotectant. Since then a variety of cells and tissues have been frozen. Consequently, the efficient storage of sperm from genetically superior animals has allowed significant advancements in agriculture, including the

international exchange of genetics and further development of reproductive biotechnologies (Vishwanath, 2003; Woods et al., 2004).

Semen cryopreservation has resulted in increased use of AI in beef and dairy cattle. Despite loss of viable sperm to cryopreservation, the cryopreservation protocol hasn't changed significantly in the last 40 years (Gunasena and Critser, 1997; Parrish et al., 1986; Robbins et al., 1976). Nevertheless, the use of cryopreserved semen in AI and IVF has shown satisfactory fertility rates in many species, including the bovine and humans (Holt, 2000).

The use of AI in *Bos indicus* cows, especially in Brazil, following estrous detection is challenging. According to Meneghetti and Vasconcelos (2008) *Bos indicus* cattle have a longer anestrous period postpartum compared with *Bos taurus* which contributes to poor reproductive efficiency. Also, estrous duration is shorter in *Bos indicus* than *Bos taurus* (Pinheiro et al., 1998). Combined, the increase in postpartum anestrous period and shorter duration of estrus lead to an increase in calving interval. The introduction of estrous synchronization protocols with fixed-timed AI (TAI), however, has eliminated the necessity for estrous detection in beef cattle and has the potential to shorten the calving season (Lamb et al., 2010) by allowing for insemination of both anestrous and cycling cows via TAI (Vasconcelos et al., 2017).

The most common TAI protocol in Brazil begins with the use of an intravaginal progesterone insert and an injection of estradiol benzoate on the first day of the protocol (Bó et al., 2007; Meneghetti et al., 2009; Sá Filho et al., 2010; 2011; Sá Filho et al., 2009). Progesterone insert removal usually occurs eight (Sá Filho et al., 2010; 2011) or nine days (Meneghetti et al., 2009; Sá Filho et al., 2009) after insertion, and is coupled with an injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and estradiol cypionate. Timed AI occurs 48 to 60 hours after

progesterone insert removal (Meneghetti et al., 2009; Sá Filho et al., 2010; 2011; Sá Filho et al., 2009).

Meneghetti et al. (2009) reported an increase in pregnancy rates for cyclic cows (presence of corpus luteum at the beginning of the protocol) when the prostaglandin injection of the protocol was administered on day seven compared to day nine. Improved fertility at TAI (Sá Filho et al., 2009) has also been reported in lactating *Bos indicus* cows following a) temporary calf removal (approximately 48 to 60 h) from progesterone device withdrawal to TAI, or b) the injection of equine chorionic gonadotropin (eCG) at progesterone insert removal. Although TAI protocols do not require estrous detection, estrous expression prior to TAI has been reported to positively influence pregnancy results (Richardson et al., 2017; Sá Filho et al., 2010; 2011). For example, Sá Filho et al. (2010) reported cows that displayed estrus prior to TAI were 3.3 times more likely to become pregnant than those that did not display estrus. Other factors have been reported to influence fertility at TAI including body condition score (BCS; Meneghetti et al., 2009; Sá Filho et al., 2010; Sá Filho et al., 2009; Vasconcelos et al., 2017), follicle diameter at TAI (Meneghetti et al., 2009; Sá Filho et al., 2010; Sá Filho et al., 2009), breed (Sá Filho et al., 2009), parity (multiparous vs. primiparous vs. heifers; Sá Filho et al., 2009), sire (Flowers, 2013; Sá Filho et al., 2009) and AI technician (Dalton et al., 2004; Sá Filho et al., 2009).

Sperm evaluation

Fertility is multifactorial and depends on a complex series of events, including the ability of sperm to survive, navigate the female barriers and complete fertilization (Rodríguez-Martínez, 2003; Saacke, 2008; Vincent et al., 2012). For successful fertilization and acceptable fertility, sperm must have normal morphology, high progressive motility, intact

membranes, stable DNA, and the ability to undergo capacitation (Garner, 2014; Malama et al., 2017; Rodríguez-Martínez, 2003; Saacke, 2008; Vincent et al., 2012). The efficiency or ability of sperm to fertilize the oocyte after AI is also dependent on many factors including appropriate semen storage and handling, accurate timing and method of insemination, female health and management, and cryopreservation and thawing (Dalton et al., 2017; Saacke, 2008; Vincent et al., 2012).

It is the responsibility of AI centers to accurately evaluate and assure the quality of sperm to be sold, before and after cryopreservation (DeJarnette, 2012; Vincent et al., 2012). Pre-freeze evaluations of an ejaculate generally include volume (based on weight), sperm concentration and progressive motility (Rodríguez-Martínez, 2003). Pre-freeze evaluations influence the number of insemination doses that may be produced from an ejaculate (DeJarnette, 2005). The rate of cooling and the quality and type (e.g., egg-yolk or milk-based) of extenders used are important, and affect the quality of sperm after thawing (Karabinus et al., 1991; Vincent et al., 2012; Zorzetto et al., 2017). Post-thaw evaluations generally include progressive motility, morphology, and membrane and DNA integrity (Rodríguez-Martínez, 2003; Garner, 2014).

Basic requirements for semen analyses are objectivity, repeatability and accuracy (Rodríguez-Martínez, 2003). Even newly developed computerized systems are not exempt from human bias, which contributes to a decrease in objectivity. Repeatability is increased when the number of sperm analyzed increases, e.g., thousands of sperm in a flow cytometer (FC) compared to a few hundred with a microscope. Accuracy increases when more attributes are assayed in the same population compared to a single measurement, e. g, the use of multiple fluorescent probes in FC compared to a single probe, or the use of a combination of

morphology, computer-assisted sperm analyses (CASA) and FC to evaluate the same sample (Rodríguez-Martínez, 2003).

Subjective assessment of sperm characteristics through the use of light microscopes has been the standard for commercial AI centers for many years (DeJarnette, 2005; Vincent et al., 2012). Recently, the use of more sophisticated technologies such as FC and CASA has been adopted to decrease subjectivity (Malama et al., 2017; Vincent et al., 2012). Also, the analysis of sperm becomes more representative of the population and more accurate with an increase in the number of sperm evaluated, from 100 to 200 with the light microscope, to several hundred with CASA, and several thousand with the FC (Garner, 2014). The adoption of new technologies (CASA and FC), however, must be considered carefully. As argued by DeJarnette (2005; 2012) "does the new technology result in additive value over prior technology, or is the new technology only a more expensive way to measure the same characteristic?" Commercial AI centers and producers must remember it is important to provide a product that will achieve the producer's expectation of fertility, while also producing enough straws that will meet demand (DeJarnette, 2005; 2012). Therefore, the number of sperm per dose must meet fertility expectations and at the same time be profitable for the seller (DeJarnette, 2005; 2012). Consequently, AI centers strive to efficiently use collected sperm and maximize fertility of each bull while avoiding an excess in sperm number per dose that may limit supply (Amann and DeJarnette, 2012; Dalton et al., 2017). This must be done while remembering the minimum number of viable sperm required for maximum fertility differs among bulls, as does the rate at which maximum fertility is achieved with increasing sperm dosage (den Daas et al., 1998; Salisbury and VanDemark, 1961).

Even with the most current sperm analyses we are only able to explain approximately 50-60% of the variability in bull fertility (Saacke, 2008). Previous authors have reported correlations of semen characteristics with bull fertility (reviewed by Rodríguez-Martínez, 2003); however, the results are variable. The estimation, but not prediction, of a bull's fertility potential may be partially achieved with laboratory analyses, however, the use of hundreds of inseminations and the precise recording and analyses of field fertility data are still necessary (Foote, 2003; Rodríguez-Martínez, 2003; Saacke, 2008; Utt, 2016).

Commercial AI centers use quality control analyses to assure that consumers, either beef or dairy, constantly receive semen that meets or exceeds their expectations (DeJarnette, 2012). Techniques to predict bull fertility have been studied for nearly a century and we still have not achieved the goal of fertility prediction (Amann and Hammerstedt, 1993). In order to determine whether a bull or ejaculate is acceptable, commercial AI centers routinely evaluate sperm for motility and morphology which are known to be associated to fertility. While the use of sperm analyses can currently determine infertile or subfertile bulls (or ejaculates) due to known characteristics, difficulty remains in the prediction of fertility because of unknown characteristics important to fertility that may not be assessed (DeJarnette, 2005; Dalton, 2010).

Sperm morphology can be assayed by multiple methods, however, it is most common to use differential interference contrast (DIC) microscopy (Barth and Oko, 1989). Saacke (2008) stated that morphologically abnormal sperm may represent the "tip of an iceberg" in cattle reproduction, as we remain unsure what lies below the surface. Saacke et al. (1998) provided evidence that sperm with misshapen heads, and impaired or abnormal sperm motility based on head morphology, are unable to reach the fertilization site. Although

morphologically abnormal sperm may contribute to decreased fertility, misshapen heads, tail and midpiece defects are considered "compensable," as fertility may be increased by an increase in the total number of sperm inseminated (Saacke, 2008). Compensable seminal traits, therefore, are related to sperm transport as well as the capacity to initiate fertilization and prevent polyspermy (Saacke et al., 2000).

Sperm head evaluation is important because of the presence of DNA, which is necessary for formation of the zygote and further embryo development (Garner, 2014; Saacke, 2008). There is evidence both morphologically abnormal and normal sperm may exhibit defective DNA, and therefore be a cause of subfertility in bulls (Garner, 2014; Rodríguez-Martínez, 2003; Saacke, 2008). Defective DNA is considered to be an "uncompensable" trait as fertility remains independent of the number of sperm inseminated (Saacke, 2008). In the case of uncompensable traits, affected sperm are able to reach the oocyte, penetrate the zona pellucida, and prevent polyspermy, however, the ability of the fertilizing sperm to complete fertilization (decondensation of chromatin and formation of a pronucleus) or sustain embryogenesis is impaired (Saacke, 2008). To summarize compensable and uncompensable traits, differences in fertility among males responsive to increased sperm dosage are considered "compensable," whereas those not responsive to increased sperm dosage are considered "uncompensable," as originally described by Saacke et al. (1994).

One of the newest methods of sperm evaluation is CASA. In order to detect sperm movement, CASA takes consecutive pictures (30 - 60 pictures per second) in multiples frames (3 to 8), and analyzes hundreds of sperm by following movement across the images. Numerous sperm motion characteristics are reported by CASA including percentage of total motile sperm, and progressively motile sperm; three types of velocity, average pathway

(VAP), curvilinear (VCL) and straight line (VSL); lateral head displacement (ALH), beat cross-frequency (BCF) and the percentage of linearity (LIN; VSL/VCL × 100), straightness (STR; VSL/VAP × 100) and wobble (WOB; VAP/VCL × 100; Mortimer, 2000; Vincent et al., 2012).

Although CASA may be more accurate than subjective assessment of motility with a light microscope, some limitations have to be considered when working with CASA. For example, the type of chamber, as well as the temperature at which analyses are carried out may give different results, so, it is important to keep chamber type and temperature constant across samples. Results from CASA may also be affected by the extender used (Vincent et al., 2012). In some cases, it is necessary to use a fluorescent probe (e.g. Hoechst 33342) to stain the DNA in order to discriminate sperm from extender debris (Vincent et al., 2012). The concentration of the sample tested also must be considered, i.e., a low dose (less than 20×10^6 sperm/mL) or high dose (more than 50×10^6 sperm/mL) may overestimate or underestimate motility, respectively (Vincent et al., 2012).

The use of fluorescent probes permits the assessment of various sperm functions with fluorescence microscopy and FC. The advantage of using FC over traditional microscopy is the ability to interrogate several thousand sperm regarding multiple attributes on the same sperm in contrast to a few hundred sperm (Garner, 2014). Lasers and photomultipliers allow the FC to rapidly read fluorescently labelled cells (Garner, 2014; Vincent et al., 2012).

The sperm is divided into several compartments (nuclear, acrosomal and plasma membranes, midpiece, tail and cytoplasmic droplet) each with a distinct function and method of analysis (Garner, 2014). Common FC analyses include: integrity of plasma membrane, acrosome and DNA; calcium influx; mitochondrial membrane potential; and reactive oxygen

species (Garner, 2014; Malama et al., 2017; Rodríguez-Martínez, 2003; Vincent et al., 2012). An intact plasma membrane is important because of its role in cell boundary and cell-to-cell interaction. An intact acrosomal membrane is important for interaction between the sperm and the zona pellucida of the oocyte. Calcium influx is used to estimate the level of early capacitation caused by the cryopreservation process (Garner, 2014; Vincent et al., 2012), while mitochondrial membrane potential and reactive oxygen species are related to longevity and the ability of sperm to undergo hyperactivation (Aitken et al., 2014; Amaral et al., 2013; Marques et al., 2014; O'Flaherty et al., 2006).

The analyses of multiple sperm characteristics simultaneously through the use of multiple probes increases the ability of detection of subfertile semen samples. For example, the use of plasma membrane and acrosomal membrane probes [Propidium iodide + fluorescein isothiocyanate-conjugated peanut agglutinin-647(PNA)] results in four distinct populations: 1) plasma membrane and acrosomal membrane intact, 2) plasma membrane and acrosomal membrane disrupted, 3) plasma membrane intact and acrosomal membrane disrupted, and 4) plasma membrane disrupted and acrosomal membrane intact (Garner, 2014; Malama et al., 2017). Although it is possible to use more probes together, there is a practical limitation to the number of lasers, photomultipliers and excitability of the probes to be used, due to the fact that different probes may emit the same color at the same wave length, causing difficulty in understanding the results (Garner, 2014).

Literature cited

Aitken, R. J., T. B. Smith, M. S. Jobling, M. A. Baker, and G. N. De Iuliis. 2014. Oxidative stress and male reproductive health. Asian J. Androl. 16:31-38. doi:10.4103/1008-682X.122203

Almquist, J. O., W. T. S. Thorp, and C. B. Knodt. 1946. The effect of penicillin upon the livability, fertility and bacterial content of bull semen. J. Anim. Sci. 5:400.

Almquist, J. O., P. J. Glantz, and H. E. Shaffer. 1949. The effect of a combination of penicillin and streptomycin upon the livability and bacterial content of bovine semen. J. Dairy Sci. 32:183.

Amann, R. P., and R. H. Hammerstedt. 1993. In vitro evaluation of sperm quality: An opinion. J. Androl. 14(6):397-406.

Amann, R. P., and J. M. DeJarnette. 2012. Impact of genomic selection of AI dairy sires on their likely utilization and methods to estimate fertility: A paradigm shift. Theriogenology. 77:795–817. doi:10.1016/j.theriogenology.2011.09.002

Amaral, A., B. Lourenço, M. Marques, and J. Ramalho-Santos. 2013. Mitochondria functionality and sperm quality. Reproduction. 146:R163-R174. doi:10.1530/REP-13-0178

Associação Brasileira de inseminação artificial (ASBIA). Index ASBIA 1º semestre 2016 (press release, received in December 21, 2017). http://www.asbia.org.br/

Associação Brasileira dos criadores de Nelore (ABCN). http://www.nelore.org.br/Raca/ Accessed April 3, 2018.

Associação Brasileira das indústrias exportadoras de carne (ABIEC). Brazilian livestock profile. http://www.newsprime.com.br/img/upload2/perfil-abiec-set2015-ingles.pdf/ Accessed April 3, 2018.

Associação Brasileira das indústrias exportadoras de carne (ABIEC). Brazilian livestock profile. http://www.newsprime.com.br/img/upload2/2016_FolderPerfil_EN.pdf/ Accessed April 3, 2018.

Barth, A. D., and J. Oko. 1989. Abnormal morphology of bovine spermatozoa. Iowa State University Press, Ames.

Bó, G. A., L. Cutaia, L. C. Peres, D. Pincinato, D. Maraña, and P. S. Baruselli. 2007. Technologies for fixed-time artificial insemination and their influence on reproductive performance of *Bos Indicus* cattle. Soc. Reprod. Fert. Suppl. 64:223-236.

Dalton, J. C., A. Ahmadzadeh, B. Shafii, W. J. Price, and J. M. DeJarnette. 2004. Effect of thawing multiple 0.5-mL semen straws and sequential insemination number on conception rates in dairy cattle. J. Dairy Sci. 87:972-975.

Dalton, J.C. 2010. Semen evaluation and fertility in the field. In: Proc. 4th International Symposium on Applied Animal Reproduction, Londrina, Paraná, Brazil, p. 101-116.

Dalton, J. C., J. M. DeJarnette, R. G. Saacke, and R. P. Amann. 2017. The male component of dairy herd fertility. In: D.K. Beede, editor, Large Dairy Herd Management, third edition, American Dairy Science Association, Champaign, IL, p. 565-578.

DeJarnette, J. M. 2005. The effect of semen quality on reproductive efficiency. Vet. Clin. Food Anim. 21:409-418.

DeJarnette, J. M. 2012. Semen quality control and quality assurance in AI centers. In: Proc. Association for Applied Animal Andrology Conference, Vancouver, BC, Canada.

Den Daas, J. H. G., G. De Jong, L. M. T. E. Lansbergen, and A. M. Van Wagtendonk-De Leeuw. 1998. The relationship between the number of spermatozoa inseminated and the reproductive efficiency of individual dairy bulls. J. Dairy Sci. 81:1714–1723. doi:10.3168/jds.S0022-0302(98)75739-X

Duarte, M. S., T. S. Martins, P. V. R. Paulino, M. L. Chizotti, M. M. Ladeira, R. A. T. Filho, T. I. R. C. Alavarenga, and R. A. Gomes. 2013. Calpastatin activity and beef tenderness of Nellore and Angus cattle fed two feeding strategies. In Proc. 59th Int'l. Cong. of Meat Sci. Tech, Izmir, Turkey.

Flowers, W. L. 2013. Triennial reproduction symposium: Sperm characteristics that limit success of fertilization. J. Anim. Sci. 91:3022-3029. doi:10.2527/jas2012-5945

Foote, R. H. 2003. Fertility estimation: a review of past experience and future prospects. Anim. Reprod. Sci. 75:119-139. doi:S0378-4320(02)00233-6

Garner, D. 2014. Use of flow cytometry for semen evaluation. In: Proc. 25th Tech. Conf. Artif. Insem. and Reprod., Columbia, MO: National Association of Animal Breeders. p. 114-121.

Gunasena, K. T., and J. K. Critser. 1997. Utility of viable tissues ex vivo. In: A.M. Karow, J.K. Critser (Eds.), Reproductive Tissue Banking, Academic Press, San Diego, CA, p. 1–20.

Holt, W. V. 2000. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology. 53:47–58.

Instituto Brasileiro de geografia e estatística (IBGE). Estatisticas da produção pecuária (Statistics of livestock production). March, 2016.

https://biblioteca.ibge.gov.br/visualizacao/periodicos/2380/epp_2016_mar.pdf/ Accessed April 3, 2018.

Karabinus, D. S., D. P. Evenson, and M. T. Kaproth. 1991. Effects of egg yolk- and milk extenders on chromatin structure and viability of cryopreserved bull sperm. J. Dairy Sci. 74:3836-3848.

Lage, J. F., P. V. R. Paulino, S. C. Valadares Filho, E. J. O. Souza, M. S. Duarte, P. D. B. Benedeti, N. K. P. Souza, and R. B. Cox. 2012. Influence of genetic type and level of concentrate in the finishing diet on carcass and meat quality traits in beef heifers. Meat Sci. 90:770-774. doi:10.106/j.meatsci.2011.11.012

Lamb, G. C., C. R. Dahlen, J. E. Larson, G. Marquezini, and J. S. Stevenson. 2010. Control of estrous cycle to improve fertility for fixed-timed artificial insemination in beef cattle: A review. J. Anim. Sci. 88:E181-E192. doi:10.2527/jas.2009-2349

Marques, M., A. P. Sousa, A. Paiva, T. Almeida-Santos, and J. Ramalho-Santos. 2014. Low amounts of mitochondrial reactive oxygen species define human sperm quality. Reproduction. 147:817-824. doi:10.1530/REP-13-0644

Meat Import Council of America (MICA) http://www.micausa.org/2017-world-beef-trade-major-exporters/ Accessed February 7, 2018.

Malama, E., Y. Zeron, F. Janett, M. Siuda, Z. Roth, and H. Bollwein. 2017. Use of computer-assisted sperm analysis and flow cytometry to detect seasonal variations of bovine semen quality. Theriogenology. 87:79-90. doi: 10.1016/j.theriogenology.2016.08.002

Martins, T. A., L. M. P. Sanglard, W. Silva, M. L. Chizzotti, L. N. Rennó, N. V. L. Serão, F. F. Silva, S. E. F. Guimarães, M.M. Ladeira, M. V. Dodson, M. Du, and M. S. Duarte. 2015. Molecular factors underlying the deposition of intramuscular fat and collagen in skeletal muscle of Nellore and Angus cattle. PLoS ONE 10(10):e0139943. doi:10.1371/journal.pone.0129943

Meneghetti, M., and J. L. M. Vasconcelos. 2008. Calving date, body condition score, and response to a timed artificial insemination protocol in first-calving beef cows. Arq Bras Med Vet Zootec. 60:4 ISSN: 1678-4162. doi:10.1590/S0102-09352008000400002

Meneghetti, M., O. G. Sá Filho, R. F. G. Peres, G. C. Lamb, and J. L. M. Vasconcelos. 2009. Fixed-time artificial insemination with estradiol and progesterone for Bos indicus cows I: Basis for development of protocols. Theriogenology. 72:179–189. doi: 10.1016/j.theriogenology.2009.02.010

Miller, D., M. Brinkworth, and D. Iles. 2010. Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. Reproduction 139(2):287-301. doi:10.1530/REP-09-0281

Mortimer, S. T. 2000. CASA – Practical aspects. J. Androl. 21(4):515-524. doi:10.1002/j.1939-4640.2000.tb02116.x

O'Flaherty, C., E. de Lamirande, and C. Gagnon. 2006. Positive role of reactive oxygen species in mammalian sperm capacitation: Triggering and modulation of phosphorylation events. Free Radical Biol and Medicine. 41:528-540. doi:10.1016/j.freeradbiomed.2006.04.027

Oklahoma State University Department of Animal Science (OK State) http://www.ansi.okstate.edu/breeds/cattle/nelore/index.html/ Accessed February 7, 2018.

Parrish, J. J., J. K. Susko-Parrish, M. L. Leibried-Rutlage, E. S. Critser, W. H. Eyestone, and N. L. First. 1986. Bovine in vitro fertilization with frozen thawed semen. Theriogenology. 25:591–600.

Phillips. P.H., and H.A. Lardy. 1940. A yolk-buffer pabulum for the cryopreservation of bull semen. J. Dairy Sci. 23:399.

Pinheiro, O., C. Barros, R. Figueiredo, E. Do Valle, R. Encarnação, and C. Padovani. 1998. Estrous behavior and the estrus-to-ovulation interval in Nelore cattle (*Bos indicus* with natural estrus or estrus induced with prostaglandin $F_{2\alpha}$ or norgestomet and estradiol valerate. Theriogenology. 49:667–681.

Polge, C., A. U. Smith, and A. S. Parkes. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature. 164:666–676.

Polge, C., and L. E. A. Rowson. 1952. Fertilizing capacity of bull spermatozoa after freezing at -79 degrees C. Nature. 169:626.

Robbins, R. K., R. G. Saacke, and P. T. Chandler. 1976. Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in French Straws. J. Anim. Sci. 42:145–154. doi:10.2527/jas1976.421145x

Rodrigues, R. T. S., M. L. Chizzotti, C. E. Vital, M. C. Baracat-Pereira, E. Barros, K. C. Busato, R. A. Gomes, M. M. Ladeira, and T. S. Martins. 2017. Differences in beef quality between Angus (*Bos Taurus taurus*) and Nellore (*Bos Taurus indicus*) cattle

through a proteomic and phosphoproteomic Approach. PLoS ONE 12(1): e0170294. doi:10.1371/journal.pone.0170294

Rodríguez-Martínez, H. 2003. Laboratory semen assessment and prediction of fertility: still Utopia? Reprod. Domest. Anim. 38(4):312-318. doi: 10.1046/j.1439-0531.2003.00436.x

Sá Filho, O.G., M. Meneghetti, R. F. G. Peres, G. C. Lamb, and J. L. M. Vasconcelos. 2009. Fixed-time artificial insemination with estradiol and progesterone for *Bos indicus* cows II: Strategies and factors affecting fertility. Theriogenology. 72.210–218. doi:10.1016/j.theriogenology.2009.02.008

Sá Filho, M. F., A. M. Crespilho, J. E. P. Santos, G. A. Perry, and P. S. Baruselli. 2010. Ovarian follicle diameter at timed insemination and estrous response influence likelihood of ovulation and pregnancy after estrous synchronization with progesterone or progestin-based protocols in suckled *Bos indicus* cows. Anim. Reprod. Sci. 120:23-30. doi:10.1016/j.anireprosci. 2010.03.007

Sá Filho, M. F., J. E. P. Santos, R. M. Ferreira. J. N. S. Sales, and P. S. Baruselli. 2011. Importance of estrus on pregnancy per insemination in suckled *Bos indicus* cows submitted to estradiol/progesterone-based timed insemination protocols. Theriogenology. 76:455-463. doi:10..1016/j.theriogenology.2011.02.022

Saacke, R. G., S. Nadir, and R. L. Nebel. 1994. Relationship of semen quality to sperm transport, fertilization, and embryo quality in ruminants. Theriogenology. 41:45-50. doi:10.1016/S0093-691X(05)80047-0

Saacke, R. G., J. M. DeJarnette, J. H. Bame, D. S. Karabinus, and S. Whitman. 1998. Can spermatozoa with abnormal heads gain access to the ovum in artificially inseminated

super- and single-ovulating cattle? Theriogenology. 50:117-128. doi:10.1016/S0093-691X(98)00119-8

Saacke, R. G., J. C. Dalton, S. Nadir, R. L. Nebel, and J. H. Bame. 2000. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. Anim. Reprod. Sci. 60-61:663-77. doi:10.1016/S0378-4320(00)00137-8

Saacke R. G. 2008. Sperm morphology: Its relevance to compensable and uncompensable traits in semen. Theriogenology. 70:473-478. doi:10.1016/j.theriogenology.2008.04.012

Salisbury, G. W., and N. L. VanDemark. 1961. Significance of semen quality. In: Physiology of Reproduction and Artificial Insemination in Cattle, first edition, W. H. Freeman and Co., San Francisco, CA, p. 358-379.

Senger, P. L. 2012. Male endocrinology and spermatogenesis. In: Pathways to Pregnancy and Parturition, 3rd ed., Redmond, OR: Current Conceptions, Inc.

Utt, M. D. 2016. Prediction of fertility. Anim Reprod Sci. 169:37-44. doi:10.1016/j.anireprosci.2015.12.011

Vasconcelos, J. L. M., R. Carvalho, R. F. G. Peres, A. D. P. Rodrigues, I. Claro Junior, M. Meneghetti, F. H. Aono, W. M. Costa, C. N. Lopes, R. F. Cooke, and K. G. Pohler. 2017. Reproductive programs for beef cattle: incorporating management and reproductive techniques for better fertility. In: Proc. 31st Annual Meeting of the Brazilian Embryo Technology Society (SBTE), Cabo de Santo Agostinho, PE, Brazil. doi:10.21451/1984-3143-AR998

Vincent, P., S. L. Underwood, C. Dolbec, N. Bouchard, T. Kroetsch, and P. Blondin. 2012. Bovine semen quality control in artificial insemination centers. Anim. Reprod. 9(3):153-165.

Vishwanath, R. 2003. Artificial insemination: the state of the art. Theriogenology. 59:571-584. doi:10.1016/S0093-691X(02)01241-4

Woods, E. J., J.D. Benson, Y. Agca, and J. K. Critser. 2004. Fundamental cryobiology of reproductive cells and tissues. Cryobiology 48:146-156

Zorzetto, M. F., I. Martin, Y. F. R. Sancler-Silva, S. Zoca, C. P. Freitas-Dell'Aqua, F. O. Papa, A. A. Ramos, J. F. Nunes, C. C. M. Salgueiro, and E. Oba. 2018. Comparison of three different extenders on Murrah buffaloes (*Bubalus bubalis*) semen freezability. Andrologia. 50:e12830. doi:10.1111/and.12830

CHAPTER 2

"Effect of sperm dosage on pregnancy per timed-AI in Brazilian beef cattle"

Objectives

The primary objective of this study was to determine if beef bull fertility varied by number of sperm inseminated. A secondary objective was to characterize the potential impact of random variation through the use of two identical sperm per dose treatments, which differed only by straw color.

Hypothesis

We hypothesized that beef bull fertility at first service timed AI will not differ due to the sperm dosage used in this experiment.

Abstract

Ejaculates from five Angus bulls were collected, extended, and cryopreserved at 10, 20, 20 or 40 × 10^6 sperm per dose in color-coded 0.5-mL French straws. Straws were distributed to ten Brazilian farms. Multiparous cows (n = 4,866) were synchronized for first service timed artificial insemination (TAI), and bull identification and straw color were recorded at TAI. Pregnancy was diagnosed by transrectal ultrasonography 30 to 90 days after TAI. Pregnancy per timed AI (P/TAI) were analyzed using a generalized linear mixed model assuming a beta distribution with bull, sperm dose, and their respective interactions as fixed effects and farms as a random blocking effect. There was no interaction between bull and sperm dose (P = 0.53). Also, P/TAI was not significantly different between sperm doses (43.8,

45.3, 43.8 and 47.1% for 10, 20, 20 or 40 × 10^6 sperm respectively; P = 0.31). The P/TAI, however, was different between bulls (P < 0.01). Bulls A and B exhibited greater P/TAI relative to bull C (48.1 and 47.7 vs. 40.7 % respectively), whereas bulls D and E were intermediary (45.5 and 43.1%, respectively). Although the overall P/TAI between the two control groups (20-a: 45.3%; 20-b: 43.8%) were not different, the numerical variation (for these sperm doses) within bull ranged from 0.5 (bull E) to 4.9 percentage points (bull D), providing evidence that variation in reproductive field trials should not be ignored. Estrous behavior was observed in a subset of cows (n = 1,061) during the period immediately following progesterone insert removal until TAI; 64.4% (683/1061) of cows were determined to be in estrus. The P/TAI for cows detected in estrus was 44.5%, as compared to 24.6% for cows not in estrus (P < 0.01). In conclusion, although fertility differences between bulls were detected, fertility following TAI with 10, 20, 20 or 40 × 10^6 sperm per dose resulted in similar P/TAI at first service in synchronized beef cattle.

Introduction

The AI industry strives to efficiently utilize collected sperm and maximize fertility of each bull while avoiding an excess in sperm number per dose that may limit supply (Amann and DeJarnette, 2012; Dalton et al., 2017). The minimum number of viable sperm required for maximum fertility differs among bulls, as does the rate at which maximum fertility is achieved with increasing sperm dosage (Salisbury and VanDemark, 1961; Den Daas et al. 1998). Differences in fertility among bulls responsive to increased sperm dosage are considered “compensable” whereas those not responsive to increased sperm dosage are considered “uncompensable,” as originally described by Saacke et al. (1994).

Compensable seminal traits are related to sperm transport as well as the capacity to initiate fertilization and prevent polyspermy (Saacke et al., 2000). In contrast, uncompensable seminal traits are related to competence of fertilizing sperm to complete fertilization and maintenance of early embryogenesis (Saacke et al., 2000).

Timed artificial insemination **(**TAI**)** is an efficient management strategy to administer AI in large groups of beef cattle. The hypothesis of this study was that beef bull fertility, following TAI with 10, 20, 20 or 40 × 10^6 sperm per dose would result in similar pregnancy per TAI **(**P/TAI**)** at first service in synchronized beef cattle. The primary objective, therefore, was to determine if beef bull fertility varied by number of sperm inseminated, whereas a secondary objective was to characterize the potential impact of random variation through the use of two 20 × 10^6 sperm per dose treatments.

Materials and methods

Animals and reproductive management

All procedures were approved by the University of Idaho Animal Use and Care Committee (Appendix 1). This study was conducted in ten farms located in Acre, Goiás and Mato Grosso, Brazil (Table 2.1). All cows were maintained on *Brachiaria decumbens* pasture with ad libitum access to water and mineralized salt during the experimental period. Multiparous *Bos indicus* or *Bos indicus* × *Bos taurus* cows (n=4,866), 40 to 60 days postpartum, were evaluated for body condition score [BCS; one (emaciated) to five scale (obese); Houghton et al., 1990; Ayres et al., 2009] and enrolled in a first-service TAI program. All cows were synchronized for TAI using an intravaginal progesterone device and estradiol-based protocol as previously described (Figure 2.1; Sá Filho et al., 2010; 2011; Sá Filho et al.,

2009; Meneghetti et al., 2009). Products used in TAI were from multiple suppliers: intravaginal progesterone devices [CIDR® (1.9 g progesterone, Zoetis Indústria de Produtos Veterinários Ltda, Brazil), DIB® (1.0 g progesterone, Zoetis Indústria de Produtos Veterinários Ltda, Brazil), Fertilcare® (1.2 g progesterone, Von Franken S. A. I. C., Argentina), or Prociclar® (750 mg progesterone, Hertape Saúde Animal S.A., Brazil)]; estradiol benzoate [2 mg per cow; Gonadiol (1 mg per mL - Zoetis Indústria de Produtos Veterinários Ltda, Brazil) or Estrogin (1 mg per mL – Biofarm Química e Farmacêutica Ltda, Brazil)], estradiol cypionate [1 mg per cow; ECP® (2 mg/mL - Zoetis Indústria de Produtos Veterinários Ltda, Brazil)], equine chorionic gonadotropin [300 IU per cow; Novormon (200 IU per mL - Zoetis Indústria de Produtos Veterinários Ltda, Brazil) or Folligon (200 IU per mL – MSD Saúde Animal, Brazil)], and prostaglandin $F_{2\alpha}$ [either 12.5 mg per cow of Lutalyse (dinoprost trometamina; 5 mg per mL - Zoetis Indústria de Produtos Veterinários Ltda, Brazil) or 0.5 mg per cow of Sincrocio (Cloprostenol sódico; 0.25 mg/mL – Ourofino Saúde Animal Ltda, Brazil)].

Detection of estrus was performed using an Estrotect patch (Estrotect, Spring Valley, WI, USA) on two farms (farms 8 and 10; n = 1,061 cows). Estrotect patches were administered to cattle immediately upon removal of the intravaginal progesterone device to facilitate detection of estrus. All cows received TAI regardless of Estrotect patch activation status.

Semen straws were thawed in 37ºC water for a minimum of 30 seconds. At TAI, straw color and sire identification were recorded. Pregnancy was diagnosed by transrectal ultrasonography 30 to 90 days after TAI, according to each farm's standard operating procedure. A total of 1,228 cows were diagnosed for pregnancy between 30 to 45 days, 3,424 cows between 50 to 70 days and 214 cows at 90 days after TAI (Table 2.2). On farms 8 and

10, cows that received Estrotect patches were subjected to pregnancy diagnosis by ultrasound between 50 and 70 days after TAI.

Semen collection, evaluation, extension and cryopreservation

Ejaculates from Angus bulls (n = 5; range in age: 2 to 4 years) housed at Select Sires, Inc. (Plain City, OH, USA) were collected by artificial vagina over a period of 2 to 4 days between May 13, 2014 to May 30, 2014. Two ejaculates in succession were collected, with each ejaculate preceded by two false mounts separated by 2 min of restraint.

Having met the minimum criteria of 65% motility in a subjective assessment, the two ejaculates collected in succession were pooled. Semen was processed by diluting to the correct, respective dose concentration (20, 40, 40 and 80 × 10^6 sperm per mL) using a two-step, proprietary milk-based extender (Select Sires, Inc.). Semen was packaged in 0.5-mL French straws (Instruments de Médecine Vétérinaire, l'Aigle, France) which contained each bull's identification and freezing code. Thus, four insemination dose groups were created, 10, 20-a, 20-b and 40 × 10^6 sperm per straw. Straws were color-coded (brown, purple, red, yellow) and straw color alternated across bulls and between sperm doses to ensure AI personnel were blind to sperm dosage for each straw. Samples were frozen using a static vapor freeze while suspended above liquid nitrogen before being plunged and stored in liquid nitrogen (Robbins et al., 1976). The use of two 20 × 10^6 (20-a and 20-b) sperm per straw treatments was an attempt to illustrate the potential impact of random variation on P/TAI, as these treatments differed only in straw color.

Sample straws from each bull were thawed in 37°C water for 1 minute before post-thaw evaluation of progressive sperm motility (DeJarnette et al., 2010). Briefly, motility was

evaluated subjectively using a minimum of five random fields of view (avoiding slide perimeter) at 200× magnification using phase-contrast optics and rounded to the nearest 5% (DeJarnette et al., 2010). A minimum of 60% post-thaw motility was required for inclusion in the study (Table 3.3). To evaluate sperm plasma membrane viability post-thaw, flow cytometry was used. Two µL of extended semen was diluted in buffer (198 µL) containing Hoechst 33342 (15.5 µg/mL) and propidium iodide (PI; 5 µg/mL). Subsequently, samples were incubated for 20 minutes at 35ºC without presence of light (Garner et al., 1995). Samples were prepared in round bottom 96-well plates and a total of 5000 cells/sample were analyzed using a MACSQuant Analyzer 10 (Miltenyi Biotec, Auburn, CA, USA). Hoescht 33342 was excited via a 405 nm laser and emission spectra were collected with a 450/50 nm band pass filter. A 488 nm laser was used to excite PI and emission spectra were collected with a 655-730 nm band pass filter. Flow cytometric data was analyzed with FLOWJo (FLOWJo, LLC, Ashland, OR, USA). The percentage of viable sperm was calculated for each sample (Table 2.3).

Sperm morphology was evaluated post-thaw using differential interference contrast microscopy at 1000× magnification after fixing sperm with 0.2% formalin and wet mount preparation (Richardson et al., 2017). One hundred cells were evaluated per bull. Sperm head defects were classified as primary defects, while sperm tail defects were classified as secondary defects (Barth and Oko, 1989). A minimum of 65% normal sperm morphology was required for inclusion in the study.

Following post-thaw quality control analyses, sperm doses were distributed to ten Brazilian farms. Distribution was facilitated by Select Sires do Brasil (Porto Alegre, Brazil) and each farm received all bull and sperm dose combinations; however, collection date was

randomly assigned for each location, and therefore, considered as a random effect within farms.

Statistical methods

Pregnancy per timed AI (P/TAI) were analyzed using a generalized linear mixed model assuming a beta distribution (GLIMMIX procedure; SAS version 9.4) with bull, sperm dose, and their respective interactions as fixed effects and farms as a random blocking effect (Stroup, 2014). Body condition score (BCS) was analyzed with the same model, but was considered a normal distribution following assessment of univariate analyses. Differences in means for significant effects were assessed using pair-wise comparisons with statistical significance established at $P \leq 0.05$. All statistical computations were carried out using SAS version 9.4 (SAS Inst. Inc., Cary, NC).

Results and discussion

In beef cows BCS at TAI is a critical factor in TAI success (Meneghetti et al., 2009; Sá Filho et al., 2009; Vasconcelos et al., 2017). The overall mean BCS ± SD was 2.84 ± 0.34 (all cows) at the beginning of the TAI protocol. Body condition score was uniform within bull, sperm dose and bull by sperm dose interaction; consequently, no effect of BCS on bull ($P = 0.19$), sperm dose ($P = 0.91$) and their respective interaction ($P = 0.35$) was identified (Table 2.4). Body condition score mean ± SE ranged from 2.80 ± 0.06 to 2.90 ± 0.06. For this reason, BCS was not included in the statistical model for P/TAI.

Salisbury and VanDemark (1961) first proposed that fertility increases with increasing number of viable sperm inseminated up to a certain threshold at which the fertility level of the female population becomes the limiting factor. In the present study, there was no difference

for P/TAI in the bull by sperm dosage interaction (P = 0.53; Table 2.5). There was also no effect of sperm dose on P/TAI (P = 0.31). For the sperm dosages used in this study, it appears the bulls had few, if any compensable traits, as no difference in P/TAI was detected between sperm doses (DeJarnette, 2005; Dalton, 2010). Den Daas et al. (1998), in a study with 20 mature bulls, estimated a dose of 4.0 to 5.3 × 10^6 total sperm were necessary for bulls to achieve 95% of the optimum conception rate, thereby satisfying the compensable component for the bulls used in their study. Further, Den Daas (1998) reported only one of the 20 bulls required more than 10 × 10^6 total sperm (11.8 × 10^6) to achieve 95% of the optimum conception rate. Our results provide further evidence that 10 × 10^6 sperm per dose was sufficient to achieve similar fertility among the bulls at all dosages used in this study (Table 2.5). This provides evidence the compensable component was satisfied as described by Den Daas (1998). Similar to Den Daas (1998), however, there was one bull in the present study that appeared to need more sperm in the insemination dose. Although no significant bull by sperm dosage interaction was detected in this study, P/TAI for bull E appears to be numerically lower at 10 × 10^6 sperm per straw (36.6%) as compared to 20-a (44.7%), 20-b (45.2) and 40 × 10^6 (45.9%) sperm per straw, respectively. It is possible that bull E has a higher threshold level of compensable traits, as P/TAI increased from 10 × 10^6 (36.6%) and plateaued at 20 to 40 × 10^6 sperm per dose (approximately 45%).

Clay and McDaniel (2001) and DeJarnette (2005) argue more than 90% of semen from commercial bulls are within ± 3% of average fertility, with no statistical significance between bull fertility to be expected in large samples. In contrast, our results demonstrate a significant difference between bulls A, B, and C (P < 0.01) in which bulls A and B have greater P/TAI as compared to bull C (48.1 and 47.7 vs. 40.7 % respectively). In contrast, research conducted

in Brazil (Oliveira et al., 2012) using Nelore cows (n = 944) and three Angus bulls showed no difference in P/TAI for bulls, and results appeared to be similar to the greater fertility bulls A, B, and D in the present study.

All ejaculates from bulls in the present study passed pre-freeze and post-thaw quality control analyses, including motility, morphology, and membrane integrity; therefore, it was not expected that bull C would exhibit lower overall fertility than other bulls in the current study. Nevertheless, as described by DeJarnette (2005), bull fertility should be considered questionable even when sufficient levels of known semen characteristics are reached, because of unknown or unmeasured characteristics that could affect fertility. For example, bull C may have had uncompensable seminal traits which affected fertility at all doses studied (DeJarnette, 2005; Saacke et al., 1998). Further analyses (e.g. CASA and flow cytometry), as detailed in the next chapter are necessary to attempt to describe seminal traits that may be associated to fertility observed in this study. Nevertheless, unknown or unmeasured characteristics may impede the explanation of the differences in bull fertility detected in this study.

A secondary objective was to characterize the potential impact of random variation through the use of two 20×10^6 sperm per dose treatments, which differed only by straw color. Although the overall P/TAI between the two control groups (20-a: 45.3%; 20-b: 43.8%) were not different, the numerical variation within bull ranged from 0.5 (bull E) to 4.9 percentage points (bull D), providing evidence that random variation in reproductive field trials should not be ignored. Thus, random variation is an additional factor to be considered when prediction of bull fertility is intended (Amann and DeJarnette, 2012; Utt, 2016).

Estrotect patches were used on a subset of animals to facilitate detection of estrus. During the period immediately following progesterone insert removal and TAI, 64.4% of cows (683/1061) were detected in estrus. Richardson et al. (2017) reported cows that expressed estrus had a greater P/TAI than cows that did not show estrus regardless of semen type (frozen or liquid). In agreement, the P/TAI for cows detected in estrus in the present study was 44.7% compared with 20.9% for cows not detected in estrus ($P < 0.01$). In similar studies, Sá Filho et al. (2010; 2011) reported that 57.8% and 57.4% of cows were detected in estrus, with P/TAI for cows detected in estrus prior to TAI of 67.7% and 61.9%, compared to P/TAI for cows not detected in estrus of 36.2% and 41.4%, respectively. In the present study, a greater percentage of cows appears to have been detected in estrus as compared to Sá Filho et al. (2010; 2011); however, fertility in the present study appears to be depressed in relation to Sá Filho et al. (2010; 2011). A difference in time to pregnancy determination may play a role in understanding the relative fertility reported in these studies, as pregnancy status was determined by Sá Filho et al. (2010; 2011) 30 d after TAI compared with between 50 and 70 d after TAI in the estrous detection subset in the present study, allowing increased time for pregnancy loss to occur (Aono et al., 2013; Pohler et al., 2016).

Conclusions

In conclusion, although fertility differences between bulls were detected, fertility following TAI with 10, 20, 20 or 40 × 10^6 sperm per dose resulted in similar P/TAI at first service in synchronized beef cattle. Not surprisingly, P/TAI was greater in cows that expressed estrus before TAI. The use of two 20 × 10^6 sperm per dose treatments, which differed only by straw color, revealed numerical variation in P/TAI within bull ranged from 0.5 to 4.9 percentage points. The results of the present study provide evidence the use of 10 × 10^6 sperm

per dose (bulls A, B, C, D) to 20×10^6 sperm per dose (bull E) results in acceptable fertility, with enough sperm per dose to overcome compensable traits. Further studies are necessary to describe whether a dose lower than 10×10^6 sperm would result in similar fertility to 10×10^6 and 20×10^6 sperm per dose.

Table 2.1 Total number of cows inseminated within ten farms in three different Brazilian states (BR-State) and timed AI protocol used.

Farm	*n*	BR-State[1,2,3]	Protocol[4]
1	131	AC[1]	3rd
2	135	AC	3rd
3	374	AC	3rd
4	574	GO[2]	2nd
5	218	MT[3]	2nd
6	359	MT	2nd
7	513	MT	2nd
8	595	MT	2nd
9	316/384	MT	2nd/3rd[5]
10	1,267	MT	1st
Total	4,866	3	-

[1]AC: Acre
[2]GO: Goias
[3]MT: Mato Grosso
[4]Protocols are depicted in Figure 2.1
[5]Farm used two protocols according to veterinary prescription

Table 2.2 Pregnancy diagnoses [days (d) after TAI], number of cows, and proportion of cows pregnant (P/TAI) per farm (1 to 10).

Farm	30-45 d	50-70 d	90 d	P/TAI, %
1	131			48.1
2	135			53.3
3	374			49.5
4		574		40.8
5		218		40.4
6		359		50.4
7		513		46.2
8		595		42.0
9		700		46.1
10	588	465	214	39.0
Total	**1228**	**3424**	**214**	43.7

Table 2.3 Semen evaluation post-thaw for quality control prior to field fertility trial of five Angus bulls.

Bull	Motility, %	Viability, %
A	65.4	57.3
B	68.1	58.7
C	68.1	48.0
D	70.0	69.7
E	63.1	36.2

Table 2.4 Mean body condition score (BCS) of cows by AI service sire (bull) and sperm dose ($P > 0.1$).

Bull	Dose				Bull mean[3]
	10×10^6	20-a[2] $\times 10^6$	20-b[2] $\times 10^6$	40×10^6	
A	2.86	2.85	2.86	2.89	2.87
B	2.84	2.85	2.85	2.88	2.86
C	2.88	2.90	2.84	2.86	2.86
D	2.85	2.89	2.86	2.84	2.86
E	2.85	2.80	2.85	2.84	2.83
Dose mean[1]	2.86	2.86	2.85	2.86	2.84

[1]Dose mean = overall BCS of cows used per sperm dosage
[2]20-a and 20-b treatments differ only in straw color
[3]Bull mean = overall BCS of cows used per bull

Table 2.5 Proportion of cows pregnant per timed AI (P/TAI; % ± SEM) across sperm dosages and bulls in Brazilian beef cattle.

Bull	Dose, × 10^6								Bull mean[3]	*n*
	10	*n*	20-a[1]	*n*	20-b[1]	*n*	40	*n*		
A	48.5 ± 3.3	274	50.2 ± 3.5	240	48.7 ± 3.4	261	45.1 ± 3.3	275	48.1 ± 2.0[a]	1,050
B	47.2 ± 3.3	270	44.4 ± 3.5	241	46.7 ± 3.3	275	52.7 ± 3.3	272	47.7 ± 2.0[a]	1,058
C	41.4 ± 3.2	297	41.1 ± 3.0	334	37.3 ± 3.3	263	43.1 ± 3.1	312	40.7 ± 1.9[c]	1,206
D	45.2 ± 4.0	179	46.4 ± 3.8	195	41.5 ± 3.8	190	48.9 ± 4.0	183	45.5 ± 2.3[ab]	747
E	36.6 ± 3.6	211	44.7 ± 3.8	194	45.2 ± 3.8	193	45.9 ± 3.7	207	43.1 ± 2.2[bc]	805
Dose mean[2]	43.8 ± 2.0	1,231	45.3 ± 2.0	1,204	43.8 ± 2.0	1,182	47.1 ± 2.0	1,249	-	4,866

[a,b,c] Different letters in the same column denotes statistical difference ($P < 0.05$)
[1]20-a and 20-b treatments differ only in straw color ($P > 0.1$)
[2]Dose mean = overall mean of P/TAI per sperm dosage
[3]Bull mean = overall mean of P/TAI per bull

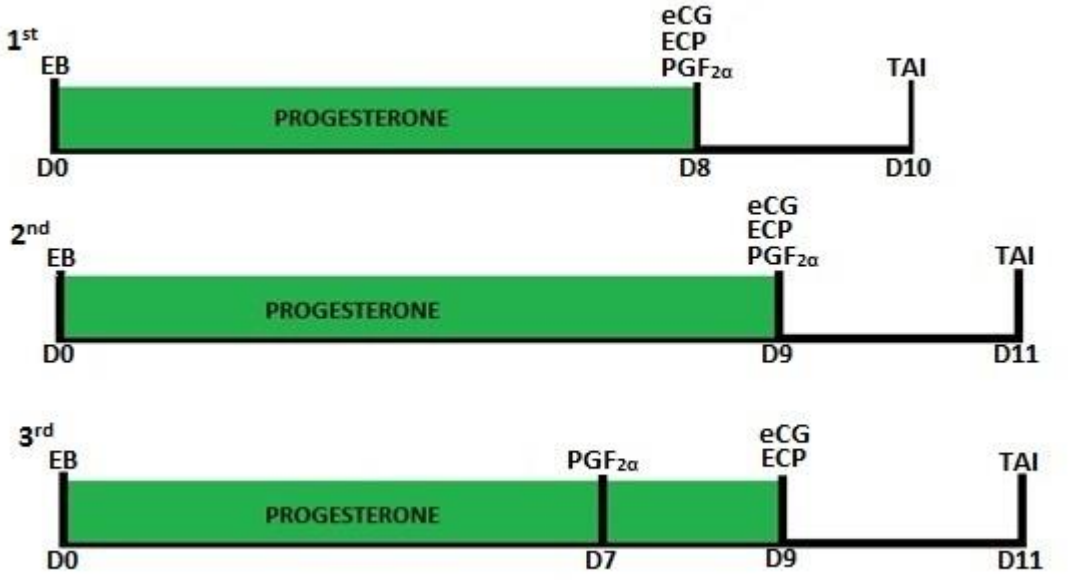

Figure 2.1 Protocols used for timed AI (TAI) in ten Brazilian beef cattle farms. 1st protocol: Intravaginal progesterone insert and administration of estradiol benzoate (EB) on day 0 (D0), withdrawal of insert on day 8 (D8) coupled with administration of estradiol cypionate (ECP), equine chorionic gonadotropin (eCG) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), TAI on day 10 (D10; Sá Filho et al., 2011). 2nd protocol: Intravaginal progesterone insert and administration of EB on D0, withdrawal of progesterone insert on day 9 (D9) coupled with administration of ECP, eCG and $PGF_{2\alpha,}$ TAI on day 11 (D11; Sá Filho et al., 2009; Meneghetti et al., 2009). 3rd protocol: Intravaginal progesterone insert and administration of EB on D0, administration of $PGF_{2\alpha}$ on day 7 (D7), withdrawal of progesterone insert on D9 coupled with administration of ECP and eCG, TAI on D11 (Sá Filho et al., 2009; Meneghetti et al., 2009). The green bar represents time in which cows were exposed to the progesterone insert (D0 to D8 or D9).

Literature cited

Amann, R. P., and J. M. DeJarnette. 2012. Impact of genomic selection of AI dairy sires on their likely utilization and methods to estimate fertility: A paradigm shift. Theriogenology. 77:795–817. doi:10.1016/j.theriogenology.2011.09.002

Aono, F. H., R. F. Cooke, A. A. Alfieri, and J. L. M. Vasconcelos. 2013. Effects of vaccination against reproductive diseases on reproductive performance of beef cows submitted to fixed-timed AI in Brazilian cow-calf operation. Theriogenology. 79:242-248. doi:10.1016/j.theriogenology.2012.08.008

Ayres, H., R. M. Ferreira, J. R. S. Torres-Júnior, C. G. B. Demétrio, C. G. de-Lima, and P. S. Baruselli. 2009. Validation of body condition score as a predictor of subcutaneous fat in Nelore (*Bos indicus*) cows. Livestock Sci. 123:175–9.

Barth, A. D., and J. Oko. 1989. Abnormal morphology of bovine spermatozoa. Iowa State University Press, Ames.

Clay, J. S., and B. T. McDaniel. 2001. Computing mating bull fertility from DHI nonreturn data. J. Dairy Sci. 84;1238-45.

Dalton, J. C. 2010. Semen evaluation and fertility in the field. In: Proc. 4th International Symposium on Applied Animal Reproduction, Londrina, Paraná, Brazil, p. 101-116.

Dalton, J. C., J. M. DeJarnette, R. G. Saacke, and R. P. Amann. 2017. The male component of dairy herd fertility. In: D. K. Beede, editor, Large Dairy Herd Management, third edition, American Dairy Science Association, Champaign, IL, p. 565-578.

DeJarnette, J. M. 2005. The effect of semen quality on reproductive efficiency. Vet. Clin. Food Anim. 21:409-418.

DeJarnette, J. M., C. R. McCleary, M. A. Leach, J. F. Moreno, R. L. Nebel, and C. E. Marshall. 2010. Effects of 2.1 and 3.5 × 10^6 sex-sorted sperm dosages on conception rates of Holstein cows and heifers. J. Dairy Sci. 93:4079–4085.

Den Daas, J. H. G., G. De Jong, L. M. T. E. Lansbergen, and A. M. Van Wagtendonk-De Leeuw. 1998. The relationship between the number of spermatozoa inseminated and the reproductive efficiency of individual dairy bulls. J. Dairy Sci. 81:1714–1723.

Garner, D. L., J. R. Dobrinsky, G. R. Welch, and L. A. Johnson. 1995. Porcine sperm viability, oocyte fertilization and embryo development after staining spermatozoa with SYBR-14. Theriogenology. 45:1103-1113. doi:10.1016/0093-691X (96)00067-2

Houghton, P. L., R. P. Lemenager, G. E. Moss, and K. S. Hendrix. 1990. Prediction of post partum beef cow body composition using weight to height ratio and visual body condition score. J. Anim. Sci. 68:1428–37.

Meneghetti, M., O. G. Sá Filho, R. F. G. Peres, G. C. Lamb, and J. L. M. Vasconcelos. 2009. Fixed-time artificial insemination with estradiol and progesterone for *Bos indicus* cows I: Basis for development of protocols. Theriogenology. 72:179–189. doi: 10.1016/j.theriogenology.2009.02.010

Oliveira, L. Z., R. P. de Arruda, A. F. C. de Andrade, E. C. C. Celeghini, R. M. dos Santos, M. E. Beletti, R. F. G. Peres, C. S. Oliveira, and V. F. M. H. de Lima. 2012. Assessment of field fertility and several in vitro sperm characteristics following the use of different Angus sires in a timed-AI program with suckled Nelore cows. Livestock Sci. 146:36-46. doi:10.1016/j.livsci.2012.02.018

Pohler, K. G., M. H. C. Pereira, F. R. Lopes, J. C. Lawrence, D. H. Keisler, M. F. Smith, J. L. M. Vasconcelos, and J. A. Green. 2016. Circulating concentration of bovine

pregnancy-associated glycoproteins and late embryonic mortality in lactating dairy herds. J. Dairy Sci. 99:1584-1594. doi:10.3168/jds.2015-10192

Richardson, B. N., E. L. Larimore, J. A. Walker, M. D. Utt, J. M. DeJarnette, and G. A. Perry. 2017. Comparison of fertility of liquid or frozen semen when varying the interval from CIDR removal to insemination. Anim. Reprod. Sci. 178:61-66. doi:10.1016/j.anireprosci.2017.01.010

Robbins, R. K., R. G. Saacke, and P. T. Chandler. 1976. Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in French Straws. J. Anim. Sci. 42:145–154.

Saacke, R. G., S. Nadir, and R. L. Nebel. 1994. Relationship of semen quality to sperm transport, fertilization, and embryo quality in ruminants. Theriogenology. 41:45-50.

Saacke, R. G., J. M. DeJarnette, J. H. Bame, D. S. Karabinus, and S. S. Whitman. 1998. Can spermatozoa with abnormal heads gain access to the ovum in artificially inseminated super- and single-ovulating cattle? Theriogenology. 50:117-128. doi:10.1016/S0093-691X(98)00119-8

Saacke, R. G., J. C. Dalton, S. Nadir, R. L. Nebel, and J. H. Bame. 2000. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. Anim. Reprod. Sci. 50-61:663-77.

Sá Filho, O.G., M. Meneghetti, R. F. G. Peres, G. C. Lamb, and J. L. M. Vasconcelos. 2009. Fixed-time artificial insemination with estradiol and progesterone for *Bos indicus* cows II: Strategies and factors affecting fertility. Theriogenology. 72.210–218. doi:10.1016/j.theriogenology.2009.02.008

Sá Filho, M. F., A. M. Crespilho, J. E. P. Santos, G. A. Perry, and P. S. Baruselli. 2010. Ovarian follicle diameter at timed insemination and estrous response influence likelihood of ovulation and pregnancy after estrous synchronization with progesterone or progestin-based protocols in suckled *Bos indicus* cow. Anim. Reprod. Sci. 120:23–30.

Sá Filho, M. F., J. E. P. Santos, R. M. Ferreira, J. N. S Sales, and P. S. Baruselli. 2011. Importance of estrus on pregnancy per insemination in suckled *Bos indicus* cows submitted to estradiol/progesterone-based timed insemination protocols. Theriogenology. 76:455-463. doi:10.1016/j.theriogenology.2011.02.022

Salisbury, G. W., and N. L. VanDemark. 1961. Significance of semen quality. In: Physiology of Reproduction and Artificial Insemination in Cattle, first edition, W. H. Freeman and Co., San Francisco, CA, p. 358-379.

Stroup, W. W. 2014. Rethinking the analysis of non-normal data in plant and soil science. Agron. J. 106:1–17. doi:10.2134/agronj2013.03.

Utt, M. D. 2016. Prediction of bull fertility. Anim. Reprod. Sci. 169:37-44. doi:10.1016/j.animreprosci.2015.12.011

Vasconcelos, J. L. M., R. Carvalho, R. F. G. Peres, A. D. P. Rodrigues, I. Claro Junior, M. Meneghetti, F. H. Aono, W. M. Costa, C. N. Lopes, R.F. Cooke, and K. G. Pohler. 2017. Reproductive programs for beef cattle: incorporating management and reproductive techniques for better fertility. In: Proc. 31st Ann. Meeting of the Brazilian Embryo Technology Society (SBTE), Cabo de Santo Agostinho, PE, Brazil. doi:10.21451/1984-3143-ar998

CHAPTER 3

"Use of computer-assisted sperm analysis and flow cytometry to explain field fertility differences of five Angus bulls"

Objective

Based on the field fertility trial, the overall objective of the *in vitro* sperm analyses was to identify semen characteristics that may be associated with fertility differences between bulls.

Hypothesis

We hypothesized that sperm of lower fertility bulls will show lower levels of desirable characteristics and higher levels of undesirable characteristics in the *in vitro* sperm analyses.

Abstract

The primary objective was to evaluate whether computer-assisted sperm analysis (CASA) and flow cytometry (FC) were able to explain the difference in field fertility of five Angus bulls. The secondary objective was to investigate the pattern and variability of *in vitro* sperm characteristics using principal component analysis (PCA). Two 0.5-mL semen straws from each bull, of the same dose (10, 20, 20 or 40 × 10^6 sperm/straw) and same collection date were thawed simultaneously, pooled, and assayed in duplicate. Semen samples used for CASA were stained to evaluate total motility (TM), progressive motility (PM), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), average path, curvilinear and straight-line velocities (VAP, VCL, VSL, respectively), linearity (LIN), straightness (STR) and wobble (WOB). In FC analyses, values for plasma membrane integrity (viable), acrosome integrity (acrosome), viable with acrosome (VA), viable with normal calcium (VNCa),

acrosome and normal calcium within viable (ANCa), and DNA fragmentation index (DFI) were assayed with multiple stains. After FC analyses, the calculation of the overall population of sperm with intact plasma and acrosomal membranes with normal calcium influx (VANCa) were calculated by multiplying viable and ANCa. A generalized linear mixed model was used (SAS 9.4) to analyze the data. Fixed effects included bull, duplicate, and bull by duplicate interaction. Collection date, and collection date by sperm concentration per duplicate were considered random effects. Principal component analysis was used to evaluate variability of sperm analyses within and between bulls. Sixteen characteristics were described (TM, PM, ALH, BCF, VAP, VCL, VSL, LIN, STR, WOB, viable, acrosome, VA, VNCa, ANCa and DFI) and principal component axes with eigenvalues > 1.0 were retained for further consideration and interpretation. There was no effect of duplicate or bull by duplicate interaction ($P > 0.1$) in CASA and FC analyses, and BCF, LIN and WOB were not different between bulls ($P > 0.1$). All other parameters were different between bulls ($P < 0.05$). Bulls with the greatest fertility (A and B) did not display the greatest values of TM, PM and VANCa, nor the smallest value of DFI. Bull C, which had the lowest field fertility, did not present the lowest values in sperm analyses. Bulls D and E, which did not differ in field fertility, showed the highest and lowest values for all analyses, respectively. Three principal components presented an eigenvalue > 1.0 (Prin1, Prin2 and Prin3) and accounted for 88.7% of the total data variability. Principal component 1, Prin2 and Prin3 explained 47, 26 and 15.7% of the variability, respectively. Principal component 1 was most influenced by sperm viability and DFI (TM, acrosome, viable, VA, VNCa and DFI), Prin2 by sperm movement pattern (BCF, LIN, STR and WOB) and Prin3 by motility (PM, VAP, VCL and VSL). In conclusion, the use of multiparametric sperm analyses did not explain the difference in field fertility.

Principal component analysis simplified the complexity of data from CASA and FC analyses, and allowed for the identification of key sperm characteristic variables.

Introduction

Semen quality is an integral component of fertility; however, fertility is a multifactorial phenomenon and relies on multiple events that must occur in a determined order and time (Dalton et al., 2017; Flowers, 2012; Rodríguez-Martínez, 2001; 2003; Saacke, 2008; Vincent et al., 2012). It has been reported that beef cattle fertility is more influenced by the male than the female (Flowers, 2012). The greater influence of the male in beef cattle fertility has been attributed to the use of natural service, as breeding soundness exams are not conducted routinely by most producers (Flowers, 2012). In contrast, Clay and McDaniel (2001) and DeJarnette (2005) argued that more than 90% of bulls from commercial AI studs are within ± 3% of average fertility, with no statistical significance between bull fertility to be expected in large samples from AI bulls.

Differences in fertility among males responsive to increased sperm dosage are considered "compensable" fertility traits whereas those not responsive to increased sperm dosage are considered "uncompensable," as originally described by Saacke et al. (1994b). The minimum number of viable sperm required for maximum fertility differs among bulls, as does the rate at which maximum fertility is achieved with increasing sperm dosage (Salisbury and VanDemark, 1961; Den Daas et al., 1998). For maximal fertility, the inseminate must contain a sufficient number of sperm with all the desirable and necessary characteristics (Amann and Hammerstedt, 1993; Rodriguez-Martinez, 2001). Den Daas et al. (1998) estimated a dose of 4.0 to 5.3 $\times$ 10^6 total sperm were necessary for bulls to achieve 95% of the optimum conception rate, thereby satisfying the compensable component for bulls used in their study.

Compensable traits, as suggested by Flowers (2012) include motility, normal morphology, and ability to undergo capacitation, initiate the acrosome reaction and penetrate the zona pellucida. Uncompensable traits include morphology (Thundathil et al., 2000), plasma membrane characteristics and DNA integrity (Saacke et al., 2000).

The first study reported in Chapter 2 investigated the effect of sperm dose on pregnancy per timed AI (P/TAI) in Brazilian beef cattle (n = 4,866 cows on 10 farms). In summary, there was no effect of sperm dose on P/TAI, however, fertility differences between bulls were detected. Semen from all bulls used in the first study passed routine pre-freeze and post-thaw quality control analyses at Select Sires prior to distribution for use. Nevertheless, considering the heterogeneous population of sperm in an ejaculate, Garner (2014) and Rodriguez-Martinez (2001; 2003) argue further analyses may be necessary to detect potential fertility differences and subfertile males. Therefore, the overall objective of the second study described in this chapter was to identify semen characteristics that may be associated with fertility differences between bulls using computer-assisted sperm analysis (CASA) and flow cytometry (FC). The hypothesis was that high fertility bulls would exhibit the highest values for all *in vitro* analyses, except for DNA fragmentation index (DFI), while lower fertility bulls would exhibit the lowest values for all *in vitro* analyses, except for DNA fragmentation index.

Materials and methods

In this study, semen collections (n = 14) from Angus bulls (n = 5) used in the previous field fertility trial were used for *in vitro* sperm analyses (Table 3.1). Representative semen samples from each bull, dose and collection date were analyzed in duplicate for subjective motility (SM), CASA, FC and morphology.

For all analyses, two 0.5-mL semen straws from the same bull, dose and collection date were thawed simultaneously in a water bath at 37°C for a minimum of 60 seconds and no more than 180 seconds before assessment (DeJarnette et al., 2010). The two straws were pooled in a 1.5 mL Eppendorf vial (Eppendorf Safe-Lock Tube; USA Scientific, Ocala, FL), and then briefly vortexed for 2 seconds to homogeneity.

Reagents for FC and CASA analyses were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and included propidium iodide (PI), acridine orange (AO), tris buffer and bovine serum albumin (BSA) fraction V. Fluorescein isothiocyanate-conjugated peanut agglutinin-647 (PNA), fluo-3-acetomethoxy ester (Fluo-3) and Hoechst 33342 were obtained from Invitrogen (Thermo Fisher Scientific - Life Technologies Corporation, Eugene, OR, USA).

As suggested by Seidel (2012), SM was estimated at 200× magnification using phase contrast optics by two trained technicians (in duplicate), and the estimates were averaged. Two different samples (e.g., two bulls or two collection dates) were placed in four different numbered 1.5 mL Eppendorf vials (numbered 1 to 92) for blind assessment. Aliquots from the same samples used for SM were also used for CASA.

Computer-assisted sperm analysis was accomplished with the IVOS II (Hamilton Thorne, Beverly, MA, USA). Semen samples used for CASA were diluted to approximately 20×10^6 sperm per mL using tris buffer at 37°C. A proprietary milk-based extender was used for cryopreservation, therefore, all samples were incubated with Hoechst 33342 (80 µg per mL) for 10 minutes without the presence of light, to allow for the detection of individual sperm by CASA (Richardson et al., 2017). Samples were loaded in Leja slides (Leja Standard Count 4-Chamber 20 micron, Ref# SC 20-01-04-B, Lot# 081715B1, Nieuw-Vennep, Netherlands).

Ten fields from each chamber were analyzed using auto-capture and run in duplicate on different chambers of the same slide. The variables analyzed were total motility (TM, %), progressive motility (PM, %), amplitude of lateral head displacement (ALH, μm: the average value of the extreme side-to-side movement of the sperm head in each cycle), beat cross frequency (BCF, Hz: the frequency with which the actual sperm trajectory crosses the average path trajectory), straight-line velocity (VSL, μm/sec: the average path velocity of the sperm head along a straight line from its first to its last position), average path velocity (VAP, μm/sec: the average velocity of the sperm head along its average trajectory), curvilinear velocity (VCL, μm/sec: the average path velocity of the sperm head along its actual trajectory), linearity (LIN, %: the ratio between VSL and VCL), straightness (STR, %: the ratio between VSL and VAP) and wobble (WOB, %: the ratio between VAP and VCL).

For the initial FC analysis, 0.5mL-semen straws from six different samples were simultaneously thawed and run in duplicate in different wells. A 2 μL sample of extended semen was incubated for 20 minutes at 35ºC without the presence of light in a tris buffer solution containing 5 ng per μL PI and 15.5 ng per μL Hoechst 33342 (Garner et al., 1995). Samples were prepared in round bottom 96-well plates and a total of 5000 cells per sample were analyzed using a MACSQuant Analyzer 10 (Miltenyi Biotec, Auburn, CA, USA). Hoechst 33342 was excited via a 405 nm laser and detected with a 450/50 nm bandpass filter. A 488 nm laser was used to excite PI which was detected with 655-730 nm. Flow cytometric data were analyzed with FLOWJo (FLOWJo, LLC, Ashland, OR, USA). The percentage of viable sperm with intact plasma membranes (viablePI; PI negative) was calculated by FLOWJo for each sample based on a histogram.

A flow cytometric analysis, using four different stains simultaneously, was also conducted and the proportion of sperm with intact plasma membranes (viable; PI negative as determined from a histogram), intact acrosome (acrosome; PNA negative as determined from a histogram), viable with acrosome (VA; PI and PNA negative as determined from dot plot gating), viable with normal calcium (VNCa; PI and Fluo-3 negative as determined from dot plot gating), and acrosome with normal calcium within viable (ANCa; PI negative as determined from a histogram, and PNA and Fluo-3 negative as determined from a dot plot within viable) were determined. Briefly, a 2 µL sample of extended semen was incubated for 20 minutes at 35ºC without light in a tris buffer solution containing 5 ng/µL PI, 15.5 ng/µL Hoechst 33342, 12.5 ng/µL PNA, and 11 µM Fluo-3 (adapted from Garner et al., 1995; Gualtieri et al., 2005; Landim-Alvarenga et al., 2004; Odhiambo et al., 2011; Purdy and Graham, 2004). Samples were prepared in round bottom 96-well plates and a total of 5000 cells per sample were analyzed using a MACSQuant Analyzer 10 (Miltenyi Biotec, Auburn, CA, USA). Hoechst 33342 was excited via a 405 nm laser and detected with a 450/50 nm bandpass filter. A 488 nm laser was used to excite PI and Fluo-3 which were detected with 655-730 nm and 525/50 nm filters, respectively. A red 635 nm laser was used to excite PNA-647 which was detected with a 655-730 nm bandpass filter. Flow cytometric data was analyzed with FLOWJo (FLOWJo, LLC, Ashland, OR, USA). To determine the proportion of total sperm analyzed that possess all the desirable characteristics assayed, the overall population of sperm with intact plasma and acrosome membranes with normal calcium influx (VANCa) were calculated by multiplying viable and ANCa (VANCa = viable × ANCa).

To analyze the chromatin structure of sperm, 20 µL of semen was diluted in 180 µL of TNE buffer (0.01 M TRIS-HCl, 0.15 M NaCl, and 1mM disodium EDTA, pH 7.4). Next

acid detergent solution (400 µL; pH 1.2) was added followed by incubation for 30 seconds at room temperature. A staining solution was added (1.2 mL AO) next followed by incubation for 3 minutes at room temperature (Evenson, 2013). A 488 nm laser was used to excite AO, and red fluorescence (single stranded DNA) was detected with a 630/50 nm filter while green (double stranded DNA) was detected with a 515/30 nm filter. A total of 5000 cells per sample were analyzed using a MACSQuant Analyzer 10 (Miltenyi Biotec, Auburn, CA, USA). DNA stability was reported as the percentage of single stranded DNA and calculated as % DFI = (Single Stranded) / (Single Stranded + Double Stranded) × 100 (Evenson, 2013; 2016).

Sperm morphology was evaluated post-thaw using differential interference contrast microscopy at 1000 × magnification after fixing sperm with 0.2% formalin and wet mount preparation (Richardson et al., 2017). One hundred cells were evaluated per bull and collection date. Sperm head defects were classified as primary defects, while sperm tail defects were classified as secondary defects (Barth and Oko, 1989).

Statistical methods

A generalized linear mixed model was used to analyze the data (SAS 9.4). In the analyses, proportions (SM, TM, PM, LIN, STR and WOB, and flow cytometer characteristics) were assumed to follow a beta distribution, whereas VSL, VAP, VCL, ALH and BCF were considered as normally distributed responses following assessment of univariate analyses. Statistical differences were noted at $P \leq 0.05$. Bull, duplicate, and bull by duplicate interaction were considered fixed effects, while collection date, and collection date by sperm concentration per duplicate were considered random effects (Stroup, 2014). Mean comparisons were assessed using pair-wise tests for SM, CASA and FC results. An additional generalized linear mixed model assuming a beta distribution was also used to analyze

morphology, with bull as a fixed effect, and collection date and collection date by bull as random effects. Means were also compared using pair-wise tests. A Spearman correlation analysis (CORR, SAS 9.4) was used to investigate the association between the evaluation of sperm plasma membrane integrity as a single analysis (viablePI) or part of a multiple parameter analysis (viable).

To investigate the pattern and variability of *in vitro* sperm characteristics, a principal component analysis was used. In this analysis, 16 characteristics were investigated: TM, PM, ALH, BCF, VAP, VCL, VSL, LIN, STR, WOB, viable, acrosome, VA, VNCa, ANCa and DFI. Duplicative parameters were not included in the analyses, e.g., SM and viablePI. Further, because VANCa was calculated based on the analyses of viable and ANCa, it was also excluded from PCA. Principal component axes with eigenvalues > 1.0 were retained for further consideration and interpretation.

Results and Discussion

Sperm morphology has been reported to affect embryo quality (DeJarnette et al., 1992; Saacke et al., 1994a) and embryonic survival (Kidder et al., 1954; Bearden et al., 1956). Morphologic defects of the sperm tail and acrosomal membrane are considered compensable traits, as deficiencies in fertility may be overcome with an increase in sperm number per insemination dose (DeJarnette, 2005). In contrast, abnormalities in sperm head shape are generally considered uncompensable traits, which limit the maximum fertility threshold (DeJarnette, 2005; Saacke, 2008) and cannot be overcome by increasing the number of sperm per insemination dose. Saacke et al. (1998), however, reported that morphologically abnormal sperm are excluded from the accessory sperm population in the ovum based on the severity of sperm head shape distortion. Given the importance of morphology and its association with

fertility, semen from all bulls in this study were required to have a minimum of 65% morphologically normal sperm to be included in the field fertility study, which was conducted before the *in vitro* study. Consequently, it is not surprising that morphology results showed no differences between bulls in the proportion of morphologically normal and abnormal sperm (Table 3.2.; $P = 0.7$).

There was no effect of duplicate or duplicate by bull interaction ($P > 0.1$) in both CASA and FC analyses. In addition, no differences in BCF, LIN and WOB (Table 3.3; $P > 0.1$) were found; however, all other *in vitro* sperm characteristics were significantly different between bulls for CASA, SM (Table 3.3; $P < 0.05$) and FC (Table 3.4; $P < 0.05$). Field fertility (P/TAI) of each bull is reported at the top of Tables 3.3 and 3.4 to facilitate comparison.

The visual assessment of sperm motility, i.e. subjective motility as estimated with light microscopy, is a classic measure of sperm quality (DeJarnette, 2005). Seidel (2012) suggested that progressive motility is the most appropriate way to evaluate motility and a good measure of potential fertility. In the present study, we used two well-trained evaluators who estimated progressive motility in blind samples, and in duplicate, as suggested by Seidel (2012). Farrell et al. (1997) compared SM and CASA motility for fresh semen and CASA motility had larger variation (52-82% PM) than SM (62- 69% PM). It appears that SM was overestimated in the present study, as compared to CASA results (TM and PM; Table 3.3). Surprisingly, the results for SM appeared to be numerically more closely related to total motility of CASA, rather than PM, which is the goal of the visual subjective analysis. These findings may be related to the inherent differences in the estimation of motility by each method, i.e., human subjectivity (SM) and computer objectivity and subsequent calculation (CASA TM and PM).

The ejaculate of the bull contains a heterogenous population of sperm (Rodriguez-Martinez, 2001). Sperm subpopulations have been reported in bulls (Muiño et al., 2008) and rams (Luna et al., 2017). Four sperm subpopulations were described by Muiño et al. (2008) in Holstein bulls with the use of CASA. The first subpopulation had relatively low velocity (medium VCL, VSL and VAP) but exhibited highly progressive traits (high LIN, STR, WOB and BCF, and low ALH). The second subpopulation was characterized as highly active but non-progressive sperm (high values of VCL and ALH with low values of LIN and STR, and moderate BCF), suggesting hyperactivated-like motility (Muiño et al., 2008). The third subpopulation contained poorly motile and non-progressive sperm (low VAP, VCL, VSL, BCF, ALH, LIN, STR and WOB), whereas the fourth subpopulation, exhibited mostly rapid and progressive sperm movement (highest values of VCL, VSL, VAP, BCF, VCL, VSL, VAP and BCF and moderate ALH; Muiño et al., 2008).

Bull E appears to more closely fit the second subpopulation described by Muiño et al. (2008) as evidenced by high VCL and ALH values. Nevertheless, bull E does not exhibit low LIN, STR, and moderate BCF which were also included in the traits of the second sperm subpopulation by Muiño et al. (2008). Sperm from bull E, however, exhibited the lowest TM and highest VCL, VAP, VSL and ALH values, which when taken together with the lowest percentage of intact acrosomes (53%; Table 3.4), may be indicative of early capacitation (Rodriguez-Martinez, 2003), hyperactivated-like motility (Muiño et al., 2008), and shortened lifespan in the reproductive tract.

A Spearman correlation analysis to investigate the association between sperm plasma membrane evaluation with either multiple parameters analyzed simultaneously (viable; Hoechst 33342, PI, PNA and Fluo-3) or a single parameter (viablePI; Hoechst 33342 and PI)

revealed a high correlation ($r = 0.91$; $P < 0.05$; Figure 3.1). The high correlation between viablePI (Hoechst 33342 and PI) and viable (multiple stains) for plasma membrane integrity assessment is demonstrated by an almost linear relationship as shown in Figure 3.1. This relationship provides evidence of the reliability of our results for multiple assessments using multiple stains simultaneously.

The sperm population in an ejaculate and frozen-thawed semen is heterogeneous, and possesses diversified genetics, somewhat different morphology and physiological status (Rodriguez-Martinez, 2001). The process of cryopreservation can cause irreversible damage to sperm which may affect the ability of sperm to fertilize the ovum (Muiño et al., 2008; Richardson et al., 2017; Rodriguez-Martinez, 2001). Although an intact plasma membrane is an important sperm characteristic, a wide range in the correlation between fertility and plasma membrane integrity as measured with fluorescent probes has been reported ($r = 0.05$ to $r = 0.56$; Alm et al., 2001; Anzar et al., 2002, Januskauskas et al., 2001; 2003). In the present study, fertility appears to be associated more closely with FC results for viable, VNCa and VANCa, in which bulls A, B and D have the greatest values. Further, bulls B and D also exhibit the greatest values for acrosome and VA, and the smallest for DFI, which may be related to their greater fertility as compared with bull C. A comparison of FC values for bulls A and C, the greatest and smallest fertility bulls from the field trial, respectively, is confusing as bulls A and C are similar in 5 of 8 FC values (Table 3.4).

Several studies have reported an inverse relationship between DFI and fertility in a variety of species (as DFI increases, fertility decreases; Ballachey et al., 1987; 1988; Evenson, 2016; Gliozzi et al., 2017; Januskauskas et al., 2003; Waterhouse et al., 2006). The results reported here do not agree with those studies, as bull A and C, the highest and lowest fertility

bulls, respectively, showed similar DFI values (37.7 vs. 36.6%, respectively). Evenson (2016) argued bulls with DFI greater than 10 – 20% would have reduced fertility. In the present study, however, bulls A and B had acceptable field fertility (48.1 and 47.7% P/TAI) with greater than recommended DFI (37.7 and 26.1%), respectively. Richardson et al. (2017) also described acceptable fertility while reporting higher values of DFI (33 and 41%) for two Angus bulls.

As stated by Amann and Hammerstedt (1993), an inseminate should have enough sperm with all the necessary characteristics in order for fertility to reach the maximum potential of the female population. Included in these necessary characteristics would be the ability to complete fertilization and embryonic development (Rodriguez-Martinez, 2001), i.e., low levels of uncompensable traits (Saacke, 2008). Further, DeJarnette (2005) argued that bull fertility should be questioned even after passing all *in vitro* tests because of unmeasured or unknown sperm characteristics. In our study, bulls A and B exhibited the greatest fertility and therefore appear to have enough of the necessary characteristics, despite intermediary FC results. Bull D and E, however, had similar fertility and were opposites relative to FC sperm analyses. Consequently, our FC findings with bull D corroborate the concerns of DeJarnette (2005), where the highest FC values coupled with the lowest DFI did not result in greater fertility for bull D as compared to bulls A and B.

When a large number of measurements are available, the use of PCA is suggested to evaluate the possibility of their replacement with fewer measurements without losing valuable information (Rao, 1964). Recently in sperm analyses, PCA has been used for grouping subpopulations within CASA analyses in rams (Luna et al., 2017); to evaluate the relationship of sperm analyses with recurrent pregnancy loss in humans (Gil-Villa et al., 2010); and as a

method to create an index for CASA and morphological parameters to evaluate their influence in dog semen freezeability (Núñez Martínez et al., 2006).

In our study, PCA revealed 88.7% of total data variability was accounted for by three principal components. The first principal component (Prin1) accounted for 47.1% of total data variability and was most influenced by sperm viability (TM, viable, acrosome, VA, VNCa) and DFI. The second principal component (Prin2) accounted for 25.9% of total data variability and was most influenced by sperm movement pattern (BCF, LIN, STR and WOB). The third principal component (Prin3) represented 15.7% of the total data variability and was most influenced by motility (PM, VAP, VCL and VSL). Across the three principal components with eigenvalues > 1.0, two parameters were consistently not influential in the variability (ALH and ANCa); thus, ALH and ANCa are not included in Prin1, Prin2 or Prin3 (Appendix 2).

A biplot of Prin1 vs. Prin2 (Figure 3.2), accounting for 73% of the total data variability, shows patterns related to each bull. In Prin1, the relative correlation of parameters moved samples along the X axis, whereas in Prin2 the relative correlation of parameters moved samples along the Y axis. Together, Prin1 and Prin2 formed 4 quadrants (Figure 3.2): top right, with positive values on Prin1 and Prin2, bottom right, with positive values of Prin1 and negative values on Prin2, top left, with negative values in Prin1 and positive values on Prin2, and bottom left, with negative values on Prin1 and Prin2. In Prin1, higher values of TM, viable, acrosome, VA and VNCa, and lower values of DFI influenced the movement of samples to the right of the X-axis. Hence, bulls with higher values of TM, viable, acrosome, VA and VNCa, and lower values of DFI are seen at the right of Figure 3.2. In Prin2, higher

values of BCF, LIN, STR and WOB influenced the movement of samples down the Y axis (Figure 3.2).

Bulls B and D are in the top right quadrant with the lowest variability as samples cluster together, followed by bull A in the lower right quadrant. Bull E exhibited the largest variability in Prin1 and Prin2 covering portions of both the lower and upper left quadrants. In contrast to bull E, bull C showed lower variability in Prin1 and similar variability in Prin2.

The use of PCA allows the analysis of variability and provides a summary with fewer variables. Thus, in our results, we were able to summarize 16 parameters from CASA and FC analyses with 3 principal components. When comparing PCA results (Figure 3.2) and the field fertility data, we can clearly detect that bulls with higher fertility (A, B and D) were located in the right quadrants of the plot and had lower variability in both Prin1 and Prin2. The lowest fertility bull (C) was located in the left quadrants, displayed the smallest variation in Prin1 (which accounted for the largest proportion of total data variability), and had higher variability in Prin2. Interestingly, bull E showed high variability in both Prin1 and Prin2, was located primarily in the left quadrants, yet had fertility similar to both bull C and bull D.

Conclusions

In conclusion, morphology, CASA and FC were not able to explain the difference in field fertility between bulls, further illustrating the difficulties in predicting bull fertility. Principal component analysis, however, simplified the complexity of CASA and FC data allowing for the grouping and identification of key sperm characteristics. In the data presented here, the greatest variability was accounted for by sperm viability and DFI, followed by movement patterns and motility; however, the relative influence of the characteristics varied by bull.

Table 3.1 Bull age (years), number of collection dates and sperm doses (10, 20, 20, 40 × 10^6 sperm per straw) used for field fertility, and number of samples analyzed for subjective motility (SM), computer-assisted sperm analyses (CASA), flow cytometer (FC) and morphology per bull.

Bull	Age	Collections	Dose	CASA	SM	FC	Morphology
A	2	3	4	14	14	14	3
B	4	2	4	12	12	12	2
C	3	2	4	16	16	16	2
D	2	3	4	20	20	20	3
E	2	4	4	30	30	30	4

Table 3.2 Estimated percentage sperm morphology assessed with differential interference contrast (DIC) microscopy for five Angus bulls used in a timed AI program[1].

Bull	Normal[2], %	Primary[3], %	Secondary[4], %
A	66.7	29.6	3.7
B	72.5	13.5	14
C	70.5	18.5	11
D	75.7	16	8.3
E	70.8	26.5	2.7

[1]One hundred cells were evaluated per bull and collection date.
[2]Normal: total normal sperm (P = 0.7).
[3]Primary: Sperm head defects
[4]Secondary: Sperm tail defects (Barth and Oko, 1989).

Table 3.3 Field fertility (%), subjective motility (SM; mean ± SEM) and computer-assisted sperm analyses (CASA; mean ± SEM) of representative semen samples from bulls with different fertility in a timed AI program.

Variables	Bull A	Bull B	Bull C	Bull D	Bull E
Fertility[1], %	48.1[a]	47.7[a]	40.7[c]	45.5[ab]	43.1[bc]
SM, %	42.1 ± 2.3[c]	54.8 2.5[ab]	50.8 ± 2.2[b]	60.6 ± 1.9[a]	38.3 ± 1.5[c]
CASA					
TM[2], %	31.8 ± 2.2[bc]	33.0 ± 2.4[b]	26.5 ± 2.0[cd]	51.6 ± 2.0[a]	24.2 ± 1.4[d]
PM[3], %	21.8 ± 2.0[b]	23.7 ± 2.2[b]	19.8 ± 1.8[b]	36.5 ± 2.0[a]	19.0 ± 1.3[b]
ALH[4], µm	7.2 ± 0.3[c]	7.6 ± 0.3[bc]	8.2 ± 0.3[b]	7.7 ± 0.2[bc]	9.1 ± 0.2[a]
BCF[5], Hz	30.0 ± 0.9	28.4 ± 0.9	31.0 ± 0.8	30.8 ± 0.7	29.6 ± 0.6
VAP[6], µm/s	87.3 ± 2.4[d]	93.3 ± 2.5[cd]	107.1 ± 2.2[b]	99.5 ± 2.0[c]	114.0 ± 1.6[a]
VCL[7], µm/s	154.3 ± 5.3[d]	165.3 ± 5.7[cd]	192.4 ± 5.0[b]	177.5 ± 4.5[c]	206.4 ± 3.7[a]
VSL[8], µm/s	73.7 ± 2.1[c]	76.9 ± 2.2[c]	89.9 ± 2.0[a]	84.1 ± 1.7[b]	92.8 ± 1.4[a]
STR[9], %	83.8 ± 0.9[abc]	82.4 ± 1.0[bc]	85.3 ± 0.8[a]	84.6 ± 0.7[ab]	82.1 ± 0.6[c]
LIN[10], %	50.9 ± 1.2	49.1 ± 1.3	51.0 ± 1.1	50.4 ± 1.0	48.4 ± 0.8
WOB[11], %	59.2 ± 0.9	58.2 ± 0.9	58.8 ± 0.8	58.4 ± 0.7	57.9 ± 0.6

[a-d] Values within the same row not sharing a common superscript differ $P \leq 0.05$
[1]Fertility: proportion of cows pregnant per timed AI in ten Brazilian beef farms
[2]TM: total motility
[3]PM: progressive motility
[4]ALH: amplitude of lateral head displacement
[5]BCF: beat-cross frequency
[6]VAP: average path velocity
[7]VCL: curvilinear velocity
[8]VSL: straight-line velocity
[9]STR: straightness
[10] LIN: linearity
[11]WOB: wobble

Table 3.4 Field fertility (%) and flow cytometer (mean % ± SEM) analyses of semen of bulls with different fertility in a timed AI program[1].

Variable	Bull A	Bull B	Bull C	Bull D	Bull E
Fertility[2], %	48.1[a]	47.7[a]	40.7[c]	45.5[ab]	43.1[bc]
Flow Cytometer					
ViablePI[3]	48.3 ± 1.8[c]	56.1 ± 1.8[b]	46.5 ± 1.7[c]	66.7 ± 1.4[a]	34.8 ± 1.2[d]
Viable[4]	48.4 ± 2.0[c]	55.1 ± 2.1[b]	41.6 ± 1.8[d]	66.2 ± 1.6[a]	30.5 ± 1.2[e]
Acrosome[5]	59.7 ±2.1[b]	76.7 ± 1.8[a]	64.3 ± 1.9[b]	77.5 ± 1.4[a]	53.2 ± 1.5[c]
VA[6]	49.4 ± 2.0[c]	58.1 ± 2.2[b]	44.8 ± 1.9[c]	68.1 ± 1.6[a]	31.8 ± 1.3[d]
VNCa[7]	45.9 ± 2.1[c]	53.2 ± 2.3[b]	40.1 ± 2.0[d]	62.9 ± 1.7[a]	29.1 ± 1.3[e]
ANCa[8]	91.6 ± 0.9[a]	92.1 ± 0.9[a]	91.7 ± 0.8[a]	91.8 ± 0.7[a]	87.7 ± 0.8[b]
VANCa[9]	44.1 ± 2.0[c]	50.7 ± 2.2[b]	38.1 ± 1.8[d]	60.6 ± 1.7[a]	27.1 ± 1.2[e]
DFI[10]	37.7 ± 2.0[c]	26.1 ± 1.8[b]	36.6 ± 1.9[c]	21.5 ± 1.4[a]	47.8 ± 1.5[d]

[a-e] Values within the same row not sharing a common superscript differ between bulls $P \leq 0.05$

[1]Flow cytometric data were analyzed with FLOWJo software. The proportion of viable and acrosome for each sample were determined based on the histogram generated. The proportion of VA and VNCa for each sample were determined based on a dot plot and the proportion of ANCa was determined by a dot plot within the viable population, which was determined based on a histogram.

[2]Fertility: proportion of cows pregnant per timed AI in ten Brazilian beef farms

[3]ViablePI: membrane integrity dual stain [Hoechst 33342 and propidium iodide(PI)]

[4]Viable: membrane integrity

[5]Acrosome: acrosome integrity

[6]VA: viable with intact acrosome

[7]VNCa: viable with normal Ca influx

[8]ANCa: within viable with intact acrosome and normal Ca

[9]VANCa: viable with intact acrosome and normal calcium with multiple stains [Hoechst 33342, PI, fluorescein isothiocyanate-conjugated peanut agglutinin-647 (PNA), fluo-3-acetomethoxy ester (Fluo-3)]

[10]DFI: proportion of single stranded DNA [acridine orange (AO)]

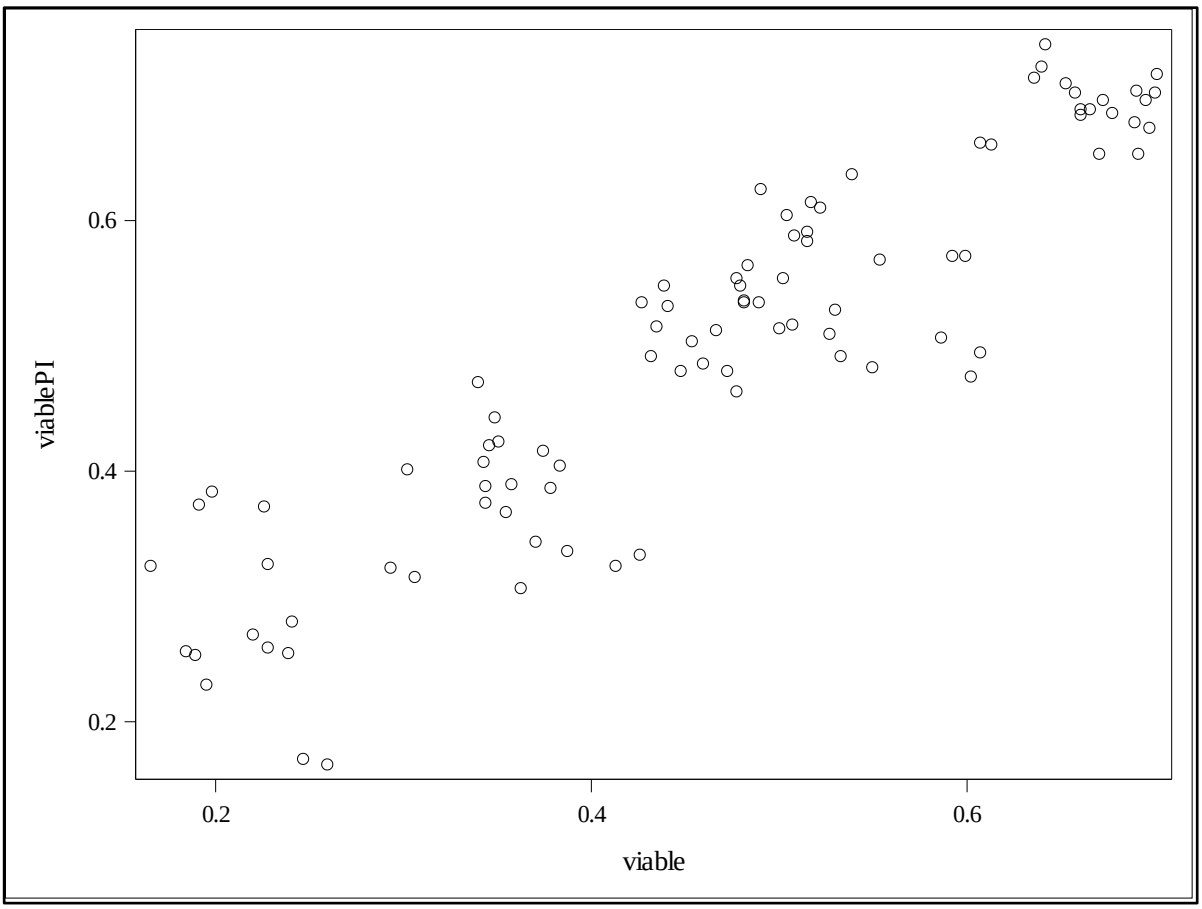

Figure 3.1 Spearman correlation between sperm plasma membrane analysis with multiple parameters analyzed simultaneously (viable; Hoechst 33342, PI, PNA and Fluo-3) or single parameter (viablePI; Hoechst 33342 and PI). X axis: proportion of viable sperm analyzed with multiple stains simultaneously. Y axis: proportion of viablePI with dual staining for membrane integrity ($r = 0.91$; $P < 0.01$).

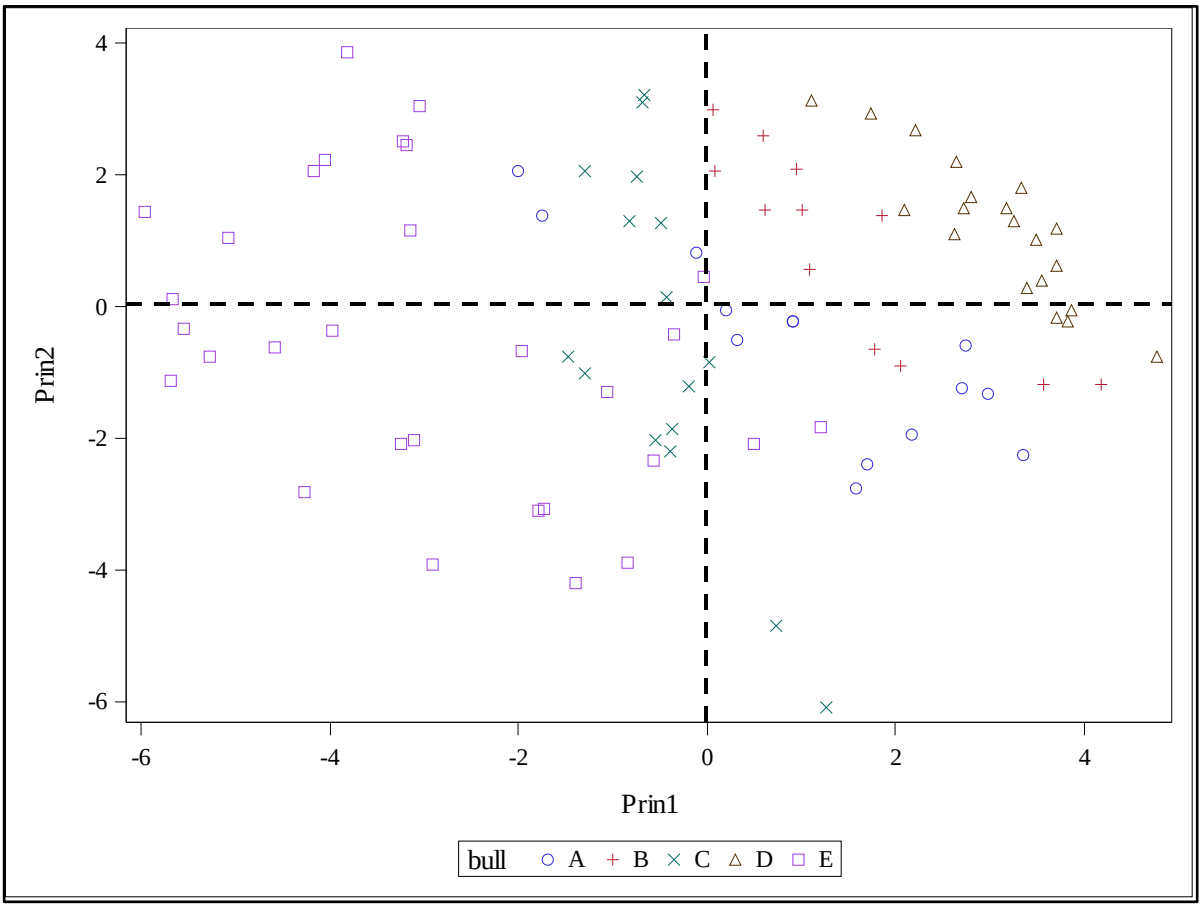

Figure 3.2 Principal component 1 (Prin1) and 2 (Prin2) for 16 *in vitro* sperm characteristics of five Angus bulls with different fertility used in a timed AI program. X axis: Principal component 1 (Prin1; 47.1% of total variability) most influenced by sperm viability and DFI. Y axis: Principal component 2 (Prin2; 25.9% of total variability) most influenced by sperm movement pattern. Prin1 and Prin2 account for 73% of the total variability associated with 16 *in vitro* sperm characteristics (total motility, progressive motility, amplitude of lateral head displacement, beat cross frequency, average path velocity, curvilinear velocity, straight line velocity, linearity, straightness, wobble, membrane integrity, acrosome integrity, membrane and acrosome intact, membrane integrity with normal Ca influx, membrane and acrosome intact with normal Ca influx and DNA fragmentation index). Symbols (as described in the legend) represent observations from the five bulls for each sample.

Literature cited

Alm, K., J. Taponen, M. Dahlbom, E. Tuunainen, E. Koskinen, and M. Andersson. 2001. A novel automated fluorometric assay to evaluate sperm viability and fertility in dairy bulls. Theriogenology. 56:677-684. doi:10.1016/S0093-691X(01)00599-4

Amann, R. P., and R. H. Hammerstedt. 1993. *In vitro* evaluation of sperm quality: An opinion. J. Androl. 14(6):397-406.

Anzar, M., L. He, M. M. Buhr, T. G. Kroetsch, and K. P. Pauls. 2002. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol. Reprod. 66:354-360. doi:10.1095/biolreprod66.2.354

Barth, A. D., and J. Oko. 1989. Abnormal morphology of bovine spermatozoa. Iowa State University Press, Ames.

Ballachey, B. E., W. D. Hohenboken, and D. P. Evenson. 1987. Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. Biol. Reprod. 36:915-925.

Ballachey, B. E., R. G. Saacke, and D. P. Evenson. 1988. The sperm chromatin structure assay: relationship with alternate tests of sperm quality and heterospermic performance of bulls. J. Androl. 9:109-115.

Bearden, H. J., W. M. Hansel, and R. W. Bratton. 1956. Fertilization and embryonic mortality rates of bulls with histories of either low or high fertility in artificial breeding. J. Dairy Sci. 39:312-318. doi:10.3168/jds.s0022-0302(56)94750-6

Clay, J. S., and B. T. McDaniel. 2001. Computing mating bull fertility from DHI nonreturn data. J. Dairy Sci. 84;1238-45.

Dalton, J. C., J. M. DeJarnette, R. G. Saacke, and R. P. Amann. 2017. The male component of dairy herd fertility. In: D.K. Beede, editor, Large Dairy Herd Management, third edition, American Dairy Science Association, Champaign, IL, p. 565-578.

DeJarnette, J. M., R. G. Saacke, J. Bame, and C. J. Vogler. 1992. Accessory sperm: their importance to fertility and embryo quality, and attempt to alter their number in artificially inseminated cattle. J. Anim. Sci. 70:484-491. doi:10.2527/1992.742484x

DeJarnette, J. M. 2005 The effect of semen quality on reproductive efficiency. Vet. Clin. Food Anim. 21:409-418.

DeJarnette, J. M., C. R. McCleary, M. A. Leach, J. F. Moreno, R. L. Nebel, and C. E. Marshall. 2010. Effects of 2.1 and 3.5×10^6 sex-sorted sperm dosages on conception rates of Holstein cows and heifers. J. Dairy Sci. 93:4079–4085.

Den Daas, J. H. G., G. De Jong, L. M. T. E. Lansbergen, and A. M. Van Wagtendonk-De Leeuw. 1998. The relationship between the number of spermatozoa inseminated and the reproductive efficiency of individual dairy bulls. J. Dairy Sci. 81:1714–1723.

Evenson, D. P. 2013. Sperm Chromatin Structure Assay (SCSA®). In: Carrell D., Aston K. (eds) Spermatogenesis. Methods in Molecular Biology (Methods and Protocols), Vol 927. Humana Press, Totowa, NJ. doi:10.1007/978-1-62703-038-0_14

Evenson, D. P. 2016. The sperm chromatin structure assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. Anim. Reprod. Sci. 169:56-75. doi:10.1016/j.anireprosci.2016.01.017.

Farrell, P. B., G. A. Presicce, C. C. Brockett, and R. H. Foote. 1998. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenology. 49:871-879. doi:S0093-691X(98)00036-3

Flowers, W. L. 2013. Triennial reproduction symposium: Sperm characteristics that limit success of fertilization. J. Anim. Sci. 91:3022-3029. doi:10.2527/jas2012-5945.

Garner, D. L., J. R. Dobrinsky, G. R. Welch, and L. A. Johnson. 1995. Porcine sperm viability, oocyte fertilization and embryo development after staining spermatozoa with SYBR-14. Theriogenology. 45:1103-1113. doi:10.1016/0093-691X (96)00067-2

Garner, D. 2014. Use of flow cytometry for semen evaluation. In: Proc. 25th Tech. Conf. Artif. Insem. and Reprod. Columbia, MO: National Association of Animal Breeders. p. 114-121.

Gil-Villa, A. M., W. Cardona-Maya, A. Agarwal, R. Sharma, and A. Cadavid. 2010. Assessment of sperm factors possibly involved in early recurrent pregnancy loss. Fertil. Steril. 94:4:1465-1472. doi:10.1016/j.fertnstert.2009.05.042

Gliozzi, T. M., F. Turri, S. Manes, C. Cassinelli, and F. Pizzi. 2017. The combination of kinetic and flow cytometric semen parameters as a tool to predict fertility in cryopreserved bull semen. Anima. 11:11:1975-1982. doi:10.1017/S1751731117000684

Gualtieri, R., R. Boni, E. Tosti, M. Zagami, and R. Talevi. 2005. Intracellular calcium and protein tyrosine phosphorylation during the release of bovine sperm adhering to the fallopian tube epithelium *in vitro*. Reproduction. 129:51-60. doi:10.1530/rep.1.00374

Januskauskas, A., A. Johannisson, and H. Rodríguez-Martínez. 2001. Assessment of sperm quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen-thawed semen from Swedish AI bulls. Theriogenology. 55:947-961. doi:10.1016/S0093-691X(01)00456-3

Januskauskas, A., A. Johannisson, and H. Rodríguez-Martínez. 2003. Subtle membrane changes in cryopreserved bull semen in relation to sperm viability, chromatin structure and field fertility. Theriogenology. 60:743-758. doi:10.1016/S0093-691X(03)00050-5

Kidder, H. E., W. G. Black, J. N. Wiltbank, L. C. Ulberg, and L. E. Casida. 1954. Fertilization rates and embryonic death rates of cows bred to bulls of different levels of fertility. J. Dairy Sci. 37:691-697. doi:103168/jds.s0022-0302(54)92314-4

Landim-Alvarenga, F. C., J. K. Graham, M. A. Alvarenga, and E. L. Squires. 2004. Calcium influx into equine and bovine spermatozoa during *in vitro* capacitation. Anim. Reprod. 1: 96–105.

Luna, C., M. Yeste, M. M. Rivera del Alamo, J. Domingo, A. Casao, J. E. Rodriguez-Gil, R. Pérez-Pé, J. A. Cebrián-Pérez, and T. Muiño-Blanco. 2017. Effect of seminal plasma proteins on the motile sperm subpopulations in ram ejaculates. Reprod. Fert. Devel. 29:394-405. doi:10.1071/RD15231

Muiño, R., C. Tamargo, C. O. Hidalgo, and A. I. Peña. 2008. Identification of sperm subpopulation with defined motility characteristics in ejaculates from Holsteins bulls: Effect of Cryopreservation and between bull variation. Anim. Reprod. Sci. 109:27-39. doi:10.1016/j.anireprosci.2007.10.007

Núñez Martínez, I., J. M. Morán, and F. J. Peña. 2006. Two-step cluster procedure after principal component analysis identifies sperm subpopulations in canine ejaculates and its relation to cryoresistance. J. Androl. 27:596-603. doi:10.2164/jandrol.05153

Odhiambo, J. F., M. Sutovsky, J. M. DeJarnette, C. Marshall, and P. Sutovsky. 2011. Adaptation of ubiquitin-PNA based sperm quality assay for semen evaluation by a

conventional flow cytometer and a dedicated platform for flow cytometric semen analysis. Theriogenology. 76:1168-1176. doi:10.1016/j.theriogenology.2011.05.009

Purdy, P.H., and J. K. Graham. 2004. Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction and fertility. Biol. Reprod. 71, 522–527.

Rao, C. R. 1964. The use and interpretation of principal component analysis in applied research. Sankhyā: The Indian Journal of Statistics, Serie A (1961-2002). 26:4:329.358.

Richardson, B. N., E. L. Larimore, J. A. Walker, M. D. Utt, J. M. DeJarnette, and G. A. Perry. 2017. Comparison of fertility of liquid or frozen semen when varying the interval from CIDR removal to insemination. Anim. Reprod. Sci. 178:61-66. doi:10.1016/j.anireprosci.2017.01.010

Rodríguez-Martínez, H. 2001. Sperm function in cattle and pigs: morphological and functional aspects. Arch. Animal Breed. 44:102-113.

Rodríguez-Martínez, H. 2003. Laboratory semen assessment and prediction of fertility: still Utopia? Reprod. Domest. Anim. 38(4):312-318. doi:10.1046/j.1439-0531.2003.00436.X

Saacke, R. G., S. Nadir, and J. Dalton. 1994a. Accessory sperm evaluation and bull fertility (an update). In: Proc. 15th Tech. Conf. Artif. Insem. and Reprod. Columbia, MO: National Association of Animal Breeders. p. 57-67.

Saacke, R. G., S. Nadir, and R. L. Nebel. 1994b. Relationship of semen quality to sperm transport, fertilization, and embryo quality in ruminants. Theriogenology. 41:45-50.

Saacke, R. G., J. M. DeJarnette, J. H. Bame, D. S. Karabinus, and S. S. Whitman. 1998. Can spermatozoa with abnormal heads gain access to the ovum in artificially inseminated

super- and single-ovulating cattle? Theriogenology. 50:117-128. doi:10.1016/S0093-691X(98)00119-8

Saacke, R. G., J. C. Dalton, S. Nadir, R. L. Nebel, and J. H. Bame. 2000. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. Anim. Reprod. Sci. 50-61:663-77.

Saacke R. G. 2008. Sperm morphology: Its relevance to compensable and uncompensable traits in semen. Theriogenology. 70:473-478. doi:10.1016/j.theriogenology.2008.04.012

Seidel Jr., G. E. 2012. Several insights on evaluation of semen. Anim. Reprod. 9(3):329-332.

Stroup, W. W. 2014. Rethinking the analysis of non-normal data in plant and soil science. Agron. J. 106:1–17. doi:10.2134/agronj2013.03

Thundathil, J., R. Meyer, A. T. Palasz, A. D. Barth, and R. J. Mapletoft. 2000. Effect of the knobbed acrosome defect in bovine sperm on IVF and embryo production. Theriogenology. 54:921-934. doi:10.1016/S0093-691X(00)00402-7

Vincent P., S. L. Underwood, C. Dolbec, N. Bouchard, T. Kroetsch, and P. Blondin. 2012. Bovine semen quality control in artificial insemination centers. Anim. Reprod. 9(3):153-165.

Waterhouse, K. E., T. Haugan, E. Kommisrud, A. Tverdal, G. Flatberg, W. Farstad, D. P. Evenson, and P. M. De Angelis. 2006. Sperm DNA damage is related to field fertility of semen from young Norwegian Red bulls. Reprod. Fertil. Devel. 18:781-788.

CHAPTER 4

"Discussion: Influence of bull, sperm dosage and semen evaluation on beef cattle fertility"

The use of crossbreeding, as was done in the field fertility trial described in Chapter 1, is an important tool for the Brazilian beef market because of its impact on meat quality (Lage et al., 2012). Researchers have studied the effect of the female in the Brazilian beef cattle industry in crossbreeding programs (*Bos indicus* × *Bos taurus*; Sá Filho et al. 2010; 2011). Further, although beef cattle fertility has been studied for many years and the effect of the bull on cattle fertility has been described (Flowers, 2013), the literature lacks information about the influence of sperm dosage and sperm evaluation of Angus bulls in crossbreeding programs in Brazil (Oliveira et al., 2012; 2013).

Higher fertility levels improve the viability of crossbreeding and AI programs. Unfortunately, correlations between sperm analyses and fertility have been variable (Foote, 2003; Rodríguez-Martínez, 2003) with single sperm parameters having low or no correlation with fertility whereas multiple sperm parameters have demonstrated higher correlations to fertility (Farrell et al., 1998; Tardiff et al., 1998). The estimation of bull fertility is more accurate for a single ejaculate than a lifetime estimation, because bull fertility can vary by season (Malama et al., 2017), age, and health status which makes the long-term prediction of fertility based on sperm analyses nearly unachievable (Amann and DeJarnette, 2012).

In Chapter 2 the influence of bull, but not sperm dosage (10, 20 and 40 × 10^6 sperm per dose) on pregnancy per timed-AI (P/TAI) was detected in Brazilian beef cattle (*Bos*

indicus and *Bos indicus* × *Bos taurus* crossbred). Bull E may have had higher levels of compensable traits, as evidenced by P/TAI of 36.6% for 10×10^6 sperm inseminated, followed by a plateau of P/TAI of 44.7 to 45.9% for 20 and 40×10^6 sperm inseminated, respectively. Bull E exhibited the lowest values for motility and viability in Chapter 3, which agrees with the argument in Chapter 2 regarding potential compensable traits, as previously described by DeJarnette (2005) and Saacke et al. (2000).

Using the data from the flow cytometric analysis in Chapter 3, the proportion of viable sperm with acrosome and normal calcium (VANCa) was calculated. The insemination doses of 10, 20 and 40×10^6 sperm per straw for bull E correspond to approximately 2.7, 5.4 and 10.8×10^6 viable sperm per straw. This agrees with Den Daas et al. (1998) who estimated a dose of 4.0 to 5.3×10^6 total viable sperm was necessary for bulls to achieve 95% of the optimum conception rate, thereby satisfying the compensable component for bulls used in their study.

Interestingly, bull E showed the greatest percentage DNA fragmentation index (DFI), which is considered an uncompensable trait (DeJarnette, 2005; Saacke et al. 2000). However, the overall fertility of bull E could be considered intermediary relative to all bulls in the study (Table 2.5). It was proposed in Chapter 2 that bull C may have had higher levels of uncompensable traits because of the lower fertility exhibited across all dosages. However, the level of uncompensable characteristics (DFI and morphology; Table 3.4 and 3.2, respectively) for bull C were not statistically different from the highest fertility bull (bull A). The results from bull E and C when taken together, agree with the argument by DeJarnette (2005) that samples with sufficient levels of known characteristics should still be considered of

questionable fertility because of unknown or unmeasured characteristics that may affect fertility.

Although it appears far from possible, the accurate prediction, rather than estimation, of fertility remains the goal for semen evaluation. Technological methods of semen evaluation, such as FC and CASA, have been included or are in the process of inclusion in routine quality control analyses at AI centers (DeJarnette, 2012). Our results of principal component analyses (Figure 3.2), however, provide evidence progress can be made in understanding variability within and between bulls, as PCA simplified the complexity of CASA and FC data allowing for the grouping and identification of key sperm characteristics. Unfortunately, we currently do not know, how "much" of each sperm characteristic is "enough" for successful completion of fertilization and an acceptable fertility outcome (Amann and Hammerstedt, 1993).

Conclusions

Morphology, CASA and FC were not able to explain the difference in field fertility between bulls, further illustrating the difficulties in predicting bull fertility. This conclusion, unfortunately, agrees with previous authors' contention that male fertility is multifactorial, and the insemination dose must contain enough of all necessary characteristics, known and unknown, in proportion and number, to reach the maximal fertility of the female population. The use of CASA and FC to assess multiple sperm characteristics simultaneously is promising, especially when considering the large numbers of cells analyzed as compared to routine subjective analyses with microscopy. The use of principal component analysis to simplify the complexity of CASA and FC data should be investigated further, as it allows for

the grouping and identification of key sperm characteristics, and provides a visual aspect to understanding variability.

Literature cited

Amann, R. P., and R. H. Hammerstedt. 1993. In vitro evaluation of sperm quality: An opinion. J. Androl. 14(6):397-406.

Amann, R. P., and J. M. DeJarnette. 2012. Impact of genomic selection of AI dairy sires on their likely utilization and methods to estimate fertility: A paradigm shift. Theriogenology. 77:795–817. doi:10.1016/j.theriogenology.2011.09.002

DeJarnette, J. M. 2005. The effect of semen quality on reproductive efficiency. Vet. Clin. Food Anim. 21:409-418.

DeJarnette, J. M. 2012. Semen quality control and quality assurance in AI centers. In: Proc. Association for Applied Animal Andrology Conference, Vancouver, BC, Canada.

Den Daas, J. H. G., G. De Jong, L. M. T. E. Lansbergen, and A. M. Van Wagtendonk-De Leeuw. 1998. The relationship between the number of spermatozoa inseminated and the reproductive efficiency of individual dairy bulls. J. Dairy Sci. 81:1714–1723.

Farrell, P. B., G. A. Presicce, C. C. Brockett, and R. H. Foote. 1998. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenology. 49:871-879. doi:S0093-691X(98)00036-3

Flowers, W. L. 2013. Triennial reproduction symposium: Sperm characteristics that limit success of fertilization. J. Anim. Sci. 91:3022-3029. doi:10.2527/jas2012-5945

Foote, R. H. 2003. Fertility estimation: a review of past experience and future prospects. Anim. Reprod. Sci. 75:119-139. doi:S0378-4320(02)00233-6

Lage, J. F., P. V. R. Paulino, S. C. Valadares Filho, E. J. O. Souza, M. S. Duarte, P. D. B. Benedeti, N. K. P. Souza, and R. B. Cox. 2012. Influence of genetic type and level of

concentrate in the finishing diet on carcass and meat quality traits in beef heifers. Meat Sci. 90:770-774. doi:10.106/j.meatsci.2011.11.012

Malama, E., Y. Zeron, F. Janett, M. Siuda, Z. Roth, and H. Bollwein. 2017. Use of computer-assisted sperm analysis and flow cytometry to detect seasonal variations of bovine semen quality. Theriogenology. 87:79-90. doi: 10.1016/j.theriogenology.2016.08.002

Oliveira, L. Z., R. P. de Arruda, A. F. C. de Andrade, E. C. C. Celeghini, R. M. dos Santos, M. E. Beletti, R. F. G. Peres, C. S. Oliveira, and V. F. M. H. de Lima. 2012. Assessment of field fertility and several in vitro sperm characteristics following the use of different Angus sires in a timed-AI program with suckled Nelore cows. Livestock Sci. 146:36-46. doi:10.1016/j.livsci.2012.02.018

Oliveira, L. Z., R. P. de Arruda, A. F. C. de Andrade, E. C. C. Celeghini, P. D. Reeb, J. P. N. Martins, R. M. dos Santos, M. E. Beletti, R. F. G. Peres, F. M. Monteiro, and V. F. M. H. de Lima. 2013. Assessment of *in vitro* sperm characteristics and their importance in the prediction of conception rate in a bovine timed-AI program. Anim. Reprod. Sci. 137:145-155. doi:10.1016/j.anireprosci.2013.01.010

Rodríguez-Martínez, H. 2003. Laboratory semen assessment and prediction of fertility: still Utopia? Reprod. Domest. Anim. 38(4):312-318. doi:10.1046/j.1439-0531.2003.00436.x

Saacke, R. G., J. C. Dalton, S. Nadir, R. L. Nebel, and J. H. Bame. 2000. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. Anim. Reprod. Sci. 50-61:663-77.

Sá Filho, M. F., A. M. Crespilho, J. E. P. Santos, G. A. Perry, and P. S. Baruselli. 2010. Ovarian follicle diameter at timed insemination and estrous response influence

likelihood of ovulation and pregnancy after estrous synchronization with progesterone or progestin-based protocols in suckled *Bos indicus* cows. Anim. Reprod. Sci. 120:23-30. doi:10.1016/j.anireprosci.2010.03.007

Sá Filho, M. F., J. E. P. Santos, R. M. Ferreira. J. N. S. Sales, and P. S. Baruselli. 2011. Importance of estrus on pregnancy per insemination in suckled *Bos indicus* cows submitted to estradiol/progesterone-based timed insemination protocols. Theriogenology. 76:455-463. doi:10.1016/j.theriogenology.2011.02.022

Tardiff, A. L., P. B. Farrell, V. Trouern-Trend, M. E. Simkin, and R. H. Foote. 1998. Use of Hoechst 33342 stain to evaluate live, fresh and frozen bull sperm by computer assisted analysis. J. Androl. 19:201-206.

APPENDIX 1

"Institutional Animal Care and Use Committee Approval"

University of Idaho
Institutional Animal Care and Use Committee

Date: Monday, June 6, 2016
To: Joseph Dalton
From: University of Idaho
Institutional Animal Care and Use Committee
Re: Protocol 2016-24
Beef Semen Dose Titration

Your animal care and use protocol for the project shown above was reviewed and approved by the Institutional Animal Care and Use Committee on Monday, June 6, 2016.

This protocol was originally submitted for review on: Thursday, April 14, 2016
The original approval date for this protocol is: Monday, June 6, 2016
This approval will remain in effect until: Tuesday, June 6, 2017
The protocol may be continued by annual updates until: Thursday, June 6, 2019

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Barrie Robison, IACUC Chair

APPENDIX 2

"Eigenvectors"

Table 1. Eigenvectors for principal component 1(Prin1), principal component 2 (Prin2) and principal component 3 (Prin3).

Parameters	Prin1	Prin2	Prin3
TM[1]	0.30	0.06	0.24
PM[2]	0.27	0.04	0.35
ALH[3]	-0.27	0.28	0.19
BCF[4]	0.15	-0.39	0.10
VAP[5]	-0.23	0.10	0.47
VCL[6]	-0.26	0.20	0.36
VSL[7]	-0.16	-0.01	0.56
LIN[8]	0.19	-0.39	0.16
STR[9]	0.19	-0.31	0.25
WOB[10]	0.17	-0.40	0.11
Viable[11]	0.32	0.23	0.03
Acrosome[12]	0.29	0.24	0.06
VA[13]	0.32	0.23	0.03
VNCa[14]	0.31	0.25	0.02
ANCa[15]	0.13	0.23	-0.08
DFI[16]	-0.31	-0.18	-0.07

[1]TM: total motility
[2]PM: progressive motility
[3]ALH: amplitude of lateral head displacement
[4]BCF: beat-cross frequency
[5]VAP: average path velocity
[6]VCL: curvilinear velocity
[7]VSL: straight-line velocity
[8]LIN: linearity
[9]STR: straightness
[10]WOB: wobble
[11]Viable: membrane integrity
[12]Acrosome: acrosome integrity
[13]VA: viable with intact acrosome
[14]VNCa: viable with normal Ca influx
[15]ANCa: within viable with intact acrosome and normal Ca
[16]DFI: proportion of single stranded DNA.