South Dakota State University
Open PRAIRIE: Open Public Research Access Institutional Repository and Information Exchange

Electronic Theses and Dissertations

2021

Search for New Fertility Markers in Bull Sperm

Saulo Menegatti Zoca
South Dakota State University, saulo_mzoca@hotmail.com

Follow this and additional works at: https://openprairie.sdstate.edu/etd2

Part of the Animal Sciences Commons, and the Physiology Commons

Recommended Citation

Menegatti Zoca, Saulo, "Search for New Fertility Markers in Bull Sperm" (2021). *Electronic Theses and Dissertations*. 221.
https://openprairie.sdstate.edu/etd2/221

This Dissertation - Open Access is brought to you for free and open access by Open PRAIRIE: Open Public Research Access Institutional Repository and Information Exchange. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of Open PRAIRIE: Open Public Research Access Institutional Repository and Information Exchange. For more information, please contact michael.biondo@sdstate.edu.

SEARCH FOR NEW FERTILITY MARKERS IN BULL SPERM

BY

SAULO MENEGATTI ZOCA

A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Animal Science

South Dakota State University

2021

DISSERTATION ACCEPTANCE PAGE

Saulo Menegatti Zoca

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Julie Walker
Advisor Date

Joseph P Cassady
Department Head Date

Nicole Lounsbery, PhD
Director, Graduate School Date

I would like to dedicate this dissertation to all people who have made it possible, in special to my family, advisors, mentors and friends. Primarily, I would like to thank and dedicate this dissertation to the most important people in my life, my daughter Luiza Mattiello Zoca, my father Moises Zoca, my mother Regina Aparecida Menegatti Zoca, my partner Samara Paula Mattiello, and my siblings, Samuel Menegatti Zoca and Sara Menegatti Zoca, who have helped and supported me in many ways during my academic path since I started my studies, especially during my time in the USA. Words cannot describe my gratitude for their support.

Secondarily, I would like to dedicate this dissertation to my advisors and my mentors, Dr. George Perry, Dr. Julie Walker, Dr. Robert Cushman and Dr. Tom Geary, whose advise, mentorship, and support have helped me accomplish my goal to become a Doctor of Philosophy. Finally, I would like to dedicate this dissertation to all my friends and stepfamily, in special to Kaitlin Epperson, Adalaide Kline, Taylor Andrews, Lacey Quail, Jaclyn Ketchum, and Dr. Jerica Rich who have helped and supported me throughout my program.

ACKNOWLEDGEMENTS

I would like to thank and acknowledge all the people that have made it possible for me to accomplish this degree. First and foremost, I would like to thank my advisor Dr. George Perry. Dr. Perry has been a very supportive, kind, and friendly advisor, who has helped me tailor my graduate program to accomplish my career goals, some of those goals he envisioned in my path long before I did. I would also like to thank Dr. Perry for all the mentoring in my scientific development, in all my projects and for the freedom he has provided to me in my scientific discovery, that sometimes was frustrated by bad results, but followed by lots of learning. Dr. Perry, thank you for all these years of support, both scientifically and personally, and thank you for providing me this amazing scientific family that you literally treat as if we were one of your own kids.

Secondly, I would like to thank Dr. Julie Walker, who has been my advisor since Dr. Perry moved to his new position at Texas A&M University. Dr. Walker who prefers to be called Julie, but yes, she is Dr. Walker, is one of the toughest people I have ever met. Julie is always doing at least 110%, even with a broken foot or arm, and demonstrating by her example that hard work always pays off. Julie is an example of a mentor who mixes toughness and kindness in the correct amount. For the rest of my professional career and personal life, Julie will always be an example to be followed in work ethic and kindness. Of course, Luiza is very fortunate to have Julie as another grandmother in her life.

Thirdly, I would like to thank Dr. Robert Cushman for all his scientific training, patience with my numerous SAS questions, and putting up with my rebellious “Thank

you". I cannot thank you enough, even though you always say we should not thank you because it is your job as our mentor to help us. I prefer to disregard your stance on receiving "Thank you" and I prefer to follow the other part of your teaching style by being rebellious and saying "Thank you" as many times as I can. Since you will be reading this before my defense, these many "Thank you" in one paragraph directed towards you may backfire on me. However, I would like to finish this paragraph with a sentence starting with a "however" to demonstrate that your teachings are always appreciated. Thank you, Dr. Robert Bob Cushman (yes Bob, that was on purpose).

Fourthly, I would like to thank Dr. Tom Geary, who has been an amazing mentor and a friend. Tom opened his lab to me in a moment when I had my back against the wall, and luckily with Tom and Abby's assistance we were able to accomplish many projects in a short period of time. I am very thankful for the opportunity and mentorship you have provided me. Tom, at first site you may have an intimidating face, but it is quickly noticed that your heart does not fit inside your chest. You are an amazing and caring person and I appreciate all of your advice throughout the years, thank you very much. Abby, thank you very much for your assistance with lab work and for your friendship, I am very grateful for all you have done for me.

Fifthly, I would like to acknowledge two very special mentors who have helped me beyond any word of gratitude, Dr. Joseph Dalton, and Dr. Vasconcelos (Zequinha). To put in some words, Zequinha was my mentor during my undergrad who showed me the path of science and the passion for reproductive physiology, he is also responsible for putting Dr. Dalton and Dr. Perry in my path. Dr. Dalton accepted me as an intern when I was finishing my undergrad and he was my master's advisor. Both Zequinha and Dr.

Dalton are amazing people and friends who have helped me directly in my accomplishments presented in this dissertation by their mentorship, teachings, and passion for science.

Finally, but not least, I would like to thank all my scientific siblings, especially Kaitlin Epperson, Adalaide Kline, Taylor Andrews, Lacey Quail, Jaclyn Ketchum, and Dr. Jerica Rich, who are more than just friends, they are the people who I have shared the most amazing and frustrating moments of my program with and I theirs. These very supportive, kind, smart, and absolutely crazy people helped me throughout my program and without their assistance I would not be able to accomplish what I have accomplished.

CONTENTS

ABBREVIATIONS ix

LIST OF FIGURES xviii

LIST OF TABLES xxii

ABSTRACT xxiv

CHAPTER 1 – Literature Review 1

INTRODUCTION 1

SPERMATOZOA: FROM SPERMATOGENESIS TO EJACULATION 2

SPERMATOZOA: FROM EJACULATION TO FERTILIZATION 14

BULL FERTILITY EVALUATION 28

SUMMARY 44

CHAPTER 2 – Proteomic analyses identify differences between bovine epididymal and ejaculated spermatozoa that contribute to longevity 46

ABSTRACT 46

INTRODUCTION 47

MATERIALS AND METHODS 49

RESULTS 55

DISCUSSION 60

TABLES 67

FIGURES 70

CHAPTER 3 – Relationship between sire conception rate, sperm parameter, DAG1 and SERPINA5 proteins, and embryo *in vitro* production 79

ABSTRACT....79

INTRODUCTION....80

MATERIALS AND METHODS....83

RESULTS....88

DISCUSSION....90

TABLES....97

FIGURES....101

CHAPTER 4 - Bull field fertility differences can be estimated with *in vitro* sperm capacitation and flow cytometry....114

ABSTRACT....114

INTRODUCTION....115

MATERIALS AND METHODS....118

RESULTS....126

DISCUSSION....133

TABLES....143

FIGURES....148

LITERATURE CITED....174

APPENDIX....211

ABBREVIATIONS

ºC	Degrees Celsius
µg	Microgram
µL	Microliter
µm	Micrometer
ABP	Androgen-binding protein
ACE	Angiotensin converting enzyme
ACN	Acetonitrile
ADAM	A desintegrin and metalloprotease (superfamily)
AI	Artificial insemination
AKAP4	A-kinase anchor protein-4
AKR1A1	Alcohol dehydrogenase
ALDH2	Aldehyde dehydrogenase
ALDOA	Fructose-bisphosphate aldolase A
ALH	Amplitude of lateral head displacement
ANTE(1)	First-order ante-dependent
ANXA2	Annexin A2
AR(1)	First-order autoregressive
ARH(1)	Heterogeneous first-order autoregressive
ATP	Adenosine triphosphate

ATP6AP1	V-type proton ATPase subunit S1
au	Arbitrary unit
BCF	Beat-cross frequency
BCS	Body condition score
BIC	Bayesian information criterion
BL	Blastocyst rate
bNCM	Bovine non-capacitation media
BSA	Bovine serum albumin - fatty-acid free
BSE	Breeding soundness exam
BSP	Binder of sperm protein
Ca	Calcium
CALM1	Calmodulin
CALR3	Calsperin
CALR	Calreticulin receptor
CAMK2	Calmodulin kinase II
cAMP	Cyclic adenosine monophosphate
CASA	Computer-assisted sperm analysis
CATSPER	Cation channel sperm-associated protein
CL	Cleavage rate
CLGN	Calmegin

cm	Centimeter
CM	Capacitation media
CO_2	Carbon dioxide
COC	Cumulus-oocyte complex
CRISPs	Cysteine-rich secretory proteins
CS	Compound symmetry
CSH	Heterogeneous compound symmetry
CTSA	Lysosomal protective protein
CTSB	Cathepsin B
CTSD	Cathepsin D
d	Day
Da	Dalton
DAG1	Dystroglycan
dead	Sperm with disrupted plasma membrane
dead CD9-	Sperm with disrupted plasma membrane not labeled for CD9 protein
dead CD9+	Sperm with disrupted plasma membrane positive labeled for CD9 protein
dead disrupted	Sperm with disrupted plasma membrane and disrupted acrosome
dead intact	Sperm with disrupted plasma membrane and intact acrosome
dead ROS-	Sperm with disrupted plasma membrane negative for reactive oxygen species measured by Easykit 3

dead ROS+	Sperm with disrupted plasma membrane positive for reactive oxygen species measured by Easykit 3
DIC	Differential interface contrast
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
Ejac-F	Ejaculated fluid; seminal plasma
Ejac-S	Ejaculated sperm protein
Epid-F	Epididymal fluid
Epid-S	Epididymal sperm protein
FA	Formic acid
FAA	Fertility-associated antigen
FDR	False discovery rate
FI	Fluorescence intensity
FITC	Fluorescein isothiocyanate
FSH	Follicle-stimulating hormone
FTAI	Fixed-time artificial insemination
FW	Formula weight
FZ3	Fluozin-3, AM
GAA	Lysosomal alpha-glucosidase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBA	Glucosylceramidase

GLB1	Beta-galactosidase
GnRH	Gonadotropin releasing hormone
GNS	N-acetylglucosamine-6-sulfatase
GPI	Glycosylphosphatidylinositol
GPR1L1	Glioma pathogeneses-related protein 1
GSTM1	Glutathione S-transferase Mu
GSTP1	Glutathione S-transferase P
h	Hour
H	Hydrogen
HCO_3	Bicarbonate
HPSE	Heparanase
HVCN1	Voltage-sensitive proton channel
Hz	Hertz
ITPR1	Inositol 1,4,5-triphosphate receptor
IVF-TALP	Fertilization media
kDA	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	Kilogram
LCMS/MS	Liquid chromatography with tandem mass spectrometry analysis
LDHB	L-lactate dehydrogenase B chain

LGMN	Legumain
LH	Luteinizing hormone
LIN	Linearity
LY6K	Lymphocyte antigen 6K
MIF	Macrophage migration inhibitory factor
min	Minute
mito-potential	Mitochondrial membrane energy potential
mL	Milliliter
mm	Millimeter
mM	Millimolar
mo	Month
MS/MS	Tandem mass spectrometry analysis
Na	Sodium
NAAB-CSS	National Association of Animal Breeders - Certified Semen Services
NAGLU	N-acetyl-alpha-glucosaminidase
ng	Nanogram
nL	Nanoliter
nM	Nanomolar
P/AI	Pregnancy per artificial insemination
PBS	Phosphate buffer saline
PDILT	Protein disulfide isomerase

PEPBP4	Phosphatidylethanolamine-binding protein 4
PI	Propidium iodide
PKA	Protein kinase A
PNA	Fluorescein isothiocyanate-conjugated to peanut agglutinin
PPI	Protein-protein interaction
ppm	Parts per million
PPT1	Palmitoyl-protein thioesterase 1
PROG	Progressive motility
PSAP	Prosaposin
PTK	Protein tyrosine kinases
PTP	Protein tyrosine phosphatases
PVA	Polyvinyl alcohol
RNA	Ribonucleic acid
ROS	Reactive oxygen species
s	Second
SCR	Sire conception rate
SD	Standard deviation
SDHA	Succinate dehydrogenase [ubiquinone] flavoprotein subunit
SE	Standard error
SEM	Standard error of the mean

SERPIN	Serine protease inhibitor (superfamily)
SERPINA5	Plasma serine protease inhibitor, protein C inhibitor
SERPINE2	Glia-derived nexin or protease nexin 1
signature 1 + 2	Combination of zinc signature 1 and zinc signature 2
SLC9A10	Sperm-specific Na+-H+ exchange regulated by membrane potential
SOF-BE2	Culture media
SPA17	Surface protein Sp17
SPADH2	Spermadhesin Z13
SPAM1	Sperm adhesion molecule 1
STR	Straightness
TALP	Tyrode's albumen lactate pyruvate
TCA	Tricarboxylic acid
TMOT	Total motility
TOEP	Toeplitz
TPST2	Tyrosyl protein sulfotransferase-2
UTJ	Utero-tubular junction
v	volume
VAP	Average path velocity
VC	Variance components
VCL	Curvilinear velocity

viability	Plasma membrane integrity
viable	Sperm with intact plasma membrane
viable CD9-	Sperm with intact plasma membrane not labeled for CD9 protein
viable CD9+	Sperm with intact plasma membrane positive labeled for CD9 protein
viable disrupted	Sperm with intact plasma membrane and disrupted acrosome
viable intact	Sperm with intact plasma membrane and intact acrosome
viable ROS-	Sperm with intact plasma membrane negative for reactive oxygen species measured by Easykit 3
viable ROS+	Sperm with intact plasma membrane positive for reactive oxygen species measured by Easykit 3
VSL	Straight-line velocity
Wk	Week
w/w	Weight per weight
yr	Year
Zn	Zinc

LIST OF FIGURES

Figure 1.1. Diagram representing the steps of spermatogenesis in the seminiferous tubules of the bull..6

Figure 2.1. Percentage of total motility for epididymal sperm culture at pH 5.8, 6.8, and 7.3 (Study I) 70

Figure 2.2. Percentage of progressive motility for epididymal sperm cultured at pH 5.8, 6.8, and 7.3 (Study I) 71

Figure 2.3. Percentage of viable epididymal sperm cultured at pH 5.8, 6.8, and 7.3 (Study I)..72

Figure 2.4. Percentage of total motility for epididymal and ejaculated sperm cultured at pH 5.8, 6.8, and 7.3 (Study II) 73

Figure 2.5. Percentage of progressive motility for epididymal and ejaculated sperm cultured at pH 5.8, 6.8, and 7.3 (Study II) 74

Figure 2.6. Percentage of viable for epididymal and ejaculated sperm cultured at pH 5.8, 6.8, and 7.3 (Study II) 75

Figure 2.7. Venn diagram for the number of proteins detected in epididymal fluid, epididymal sperm, ejaculated fluid, and ejaculated sperm. 76

Figure 2.8. Protein interaction analyzed by STRING database of proteins present only in the ejaculated fluid or epididymis fluid from the metabolic pathway 77

Figure 2.9. Protein interaction analyzed by STRING database of proteins present only in the ejaculated sperm or epididymis sperm from the metabolic pathway...........................78

Figure 3.1. Representative picture of bovine sperm labeled with anti-SERPINA5 on the sperm head and sperm tail, and bright field of A...101

Figure 3.2. Percentage of sperm tail labeled with anti-SERPINA5 in beef bulls (Study I). 102

Figure 3.3. Distribution of SERPINA5 fluorescence intensity on sperm head of bulls (Study I). 103

Figure 3.4. Percentage of sperm tail labeled with anti-SERPINA5 per ejaculate of dairy bulls (Study II). 104

Figure 3.5. Distribution of SERPINA5 fluorescence intensity on sperm head of bulls (Study II)...105

Figure 3.6. Representative picture of bovine sperm labeled with anti-DAG1 on the sperm head, and bright field of A 106

Figure 3.7. Distribution of sperm head DAG1 fluorescence intensity per bull (Study II). 107

Figure 3.8. Correlation between sperm total and progressive motility for 22 dairy bulls. 108

Figure 3.9. Correlation between proportion of sperm tail labeled for SERPINA5 and proportion of sperm with an intact plasma membrane for 20 dairy bulls. 109

Figure 3.10. Correlation between sperm SERPINA5 relative concentration and cleavage rate for 19 dairy bulls. 110

Figure 3.11. Correlation between sperm SERPINA5 and DAG1 relative concentration for 22 dairy bulls...111

Figure 3.12. Correlation between proportion of sperm tail labeled for SERPINA5 and cleavage rate for 19 dairy bulls. 112

Figure 3.13. Correlation between cleavage rate and blastocyst rate for 19 dairy bulls. ..113

Figure 4.1. Effect of time and treatment by time interaction on sperm total and progressive motility 148

Figure 4.2. Effect of time and treatment by time interaction on sperm plasma membrane integrity 149

Figure 4.3. Effect of treatment by time interaction on the percentage of sperm with intact plasma membrane (viable) and disrupted acrosome, sperm with disrupted plasma membrane (dead) and disrupted acrosome, viable sperm with intact acrosome, and dead sperm with intact acrosome 150

Figure 4.4. Effect of treatment and time interaction on sperm plasma membrane integrity and reactive oxygen species...151

Figure 4.5. Effect of treatment by time interaction on sperm plasma membrane integrity and zinc concentration 152

Figure 4.6. Effect of time, and treatment by time interaction on sperm with intact plasma membrane and high and low zinc concentration combined (signatures 1 and 2 combined) 153

Figure 4.7. Effect of time on the percentage of sperm with intact plasma membrane (viable) and disrupted acrosome, sperm with disrupted plasma membrane (dead) and disrupted acrosome, viable sperm with intact acrosome, and dead sperm with intact acrosome 154

Figure 4.8. Effect of time on sperm plasma membrane integrity and reactive oxygen species 155

Figure 4.9. Effect of time on sperm plasma membrane integrity and zinc concentration... 156

Figure 4.10. Effect of time and bull by time interaction on sperm total and progressive motility 157

Figure 4.11. Effect of bull by time on the percentage of sperm with intact plasma membrane (viable) and disrupted acrosome, sperm with disrupted plasma membrane (dead) and disrupted acrosome, viable sperm with intact acrosome, and dead sperm with intact acrosome 158

Figure 4.12. Effect of bull by time interaction on sperm plasma membrane integrity and reactive oxygen species 159

Figure 4.13. Effect of time and bull by time interaction on sperm mitochondrial membrane energy potential 160

Figure 4.14. Effect of bull by time interaction on sperm plasma membrane integrity and zinc concentration 161

Figure 4.15. Effect of bull by time interaction on sperm plasma membrane integrity and CD9 protein 162

Figure 4.16. Effect time and bull by time interaction on sperm plasma membrane integrity 163

Figure 4.17. Effect of time and bull by time interaction on the percentage of sperm with an intact plasma membrane with high (signature 1) and low (signature 2) zinc concentration combined (signature 1 + 2) 164

Figure 4.18. Effect of time on sperm plasma membrane integrity and reactive oxygen species 165

Figure 4.19. Effect of time on the percentage of sperm with intact plasma membrane (viable) and disrupted acrosome, sperm with disrupted plasma membrane (dead) and disrupted acrosome, viable sperm with intact acrosome, and dead sperm with intact acrosome 166

Figure 4.20. Effect of time on sperm plasma membrane integrity and zinc concentration 167

Figure 4.21. Effect of time on sperm plasma membrane integrity and CD9 protein.......168

Figure 4.22. Sperm plasma membrane integrity and positive or negative for CD9 protein 169

Figure 4.23. Sperm with an intact plasma membrane and negative or positive for CD9 protein 170

Figure 4.24. Sperm with a disrupted plasma membrane and negative or positive for CD9 protein 171

Figure 4.25. Effect of bull, time and bull by time interaction on fluorescence intensity of sperm with intact plasma membrane or disrupted plasma membrane 172

LIST OF TABLES

Table 2.1. The effect of treatment (ejaculated sperm at pH 7.3 and epididymis sperm at pH 5.8 or 7.3) on motility and viability parameters measured by CASA..........................67

Table 2.2. Number of proteins and level of significance for the Kyoto Encyclopedia of Genes and Genomes pathway for proteins identified in epididymal and ejaculated fluid..... 68

Table 2.3. Number of proteins and level of significance for the Kyoto Encyclopedia of Genes and Genomes pathway for proteins stripped from the sperm and identified in epididymal and ejaculated samples...69

Table 3.1. Description of sire conception rate (SCR), blastocyst rate (BL), field fertility classification based on SCR value, and field and *in vitro* classification based on SCR and BL per bull 97

Table 3.2. Pearson's correlation coefficient and significance level between sire conception rate, total motility, progressive motility, sperm plasma membrane integrity, SERPINA5 mean relative concentration, percentage of sperm tail positive for SERPINA5, *in vitro* produced embryos cleavage and blastocyst rate, and DAG1 mean relative concentration...98

Table 3.3. Relationship of sire conception rate fertility classification on total motility, progressive motility, sperm plasma membrane integrity, *in vitro* produced embryos cleavage and blastocyst rate, SERPINA5 mean relative concentration, percentage of sperm tail positive for SERPINA5, and DAG1 mean relative concentration...................99

Table 3.4. Relationship of field and *in vitro* fertility classification on total motility, progressive motility, sperm plasma membrane integrity, *in vitro* produced embryos cleavage rate and blastocyst rate, SERPINA5 mean relative concentration, percentage of sperm tail positive for SERPINA5, and DAG1 mean relative concentration.................100

Table 4.1. Bulls A-E P/AI, number of AI per research, number of collection dates used, range of d between first and last collection date evaluated and field fertility level assignment...143

Table 4.2. Effect of treatment on sperm total and progressive motility, plasma membrane integrity, acrosome integrity, reactive oxygen species, and zinc signatures. 144

Table 4.3. Effect of bull on sperm total and progressive motility, plasma membrane integrity, acrosome integrity, reactive oxygen species, mitochondrial membrane energy potential, zinc signatures, and CD9 populations...145

Table 4.4. Pearson's correlation coefficient and significance level between CD9 populations and fluorescence intensities..146

Table 4.5. Pearson's correlation coefficient of CD9 populations and fluorescence intensities with sperm total and progressive motility, viability, acrosome integrity, reactive oxygen species, mitochondrial membrane potential, and zinc signatures..........147

ABSTRACT

SEARCH FOR NEW FERTILITY MARKERS IN BULL SPERM

SAULO MENEGATTI ZOCA

2021

Sperm are stored for extended periods of time in the epididymis, but upon ejaculation motility is increased and lifespan is decreased. After insemination, sperm must traverse the female barriers and undergo capacitation to complete fertilization; however, there are differences in fertility even among bulls that successfully pass a breeding soundness exam. For any potential marker of fertility, there must be variability expressed among animals. The series of studies in this dissertation had the objective 1) to evaluate differences between epididymal and ejaculated sperm and respective fluids proteins to understand increased longevity of epididymal sperm; 2) to evaluate the potential of dystroglycan (DAG1) and plasma serine protease inhibitor (SERPINA5) as fertility markers; and 3) to evaluate whether post *in vitro* capacitation changes in sperm could be used to estimate fertility differences between bulls. In summary, it was observed that 1) epididymal sperm was able to maintain viability longer than ejaculated sperm when cultured under the same conditions, also, sperm energy metabolism appears to be more glycolytic compared to sperm in the ejaculate, based on the greater number of proteins related to this pathway only present in epididymal samples. Sperm also has a greater number of antioxidants available in the epididymis that is likely to be maintaining reactive oxygen species (ROS) at low concentrations to inhibit premature sperm activation. This is supported by a greater mitochondrial membrane potential of epididymal sperm compared to ejaculated sperm and the fact that epididymal sperm was

able to maintain viability longer than ejaculated when cultured under the same conditions. Then, it was observed that 2) DAG1 and SERPINA5 proteins, that are associated with cell to cell interactions, were localized on the bovine sperm head, also, SERPINA5 was localized on the sperm tail, and concentrations of DAG1 and SERPINA5 on the sperm head were correlated with each other ($P = 0.01$, $r^2 = 0.54$), also, SERPINA5 was correlated with embryo cleavage rate ($P = 0.04$, $r^2 = 0.48$), and the percentage of tail labeled for SERPINA5 was correlated with sperm viability ($P = 0.05$, $r^2 = 0.44$); however, neither protein was associated with sire conception rate (SCR; i.e., field fertility). Thus, SERPINA5 may be related with sperm protection and/or oocyte fertilization while DAG1 may be related to sperm transport or formation of the sperm reservoir in the oviduct. Lastly, it was observed that 3) multiple analyses over time in capacitation media of viability, zinc signature 2, zinc signature 1 + 2, and dead CD9+ were able to estimate differences between low fertility bulls to high and intermediary fertility bulls. The inclusion of a viability, a zinc signature, or CD9 protein assay in artificial insemination (AI) studs' quality control measurements may have the potential to predict bull fertility; however, a larger number of bulls with known fertility need to be evaluated to validate these results. In conclusion, more research is necessary to understand: 1) the role of the proteins only identified in epididymis and their role in increased sperm longevity; 2) the role of SERPINA5 and DAG1 *in vivo*; and 3) the potential of viability, zinc signature and CD9 protein analyses post sperm capacitation as predictors of bull fertility.

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Bull fertility is affected by several factors; in a breeding soundness exam (BSE), physical soundness, scrotal circumference and sperm quality are evaluated to predict bull fertility (Barth and Oko, 1989; Barth and Waldner, 2002; Barth, 2007; 2018; Koziol and Armstrong, 2018). Further, sperm quality may be affected by spermatogenesis, sperm maturation, and sperm interaction with testis, epididymis and seminal plasma milieu, especially their proteins (Barth and Oko, 1989; Amann and Hammerstedt, 1993; Johnson et al., 2000; Barth and Waldner, 2002; Saez et al., 2003; Sullivan et al., 2005; Barth, 2007; 2018; Saacke, 2008; Caballero et al., 2011; Sullivan and Saez, 2013; Sullivan, 2016; Dalton et al., 2017; Sullivan and Belleannée, 2017; Amann et al., 2018; Staub and Johnson, 2018). Also, when sperm is processed for artificial insemination (AI), semen handling plays a very important role in maintaining sperm quality, especially in cryopreserved semen, because sperm have to endure the process of freezing and thawing (Januskauskas et al., 2001; 2003; Rodriguez-Martinez, 2003; DeJarnette, 2005; 2012; Dalton et al., 2017; Harstine et al., 2018; Dalton, 2019; DeJarnette et al., 2021). In the female, vaginal, uterine, and oviductal environment affect sperm transport, hyperactivation, capacitation and sperm-egg interaction (Austin, 1951; 1952; 1975; Chang, 1951; Yanagimachi, 1994; Suarez, 2006; 2008; 2015; 2016; Suarez and Pacey, 2006; Sutovsky, 2009; 2018; Florman and Fissore, 2015). Thus, bull fertility and sperm

quality are very complex and depend on a multitude of events that must occur in an orderly fashion in order for a viable pregnancy to be established.

Bull fertility has been measured by evaluation of sperm morphology and motility for many decades, several tests have been developed during this time to evaluate and predict bull fertility; however, bull fertility prediction has not changed considerably and most of industry still relies on motility and morphology to predict sperm fertility (Barth and Oko, 1989; Utt, 2016; Dalton et al., 2017; Barth, 2018; Harstine et al., 2018; DeJarnette et al., 2021). Thus, the development of new fertility markers that can improve bull fertility prediction is necessary in order to advance cattle reproduction.

In this literature review, the objective is to explain the process by which sperm is formed (spermatogenesis and sperm maturation), transported and fertilize the oocyte, followed by methods of evaluation of bull fertility that will aid in the understanding of the objectives and results of the following chapters. Also, physiological factors and analysis of sperm characteristics that may influence bull fertility are discussed, in which other species such as, but not limited to, guinea pig, human, mice, and porcine were used to fill the gaps of physiological knowledge where bovine data is not available.

SPERMATOZOA: FROM SPERMATOGENESIS TO EJACULATION

Under normal physiological conditions, a mammalian animal sex is determined by the sperm that fertilizes the oocyte. In bovine, if the fertilizing sperm was bearing an Y sex-chromosome, then the calf is deemed to be a male and if the fertilizing sperm was

bearing an X sex-chromosome the calf will be a female (Wallis et al., 2008; McGowan et al., 2018). Physical sexual differentiation, also, depends on a series of physiological events that must occur in order for that genetically male or female calf to become phenotypically male or female (Wallis et al., 2008; McGowan et al., 2018).

In bulls, puberty is defined as the ability to ejaculate at least 50 million sperm with a minimum of 10% motile sperm (Wolf et al., 1965). The age that bulls reach puberty (*Bos taurus*) range from 264 to 326 d-old with an average of 294 ± 4 d; while the average scrotal circumference and body weight at puberty was described to be 27.9 ± 0.2 cm and 273 ± 4 kg, respectively (Lunstra et al., 1978; Harstine et al., 2015). The reproductive anatomy of the bull, that is considered normal anatomy, includes two symmetrical testes, epididymides, vas deferens (ductus deferens), spermatic cords, ampullae, vesicular glands, and bulbourethral glands, also, one prostate gland (corpus and disseminate) and a fibroelastic penis with a sigmoid flexure (Ashdown and Hancock, 1974; Chenoweth and Kastelic, 2007; Barth, 2013; Koziol and Armstrong, 2018). The testes, and epididymides are located outside the abdominal cavity, suspended in the scrotum which is necessary for thermoregulation and normal spermatogenesis (Ashdown and Hancock, 1974; Barth, 2007; 2013; Chenoweth and Kastelic, 2007; Koziol and Armstrong, 2018).

The anatomy of testis parenchyma is composed of 70 to 90% Sertoli cells and germ cells, while the remainder is composed of Leydig cells, lymph and blood vessels, and connective tissue (Johnson et al., 2000; Harstine et al., 2015; Staub and Johnson, 2018). The seminiferous tubules, where spermatogenesis occur, are drained to the mediastinum, located in the central region of the testis (Ashdown and Hancock, 1974).

Sperm are carried from the seminiferous tubules to the epididymis through the rete testis, then mediastinum, connected to the head of epididymis by the efferent ducts (White, 1974). Testicular parenchyma is covered by a connective tissues layer called tunica albuginea that is covered by a thin layer of serous membrane called visceral tunica vaginalis (Ashdown and Hancock, 1974). Testicular germ cells are protected from blood through the blood-testis barrier formed by specialized junctions between Sertoli cells which divide the seminiferous tubules into basal and adluminal compartments (Dym and Fawcett, 1970; Russell and Griswold, 1993). Spermatogonia lie between Sertoli cells and tubule basement membrane, and other germ cell lines are located either in crypts from Sertoli cells or in the space between two adjacent Sertoli cells (Dym and Fawcett, 1970; Chenoweth and Kastelic, 2007). The number of sperm produced during spermatogenesis is directly correlated with the number of Sertoli cells (Hochereau-de Reviers et al., 1987).

Spermatogenesis is a series of processes by which a round diploid cell (spermatogonia stem cell) goes through until it becomes a very specialized haploid cell (spermatozoon) that contains a head and a flagellum (Ortavant, 1959; Amann, 1962; Barth and Oko, 1989; Staub and Johnson, 2018). Spermatogenesis is under endocrine control of the hypothalamus-pituitary-gonadal axis, mainly through gonadotropin releasing hormone (GnRH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and testosterone from hypothalamus, anterior pituitary and gonads, respectively (Niswender et al., 1974; Amann and Schanbacher, 1983; Bardin et al., 1994; Hall, 1994; Sharpe, 1994; Smith and Walker, 2015). The hypothalamus secretes GnRH which stimulates the production and release of FSH and LH from the anterior pituitary gland, these protein hormones then act on Sertoli and Leydig cells in the testis, respectively,

which stimulates the production of androgen-binding protein (ABP), inhibin and activin in Sertoli cells and testosterone in Leydig cells (Niswender et al., 1974; Amann and Schanbacher, 1983; Bardin et al., 1994; Hall, 1994; Sharpe, 1994; Smith and Walker, 2015). Testosterone secreted in the blood by Leydig cells has negative feedback to the level of the pituitary decreasing the secretion of LH, also, an elevated level of testosterone has a negative feedback to the level of hypothalamus and pituitary to suppress the release of GnRH and LH, respectively (Amann and Schanbacher, 1983; Smith and Walker, 2015). Sertoli cells secrete large quantities of ABP in the lumen of seminiferous tubules, this protein binds to testosterone and maintains elevated concentrations of testosterone inside the seminiferous tubules which is necessary for normal spermatogenesis (Bardin et al., 1994; Sharpe, 1994; Smith and Walker, 2015). Sertoli cells, also, produce inhibin that is released in the blood and acts as a direct inhibitory factor on the anterior pituitary for FSH secretion, and activin that stimulates the secretion of FSH from the anterior pituitary (Bardin et al., 1994; Sharpe, 1994; Smith and Walker, 2015).

Spermatogonia stem cells go through several mitotic divisions to form A spermatogonia, intermediate spermatogonia, and B spermatogonia (depicted in Fig. 1.1). Then preleptotene spermatocytes are formed from B spermatogonia and move from the basement membrane of seminiferous tubules to the adluminal portion where they go through cell differentiation (preleptotene, leptotene, zygotene, pachytene and diplotene, respectively) before meiotic division and morphological changes occur and it becomes a spermatozoon (Fig. 1.1). These processes are specifically called spermatocytogenesis, meiosis, and spermiogenesis, respectively (Fig. 1.1; Ortavant, 1959; Amann, 1962;

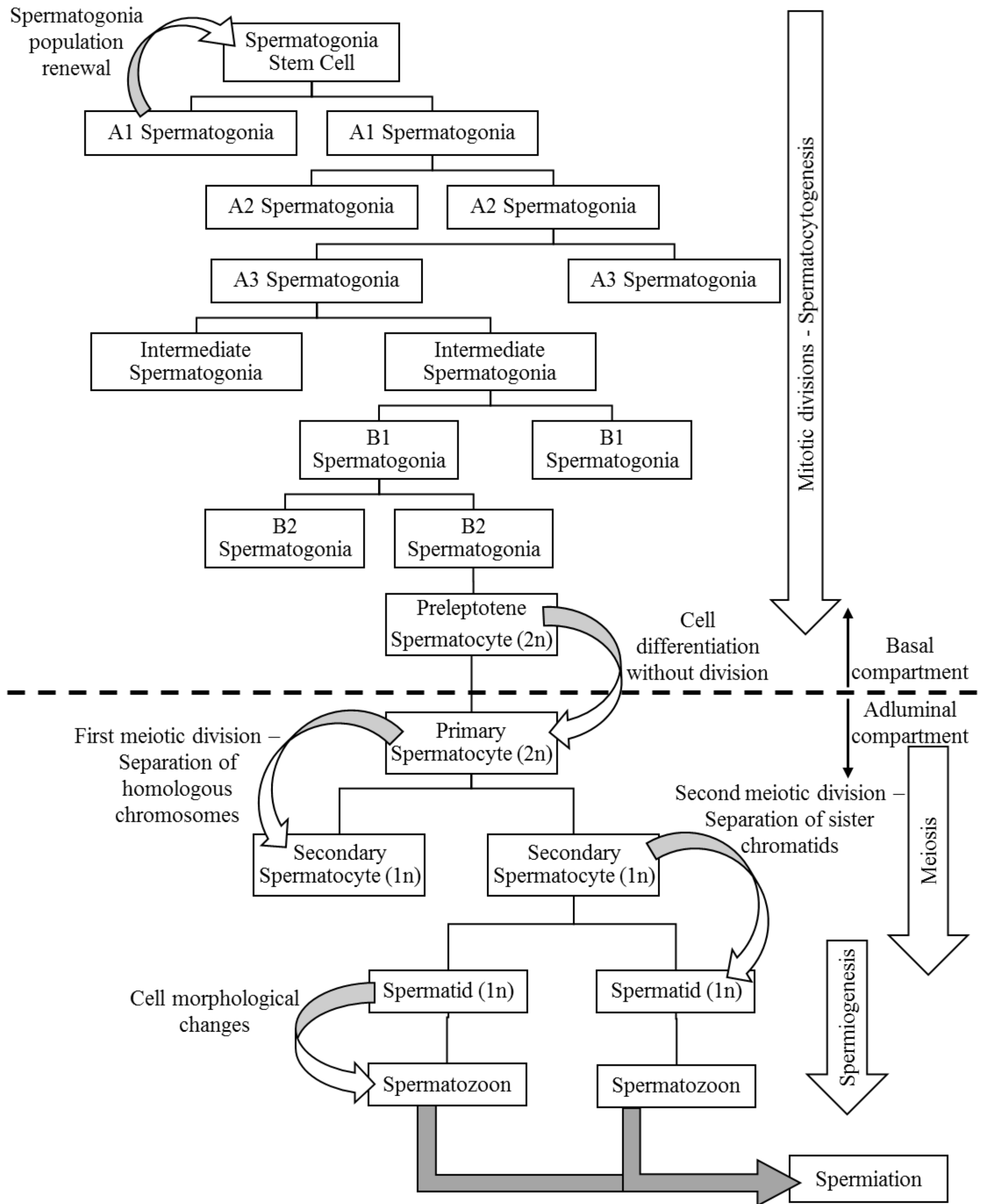


Figure 1.1. Diagram representing the steps of spermatogenesis in the seminiferous tubules of the bull. Spermatocytogenesis happen in the basal compartment of the tubules with migration of preleptotene spermatocyte to the adluminal compartment of tubules inside the blood-testis barrier (dashed line) where meiosis and spermiogenesis occur (Adapted from Ortavant, 1959; Amann, 1962; Curtis and Amann, 1981; Amann and Schanbacher, 1983; Johnson et al., 2000; Staub and Johnson, 2018).

Curtis and Amann, 1981; Amann and Schanbacher, 1983; Johnson et al., 2000; Smith and Walker, 2015; Staub and Johnson, 2018). Spermiogenesis is divided into four phases, Golgi, cap, acrosomal, and maturation, respectively, and ends with spermiation which is the release of sperm from Sertoli cells to the lumen of seminiferous tubules (Barth and Oko, 1989; Goossens and Tournaye, 2017).

In the bull, it takes 21, 23 and 17 d for spermatocytogenesis, meiosis and spermiogenesis, respectively, to occur which total 61 d for a round diploid spermatogonia to become a mature spermatozoon (Amann, 1962; Curtis and Amann, 1981; Amann and Schanbacher, 1983; Johnson et al., 2000; Chenoweth and Kastelic, 2007; Staub and Johnson, 2018). Spermatogenesis happens in cycles which was described at the end of the 19th century (reviewed by Lonergan, 2018; Staub and Johnson, 2018). In the bull, it has been demonstrated that each cycle has eight (Ortavant, 1959; Amann, 1962) or 12 stages (Berndston and Desjardins, 1974) that take 13.5 d to be completed, independent of classification, and 4.5 cycles for full spermatogenesis (from a round spermatogonia to spermiation), thus completing the 61 d of spermatogenesis (Ortavant, 1959; Amann, 1962; Berndston and Desjardins, 1974; Amann and Schanbacher, 1983).

Spermatozoa are then released into the lumen of the seminiferous tubules (spermiation) and further matured while traversing through the epididymis (Amann and Schanbacher, 1983; Barth and Oko, 1989; Robaire and Hinton, 2015). In the efferent ducts (connection from rete testis to epididymis) and initial portion of the head of epididymis most of the testicular fluid is resorbed and sperm is concentrated (Amann and Schanbacher, 1983). At this point, most of the cytoplasm of the sperm has been removed, however, a small portion is still retained as a proximal droplet (Barth and Oko, 1989).

During the transit of sperm through head and body of the epididymis, proximal droplets are moved to a distal position, sperm also acquire progressive motility and fertilizing capacity at the end of corpus and beginning of epididymal tail, while sperm chromatin is further and tighter condensed with replacement of histone for protamine (Igboeli and Foote, 1968; Balhorn, 1982; Acott et al., 1983; Amann and Schanbacher, 1983; Pholpramool et al., 1985; Barth and Oko, 1989; Goossens and Tournaye, 2017). The epididymis has several cytological divisions; however, the three main distinct functional divisions are the head (or caput), body (or corpus), and tail (or cauda) which are stimulated by androgens that promote protein secretion (Amann and Schanbacher, 1983; Goyal, 1985; Cooper et al., 1986; Robaire and Viger, 1995; Robaire and Hinton, 2015). The transit through the epididymis takes approximately 8 to 11 d which is divided into 2 to 3 d for passage through the head, 2 to 3 d through the body and 4 to 5 d through the tail (Koefoed-Johnsen, 1960; Amann and Schanbacher, 1983; Barth and Oko, 1989). The epididymal epithelium is mostly composed of principal cells (~85%) with a highly active protein secretion activity, other cells present in the epididymis are clear cells, basal cells, and halo cells (Hermo et al., 1991; Belleannée et al., 2012; Sullivan and Belleannée, 2017). Principal cells have pseudociliary projections that optimize protein secretion while tight junctions connect these cells forming a blood-epididymal barrier, similar to what happens in the testis, which allow the formation of a unique intraluminal environment and provide immune protection for sperm (Cyr, 2011; Belleannée et al., 2012; Sullivan, 2016).

Sperm become dependent on their environment to survive and function, because during final maturation, sperm lose their ability to biosynthesize, repair, grow and divide,

and become very simple in their metabolic function (Hammerstedt, 1993). A remarkable characteristic of the epididymis is the variability in the protein content of the epididymal fluid through different regions (Cooper, 1998; Dacheux et al., 2009; Belleannée et al., 2011). A subset of proteins released in the epididymal fluid were believed to be added to the sperm while others are modified or removed (Cooper, 1998). Extracellular microvesicles are released in the lumen of epididymis, these microvesicles are named epididymosomes and play a role on sperm protein modification (Yanagimachi et al., 1985; Frenette et al., 2002; 2003; Saez et al., 2003; Sullivan et al., 2007; Caballero et al., 2011). Goyal (1985) reported the presence of vacuoles in the cytoplasm of principal cell and suggested that these vacuoles may be associated with exocytosis, this may have been the first description of epididymosomes in bovine. There is evidence of other molecules, beside proteins, being transported, modified, and incorporated (intracellular or to the membrane) to the sperm by epididymosomes during epididymal transit, such as microRNA, and phospholipids (Sullivan and Saez, 2013; Sullivan, 2016; Sullivan and Belleannée, 2017), however, this literature review will focus only on proteins.

Epididymosomes from the head and tail epididymis were reported to have 555 and 438 proteins, respectively, which 231 were common between the two regions (Girouard et al., 2011). Some of the proteins in epididymosomes are involved in sperm maturation, as these proteins have been reported to be involved with sperm-egg interaction and sperm motility (Frenette et al., 2003; Girouard et al., 2011). Further, epididymosomes contain enzymes involved in glycosylation and in the acquisition of the glycosylphosphatidylinositol-anchoring (GPI-anchoring) process (Girouard et al., 2011). It has also been reported that epididymosomes molecules transfer to the maturing sperm

was mediated by CD9-positive vesicles (Caballero et al., 2013). Epididymosomes-associated proteins that have been demonstrated to be transferred to the bull sperm that are involved with fertilization included: P25b (Frenette and Sullivan, 2001), sperm adhesion molecule 1 (SPAM1; Morin et al., 2005; 2010; Martin-DeLeon, 2006), and glioma pathogeneses-related protein 1 (GPR1L1; Caballero et al., 2012). Other proteins have also been reported to be transferred to the sperm using epididymosomes, such as, ubiquitin, macrophage migration inhibitory factor (MIF), and other (Sutovsky et al., 2001; Frenette et al., 2003; Sullivan and Saez, 2013; Sullivan, 2016; Sullivan and Belleannée, 2017). During epididymal transit, proteins are incorporated to the sperm not only by epididymosomes, these proteins secreted by epididymal epithelial cells can be loosely attached to the sperm by electrostatic interactions, while other proteins can be GPI-anchored (may or may not involve epididymosomes) or behave as integral membrane proteins (Kirchhoff, 1994; Kirchhoff and Hale, 1996; Cooper, 1998; Saez et al., 2003; Sullivan, 2016).

As previously described, sperm that reach the tail of epididymis are mature and have acquired motility and fertilizing capacity (sperm still needs to go through capacitation to be able to fertilize an oocyte; sperm capacitation will be discussed in detail within later sections), thus the tail of the epididymis serves as a reservoir of ready to be ejaculated sperm (Amann and Schanbacher, 1983). A study with ovine demonstrated that embryo survival was greater from sperm obtained from the tail versus the end of the body of the epididymis (Fournier-Delpech et al., 1979) suggesting that further maturation occurs in the beginning of the epididymis tail that supports embryo survival. Epididymal tail may store viable sperm of an equivalent to 7 d of daily sperm

production (Amann and Schanbacher, 1983; Chenoweth and Kastelic, 2007), this is possible because sperm are maintained in a quiescent mode until they are ejaculated (Acott and Carr, 1984; Carr and Acott, 1984). As reviewed by Jones and Murdoch (1996), sperm metabolism and motility, in the epididymis, has been reported to be controlled by Ca^{2+}, cyclic adenosine monophosphate (cAMP), and pH as intracellular messengers, however, no first messenger has been identified. Epididymal fluid pH range from 5.8 to 6.8 in bulls, further, lower pH has been reported to inhibit sperm motility while greater pH improved sperm motility (Wales et al., 1966; Acott and Carr, 1984; Carr et al., 1985). Uterine pH decreased (~7.1 to ~6.8) at the initiation of standing estrus (Elrod and Butler, 1993) and was also decreased in animals that exhibited standing estrus (~6.8) prior to fixed-time AI compared to animals not exhibiting standing estrus (~7.1; Perry and Perry, 2008a, b). Estrus expression prior to fixed-time insemination increased the number of spermatozoa that reached the site of fertilization (Larimore et al., 2015) and had a linear relationship with pregnancy success (Grant et al., 2011). It is hypothesized that the decrease in pH at onset of estrus would increase sperm longevity and the rise in pH prior to ovulation would increase sperm motility (Perry and Perry, 2008a, b).

During ejaculation, epididymal sperm is mixed with seminal fluid from the accessory sex glands while epididymal fluid that kept sperm in a quiescent mode is diluted, consequently, sperm motility increased to a predominantly progressive motility pattern (Cheng et al., 1947; 1949; Acott and Carr, 1984; Carr and Acott, 1984). It has been reported that besides dilution of epididymal fluid, seminal plasma proteins, originated from the accessory sex glands, are attached to the sperm during ejaculation

(Gwathmey et al., 2003; 2006). Similar to epididymal fluid, seminal plasma has extracellular microvesicles, called prostasomes, due to its first description from human prostate secretion (Ronquist et al., 1978a, b); prostasomes have been described in bull semen as well; however, its source is the seminal vesicles instead of the prostate (Agrawal and Vanha-Perttula, 1987; Frenette and Sullivan, 2001). Prostasomes are important for protein transfer to the sperm when epididymal sperm get in contact with seminal plasma from the accessory sex glands. Several proteins are transferred to bull sperm through prostasomes during ejaculation, for example, P25b, MIF, and BSP1 (Girouard et al., 2008); however, prostasomes have been studied further in humans. In humans, prostasomes have been reported to work on sperm protection against immune system complement attack, increase sperm motility, influence sperm capacitation, and plasma membrane stabilization (Stegmayr and Ronquist, 1982; Rooney et al., 1993; Carlsson et al., 1997; Cross and Mahasreshti, 1997; Saez et al., 2003). In proteomic analysis of bulls' accessory sex glands fluid, it was reported that high fertility bulls had increased concentration of osteopontin and phospholipase A2 (Moura et al., 2006). Osteopontin has been suggested to work on fertilization and the block of polyspermy (Erikson et al., 2007), while phospholipase A2 is involved with acrosome reaction, sperm-egg fusion, and possibly sperm motility (Yuan et al., 2003; Bao et al., 2004). Further, protein concentration in human semen has been associated with unexplained male infertility (Panner Selvam et al., 2019). It was reported that surface protein Sp17 (SPA17) and plasma serine protease inhibitor (SERPINA5) were lowly expressed and annexin A2 (ANXA2) was overexpressed in infertile men compared to fertile men (Panner Selvam et al., 2019). In mice, SERPINA5 has been reported to influence male

fertility, since knock out mice for SERPINA5 had only 5% morphologically normal sperm and 12.5% motile sperm, also, *in vivo* and *in vitro* pregnancy rates were 0.5% and 0%, respectively (Uhrin et al., 2000). The ability of human sperm to bind to human zona pellucida was evaluated in the presence of different concentrations of anti-SERPINA5 or SERPINA5 in the media (Elisen et al., 1998). It was reported that lower concentrations of anti-SERPINA5 increased the ability of sperm to bind to the zona pellucida; however, an increase of SERPINA5 concentration in the media decreased the ability of sperm to bind to the zona pellucida (Elisen et al., 1998). Another member of the serine protease inhibitor (SERPIN) superfamily called glia-derived nexin or protease nexin 1 (SERPINE2) that is secreted by seminal vesicles, has been reported to act as a decapacitating factor in mice (Lu et al., 2011). Another protein that has been described to be present in the human seminal plasma was dystroglycan (DAG1), but this protein has not been reported to be present in human sperm (Jodar et al., 2016). In mice and guinea pig, DAG1 has been reported to be present in the tail middle piece and post-acrosomal region (Hernández-González et al., 2001; 2005). Further, mice with DAG1 deficiency in the sperm had decreased percentage of morphologically normal sperm compared to mice with normal DAG1 (Hernández-González et al., 2005).

In summary, a round spermatogonia stem cell goes through a series of changes to make highly differentiated cells that have head and flagellum, spermatozoa. After its formation, sperm go through further maturation, especially in the protein content of both head and tail, that allow sperm to become motile, traverse the female barriers, undergo hyperactivation and capacitation, and fertilize an oocyte. Upon ejaculation, epididymal sperm is diluted with seminal plasma and another change in the sperm proteomics occur

which increase sperm ability to survive and navigate through the female reproductive tract and form the sperm reservoir as will be discussed in the following section.

SPERMATOZOA: FROM EJACULATION TO FERTILIZATION

Sperm transport through the female reproductive tract

Sperm interacts with the female reproductive tract in two main ways: physical and molecular. Physical interaction is related to the swimming response of the sperm through the cervix, uterus and oviduct microarchitectures of the wall and fluids (flow and viscoelasticity; Suarez, 2016). Molecular interaction involves the interaction of sperm surface molecules such as proteins and glycoproteins with their receptor in the epithelial lining of the tract (Suarez, 2016).

Following natural service, a bull deposits semen in the anterior vagina. Sperm must then travel from the anterior vagina to the uterus through the cervix. When a female is in estrus the cervix produces mucus. This mucus production is regulated by several mucin genes (Pluta et al., 2012), and this mucus is believed to assist in sperm orientation with sperm orienting themselves along the long axis of threads (Tampion and Gibbons, 1962). *In vitro* experiments demonstrated that sperm swim against the fluid flow (rheotaxis) in viscoelastic fluids (mucus) at a specific range (2 to 5 μL/min), however, below (≤ 1 μL/min) that range sperm swimming was unaffected (maintained a random pattern) and above (> 5 μL/min) sperm were swept downstream (Miki and Clapham, 2013; Kantsler et al., 2014; Tung et al., 2014; 2015). In addition to mucosal flow, the bovine cervix has microgrooves which are believed to provide a safe pathway for sperm

toward the uterus, protecting sperm from strong estrus outward mucosal flow (Mullins and Saacke, 1989). This hypothesis is supported by previous findings (Mattner, 1968) which identified that half of the sperm inseminated after natural mating were recovered by flushing the cervix of cows and goats and the other half was found in mucosal grooves in the cervix. These grooves are also believed to provide protection to the sperm against the female immune system, specifically leukocytes (Mattner, 1968). The hypothesis proposed by Mullins and Saacke (1989) was latter demonstrated within an *in vitro* study which sperm entered microgrooves when a flow was applied and sperm continued swimming against the flow (Tung et al., 2015).

After entering the uterus, sperm need to travel to the oviduct. Sperm transport through this part of the female reproductive tract has been divided into two types, rapid phase and prolonged phase (Overstreet and Cooper, 1978; Overstreet et al., 1978; Hawk, 1983). In rapid sperm transport, sperm is likely moved to the oviduct through uterine contractions (VanDemark and Hays, 1952), as demonstrated by pro-ovarian contraction of the myometrium layer closer to the lumen of the uterus in women during follicular phase (Lyons et al., 1991). Sperm have been found in the oviduct of cattle as soon as 3 or 4 min after AI or natural mating (Moeller and VanDemark, 1951; VanDemark and Moeller, 1951). When sperm transported by rapid transport were evaluated in rabbits, most of the sperm were nonmotile, had damaged membranes, and were most likely dead (Overstreet and Cooper, 1978). Thus, it is unlikely that any sperm found in the oviduct due to rapid phase transport participates in fertilization (Hunter and Wilmut, 1984; Wilmut and Hunter, 1984). In the cow, the uterine body and uterine horn are approximately 23 – 44 cm long. Sperm would take approximately 30 min to 1 h to reach

the oviduct with an average swimming rate of 7 mm/min (aqueous media) or 3 mm/min (viscoelastic media; Tung et al., 2015) and swimming in a straight line. Taking the length of the uterus and sperm swimming speed into consideration, it is unlikely that sperm found in the oviduct prior to 30 min to 1 h would have reached the oviduct through self-propulsion (swimming). During the prolonged phase of sperm transport, it is believed that sperm reach the oviduct through swimming rather than myometrial contractions. It has been reported that sperm take approximately 8 to 12 h to reach the oviduct in sufficient quantities to sustain normal fertilization rates (Hunter and Wilmut, 1984; Wilmut and Hunter, 1984). Several studies have evaluated sperm transport through the cattle reproductive tract (Mattner, 1968; Dobrowolski and Hafez, 1970; Suga and Higaki, 1971; Larsson and Larsson, 1985; Mitchell et al., 1985). Heifers AI'ed in the exterior cervical os with 2 billion sperm had 24,000, 200,000, and 15,000 sperm in the oviduct at 1, 8 and 24 h, respectively (Dobrowolski and Hafez, 1970). Cows AI'ed with 300 million sperm in the uterine body had 30,000, 142, 21, 194, 319 sperm in the oviduct at 3 to 30 min, 30 to 60 min, 1 to 2 h, 2 to 3 h, and 3 to 5 h post insemination, respectively (Suga and Higaki, 1971). Cows AI'ed with 1 billion or 412 million sperm in the uterine body had 30,000 and 8,000 sperm in the oviduct at 12 h post insemination, respectively (Mitchell et al., 1985). In combination, these studies demonstrate that the number of sperm decreases from the site of sperm deposition as it moves in the female's reproductive tract toward the site of fertilization, from billions or millions of sperm inseminated to tens to hundreds of thousands reaching the oviduct and an even smaller number reaching the site of fertilization.

Except for during rapid phase transport, when sperm is assumed to pass through the utero-tubular junction (UTJ) by myometrial contraction, sperm passage through UTJ is believed to be highly regulated, especially through protein interactions. The entrance of UTJ in cattle has several mucosal folds that make it very difficult for sperm passage (Wrobel et al., 1993). Large and small mucosal folds form blind pockets with openings that face towards the uterus (Yániz et al., 2000), and are very small (Suarez et al., 1997), thus creating a complex maze that sperm need to interact with and traverse. The passage through the UTJ has been reported, in hamsters, to be optimized by sperm progressive motility (Shalgi et al., 1992). However, male mice lacking the proteins: calmegin (CLGN; Ikawa et al., 1997; Yamagata et al., 2002), a testis-specific angiotensin converting enzyme (ACE; Krege et al., 1995; Hagaman et al., 1998), a desintegrin and metalloprotease 3 (ADAM3; Yamaguchi et al., 2009), fertilin β (ADAM2; Cho et al., 1998), ADAM1a (Nishimura et al., 2004), calsperin (CALR3; Ikawa et al., 2011), tyrosyl protein sulfotransferase-2 (TPST2; Marcello et al., 2011) or protein disulfide isomerase (PDILT; Tokuhiro et al., 2012) were infertile. The reason for infertility in these mice was an inability of sperm to pass through the UTJ regardless of normal motility and morphology. All the proteins aforementioned have been reported to directly or indirectly influence ADAM3 correct placement in the membrane (Suarez, 2015). Nevertheless, Fujihara et al. (2014) identified that knockout mice that lacked lymphocyte antigen 6K (LY6K) had normal distribution of ADAM3; however, sperm were unable to pass through the UTJ. Taken together, the required molecule for mice sperm to enter the UTJ is not yet known, even though ADAM3 seems a very important molecule in this process. Also, these results prove that sperm passage through the UTJ is regulated, most likely

through protein interactions and motility alone is not sufficient for sperm to enter the oviduct, at least in mice.

The passage from the uterus to the oviduct in bovine has not been studied in detail as it has in mice; however, conserved mechanisms regulating the passage through the UTJ between mice and bovine is possible. After entering the oviduct, sperm interact with cells of oviductal isthmus to form a sperm reservoir. The sperm reservoir was first described in hamsters (Yanagimachi and Chang, 1963) and was first described in cattle by Hunter and Wilmut (1984) then characterized by Lefebvre et al. (1995). The oviduct provides a safe place for sperm. Unlike the vagina, cervix and uterus, leukocyte infiltration in the oviduct following insemination was not present (Rodriguez-Martinez et al., 1990). Besides protection against leukocytes, when sperm are bound to the oviductal epithelial cells, sperm viability is maintained for longer periods of time with fertilization ability having been estimated to be approximately 30 h (Pollard et al., 1991; Chian and Sirard, 1995). Similar to what has been reported for sperm to enter the oviduct, sperm interaction with oviductal isthmus is regulated by protein interactions. The exact proteins that sperm interact with in the oviduct are not fully understood, however, it is known that sperm interact with carbohydrate molecules attached to protein(s) in the oviductal epithelial wall to form the sperm reservoir (Lefebvre et al., 1997; Suarez et al., 1998). Annexins have been proposed to be the receptor for bull sperm in the oviduct (Ignotz et al., 2007). They have been identified on the surface of oviductal epithelial cells (Ignotz et al., 2007) which is the region sperm bind to (Lefebvre et al., 1995) and antibodies against annexins reduced sperm binding *in vitro* (Ignotz et al., 2007).

On the sperm, proteins related to bovine sperm interaction with the oviductal epithelial cells are Binder of Sperm Proteins (BSP; Ignotz et al., 2001; Gwathmey et al., 2003; 2006). These proteins are attached to the sperm membrane during ejaculation when epididymal sperm come into contact with seminal plasma, especially secretions from the seminal vesicles which are abundant in BSP (Desnoyers and Manjunath, 1992; Müller et al., 1998; Nauc and Manjunath, 2000). Nevertheless, epididymal sperm can bind to oviductal cells but in very low numbers; however, when epididymal sperm were incubated with BSP1 prior to incubation with oviductal cells, the number of epididymal sperm binding to oviductal cells increased similar to that observed with ejaculated sperm (Gwathmey et al., 2003). Furthermore, it has been demonstrated that BSP1 increased sperm viability by stabilizing the plasma membrane, decreasing membrane fluidity and immobilizing cholesterol (Greube et al., 2001; Müller et al., 2002).

To reach the site of fertilization sperm need to detach from the oviduct and travel to the ampullary region of the oviduct where fertilization occurs. It has been reported that sperm bind with the same affinity to oviductal epithelial cells during different stages of the estrous cycle (Lefebvre et al., 1995), thus the process of sperm detachment from the oviduct is likely to occur through sperm changes rather than oviductal changes in receptor abundance (Suarez and Pacey, 2006). Sperm capacitation may play a key role in detachment from the oviduct, more specifically, surface protein modification and sperm hyperactivated motility. When bull sperm were capacitated prior to incubation with oviductal epithelial cells, the number of sperm that bound to epithelial cells was significantly reduced compared to non-capacitated sperm (Lefebvre and Suarez, 1996) and similar results were reported in hamsters (Smith and Yanagimachi, 1991). In bovine,

sperm were not hyperactivated (Lefebvre and Suarez, 1996); thus, Lefebvre and Suarez (1996) concluded that sperm capacitation decreased binding affinity to the oviduct; however, only hyperactivated sperm were observed detaching from the oviduct in mice (Demott and Suarez, 1992; Chang and Suarez, 2012). Together, the release of the sperm from the oviduct is likely due to both sperm capacitation and hyperactivated motility providing the pulling force necessary for detachment. Chang and Suarez (2012) demonstrated that mouse sperm moved forward towards the ampulla by detaching and attaching again to the oviductal epithelium, the detachment was aided by oviductal contraction, but contractions were not required for sperm release, these findings agree with Lefebvre et al. (1995) who reported that *in vitro* bull sperm attached to ampullary and isthmus epithelial cell with similar affinity.

Closer to the time of ovulation, the oviduct secretes molecules that enhance sperm capacitation (Parrish et al., 1985; 1988; 1989; Mahmoud and Parrish, 1996; Bergqvist et al., 2006). Capacitation is a required process that sperm must undergo in order to acquire the ability to undergo the acrosome reaction and fertilize the oocyte (Austin, 1951; Chang, 1951). Glycosaminoglycan substances are secreted by the oviduct which induced changes in the sperm that leads to sperm capacitation (Handrow et al., 1982; Parrish et al., 1988; Bergqvist et al., 2006). Sperm proteins BSP3 and BSP5 have heparin (i.e., glycosaminoglycan) binding sites (Gwathmey et al., 2006) and there is evidence that BSP1 is removed from sperm during capacitation (Thérien et al., 2001). Altogether, changes in sperm during capacitation together with hyperactivation aid in the sperm detachment and transport through the oviduct from the sperm reservoir to the site of fertilization in the ampulla.

Sperm hyperactivation, capacitation and fertilization

Mature epididymal or ejaculated spermatozoa do not have immediate capacity to fertilize an oocyte. It was described that sperm needed to reside in the female reproductive tract for approximately 5 h before fertilization could occur (Austin, 1951; Chang, 1951), these were the first reports of what was later called sperm capacitation (Austin, 1952). One of the classic measures of sperm capacitation was by oocyte fertilization, since only capacitated sperm are capable of fertilizing an oocyte; however, some capacitated sperm are still not able to fertilize an oocyte (Yanagimachi, 1994). Sperm capacitation is stimulated by different molecules in different species and is temperature sensitive (Bedford, 1970; Yanagimachi, 1994); for example, a temperature of 37 to 38 ºC is adequate to support sperm capacitation in most species *in vitro*; however, swine and ovine seem to benefit when temperatures are elevated closer to body temperature 39 ºC (Yanagimachi, 1994). Thus, sperm hyperactivation, capacitation and oocyte fertilization described herein will focus on bovine with knowledge of other species when necessary.

Capacitated sperm have gone through biochemical modifications that allow sperm to undergo the acrosome reaction when in contact or stimulated by the zona pellucida, cumulus cells, or other substances associated with *in vitro* or *in vivo* (ovulated) matured oocytes (Yanagimachi and Usui, 1974; Florman and First, 1988a; Parrish, 1989; Mahmoud and Parrish, 1996). Sperm capacitation is a terminal event that leads to oocyte fertilization or sperm death (Suarez, 2016). It was believed that sperm capacitation and sperm hyperactivation belonged to the same cascade of events; however, sperm hyperactivation has been demonstrated to be independent of capacitation (Lefebvre and

Suarez, 1996; Marquez and Suarez, 2004). When bull sperm was incubated with caffeine or procaine, hyperactivation was induced; however, sperm did not become capacitated (Ho and Suarez, 2001a, 2003). Sperm hyperactivation is characterized by changes in flagellar (tail) beating which has an increased bending amplitude and asymmetric movement, causing sperm to swim in circular pattern, in aqueous media, resulting in loss of progressive motility (Yanagimachi, 1994). Also, sperm hyperactivation increased the ability of sperm to swim through mucus, which is present in the oviductal lumen (Suarez et al., 1997), and hyperactivation is required for sperm to penetrate the zona pellucida (Stauss et al., 1995). Additionally, hyperactivation has been reported to be involved in sperm release from the oviductal reservoir, at least in mice (Demott and Suarez, 1992). Nevertheless, sperm hyperactivation is transient and sperm can switch from progressive to hyperactivated motility and back to progressive (Suarez and Osman, 1987; Suarez et al., 1987) while sperm capacitation is not reversible.

The mechanism that regulates sperm hyperactivation is complex; however, sperm hyperactivation is dependent on Ca^{2+} (Ho and Suarez, 2001b; Ho et al., 2002; Marquez and Suarez, 2007). Intracellular levels of Ca^{2+} regulates sperm motility, when intracellular Ca^{2+} is elevated (~100 – 400 nM) sperm become hyperactivated (Ho and Suarez, 2001b; Ho et al., 2002). It has been reported that Ca^{2+} acts in combination with calmodulin (CALM1) to activate calmodulin kinase II (CAMK2) which are both present in the sperm tail (Ignotz and Suarez, 2005). Intracellular concentrations of Ca^{2+} are highly regulated by intracellular storage and membrane channels (Carafoli and Crompton, 1978). In sperm, Ca^{2+} concentrations are regulated through cation channel sperm-associated proteins (CATSPER) present on the sperm tail principal piece (Ren et

al., 2001; Johnson et al., 2017); CATSPER is a complex of four subunits that are weakly voltage-dependent, Ca^{2+}-selective, and pH-sensitive ion channels that control the entry of positively charged Ca^{2+} ions into the sperm (Singh and Rajender, 2015). Increases in intracellular pH occurred during sperm capacitation (Parrish et al., 1989; Vredenburgh-Wilberg and Parrish, 1995) and induced CATSPER activation (Kirichok et al., 2006). Sperm alkalinization can be induced through several pathways such as activation of a sperm-specific Na^{+}-H^{+} exchange regulated by membrane potential (SLC9A10), a voltage-sensitive proton channel (HVCN1), and HCO^{3-} (bicarbonate) entry pathways and formation (Florman and Fissore, 2015). Also, CATSPER can be activated by progesterone, prostaglandins, some odorants, and other small molecules (Florman and Fissore, 2015). The intracellular level of Ca^{2+} was also increased by internal release of Ca^{2+} from the base of the flagellum when inositol 1,4,5-triphosphate receptor (ITPR1) and calreticulin receptor (CALR) were stimulated pharmacologically (Ho and Suarez, 2001b, 2003). These changes in sperm during hyperactivation and capacitation increased sperm consumption of energy substrates, producing high levels of adenosine triphosphate (ATP) through oxidative phosphorylation (Gibb et al., 2016). Oxidative phosphorylation produced reactive oxygen species (ROS), which in moderate concentrations have been reported to be involved with triggering the start of the capacitation cascade, most likely through involvement in protein modification and activation that leads to Ca^{2+} uptake and increased cAMP (Aitken et al., 2015; Aitken, 2017). At elevated concentrations, ROS can cause damage to the sperm, especially chromatin (Aitken et al., 2015; Aitken, 2017). The effects of ROS in male reproduction has been well reviewed (Gibb et al., 2020).

Sperm capacitation was induced by glycosaminoglycans present in the oviductal and follicular fluid, especially by heparin (Lee and Ax, 1984; Parrish et al., 1986; 1988; 1989; Florman and First, 1988a, b). When sperm was incubated with heparin prior to *in vitro* fertilization, there was an increase in oocyte fertilization (Parrish et al., 1986; 1988). It was believed that sperm capacitation occurred by simple removal of decapacitating factor (Brackett and Oliphant, 1975); however, it has since been reported to be more complex and involves a series of biochemical and biophysical transformations in the sperm head and tail. The removal of decapacitating factors is believed to be the first step that leads to a series of biochemical events. Sperm lose adsorbed proteins during the first steps of capacitation, which stabilizes the plasma membrane (Lefebvre et al., 1995; 1997; Lefebvre and Suarez, 1996; Suarez et al., 1998; Ignotz et al., 2001; Gwathmey et al., 2003; 2006). For example, heparin binds to BSP proteins that are attached to the sperm during ejaculation, when epididymal sperm are diluted in seminal plasma (Manjunath and Thérien, 2002), and BSP were completely (BSP1, BSP5) or partially (BSP3) removed during sperm capacitation (Ignotz et al., 2001; Thérien et al., 2001; Gwathmey et al., 2003; 2006; Hung and Suarez, 2012), allowing sperm to be released from the sperm reservoir. In addition to the loss of decapacitating factors, capacitation encompass redistribution of plasma membrane proteins, changes in the amount of certain proteins, lipid diffusion, change in phospholipids distribution, affects cholesterol efflux, changes in cAMP metabolism, increase in tyrosine phosphorylation, hyperpolarization of the plasma membrane, increase of internal pH and Ca^{2+} influx of sperm head and tail (Davis, 1981; Wolf et al., 1986; Carr and Acott, 1989; Zeng et al., 1995; Galantino-Homer et al., 1997; Vijayaraghavan et al., 1997; Arnoult et al., 1999; Hess et al., 2005; Boerke et al., 2008;

Gadella, 2008; Nixon and Aitken, 2009; Lishko et al., 2012; Parrish, 2014). Additionally, Zn^{2+} has been reported to play a role in sperm capacitation; this role has been discussed by several authors (Clapper et al., 1985; Riffo et al., 1992; Andrews et al., 1994; Eickhoff et al., 2004; Michailov et al., 2014; Kerns et al., 2018; 2020; Sutovsky et al., 2019). The active removal of Zn^{2+} has been reported to be a prerequisite for sperm capacitation (Andrews et al., 1994) and recently it was demonstrated that sperm Zn^{2+} signature can be used to characterize sperm capacitation (Kerns et al., 2018; 2020; Sutovsky et al., 2019).

Briefly, after losing decapacitating factors, the next step is the efflux of cholesterol which increases sperm membrane fluidity (Davis, 1981; Gadella, 2008), allowing for an influx of HCO_3^+. This increase in HCO_3^+, cause an increase in intracellular pH, and Ca^{2+}, which regulate the activity of soluble adenylyl cyclase culminating in an increase of cAMP (Garty and Salomon, 1987; Vredenburgh-Wilberg and Parrish, 1995; Vijayaraghavan et al., 1997; Arnoult et al., 1999; Hess et al., 2005; Lishko et al., 2012). The increase in intracellular cAMP activates protein kinase A (PKA) that phosphorylates two types of proteins, protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP) which activate and inhibit their activities, respectively; thus increasing protein tyrosine phosphorylation in the sperm head and tail which is a sperm capacitation hallmark (Carr and Acott, 1989; Galantino-Homer et al., 1997; Vijayaraghavan et al., 1997; Baker et al., 2006). Low levels of intracellular Zn^{2+} seems necessary for the activation of PKA pathway, stimulated by epidermal growth factor receptor (EGFR), leading to protein tyrosine phosphorylation and capacitation; however, high levels of Zn^{2+} are found in sperm that have not started capacitation and complete

removal of Zn^{2+} has been reported to inhibit sperm motility (Michailov et al., 2014; Kerns et al., 2018).

Following these series of events, sperm are hyperactivated and capacitated; thus, have acquired the ability to undergo the acrosome reaction. The acrosome reaction is an exocytotic process that releases the acrosomal content that aid in sperm passage through the cumulus oophorus (remaining granulosa cells surrounding the oocyte) and zona pellucida (Florman and First, 1988a, b; Florman et al., 1989; 2004; Dandekar et al., 1992; Florman and Fissore, 2015). Similar to the processes of hyperactivation and capacitation, there is a rise in intracellular Ca^{2+} that induces the acrosome reaction which is stimulated by sperm interaction with cumulus cells or the zona pellucida (Yanagimachi, 1994; Fraser et al., 1995; Florman and Fissore, 2015). Further, the acrosome reaction can be activated by G-protein-coupled receptors that induce activation of EGFR through PKA, PTK (more specifically SRC) and Zn-dependent metalloproteinases which were reported to trigger a downstream effector that culminated in increased intracellular Ca^{2+} and downstream activation of the acrosome reaction (Fraser et al., 1995; Etkovitz et al., 2009; Michailov et al., 2014). The zona pellucida is composed of three heavily glycosylated proteins ZPA, ZPB, and ZPC, these are homologous proteins to the human ZP2, ZP4/ZP3β, and ZP3/ZP3α, respectively, which ZPB and ZPC serve as sperm receptors that induce the acrosome reaction (Wassarman, 1990; Yonezawa, 2014) and ZPA/ZP2 is believed to be of secondary or sustained sperm-zona binding (Gadella, 2011). In the mouse, it was proposed that the acrosome reaction starts during sperm migration through the female reproductive tract (La Spina et al., 2016); however, it is not known if that happens in bovine. Acrosome reacted sperm, after traversing the zona pellucida, found in

the perivitelline space, interacts with the plasma membrane of the oocyte through the equatorial region which has oolemma-binding proteins that were exposed during acrosomal exocytosis (Cuasnicú et al., 2016).

In mouse studies, it has been demonstrated that sperm proteins present on the equatorial region were required for sperm adhesion or fusion with the oocyte plasma membrane. These proteins are equatorin (or MN9 antigen), CD9, and IZUMO1 (Toshimori et al., 1998; Manandhar and Toshimori, 2001; Inoue et al., 2005; Ito et al., 2010; Satouh et al., 2012). Also, oocyte JUNO (IZUMO1 receptor), and tetraspanins CD9 and CD81 have been demonstrated to be required for mouse fertilization (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Rubinstein et al., 2006; Bianchi et al., 2014). The proteins CD9, JUNO and IZUMO1 have been reported to be present on bovine gametes (Zhou et al., 2009; Fukuda et al., 2016; Zhao et al., 2018). When zona-free oocytes were incubated with anti-CD9 antibodies, oocyte fertilization was significantly decreased (41.6% vs. 81.3%; Zhou et al., 2009); however, the requirement of JUNO and IZUMO1 in bovine fertilization has not been demonstrated. Other proteins have also been proposed to be involved with mammalian fertilization either on the oocyte or on the sperm, such as ADAM2, ADAM3, cysteine-rich secretory proteins (CRISPs), and integrin α6β1; however, their requirement for fertilization has been questionable (Sutovsky, 2009).

After sperm-egg fusion, sperm induce the block of polyspermy that involves release of proteases (ovastacin), Zn^{2+} (called zinc spark), and Ca^{2+}, that acts in zona hardening, release of cortical granules and polarization of the oocyte membrane, blocking other sperm from penetrating the zona pellucida, adhering or fusing with the oocyte

(Florman and Fissore, 2015; Que et al., 2015; Zhang et al., 2016; Bianchi and Wright, 2020). If fertilization and block of polyspermy are successful, a viable embryo may be developed.

BULL FERTILITY EVALUATION

Breeding soundness exam

The guidelines for the bull breeding soundness examination (BSE) are set by the Society for Theriogenology (Koziol and Armstrong, 2018). A bull BSE evaluates the physical soundness, estimates sperm production, and assesses the quality of the sperm produced. After evaluation, a bull is classified as “Satisfactory potential breeder”, “Unsatisfactory potential breeder” or “Classification deferred” (pending a new evaluation to be determine by the veterinarian).

According to the manual for BSE of the bull (Koziol and Armstrong, 2018) the physical soundness exam evaluates the bull’s eyes, feet, legs, mouth, scrotum, testes, internal and external genitalia, body condition, locomotion, i.e., the bull’s general health. A comprehensive physical exam of the bull is not required in a BSE; however, detection of physical deficiencies that affects breeding potential of the bull is necessary. For example, injury to the eye may affect the bull’s ability to identify sexually active groups of females (Geary and Reeves, 1992), feet and leg problems may affect ability to mount (serve) females (Barth and Waldner, 2002), and problems with the mouth may affect ability to maintain good body condition. Further, optimum body condition score (BCS)

for beef bulls prior to a breeding season is between 5 and 7 (score 1 to 9; Whitman, 1975; Barth and Waldner, 2002). Bulls developed under high energy diets (80% grain, 20% forage) had greater body weight, back fat, and scrotal circumference compared to bulls developed under moderate energy diet (100% forage); however, bulls fed high energy diet had decreased testicular tone, sperm motility and percentage of morphologically normal sperm cells, greater percentage of primary and secondary defects, greater testicular temperature, and decreased epididymal sperm reserve (Coulter et al., 1997). Effects on sperm quality observed among bulls developed on high-energy diets are caused by insulation of the spermatic cord due to fat deposition which decreased the ability of the testes to regulate temperature (Kastelic et al., 1996; Coulter et al., 1997).

The scrotum and its contents should be evaluated by visual observation of the skin and testes placement, and by palpation of the spermatic cords, testes, and epididymides. A normal scrotum has both testes, with ability to move (not attached to the skin) upward (towards the body) and downward (away from the body); spermatic cords should be smooth and symmetrical with minimal fat deposition around the scrotum neck. Epididymal head should be firm, flattened, U-shaped, approximately 5 mm thick and located at the dorsal pole of the testis (closer to the body), epididymal body runs from the head to the tail of epididymis along the axial surface, tail of epididymis is located at the bottom of the testis and represents the storage of sperm ready to be ejaculated (Koziol and Armstrong, 2018). Testes should be symmetrical (both testes should be of similar sizes), turgid but resilient (similar tone to a semi-flexed forearm), free of swelling and not sensitive to the touch (Koziol and Armstrong, 2018). These evaluations are followed by a scrotal circumference evaluation. Animals under 15 mo of age should have a scrotal

circumference greater than 30 cm, animals between 15 and 18 mo of age should have at least 31 cm, 18 and 21 mo of age 32 cm, 21 and 24 mo of age 33 cm, and animals greater than 24 mo of age should have scrotal circumference above 34 cm to be classified as "Satisfactory potential breeder" (Koziol and Armstrong, 2018). This is because testis continually grow until the animal is 4-yr old, with approximately 90% of the testicular growth occurring up until 24 mo of age (Coulter, 1986). Also, scrotal circumference has been reported to be positively correlated with age of puberty of female offspring (Toelle and Robison, 1985). Further, scrotal circumference is a reliable measure to estimate daily sperm production (greater scrotal circumference equals greater daily sperm production), progressive motile sperm (greater scrotal circumference equals greater percentage of sperm with progressive motility), epididymal reserves (greater scrotal circumference equals greater epididymal reserve), and primary sperm abnormalities (greater scrotal circumference equals lower number of sperm with primary morphological abnormalities; Almquist et al., 1976; Coulter and Foote, 1979; Smith et al., 1981; Kastelic et al., 2001; Silva et al., 2013).

Following physical and scrotal examination, the next step in a breeding soundness exam is the evaluation of the accessory sex glands through transrectal palpation (Koziol and Armstrong, 2018). This should be accomplished prior to electroejaculation, one should evaluate prostate gland, vesicular gland (seminal vesicles), and ampullae. This is also the order of appearance when transrectally palpating, it is important to notice that bulls possess bulbourethral gland, and a disseminate portion of prostate that are not palpable. The corpus prostate (palpable section of bull's prostate) has been reported to feel like a "ring on a giant's finger" located caudally to the vesicular gland and ampullae

(Koziol and Armstrong, 2018). Vesicular glands are 8 to 15 cm long and 2 to 3 cm thick located craniolaterally to the corpus prostate (bilateral) and lateral to the neck of the bladder and ampullae, it should be uniform in size, turgid, lobulated, and movable (Barth, 2013). Normal ampullae (bilateral) are 10 to 15 cm long with 5 to 8 mm diameter each, which can be felt cranial to the corpus prostate; however, they are not very distinct structures to palpate (Barth, 2013; Koziol and Armstrong, 2018). Lastly, the inguinal ring should be evaluated, which normal structure should allow the entrance of one to two fingers and rarely three (Koziol and Armstrong, 2018). Also, the massage of accessory sex gland may enhance the ability of semen collection through electroejaculation (Koziol and Armstrong, 2018).

Semen can be collected by several methods, including electroejaculation, artificial vagina, vaginal aspiration, and transrectal massage (Seidel Jr and Foote, 1969a, b; Barth et al., 2004; Palmer et al., 2005; Barth, 2007). Semen evaluation should consist of visual observation of color, semen should be free of urine and blood, normal color ranges from white creamy to a skim-milk like color and some breeds such as Jersey and Angus may present a light-yellow to gold color (Koziol and Armstrong, 2018). Sperm concentration may be assessed by a hemocytometer ("gold standard"), spectrophotometer, flow cytometer, computer-assisted sperm analysis (CASA), and others (DeJarnette, 2012). Followed by motility and morphology evaluation, sperm motility should be above 30% progressive motile evaluated by a microscope (bright field or phase-contrast) in a subjective motility evaluation or by using a CASA for a bull to be classified as "Satisfactory potential breeder" (Thundathil et al., 2016; Lone et al., 2017; Koziol and Armstrong, 2018). For morphology, the ejaculate must have greater than 70% of sperm

with normal morphology. Sperm morphology can be performed by brightfield microscopy utilizing stained sperm, phase contrast or differential interface contrast (DIC) with sperm fixed in formaldehyde. All morphology analyses should be performed under oil immersion with x 1000 magnification or greater (Barth and Oko, 1989; Thundathil et al., 2016; Lone et al., 2017; Koziol and Armstrong, 2018). Morphological defects usually are classified as primary and secondary defects, usually head and tail defects, respectively. Specific classification and illustration of each morphological defect of the sperm can be found in Barth and Oko (1989), Barth (2007), and Koziol and Armstrong (2018). The degree of deviation from normal of each section of the BSE may affect bull's BSE classification. From "Satisfactory potential breeder" to "classification deferred", depends on whether the deviation or insult detected can be reversed and animal possibly return to normal function and sperm production and be classified as "Satisfactory potential breeder" pending a new evaluation after the problem is solved. From "Satisfactory potential breeder" to "Unsatisfactory potential breeder", reflects an inability of the animal to return to normal function of the testis (Thundathil et al., 2016; Koziol and Armstrong, 2018).

A "Satisfactory potential breeder" classification does not guarantee that the bull will be a satisfactory breeder for its whole life; more accurately, a single BSE result is a snapshot of a bull's fertility that represents the samples collected that day. Thus, any alteration with the bull's health or physical soundness could affect its BSE results. Further, it has been estimated that approximately 20% of bulls in an unselected population would be classified as unsatisfactory potential breeders because of physical soundness, semen quality or a combination of those problems (Carroll et al., 1963;

Elmore et al., 1975). In the previous mentioned studies, serving capacity was not measured (not a BSE requirement); thus, it is likely that approximately 25% of bulls in an unselected population would fail a BSE if serving capacity were included (Barth, 2018). Bulls that pass a BSE have been reported to have 5% or greater fertility compared to unevaluated bulls (Wiltbank and Parish, 1986). Also, calf crop was increased for bulls with greater than 70% morphologically normal sperm compared to bulls with less than 50% normal sperm (Fitzpatrick et al., 2002). Moreover, the economic benefit per cost ratio of a BSE has been estimated at 36:1 in Brazil (Menegassi et al., 2011) and 17:1 in USA beef cattle herds (Chenoweth, 2000).

Bull breeding capacity and libido are not routinely assessed in a BSE; however, producers are encouraged to test their bulls' libido and breeding capacity. Libido is the bull's willingness to serve, and breeding capacity is the ability to serve (mount female and penis insertion into the vagina). Both, libido and serving capacity, can be tested by observing bulls' behavior with females in estrus or not and restrained or not; it is preferred that females are restrained to reduce variability in the results (Coulter and Kozub, 1989; Barth et al., 2004; Barth, 2018; Koziol and Armstrong, 2018). Barth et al. (2004) demonstrated that 4.8% of bulls that were classified as satisfactory potential breeders by semen evaluation were unable to serve cows. Also, bulls that were unable or unwilling to serve cows during a libido test had lower pregnancy rates (Blockey, 1980; Coulter and Kozub, 1989). Little has changed in the BSE over the past several years and the ability to identify subfertile bulls has not improved greatly (Kennedy et al., 2002); however, with the tools available for BSE, it is possible to greatly decrease the use of lowly fertile bulls even though some "Unsatisfactory potential breeder" bulls are still

classified as "Satisfactory potential breeders" due to limitation of time and money that producers are willing to invest in testing bulls (Barth, 2018).

Sperm Preservation, Evaluation, and Fertility Potential

In 1940, bovine semen was successfully cooled after being diluted with an egg-yolk extender and sperm survived for several days (Phillips and Lardy, 1940). Later, the use of antibiotics was reported to improve pregnancy rates (~10% improvement), and the discovery of glycerol as cryoprotectant allowed for cryopreservation of sperm and successful long-term storage of sperm (Almquist et al., 1946; 1949; Polge et al., 1949; Almquist, 1951; Polge and Rowson, 1952). These advances in sperm storage were key components for the success and dissemination of AI in cattle. Presently, 90% of dairy cattle and approximately 15% of beef heifers and 5% of beef cows are AI'ed (USDA, 2018, 2020). The protocols for bovine sperm cryopreservation have not changed considerably in ~45 years, despite the fact that sperm viability is greatly affected in the process resulting in approximately 50% of sperm death during freezing and thawing (Robbins et al., 1976; Parrish et al., 1986; Gunasena and Critser, 1997). Nevertheless, fertility of frozen sperm reaches acceptable successful rates for both AI and *in vitro* fertilization in cattle (Holt, 2000).

Evaluation of fresh ejaculated semen at AI centers that is destined for cryopreservation has a greater motility threshold (≥ 60% progressive motility) compared to semen evaluation during a BSE (≥ 30% progressive motility), in a subjective assessment by light microscopy (DeJarnette, 2005; 2012; Lone et al., 2017; Harstine et

al., 2018; Koziol and Armstrong, 2018; DeJarnette et al., 2021). Conversely, the percentage of morphologically normal sperm is similar (≥ 70%) between a BSE and quality control analysis at AI centers, as samples above this threshold have small to no correlation to fertility (DeJarnette, 2005; 2012; Harstine et al., 2018; DeJarnette et al., 2021). The cryopreservation process is the reason for pre-freeze higher standards in AI centers. Sperm are damaged during the freezing and thawing process (~50% loss); additionally, lower insemination doses (10 to 40 million sperm per insemination dose) are used for AI, in the uterine body, compared to greater insemination dose (several billions) being deposited in the female vagina during natural service by the bull (Zoca et al., 2020; DeJarnette et al., 2021).

Sperm dose used for AI has been reported to affect pregnancy rates. Pregnancy rates increase with increased sperm per dose until a plateau is reached which is dependent on the maximum fertility of the female population and/or the sire (Salisbury and VanDemark, 1961; Saacke et al., 1994; Den Daas et al., 1998; Saacke, 2008). Den Daas et al. (1998) demonstrated that the insemination dose at which each bull reaches its maximum fertility varied from ~1 million to ~10 million viable sperm per dose, which is consistent with sperm doses commercially available between 10 and 40 million sperm per dose (DeJarnette, 2005; 2012; Harstine et al., 2018). A more recent study has reported no effect of sperm insemination dose when varying from 10 to 40 million sperm per dose, agreeing with previous reports (Zoca et al., 2020). The reason different bulls reach their maximum fertility at different doses is explained by “compensable” and “uncompensable” sperm traits (originally described by Saacke et al., 1994). Compensable sperm traits refer to the inability of sperm to fertilize an oocyte which is associated with

failure of sperm transport and initiation of fertilization. Examples of compensable sperm traits include lower percentage of progressive motile sperm and high percentage of sperm with disrupted plasma membrane (Saacke et al., 1994; Saacke, 2008; Amann et al., 2018). Uncompensable sperm traits pertain to sperm that successfully initiate fertilization, however, are unable to support development of viable embryos, an example of an “uncompensable” trait is sperm with damaged DNA (Eid et al., 1994; Saacke et al., 1994; Saacke, 2008; Amann et al., 2018).

Only one sperm is required for successful fertilization of an oocyte. The fertilizing sperm that reaches the site of fertilization and successfully completes fertilization, must have normal morphology, progressive motility, intact membranes (plasma and acrosomal membrane), stable and intact DNA, be able to be capacitated and hyperactivated, and be able to fertilize and activate the oocyte (Rodriguez-Martinez, 2003; Saacke, 2008; Vincent et al., 2012; Garner, 2014). One single sperm containing all these characteristics is sufficient for fertilization; however, for a bull to be highly fertile (great proportion of females pregnant by single insemination), a great proportion of inseminated sperm need to display these desired characteristics. Increases in pregnancy rates by increasing insemination dose, previously mentioned, is due to sufficing the “compensable” characteristics of the sperm (Saacke, 2008). Further, the maximum fertility of a bull or the level at which a bull’s fertility plateaus (considering that the female population fertility is optimum) is determined by its “uncompensable” characteristics (Saacke, 2008). Finally, the ejaculate is composed of three sperm populations, 1) sperm that cannot initiate fertilization (compensable), 2) sperm that initiate fertilization but do not generate a viable embryo (uncompensable), and 3) fully

competent sperm that can generate a viable embryo; therefore, each bull's fertility is dependent on the proportion of each of these populations in an ejaculate or insemination dose (Amann et al., 2018). Nevertheless, pregnancy rates following AI are dependent on multiple factors beside the quality of an ejaculate or insemination dose, including female's health and management, semen storage and handling, and proper AI technique (Saacke, 2008; Vincent et al., 2012; Dalton et al., 2017; Amann et al., 2018). It is also necessary that the fertilizing spermatozoon be at the correct place (ampullae or ampullary isthmus junction) and at the correct time (when the oocyte reaches the right place; Amann et al., 2018). This becomes a concern especially when implementing AI and females are AI'ed at different intervals after the onset of estrus. It was reported that females AI'ed shortly after the beginning of behavioral estrus had decreased fertilization rates compared to females AI'ed toward the end of estrus; however, embryo quality was greater in those females AI'ed at the beginning of estrus compared to those AI'ed towards the end of estrus (Saacke et al., 2000; Dalton et al., 2001). This is an even greater challenge when fixed-time AI (FTAI) is used, because females are AI'ed regardless of expression of behavioral estrus. Females that expressed behavioral estrus, i.e., they were exposed to high levels of estradiol prior to FTAI, had conception rates, on average, 27% greater than those that did not expressed behavioral estrus (Perry and Perry, 2008b; Larimore et al., 2015; Richardson et al., 2016). Thus, the use of an insemination dose of high sperm quality that can maximize pregnancy rates with one single insemination is necessary for optimization of beef cattle production.

The delivery of a sperm dose of high quality, that meet or exceed the industry standards is the responsibility of AI centers which is accomplished by meeting or

exceeding the guidelines established by the National Association of Animal Breeders-Certified Semen Services (NAAB-CSS; DeJarnette, 2012; Mitchell, 2012). Sperm analysis improved greatly with the use of more objective methods such as CASA and flow cytometry compared to previous subjective methods (microscopy) mainly because of the increase in number of individual sperm evaluated by these machines (thousands) compared to microscopy (few hundreds); however, microscopy is still the main method of sperm analyses for a BSE and morphology (Rodriguez-Martinez, 2003; Barth, 2007; 2018; Vincent et al., 2012). A semen quality control was added to the NAAB-CSS audit program in 2011; however, quality control minimum standards and procedures are held private (Mitchell, 2012). Although each company may have different standards, quality control minimum standard procedures for pre-freeze and post-thaw (usually motility and morphology) must be approved by an NAAB-CSS auditor (Mitchell, 2012). Commonly used methods of quality control in AI centers involve: morphology (not done in all ejaculates, but ≥ 70% normal sperm is usually used), motility (≥ 60% for pre-freeze in a subjective assessment and ≥ 30% for post-thaw in an objective assessment are usually used), viability (usually only post-thaw and a threshold for this characteristic was not clearly identified; Rodriguez-Martinez, 2003; DeJarnette, 2012; Garner, 2014; Thundathil et al., 2016; Harstine et al., 2018; DeJarnette et al., 2021).

The search for prediction of male fertility has been the aim of several investigators, probably since the first reports of a sperm by Hamm and Leeuwenhoek in 1677 (Amann and Hammerstedt, 1993; Rodriguez-Martinez, 2003; Utt, 2016; Smith et al., 2018). Male fertility depends on multiple factors as discussed previously. Each ejaculate/insemination dose must contain “enough” of all required characteristics that

allow sperm to reach the site of fertilization, fertilize an oocyte and produce a viable embryo; however, it is not known what is "enough" for all sperm traits (Amann and Hammerstedt, 1993; Rodriguez-Martinez, 2003). Several sperm characteristics have been evaluated and shown to be correlated with bull fertility, some positive and others negative; however, results across studies are not consistent, as demonstrated by great variation in correlations, from strong to weak or no correlation (Rodriguez-Martinez, 2003; Utt, 2016). Even though motility is one of the key components utilized in BSE and AI centers to predict bull fertility, with strong correlation in an unselected population, in bulls that have above minimum standards for motility, correlation with fertility is variable; from non-significant to significant; $r^2 = 0.01$ to $r^2 = 0.63$ (Kjoestad et al., 1993; Stålhammar et al., 1994; Farrell et al., 1998; Gillan et al., 2008; Kathiravan et al., 2008; DeJarnette et al., 2021).

As mentioned throughout this review, bull fertility is multifactorial and dependent on a series of sperm characteristics and biological functions that sperm is required to go through in order to develop a viable embryo. Current sperm analysis can explain 50 to 60% of fertility variation of bulls (Saacke, 2008). It is unlikely that a single sperm characteristic will explain most of the variation between bulls, thus, a multivariate approach is more likely to accurately estimate the fertility level of a bull or ejaculate. When multiple CASA parameters were included in a regression analysis, the correlation with fertility was increased from $r^2 = 0.34$ with only total motility in the model to $r^2 = 0.68$ with amplitude of lateral head displacement (ALH) and progressive motility, and was reported to be as high as $r^2 = 0.98$ when five CASA characteristics were included in the model (Farrell et al., 1998). In another study, single sperm characteristic correlation

with fertility varied from $r^2 = 0.28$ to $r^2 = 0.45$, while multiple (4 or 5) characteristics ranged from $r^2 = 0.50$ to $r^2 = 0.58$ (Januskauskas et al., 2001). Researchers, AI industry personnel and veterinarians aim to predict bull fertility by identifying a threshold for sperm characteristics that will correctly predict a bull's fertility, such as ≥ 70% normal sperm and ≥ 30% motile sperm; however, with such variability in correlation with fertility it makes it very difficult to develop an accurate predictive model. Also, several of these characteristics were strongly correlated with motility, morphology, or both (Rodriguez-Martinez, 2003; Utt, 2016). Consideration should be taken in the selection of sperm parameters that will be included in a multiple regression analysis. It is important that sperm parameters are not correlated or the correlation between sperm parameters is weak (Utt, 2016). As described by Utt (2016), when parameters in a multiple regression analysis are correlated, it can lead to an incorrect estimation of fertility by the regression model which decrease the predictive ability of the model when applied in a greater population. Thus, when developing a fertility predictor/estimator multiple regression model, researchers or industry must first evaluate whether parameters included are measuring correlated characteristics or not.

In combination, predicting bull fertility based on sperm analyses is extremely complex. The correct prediction of a bull's fertility (more likely on the ejaculate level than the whole animal's life), if possible, will require the development of new markers of fertility that are not correlated with motility and morphology, and most likely will involve a combination of several sperm characteristics in a multifactorial equation. In the near future, it is more likely that scientists improve the ability to detect lowly fertile animals

through new tests compared to predicting highly fertile ones (DeJarnette, 2005; Dalton, 2019).

Use of sperm proteins as fertility marker

Sperm biological processes are regulated through proteins, such as sperm hyperactivation and capacitation, formation of the sperm reservoir, induction of the acrosome reaction, and fertilization (Sutovsky, 2009; 2018; Florman and Fissore, 2015; Suarez, 2015, 2016; Saint-Dizier et al., 2020; Mahé et al., 2021). Variation in protein expression between bulls may serve as fertility markers and aid in the selection of highly fertile bulls, as several studies have reported their correlation or association with field or *in vitro* fertility.

Several sperm proteins have been tested as fertility markers and demonstrated promising results. A study focused on a sperm protein of 25 kDa (called P25b) showed that this 25 kDa sperm protein was lowly expressed in some subfertile bulls compared to high fertility and some low fertility bulls; also, this protein was present on the acrosomal region and the principal piece of sperm tail (Parent et al., 1999). Others have described that osteopontin (Ca^{2+}-binding protein) was more abundant in semen samples from high fertility bulls compared to low fertility bulls (Killian et al., 1993; Cancel et al., 1997; Moura et al., 2006) and when osteopontin was added to fertilization media (10 μg/mL) cleavage and blastocyst rate of *in vitro* fertilized oocytes were improved compared to control media without osteopontin (Monaco et al., 2009). Further, phospholipase A2 was more abundant and spermadhesin Z13 (SPADH2) was less abundant in high fertility bulls

(Moura et al., 2006); however, in a different study, it was reported that calmodulin (CALM1), SPADH2 and phosphatidylethanolamine-binding protein 4 (PEBP4) were in greater concentration in semen samples from bulls of high fertility compared to low fertility and BSP1 was more abundant in low fertility bulls (Somashekar et al., 2015; 2017). Ibrahim et al. (2000) investigated the relationship of sperm protein clusterin with bull fertility and other sperm parameters, although clusterin was negatively correlated with nonreturn rates (raw and adjusted; $r^2 = 0.09$ and $r^2 = 0.33$, respectively) and estimated relative conception rate ($r^2 = 0.36$), it was also negatively correlated with motility and positively correlated with morphology abnormalities ($r^2 = 0.10$ to $r^2 = 0.60$).

A protein called fertility-associated antigen (FAA; previously called heparin-binding proteins) was characterized on ejaculated sperm and demonstrated association with bull fertility (natural service). Bulls with sperm positive for FAA had greater pregnancy rates (9 to 40 percentage points) compared to bulls that sperm lacked FAA (Bellin et al., 1994; 1996; 1998). Bulls used for AI with sperm positive for FAA had greater pregnancy rates (7 to 9 percentage points) compared to bull with sperm negative for FAA (Sprott et al., 2000); however, in a different study, pregnancy rates of bulls with sperm positive for FAA were not different (41.5% vs. 39.3%, respectively) or were lower (33.7% vs. 40.7%, respectively) than bulls with sperm FAA negative (Dalton et al., 2012). Additionally, it was described that A-kinase anchor protein-4 (AKAP4) was present on the sperm principal piece and this protein was related to sperm motility (Moss et al., 1999). Later it was reported that the expression level of this protein differed between high and low fertility bulls with high fertility bulls having greater expression

(Peddinti et al., 2008) and a commercial kit is available for testing AKAP4 on sperm samples (Sergeant et al., 2019).

Ubiquitin has been intensively studied and was reported as a negative marker of fertility, as bulls with greater sperm ubiquitination was associated with greater numbers of sperm defects (Sutovsky et al., 2001; 2002; 2007; Odhiambo et al., 2011; Kennedy et al., 2014). In an *in vitro* trial, nanopurification of sperm with anti-ubiquitin improved fertilization rates in comparison to control; however, when sperm was nanopurified with anti-ubiquitin and used for AI, there was a decrease in pregnancy rates when nanopurified sperm at 10 million sperm per dose was compared with control sperm at 20 million sperm per dose, but no difference in fertility was detected with the same insemination dose between nanopurified and control (10 million sperm per dose; Odhiambo et al., 2014).

These are some examples of proteins that have been used as fertility markers in bovine. Several other examples can be found in bovine, laboratory animals, men and other livestock species and poultry. If the words “sperm” and “protein” and “fertility” are entered in https://pubmed.ncbi.nlm.nih.gov/ search, almost 8,000 results are found and approximately 6,000 of those publications happened in the past 20 yr and 4,000 in past 10 yr. In combination, these studies, demonstrate the challenge in identifying a new biomarker of bull sperm fertility. Some biomarkers are associated or predictive of bull fertility in a small group but not in the population, as illustrated by Sprott et al. (2000) and Dalton et al. (2012) results. Others are correlated with motility and morphology and the additive predictive value of those biomarkers are debatable (Ibrahim et al., 2000). Further, some demonstrate promising results in laboratory studies but not in the field (Odhiambo et al., 2014). Some lack field validation or are not used by the industry as a

fertility marker. In order to improve identification of higher fertility animals, and, consequently, removal of subfertile animals from the breeding population, it is necessary to validate biomarkers of fertility already identified or newly developed. Also, new tests of fertility need to be easy to perform by AI industry personnel and veterinarians in the field.

SUMMARY

In summary, sperm is produced in the testis through mitotic, meiotic and cell differentiation, then, it is released from testicular parenchyma and travel through testis and epididymal tubules where it further matures. Sperm are stored in the tail of the epididymis in a quiescent status and upon ejaculation, sperm progressive motility is increased. During ejaculation, epididymal sperm is diluted with seminal plasma from the accessory sex glands. In this process, there are changes in surface proteins of the sperm.

In natural service, sperm is deposited in the vagina, and uterine contractions and sperm motility moves the sperm through the cervix to the uterus. In both, natural service and AI, sperm move through the uterus by self-propulsion to the oviduct by interaction with the utero tubular junction. In the oviduct, sperm quickly binds to the oviductal epithelial cells to form the sperm reservoir. To be released from the sperm reservoir, sperm goes through hyperactivation and capacitation. During sperm capacitation, sperm lose surface proteins, and a series of biochemical and biophysical transformations in the sperm head and tail allow the sperm to go through the acrosome reaction. When sperm

encounters the oocyte and its vestments (cumulus cells and zona pellucida) the acrosome reaction is induced, and fertilization may occur.

The current evaluation of bull fertility is through a BSE which evaluates the physical soundness, scrotal circumference, and sperm quality of a bull. For a bull to have potential high fertility, it must be healthy, produce an ejaculate with a great proportion of sperm with high levels of desirable traits, and a scrotal circumference sufficient for daily sperm production during the breeding season. Other areas that are involved in fertility but not measured in a breeding soundness exam are sperm membrane integrity, stability of the DNA, ability to undergo hyperactivation, capacitation, and the acrosome reaction, traverse the female barriers, fertilize the oocyte and generate a viable pregnancy.

Methods of bull fertility evaluation have not changed significantly in the past several years. Identification of new fertility markers may improve the ability of industry to detect lower fertility bulls that need to be culled before they enter the breeding season or predict which bulls will have high fertility. Proteins found on the sperm may play a key role in sperm fertilizing ability. Since sperm population is heterogeneous, even within an ejaculate, and sperm proteomics have been shown to change in sperm that have just been released in the seminiferous tubules, to epididymal sperm, to ejaculated sperm, to sperm in the female reproductive tract; thus, sperm proteins may serve as fertility biomarkers.

CHAPTER 2

PROTEOMIC ANALYSES IDENTIFY DIFFERENCES BETWEEN BOVINE EPIDIDYMAL AND EJACULATED SPERMATOZOA THAT CONTRIBUTE TO LONGEVITY

ABSTRACT

Sperm are stored for extended periods of time in the epididymis, but upon ejaculation motility is increased and lifespan is decreased. The objective of this study was to identify differences in proteins between epididymis and ejaculated samples that are associated with longevity. Ejaculated semen was collected from mature Angus bulls (n = 9); bulls were slaughtered and epididymal semen was collected. Epididymal and ejaculated semen were centrifuged to separate sperm and fluid. Fluids were removed and sperm pellets were resuspended in a high ionic solution and vortexed to remove loosely attached proteins. Sperm samples were centrifuged, and the supernatant was removed; both fluid and sperm samples were snap frozen in liquid nitrogen and stored at -80 ºC. Protein analysis was performed by liquid chromatography with tandem mass spectrometry (LCMS/MS). A different group of yearling Angus cross bulls (n = 40) were used for sperm cultures. Ejaculated (n = 20) and epididymal (n = 20) sperm were diluted and cultured in a commercial media at pH 5.8, 6.8 and 7.3, at 4 ºC and evaluated for motility and viability every 24 h until motility was below than 20%. There was an effect of pH, time and pH by time interaction for motility and viability for both ejaculated and epididymal sperm ($P \leq 0.05$). At 216 h of incubation epididymal sperm at pH 7.3 and

ejaculated sperm at pH 6.8 reached motility below 20%. A total of 458 unique proteins were identified; 178, 298, 311, and 344 proteins were identified in ejaculated fluid, ejaculated sperm, epididymal fluid and epididymal sperm, respectively. There were 8, 24, 10, and 18 significant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (FDR < 0.05) for ejaculated fluid, epididymal fluid, ejaculated sperm, and epididymal sperm, respectively. The metabolic pathway was identified as the most important KEGG pathway; glycolysis/gluconeogenesis, pentose phosphate, and glutathione metabolism pathways were significant among proteins only present in epididymal samples within the metabolic pathway. Other proteins identified that may be related to epididymal sperm's increased longevity were peroxidases and glutathione peroxidases for their antioxidant properties. In summary, energy metabolism in the epididymis appears to be more glycolytic compared to ejaculated and epididymis sperm has a larger number of antioxidants available which may be helping to maintain sperm in a quiescent state. In addition, epididymal sperm was able to maintain viability longer than ejaculated sperm when cultured under the same conditions.

INTRODUCTION

During final maturation, spermatozoa lose their ability to biosynthesize, repair, grow, and divide, and become very simple in their metabolic function (Hammerstedt, 1993). This results in spermatozoa becoming completely dependent on their external environment to survive and function. While in the epididymis, spermatozoa are stored for a long period of time in a relatively quiescent state. It is hypothesized that this is due to

both quiescence and prevention of premature activation of the spermatozoa prior to ejaculation (Sullivan et al., 2005). Upon ejaculation or dilution of the fluid of the caudal epididymis, motility is increased (Acott and Carr, 1984; Carr and Acott, 1984). A consequence of this increased motility is a reduction in viability to only several hours in most species (Austin, 1975).

Several studies have reported that the plasma membrane of spermatozoa is coated with glycoproteins (Magargee et al., 1988; Mahmoud and Parrish, 1996; Geussova et al., 1997), and several proteins that have been identified in the epididymal fluid are enzymes that are able to modify proteins or lipids at the spermatozoa surface. A subset of these proteins are implicated in spermatozoa protection (e.g. members of the Glutathione S-transferase family or peroxiredoxin isoforms; Girouard et al., 2011), and some of the proteins that are transferred to the spermatozoa are also proposed to modulate motility (Frenette et al., 2003; 2004; 2005). Macrophage migration inhibitory factor (MIF) protein, present in the epididymis, associates with the spermatozoa flagella, and may influence thiol; therefore, impacting acquisition of spermatozoa motility (Eickhoff et al., 2004). A negative correlation has been reported between concentration of MIF and spermatozoa motility (Sullivan et al., 2005). Thus, the objective of this experiment was to identify differences in proteins that are both in the environment (fluid) and loosely attached to spermatozoa in both the epididymis and following ejaculation measured by liquid chromatography with tandem mass spectrometry analysis (LCMS/MS). A secondary objective was to evaluate the effect of pH on sperm longevity. The hypotheses were that proteins differentially expressed between ejaculated and epididymis samples

would be correlated to sperm longevity and sperm incubated in uterine pH at estrus (pH = 6.8) would have greater motility and longevity.

MATERIALS AND METHODS

All procedures were approved by the South Dakota State University Institutional Animal Care and Use Committee.

Experimental design:

Study I:

Semen from nine sexually mature (4-yr old) Angus bulls with a history of successful breeding were collected by electro-ejaculation. Weekly ejaculates were collected for two weeks and discarded (2 ejaculates); bulls were rested for one week before a third weekly ejaculate was collected. After the third semen collection, bulls were rested for six weeks to renormalize epididymal reserves and then slaughtered. Testes and epididymides were collected and transported back to the laboratory. Epididymides were dissected and epididymal fluid and spermatozoa were collected from the caudal section of the epididymis. Ejaculated and epididymal sperm were diluted ($\sim 3 \times 10^9$ sperm/mL) and evaluated for motility, viability, and mitochondrial membrane potential at the time of semen collection. Ejaculated sperm was evaluated at pH 7.3 (most semen extender pH and uterine pH before and after estrus) and epididymal sperm was evaluated at physiological pH (5.8) and at pH 7.3. Epididymal semen from a subset of bulls (n = 3)

were cultured for 310 h at 4 °C in three different pH; physiological pH 5.8, pH 7.3 mimicking uterine pH before and after estrus, and pH 6.8 which has been reported to be the uterine pH at estrus (Perry and Perry, 2008a, b). The remainder of samples were processed for protein analysis.

Study II:

Semen from 20 yearling (12- to 15-mo old) Angus crossed bulls were collected by electro-ejaculation, diluted (~42×10^6 sperm/mL) and incubated at three different pH (5.8, 6.8, and 7.3). Twenty different yearling (12- to 15-mo old) Angus crossed bulls were slaughtered and testes and epididymides were collected at a commercial slaughter facility. Epididymides were dissected and epididymal fluid and spermatozoa were collected from the cauda section of the epididymis. Epididymal semen was diluted (~60×10^6 sperm/mL) and incubated at three different pH (5.8, 6.8, and 7.3) and transported back to the laboratory in culture, thus, first evaluation at 24 h incubation. Samples were evaluated every 24 h, until total motility were below 20%, then no further evaluation was made for total motility, progressive motility, or viability.

Sperm culture and analyses:

Aliquots of each sample (ejaculated and epididymal) were evaluated at collection, 0 h for ejaculated and 24 h after slaughter for epididymal. Samples were stained with Hoechst 33258 and evaluated for motility and viability (plasma membrane permeability to Hoechst 33258) by a computer-assisted sperm analysis machine (CASA; Hamilton

Thorne IVOS II, Beverly, MA), and mitochondrial membrane potential procedure (study I only), by MitoTracker red (Thermo Fisher, Eugene, OR) staining following manufacturer's label. Mitochondrial membrane potential was evaluated using a Nikon Fluorescence microscope, and the NIS-Elements software package was used to outline 100 individual spermatozoa and fluorescence intensity was determined.

Samples were diluted (42×10^6 to 3×10^9 sperm per mL; vide experimental design) and cultured in a commercially available media (OPTIXcell, IMV technologies, France) and adjusted to different pH (5.8, 6.8, or 7.3), at 4 °C. Samples were evaluated every 24 h, from 24 h after collection (due to transport to the lab a true 0 h was not possible) until 310 h incubation for motility and viability. At each evaluation, 50 µL of each culture was removed and diluted with 150 µL of tris buffer. Samples were then stained with Hoechst 33258 (final concentration 10 ng/mL) for 2 min at 37 °C and evaluated on a CASA for motility and viability (samples and machine maintained at 37 °C).

Protein isolation:

Samples were centrifuged (700 × g for 10 min) to separate spermatozoa and fluids (epididymal fluid or seminal plasma) for protein analysis. Fluids were removed and snap frozen in liquid nitrogen and stored at -80 °C until analyzed. Spermatozoa pellets were then washed with a high ionic solution (Rifkin and Olson, 1985) and vortexed for 1 min to remove proteins loosely attached to the spermatozoa. Samples were then centrifuged (700 × g for 10 min) to separate spermatozoa from stripped proteins. Stripped proteins were removed, snap frozen in liquid nitrogen and stored at -80 °C until analyzed. This

resulted in four types of samples: 1) epididymal fluid, 2) ejaculated fluid, 3) epididymal sperm stripped proteins (epididymal sperm), and 4) ejaculated sperm stripped proteins (ejaculated sperm).

Liquid chromatography mass spectrometry analysis:

Protein samples were shipped to the University of Minnesota Mass Spectrometry facility for identification by LCMS/MS. Samples were processed by trypsin digestion and cleaned by gel purification. Approximately 400 ng of reconstituted peptide were analyzed by capillary LCMS/MS on an Orbitrap Velos mass spectrometer system as previously described (Lin-Moshier et al., 2013) with the following modifications: the capillary column diameter was 100 µm, the gradient elution profile was of 8 – 35% B Solvent over 67 min at 330 nL/min, where A Solvent was 98:2:0.01, H_2O:acetonitrile (ACN):formic acid (FA); and B Solvent was 98:2:0.01, ACN:H_2O:FA, lock mass was not employed; dynamic exclusion settings were: repeat count = 1, exclusion list size was 200, exclusion duration = 12 s, exclusion mass width (high and low) was 15 ppm and early expiration was disabled.

All LCMS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 2.1.0.81). Sequest was set up to search the bovine (taxid 9913) protein sequence database from Uniprot.org with canonical and isoforms included, downloaded on March 6, 2013 and merged with the common lab contaminant protein database (thegpm.org/crap/index, 109 proteins). Sequest was searched with the digestion enzyme trypsin, fragment ion mass tolerance 0.100 Da and precursor tolerance of 50

ppm. Oxidation and di-oxidation of methionine, deamidated of asparagine and glutamine and pyroglutamic acid were set as variable peptide modifications, N-terminal protein acetylation was set as a variable modification and carbamidomethyl cysteine was set as a fixed modification.

Scaffold (version Scaffold_5.0.0, Proteome Software Inc., Portland, OR) was used to validate LCMS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 99.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 7.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on tandem mass spectrometry (MS/MS) analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

Further analysis was conducted using peptides identified as exclusive and unique to each protein. Total spectrum counts for proteins were used for abundance comparisons (proteins found in one sample but not the other or found in both samples) and statistical analysis. Comparisons were made for total spectrum counts between epididymal and ejaculated fluid proteins and between epididymal and ejaculated spermatozoa surface proteins. Significant *P*-values were adjusted for multiple testing using the Benjamini-Hochberg calculation to correct the FDR. For each comparison, proteins that were identified in the samples were entered into DAVID v 6.8 (Huang et al., 2008, 2009) using their official gene names to determine the top Kyoto Encyclopedia of Genes and

Genomes (KEGG) pathways associated with those proteins. For the significant KEGG pathways within each sample, that had a physiological meaning for the study objective, the proteins contributing to that KEGG that were exclusive for one of the samples were entered into the STRING database (Szklarczyk et al., 2015; Hu et al., 2018) to determine the network interactions of those proteins.

Statistical analyses:

Study I:

Differences of spermatozoa parameters between ejaculated and epididymal (pH 5.8 and 7.3) at collection were evaluated using the GLIMMIX procedures of SAS (v 9.4) proportions were assumed to have a beta distribution; velocities, Hz, µm and fluorescence intensity data were assumed to have a Gaussian distribution. The model used was treatment (ejaculated, epididymis 5.8, epididymis 7.3) as a fixed effect and bull as a random effect. For fluorescence intensity, area measured was included as a covariate. Cultured spermatozoa parameters were evaluated using the GLIMMIX procedure of SAS (v 9.4) and data were assumed to have a beta distribution. The model included the fixed effect of treatment (pH 5.8, 6.8, and 7.3), time of incubation and the interaction. Three random statements were used; the first random statement was used to model the R-side of residuals to analyze the data as repeated measures. The subject was bull with covariate structures selected based on the smaller -2 Res Log Pseudo-Likelihood. For total and progressive motility, the covariate structure was Compound Symmetry (CS); for viability, the covariate structure was Toeplitz (TOEP). Time points 286 and 310 h incubation were

removed from progressive motility analysis because all values equaled zero. When a significant effect ($P \leq 0.05$) or tendency ($P \leq 0.10$) of treatment was detected, the pairwise comparisons from the analysis were used to determine level of significance.

Study II:

Ejaculated and epididymal sperm parameters were evaluated separately. Total motility, progressive motility and viability were evaluated using the methods described above for repeated measures. For ejaculated sperm data the covariate structure for total and progressive motility was TOEP; and for viability, the covariate structure was First-Order Ante-dependence [ANTE(1)]. The covariate structure for epididymis total motility was TOEP; for progressive motility it was ANTE(1), and for viability, it was Heterogeneous Compound Symmetry (CSH). When a significant effect ($P \leq 0.05$) or tendency ($P \leq 0.10$) of treatment was detected, the pairwise comparisons from the analysis were used to determine level of significance.

RESULTS

Spermatozoa culture and analysis:

In study I, there was an effect of treatment (ejaculated pH 7.3, epididymis pH 5.8, and epididymis pH 7.3) on the mitochondrial membrane potential ($P < 0.0001$). Ejaculated sperm had decreased ($P \leq 0.0015$) mitochondrial membrane potential compared to both epididymis sperm at pH 5.8 and 7.3 (1.79 ± 0.32, 4.30 ± 0.34 and 3.41

± 0.32, fluorescence intensity, respectively). Epididymis sperm at pH 5.8 tended (P = 0.07) to have greater mitochondrial membrane potential than epididymis sperm at 7.3. Treatment influenced percentage of total motility, progressive motility, viability, viable linearity, and viable straightness ($P \leq 0.001$; Table 2.1); however, motile linearity and motile straightness did not differ ($P \geq 0.11$; Table 2.1). There was also an effect of treatment on average path velocity, curvilinear velocity, straight-line velocity, amplitude of lateral head displacement, and beat cross frequency for both motile and viable sperm ($P \leq 0.01$; Table 2.1). When epididymal sperm were cultured at pH 5.8, 6.8 and 7.3 there was an effect of pH by time interaction on total motility and progressive motility ($P \leq$ 0.05; Fig. 2.1 and 2.2); however, the pH by time interaction was not significant for viability ($P = 0.16$; Fig. 2.3). There was an effect of pH on total motility ($P < 0.0001$) and viability ($P < 0.0001$; Fig. 2.3), but there was no effect of pH on the percentage of progressive motility ($P = 0.59$). There was also an effect of time on total motility, progressive motility, and viability ($P < 0.0001$).

In study II, there was an effect of pH, time and pH by time interaction for total motility (Fig. 2.4), progressive motility (Fig. 2.5) and viability (Fig. 2.6) for both ejaculated and epididymal sperm ($P \leq 0.05$). Ejaculated sperm at pH 6.8 and epididymal sperm at pH 7.3 maintained total motility above 20% longer than the other samples, at 216 h of incubation motility decreased below 20% for both ejaculated 6.8 and epididymal 7.3 (17.1% and 18.9%, respectively). The percentage of sperm displaying progressive motility at 216 h was 1.6% and 1.1%, and viability 51.3% and 95.4%, for ejaculated 6.8 and epididymal 7.3, respectively.

Protein identification:

An overall total of 458 unique proteins were detected between all samples (Fig. 2.7), 178 proteins were detected in ejaculated fluid (seminal plasma) and 298 proteins were identified stripped from ejaculated sperm. In epididymal samples, 311 proteins were identified in epididymal fluid, and 334 proteins were identified stripped from epididymal sperm (Fig. 2.7). There were 103 proteins detected in the fluids that were present in both ejaculated and epididymal samples, ten proteins had increased abundance in ejaculated fluid ($P \leq 0.05$; A5D9E8, CLUS, Cytokeratin-9, F1MK08, IPSP, LG3BP, Q58DP6, RNS, SFP1, SPAD1) and 29 had increased abundance in epididymal fluid ($P \leq 0.05$; A6QLB0, ACTB, ACTC, ACTS, CBPQ, DHSO, ENOA, F16P1, F1N0E5, F1N5M2, G3X6N3, G3X757, HBA, HBB, HEMO, HS90A, K2C8, KAD1, KAP0, PARK7, PEBP1, PRDX5, SPA31, SPA37, TBA8, TBB4A, TBB4B, TBB5, TRFE). There were 221 proteins detected in the sperm samples that were present in both ejaculated and epididymal samples, 12 proteins had increased abundance in the ejaculated sperm ($P \leq 0.05$; B2MG, CLUS, F1MK08, F1MTI7, F1MXP8, Q4R0H2, Q58DP6, RNS, SFP1, SPAD1, Trypsin precursor, Z13) and 109 proteins had increased abundance ($P \leq 0.05$) in the epididymal sperm.

Pathway analysis:

There were eight significant KEGG pathways (FDR < 0.05) for ejaculated fluid proteins and 24 KEGG pathways for epididymal fluid proteins (Table 2.2). There were ten significant pathways for ejaculated and 18 for epididymal proteins that were stripped

from the sperm (Table 2.3). The metabolic pathway was identified as the most important KEGG pathway for this data set. This was expected as sperm are maintained in a quiescent mode in the epididymis which allows sperm to be stored for several days.

Metabolic pathway associated proteins in the fluid samples included: 15 proteins that were present in both ejaculated and epididymis samples (AK1, ENO1, FBP1, FH, GALK1, GAPDHS, GLB1, GNS, GPI, LDHA, MDH2, PGAM1, PGAM2, PTGDS, SORD), nine proteins that were only present in ejaculated fluid and 55 proteins that were only present in epididymis fluid (Fig. 2.8). The proteins related to the metabolic pathway only present in ejaculated fluid were not highly related as seen by few connections between proteins; however, the proteins Heparanase (HPSE) and N-acetyl-alpha-glucosaminidase (NAGLU) participate in glycosaminoglycan degradation (Fig. 2.8 A). Two other proteins were detected in this pathway but were not related to the metabolic pathway. They were Beta-galactosidase (GLB1) and N-acetylglucosamine-6-sulfatase (GNS). The proteins Glucosylceramidase (GBA), Lysosomal alpha-glucosidase (GAA), NAGLU, Palmitoyl-protein thioesterase 1 (PPT1), and V-type proton ATPase subunit S1 (ATP6AP1) participate in lysosome pathway (Fig. 2.8 A). Five other proteins were identified to the lysosome pathway but were not related to the metabolic pathway, they were Lysosomal protective protein (CTSA), Cathepsin B (CTSB), Cathepsin D (CTSD), Legumain (LGMN), and Prosaposin (PSAP).

The proteins present only in epididymis fluid and related to metabolic pathway (Fig. 2.8 B) differed from ejaculated fluid and were highly interactive as demonstrated by a complex network. Eleven proteins in this network were related to glycolysis/gluconeogenesis pathway (AKR1A1, ALDH2, ALDH9A1, ALDOA, GALM,

GAPDH, LDHB, PGK1, PGM1, PGM2, TPI1), five proteins were related to the oxidative phosphorylation pathway (ATP6V1H, ATP6V1B2, ATP6V1A, ATP5A1, ATP5B), eight were related to the pentose phosphate pathway (ALDOA, G6PD, PGM1, PGM2, PRPS1, TALDO1, TKT, TKTL1), and four proteins were related to fructose and mannose metabolism (AKR1B1, ALDOA, MPI, TPI1).

There were 36 proteins present in both ejaculated and epididymis sperm samples related to the metabolic pathway (AK1, AKR1B1, ALDH2, ALDOA, AOX1, APRT, ATIC, ATP5A1, ATP5B, ATP6V1E2, ATP6V1H, DCXR, ENO1, FBP1, FH, GALK1, GAPDHS, GLB1, GLUL, GNS, GPI, IDH1, ISYNA1, LDHA, MDH2, NME2, PGAM1, PGAM2, PGK1, PGLS, PGM2, PTGDS, QDPR, RPN2, SMS, SORD). Nevertheless, 11 proteins were only present on ejaculated sperm (Fig. 2.9 A) and 32 were only present on epididymal sperm (Fig. 2.9 B). Proteins related to the metabolic pathway only present in ejaculated sperm were not highly related as seen by few connections between proteins, similarly to proteins only present in ejaculated fluid (Fig. 2.9 A). The same proteins were detected in the glycosaminoglycan degradation and lysosome pathway between ejaculated fluid and sperm. Similar to ejaculated fluid, there were proteins not related to metabolic pathway that were also present in the lysosome pathway (CTSB, CTSD and LGMN); however, glycosaminoglycan degradation was not detected as a significant pathway for sperm proteins. Interestingly, two proteins were detected to be part of the oxidative phosphorylation pathway, Succinate dehydrogenase [ubiquinone] flavoprotein subunit (SDHA) and ATP6AP1.

The proteins only present in the epididymis sperm samples and related to the metabolic pathway (Fig. 2.8 B), different from ejaculated sperm and similarly to those

from epididymis fluid, were highly interactive as demonstrated by a complex network. There were seven proteins related to the pentose phosphate pathway (G6PD, PGD, PGM1, PRPS1, TALDO1, TKT, TKTL1), five proteins related to glycolysis/gluconeogenesis pathway (AKR1A1, LDHB, GAPDH, PGM1, TPI1) and two proteins related to the fructose and mannose metabolism pathway (MPI, TPI1) that were present only in the epididymis sperm samples compared to the ejaculated sperm samples.

DISCUSSION

Efficient transportation of spermatozoa through the female reproductive tract from the site of deposition to the site of fertilization requires that the female be in estrus or under the influence of estrogen (Hawk, 1983). Estrogen may influence fertilization rates through both spermatozoa transport and fertilization efficiency by altering the uterine environment (pH). Uterine pH decreased at the initiation of standing estrus (Elrod and Butler, 1993) and was also decreased in animals that exhibited standing estrus prior to fixed-time AI compared to animals not exhibiting standing estrus (Perry and Perry, 2008a, b). Estrus expression prior to fixed-time insemination increased the number of spermatozoa that reached the site of fertilization (Larimore et al., 2015) and had a linear relationship with pregnancy success (Grant et al., 2011). It is hypothesized that the decrease in pH at onset of estrus would increase sperm longevity and the rise in pH prior to ovulation would increase sperm motility (Perry and Perry, 2008a, b). Thus, sperm was incubated at three different pH: physiological pH of the epididymis (5.8), physiological

pH upon ejaculation which is similar to the uterine pH at time of ovulation (7.3) and uterine pH at onset of estrus (6.8). In study I, epididymal sperm was able to maintain motility for a longer period of time when it was incubated at pH 6.8 compared to pH 5.8 or 7.3. This is consistent with the hypothesis that a decrease in uterine pH at the onset of estrus would increase sperm longevity. In study II, this hypothesis held true for ejaculated sperm (pH 6.8 had the greatest longevity); however, epididymal sperm at pH 7.3 had greater longevity (total motility) compared to sperm at pH 6.8. Animals in study I and study II were different, the main differences between the two groups of bulls (age) may have caused the observed differences. In study I, animals were mature bulls (4-yr old) with proven fertility and study II animals were 12- to 15-mo old that had just reached puberty and passed a breeding soundness exam.

Upon dilution of caudal epididymis fluid motility was increased (Acott and Carr, 1984; Carr and Acott, 1984), but when epididymal sperm was incubated in caudal epididymal fluid, motility was inhibited (Carr and Acott, 1984). When epididymal sperm was diluted and the pH altered to 7.3 there was an increase in sperm motility. The washing and dilution of caudal epydidimal fluid were sufficient to cause an increase in sperm motility which explains the lack of statistical difference between epididymis sperm at pH 5.8 and ejaculated sperm at pH 7.3 in study I and is similar to what has been reported by others (Acott and Carr, 1984; Carr and Acott, 1984; Carr et al., 1985). Interestingly, when the pH of epididymal sperm was adjusted to 7.3 total motility and progressive motility were increased to above ejaculated sperm. This is consistent with the increased mitochondrial membrane potential of epididymal sperm and agrees with the increase in sperm motility reported by others (Ericsson et al., 1993).

When comparing sperm viability (study II) between ejaculated and epididymal sperm, even though sperm motility decreased during incubation, epididymal sperm had at least 15 percentage points more viable sperm compared to ejaculated sperm at any time point regardless of media pH (Fig. 2.6). In the cauda epididymis, sperm are stored for a long period of time. After differentiation and maturation, sperm has a relatively simple metabolism and is highly dependent on its environment (Hammerstedt, 1993). Sullivan et al. (2005) hypothesized that the increased longevity of epididymal sperm is due to both quiescence and prevention of premature activation of the spermatozoa. In agreement with Sullivan et al. (2005), the increased viability of epididymal spermatozoa compared to the ejaculated, was not only due to the relatively quiescent state it was in, but also due to proteins associated with these spermatozoa, because even after dilution and initiation of motility epididymal sperm had increased viability compared to ejaculated sperm in the present study.

Proteins were identified in ejaculated and epididymal samples in order to investigate which proteins may be involved in increased viability of epididymal sperm. There were 153 proteins identified in epididymis samples only, and 74 were only identified in ejaculated samples. When comparing proteins stripped from the sperm and in the fluids between ejaculated and epididymis samples, the metabolic pathway had the greatest number of proteins. The KEGG metabolic pathway can be subdivided into other pathways, as proteins may have function in multiple pathways [e.g., Fructose-bisphosphate aldolase A (ALDOA) is present in the metabolic pathway, glycolysis/gluconeogenesis pathway, pentose phosphate pathway and, fructose and mannose pathway]. The total number of proteins identified in ejaculated (n = 305) and

epididymal sperm (n = 384) suggests that epididymal sperm metabolism and environment are more regulated by proteins than ejaculated sperm, especially, since 153 proteins were present only in epididymal samples compared to 74 proteins present in ejaculated samples only.

Bovine sperm can utilize both anaerobic and aerobic methods of energy production to maintain similar levels of motility (Krzyzosiak et al., 1999). Proteins only in the epididymal samples that were involved in the glycolysis/gluconeogenesis pathway (11 and five in fluid and sperm, respectively) and that were associated with oxidative phosphorylation (five proteins identified in fluid) were identified. Two different proteins were present in ejaculated sperm that related to oxidative phosphorylation. The glycolysis/gluconeogenesis pathway had seven and 11 proteins that were present in both ejaculated and epididymis samples, fluid and sperm, respectively. Interestingly, the oxidative phosphorylation pathway was not detected when all proteins from each sample were analyzed by DAVID; however, proteins in this pathway were identified when the metabolic pathway proteins were entered in the STRING database.

Human patients with asthenozoospermia had increased levels of ALDOA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), alcohol dehydrogenase (AKR1A1), L-lactate dehydrogenase B chain (LDHB) in seminal plasma compared to control patients (Wang et al., 2009). In this data set, the proteins elevated in seminal plasma of patients with asthenozoospermia, ALDOA, GAPDH, and AKR1A1 were only detected in epididymal fluid. Also, AKR1A1, LDHB and GAPDH were only present on epididymal sperm (ALDOA was present in ejaculated sperm but in lower abundance compared to epididymal sperm). Our results suggest that energy production, specifically

through glycolysis, in the epididymis is more regulated compared to after ejaculation. Thus, these proteins only detected in epididymal samples may need to be removed or diluted to undetectable levels to facilitate and promote energy production and, consequently, sperm motility.

It is possible that the increased number of proteins related to glycolysis in epididymal samples is a mechanism to reduce the production of reactive oxygen species (ROS) which is a by-product of oxidative phosphorylation and not produced during glycolysis. Reactive oxygen species are oxygen containing molecules that can be found as free radicals or non-radical oxidants, these molecules remove electrons from specific reactants. The presence of ROS is necessary for normal sperm function; however, the deleterious capacity of ROS is determined by its concentration. The increase in sperm intracellular cyclic adenosine monophosphate (cAMP) is caused by ROS which leads to a cascade of biochemical events that lead to sperm capacitation (Aitken et al., 2015; Aitken, 2017); however, when in elevated concentrations, ROS can cause oxidative stress which leads to lipid peroxidation. Lipid peroxidation has been reported to increase DNA fragmentation, decrease plasma membrane integrity (viability), and reduce motility in bovine sperm (Kasimanickam et al., 2007). Antioxidant proteins can remove ROS from the media and prevent harmful elevated concentrations of ROS. Aldehyde dehydrogenase (ALDH2) was identified in all samples except for ejaculated fluid, this protein was identified in the metabolic pathway, and it has been reported to provide antioxidant properties in the stallion sperm (Gibb et al., 2016; Hall et al., 2017). The glutathione metabolism pathway was present in epididymal fluid but not in ejaculated fluid and was present in both ejaculated and epididymal sperm. The glutathione S-transferases proteins

have been reported to be antioxidants in stallion (Gibb et al., 2016; Hall et al., 2017) and have been suggested to be involved in bovine sperm protection (Girouard et al., 2011). It was identified that the protein glutathione S-transferase P (GSTP1) was only present in epididymal fluid and sperm; however, glutathione S-transferase Mu (GSTM1) was present in epididymal fluid and both epididymal and ejaculated sperm. Another pathway that has been reported to have antioxidant properties is the pentose phosphate pathway (Williams and Ford, 2004). Peroxidases (PRDX1, PRDX2, PRDX4, and PRDX6) are important antioxidants that have been reported to protect sperm from oxidative stress, and they were identified in epididymal samples but not ejaculated samples. Additionally, glutathione peroxidases were identified in both ejaculated and epididymal samples (GPX5) or only in ejaculated samples (GPX6; O'Flaherty, 2019).

In summary, in the epididymis, sperm energy metabolism appears to be more glycolytic compared to sperm in the ejaculate, based on the greater number of proteins related to this pathway only present in epididymal samples. Sperm also has a greater number of antioxidants available in the epididymis that is likely to be maintaining ROS at low concentrations to inhibit premature sperm activation. This is supported by a greater mitochondrial membrane potential of epididymal sperm compared to ejaculated sperm and the fact that epididymal sperm was able to maintain viability longer than ejaculated when cultured under the same conditions. In addition, when both ejaculated and epididymal sperm were cultured at uterine pH (7.3), epididymal sperm had greater motility. More research is necessary to better understand the specific roles of the proteins only identified in the epididymis with the increase in sperm longevity, regulation of

sperm activation, and their possible role in bull fertility and ability to dominate a breeding pasture (Abell et al., 2017; Bennett et al., 2021).

Table 2.1. The effect of treatment (ejaculated sperm at pH 7.3 and epididymis sperm at pH 5.8 or 7.3) on motility and viability parameters measured by CASA (mean ± SEM).

Variables	Ejaculated pH 7.3	Epididymis pH 5.8	Epididymis pH 7.3
Total motility, %	48.0 ± 4.5^{b}	39.4 ± 5.4^{b}	71.5 ± 4.0^{a}
Progressive motility, %	5.5 ± 1.4^{b}	5.0 ± 1.7^{b}	16.2 ± 2.5^{a}
Viable, %	72.4 ± 4.7^{b}	95.8 ± 2.0^{a}	94.1 ± 2.0^{a}
LIN[1] - motile, %	31.9 ± 1.8	28.0 ± 1.9	29.3 ± 1.7
LIN[1] - viable, %	18.9 ± 1.5^{a}	10.9 ± 1.4^{b}	21.1 ± 1.5^{a}
STR[2] - motile, %	61.9 ± 2.9	57.9 ±3.6	61.6 ± 2.9
STR[2] - viable, %	37.5 ± 2.8^{a*}	23.0 ± 3.0^{b}	45.2 ± 2.9^{a†}
VAP[3] - motile, µm/s	68.2 ± 8.0^{ab*}	47.9 ± 9.8^{b}	90.1 ± 8.0^{a†}
VAP[3] - viable, µm/s	47.1 ± 8.6^{ab*}	19.8 ± 10.5^{b†}	70.2 ± 8.6$^{a\text{ł}}$
VCL[4] - motile, µm/s	138.5 ± 15.1^{b}	111.5 ± 18.5^{b}	206.5 ± 15.1^{a}
VCL[4] - viable, µm/s	95.9 ± 16.8^{b*}	46.2 ± 20.6^{b†}	159.9 ± 16.8^{a}
VSL[5] - motile, µm/s	41.0 ± 5.6^{ab*}	28.7 ± 6.9^{b}	56.3 ± 5.6^{a†}
VSL[5] - viable, µm/s	26.9 ± 5.2^{b*}	11.8 ± 6.4^{b†}	43.8 ± 5.2^{a}
ALH[6] - motile, µm	7.9 ± 0.5^{b}	6.5 ± 0.7^{b}	9.3 ± 0.5^{a}
ALH[6] - viable, µm	5.5 ± 0.7^{a}	2.7 ± 0.9^{b}	7.1 ± 0.7^{a}
BCF[7] - motile, Hz	28.4 ± 2.9^{b}	39.6 ± 3.7^{a}	39.5 ± 2.9^{a}
BCF[7] - viable, Hz	16.6 ± 2.1^{b}	15.7 ± 2.6^{b}	28.5 ± 2.1^{a}

[1-7] LIN = Linearity, STR = Straightness, VAP = Average path velocity, VCL = Curvilinear velocity, VSL = Straight-line velocity, ALH = Amplitude of lateral head displacement, BCF = Beat-cross frequency.

[a-b] Values within the same row not sharing a common superscript differ $P \leq 0.05$.

[*,†,ł] Values within the same row not sharing a common superscript differ $P \leq 0.10$.

Table 2.2. Number of proteins (Count) and level of significance (FDR) for the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway for proteins identified in epididymal and ejaculated fluid.

KEGG	Epididymis fluid		Ejaculated fluid	
	Count	FDR	Count	FDR
bta00010: Glycolysis/Gluconeogenesis	18	5.09E-12	7	0.006655
bta00020: Citrate cycle (TCA cycle)	5	0.047883		
bta00030: Pentose phosphate pathway	10	2.34E-07		
bta00040: Pentose and glucuronate interconversions	6	0.005755		
bta00051: Fructose and mannose metabolism	6	0.010003		
bta00052: Galactose metabolism	7	0.00169	3	0.465539
bta00330: Arginine and proline metabolism	7	0.012378		
bta00480: Glutathione metabolism	8	0.005533		
bta00500: Starch and sucrose metabolism	6	0.009208		
bta00520: Amino sugar and nucleotide sugar metabolism	7	0.010479		
bta00531: Glycosaminoglycan degradation			4	0.041254
bta00620: Pyruvate metabolism	8	6.72E-04	3	0.555899
bta00630: Glyoxylate and dicarboxylate metabolism	5	0.029481		
bta01100: Metabolic pathways	71	5.41E-11	24	0.223982
bta01130: Biosynthesis of antibiotics	39	3.84E-21	10	0.017366
bta01200: Carbon metabolism	25	6.57E-15	7	0.036405
bta01230: Biosynthesis of amino acids	16	4.33E-09		
bta03050: Proteasome	10	3.21E-05		
bta04141: Protein processing in endoplasmic reticulum	13	0.010003	6	0.465539
bta04142: Lysosome	11	0.010479	17	1.64E-10
bta04145: Phagosome	12	0.015453	7	0.154659
bta04610: Complement and coagulation cascades	15	7.21E-08	7	0.007027
bta04612: Antigen processing and presentation	9	0.006383	7	0.007027
bta04614: Renin-angiotensin system			5	0.007027
bta05134: Legionellosis	8	0.006383	5	0.069776

Table 2.3. Number of proteins (Count) and level of significance (FDR) for the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway for proteins stripped from the sperm and identified in epididymal and ejaculated samples.

KEGG	Epididymis sperm		Ejaculated sperm	
	Count	FDR	Count	FDR
bta00010: Glycolysis/Gluconeogenesis	16	1.2E-09	11	3.8E-05
bta00020: Citrate cycle (TCA cycle)	6	1.1E-02	4	2.5E-01
bta00030: Pentose phosphate pathway	12	1.1E-09	5	4.3E-02
bta00040: Pentose and glucuronate interconversions	6	6.9E-03	4	1.9E-01
bta00051: Fructose and mannose metabolism	6	1.2E-02	4	2.5E-01
bta00480: Glutathione metabolism	10	1.5E-04	6	7.7E-02
bta00620: Pyruvate metabolism	6	2.8E-02	4	3.7E-01
bta00630: Glyoxylate and dicarboxylate metabolism	5	3.8E-02		
bta01100: Metabolic pathways	69	1.3E-09	48	6.1E-04
bta01130: Biosynthesis of antibiotics	35	8.4E-17	21	8.1E-07
bta01200: Carbon metabolism	26	8.3E-16	13	1.2E-04
bta01230: Biosynthesis of amino acids	16	4.8E-09	7	6.0E-02
bta03050: Proteasome	8	2.7E-03	8	1.3E-03
bta04141: Protein processing in endoplasmic reticulum	12	3.8E-02	8	5.1E-01
bta04142: Lysosome	12	5.8E-03	18	1.5E-07
bta04610: Complement and coagulation cascades	15	8.1E-08	8	2.0E-02
bta04612: Antigen processing and presentation	9	7.8E-03	8	2.0E-02
bta04614: Renin-angiotensin system			6	4.8E-03
bta05134: Legionellosis	8	7.8E-03	6	9.1E-02

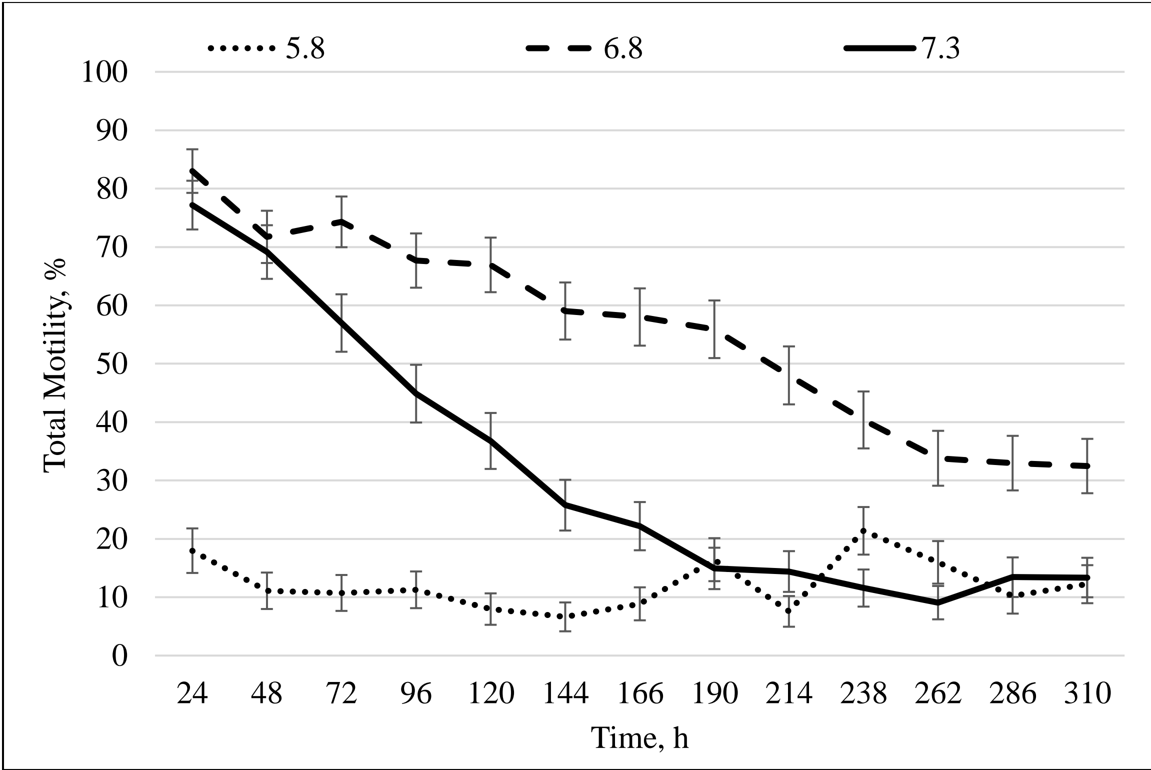


Figure 2.1. Percentage of total motility for epididymal sperm culture at pH 5.8, 6.8, and 7.3 (Study I). There was a significant pH, time, and pH by time interaction ($P < 0.0001$).

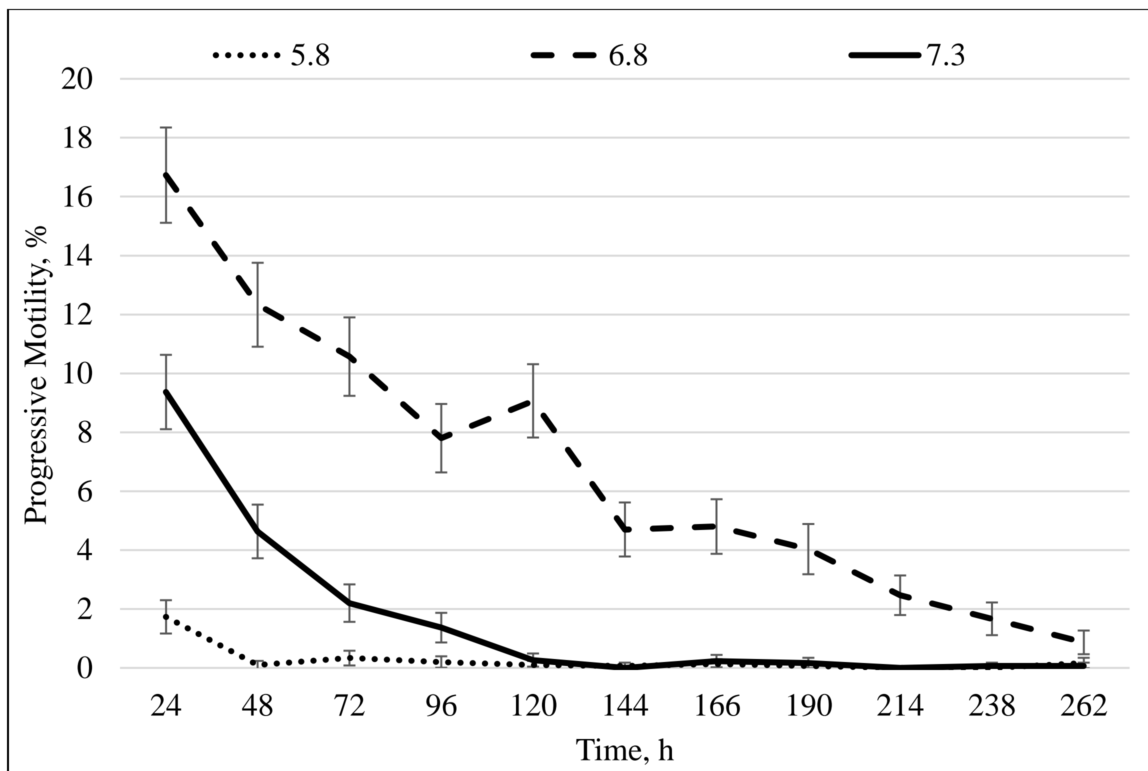


Figure 2.2. Percentage of progressive motility for epididymal sperm cultured at pH 5.8, 6.8, and 7.3 (Study I). There was a significant time and pH by time interaction ($P \leq 0.05$); pH was not significant ($P = 0.59$).

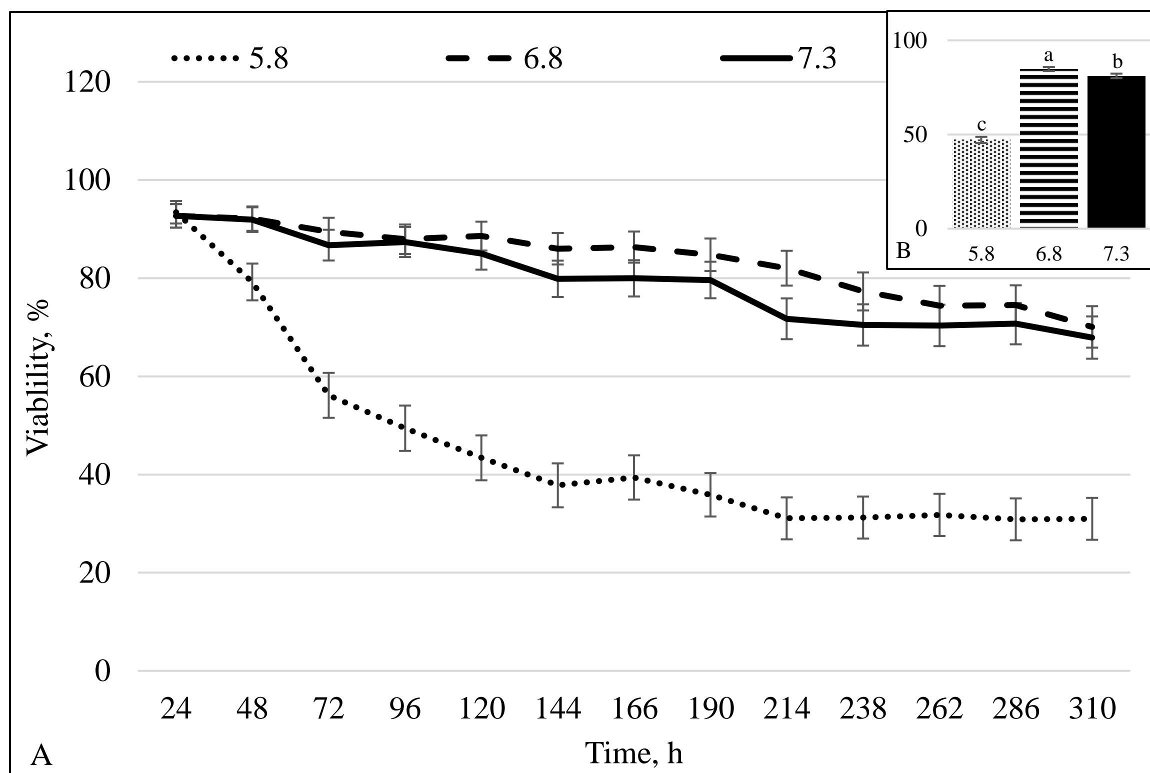


Figure 2.3. Percentage of viable epididymal sperm cultured at pH 5.8, 6.8, and 7.3 (Study I). There was not a significant pH by time interaction (A; $P = 0.16$). There was a significant pH (B; Y-axis represents viability % and X-axis represents pH treatment) and time effect ($P < 0.0001$).

[a-c] Bars within figure not sharing a common superscript differ $P \leq 0.05$.

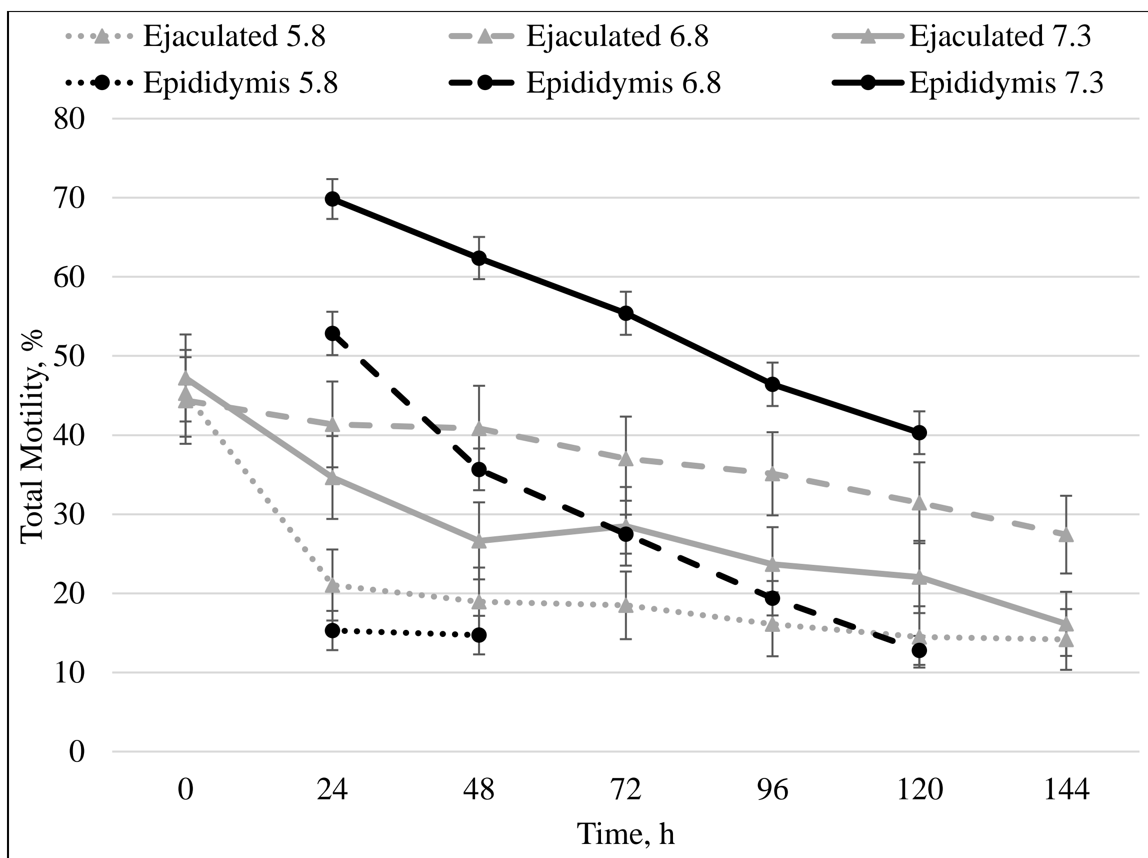


Figure 2.4. Percentage of total motility for epididymal and ejaculated sperm cultured at pH 5.8, 6.8, and 7.3 (Study II). Samples were considered non-viable when total motility decreased below 20%. There was a significant pH, time and pH by time interaction for both epididymal and ejaculated sperm ($P \leq 0.04$).

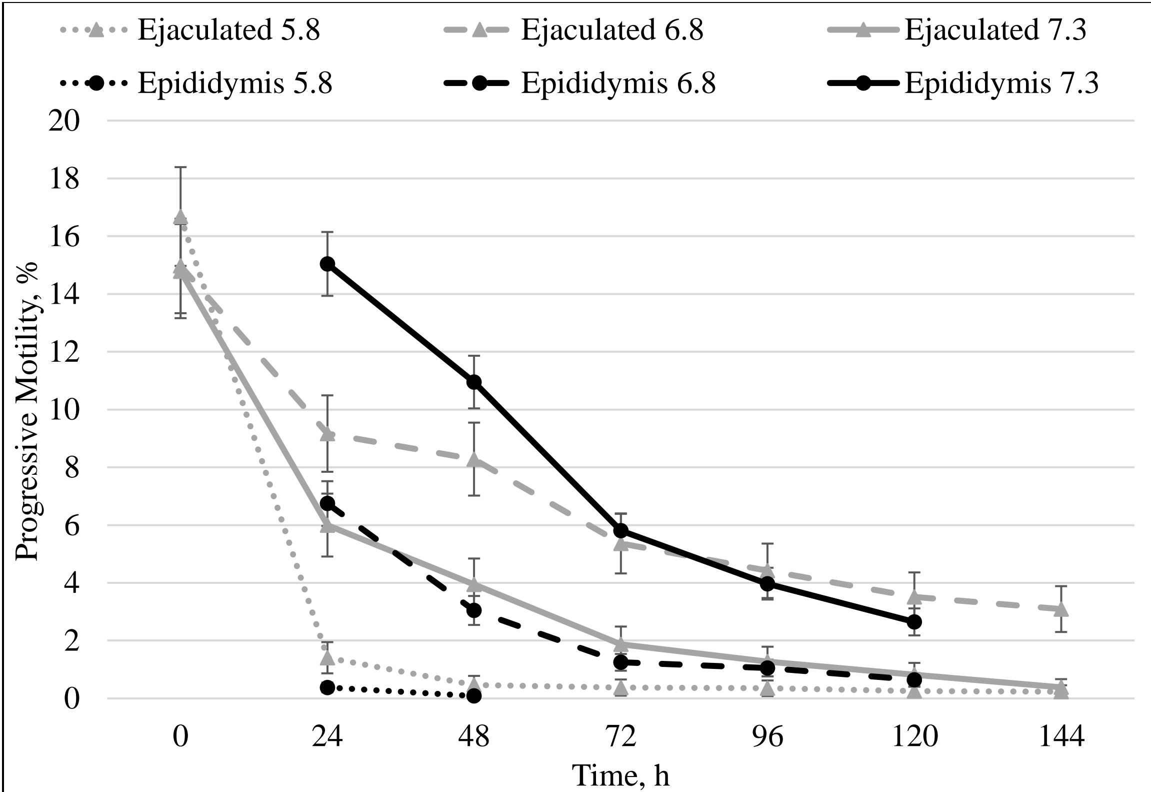


Figure 2.5. Percentage of progressive motility for epididymal and ejaculated sperm cultured at pH 5.8, 6.8, and 7.3 (Study II). Samples were considered non-viable when total motility decreased below 20%. There was a significant pH, time and pH by time interaction for both epididymal and ejaculated sperm ($P \leq 0.03$).

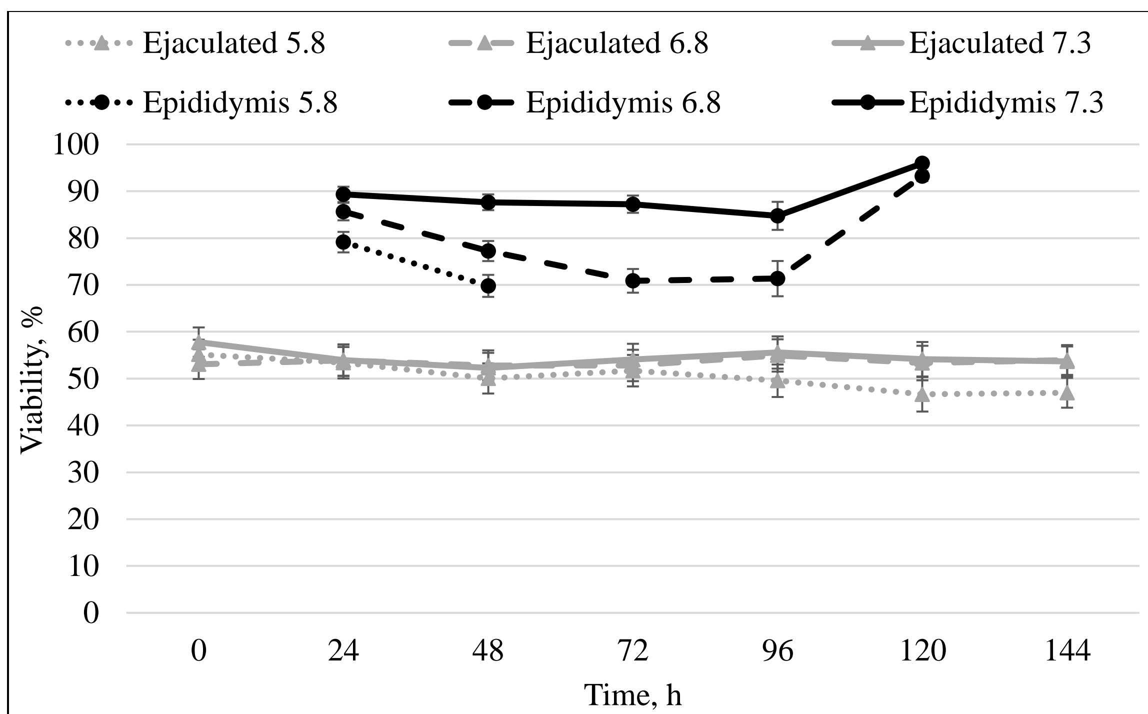


Figure 2.6. Percentage of viable for epididymal and ejaculated sperm cultured at pH 5.8, 6.8, and 7.3 (Study II). Samples were considered non-viable when total motility decreased below 20%. There was a significant pH, time and pH by time interaction for both epididymal and ejaculated sperm ($P \leq 0.02$).

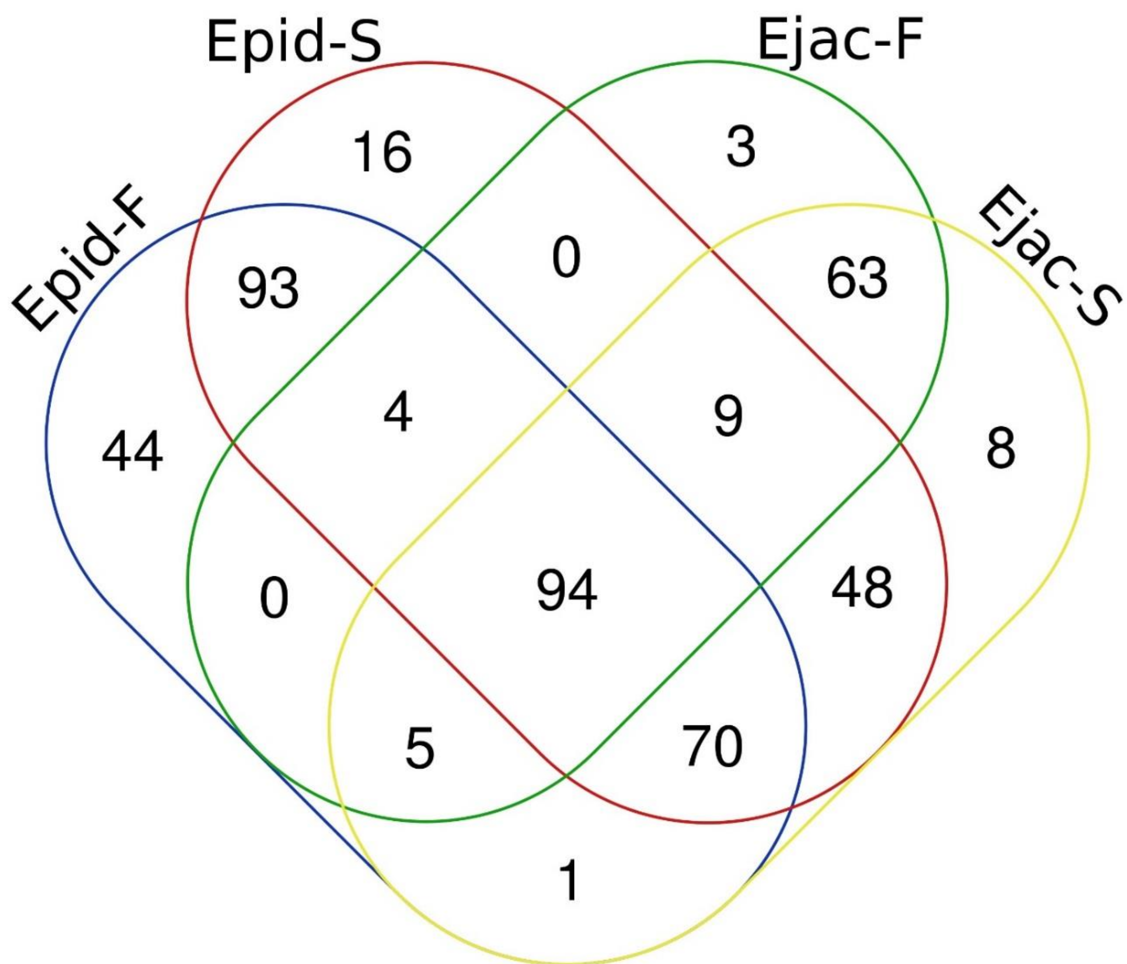


Figure 2.7. Venn diagram for the number of proteins detected in epididymal fluid (Epid-F), epididymal sperm (Epid-S), ejaculated fluid (Ejac-F), and ejaculated sperm (Ejac-S). A total of 458 unique proteins were detected by LCMS/MS. Protein identifications were accepted if a minimum of one unique peptide was identified to a known protein, and minimum of a 50% confidence in the identity of the protein was achieved. Peptide threshold was set at 95% peptide probability.

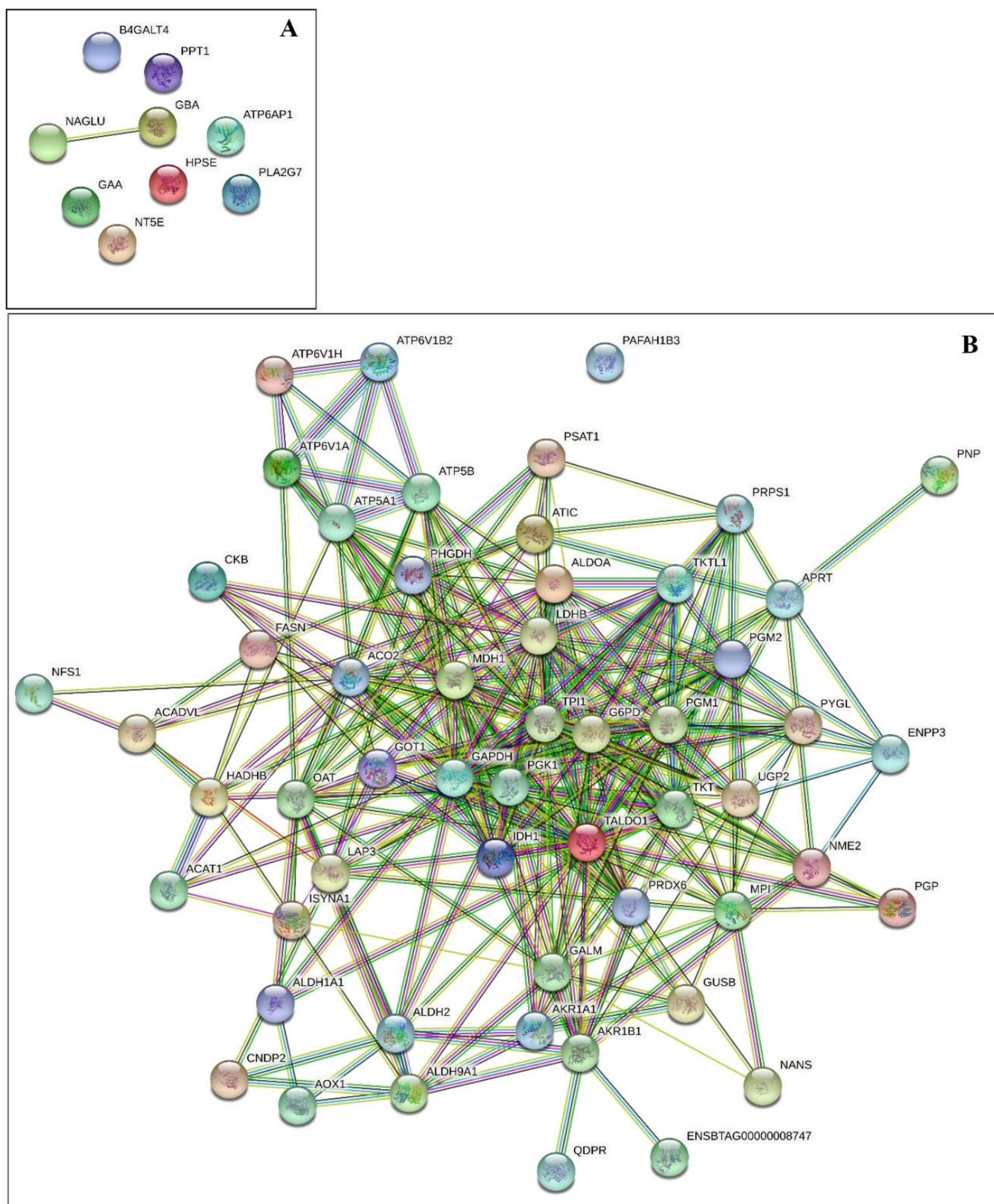


Figure 2.8. Protein interaction analyzed by STRING database of proteins present only in the ejaculated fluid (A; PPI enrichment $P = 0.22$) or epididymis fluid (B; PPI enrichment $P < 0.0001$) from the metabolic pathway (KEGG; Table 2.2).

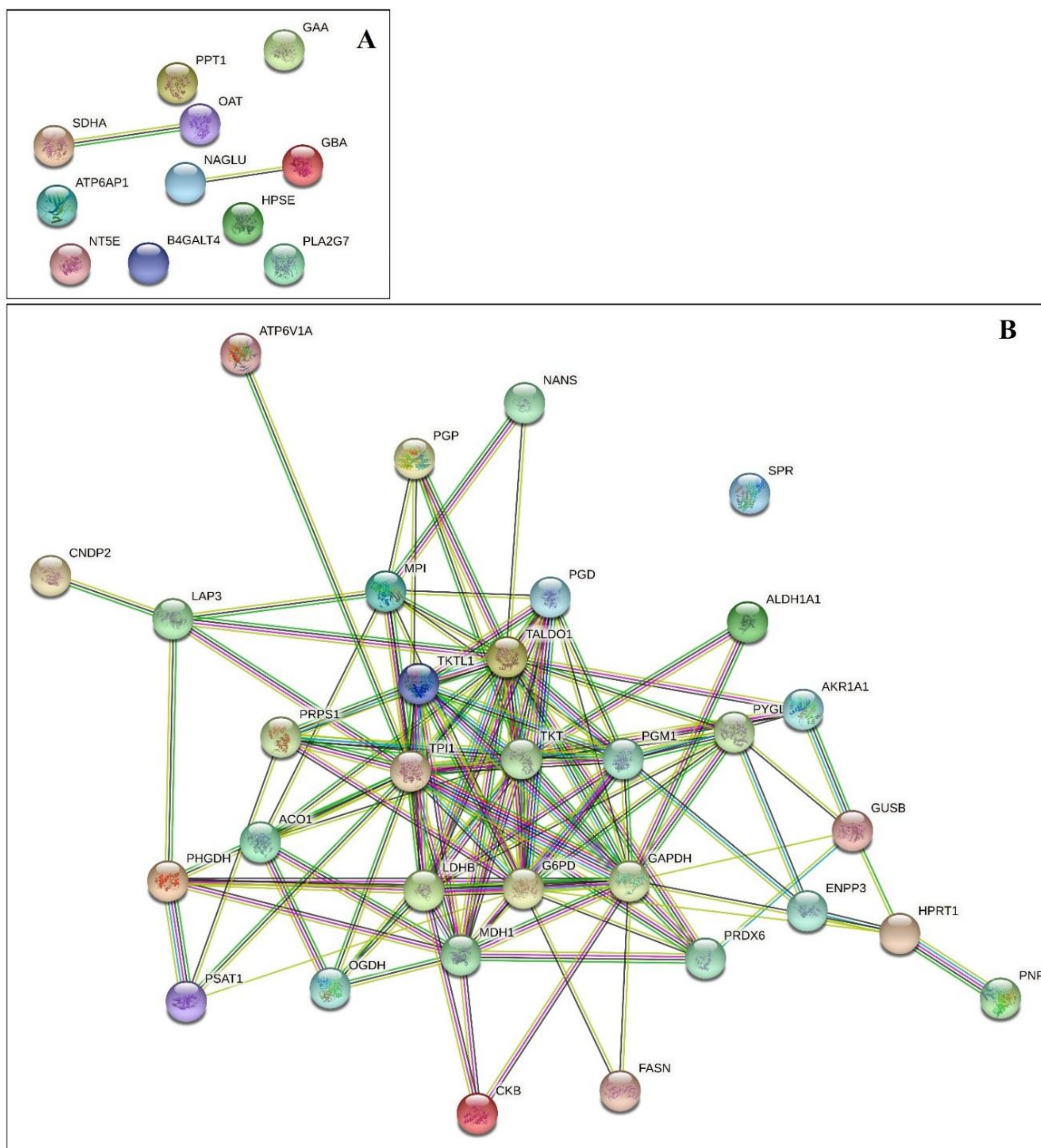


Figure 2.9. Protein interaction analyzed by STRING database of proteins present only in the ejaculated sperm (A; PPI enrichment $P = 0.09$) or epididymis sperm (B; PPI enrichment $P < 0.0001$) from the metabolic pathway (KEGG; Table 2.3). Sperm proteins were stripped from the sperm with a high ionic solution.

CHAPTER 3

RELATIONSHIP OF FIELD AND *IN VITRO* FERTILITY OF DAIRY BULLS WITH SPERM PARAMETERS, INCLUDING, DAG1 AND SERPINA5 PROTEINS

ABSTRACT

Even among bulls that successfully pass a breeding soundness exam; there are differences in fertility, but for any potential marker of fertility there must be variability expressed among animals. Thus, the first objective of these studies was to characterize dystroglycan (DAG1) and plasma serine protease inhibitor (SERPINA5) proteins localization and variability on bovine sperm, and the second objective was to investigate the relationship of DAG1 and SERPINA5 with field fertility (sire conception rate; SCR), *in vitro* fertility (*in vitro* embryo production), and sperm parameters. Dairy bulls (n = 22) were evaluated for DAG1 and SERPINA5 immunolocalization, and fluorescence intensity in two separate ejaculates. The GLM procedure in SAS was used with bull as a fixed effect to determine if variance was greater between bulls compared to within bull. Correlations were determined among DAG1 and SERPINA5 concentrations, percentage of tail labeled for SERPINA5, SCR, sperm total motility, progressive motility, and plasma membrane integrity (viability), and *in vitro* embryo produced cleavage (CL) and blastocyst (BL) rates. Both SERPINA5 and DAG1 were localized on the sperm head; however, SERPINA5 was also localized on the sperm tail. There was greater variance in concentration among bulls compared to within bull for both DAG1 ($P < 0.0001$; 69.4 vs 49.1, respectively) and SERPINA5 ($P < 0.0001$; 325.8 vs 285.4, respectively). There was

a positive correlation between concentration of DAG1 and SERPINA5 ($P = 0.01$; r = 0.54). Concentrations of SERPINA5 were also correlated with CL ($P = 0.04$; r = 0.48), and percentage of sperm tail labeled for SERPINA5 was correlated with viability ($P = 0.05$; r = 0.44) and tended to be correlated with CL ($P = 0.10$; r = 0.39). There was no relationship between SCR or BL rate classifications and DAG1 ($P \geq 0.66$), SERPINA5 ($P \geq 0.54$), or percentage of sperm tail labeled for SERPINA5 ($P \geq 0.48$). In conclusion, DAG1 and SERPINA5 were localized to the sperm head, and tail (SERPINA5). Concentrations of DAG1 and SERPINA5 on the sperm head were correlated with each other and SERPINA5 was correlated with CL. The percentage of tail labeled for SERPINA5 was correlated with sperm viability; however, neither protein was associated with SCR.

INTRODUCTION

After differentiation, sperm lose the ability to growth, divide, repair and synthesize proteins, and have limited metabolic function (Hammerstedt, 1993). After spermiation, sperm travel through testis tubules, epididymis (further maturation occurs) and are stored in the epididymis tail in a quiescent state until ejaculation (Acott and Carr, 1984; Carr and Acott, 1984; Barth and Oko, 1989). Upon ejaculation, epididymal sperm are diluted with seminal plasma from accessory sex glands and motility is initiated (Acott and Carr, 1984; Carr and Acott, 1984). Sperm with fertilizing ability reach the oviduct approximately 6-12 h after insemination, populate the isthmus portion of the oviduct and form the sperm reservoir (Hunter and Wilmut, 1984; Wilmut and Hunter, 1984; Lefebvre

et al., 1995). Sperm that bind to oviductal cells have prolonged motility and fertilization ability (~30 h) compared to sperm free in the media (Pollard et al., 1991).

Cell to cell interactions (i.e. sperm to oviduct and sperm to oocyte) are mediated through proteins; thus, these interactions are important for successful fertilization. The sperm's apical surface binds to oviductal isthmus and ampullary ciliated cells (Pollard et al., 1991; Lefebvre et al., 1995) and Binder of Sperm Proteins (BSP) has been reported to be involved with sperm reservoir formation (Ignotz et al., 2001; Gwathmey et al., 2003; 2006). There are few proteins known to be required for fertilization, and include CD9 (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000) and JUNO (Bianchi et al., 2014) on the egg, and IZUMO1 on the sperm (Inoue et al., 2005). Other proteins have been identified to be associated with mammalian fertility, but not required (see review by Sutovsky, 2009).

In chapter 2, it was identified that DAG1 and SERPINA5 were present and loosely attached to ejaculated sperm, but they were not present on epididymal sperm; however, SERPINA5 was present in both epididymal fluid and seminal plasma (increased abundance in seminal plasma) while DAG1 was only present in seminal plasma. The gene DAG1 encodes the dystroglycan precursor that generates two proteins alpha- and beta-dystroglycan through post-translational modification. Alpha-dystroglycan is an extracellular/surface protein and beta-dystroglycan is a transmembrane protein (Ibraghimov-Beskrovnaya et al., 1992). The presence of DAG1 has been reported in seminal plasma but not on human sperm (Jodar et al., 2016). Beta-dystroglycan has been reported to be localized to the tail middle piece of guinea pig sperm (Hernández-González et al., 2001) and the post-acrosomal region and middle piece of mouse sperm

(Hernández-González et al., 2005). The gene SERPINA5 encodes the plasma serine protease inhibitor. This protein is also known as serpin family A member 5, protein C inhibitor, and others. The presence of SERPINA5 protein has been reported in many body fluids, including plasma (blood), seminal plasma, follicular fluid, amniotic fluid, milk, and others (Laurell et al., 1992). In double knockout mice for SERPINA5, females were fertile and males were infertile in both *in vitro* (0.5% pregnancy) and *in vivo* (0% pregnancy) experiments. Also, sperm motility (12.5% motility) and the percentage of morphologically normal sperm (5% normal morphology) were decreased in double knockout mice (Uhrin et al., 2000). Similarly, SERPINA5 concentrations were decreased in normozoospermic infertile men compared to normozoospermic fertile men (Panner Selvam et al., 2019). Nevertheless, in men, SERPINA5 has been localized to the sperm head (Zheng et al., 1994; Elisen et al., 1998). Thus, the first objective of these studies was to characterize DAG1 and SERPINA5 immunolocalization on bovine sperm and their potential as fertility markers by evaluating variability within and amongst bulls. The second objective was to investigate the relationship of DAG1 and SERPINA5 with field fertility (sire conception rate; SCR), *in vitro* fertility (*in vitro* embryo production), and sperm parameters.

MATERIAL AND METHODS

Experimental design:

Study I:

Semen from bulls of different beef breeds (n = 17) of unknown fertility were evaluated to assess the presence, localization, fluorescence intensity (FI; relative concentration), and animal variability of SERPINA5 protein on the sperm. Sperm were also analyzed for sperm total (TMOT) and progressive (PROG) motility with a computer-assisted sperm analysis system (CASA; IVOS II; Hamilton Thorne, Beverly, MA, USA).

Study II:

Dairy bulls (n = 22) with different SCR values, ranging from -7.7 to 4.45, were classified as High (High-SCR > 1.0; n = 11) or Low (Low-SCR < -4.0; n =11) field fertility (Table 3.1). Semen from two ejaculates (140 ± 278 d between ejaculates) were used to assess sperm relative concentrations of DAG1 and SERPINA5, TMOT, PROG, and plasma membrane integrity (viability; n = 20; semen of two bulls had already been processed before viability could be assessed), and *in vitro* production of embryos (n = 19; one High-SCR and two Low-SCR bulls' semen were not available for *in vitro* production of embryos); also, Low-SCR bulls were subdivided further based on their blastocyst rate (BL) as High (Low-SCR/High-BL ≥ 31%; n = 6) or Low (Low-SCR/Low-BL ≤ 26%; n = 3; Table 3.1).

Sperm Motility and Viability Analyses:

Sperm motility analyses were performed using a CASA. Briefly, an aliquot of frozen-thawed semen was diluted in easy buffer B (IMV technologies, France) and incubated with Hoechst 33342 (final concentration 40 or 80 µg/mL, milk- or egg-yolk-based extender, respectively) at 37 °C for 10 min. After incubation, samples were loaded on a Leja slide and evaluated for sperm TMOT and PROG. Sperm plasma membrane integrity was performed by the addition of 2 µL of propidium iodide to CASA samples (after CASA analysis), and incubated for 5 min. One hundred sperm per sample in a minimum of five fields of view avoiding the edge of the slide were evaluated in a Nikon Fluorescence microscope.

Sperm Protein Analyses:

Sperm fixation procedure:

Frozen-thawed semen samples, not used for CASA analysis, were fixed in 2% formaldehyde solution [10% formaldehyde (EM grade) diluted with phosphate buffer saline (PBS)] at room temperature for 40 min (100 µL of 2% formaldehyde solution per ~450 µL of extended semen). Following incubation, samples were washed by centrifugation twice at 500 × g for 5 min, supernatant was removed with a glass Pasteur pipette and sperm pellet resuspended with PBS. Samples were diluted to 5 million sperm per mL and stored at 4 ºC until analyzed for DAG1 or SERPINA5.

Sperm DAG1 analysis:

Anti-DAG1 antibody (goat anti-human, ab136665, polyclonal, ABCAM, United Kingdom) was purified using the 10kD Spin Column (ab93349, ABCAM). Briefly, 135 μL of anti-DAG1 was diluted with 300 μL of PBS, added to 10kD Spin Column and centrifuged at 10,000 × g for 10 min. Purified antibody was resuspended in PBS to 135 μL final volume. Anti-DAG1 was conjugated to PE/R-Phycoerythrin (ab102918, ABCAM) according to manufacturer instructions and diluted with PBS to a final concentration of 0.05 μg/μL. Anti-DAG1 (5 μL) and fixed sperm (100 μL at 5×10^6 sperm per mL) were incubated in a 0.5 mL tube for 4 h at room temperature without exposure to light. After incubation, antibody reaction was stopped by the addition of 100 μL of 2% formaldehyde solution and incubated for 40 min without exposure to light. Samples were centrifuged at 700 × g for 10 min, supernatant was removed, pellet was resuspended with PBS (200 μL) and centrifuged. After second centrifugation, supernatant was removed and approximately 20 μL of fluid were remaining and 5 μL of ProLong Diamond Antifade Mountant (P36965, Thermo Fisher Scientific, Waltham, MA) was added. Samples were evaluated with a Nikon Fluorescence microscope at 400 × magnification, and the NIS-Elements software package was used to outline 100 individual spermatozoa per sample and FI was determined. Also, immunolocalization of DAG1 on the sperm was determined.

Sperm SERPINA5 analysis:

Anti-SERPINA5 antibody (rabbit anti-human, mouse, rat, PA579976, polyclonal, Invitrogen, Waltham, MA) was conjugated to Dylight 405 Fast (ab201798, ABCAM) according to manufacturer instructions and diluted with PBS to a final concentration 0.1 µg/µL. Anti-SERPINA5 (5 µL) and fixed sperm (100 µL at 5×10^6 sperm per mL) were incubated in a 0.5 mL tube for 4 h at room temperature without exposure to light. Samples were evaluated as described for DAG1. Also, immunolocalization of SERPINA5 on the sperm was determined.

In vitro embryo production:

All media for *in vitro* embryo production and *in vitro* embryo production followed previous published procedures (Ortega et al., 2016; 2018; Tríbulo et al., 2019; Stoecklein et al., 2021). Briefly, cumulus-oocyte complexes (COC) were retrieved by follicular aspiration from ovaries collected at a commercial abattoir. Cumulus-oocyte complexes with at least three layers of compact cumulus cells and homogeneous cytoplasm were placed in groups of approximately 50 COC into 2 mL glass sterile vials containing 1 mL of oocyte maturation medium equilibrated with air containing 5% (v/v) CO_2 covered with mineral oil. Tubes with COC were shipped overnight in a portable incubator (Minitube USA Inc., Verona, WI, USA) at 38.5 ºC to the University of Missouri. After approximately 24 h of maturation, groups of 100 COC were washed three times in HEPES-Tyrode's albumen lactate pyruvate (TALP) medium and placed in a 35-mm dish containing 1.7 mL of fertilization media (IVF-TALP). Each group of COC was fertilized

with sperm from a single bull. Sperm were purified from frozen-thawed straws using a gradient of Isolate [50% (v/v) and 90% (v/v); Irvine Scientific, Santa Ana, CA], washed two times by centrifugation at 100 × g using sperm-TALP and diluted in IVF-TALP to achieve a final concentration of 1×10^6 sperm per mL in the fertilization dish. To improve sperm motility and promote fertilization 80 μL of penicillamine-hypotaurine-epinephrine solution was added to each fertilization dish. Fertilization proceeded for approximately 18 h at 38.5 ºC in a humidified atmosphere of 5% (v/v) CO_2. Putative zygotes (oocytes exposed to sperm) were vortexed for 5 min in 400 μL of HEPES-TALP to denude from the surrounding cumulus cells at the end of fertilization. Embryos were then cultured in four-well dishes in groups of up to 50 embryos in 500 μL of culture medium (SOF-BE2) covered with 300 μL of mineral oil per well at 38.5 ºC in a humidified atmosphere of 5% (v/v) O_2 and 5% (v/v) CO_2. Percentage of putative zygotes that cleaved (cleavage rate; CL) was determined at day 3 of development (day 0 = day of insemination) and BL at day 8 of development.

Statistical Analysis:

Fluorescence intensity (concentration of SERPINA5 and DAG1) was analyzed using the GLM procedure in SAS (9.4) with bull as a fixed effect to determine the variance in mean protein FI between bull and within bull. Protein immunolocalization was determined based on visual characterization and statistical analysis was not performed. The CORR procedure of SAS was used to evaluate correlations (study II) between SCR, TMOT, PROG, viability, CL, BL, DAG1 and SERPINA5 relative concentration, and proportion of sperm tail labeled for SERPINA5. The GLIMMIX

procedure of SAS was used to evaluate the relationship of bull field fertility (High- and Low-SCR), and field and *in vitro* fertility (High-SCR, Low-SCR/High-BL, Low-SCR/Low-BL) classifications with sperm TMOT, PROG, viability, CL, BL, DAG1 and SERPINA5 relative concentration, and proportion of sperm tail labeled for SERPINA5. Results are presented as least square mean ± SE unless otherwise stated. Level of significance was $\alpha \leq 0.05$, when $P > 0.05$ but $P \leq 0.10$ the results were considered as tendency.

RESULTS

Study I:

Sperm TMOT and PROG for the bulls used was 34.6 ± 13.5% and 19.4 ± 9.5% (mean ± SD), respectively (total motility ranged from 14.1 to 58.9% and PROG ranged from 9.0 to 37.5%). Immunolocalization determined that SERPINA5 protein was present both on the sperm head (Fig. 3.1) and tail (Fig. 3.1). On the sperm head, the most characteristic pattern of SERPINA5 was covering the proximal region over the acrosomal cap (Fig. 3.1). There was 33.5 ± 17.0% (mean ± SD) of sperm tails that were also positive for SERPINA5 (range 5 to 62%; Fig. 3.2). Relative concentrations of SERPINA5 on the sperm head ranged from 12.9 ± 0.4 to 19.0 ± 0.4 au (Fig. 3.3) and averaged 16.0 ± 3.9 au (mean ± SD). Overall, there was a greater variance among bulls compared to within bull ($P < 0.0001$; 15.1 vs 13.0, respectively).

Study II:

Immunolocalization of SERPINA5 was similar to that reported in study I (Fig. 3.1). There was 34.2 ± 12.7% (mean ± SD) of sperm tails in dairy bulls that were positive for SERPINA5 (ranged from 6.0 to 57.4%; Fig. 3.4). Relative concentration of SERPINA5 on the sperm head ranged from 38.9 ± 1.1 to 68.4 ± 1.1 au (Fig. 3.5) with average 53.2 ± 6.6 (mean ± SD). There was greater variance among bulls compared to within bull ($P < 0.0001$; 325.8 vs 285.4, respectively).

Immunolocalization determined that DAG1 was present on the sperm head in the proximal apical region, over the acrosomal cap (Fig. 3.6). Relative concentrations of DAG1 on the sperm head ranged from 29.6 ± 0.5 to 45.7 ± 0.5 au (Fig. 3.7) and averaged 36.0 ± 4.6 au (mean ± SD). There was greater variation among bulls compared to within bull ($P < 0.0001$; 69.4 vs 49.1, respectively).

There were positive correlations between TMOT and PROG ($P < 0.01$; r = 0.82; Table 3.2, Fig. 3.8), viability and percentage of sperm tail labeled for SERPINA5 (P = 0.05; r = 0.44; Table 1, Fig. 3.9), SERPINA5 and CL ($P = 0.04$; r = 0.48; Table 3.2, Fig. 3.10) and DAG1 ($P = 0.01$; r = 0.54; Table 3.2, Fig. 3.11), percentage of sperm tail labeled for SERPINA5 and CL ($P = 0.10$; r = 0.39; Table 3.2, Fig. 3.12), and between CL and BL ($P = 0.03$; r = 0.50; Table 3.2, Fig. 3.13).

There was no difference ($P \geq 0.54$) between High- and Low-SCR bulls for TMOT, PROG, CL, BL, SERPINA5, DAG1, and percentage of sperm tail labeled for SERPINA5 (Table 3.3); however, High-SCR tended ($P = 0.06$) to have greater sperm viability compared to Low-SCR (Table 3.3). When Low-SCR bulls were sub-divided into

High- and Low-BL there was no difference (P ≥ 0.32) between High-SCR, Low-SCR/High-BL and Low-SCR/Low-BL for TMOT, PROG, viability, CL, SERPINA5, DAG1, and percentage of sperm tail labeled with anti-SERPINA5 (Table 3.4). There was a difference in BL ($P = 0.02$) when bulls were classified based on field and *in vitro* fertility; High-SCR and Low-SCR/High-BL had greater BL ($P \leq 0.01$) compared to Low-SCR/Low-BL; however, High-SCR and Low-SCR/High-BL were not different ($P = 0.37$; Table 3.4). There was a difference in mean SCR when bulls were classified by field and *in vitro* fertility ($P \leq 0.0001$); High-SCR was greater than both Low-SCR groups ($P \leq 0.0001$) as designed; interestingly, Low-SCR/Low-BL tended ($P = 0.08$) to have greater SCR compared to Low-SCR/High-BL.

DISCUSSION

Rete of genetic improvement in a herd is far more efficient through bull selection than female selection due to the larger number of offspring generated by one single bull versus one single female. This is especially true in dairy cattle, in which 90% of females are artificially inseminated (AI; Starbuck et al., 2004; Valour et al., 2015; García-Ruiz et al., 2016; Wiggans et al., 2017; USDA, 2018). Bull fertility, especially for use in AI, have been evaluated heavily or exclusively through semen quality which relies predominantly on sperm motility and morphology, and more recently sperm viability (Barth and Oko, 1989; Koziol and Armstrong, 2018; DeJarnette et al., 2021). Sire conception rate is one of the most common methods of evaluation for bull field fertility. The SCR value given to a bull is generated based on field reports of pregnancies, SCR

value represents the bull's deviation in pregnancy rates at d 70 of gestation compared to the mean pregnancy rates from all other bulls that could have been used (Kuhn et al., 2006; Norman et al., 2011). Interestingly, it was observed that some Low-SCR bulls had good BL production with no difference from High-SCR bulls; Ortega et al. (2018) reported similar findings in which one (out of three) Low-SCR bull had BL similar to High-SCR bulls. Interestingly, Low-SCR/High-BL had decreased mean SCR compared to Low-SCR/Low-BL. Sperm must endure far less challenge to fertilize an embryo *in vitro* compared to *in vivo*. *In vivo,* sperm must navigate the female reproductive tract, survive uterine contraction, overcome the utero-tubular junction, form the sperm reservoir, capacitate, "find" the ovulated oocyte to then fertilize that single oocyte (Suarez, 2015, 2016). Additionally, AI may happen in different moments during estrus/pro-estrus which sperm must survive for prolonged periods of time or capacitate "quicker", both having an effect on fertilization rate and embryo quality (Saacke et al., 2000; Dalton et al., 2001; Richardson et al., 2017). On the contrary, *in vitro*, sperm must tolerate manipulation insults such as centrifugation (Baldi et al., 2020); however, females' barriers (except those from the oocyte) are eliminated. Thus, it is possible that bulls with Low-SCR, but good BL, have sperm transport problems or are more susceptible to the timing of insemination (sperm longevity) or the uterine/oviduct environment compared to Low-SCR bulls with lower BL which the problem may be related to fertilization itself rather than sperm transport; this hypothesis is partially explained by the "compensable" and "uncompensable" characteristics of sperm previously reported (Saacke et al., 1994; Saacke, 2008; Amann et al., 2018).

The objective of the bovine AI industry is to provide semen of high quality to cattle producers; semen that passes quality control and are commercially available have met specific thresholds (Harstine et al., 2018; DeJarnette et al., 2021). With that, sperm motility, morphology and viability of commercially available semen are expected to not correlate with field fertility, especially in large samples (DeJarnette et al., 2021). Besides the industry effort to eliminate sperm parameters correlation/association with fertility, in the present study, High-SCR bulls tended to have greater viability compared to Low-SCR bulls.

Animal variation is necessary for a test to be considered as a potential fertility marker. Also, any new test must not be correlated with current evaluations of semen quality or must provide a simpler method of evaluation over current analyses (DeJarnette, 2005; Harstine et al., 2018; DeJarnette et al., 2021). In the present study, a greater variation amongst bulls compared to within bull was observed for both DAG1 and SERPINA5, fulfilling the first characteristics for a potential fertility marker. Further, DAG1 and SERPINA5 were not correlated with TMOT, PROG, or viability, fulfilling the second characteristic of a potential fertility marker; however, percentage of tail labeled for SERPINA5 was correlated with viability.

Sperm interact with the utero-tubular junction (UTJ), oviduct (formation of sperm reservoir and movement through the oviduct), and oocyte through proteins (Lefebvre et al., 1995; Gwathmey et al., 2003; 2006; Ignotz et al., 2007; Sutovsky, 2009; Suarez, 2015, 2016). The formation of the sperm reservoir in bovine involves BSP (Ignotz et al., 2001; Gwathmey et al., 2003; 2006), these groups of proteins are attached to the sperm during ejaculation when epididymal sperm come into contact with seminal plasma

(Desnoyers and Manjunath, 1992; Müller et al., 1998; Nauc and Manjunath, 2000); similarly, as observed in Chapter 2, DAG1 and SERPINA5 are attached to the sperm during ejaculation, since DAG1 and SERPINA5 were not detected in epididymal sperm samples. Liquid chromatography with tandem mass spectrometry analysis (LCMS/MS) results (Chapter 2), demonstrated that DAG1 was lowly abundant (spectra count 1.1) while SERPINA5 was highly abundant (spectra count 37.3) on the sperm. The present results (Fig. 3.1, 3.5, 3.6, and 3.7) agree with LCMS/MS findings which SERPINA5 is present in greater abundance on the sperm compared to DAG1; interestingly, DAG1 and SERPINA5 concentrations were correlated (Table 3.2; Fig. 3.11). The localization of both, DAG1 and SERPINA5, on the sperm head is interesting and consistent with the region of the sperm that attaches to oviductal epithelial cells to form the sperm reservoir (Lefebvre et al., 1995). The function of DAG1 on the sperm is not fully understood, especially alpha-dystroglycan which is more likely than beta-dystroglycan to have been measured due to the fact that alpha-dystroglycan is an extracellular/surface protein (Ibraghimov-Beskrovnaya et al., 1992). Beta-dystroglycan, a transmembrane protein (Ibraghimov-Beskrovnaya et al., 1992), has been previously reported on the tail middle piece of guinea pig sperm (Hernández-González et al., 2001) and the middle piece and acrosomal region of mice sperm (Hernández-González et al., 2005). Hernández-González et al. (2005) demonstrated that mice sperm with beta-dystroglycan deficiency had increased morphological abnormalities in the sperm tail, and the number of sperm capable of fertilization was decreased (~50% less) compared to sperm from wild-type mice. In the present study, it was identified that DAG1 was not associated with field fertility or field and *in vitro* embryo fertility which High-SCR and Low-SCR or High-

SCR, Low-SCR/High-BL and Low-SCR/Low-BL were not different, respectively. Additionally, DAG1 concentration between SCR groups was almost identical (Tables 3.2 and 3.3). Furthermore, DAG1 was not correlated with SCR, CL, or BL. Thus, DAG1 may function to stabilize the acrosomal region as a decapacitating factor, preventing premature acrosomal reaction or formation of the sperm reservoir due to its localization on the sperm (Fig. 3.6).

Abundance of SERPINA5 in the seminal plasma and loosely attached to the sperm ranked 13th and 11th based on spectra count, respectively (Chapter 2), which agree with previous reports for SERPINA5 in seminal plasma (Pisanu et al., 2012). Reference for immunolocalization of SERPINA5 in bovine or other livestock species could not be found. Thus, within human sperm, SERPINA5 was characterized covering the acrosomal region of epididymal and ejaculated sperm (Zheng et al., 1994; Elisen et al., 1998). There was no difference in SERPINA5 localization between capacitated and non-capacitated sperm; however, when acrosome reaction was induced, SERPINA5 was limited to the equatorial region (Zheng et al., 1994; Elisen et al., 1998). The immunolocalization of SERPINA5 on the bovine sperm head (Fig. 3.1) was similar to human sperm (Zheng et al., 1994; Elisen et al., 1998); however, bovine sperm also had SERPINA5 on the sperm tail diverging from human sperm.

The protease inhibitory activity of SERPINA5 has been described in multiple body tissues and fluids (España et al., 1989; Ecke et al., 1992; Christensson and Lilja, 1994; Hermans et al., 1994; Zheng et al., 1994; Elisen et al., 1998). The activity and target enzyme of SERPINA5 can be modulated by heparin and other glycosaminoglycans (Kuhn et al., 1990; Pratt and Church, 1992; Ecke et al., 1997). Heparin and

glycosaminoglycans are present in the oviduct from oviductal fluid and follicular fluid which has been shown to induce sperm capacitation (Parrish et al., 1985; 1988; Mahmoud and Parrish, 1996; Bergqvist et al., 2007). A positive correlation was observed between SERPINA5 concentration on the sperm head and CL, also, the percentage of sperm tail labeled for SERPINA5 was correlated with sperm viability and CL. When the SERPINA5 gene was disrupted in mice, male mice were infertile both *in vitro* and *in vivo* because of morphologically abnormal sperm, lower motility, and lack of sperm-egg binding (Uhrin et al., 2000). Also, normozoospermic men with unknown reason for infertility had decreased concentration of SERPINA5 compared to fertile counterparts (Panner Selvam et al., 2019). Controversially, there was no association of SERPINA5 concentration or percentage of tail labeled for SERPINA5 with field fertility or field and *in vitro* embryo fertility.

When sperm was processed for *in vitro* fertilization, the processing may have accelerated sperm capacitation and increased damage to the sperm (Baldi et al., 2020). The ability of human sperm to bind to human zona pellucida was evaluated in the presence of different concentrations of anti-SERPINA5 or SERPINA5 in the media (Elisen et al., 1998). Interestingly, a lower concentration of anti-SERPINA5 increased the ability of sperm to bind to the zona pellucida; however, the greater the concentration of SERPINA5 in the media the lower the ability of sperm to bind to the zona pellucida (Elisen et al., 1998). Another member of the serine protease inhibitor (SERPIN) family, called glia-derived nexin or protease nexin-1 (SERPINE2), has been reported to be a decapacitating factor in mice (Lu et al., 2011). Thus, it is possible to hypothesize that increased concentrations of SERPINA5 may have provided enough protection to the

sperm; and bulls with greater concentration of SERPINA5 on the sperm head, and percentage of tail labeled, had increased CL likely due to resistance to sperm processing (protection against premature capacitation). More investigation is necessary to understand whether SERPINA5 or DAG1 could be used as a fertility marker.

In conclusion, DAG1 and SERPINA5 proteins that are associated with cell-to-cell interactions were localized on the bovine sperm head, also, SERPINA5 was localized on the sperm tail. Sperm relative concentration for both proteins were correlated to each other and SERPINA5 was correlated with CL. The percentage of sperm tail labeled for SERPINA5 was correlated with CL and sperm viability; however, proteins were not associated with bull field fertility measured by SCR. Thus, SERPINA5 may be related with sperm protection and/or oocyte fertilization while DAG1 may be related to sperm transport or formation of the sperm reservoir in the oviduct.

Table 3.1. Description of sire conception rate (SCR), blastocyst rate (BL), field fertility classification based on SCR value (High or Low), and field and *in vitro* classification based on SCR and BL (High-SCR, Low-SCR/High-BL, Low-SCR/Low-BL) per bull.

Bull	**SCR**	**BL**	**Field fertility, SCR**	**Field and *in vitro*, SCR-BL**
A	4.1	25%	High	High
B	2.8	28%	High	High
C	-5.4	-	Low	-
D	4.2	44%	High	High
E	3.0	-	High	-
G	-6.1	31%	Low	Low-High
H	4.5	36%	High	High
I	3.9	37%	High	High
J	-6.4	36%	Low	Low-High
K	3.2	38%	High	High
L	-4.7	26%	Low	Low-Low
M	-4.3	24%	Low	Low-Low
N	-6.2	-	Low	-
O	-7.7	39%	Low	Low-High
P	1.1	22%	High	High
Q	4.1	41%	High	High
R	2.8	32%	High	High
S	-5.5	21%	Low	Low-Low
T	-6.7	39%	Low	Low-High
U	-6.0	33%	Low	Low-High
V	-4.2	37%	Low	Low-High
X	4.4	33%	High	High

Table 3.2. Pearson's correlation coefficient (shaded area above diagonal) and significance level (below diagonal) between sire conception rate (SCR), total motility (TMOT), progressive motility (PROG), sperm plasma membrane integrity (viability), SERPINA5 mean relative concentration (SERPINA5), percentage of sperm tail positive for SERPINA5 (SERPINA5 Tail), *in vitro* produced embryos cleavage (CL) and blastocyst (BL) rate, and DAG1 mean relative concentration (DAG1).

Correlation/ P-value	**SCR**	**TMOT**	**PROG**	**Viability**	**SERPINA5**	**SERPINA5 Tail**	**CL**	**BL**	**DAG1**
SCR		0.09	0.01	0.36	-0.13	-0.19	-0.08	0.15	-0.08
TMOT	0.69		**0.82**	0.00	0.14	0.15	0.17	0.34	-0.25
PROG	0.95	< **0.01**		0.06	0.15	-0.07	-0.04	0.22	-0.26
Viability	0.12	0.99	0.79		0.11	**0.44**	0.24	0.15	-0.10
SERPINA5	0.56	0.53	0.50	0.65		0.28	**0.48**	0.11	**0.54**
SERPINA5 Tail	0.39	0.52	0.74	**0.05**	0.21		**0.39**	0.20	0.05
CL	0.73	0.49	0.88	0.35	**0.04**	**0.10**		**0.50**	0.33
BL	0.55	0.15	0.38	0.56	0.66	0.42	**0.03**		0.32
DAG1	0.72	0.25	0.25	0.66	**0.01**	0.81	0.17	0.18	

Table 3.3. Relationship of sire conception rate (SCR) fertility classification (High-SCR vs Low-SCR) on total motility (TMOT), progressive motility (PROG), sperm plasma membrane integrity (viability), *in vitro* produced embryos cleavage (CL) and blastocyst (BL) rate, SERPINA5 mean relative concentration (SERPINA5), percentage of sperm tail positive for SERPINA5 (SERPINA5 Tail), and DAG1 mean relative concentration (DAG1).

Variable	Fertility		SEM[1]	P-value
	High	Low		
SCR, au[2]	3.4	-5.7	0.31	< 0.0001
TMOT, %	52.0	51.3	2.89	0.86
PROG, %	35.7	35.8	2.61	0.99
Viability, %	64.0	57.3	2.39	0.06
CL, %	77.4	78.3	2.39	0.81
BL, %	33.5	31.7	2.18	0.56
SERPINA5, au[2]	52.4	54.2	2.04	0.54
SERPINA5 Tail, %	32.4	35.1	3.23	0.56
DAG1, au[2]	35.6	36.5	1.41	0.66

[1] SEM = Standard error of the mean

[2] au = arbitrary unit

Table 3.4. Relationship of field (sire conception rate; SCR) and *in vitro* (blastocyst rate; BL) fertility classification (High-SCR, Low-SCR/High-BL, and Low-SCR/Low-BL) on total motility (TMOT), progressive motility (PROG), sperm plasma membrane integrity (viability), *in vitro* produced embryos cleavage rate (CL) and BL, SERPINA5 mean relative concentration (SERPINA5), percentage of sperm tail positive for SERPINA5 (SERPINA5 Tail), and DAG1 mean relative concentration (DAG1).

Variable	**Fertility**			**SEM[1]**	**P-value**
	High-SCR	**Low-SCR/ High-BL**	**Low-SCR/ Low-BL**		
SCR, au[2]	3.4[a]	-6.2[b¶]	-4.8[b*]	0.59	< 0.0001
TMOT, %	52.0	49.3	50.0	4.86	0.81
PROG, %	35.6	34.3	32.8	4.38	0.84
Viability, %	64.0	58.8	60.3	3.79	0.32
CL, %	77.5	80.3	73.9	4.23	0.43
BL, %	33.4[a]	35.9[a]	23.9[b]	2.73	0.02
SERPINA5, au[2]	52.4	52.7	56.0	4.16	0.75
SERPINA5 Tail, %	32.6	38.5	32.7	5.60	0.48
DAG1, au[2]	35.6	36.4	36.7	2.60	0.91

[1] SEM = Standard error of the mean

[2] au = arbitrary unit

[a-b] Values within the same row not sharing a common superscript differ $P \leq 0.01$

[*,¶] Values within the same row not sharing a common superscript differ $P \leq 0.08$

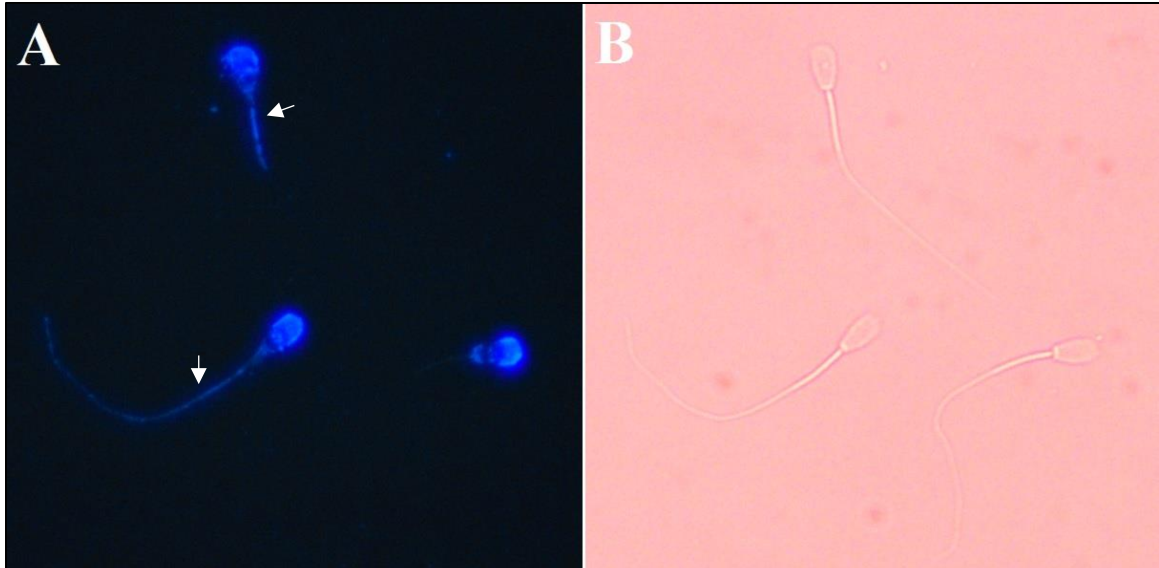


Figure 3.1. Representative picture of bovine sperm labeled with anti-SERPINA5 (PA579976, Invitrogen; conjugated to Dylight 405 Fast, ab201798, ABCAM) on the sperm head and sperm tail (A), and bright field of A (B). White arrows indicate sperm tail positive for SERPINA5. 400 × magnification.

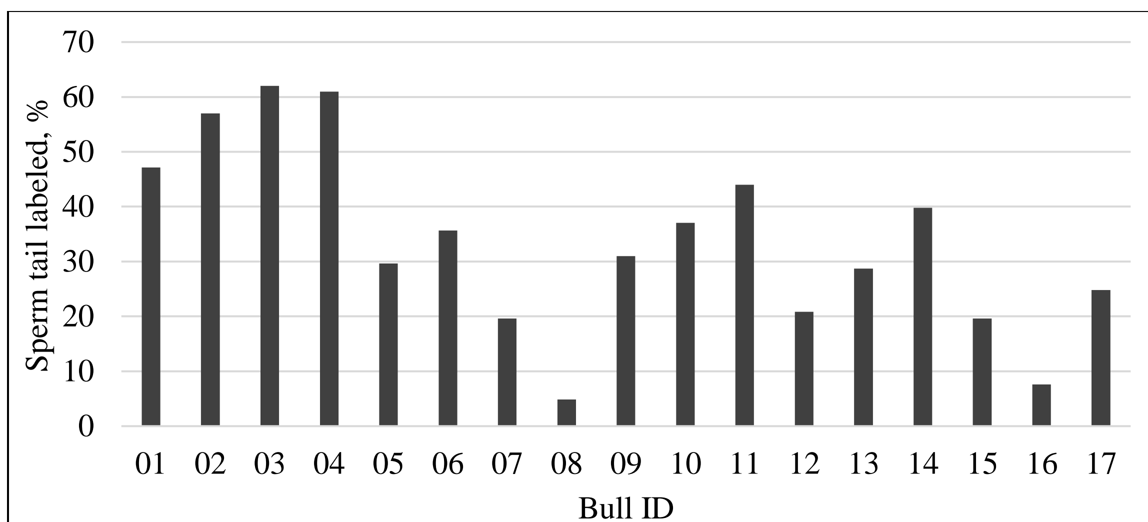


Figure 3.2. Percentage of sperm tail labeled with anti-SERPINA5 (PA579976, Invitrogen; conjugated to Dylight 405 Fast, ab201798, ABCAM) in beef bulls (Study I).

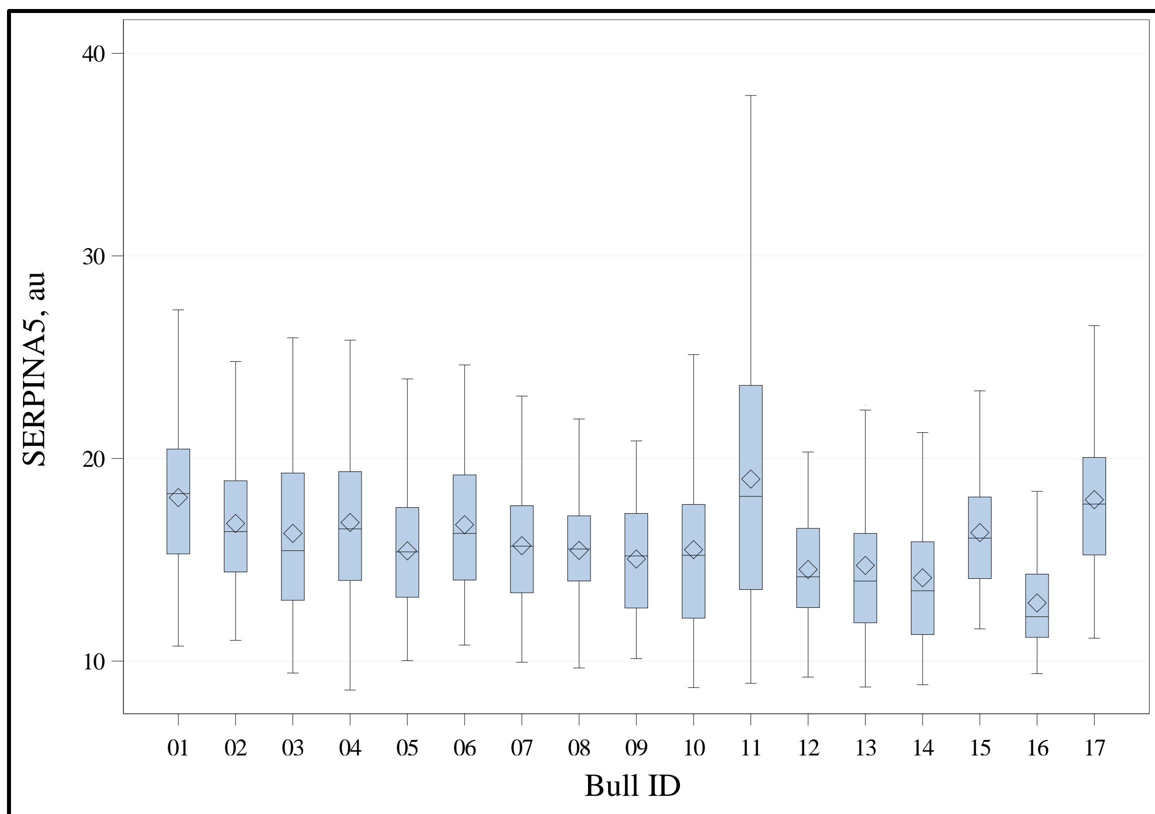


Figure 3.3. Distribution of SERPINA5 fluorescence intensity (arbitrary unit; au; PA579976, Invitrogen; conjugated to Dylight 405 Fast, ab201798, ABCAM) on sperm head of bulls. Line within box represents median and diamond shape represents mean (Study I).

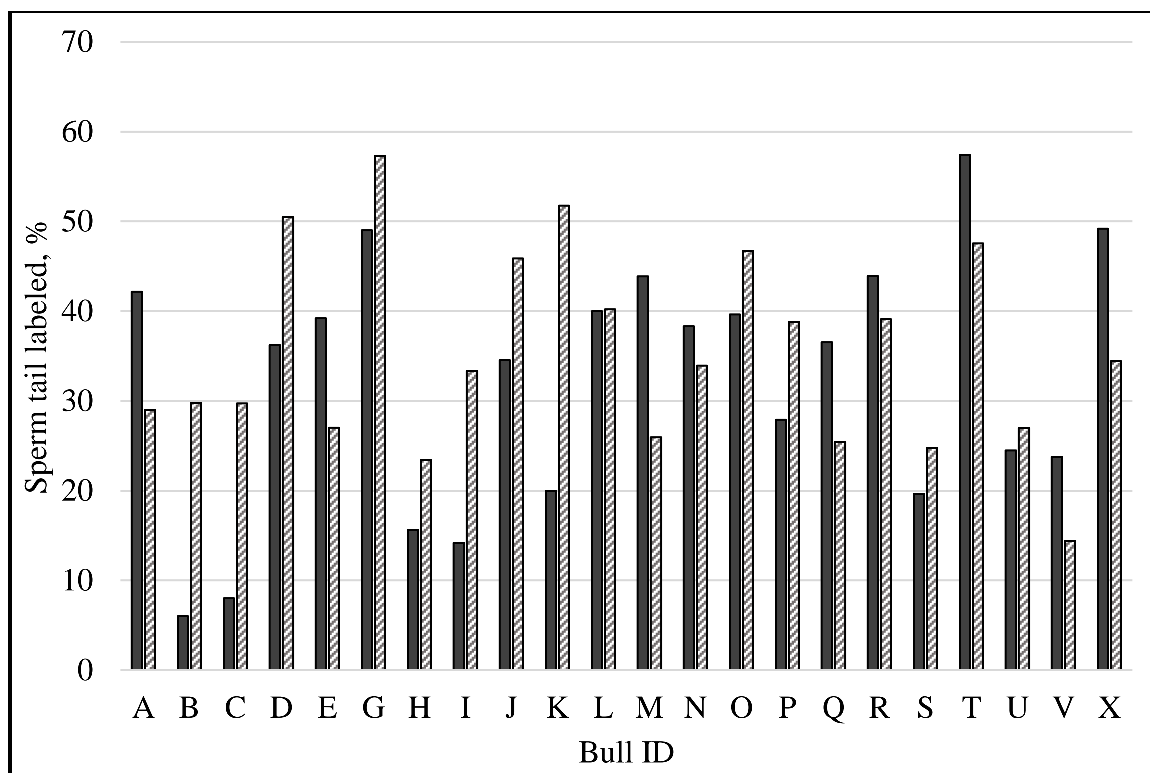


Figure 3.4. Percentage of sperm tail labeled with anti-SERPINA5 (PA579976, Invitrogen; conjugated to Dylight 405 Fast, ab201798, ABCAM) per ejaculate of dairy bulls (Study II). Solid bars represent ejaculate one and dashed bars represent ejaculate two of the same animal (140 ± 278 d between ejaculates; mean ± SD).

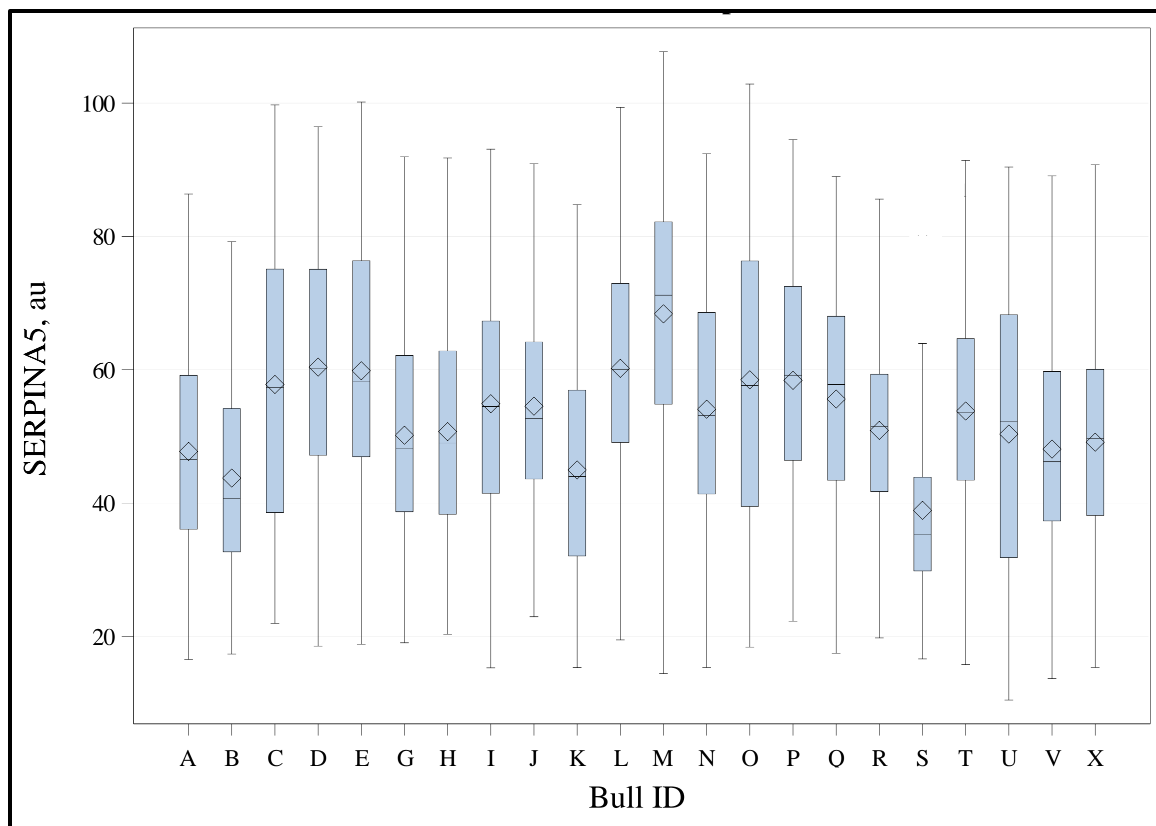


Figure 3.5. Distribution of SERPINA5 fluorescence intensity (arbitrary unit; au; PA579976, Invitrogen; conjugated to Dylight 405 Fast, ab201798, ABCAM) on sperm head of bulls. Line within box represents median and diamond shape represents mean (Study II).

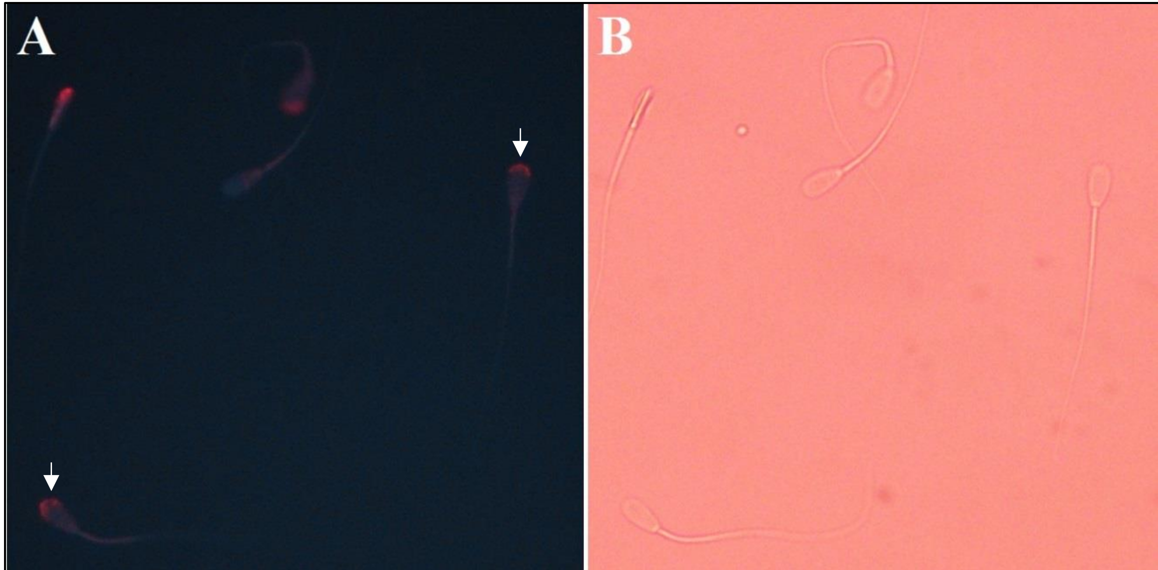


Figure 3.6. Representative picture of bovine sperm labeled with anti-DAG1 (ab136665, ABCAM; conjugated to PE/R-Phycoerythrin, ab102918, ABCAM) on the sperm head (A), and bright field of A (B). White arrows indicate sperm (on focus) positive for DAG1. 400 × magnification.

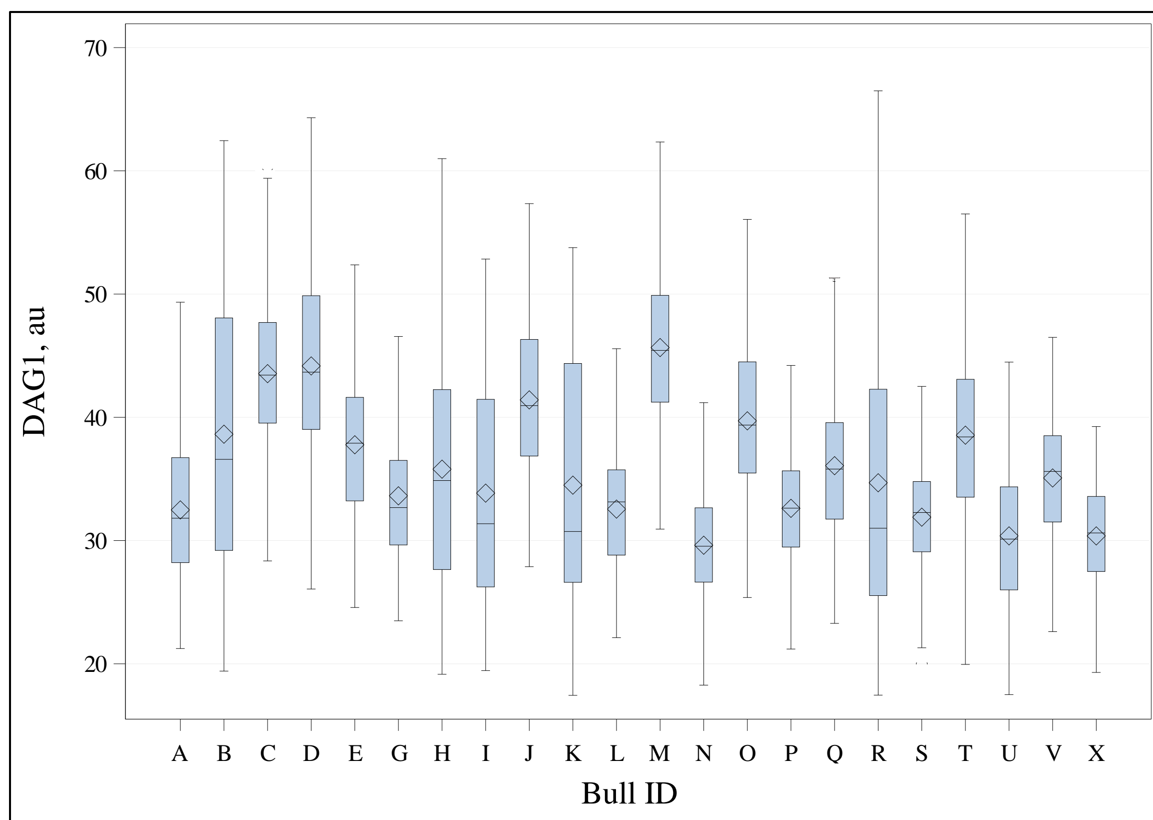


Figure 3.7. Distribution of sperm head DAG1 fluorescence intensity (arbitrary unit; au; ab136665, ABCAM; conjugated to PE/R-Phycoerythrin, ab102918, ABCAM) per bull. Line within box represents median and diamond shape represents mean (Study II).

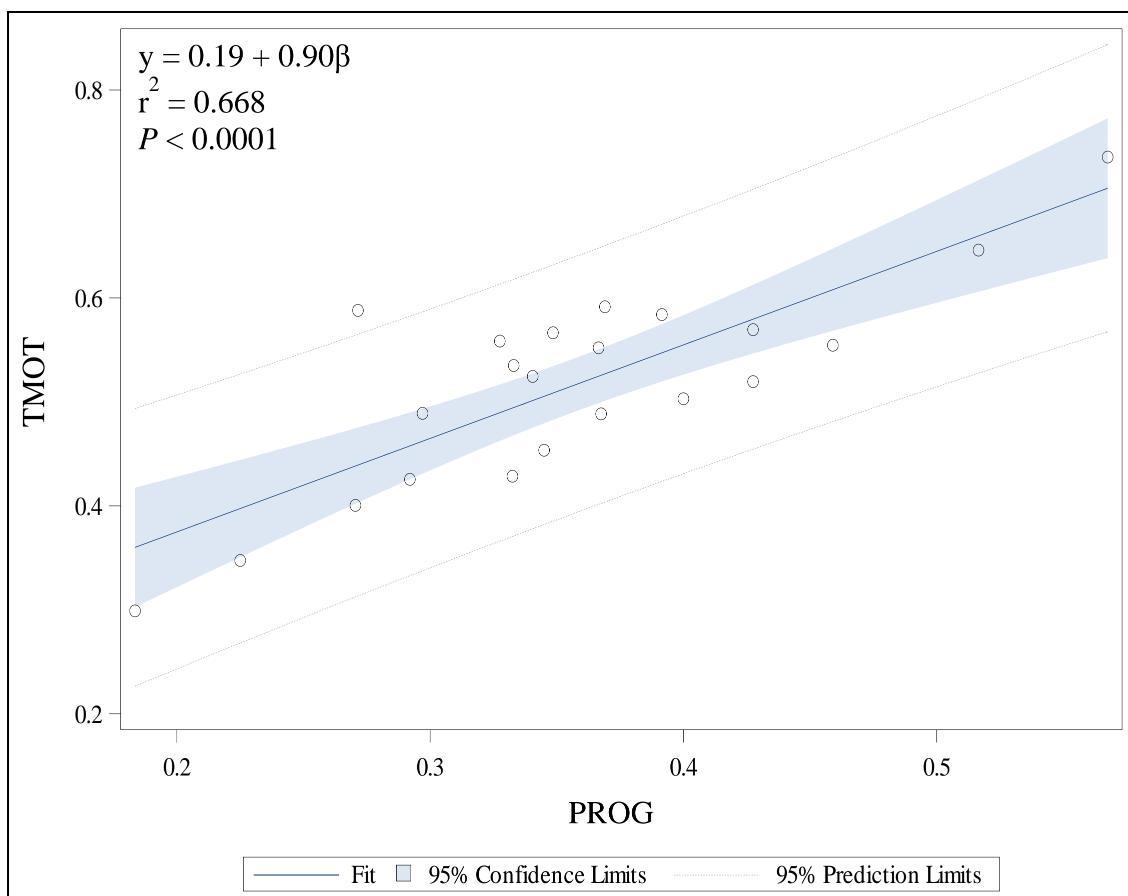


Figure 3.8. Correlation between sperm total (TMOT) and progressive (PROG) motility for 22 dairy bulls. Y- and X-axis = proportions. Circles within plot represent individual samples.

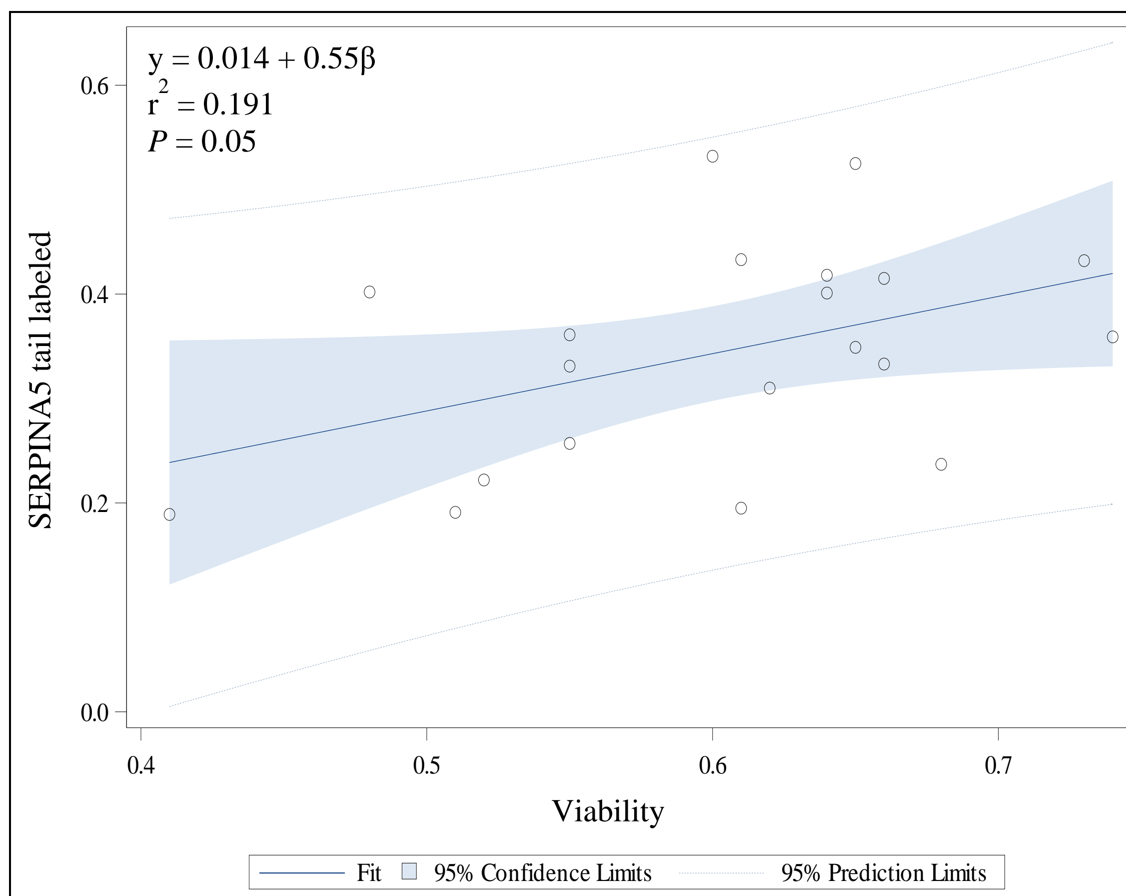


Figure 3.9. Correlation between proportion of sperm tail labeled for SERPINA5 and proportion of sperm with an intact plasma membrane (viability) for 20 dairy bulls. Y- and X-axis = proportions. Circles within plot represent individual samples.

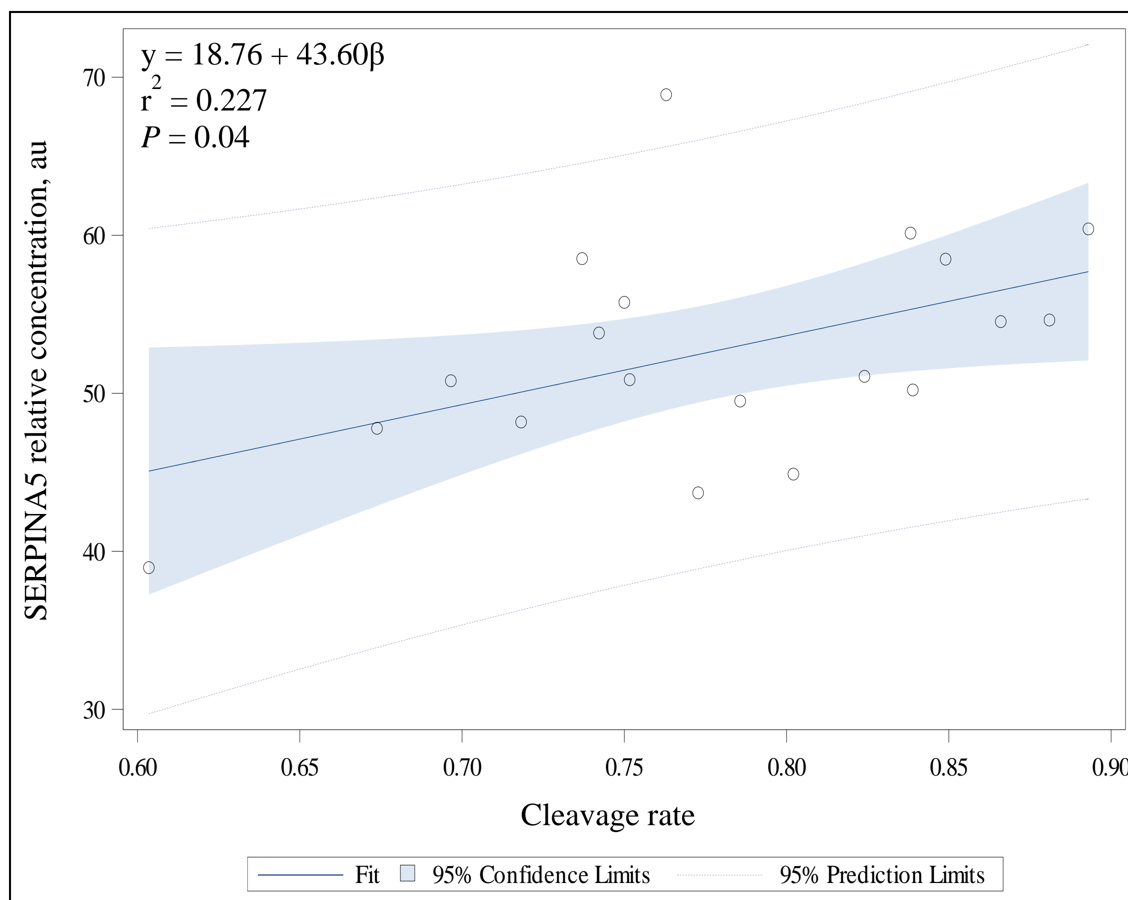


Figure 3.10. Correlation between sperm SERPINA5 relative concentration and cleavage rate for 19 dairy bulls. au = arbitrary unit; X-axis = proportions. Circles within plot represent individual samples.

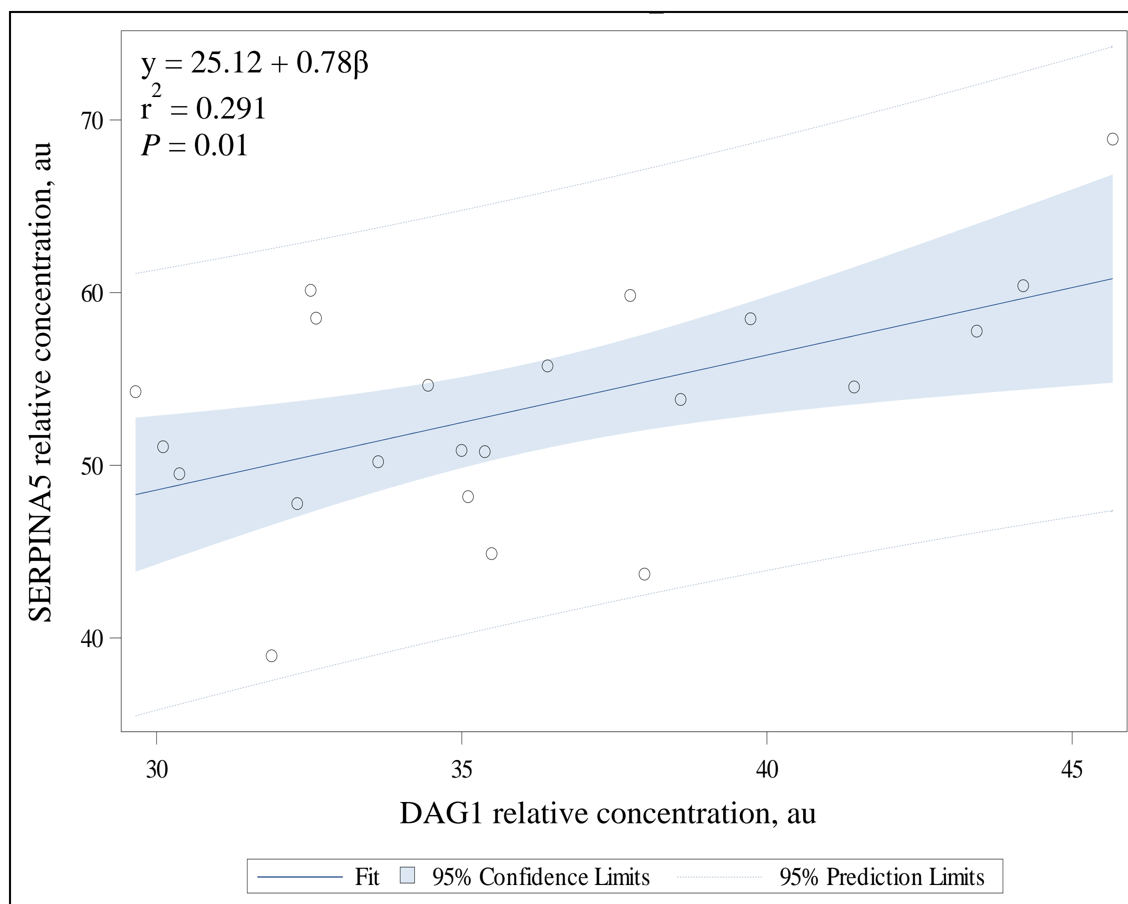


Figure 3.11. Correlation between sperm SERPINA5 and DAG1 relative concentration for 22 dairy bulls. au = arbitrary unit. Circles within plot represent individual samples.

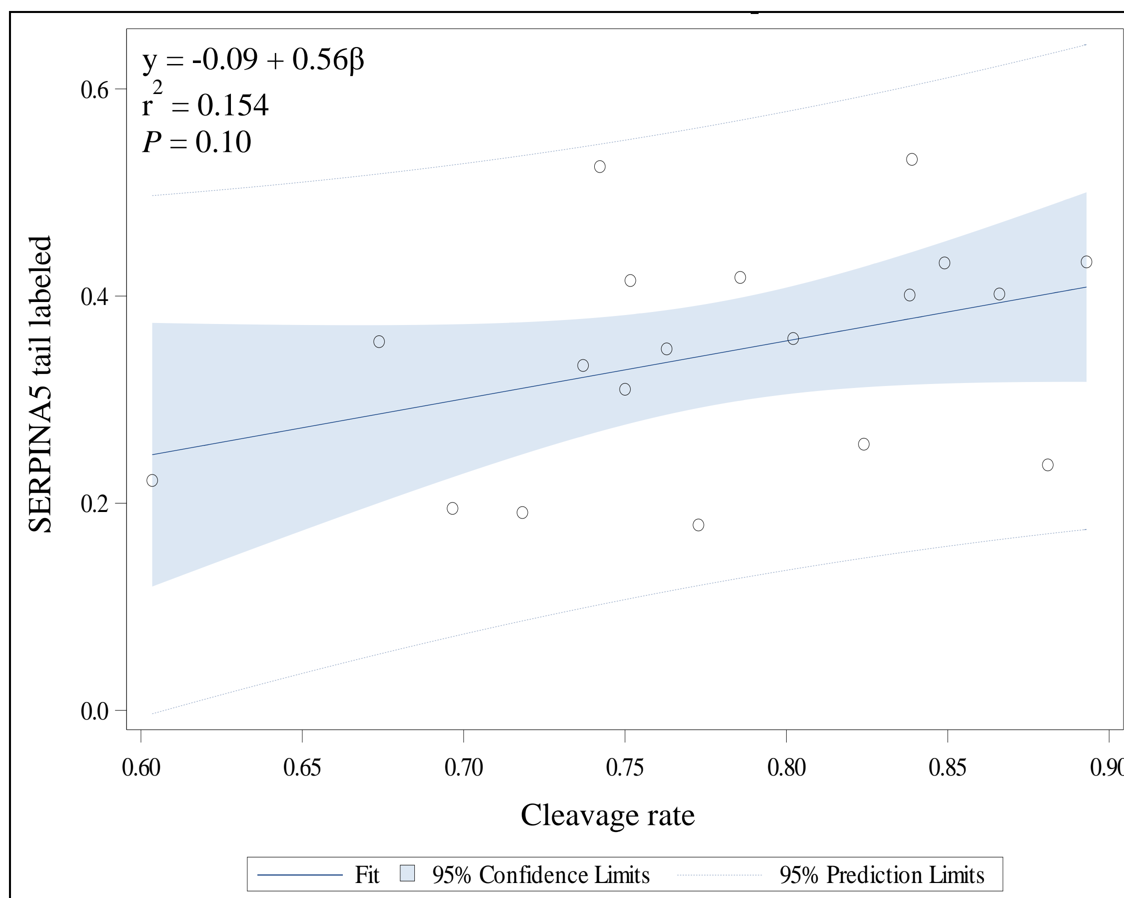


Figure 3.12. Correlation between proportion of sperm tail labeled for SERPINA5 and cleavage rate for 19 dairy bulls. Y- and X-axis = proportions. Circles within plot represent individual samples.

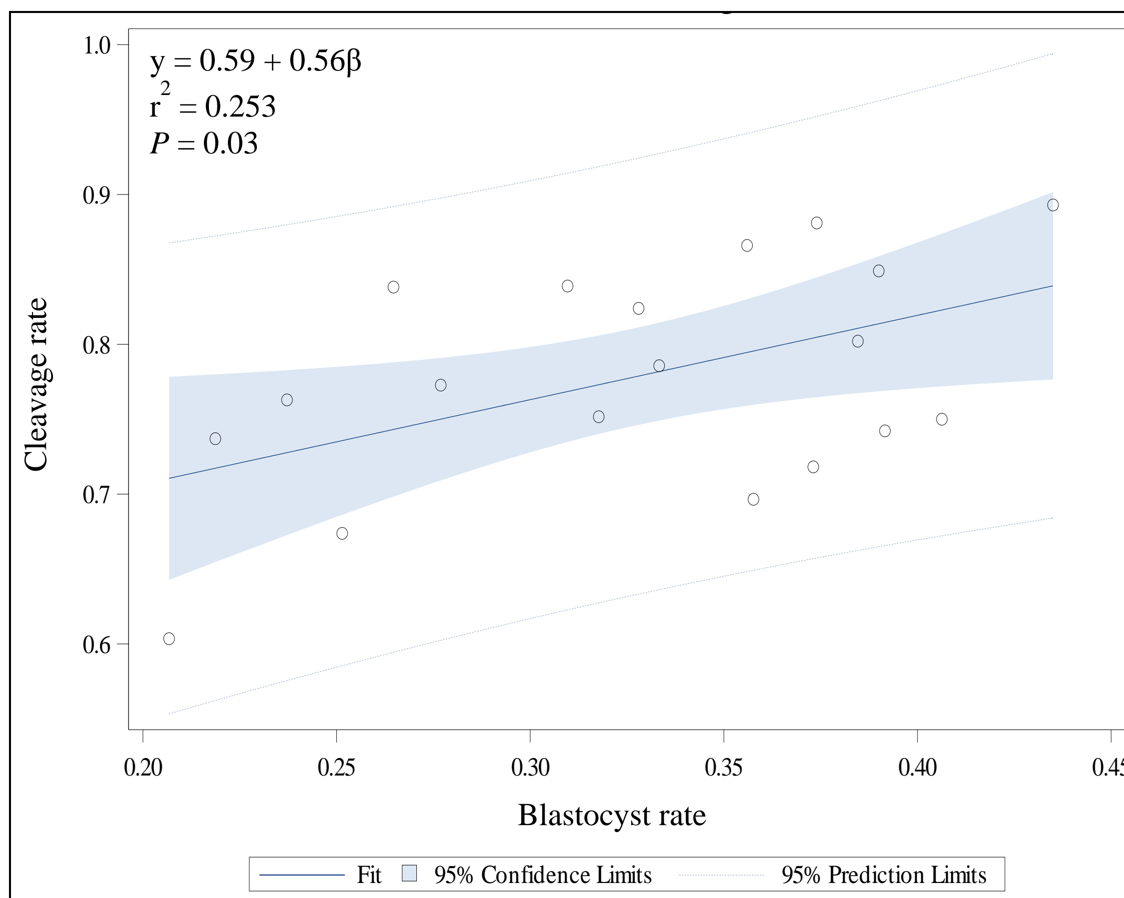


Figure 3.13. Correlation between cleavage rate and blastocyst rate for 19 dairy bulls. Y- and X-axis = proportions. Circles within plot represent individual samples.

CHAPTER 4

BULL FIELD FERTILITY DIFFERENCES CAN BE ESTIMATED WITH *IN VITRO* SPERM CAPACITATION AND FLOW CYTOMETRY

ABSTRACT

Capacitation is a biochemical cascade of events that must occur to sperm before it is capable of fertilizing an oocyte. This study evaluated whether post *in vitro* capacitation changes in sperm could be used to estimate fertility differences between bulls. Frozen-thawed semen from five bulls (two to four ejaculates per bull) previously identified as high (48.1% and 47.7%, bulls A and B, respectively), intermediary (45.5%, bull D) or low (40.7% and 43.1%, bulls C and E, respectively) fertility, based on pregnancy per AI, were evaluated for total and progressive motility, sperm plasma membrane integrity (viability), acrosome integrity (viable sperm with an intact or disrupted acrosome), reactive oxygen species (ROS; viable sperm ROS+ or ROS-), mitochondrial membrane energy potential (mito-potential), zinc signatures (signatures 1 to 4) and CD9 protein populations at pre-wash, post-wash, h 0 (diluted with non-capacitation media), and at h 0, 3, 6, and 24 after dilution with capacitation media and incubation at 37 ºC. Data were analyzed using the GLIMMIX procedure of SAS with bull, time and the interaction as fixed effects. Random statements were used to analyze the data as repeated measures by time with ejaculate per bull as subject. Bull by time interaction was significant ($P \leq 0.03$) for total motility, viability, viable sperm with disrupted acrosome, and zinc signature 3. There tended ($P = 0.06$) to be a bull by time interaction for zinc signatures 1 + 2

combined. Time was significant ($P \leq 0.003$) in all analyses, except viable ROS- (P = 0.12). There was a significant effect of bull ($P \leq 0.03$) for viability, viable sperm with disrupted acrosome, zinc signatures 1, 2 and 1 + 2, viable CD9- and dead CD9+. High and intermediary field fertility bulls had greater ($P \leq 0.04$) percentages of viable sperm (23.2 ± 1.9%, 26.8 ± 2.9%, 24.2 ± 2.2%, 16.9 ± 2.3%, 13.6 ± 1.6%, A, B, D, C and E, respectively), zinc signature 2 (18.3 ± 1.7%, 17.4 ± 2.4%, 18.9 ± 2.0%, 9.9 ± 1.9%, 10.5 ± 1.4%, A, B, D, C and E, respectively) and zinc signature 1 + 2 (21.4 ± 1.8%, 23.1 ± 2.6%, 22.7 ± 2.2%, 14.8 ± 2.2%, 11.5 ± 1.4%, A, B, D, C and E, respectively) compared to low field fertility bulls. High and intermediary fertility bulls had decreased ($P \leq 0.05$) percentage of dead CD9+ (20.3 ± 1.1%, 26.4 ± 1.8%, 20.5 ± 1.3%, 33.0 ± 1.9%, 43.0 ± 1.6%, A, B, D, C, and E, respectively) compared to low fertility bulls. Viable CD9+ differed ($P = 0.02$) and sperm with an intact acrosome and viable CD9+ tended to differ ($P = 0.06$) amongst bulls; however, association with field fertility was not observed. There was a positive correlation between pregnancy per AI and viability ($P = 0.10$; r = 0.81), zinc signature 2 ($P = 0.04$; r = 0.89), and zinc signature 1 + 2 ($P = 0.10$; r = 0.80). In summary, incubation of sperm in CM and flow cytometry analyses for viability, zinc signatures 2 and 1 + 2 combined, and dead CD9+ seems promising to estimate *in vivo* fertility differences amongst bulls.

INTRODUCTION

In order to complete fertilization, sperm must have normal morphology, progressive motility, intact membranes (e.g., acrosome and plasma membrane), stable

DNA, and the ability to undergo capacitation (Rodriguez-Martinez, 2003; Saacke, 2008; Vincent et al., 2012; Garner, 2014). An ejaculate is a heterogeneous population of sperm, thus it is normal for some sperm to display undesirable characteristics, but for a bull to have high fertility, it is important that a great proportion of the ejaculate has these desirable characteristics. Concentration and type of undesirable characteristics will determine, to some extent, the fertility of the ejaculate. Overcoming some inseminate problems can occur by increasing the insemination dose (Saacke et al., 1994).

The standard method to determine bull fertility is through a breeding soundness exam (BSE). According to the Society for Theriogenology a bull BSE evaluates physical soundness, estimates quantity of sperm produced, and evaluates quality of sperm ejaculated (Koziol and Armstrong, 2018). Conventional BSE can detect sterile and infertile animals with high accuracy; however, animals with below average or low fertility are often classified as satisfactory breeders. Even among AI sires that pass quality control analysis, it is impossible to guarantee that they will have high fertility; that is due to unknown or unmeasured semen characteristics (DeJarnette, 2005).

Sperm need to reside in the oviduct for approximately 6 h to acquire fertilization capacity. During this time, sperm undergo a series of biochemical transformations that collectively are called capacitation (Austin, 1951; Chang, 1951). Capacitation can be induced *in vitro* and has been reported to affect *in vitro* oocyte fertilization (Parrish et al., 1986; 1988). Several methods of measuring sperm capacitation have been developed (reviewed by Gillan et al., 2005). More recently intracellular Zn^{2+} was utilized to determine sperm capacitation status through changes in zinc signatures and was also

associated with boar fertility (Kerns et al., 2018). The ability of sperm to undergo capacitation may vary among bulls and may also affect fertility.

It has been demonstrated that proteins present on the sperm head were associated with sperm adhesion or fusion with the oocyte plasma membrane in mice. These proteins are equatorin (or MN9 antigen), CD9, and IZUMO1 (Toshimori et al., 1998; Manandhar and Toshimori, 2001; Inoue et al., 2005; Ito et al., 2010; Satouh et al., 2012). Also, oocyte JUNO (IZUMO1 receptor), and tetraspanins CD9 and CD81 have been demonstrated to be required for mouse fertilization (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Rubinstein et al., 2006; Bianchi et al., 2014). The proteins CD9, JUNO and IZUMO1 have been reported to be present on bovine gametes (Zhou et al., 2009; Antalíková et al., 2015; Fukuda et al., 2016; Zhao et al., 2018). When zona-free oocytes were incubated with anti-CD9 antibodies, oocyte fertilization was significantly decreased (41.6% vs. 81.3%; Zhou et al., 2009); however, the requirement of JUNO and IZUMO1 in bovine fertilization has not been demonstrated. The protein CD9 has been well characterized in oocytes (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Rubinstein et al., 2006; Sutovsky, 2009; Zhou et al., 2009). On the sperm the characterization and function of CD9 is not fully understood; however, it has been reported that CD9 was present on the sperm of mice (Rubinstein et al., 2006; Barraud-Lange et al., 2007; 2012; Ito et al., 2010), boars (Kaewmala et al., 2011), and bulls (Antalíková et al., 2015).

The aim of this study was to evaluate whether bovine sperm induced to capacitate *in vitro* and evaluated for motility and flow cytometric analyses had a relationship with field fertility. In study I, the objective was to evaluate whether different volumes of

capacitation media (CM) would affect sperm survival and capacitation over time. In study II, the objective was to evaluate whether *in vitro* capacitation of sperm could estimate fertility differences among bulls; a secondary objective was to characterize the presence of CD9 protein on sperm and its possible role as a fertility biomarker.

MATERIALS AND METHODS

Experimental design:

Study I:

Semen from six bulls (n = 6) of unknown fertility were used to establish the methodology used in Study II. Four semen straws from the same bull were thawed in a water bath at 37 °C for 60 s and combined in one 2 mL tube. An aliquot was removed for pre-wash motility analysis. Remaining semen was pipetted into four 15 mL conical tubes filled with 10 mL of bovine non-capacitation media (bNCM) pre-warmed to 37 °C; tubes were centrifuged at 500 × g for 10 min, the supernatant was removed, pellets were combined in a 2 mL tube and resuspended with approximately 200 μL of bNCM. An aliquot was removed for post-wash motility and concentration analysis. After assessment of concentration, each sample was divided into three treatments: 1) No-CM (post-wash semen was diluted to 17 million sperm per mL in bNCM); 2) Low-CM (post-wash semen was diluted to 20 million sperm per mL in bNCM and further diluted to 17 million sperm per mL in CM); 3) High-CM (post-wash semen was diluted to 40 million sperm per mL in bNCM and further diluted to 17 million sperm per mL in CM). Samples from each treatment were evaluated for total motility (TMOT) and progressive motility (PROG) by

computer-assisted sperm analysis (CASA; IVOS II; Hamilton Thorne, Beverly, MA, USA) at pre-wash, post-wash, 0 (treatment dilution), 2, 4, 6, and 24 h. Semen was evaluated by flow cytometry for plasma membrane integrity (viability), acrosome integrity, reactive oxygen species (ROS) and, zinc signatures at 0, 2, 4, 6, and 24 h. Samples were incubated at 37 °C in an incubator without exposure to light before evaluation by flow cytometry, except for assay specific temperatures. Because of semen extender mixed with semen in straws and unknown sperm concentration, evaluation of pre- and post-wash sperm were not possible by flow cytometry.

Study II:

Semen from five bulls with known field fertility as evaluated in two research trials (Richardson et al., 2017; Zoca et al., 2020) were used in this study. Bull identification was the same as Zoca et al. (2020) for bulls A through E. Bull A and D were used in both research projects (Zoca et al., 2020 and Richardson et al., 2017), thus bulls A and D represent bulls 1 and 2 from Richardson et al. (2017), respectively. A total of 15 collection dates were evaluated with a 55-d range between first and last collection. Bulls A-E pregnancy per AI (P/AI), number of breedings per research per bull, collection dates per bull evaluated, range of days between first and last semen collection, and P/AI in relationship to estrus expression as described by Richardson et al. (2017) are described in Table 4.1.

The results from study I, demonstrated that sperm capacitation induced with High-CM had a better response compared to Low-CM, specifically explained by viability

and zinc signature assays (Table 4.2); based on a greater decrease in the percentage of viable sperm and both signature 2 and signature 1 + 2 compared to No-CM. Thus, for study II, all samples were diluted using the High-CM procedure, except for 0 h that was diluted using the No-CM procedure as a baseline. Based on overall results from study I, the time intervals of 3, 6, and 24 h of incubation were used for study II. Two or three semen straws of the same bull and collection date, were thawed and washed as described in study I. Pre- and post-wash analyses were performed as described in study I. Samples were evaluated for TMOT and PROG (CASA), viability, acrosome integrity, ROS, zinc signatures, mitochondrial membrane energy potential (mito-potential), CD9 protein populations and CD9 fluorescence intensity (FI; flow cytometry) at 0 (similar to No-CM treatment), and 0 CM, 3, 6 and, 24 h incubation. Samples were also used to characterize the localization of CD9 protein on the sperm by fluorescence microscopy.

Semen from a control bull was thawed and washed as described in study I at each time point (0 and 0 CM, 3, 6, and 24 h). Semen from the control bull was diluted in bNCM as No-CM in study I and used to ensure proper machine accuracy for all analyses; therefore, the control results were used as a covariate adjustment for all analyses.

In vitro Capacitation:

In vitro capacitation was induced as described previously (Kerns et al., 2018). Briefly, bNCM was composed of NaCl (100 mM), NaH_2PO_4 (0.3 mM), KCl (3.1 mM), $MgCl_2$ $6H_2O$ (0.4 mM), Polyvinyl alcohol (PVA; 0.01 mM, FW 10,000 with unknown % hydrolyzed), Na-Pyruvate (1 mM), Na-Lactate (22 mM, 60% w/w), HEPES (40 mM),

Gentamycin 10 mg/mL stock (21 mM) and Penicillin G (0.174 mM), pH 7.20. Media was sterile filtered and stored at 4 °C for no more than 14 d. Bovine capacitation media was composed of bNCM with the following reagents added $CaCl_2$ $2H_2O$ (2.1 mM), $NaHCO_3$ (2 mM), Heparin (10 µg/mL) and bovine serum albumin – fatty acid-free (BSA; 6 g/mL), pH 7.40; CM was prepared daily. Semen was always maintained at 37 °C except for centrifugation and assay specific temperatures. Aliquots of semen were removed at each time point for analyses. All samples were analyzed in duplicate, and the average of the duplicates were used for statistical analyses.

Semen Analyses:

Sperm motility analyses were performed using a CASA. Briefly, 10 µL of semen was diluted in 10 µL of bNCM and 20 µL of Hoechst 33342 (final concentration 40 µg/mL), samples were incubated at 37 °C for 10 min. After incubation, samples were loaded on a Leja slide and evaluated for sperm concentration, TMOT and PROG.

All flow cytometric assays were performed in flat bottom polystyrene 96-well plates and evaluated with a Guava EasyCyte 5HT (IMV Technologies, France) flow cytometer; data acquisition and analyses were performed using the GuavaSoft software (version 1.0; IMV Technologies). A total of 5,000 cells per duplicate were analyzed. The flow cytometer was cleaned and EasyCheck calibration beads were used to assure proper machine performance daily.

Plasma membrane integrity was evaluated with SYBR-14 and propidium iodide assay (PI; adapted from Garner et al., 1994; 1997). Briefly, samples were incubated for

10 min with SYBR-14 (900 nM working solution), and PI (1 mg/mL). Results for viability are expressed as percentage of sperm with intact plasma membrane (viable), thus, positive for SYBR-14 and negative for PI. Sperm acrosome integrity was determined by fluorescein isothiocyanate-conjugated peanut agglutinin (PNA) as previously described (Purvis et al., 1990; Tao et al., 1993). Briefly, samples were incubated for 10 min with a stain mix (1 µL of PI, 0.5 µL of PNA, and 48.5 µL of bNCM, and filtered in a 0.22 nm filter). Results for acrosome status were expressed as percentage of viable sperm with intact acrosome (viable intact; PNA negative and PI negative) or disrupted acrosome (viable disrupted; PNA positive and PI negative), and disrupted sperm plasma membrane (dead) with intact acrosome (dead intact; PNA negative and PI positive) or disrupted acrosome (dead disrupted; PNA positive and PI positive).

Reactive oxygen species in the sperm were measured with EasyKit 3 (IMV Technologies) following manufacturer's procedures. The specific ROS measured by EasyKit 3 were H_2O_2, HOCl, ONOO- (Mahfouz et al., 2008). In this assay, sperm are challenged with H_2O_2 and sperm that react to this challenge are considered ROS positive [ROS+; green dye (proprietary information) positive], those that do not respond to the challenge are ROS negative (ROS-; green dye negative). Results for ROS were expressed as percentage of viable ROS+, viable ROS-, dead ROS+, and dead ROS-. The main population of interest in this assay are the viable sperm and ROS+, and it is worth noting that this is a 3-hour assay. Mitochondrial membrane potential (mito-potential) was evaluated with JC-1 (8 µM), diluted in ethanol (200 proof) and bNCM, and incubated for

30 min. (adapted from Garner et al., 1997; Guthrie and Welch, 2008). Results were expressed as percentage of high mito-potential.

Sperm zinc signatures are a measure of sperm capacitation and have been characterized in human, boar, and bovine by Kerns et al. (2018). The zinc signature assay used here was adapted from Kerns et al. (2018). Briefly, 90 µL of sample and 10 µL of Fluozin-3, AM (FZ3; 1:400 dilution in bNCM) were incubated at room temperature for 30 min without light exposure. After incubation, samples were centrifuged at 300 × g for 5 min, supernatant was removed and 75 µL of bNCM was added and pellet resuspended; samples were incubated at room temperature for 30 min without light exposure. After incubation, 25 µL of PI (1:50 dilution in bNCM) was added to samples and incubated at room temperature for 15 min without light exposure followed by evaluation with flow cytometry. Zinc signature results were expressed as percentage of signature 1 (viable non-capacitated sperm with high intracellular zinc), signature 2 (viable sperm in the process of capacitation with low intracellular zinc), signature 3 (dead and capacitated sperm with high intracellular zinc in the mitochondrial sheath or the acrosome region or both), and signature 4 (dead sperm without zinc). Events negative for FZ3 and PI were considered debris and removed from analyses.

For CD9 evaluation, anti-CD9 antibody (mouse anti-bovine, IVA50, monoclonal, Invitrogen, Waltham, MA) was conjugated to fluorescein isothiocyanate [FITC conjugation kit (fast) – lightning-link, ab188285, ABCAM, United Kingdom] final concentration 0.83 µg/µL. Samples (15 µL; ~250,000 sperm) were diluted with bNCM (35 µL), and incubated with 1 µL of anti-CD9/FITC and 1 µL of PI for 1 h at 37 °C (adapted from Antalíková et al., 2015). Flow cytometric CD9 and PI evaluation included

the following populations viable CD9+, dead CD9+, viable CD9-, dead CD9-, respectively. Assays were performed using 250 µL of bNCM and 5 µL of incubated sample per well. Also, CD9 FI of viable and dead population were evaluated. The localization of CD9 on the sperm was characterized by fluorescence microscopy (BZ-X710, Keyence) at 600 × magnification under oil immersion.

Statistical Procedures

Total motility, PROG, and flow cytometry measures [viability, viable intact, viable disrupted, dead intact, dead disrupted, viable ROS+, viable ROS-, dead ROS+, dead ROS-, mito-potential, zinc signatures 1, 2, 3 and 4, and combination of signature 1 and 2 (signature 1 + 2), viable CD9+, viable CD9-, dead CD9+, and dead CD9+] were evaluated with the GLIMMIX procedure of SAS (9.4). For all analyses, data was assumed beta distributed and the link function logit was used. For study II, Kenward-Roger's was used as the degrees of freedom method. Treatment (study I) or bull (study II), time, and the interaction were used as fixed effects. Three random statements were used, the first random statement was used to model the R-side of residuals to analyze the data as repeated measures. The subject was bull (study I) or collection date per bull (study II) with covariate structures selected based on the smaller -2 Res Log Pseudo-Likelihood. The covariate structures selected for each variable of study I were First-Order Ante-dependence (ANTE(1); viable intact, viable disrupted, dead intact, dead disrupted, viable ROS-, dead ROS-, and zinc signature 4), Heterogeneous First-Order Autoregressive (ARH(1); zinc signature 1), Heterogeneous Compound Symmetry (CSH; viable ROS-, and zinc signatures 1 and 3), and Toeplitz (TOEP; TMOT, PROG, viability,

and zinc signature 1 + 2); and for study II were ANTE(1) (dead intact, dead disrupted, dead ROS+, zinc signature 3, zinc signature 4, viable CD9-, and dead CD9-), First-Order Autoregressive (AR(1); viable disrupted), ARH(1) (viable intact, viable ROS- and ROS+, dead ROS-, mito-potential, zinc signature 1 + 2), CSH (viability and zinc signature 2), TOEP (TMOT, zinc signature 1, viable CD9+, and dead CD9+), and Variance Components (VC; PROG). The second random statement was the intercept and the third was the residual. Least square means were compared using the PDIFF option, and the ilink function was used to inverse transform least square means. All samples at 24 h had zero percentage results for TMOT and PROG in study I; thus, we were unable to statistically include these observations. CD9 FI was evaluated with the MIXED procedure of SAS for repeated measures with bull, time and their interaction as fixed effects. Collection date per bull was used as subject and ANTE(1) was selected as the covariate structure for both live and dead sperm FI based on the smaller BIC value. CD9 localization on the sperm was characterized; however, no statistical analysis was performed. The correlation between overall bull effect least square mean and P/AI reported by Zoca et al. (2020), as well as the correlation of CD9 population and CD9 FI with all sperm parameters were evaluated using the CORR procedure of SAS. Results are presented as mean ± SE. Level of significance was $\alpha \leq 0.05$, when $P > 0.05$ but $P \leq 0.10$ the results were considered as tendency.

RESULTS

Study I

There was no treatment by time interaction for TMOT ($P = 0.94$; Fig. 4.1 C), PROG ($P = 0.54$; Fig. 4.1 D), viability ($P = 0.68$; Fig. 4.2 B), acrosome integrity ($P \geq 0.87$; Fig. 4.3 A-D), ROS ($P \geq 0.10$; Fig. 4.4 A-D), zinc signatures 1, 2 and 4 ($P \geq 0.51$; Fig. 4.5 A, C and D) and zinc signature 1 + 2 ($P = 0.77$; Fig. 4.6 B); however, there was a treatment by time interaction for zinc signature 3 ($P = 0.04$; Fig. 4.5 B). There was no difference in zinc signature 3 ($P > 0.10$) between 0, 2, 4, 6 and 24 h incubation for No-CM. For the Low-CM, there was a tendency ($P = 0.09$) for 6 h incubation to have increased signature 3 compared to 0 h; also, 2, 4 and 6 h it was increased ($P < 0.003$) compared to 24 h. For High-CM, percentage of signature 3 at 24 h incubation was decreased ($P \leq 0.0003$) compared to 0, 2, 4, and 6 h. At 0 and 4 h, there was no difference ($P \geq 0.13$) between treatments for signature 3. At 2 h, Low-CM had greater percentage of signature 3 compared to No-CM ($P = 0.04$) and there was no difference compared to High-CM ($P = 0.18$); No-CM and High-CM were not different ($P = 0.43$). At 6 h incubation, Low-CM had greater percentage of signature 3 compared to both No-CM and High-CM ($P \leq 0.02$). At 24 h incubation, High-CM had a decreased percentage of signature 3 compared to No-CM and Low-CM ($P \leq 0.005$).

There was no effect of treatment on TMOT, PROG, acrosome integrity, viable ROS-, and dead ROS+ (Table 4.2); and there was no effect of time for dead disrupted ($P = 0.63$; Fig. 4.7 B). The overall percentage of viable sperm (viability) decreased ($P \leq 0.002$; Table 4.2) as semen was diluted with greater volume of CM. The overall

percentage of viable ROS+ decreased ($P \leq 0.03$; Table 4.2) when semen was diluted with CM and tended ($P = 0.08$) to decrease between Low- and High-CM; also, percentage of dead ROS- increased ($P < 0.0001$; Table 4.2) as semen was diluted with a greater volume of CM. There was an effect of treatment for all zinc signatures ($P \leq 0.0003$; Table 4.2). The overall percentage of zinc signature 1 decreased ($P \leq 0.04$) when semen was diluted with CM; also, signature 2 decreased ($P \leq 0.01$) as semen was diluted with greater volume of CM. Low-CM had greater ($P \leq 0.001$) signature 3 compared to No-CM and High-CM. High-CM had greater ($P \leq 0.001$) signature 4 compared to No-CM and Low-CM. There was a decrease in the overall percentage of signature 1 + 2 ($P < 0.0001$; Table 4.2) as semen was diluted with greater volume of CM.

There was a decrease over time on overall percentage of TMOT ($P < 0.01$; Fig. 4.1 A), PROG ($P < 0.02$; Fig. 4.1 B), viability ($P < 0.04$; Fig. 4.2 A), zinc signature 1 + 2 ($P < 0.02$; Fig. 4.6 A), viable intact ($P < 0.04$; Fig. 4.7 C), viable and dead ROS+ ($P \leq 0.01$; Fig. 4.8 A and B, respectively), and zinc signature 2 ($P < 0.03$; Fig. 4.9 C). There was an increase over time on the overall percentage of viable disrupted ($P < 0.02$; Fig. 4.7 A), dead intact ($P < 0.005$; Fig. 4.7 D), dead ROS- ($P \leq 0.02$; Fig. 4.8 D), and signature 4 ($P < 0.02$; Fig. 4.9 D). There was also an overall effect of time on the percentage of viable ROS- ($P \leq 0.02$; Fig. 4.8 C) and zinc signatures 1 and 3 ($P < 0.004$; Fig. 4.9 A and B, respectively).

Study II

There was no interaction between bull and time for PROG ($P = 0.36$; Fig. 4.10 D), dead disrupted ($P = 0.33$; Fig. 4.11 B), viable intact ($P = 0.82$; Fig. 4.11 C), dead intact ($P = 0.20$; Fig. 4.11 D), viable ROS+ ($P = 0.21$; Fig. 4.12 A), dead ROS+ ($P = 0.47$; Fig. 4.12 B), viable ROS- ($P = 0.93$; Fig. 4.12 C), mito-potential ($P = 0.88$; Fig. 4.13 B), zinc signature 1, 2 and 4 ($P \geq 0.16$; Fig. 4.14 A, C, and D, respectively), and CD9 populations ($P \geq 0.18$; Fig 4.15 A-D).

The effect of bull by time interaction was significant for TMOT ($P = 0.0002$; Fig. 4.10 C). All bulls had a decrease in the percentage of TMOT by time ($P \leq 0.05$); however, bulls C and E had increased TMOT at 0 h in CM compared to 0 h in bNCM. At pre-wash, bulls of high and intermediary fertility had greater percentage of TMOT ($P \leq 0.05$) than bulls of low fertility. After washing (post-wash), bull E TMOT remained lower than bulls A and D ($P \leq 0.03$) but not different than bull B ($P = 0.48$); however, bull C tended to be lower than bulls A and D ($P = 0.07$) and was not different than bull B TMOT ($P = 0.55$). At 0 h, bull A had greater TMOT than bulls C and E ($P \leq 0.03$) and at time 0 CM, 3, 6, and 24 h no differences ($P > 0.10$) were detected among bulls. Thus, overall motility measured by a CASA after thawing (pre-wash time point) of multiple ejaculates was able to estimate fertility differences between these five Angus bulls with increased percentage of TMOT for high and intermediary fertility bulls compared to low fertility bulls.

The percentage of sperm undergoing spontaneous acrosome reaction (viable disrupted) was significant for the interaction effect between bull and time ($P = 0.03$; Fig. 4.11 A). The viable disrupted percentage increased over time for bulls A, B, D, and E (*P*

≤ 0.05; Fig. 4.11 A); however, percentage of viable disrupted for bull C was not different between time points. Nevertheless, there was no association between viable disrupted differences and bull fertility. There was a significant interaction between bull and time for dead sperm ROS- ($P = 0.03$; Fig. 4.12 D). There was an increase in the percentage of dead sperm ROS- over time for all bulls ($P \leq 0.05$). At 0 h, bulls of high and intermediary fertility had lower percentage of dead ROS- compared to bull E ($P \leq 0.005$). Bull A had lower dead sperm ROS- than bull C ($P = 0.05$), and bulls B and D tended to be lower than C ($P \leq 0.08$). At 0 CM, bulls A and D were lower than C and E ($P \leq 0.04$); however, bull B was not different than bulls C and E ($P \geq 0.16$). Thus, dead ROS- was able to estimate differences in fertility at 0 h with high and intermediary fertility bulls having decreased or tending to have decreased percentage of dead ROS- compared to low fertility bulls.

There was an interaction between bull and time for sperm zinc signature 3 ($P = 0.01$; Fig. 4.14 B). High fertility bulls had an increase (bull A $P = 0.02$) or seem to have an increase (bull B $P = 0.19$) between 0 and 0 CM followed by a decrease on the percentage of zinc signature 3; however, low fertility bulls had no change between 0 and 0 CM ($P \geq 0.73$), followed by a decrease in zinc signature 3. Interestingly, bull D (intermediary fertility) had no change in zinc signature 3 from 0 to 6 h incubation, despite a numerical increase at 6 h incubation ($P \geq 0.26$); however, there was a decrease in zinc signature 3 at 24 h. There was a significant interaction on the percentage of viable sperm ($P = 0.0004$; Fig. 4.16 B). There was a decrease over time for all bulls ($P < 0.05$). Even though a numerical increase in the percentage of viable sperm appears between 0 h and 0 CM of bull B (32.5% vs 36.4%), no statistical differences were detected ($P = 0.38$). At 0

h, high and intermediary fertility bulls had greater ($P \leq 0.03$) or tended to have greater ($P = 0.09$) percentage of viable sperm compared to low fertility bulls. Greater percentage of viable sperm was observed in high and intermediary fertility bulls compared to low fertility bulls at 0 h; however, sperm diluted with CM was not able to detect differences between high fertility and low fertility in one single time point. Thus, viability at 0 h was able to estimate differences (or tendencies) in fertility between high and intermediary fertility compared to low fertility bulls.

The combination of zinc signatures 1 + 2 represents the percentage of viable cells measured by zinc signature assay (~1 h difference from viability assay). There tended to be an effect of the interaction between bull and time for zinc signature 1 + 2 ($P = 0.06$; Fig. 4.17 B). All bulls decreased the percentage of zinc signature 1 + 2 over time ($P \leq 0.05$). At time 0 h, high and intermediary fertility bulls had greater percentage of signature 1 + 2 ($P \leq 0.02$) compared to bull E, also, bulls A and D tended ($P \leq 0.10$) to be different than C; however, bull B did not differ ($P = 0.16$) from bull C. At 0 CM, high and intermediary fertility bulls had greater ($P \leq 0.003$) percentage of zinc signature 1 + 2 compared to bull E, also, bulls B and D were different ($P \leq 0.02$) and bull A tended ($P = 0.10$) to be different than bull C. Thus, zinc signature 1 + 2 at 0 CM, was able to estimate fertility differences with all high and intermediary fertility bulls having greater or tending to have greater percentage of zinc signature 1 +2 than low fertility, no other individual time point was successful in estimating high and intermediary fertility bulls.

There was no overall effect of bull on the percentage of TMOT, PROG, dead intact, viable ROS+, dead ROS+, viable ROS-, mito-potential, zinc signatures 3 and 4, and dead CD9- (Table 4.3). The overall effect of bull was significant; however, did not

detect fertility differences between bulls for viable and dead disrupted, dead ROS-, zinc signature 1, and viable CD9-; also, tended to be significant for viable intact and viable CD9+ (Table 4.3). The overall effect of bull that were significant and estimated fertility differences between bulls were viability, zinc signature 2, zinc signature 1 + 2, and dead CD9+ (Table 4.3) which high and intermediary fertility bulls had greater percentage of viable, zinc signature 2 and zinc signature 1 + 2, and lower percentage of dead CD9+ compared to low fertility bulls. There was a positive correlation between field fertility and zinc signature 2 (r = 0.89; $P = 0.04$) and there tended to be positive correlation between field fertility and viability (r = 0.81; $P = 0.10$), and zinc signature 1 + 2 (r = 0.80; $P = 0.10$); however, dead CD9+ did not correlate with fertility (r = -0.68; $P = 0.20$). Although percent dead ROS- did not estimate fertility differences between bulls, dead ROS- was negatively correlated with field fertility (r = -0.91; $P = 0.03$). There was no correlation between field fertility and other sperm parameters evaluated ($P > 0.10$).

The overall effect of time was significant for all analysis except for viable ROS- ($P = 0.12$; Fig. 4.18 C). There was a decrease over time on the percentage of TMOT ($P < 0.0001$; Fig. 4.10 A), PROG ($P < 0.0001$; Fig. 4.10 B), mito-potential ($P < 0.0001$; Fig. 4.13 A), viability ($P < 0.0001$; Fig. 4.16 A), zinc signature 1 + 2 ($P < 0.0001$; Fig. 4.17 A), viable ROS+ ($P < 0.0001$; Fig. 4.18 A), viable intact ($P = 0.003$; Fig. 4.19 C), zinc signature 2 ($P < 0.0001$; Fig. 4.20 C), and viable CD9- ($P < 0.0001$; Fig. 4.21 C). There was an increase over time on the percentage of dead ROS- ($P < 0.0001$; Fig. 4.18 D), viable disrupted ($P < 0.0001$; Fig. 4.19 A), dead intact ($P < 0.0001$; Fig. 4.19 D), zinc signature 4 ($P < 0.0001$; Fig. 4.20 D), and dead CD9- ($P < 0.0001$; Fig. 4.21 D). Other significant effects of time were dead ROS+ ($P < 0.0001$; Fig. 4.18 B), dead disrupted (P

= 0.005; Fig. 4.19 B), zinc signatures 1 and 3 ($P < 0.0001$; Fig. 4.20 A and B), and viable CD9+ and dead CD9+ ($P \leq 0.0002$; Fig 4.21 A and B).

CD9 protein was present in both viable and dead sperm on the acrosomal region (Fig. 4.22-4.24). CD9 staining varied from all acrosomal region stained to partial acrosomal region stained; however, there was no change in localization of CD9 before and after capacitation (data not shown). Nevertheless, there were changes in population percentage (Fig. 4.21) and FI of viable and dead populations (Fig. 4.25). There was no effect of bull ($P = 0.12$) and bull by time interaction ($P = 0.55$) for viable CD9 FI. There was a significant interaction bull by time for dead CD9 FI ($P = 0.03$; Fig. 4.25 F) which bull E had greatest FI in all time points compared to all other bulls, except for 0 h; which bull E was not different than bull B ($P = 0.26$) and tended to be different than bulls A, C and D ($P \leq 0.10$). There was a bull effect for dead CD9 FI (Fig. 4.25 B) which bull E had the greatest FI compared to all bulls ($P \leq 0.002$). There was an effect of time for both viable and dead CD9 FI ($P \leq 0.0004$; Fig. 4.25 C and D, respectively). Fluorescence intensity for viable sperm decreased when sperm were diluted with CM and increased during incubation period; however, dead sperm CD9 FI decreased over time (Fig. 4.25 C and D).

The correlation between CD9 population and CD9 FI were evaluated; not surprisingly, there were positive correlations between viable CD9+ and viable CD9 FI, and dead CD9+ and dead CD9 FI (Table 4.4). Also, there was a positive correlation between viable CD9- and dead CD9 FI (Table 4.4). There were negative correlations between viable CD9+ and dead CD9+, dead CD9+ and viable CD9-, viable CD9- and dead CD9-, and dead CD9- and dead CD9 FI (Table 4.4). Viable CD9+ was correlated

with viable disrupted, dead disrupted, zinc signature 2, zinc signature 3 and signature 1 + 2, and tended to be correlated with dead ROS+ (Table 4.5); dead CD9+ was correlated with viability, viable intact, viable disrupted, dead disrupted, dead ROS-, mito-potential, and zinc signatures 2, and 1 + 2, and tended to be correlated with PROG, viable ROS+, and zinc signatures 1, and 4 (Table 4.5); viable CD9- was correlated or tended to be correlated with all sperm parameters except zinc signatures 1 and 3 (Table 4.5); dead CD9- was correlated or tended to be correlated with all sperm parameters except dead disrupted, viable ROS-, and zinc signatures 1 and 3 (Table 4.5). Viable sperm CD9 FI was correlated with viable ROS+, viable ROS-, zinc signatures 2, 4 and 1 + 2 (Table 4.5); also, dead CD9 FI was correlated with all sperm parameters except for viable intact, viable ROS-, dead ROS+, and zinc signature 1 (Table 4.5).

DISCUSSION

It is well established that females must conceive in the first 21-d of the breeding season to achieve their maximum fertility potential and maximize profitability. A delay in conception will lead to decreased longevity of females in the herd, will hinder calf weaning weight, and overall production (Cushman et al., 2013). To conceive early in the breeding season and maintain a pregnancy, females must be cyclic, in good body condition and on a positive plane of nutrition; however, bull fertility also plays an important role. A BSE is essential for selection of highly fertile bulls that will contribute to early conception in a breeding season (Barth, 2018); however, passing a BSE does not

guarantee high fertility. In the beef and dairy industries, frozen semen is used for AI. It is expected that among semen from AI studs, bull fertility is not statistically different between bulls, and more than 90% of semen from these bulls be within ± 3% of average fertility (Clay and McDaniel, 2001; DeJarnette, 2005).

An ejaculate is composed of a heterogeneous population of sperm, and fertility is multifactorial (Rodriguez-Martinez, 2003). Amann and Hammerstedt (1993) suggested that an ejaculate or inseminate must have "enough" of all necessary sperm characteristics to reach a high level of fertility. In the present study, most of the sperm characteristics measured did not associate or correlate with field fertility. It has been reported that acrosome integrity, ROS, and mito-potential were associated or correlated with bull fertility (Oliveira et al., 2014; Kumaresan et al., 2017; Bernecic et al., 2021), but in the present study, they did not have an association with field fertility of the bulls evaluated. One difference between studies is the range in fertility among bulls tested. Thus, it is possible to conclude that fertility of bulls in this study were not limited by acrosome integrity, ROS, or mito-potential.

Richardson et al. (2017) and Zoca et al. (2020) demonstrated fertility differences between bulls even though they had a small sample of bulls, was contrary to what was proposed previously (Clay and McDaniel, 2001; DeJarnette, 2005). Thus, the study of semen characteristics that can better predict bull fertility is necessary. In the present study, semen from two studies (Richardson et al., 2017; Zoca et al., 2020) were analyzed to evaluate the effect of inducing capacitation *in vitro* and the ability to estimate differences between different fertility levels. It was observed that CM affected sperm viability, viable ROS+, zinc signature 1, 2, and 1 + 2 with increasing concentration of

CM decreased those sperm characteristics. Sperm capacitation is a terminal event that leads to oocyte fertilization or sperm death (Suarez, 2016) which agrees with increased dead ROS-, and zinc signature 4 when concentration of CM was increased. Thus, the greater concentrations of CM the faster capacitation happened. For study II, the greater concentration of CM was chosen because of the faster induction of capacitation.

In study II, the interaction between bull and time for TMOT was able to estimate differences between bulls that were related to field fertility; however, only at the time of pre-wash. Zoca et al. (2020) reported differences between bulls in TMOT, but bulls A (high) and C (low) did not differ (31.8% vs 26.5%, respectively). In the present study, more ejaculates were evaluated (bull A) and differences in TMOT at pre-wash between high and low fertility bulls were detected; however, the present study failed to detect differences between high fertility bulls and the intermediary fertility bull. Farrell et al. (1998) reported a moderate correlation (r = 0.58) between TMOT and bull fertility which agrees with the lack of relationship between TMOT and field fertility observed in the overall bull effect and by Zoca et al. (2020); however, when more ejaculates were added to the analysis in the present study the relationship between TMOT and field fertility was observed at pre-wash. Also, it is important to highlight that the bull with intermediary field fertility (bull D) had the greatest TMOT in the present study (38.9%) and in Zoca et al. [(2020); 51.6%] but not in Richardson et al. [(2017); bull A (1) 51%; bull D (2) 38.5%] which agrees with the moderate correlation between TMOT and fertility reported previously (Farrell et al., 1998).

It has been reported that changes in water temperature where semen straws were being held from 35 ºC to 22 ºC for only 20 min decreased sperm motility (Kaproth et al.,

2002). Further, the effect of thawing 10 straws simultaneously on sperm characteristics has been investigated (Oliveira et al., 2012; 2013). In these studies, there was an overall decrease in TMOT between the first and last straw removed from the water bath (6 min 30 s difference) of ~5 to 10% difference; however, at 2 h incubation, difference between first and last straw TMOT was ~4% with great variation between samples, also, difference between first and 2 h incubation analysis demonstrated a decrease of ~20 to 30 percentages points in TMOT (Oliveira et al., 2012; 2013). In study I, time elapsed between thawing and pre-wash analysis was ~10 to 15 min (incubation required for CASA analysis), between thawing and post-wash analysis was ~25 to 30 min, and between thawing and 0 and 0 CM h was ~1 h. During wash procedures, sperm is diluted and centrifuged with fluctuation in temperature, and centrifugation can also cause sperm damage (Baldi et al., 2020). Surprisingly, there was no difference between pre-wash and post-wash TMOT in study I; however, in study II there was a significant decrease in TMOT at post-wash. Differences between pre-wash and post-wash results from study I and study II could be related to animal-to-animal variation which agrees with great sample and animal variation reported previously (Oliveira et al., 2012; 2013).

Sperm viability also was able to estimate differences between bulls that were related to field fertility. High fertility bulls had greater overall percentage of viable sperm compared to low fertility bulls. Again, the intermediary fertility bull was not different from high fertility bulls. There was also a positive correlation between field fertility and overall bull viability. The interaction of bull by time was able to detect differences related to fertility at time 0 h in bNCM; however, no other time point was able to estimate bull differences associated with field fertility. It is possible that high and intermediary fertility

bulls required a smaller concentration of capacitating agents (i.e., heparin and bicarbonate) compared to low fertility animals, since only small changes in sperm viability were observed in low fertility bulls compared to high and intermediary bulls. This hypothesis is supported by an increase (statistically or numerically) in zinc signature 3 at 0 CM for high and at 6 h for intermediary whereas low fertility bulls had decreased zinc signature 3. Nevertheless, the induction of capacitation and incubation of sperm allowed for better separation between high and low fertility as observed in the overall effect of bull, because high and intermediary fertility bulls maintained greater (numerically or statistically) percentage of viable sperm in all time points. Bull differences in sperm viability were detected by Zoca et al. (2020), and results were similar to what was observed at 0 h. Interestingly, the intermediary fertility bull had the greatest value for viability in both studies at 0 h but this was not the case in Richardson et al. (2017); where no differences were detected between high and intermediary fertility bulls when overall viability was evaluated. The correlation between sperm plasma membrane integrity and field fertility have been widely studied; however, results varied from a weak correlation (r = 0.05 to 0.20; Alm et al., 2001; DeJarnette et al., 2021) to a moderate correlation (r = 0.41 to 0.68; Januskauskas et al., 2001; 2003; Anzar et al., 2002; Christensen et al., 2005) and a strong correlation (r = 0.85 and 0.87; Anzar et al., 2002; Kumaresan et al., 2017). In the present study, the correlation between field fertility and viability was strong. Also, both low fertility bulls had decreased viability either statistically or numerically at all time points and overall which suggests that inclusion of a viability assay as a quality control analysis for AI studs and possibly at a BSE test would assist in the prediction of highly fertile bulls.

A new marker of sperm capacitation has been recently reported. This marker uses zinc ion efflux to determine the capacitation status of the sperm and classifies sperm into 4 signatures (Kerns et al., 2018; 2020). It has been reported that non-capacitated sperm, usually found in fresh ejaculates, had elevated intracellular zinc (signature 1; Michailov et al., 2014; Kerns et al., 2018). The active removal of zinc (i.e., transition from signature 1 to signature 2) has been reported to be a prerequisite of sperm capacitation (Andrews et al., 1994); however, complete removal of zinc ceased sperm motility (Michailov et al., 2014; Kerns et al., 2018). Thus, sperm zinc signatures 1 and 2 represent the population of sperm with high fertility potential while signature 3 represents sperm that have gone through capacitation and are dead or dying and signature 4 represents sperm that are dead and may or may not have gone through capacitation before dying (Kerns et al., 2018). In boars, zinc signature 3 was increased from pre-capacitated to post-capacitated sperm among high fertility animals but no change was observed in low fertility boars (Kerns et al., 2018). In the present study, the percentage of zinc signature 3 in bull sperm decreased over time and did not seem to follow the same trend as was reported for boars (Kerns et al., 2018). Nonetheless, zinc signature 2 and zinc signature 1 + 2 were able to detect an overall difference between bulls that were associated with field fertility; similar to viability, the intermediary fertility bull was not different than the high fertility bulls, but low and high fertility bulls were different.

Richardson et al. (2017) reported differences in fertility by time between bulls A and D when cows were inseminated prior to estrus but not after the onset of estrus; however, bull D (intermediary fertility) had similar level of viability, zinc signature 2 and zinc signature 1 + 2 compared to bull A (high fertility). Thus, the mechanism for

decreased fertility of bull D compared to bull A is likely associated with other factors not related to sperm capacitation ability as measured in this study. It has been reported that pregnancy rates are increased in females that express behavioral estrus prior to AI compared to those that do not express behavioral estrus (Richardson et al., 2016). Uterine environment prior to onset of estrus may influence sperm from different bulls differently. It has been reported that uterine pH changes from ~7.3 prior to behavioral estrus to ~6.8 during behavioral estrus (under influence of elevated concentrations of estradiol) and return to ~7.3 after the end of behavioral estrus (Perry and Perry, 2008a, b). The authors hypothesized that the decrease in uterine pH during estrus would increase sperm longevity, while the increase in uterine pH after estrus would increase sperm motility (Perry and Perry, 2008a, b). In chapter 2, sperm longevity was increased when sperm was incubated in media adjusted to pH 6.8; however, bull to bull differences were not evaluated. Also, under the influence of elevated concentrations of estradiol, uterine gene expression and likely protein secretion were altered (Northrop et al., 2018; Perry et al., 2020; Northrop-Albrecht et al., 2021) and it is possible that these alterations affect sperm from different bulls differently. All these differences in the uterine environment may possibly be the reason for differences between bulls A and D. Also, differences in sperm surface proteins between bulls (Cancel et al., 1997; Dalton et al., 2012; Odhiambo et al., 2014) could be related to the previously reported differences in fertility (Richardson et al., 2017); however, results from this study cannot prove these hypotheses. Nevertheless, bull A and D had a similar reduction in the percentage of zinc signature 2 between 0 and 0 CM; but, bull A had an increase in zinc signature 3 while bull D maintained similar percentages, thus it is likely that bull A sperm was undergoing capacitation and bull D

sperm was dying, which can be observed by a numerical increase in sperm zinc signature 4 for bull D at 0 CM compared to bull A.

In the present study, the presence of, localization, and quantity of CD9 in relationship with bull fertility was evaluated. The protein CD9 has been well characterized in oocytes (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Rubinstein et al., 2006; Sutovsky, 2009; Zhou et al., 2009); however, on the sperm the characterization and function of CD9 is not fully understood. It has been reported that CD9 was present on the sperm of mice (Rubinstein et al., 2006; Barraud-Lange et al., 2007; 2012; Ito et al., 2010), boars (Kaewmala et al., 2011), and bulls (Antalíková et al., 2015). The localization of CD9 protein described here was similar and agrees with what has been previously described for bull sperm (Antalíková et al., 2015). Antalíková et al. (2015) reported that 75 to 85% of sperm were positive for CD9 with minimal change during capacitation; however, the proportion of sperm positive for CD9 observed in the present study was decreased compared to what has been reported (only 20 to 50% of sperm were positive for CD9) with the lowest percentage and the greatest percentage identified at 0 CM (bull D and E, respectively). Differences between the two studies could be related to breed (Holstein vs Angus), method of analysis (fixed samples vs "fresh" samples; primary and secondary antibody vs primary) or simply animal-to-animal variation; however, it was observed that dead sperm with a disrupted acrosome was strongly positively correlated with dead CD9+ sperm. This finding may indicate that CD9 is present in the inner portion of the acrosome and may be externalized during capacitation or CD9 can only be detected on sperm with a disrupted acrosome. This may explain the differences in CD9+ percentage identified between this and Antalíková et al.

(2015) results, since fixation of sperm can cause membrane permeabilization; however, acrosome status and CD9 were evaluated in separate assays. It was observed that one low fertility bull (bull E) had elevated concentrations of CD9 compared to other bulls among the dead sperm population; however, no differences were observed in the viable population. Fluorescence intensity of CD9 on dead sperm decreased over time which might be related to release of this protein, interestingly, viable sperm CD9 concentration greatly decreased when sperm was diluted with CM and slowly increased with incubation. Antalíková et al. (2015) reported a decrease of *in vitro* fertilization rate when sperm were treated with anti-CD9 antibodies compared to untreated sperm (64.4% vs. 89.4%, respectively). Interestingly, low fertility bulls had a greater proportion of dead CD9+ sperm compared to high and intermediary fertility bulls. Low fertility bull E had the greatest concentration of CD9; however, a correlation between field fertility and dead CD9+ was not observed, probably due to lack of power. Thus, CD9 protein assay, more specifically dead CD9+, may be a new negative marker of fertility.

In conclusion, both Low-CM and High-CM were able to induce sperm capacitation; however, at different rates, with High-CM inducing changes in sperm faster than Low-CM. Multiple analyses over time in capacitation media of viability, zinc signature 2, zinc signature 1 + 2 and dead CD9+ were able to estimate differences between low fertility bulls to high and intermediary fertility bulls. Also, TMOT at pre-wash, viability at 0 h and zinc signature 1 + 2 at 0 CM were able to estimate fertility differences between bulls. The inclusion of a viability, a zinc signature, or a CD9 protein assay in AI studs' quality control measurements may have the potential to predict bull

fertility; however, a larger number of bulls with known fertility need to be evaluated to validate these results.

Table 4.1. Bulls A-E P/AI[1], number of AI per research (breeding), number of collection dates used, range of d between first and last collection date evaluated and field fertility level assignment (adapted from Richardson et al., 2017; Zoca et al., 2020)

Bull	**P/AI[1], %**	**Breeding, n**		**Collection dates[4], n**	**Range[5], d**	**Fertility[6]**
	Zoca[2]	**Zoca[2]**	**Richardson[3]**			
A[7]	48.1[a]	1050	200	4	55	High
B	47.7[a]	1058		2	3	High
C	40.7[c]	1206		2	3	Low
D[7]	45.5[ab]	747	189	3	45	Intermediary
E	43.1[bc]	805		4	10	Low

	P/AI based on interval from estrus (0 h) to insemination from Richardson[3]								
	-26 h	-18 h	-12 h	-6 h	6 h	12 h	18 h	24 h	30 h
A, %	75	60	81	100	43	80	79	78	60
D, %	28	40	26	0	30	44	67	82	67

[1] P/AI = pregnancy per artificial insemination
[2] Zoca = numbers in the column adapted from Zoca et al. (2020)
[3] Richardson = numbers adapted from Richardson et al. (2017)
[4] Collection date = number of collection dates evaluated per bull in study II
[5] Range = range in d between first and last collection date evaluated
[6] Fertility = fertility level assigned based on Richardson et al. (2017) and Zoca et al. (2020)
[7] Bulls A and D were represented in both Richardson et al. (2017) and Zoca et al. (2020), bulls A and D represents bulls 1 and 2 from Richardson et al. (2017), respectively
[a-c] P/AI with different superscripts $P \leq 0.05$ (Zoca et al., 2020)

Table 4.2. Effect of treatment on sperm total (TMOT) and progressive (PROG) motility, plasma membrane integrity (viability), acrosome integrity (viable intact, viable disrupted, dead intact, dead disrupted), reactive oxygen species (ROS; viable ROS+, viable ROS-, dead ROS+, dead ROS-), and zinc signatures (zinc signature 1, 2, 3, 4, and 1 + 2).

Variable, %	Treatment[1]			SEM[2]	P-value
	No-CM	Low-CM	High-CM		
TMOT	8.9	9.1	9.1	2.1	0.98
PROG	2.6	3.0	3.4	1.0	0.24
Viability	21.8[a]	18.6[b]	14.6[c]	3.4	< 0.0001
Viable intact	37.3	37.8	37.4	5.7	0.92
Viable disrupted	3.5	3.9	3.8	1.1	0.30
Dead intact	30.8	29.6	30.2	4.7	0.27
Dead disrupted	26.7	26.8	26.6	2.3	0.97
Viable ROS+	9.5[a]	7.3[b*]	5.8[b¶]	2.0	0.0006
Viable ROS-	25.7	26.0	26.4	9.7	0.71
Dead ROS+	3.2	2.9	3.0	0.5	0.68
Dead ROS-	55.9[c]	58.3[b]	60.4[a]	4.6	< 0.0001
Zinc Signature 1[3]	2.1[a]	1.1[b]	0.7[b]	0.7	0.0036
Zinc Signature 2[4]	18.2[a]	13.2[b]	10.5[c]	3.2	< 0.0001
Zinc Signature 3[5]	62.1[b]	69.9[a]	63.0[b]	4.2	0.0003
Zinc Signature 4[6]	14.5[b]	12.2[b]	22.5[a]	3.6	< 0.0001
Zinc Signature 1 + 2[7]	20.9[a]	15.4[b]	12.0[c]	2.6	< 0.0001

[1] Treatments consisted of 1) semen diluted to 17 million sperm per mL with non-capacitation media (No-CM); 2) semen diluted to 20 million sperm per mL with non-capacitation media and to 17 million sperm per mL with capacitation media (CM; Low-CM); and 3) semen diluted to 40 million sperm per mL with non-capacitation media and to 17 million sperm per mL with CM (High-CM)
[2] SEM = Standard error of the means
[3] Zinc signature 1 = viable non-capacitated sperm with high intracellular zinc
[4] Zinc signature 2 = viable sperm in the process of capacitation with low intracellular zinc
[5] Zinc signature 3 = dead and capacitated sperm with high intracellular zinc in the mitochondrial sheath or the acrosome region or both
[6] Zinc signature 4 = dead sperm without zinc
[7] Zinc signature 1 + 2 = combination of signature 1 and signature 2
[a-c] Values within a row with different superscripts $P \leq 0.05$
[*,¶] Values within a row with different superscripts $P \leq 0.10$

Table 4.3. Effect of bull on sperm total (TMOT) and progressive (PROG) motility, plasma membrane integrity (viability), acrosome integrity (viable intact, viable disrupted, dead intact, dead disrupted), reactive oxygen species (ROS; viable ROS+, viable ROS-, dead ROS+, dead ROS-), mitochondrial membrane energy potential (mito-potential), zinc signatures (zinc signature 1, 2, 3, 4, and 1 + 2) and CD9 populations (viable CD9+, viable CD9-, dead CD9+, and dead CD9-).

Variable, %	**Bull**					**SEM[1]**	**P-value**
	A	**B**	**C**	**D**	**E**		
TMOT	10.0	9.1	9.1	8.7	8.5	2.7	0.98
PROG	4.4	1.9	3.1	1.3	1.1	17.5	0.92
Viability	23.2[a]	26.8[a]	16.9[b]	24.3[a]	13.6[b]	2.9	< 0.0001
Viable intact	46.3[a]	32.1[b¶]	33.3[b¶]	44.4[ab*]	33.7[b¶]	4.6	0.06
Viable disrupted	3.6[a*]	3.5[a]	3.8[a*]	2.6[a¶]	1.2[b]	0.5	< 0.0001
Dead intact	26.3	31.5	28.0	26.2	22.1	3.1	0.12
Dead disrupted	22.2[c]	33.1[b]	33.8[b]	25.4[c]	42.3[a]	2.3	< 0.0001
Viable ROS+	22.8	26.6	20.6	20.1	16.5	4.3	0.25
Viable ROS-	20.2	10.6	13.3	19.2	18.0	4.1	0.38
Dead ROS+	3.3	3.5	3.2	3.2	3.0	0.5	0.89
Dead ROS-	49.1[b]	55.4[ab]	62.5[a]	54.8[ab]	58.8[a]	3.9	0.03
Mito-potential	29.7	30.8	28.4	31.4	22.6	3.7	0.15
Zinc Signature 1[2]	2.4[b]	4.7[a]	4.9[a]	2.8[b]	1.0[c]	0.8	< 0.0001
Zinc Signature 2[3]	18.3[a]	17.4[a]	9.9[b]	18.9[a]	10.5[b]	2.4	0.001
Zinc Signature 3[4]	51.7	54.1	52.3	52.6	52.6	6.0	0.99
Zinc Signature 4[5]	19.8	19.5	24.3	22.6	29.1	7.0	0.64
Zinc Signature 1 + 2[6]	21.4[a]	23.1[a]	14.8[b]	22.7[a]	11.5[b]	2.6	< 0.0001
Viable CD9+	4.3[a]	4.0[a]	3.3[a]	3.3[ab*]	1.6[b¶]	0.8	0.06
Viable CD9-	39.5[a]	32.2[ab¶]	26.7[b]	44.4[a*]	28.5[b]	5.0	0.02
Dead CD9+	20.3[d]	26.4[c]	33.0[b]	20.5[d]	43.0[a]	1.9	< 0.0001
Dead CD9-	33.6	35.8	36.2	30.0	26.1	4.0	0.17

[1] SEM = Standard error of the means
[2] Zinc signature 1 = viable non-capacitated sperm with high intracellular zinc
[3] Zinc signature 2 = viable sperm in the process of capacitation with low intracellular zinc
[4] Zinc signature 3 = dead and capacitated sperm with high intracellular zinc in the mitochondrial sheath or the acrosome region or both
[5] Zinc signature 4 = dead sperm without zinc
[6] Zinc signature 1 + 2 = combination of signature 1 and signature 2
[a-d] Values within a row with different superscripts $P \leq 0.05$
[*,¶] Values within a row with different superscripts $P \leq 0.10$

Table 4.4. Pearson's correlation coefficient (shaded area above diagonal) and significance level (below diagonal) between CD9 populations [intact (viable) or disrupted (dead) sperm plasma membrane and CD9 positive (+) or negative (-)] and fluorescence intensities (FI; n = 70).

Correlation/ P-value	**Viable CD9+**	**Dead CD9+**	**Viable CD9-**	**Dead CD9-**	**Viable CD9 FI**	**Dead CD9 FI**
Viable CD9+		**-0.30**	0.02	-0.07	**0.61**	-0.13
Dead CD9+	**0.01**		**-0.57**	-0.16	-0.19	**0.54**
Viable CD9-	0.88	**< 0.01**		**-0.68**	-0.02	**0.21**
Dead CD9-	0.58	0.20	**< 0.01**		0.00	**-0.72**
Viable CD9 FI	**< 0.01**	0.11	0.88	0.98		0.18
Dead CD9 FI	0.27	**< 0.01**	**0.08**	**< 0.01**	0.15	

Table 4.5. Pearson's correlation coefficient of CD9 populations [intact (viable) or disrupted (dead) sperm plasma membrane and CD9 positive (+) or negative (-)] and fluorescence intensities (FI) with sperm total (TMOT) and progressive (PROG) motility, viability, acrosome integrity (viable intact, viable disrupted, dead intact, dead disrupted), reactive oxygen species (ROS; viable ROS+, viable ROS-, dead ROS-, dead ROS-), mitochondrial membrane potential (mito-potential), and zinc signatures (signature 1, signature 2, signature 3, signature 4, and signature 1 + 2).

Variables[1]	**Viable CD9+**	**Dead CD9+**	**Viable CD9-**	**Dead CD9-**	**Viable CD9 FI**	**Dead CD9 FI**
TMOT	-0.05	0.05	0.36**	-0.47**	-0.02	0.33**
PROG	0.16	-0.20*	0.48**	-0.44**	0.18	0.25**
Viability	0.16	-0.40**	0.61**	-0.42**	0.29	0.24**
Viable intact	0.15	-0.47**	0.84**	-0.63**	-0.08	0.13
Viable disrupted	0.32**	-0.40**	-0.22*	0.53**	0.17	-0.63**
Dead intact	0.13	-0.18	-0.60**	0.85**	0.17	-0.63**
Dead disrupted	-0.38**	0.87**	-0.51**	-0.07	-0.09	0.54**
Viable ROS+	0.19	-0.20*	0.51**	-0.49**	0.38**	0.36**
Viable ROS-	-0.03	-0.12	0.24**	-0.16	-0.28**	0.00
Dead ROS+	-0.23*	0.12	-0.22*	0.23*	-0.06	0.02
Dead ROS-	-0.12	0.27**	-0.65**	0.58**	-0.18	-0.38**
Mito-potential	0.09	-0.28**	0.61**	-0.50**	0.16	0.24**
Signature 1	-0.04	-0.21*	0.14	0.04	0.18	-0.11
Signature 2	0.46**	-0.48**	0.66**	-0.50**	0.53**	0.28**
Signature 3	-0.24**	0.08	0.12	-0.14	-0.04	0.23**
Signature 4	-0.04	0.20*	-0.44**	0.35**	-0.26**	-0.30**
Signature 1 + 2	0.43**	-0.50**	0.65**	-0.46**	0.54**	0.24**

[1] Ejaculates of 5 bulls evaluated at 0 h in a non-capacitation media, and 0, 3, 6 and 24 h in capacitation media (n = 70).

** $P < 0.05$

* $P \leq 0.10$

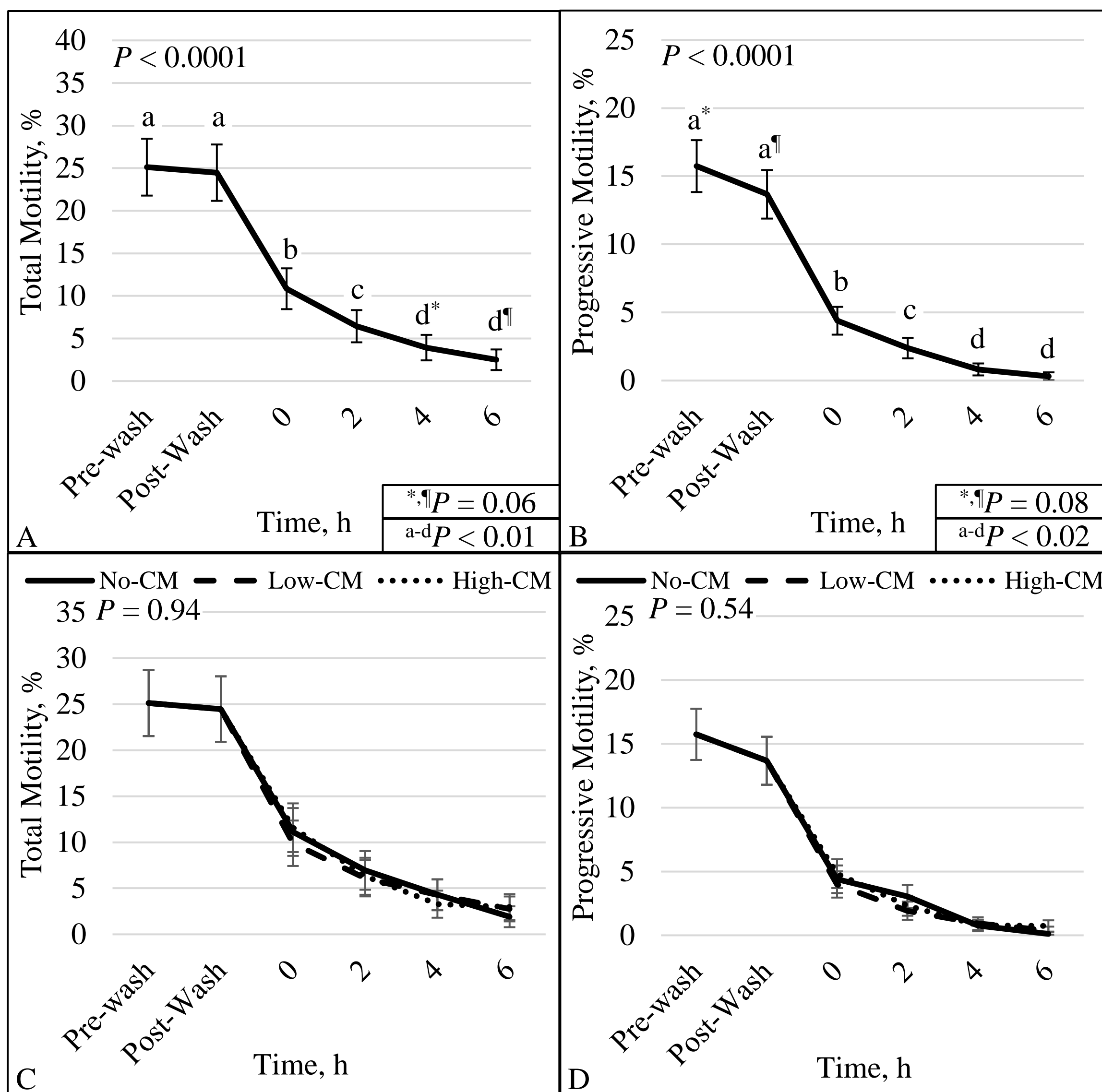


Figure 4.1. Effect of time (A, B) and treatment by time interaction (C, D) on sperm total (A, C) and progressive (B, D) motility. Treatments consisted of 1) semen diluted to 17 million sperm per mL with non-capacitation media (No-CM); 2) semen diluted to 20 million sperm per mL with non-capacitation media and to 17 million sperm per mL with capacitation media (CM; Low-CM); and 3) semen diluted to 40 million sperm per mL with non-capacitation media and to 17 million sperm per mL with CM (High-CM). Sperm were evaluated after thawing (Pre-Wash), after having been washed (Post-Wash), at dilution (0 h) and after 2, 4, 6 and 24 h incubation at 37 ºC. All samples at 24 h had zero percentage results for TMOT and PROG; thus, we were unable to statistically include these observations.

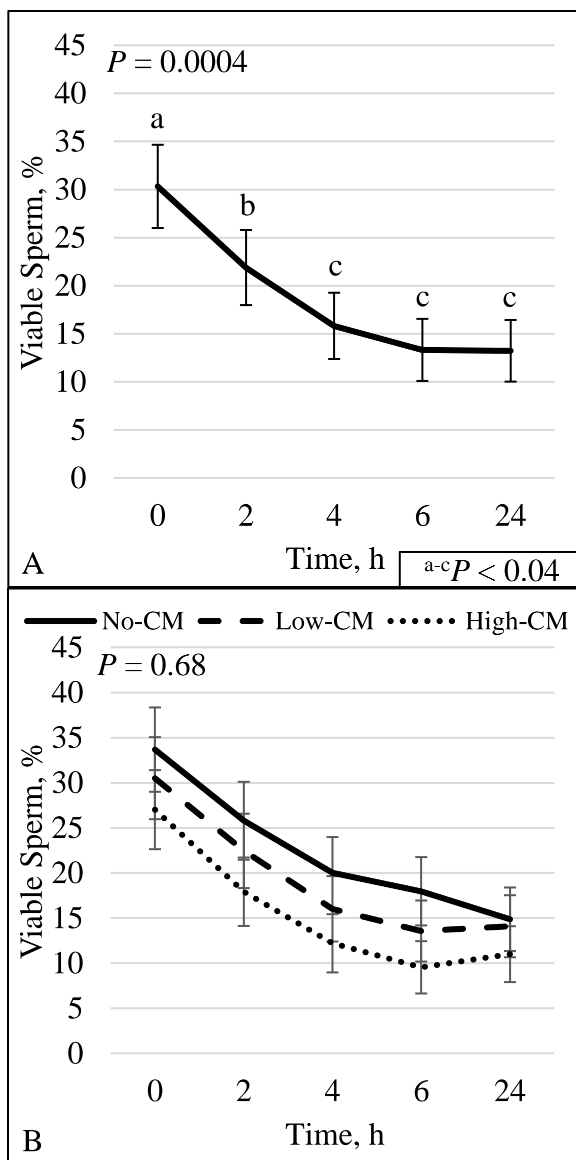


Figure 4.2. Effect of time (A) and treatment by time interaction (B) on sperm plasma membrane integrity (viability). Treatments consisted of 1) semen diluted to 17 million sperm per mL with non-capacitation media (No-CM); 2) semen diluted to 20 million sperm per mL with non-capacitation media and to 17 million sperm per mL with capacitation media (CM; Low-CM); and 3) semen diluted to 40 million sperm per mL with non-capacitation media and to 17 million sperm per mL with CM (High-CM). Sperm were evaluated at dilution (0 h) and after 2, 4, 6 and 24 h incubation at 37 ºC.

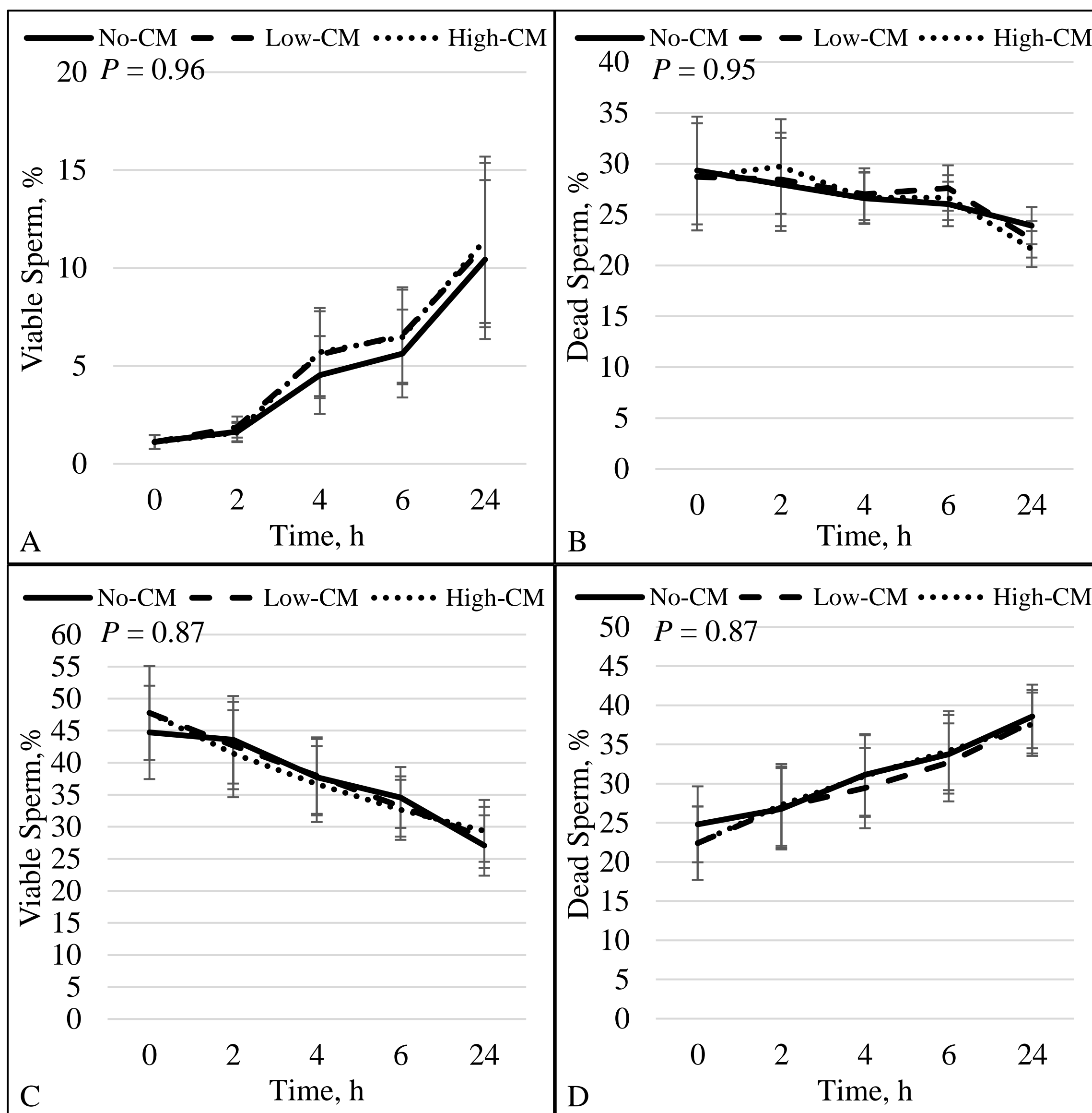


Figure 4.3. Effect of treatment by time interaction on the percentage of sperm with intact plasma membrane (viable) and disrupted acrosome (A), sperm with disrupted plasma membrane (dead) and disrupted acrosome (B), viable sperm with intact acrosome (C), and dead sperm with intact acrosome (D). Treatments consisted of 1) semen diluted to 17 million sperm per mL with non-capacitation media (No-CM); 2) semen diluted to 20 million sperm per mL with non-capacitation media and to 17 million sperm per mL with capacitation media (CM; Low-CM); and 3) semen diluted to 40 million sperm per mL with non-capacitation media and to 17 million sperm per mL with CM (High-CM). Sperm were evaluated at dilution (0 h) and after 2, 4, 6 and 24 h incubation at 37 ºC.

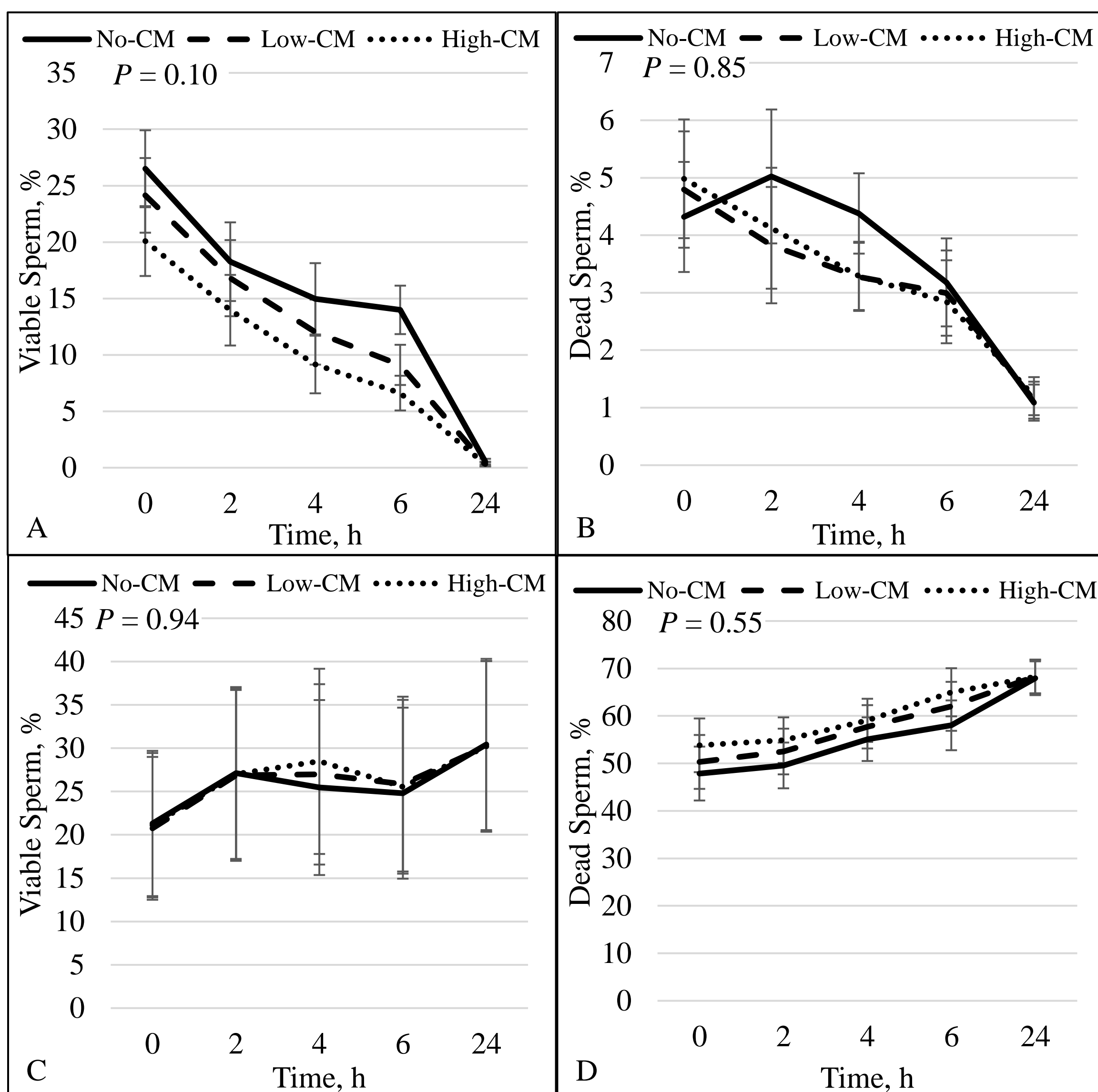


Figure 4.4. Effect of treatment and time interaction on sperm plasma membrane integrity and reactive oxygen species (ROS). The percentage of sperm with intact plasma membrane (viable) and ROS positive (A), sperm with disrupted plasma membrane (dead) and ROS positive (B), sperm viable and ROS negative (C), and sperm dead and ROS negative (D). Treatments consisted of 1) semen diluted to 17 million sperm per mL with non-capacitation media (No-CM); 2) semen diluted to 20 million sperm per mL with non-capacitation media and to 17 million sperm per mL with capacitation media (CM; Low-CM); and 3) semen diluted to 40 million sperm per mL with non-capacitation media and to 17 million sperm per mL with CM (High-CM). Sperm were evaluated at dilution (0 h) and after 2, 4, 6 and 24 h incubation at 37 ºC.

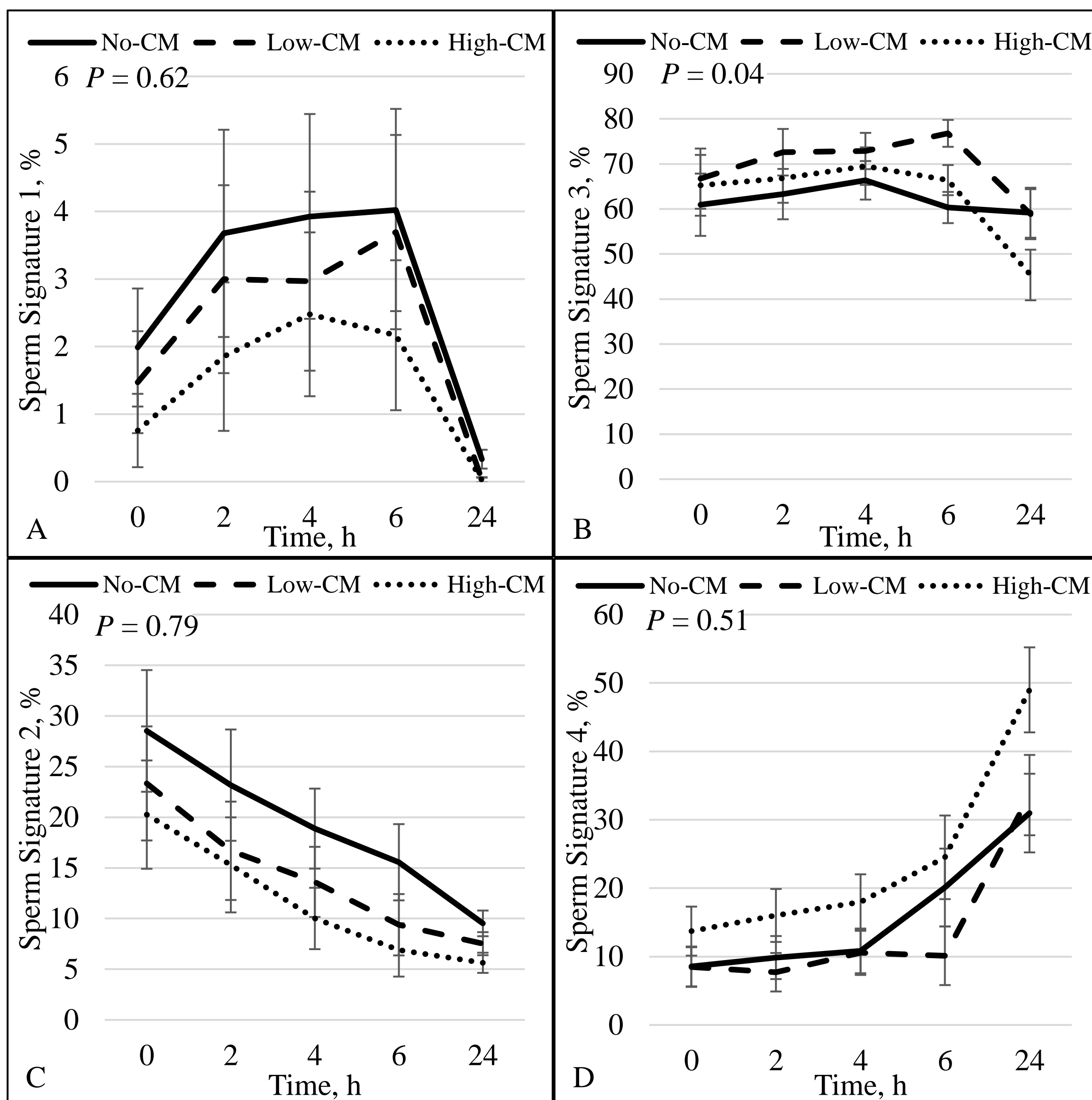


Figure 4.5. Effect of treatment by time interaction on sperm plasma membrane integrity and zinc concentration. A) The percentage of sperm with intact plasma membrane (viable) and high zinc concentration (signature 1; sperm not capacitated). B) The percentage of sperm with disrupted plasma membrane (dead) and high zinc concentration (signature 3; sperm that capacitated and died). C) The percentage of viable sperm with low zinc (signature 2; sperm undergoing capacitation). D) The percentage of dead sperm with no zinc (signature 4; dead sperm that may or may not have gone through capacitation before dying). Treatments consisted of 1) semen diluted to 17 million sperm per mL with non-capacitation media (No-CM); 2) semen diluted to 20 million sperm per mL with non-capacitation media and to 17 million sperm per mL with capacitation media (CM; Low-CM); and 3) semen diluted to 40 million sperm per mL with non-capacitation media and to 17 million sperm per mL with CM (High-CM). Sperm were evaluated at dilution (0 h) and after 2, 4, 6 and 24 h incubation at 37 ºC.

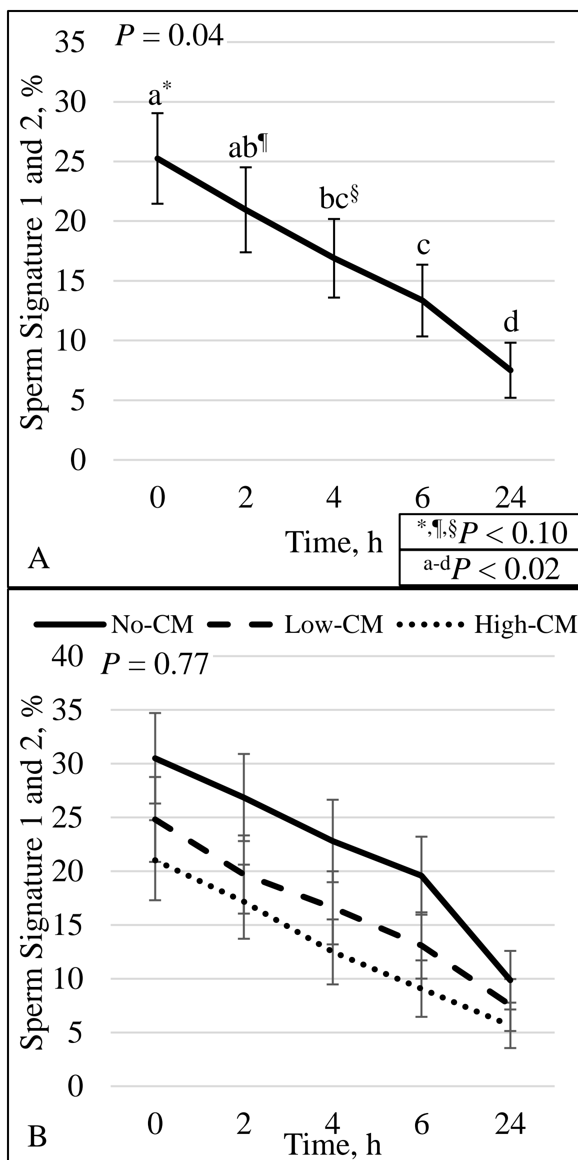


Figure 4.6. Effect of time (A), and treatment by time interaction (B) on sperm with intact plasma membrane (viable) and high and low zinc concentration combined (signatures 1 and 2 combined). Treatments consisted of 1) semen diluted to 17 million sperm per mL with non-capacitation media (No-CM); 2) semen diluted to 20 million sperm per mL with non-capacitation media and to 17 million sperm per mL with capacitation media (CM; Low-CM); and 3) semen diluted to 40 million sperm per mL with non-capacitation media and to 17 million sperm per mL with CM (High-CM). Sperm were evaluated at dilution (0 h) and after 2, 4, 6 and 24 h incubation at 37 ºC.

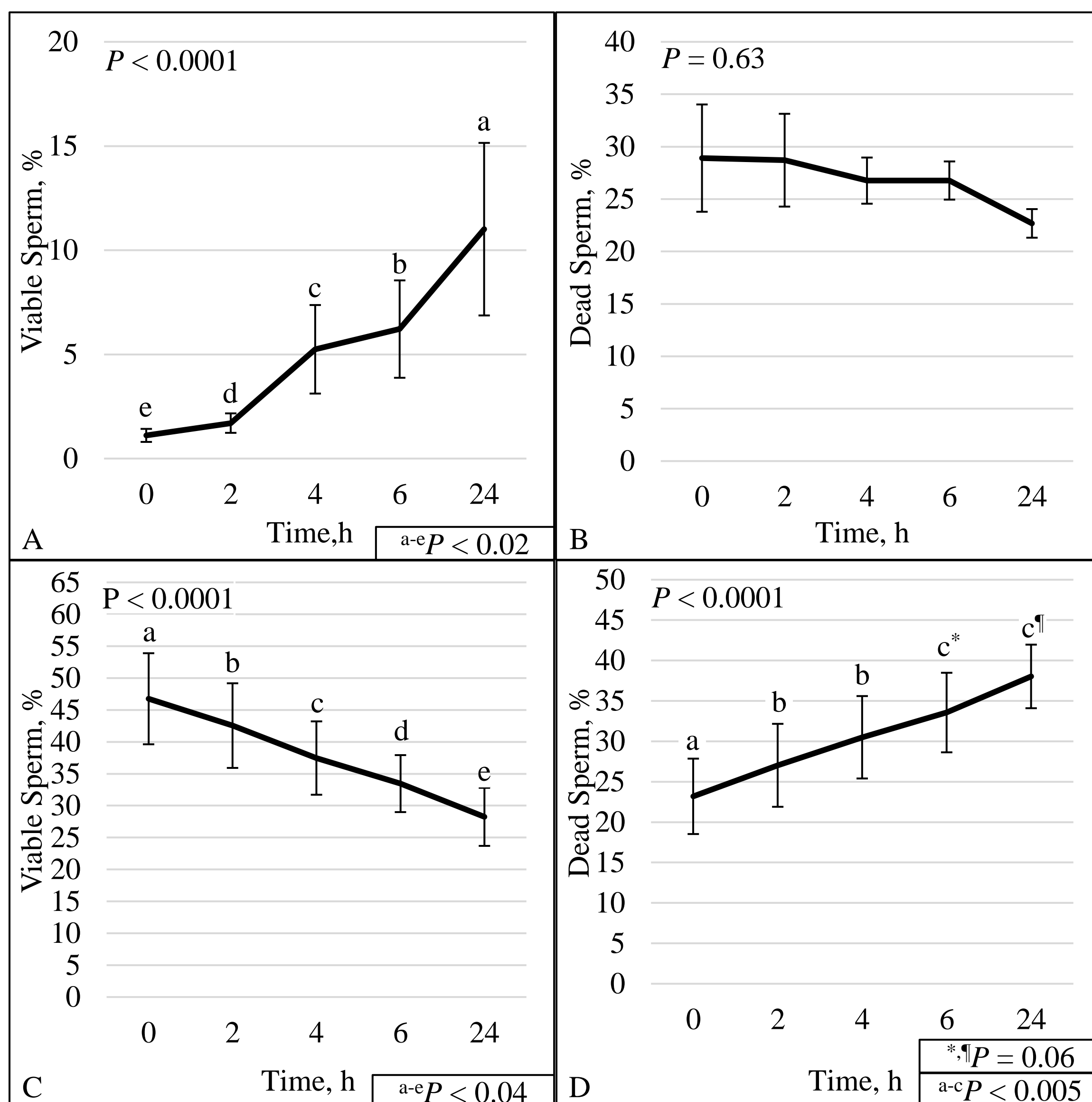


Figure 4.7. Effect of time on the percentage of sperm with intact plasma membrane (viable) and disrupted acrosome (A), sperm with disrupted plasma membrane (dead) and disrupted acrosome (B), viable sperm with intact acrosome (C), and dead sperm with intact acrosome (D). Sperm were evaluated at dilution (0 h) and after 2, 4, 6 and 24 h incubation at 37 ºC.

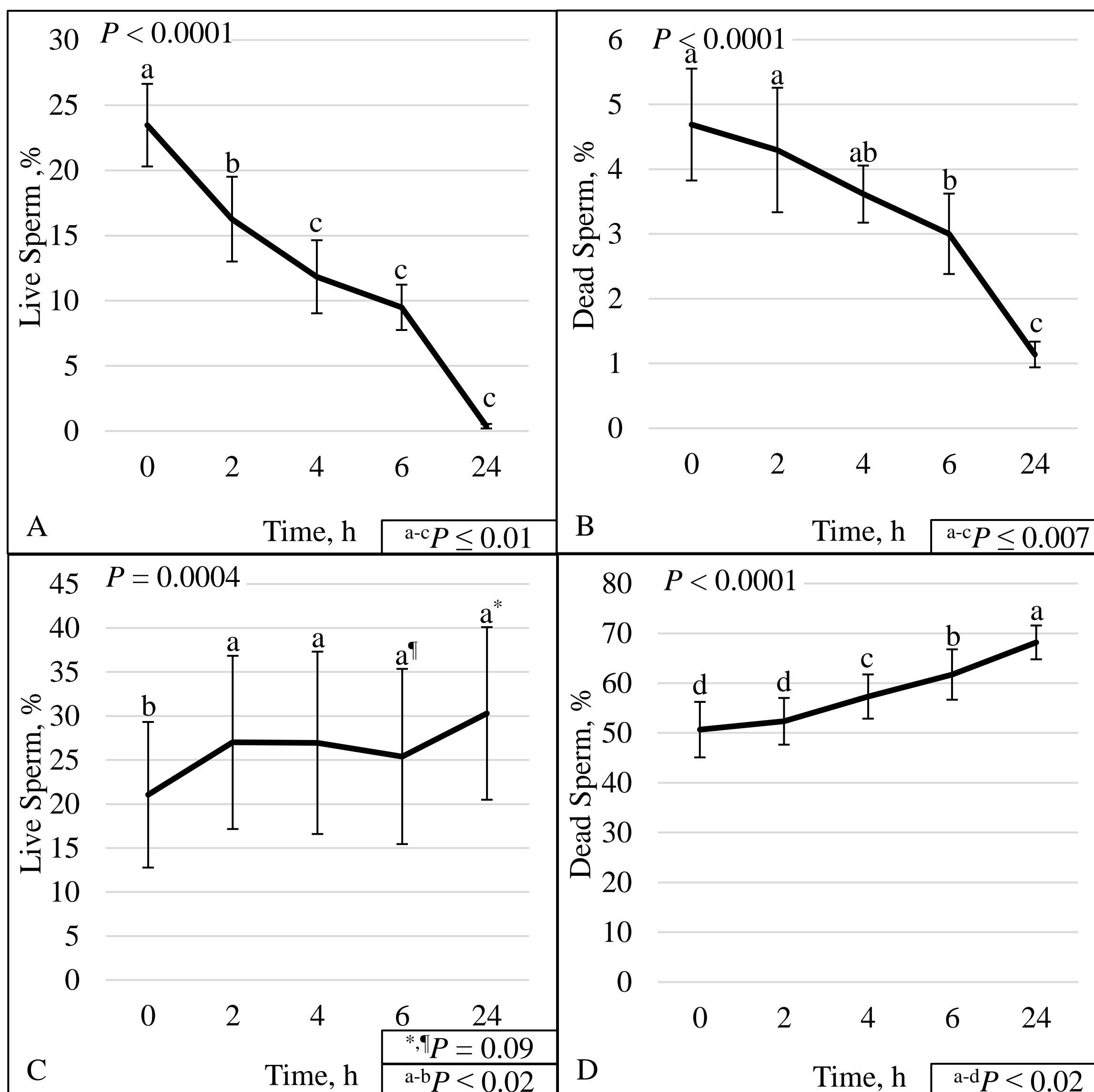


Figure 4.8. Effect of time on sperm plasma membrane integrity and reactive oxygen species (ROS). The percentage of sperm with intact plasma membrane (viable) and ROS positive (A). The percentage of sperm with disrupted plasma membrane (dead) and ROS positive (B). The percentage of sperm viable and ROS negative (C). The percentage of sperm dead and ROS negative (D). Sperm were evaluated at dilution (0 h) and after 2, 4, 6 and 24 h incubation at 37 ºC.

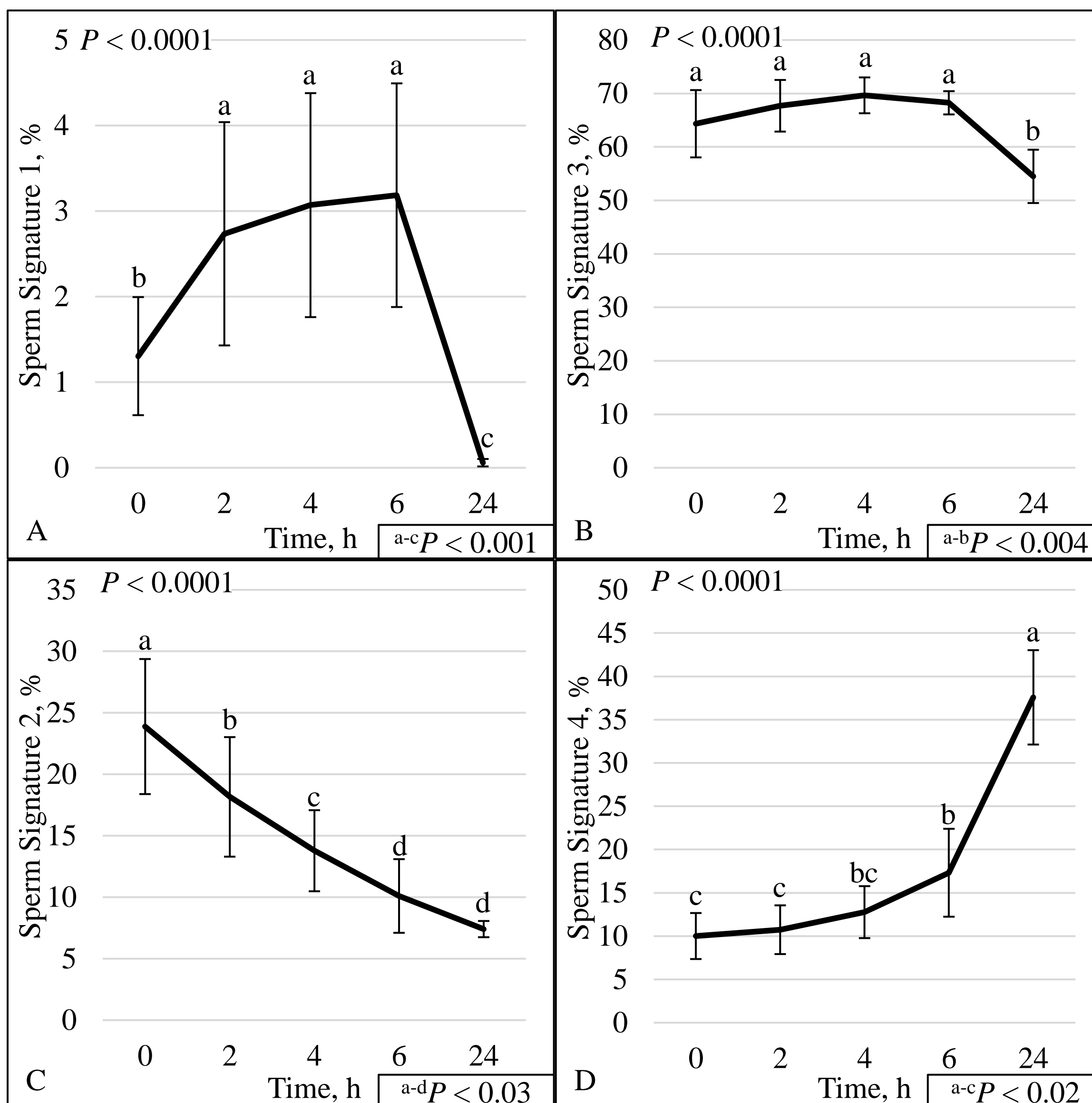


Figure 4.9. Effect of time on sperm plasma membrane integrity and zinc concentration. A) The percentage of sperm with intact plasma membrane (viable) and high zinc concentration (signature 1; sperm not capacitated). B) The percentage of sperm with disrupted plasma membrane (dead) and high zinc concentration (signature 3; sperm that capacitated and died). C) The percentage of viable sperm with low zinc (signature 2; sperm undergoing capacitation). D) The percentage of dead sperm with no zinc (signature 4; dead sperm that may or may not have gone through capacitation before dying). Sperm were evaluated at dilution (0 h) and after 2, 4, 6 and 24 h incubation at 37 ºC.

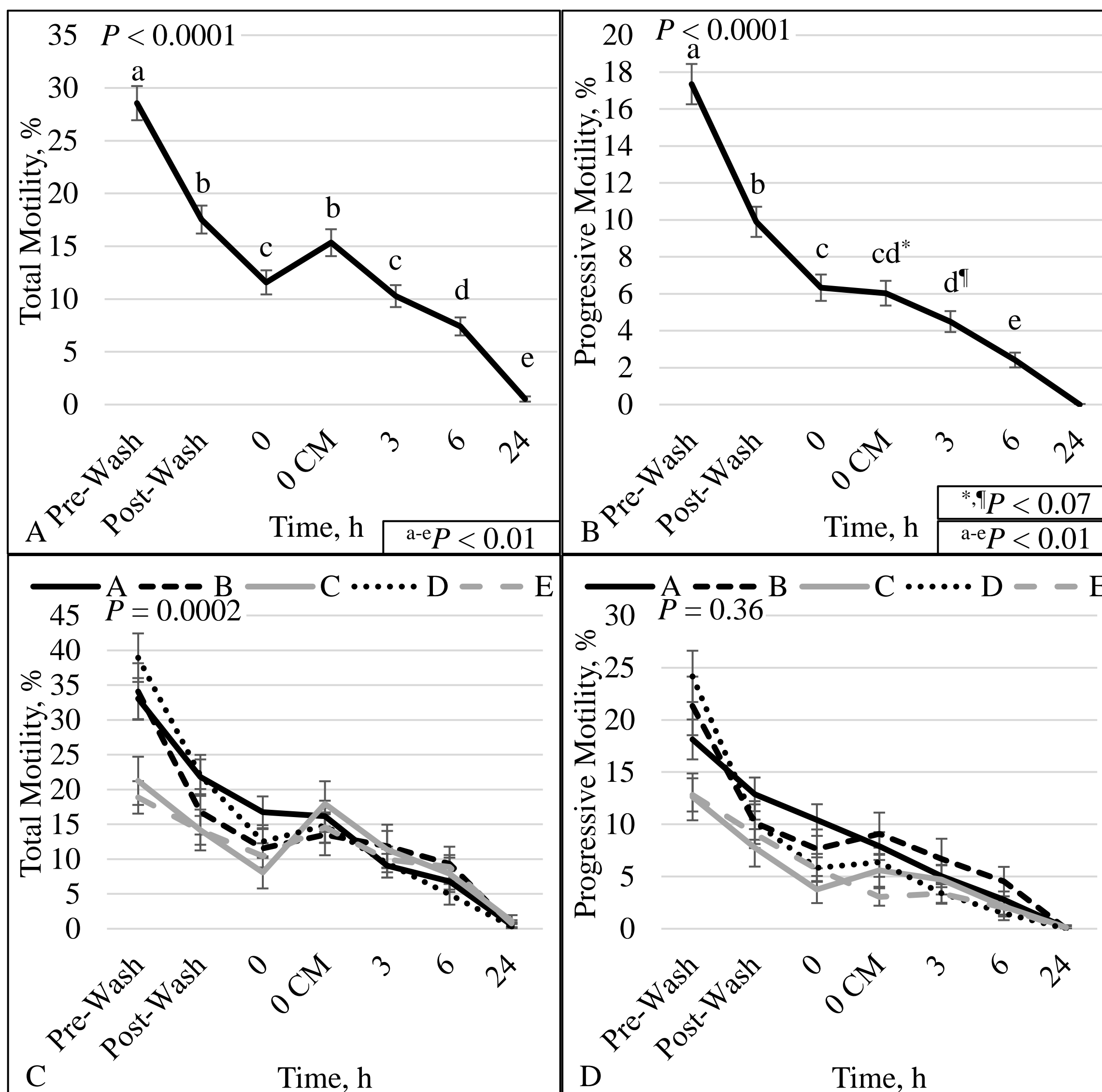


Figure 4.10. Effect of time (A, B) and bull by time interaction (C, D) on sperm total (A, C) and progressive (B, D) motility. Bulls that were previously classified as high (A, B), low (C, E), and intermediary (D) fertility were evaluated for total and progressive motility by computer-assisted sperm analysis. Sperm were evaluated after thawing (Pre-Wash), after being washed (Post-Wash), and after 0 (diluted in non-capacitation media), 0 CM (diluted in capacitation media), 2, 4, 6 and 24 h of incubation at 37 ºC.

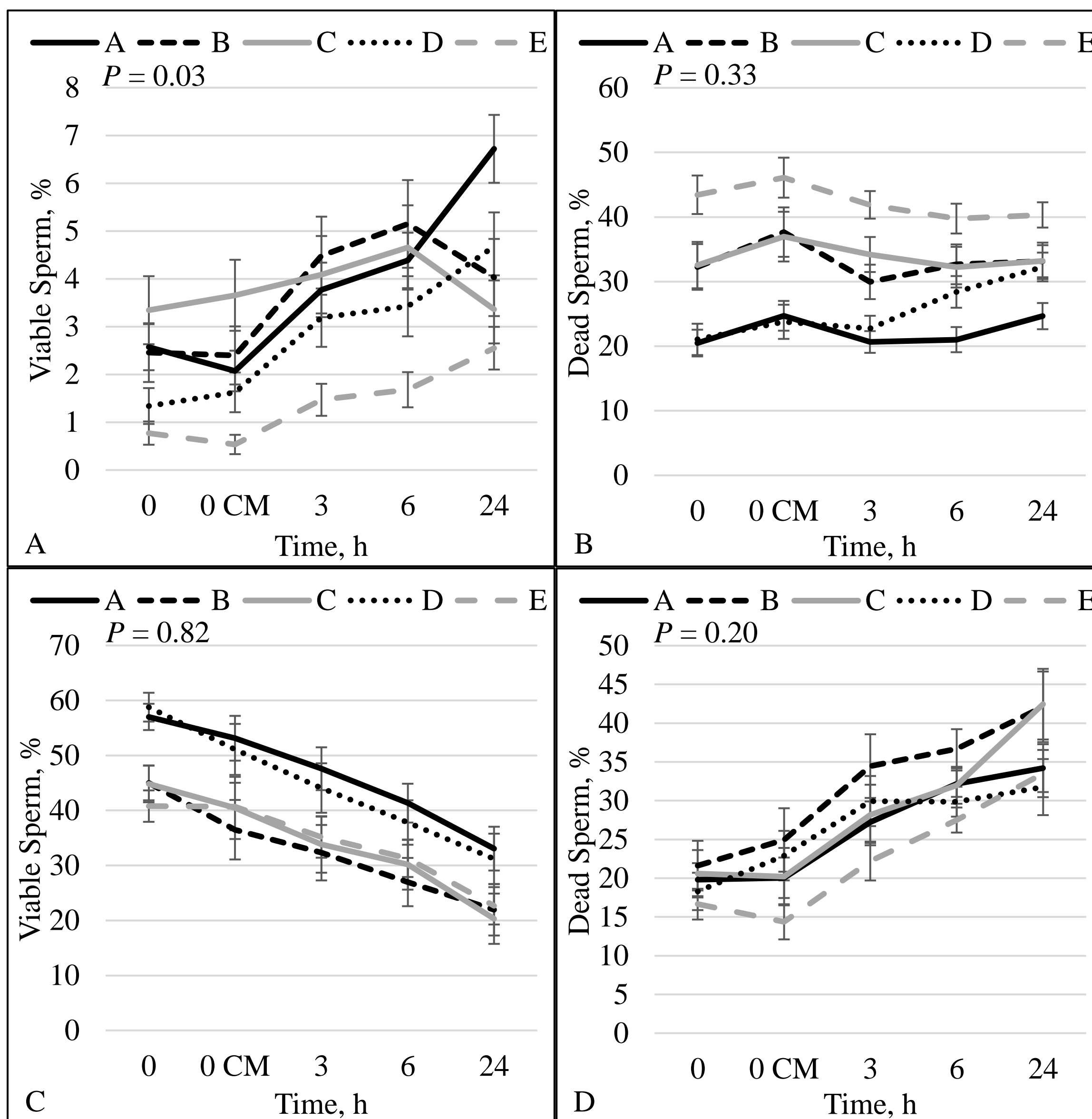


Figure 4.11. Effect of bull by time on the percentage of sperm with intact plasma membrane (viable) and disrupted acrosome (A), sperm with disrupted plasma membrane (dead) and disrupted acrosome (B), viable sperm with intact acrosome (C), and dead sperm with intact acrosome (D). Bulls that were previously classified as high (A, B), low (C, E), and intermediary (D) fertility were evaluated for sperm plasma membrane and acrosome integrity by flow cytometry. Sperm were evaluated at 0 (diluted in non-capacitation media), 0 CM (diluted in capacitation media), 2, 4, 6 and 24 h of incubation at 37 ºC.

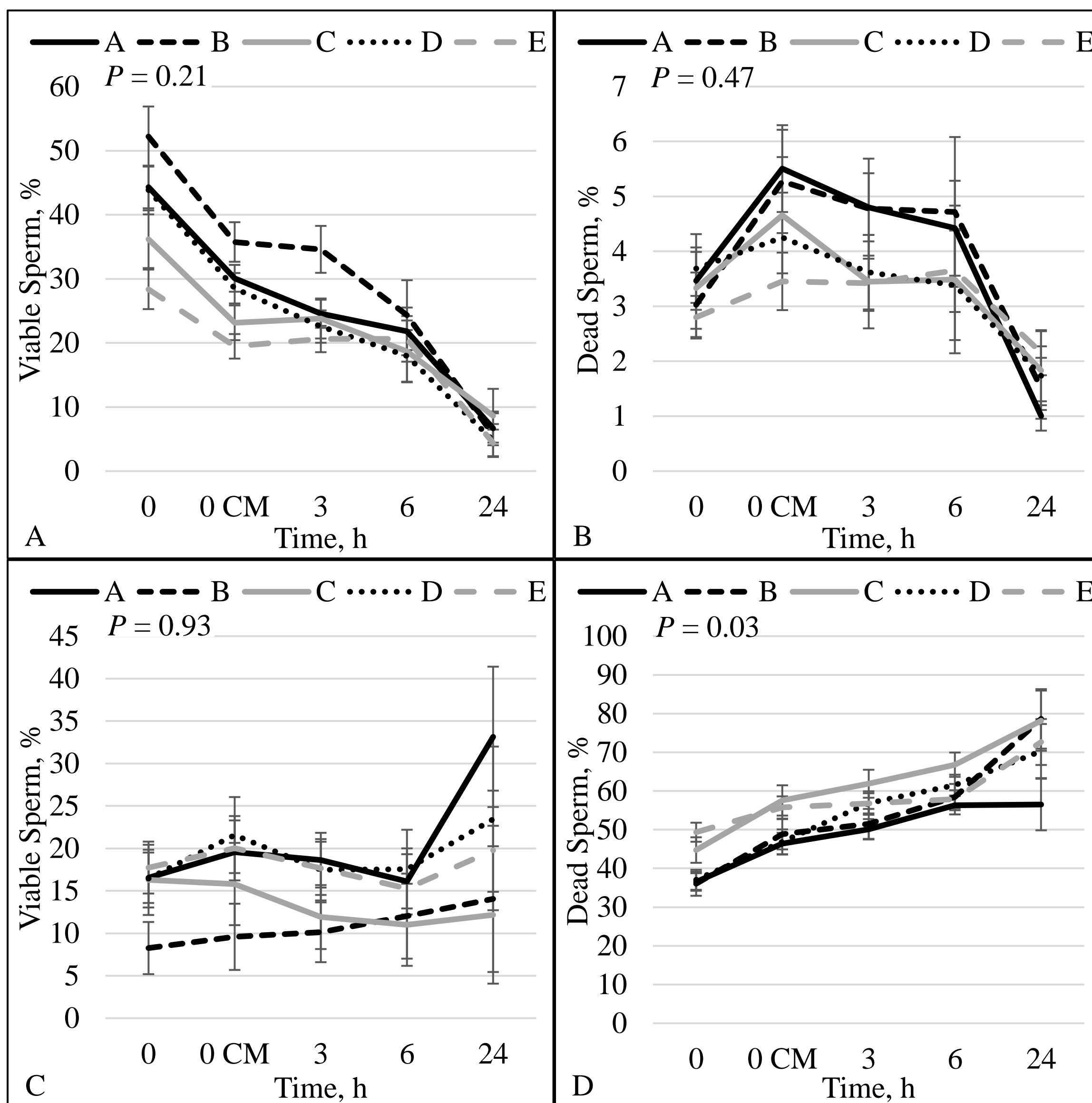


Figure 4.12. Effect of bull by time interaction on sperm plasma membrane integrity (viability) and reactive oxygen species (ROS). The percentage of sperm with intact plasma membrane (viable) and ROS positive (A), sperm with disrupted plasma membrane (dead) and ROS positive (B), sperm viable and ROS negative (C), and sperm dead and ROS negative (D). Bulls that were previously classified as high (A, B), low (C, E), and intermediary (D) fertility were evaluated for viability and ROS by flow cytometry. Sperm were evaluated at 0 (diluted in non-capacitation media), 0 CM (diluted in capacitation media), 2, 4, 6 and 24 h of incubation at 37 ºC.

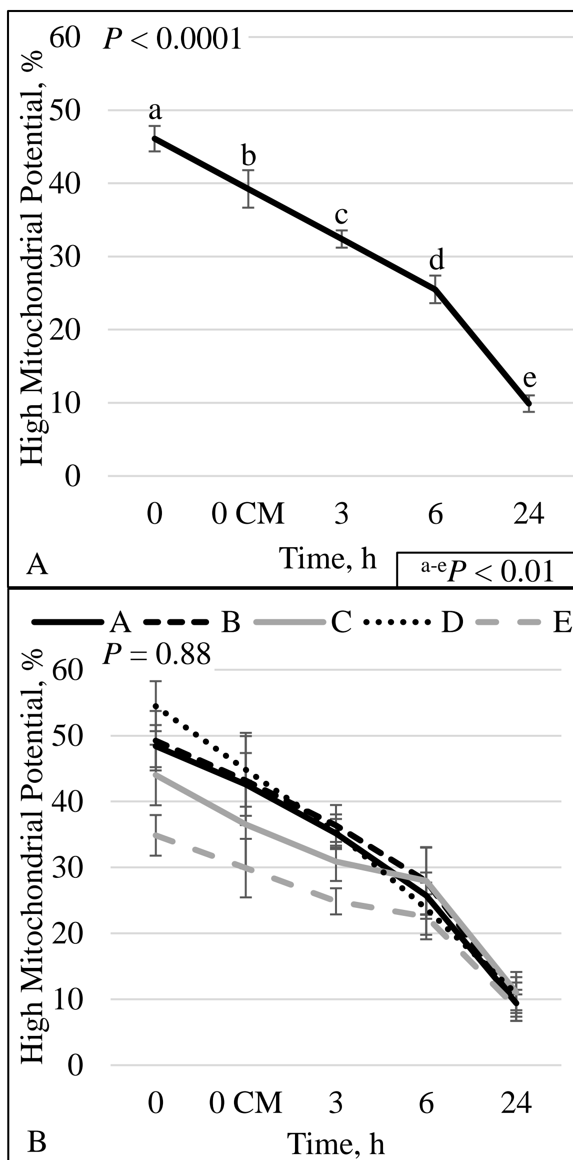


Figure 4.13. Effect of time (A) and bull by time interaction (B) on sperm mitochondrial membrane energy potential (mito-potential). Bulls that were previously classified as high (A, B), low (C, E), and intermediary (D) fertility were evaluated for mito-potential by flow cytometry. Sperm were evaluated at 0 (diluted in non-capacitation media), 0 CM (diluted in capacitation media; CM), 2, 4, 6 and 24 h of incubation at 37 ºC.

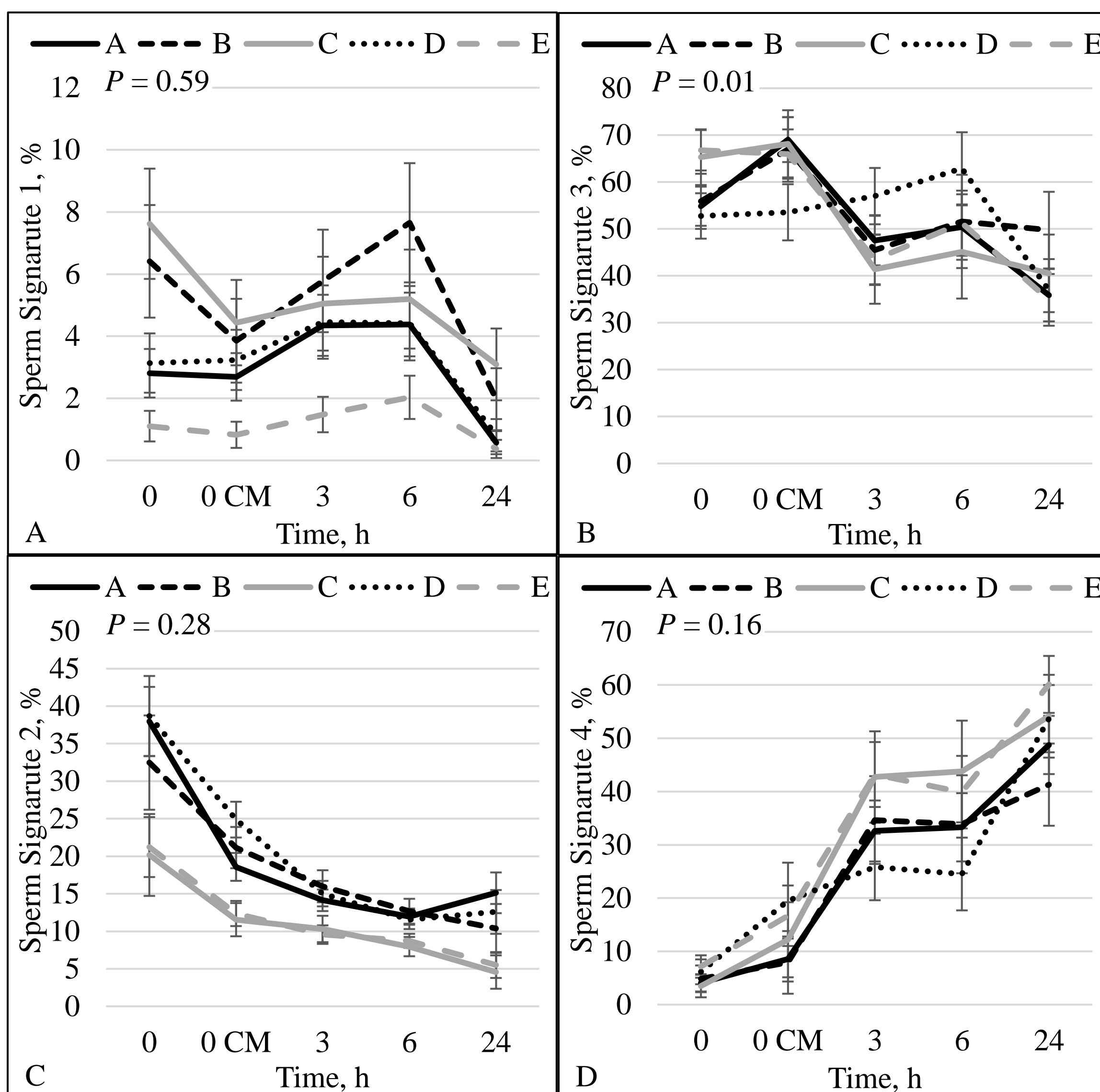


Figure 4.14. Effect of bull by time interaction on sperm plasma membrane integrity (viability) and zinc concentration. A) The percentage of sperm with intact plasma membrane (viable) and high zinc concentration (signature 1; sperm not capacitated). B) The percentage of sperm with disrupted plasma membrane (dead) and high zinc concentration (signature 3; sperm that capacitated and died). C) The percentage of viable sperm with low zinc (signature 2; sperm undergoing capacitation). D) The percentage of dead sperm with no zinc (signature 4; dead sperm that may or may not have gone through capacitation before dying). Bulls that were previously classified as high (A, B), low (C, E), and intermediary (D) fertility were evaluated for viability and zinc concentration by flow cytometry. Sperm were evaluated at 0 (diluted in non-capacitation media), 0 CM (diluted in capacitation media; CM), 2, 4, 6 and 24 h of incubation at 37 ºC.

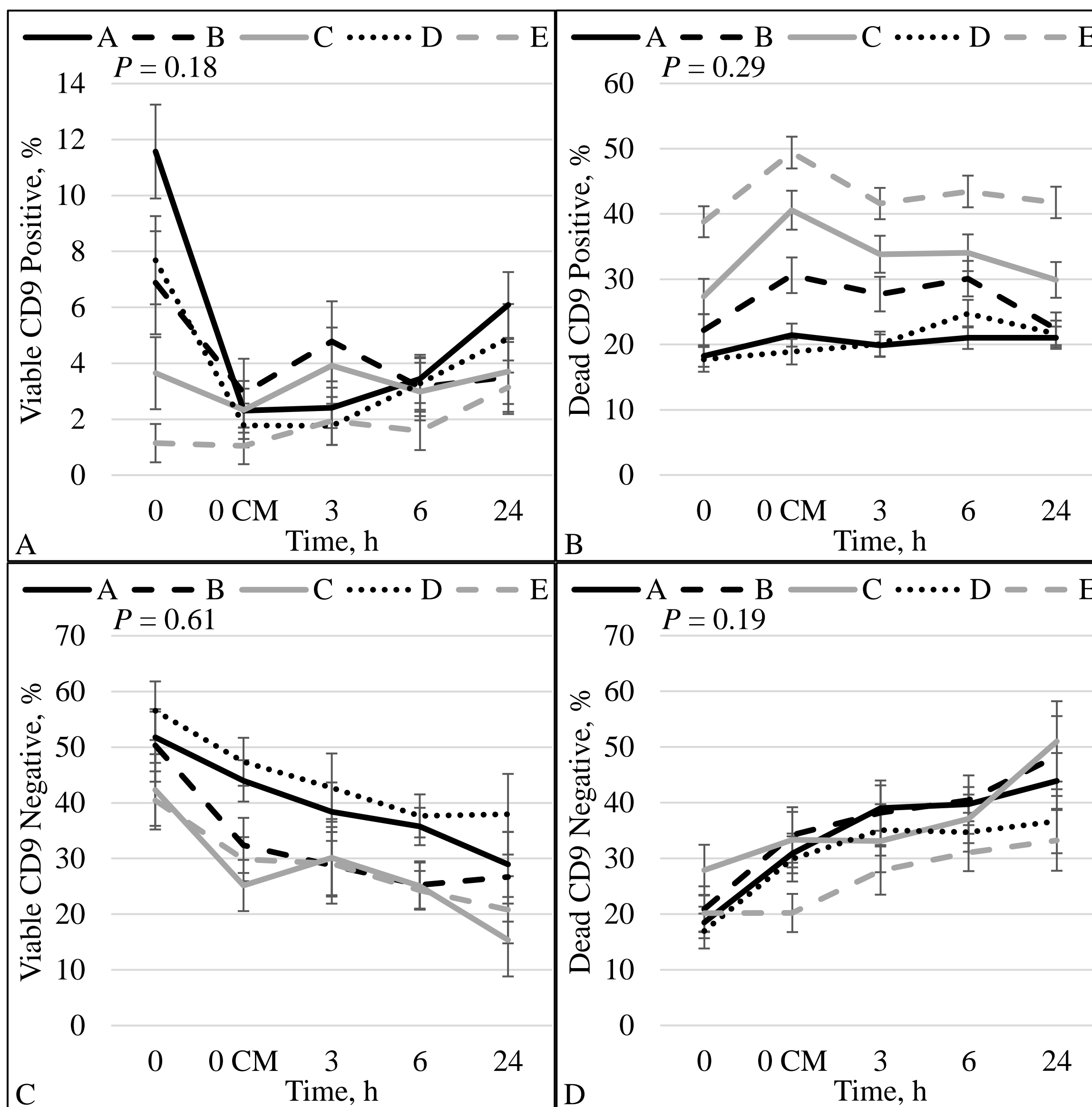


Figure 4.15. Effect of bull by time interaction on sperm plasma membrane integrity (viability) and CD9 protein. A) The percentage of sperm with intact plasma membrane (viable) and CD9 positive. B) The percentage of sperm with disrupted plasma membrane (dead) and CD9 positive. C) The percentage of viable sperm and CD9 negative. D) The percentage of dead sperm and CD9 negative. Bulls that were previously classified as high (A, B), low (C, E), and intermediary (D) fertility were evaluated for viability and CD9 protein (IVA50, Invitrogen) by flow cytometry. Sperm were evaluated at 0 (diluted in non-capacitation media), 0 CM (diluted in capacitation media; CM), 2, 4, 6 and 24 h of incubation at 37 ºC.

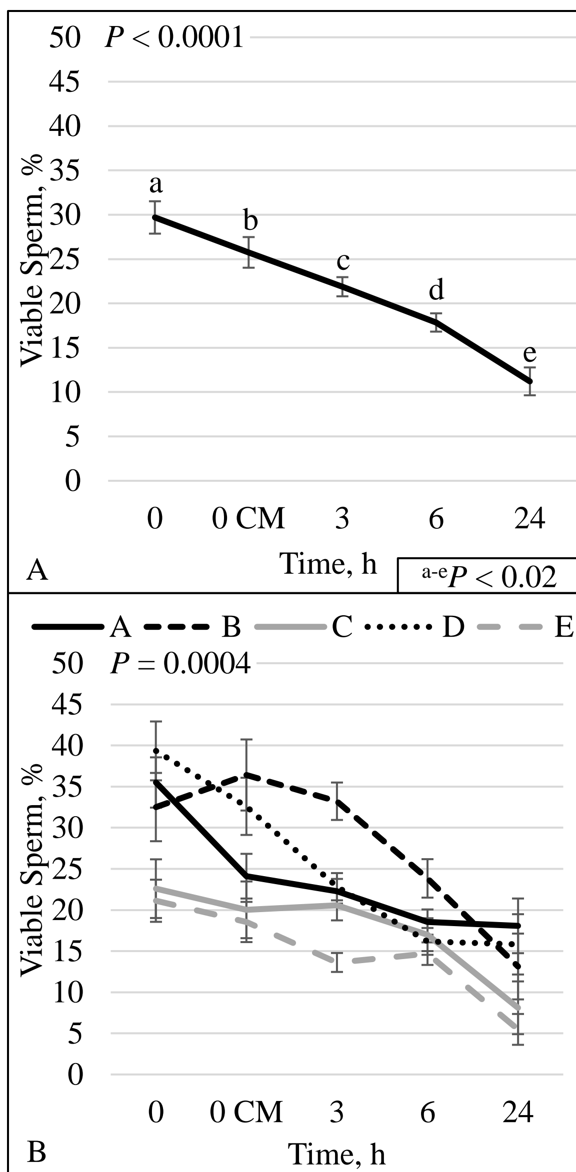


Figure 4.16. Effect time (A) and bull by time interaction (B) on sperm plasma membrane integrity (viability). Bulls that were previously classified as high (A, B), low (C, E), and intermediary (D) fertility were evaluated for viability by flow cytometry. Sperm were evaluated at 0 (diluted in non-capacitation media), 0 CM (diluted in capacitation media), 2, 4, 6 and 24 h of incubation at 37 ºC.

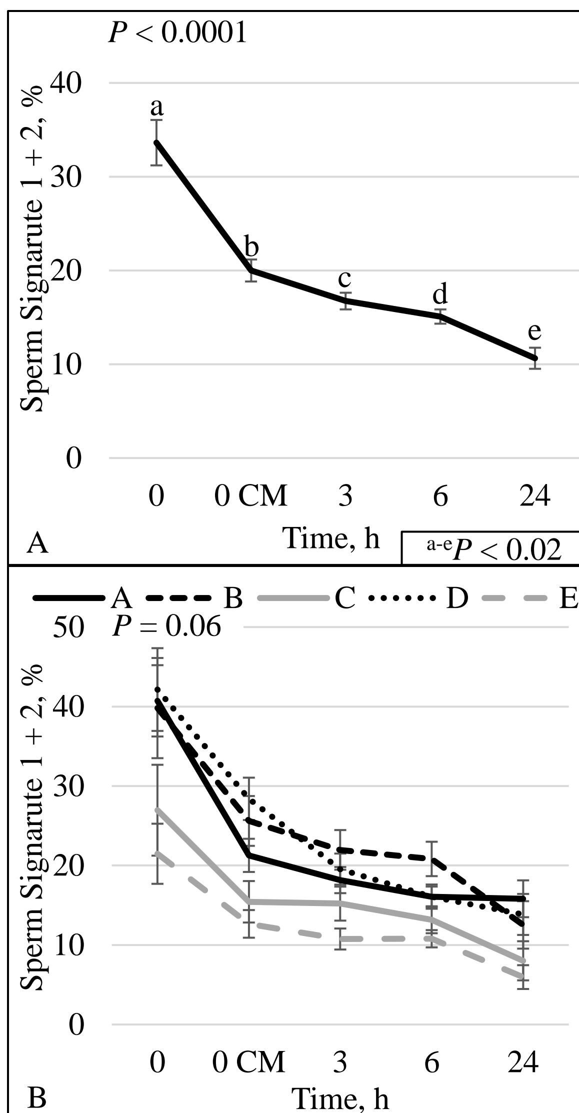


Figure 4.17. Effect of time (A) and bull by time interaction (B) on the percentage of sperm with an intact plasma membrane (viable) with high (signature 1) and low (signature 2) zinc concentration combined (signature 1 + 2). Bulls that were previously classified as high (A, B), low (C, E), and intermediary (D) fertility were evaluated for viability and zinc concentration by flow cytometry. Sperm were evaluated at 0 (diluted in non-capacitation media), 0 CM (diluted in capacitation media; CM), 2, 4, 6 and 24 h of incubation at 37 ºC.

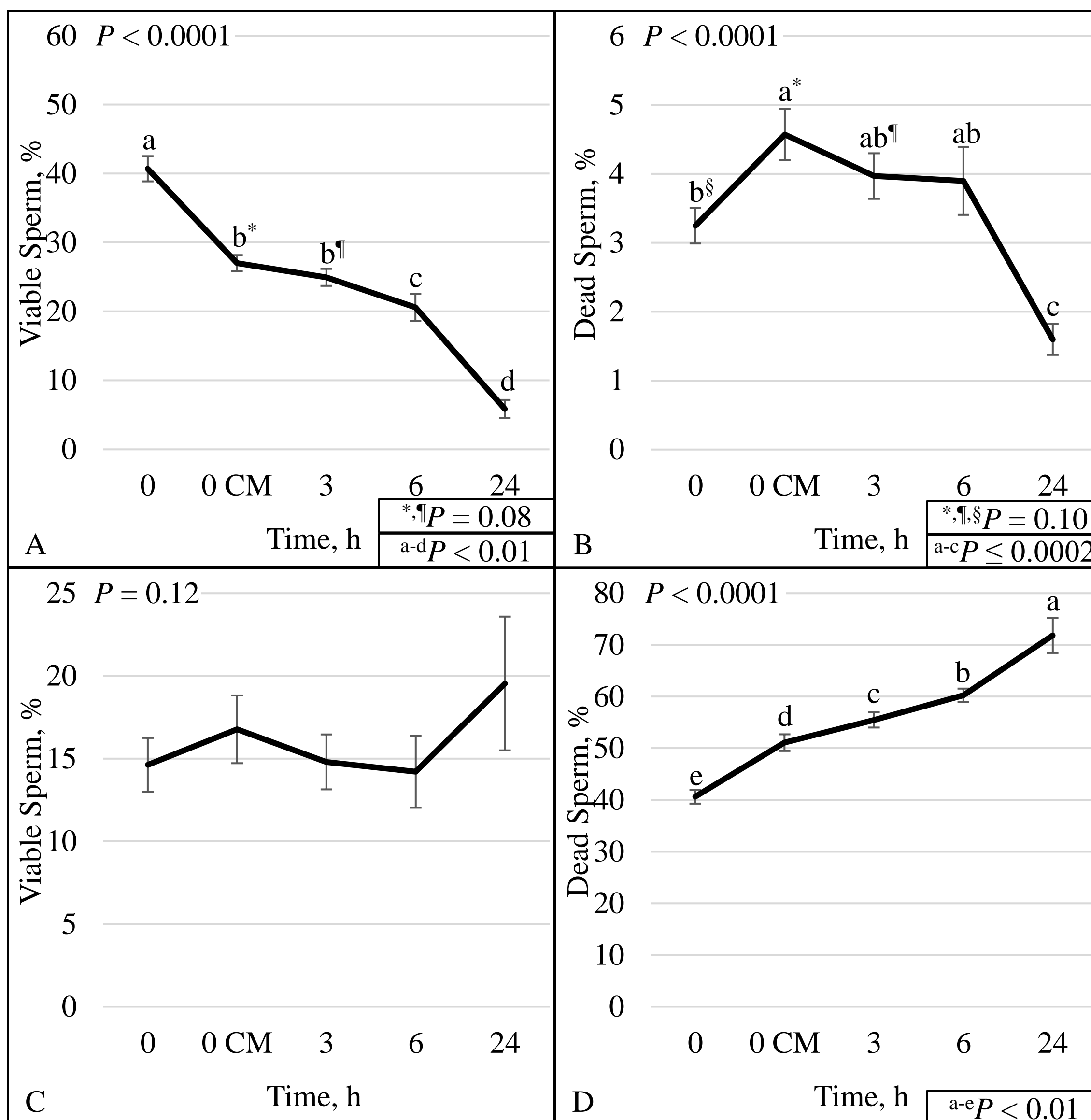


Figure 4.18. Effect of time on sperm plasma membrane integrity and reactive oxygen species (ROS). The percentage of sperm with intact plasma membrane (viable) and ROS positive (A), sperm with disrupted plasma membrane (dead) and ROS positive (B), sperm viable and ROS negative (C), and sperm dead and ROS negative (D). Sperm were evaluated by flow cytometry at 0 (diluted in non-capacitation media), 0 CM (diluted in capacitation media; CM), 2, 4, 6 and 24 h of incubation at 37 ºC.

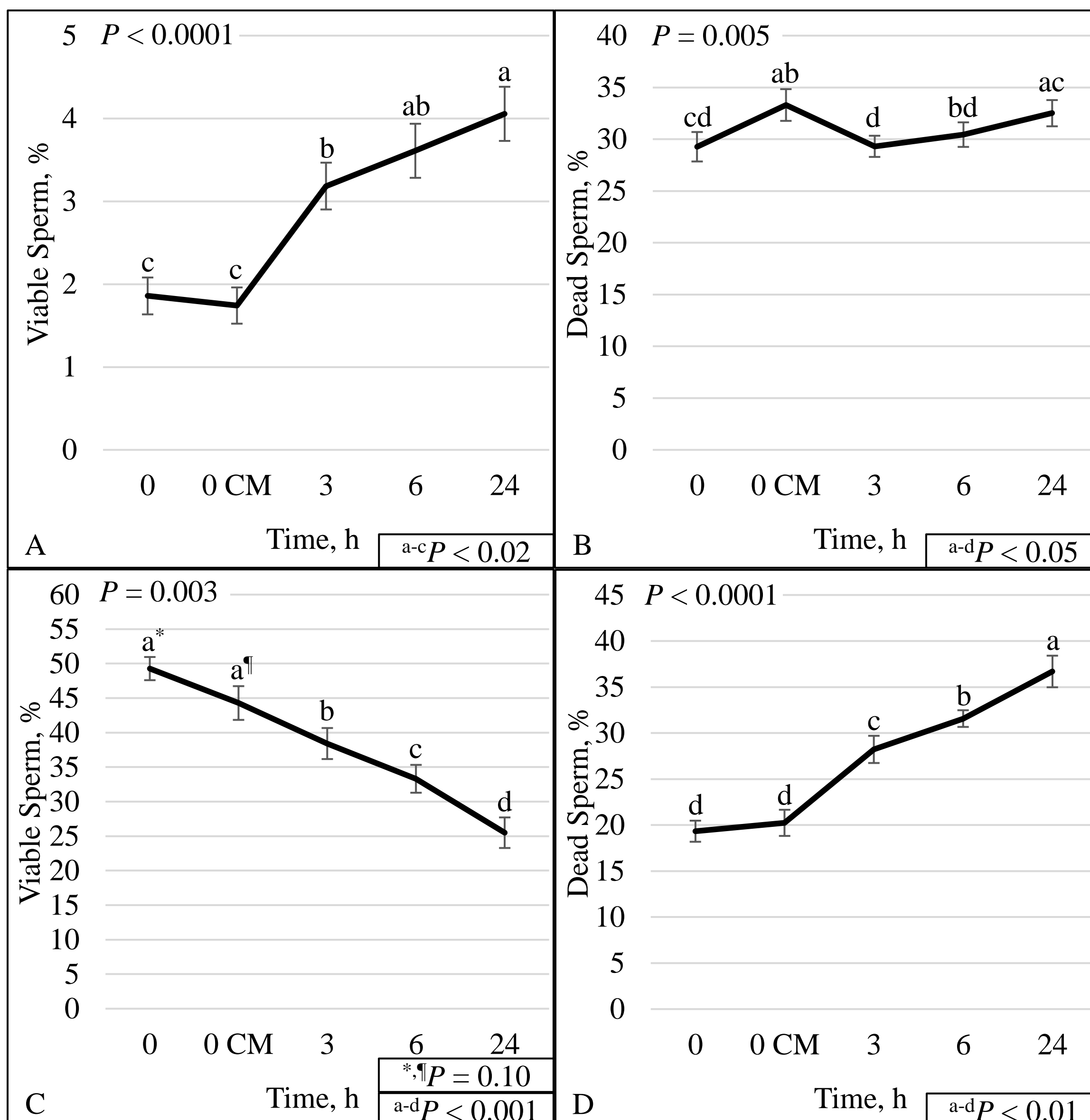


Figure 4.19. Effect of time on the percentage of sperm with intact plasma membrane (viable) and disrupted acrosome (A), sperm with disrupted plasma membrane (dead) and disrupted acrosome (B), viable sperm with intact acrosome (C), and dead sperm with intact acrosome (D). Sperm were evaluated by flow cytometry at 0 (diluted in non-capacitation media), 0 CM (diluted in capacitation media), 2, 4, 6 and 24 h of incubation at 37 ºC.

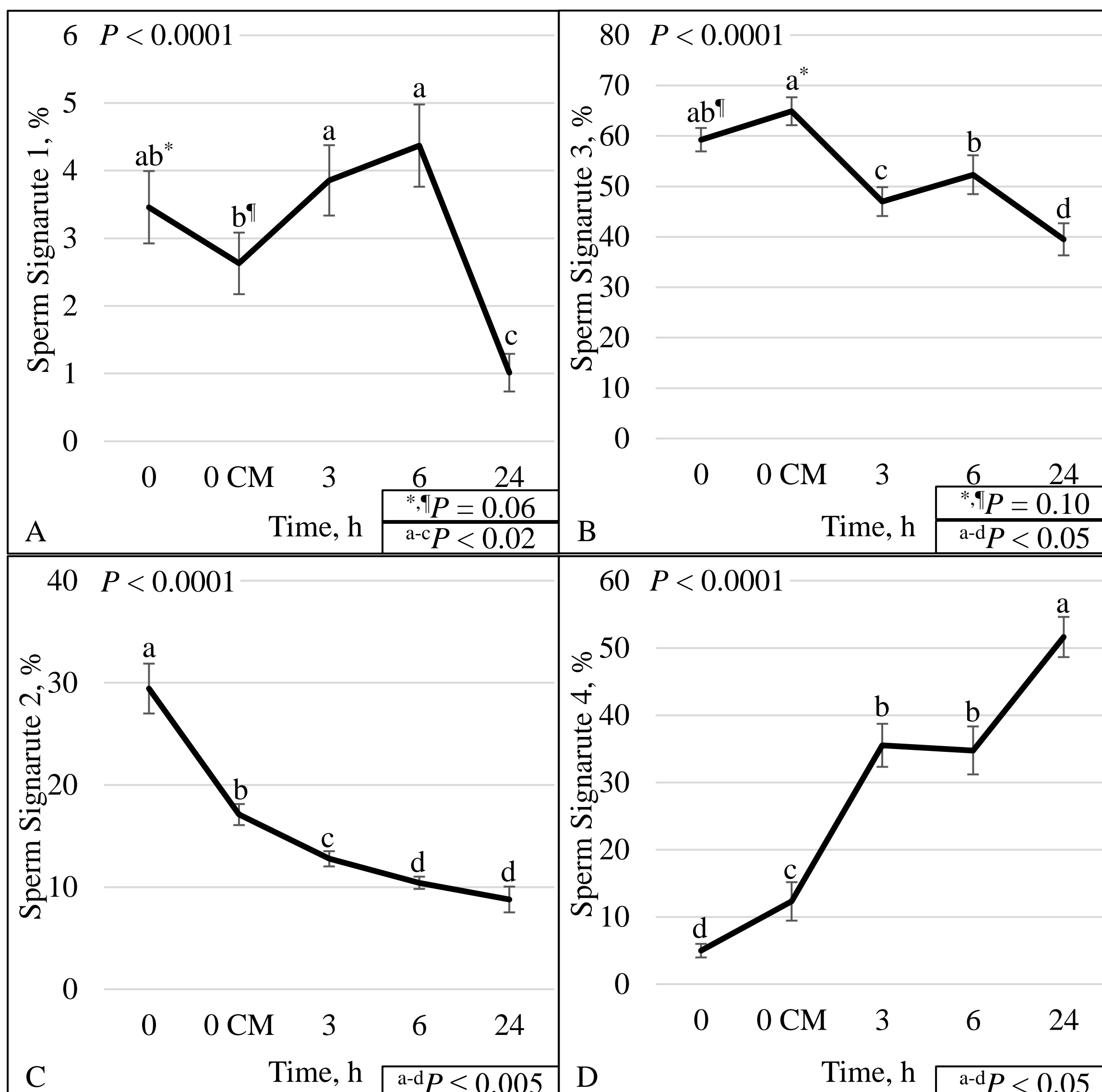


Figure 4.20. Effect of time on sperm plasma membrane integrity (viability) and zinc concentration. A) The percentage of sperm with intact plasma membrane (viable) and high zinc concentration (signature 1; sperm not capacitated). B) The percentage of sperm with disrupted plasma membrane (dead) and high zinc concentration (signature 3; sperm that capacitated and died). C) The percentage of viable sperm with low zinc (signature 2; sperm undergoing capacitation). D) The percentage of dead sperm with no zinc (signature 4; dead sperm that may or may not have gone through capacitation before dying). Sperm were evaluated by flow cytometry at 0 (diluted in non-capacitation media), 0 CM (diluted in capacitation media; CM), 2, 4, 6 and 24 h of incubation at 37 ºC.

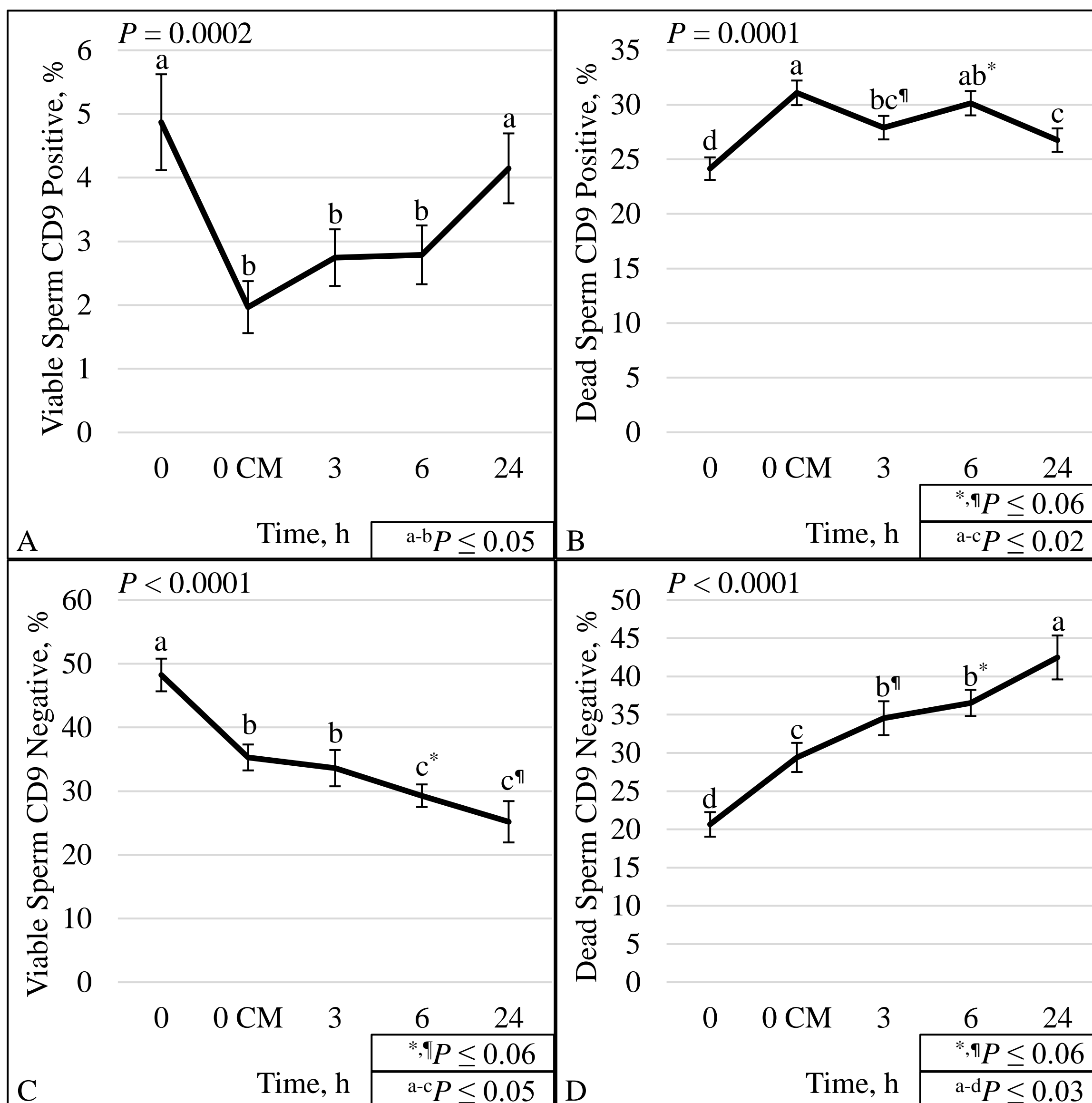


Figure 4.21. Effect of time on sperm plasma membrane integrity (viability) and CD9 protein. A) The percentage of sperm with intact plasma membrane (viable) and CD9 positive. B) The percentage of sperm with disrupted plasma membrane (dead) and CD9 positive. C) The percentage of viable sperm and CD9 negative. D) The percentage of dead sperm and CD9 negative. Sperm were evaluated for viability and CD9 protein (IVA50, Invitrogen) by flow cytometry at 0 (diluted in non-capacitation media), 0 CM (diluted in capacitation media; CM), 2, 4, 6 and 24 h of incubation at 37 ºC.

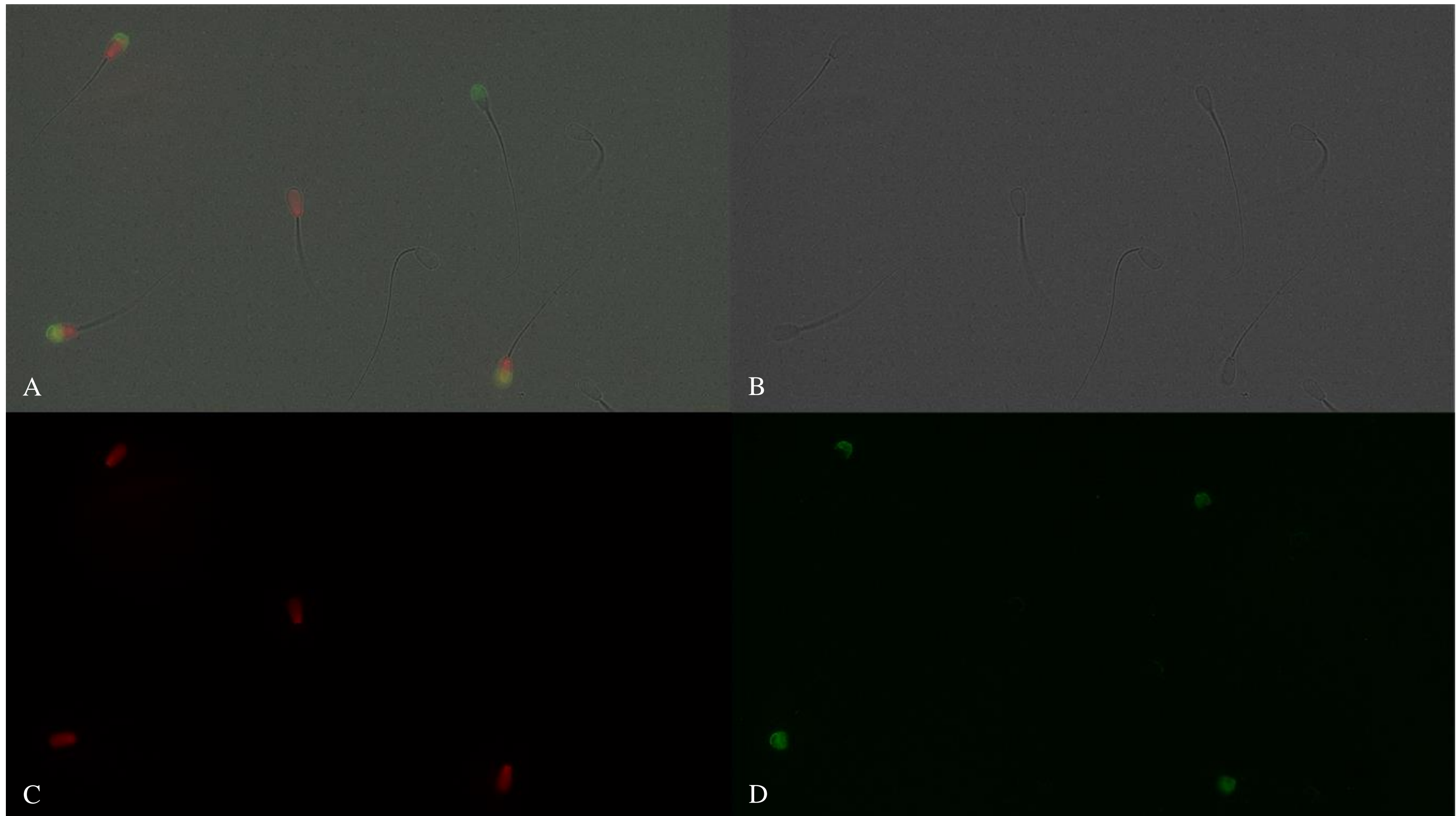


Figure 4.22. Sperm plasma membrane integrity [dead, propidium iodide positive (red) and viable, propidium iodide negative] and positive (green fluorescence) or negative for CD9 protein (IVA50, Invitrogen). A) Merged view of fields B, C, and D; B) Bright field; C) Red fluorescence = Propidium iodide; D) Green fluorescence = anti-CD9-FITC labeling. 600 × magnification under oil immersion.

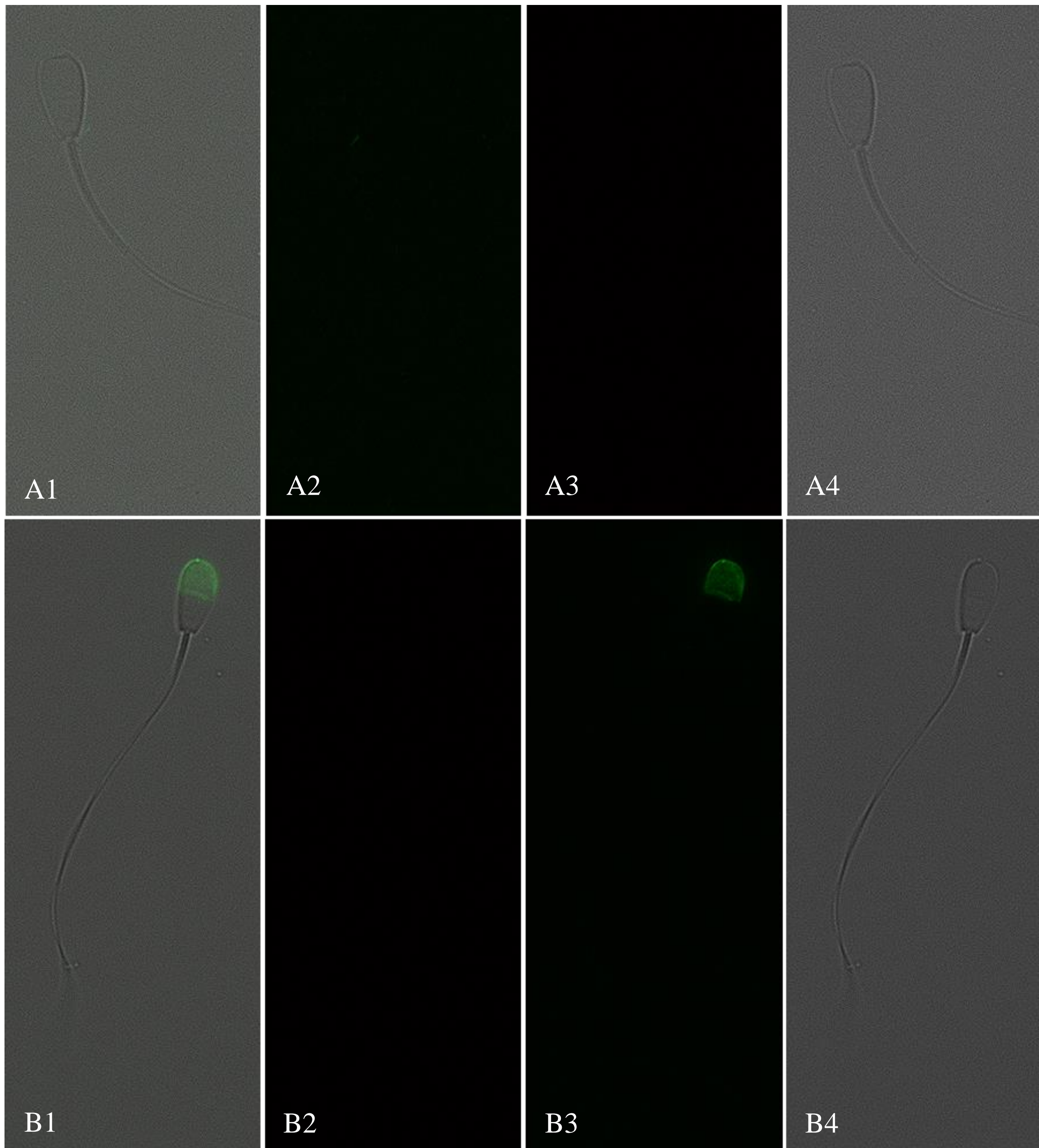


Figure 4.23. Sperm with an intact plasma membrane (viable; propidium iodide negative) and negative (A1-4) or positive (B1-4) for CD9 protein (IVA50, Invitrogen). 1) Merged view of fields 2, 3, and 4; 2) Red fluorescence = Propidium iodide; 3) Green fluorescence = anti-CD9-FITC labeling; 4) Bright field. 600 $\times$ magnification under oil immersion.

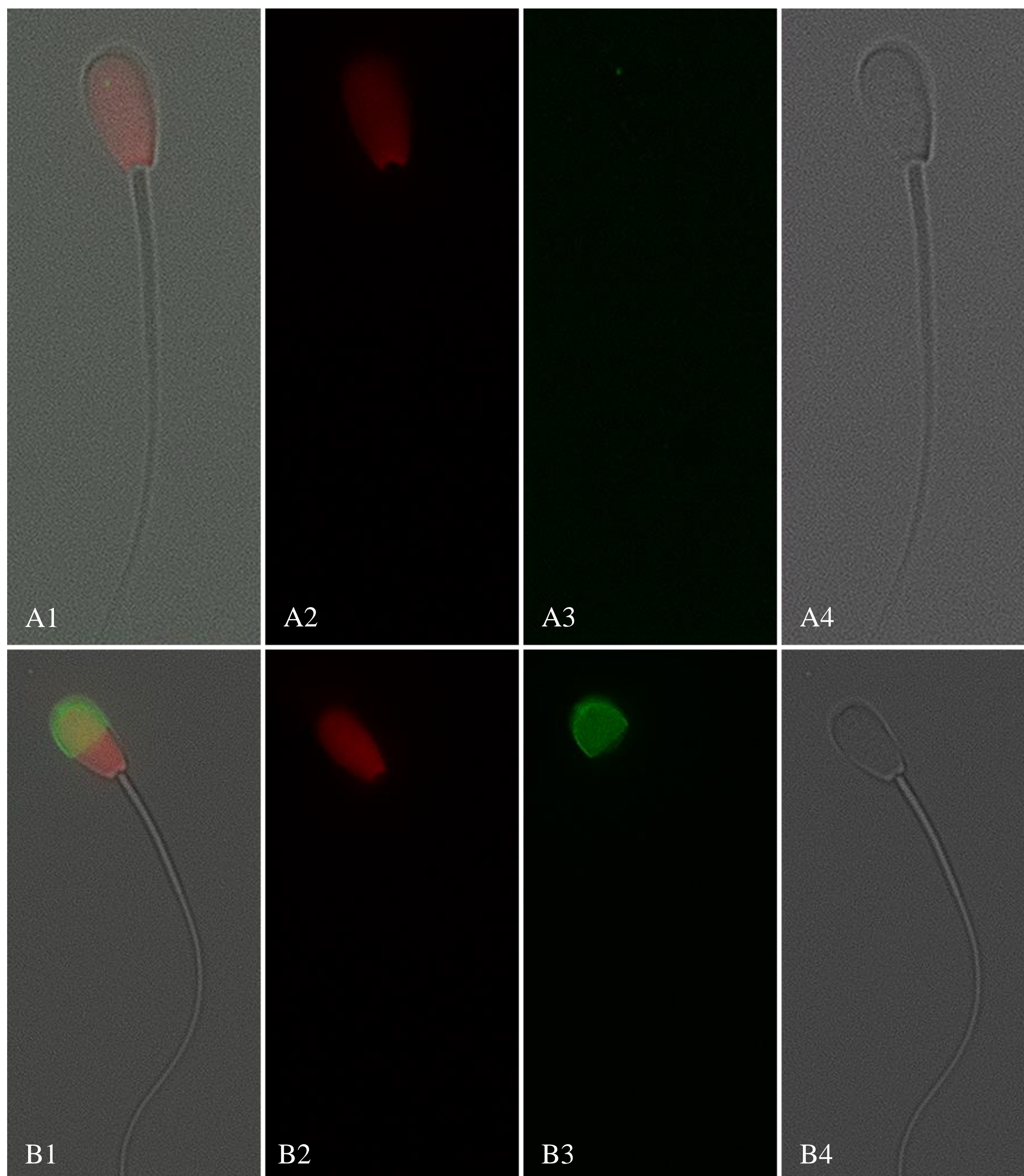
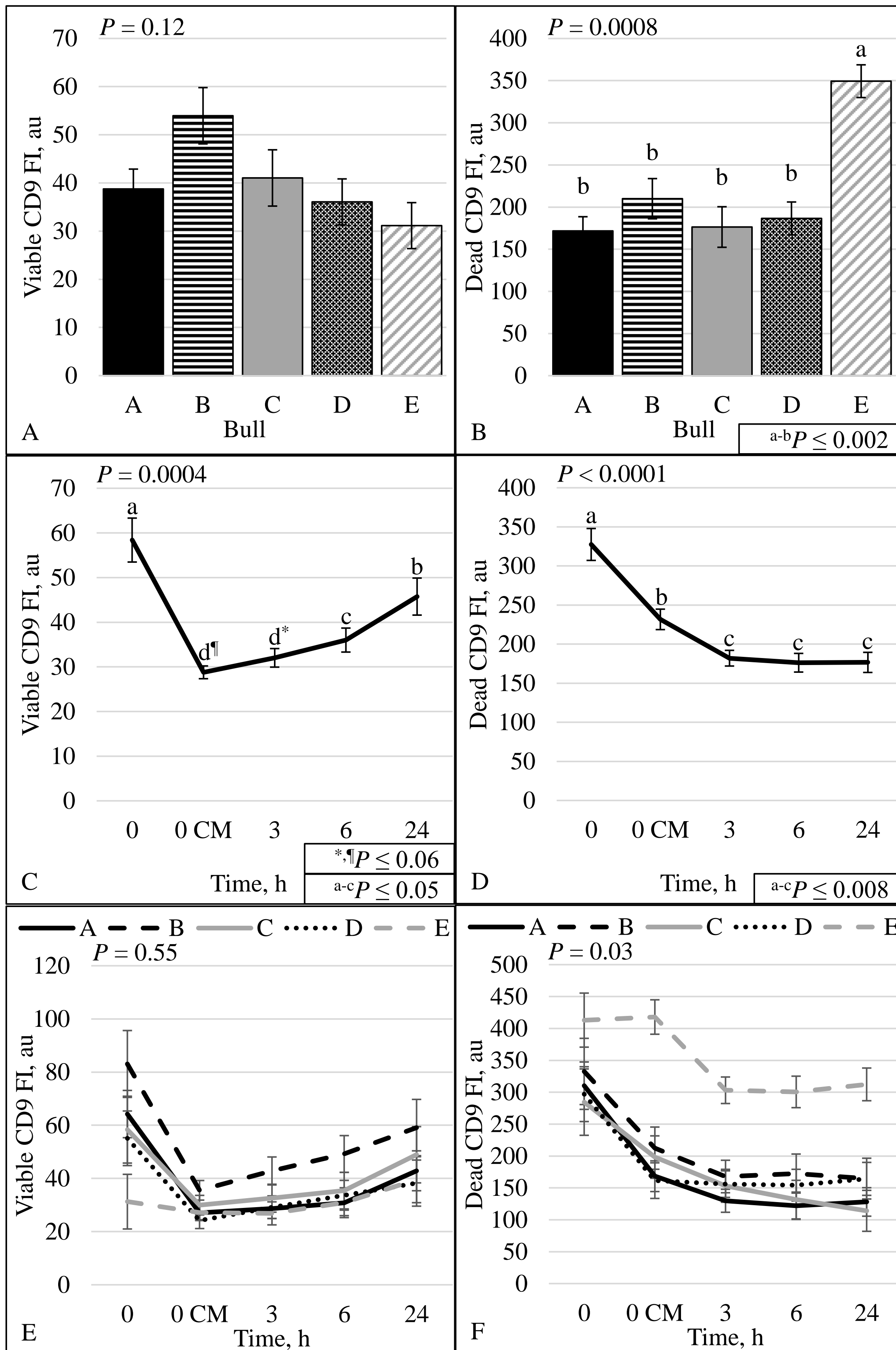


Figure 4.24. Sperm with a disrupted plasma membrane (dead; propidium iodide positive) and negative (A1-4) or positive (B1-4) for CD9 protein (IVA50, Invitrogen). 1) Merged view of fields 2, 3, and 4; 2) Red fluorescence = Propidium iodide; 3) Green fluorescence = anti-CD9-FITC labeling; 4) Bright field. 600 × magnification under oil immersion.



A
P = 0.12
Viable CD9 FI, au
Bull
B
P = 0.0008
Dead CD9 FI, au
Bull
a-bP ≤ 0.002
C
P = 0.0004
Viable CD9 FI, au
Time, h
*,¶P ≤ 0.06
a-cP ≤ 0.05
D
P < 0.0001
Dead CD9 FI, au
Time, h
a-cP ≤ 0.008
E
A B C D E
P = 0.55
Viable CD9 FI, au
Time, h
F
A B C D E
P = 0.03
Dead CD9 FI, au
Time, h

Figure 4.25. Effect of bull (A, B), time (C, D) and bull by time interaction (E, F) on fluorescence intensity (FI) of sperm with intact plasma membrane (viable; A, C, E) or disrupted plasma membrane (dead; B, D, F). Bulls that were previously classified as high (A, B), low (C, E), and intermediary (D) fertility were evaluated for viability and CD9 protein (IVA50, Invitrogen) by flow cytometry. Sperm were evaluated at 0 (diluted in non-capacitation media), 0 CM (diluted in capacitation media; CM), 2, 4, 6 and 24 h of incubation at 37 ºC.

LITERATURE CITED

Abell, K. M., M. E. Theurer, R. L. Larson, B. J. White, D. K. Hardin, R. F. Randle, and R. A. Cushman. 2017. Calving distributions of individual bulls in multiple-sire pastures. Theriogenology 93:7-11.

Acott, T. S., D. F. Katz, and D. D. Hoskins. 1983. Movement characteristics of bovine epididymal spermatozoa: effects of forward motility protein and epididymal maturation. Biology of Reproduction 29(2):389-399.

Acott, T. S., and D. W. Carr. 1984. Inhibition of bovine spermatozoa by caudal epididymal fluid: II. Interaction of pH and a quiescence factor. Biology of Reproduction 30(4):926-935.

Agrawal, Y., and T. Vanha-Perttula. 1987. Effect of secretory particles in bovine seminal vesicle secretion on sperm motility and acrosome reaction. Reproduction (Cambridge, England) 79(2):409-419.

Aitken, R. J., M. A. Baker, and B. Nixon. 2015. Are sperm capacitation and apoptosis the opposite ends of a continuum driven by oxidative stress? Asian Journal of Andrology 17(4):633.

Aitken, R. J. 2017. Reactive oxygen species as mediators of sperm capacitation and pathological damage. Molecular Reproduction and Development 84(10):1039-1052.

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(4):677-684.

Almquist, J., W. Thorp, and C. Knodt. 1946. The effect of penicillin upon the livability, fertility and bacterial content of bull semen. Journal of Animal Science. 5:400-401.

Almquist, J., P. Glantz, and H. Shaffers. 1949. The effect of a combination of penicillin and streptomycin upon the livability and bacterial content of bovine semen. Journal of Dairy Science 32(2):183-190.

Almquist, J. O. 1951. A comparison of penicillin, streptomycin and sulfanilamide for improving the fertility of semen from bulls of low fertility. Journal of Dairy Science 34(8):819-822.

Almquist, J., R. Branas, and K. Barber. 1976. Postpuberal changes in semen production of Charolais bulls ejaculated at high frequency and the relation between testicular measurements and sperm output. Journal of Animal Science 42(3):670-676.

Amann, R. P. 1962. Reproductive capacity of dairy bulls. III. The effect of ejaculation frequency, unilateral vasectomy, and age on spermatogenesis. American Journal of Anatomy 110(1):49-67.

Amann, R., and B. Schanbacher. 1983. Physiology of male reproduction. Journal of Animal Science 57(suppl_2):380-403.

Amann, R., and R. Hammerstedt. 1993. *In vitro* evaluation of sperm quality: an opinion. Journal of Andrology 14(6):397-406.

Amann, R. P., R. G. Saacke, G. F. Barbato, and D. Waberski. 2018. Measuring male-to-male differences in fertility or effects of semen treatments. Annual Review of Animal Biosciences 6:255-286.

Andrews, J. C., J. P. Nolan, R. H. Hammerstedt, and B. D. Bavister. 1994. Role of zinc during hamster sperm capacitation. Biology of Reproduction 51(6):1238-1247.

Antalíková, J., J. Jankovičová, M. Simon, P. Cupperová, K. Michalková, and Ľ. Horovská. 2015. Localization of CD 9 molecule on bull spermatozoa: its involvement in the sperm–egg interaction. Reproduction in Domestic Animals 50(3):423-430.

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. Biology of Reproduction 66(2):354-360.

Arnoult, C., I. G. Kazam, P. E. Visconti, G. S. Kopf, M. Villaz, and H. M. Florman. 1999. Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. Proceedings of the National Academy of Sciences 96(12):6757-6762.

Ashdown, R., and J. Hancock. 1974. Functional anatomy of male reproduction. In: E. S. E. Hafez, editor, Reproduction in Farm Animals. Lea & Febiger, Philadelphia, PA. p. 3-23.

Austin, C. R. 1951. Observations on the penetration of the sperm into the mammalian egg. Australian Journal of Biological Sciences 4(4):581-596.

Austin, C. R. 1952. The ‘capacitation’of the mammalian sperm. Nature 170(4321):326-326.

Austin, C. R. 1975. Sperm fertility, viability and persistence in the female tract. Journal of Reproduction and Fertility Supplement (22):75-89.

Baker, M. A., L. Hetherington, and R. J. Aitken. 2006. Identification of SRC as a key PKA-stimulated tyrosine kinase involved in the capacitation-associated hyperactivation of murine spermatozoa. Journal of Cell Science 119(15):3182-3192.

Baldi, E., L. Tamburrino, M. Muratori, S. Degl'Innocenti, and S. Marchiani. 2020. Adverse effects of *in vitro* manipulation of spermatozoa. Animal Reproduction Science 220:106314.

Balhorn, R. 1982. A model for the structure of chromatin in mammalian sperm. The Journal of Cell Biology 93(2):298-305.

Bao, S., D. J. Miller, Z. Ma, M. Wohltmann, G. Eng, S. Ramanadham, K. Moley, and J. Turk. 2004. Male mice that do not express group VIA phospholipase A2 produce spermatozoa with impaired motility and have greatly reduced fertility. Journal of Biological Chemistry 279(37):38194-38200.

Bardin, C., C. Cheng, N. Mustow, and G. Gunsalus. 1994. The Sertoli cell. In: E. Knobil and J. Neill, editors, The Physiology of Reproduction No. 1. Raven Press, New York, NY. p. 1291-1333.

Barraud-Lange, V., N. Naud-Barriant, M. Bomsel, J. P. Wolf, and A. Ziyyat. 2007. Transfer of oocyte membrane fragments to fertilizing spermatozoa. The FASEB Journal 21(13):3446-3449.

Barraud-Lange, V., C. Chalas Boissonnas, C. Serres, J. Auer, A. Schmitt, B. Lefvre, J.-P. Wolf, and A. Ziyyat. 2012. Membrane transfer from oocyte to sperm occurs in two CD9-independent ways that do not supply the fertilising ability of Cd9-deleted oocytes. Reproduction 144(1):53.

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

Barth, A. D., and C. L. Waldner. 2002. Factors affecting breeding soundness classification of beef bulls examined at the Western College of Veterinary Medicine. The Canadian Veterinary Journal 43(4):274.

Barth, A. D., A. A. Arteaga, L. F. Brito, and C. W. Palmer. 2004. Use of internal artificial vaginas for breeding soundness evaluation in range bulls: an alternative for electroejaculation allowing observation of sex drive and mating ability. Animal Reproduction Science 84(3-4):315-325.

Barth, A. D. 2007. Evaluation of potential breeding soundness of the bull. In: R. S. Youngquist and W. R. Threlfall, editors, Current Therapy in Large Animal Theriogenology. Saunders, St. Louis, MO. p. 228-240.

Barth, A. D. 2013. Physical soundness, Bull breeding soundness evaluation. Western Canadian Association of Bovine Practioners. p. 29.

Barth, A. D. 2018. The use of bull breeding soundness evaluation to identify subfertile and infertile bulls. Animal 12(s1):s158-s164.

Bedford, J. 1970. Sperm capacitation and fertilization in mammals. Biology of Reproduction (Suppl. 2):128-158.

Belleannée, C., V. Labas, A. P. Teixeira-Gomes, J. L. Gatti, J. L. Dacheux, and F. Dacheux. 2011. Identification of luminal and secreted proteins in bull epididymis. Journal of Proteomics 74(1):59-78. doi: 10.1016/j.jprot.2010.07.013

Belleannée, C., V. Thimon, and R. Sullivan. 2012. Region-specific gene expression in the epididymis. Cell and Tissue Research 349(3):717-731.

Bellin, M. E., H. E. Hawkins, and R. L. Ax. 1994. Fertility of range beef bulls grouped according to presence or absence of heparin-binding proteins in sperm membranes and seminal fluid. Journal of Animal Science 72(9):2441-2448.

Bellin, M. E., H. E. Hawkins, J. N. Oyarzo, R. J. Vanderboom, and R. L. Ax. 1996. Monoclonal antibody detection of heparin-binding proteins on sperm corresponds to increased fertility of bulls. Journal of Animal Science 74(1):173-182.

Bellin, M. E., J. N. Oyarzo, H. E. Hawkins, H. Zhang, R. G. Smith, D. W. Forrest, L. Sprott, and R. L. Ax. 1998. Fertility-associated antigen on bull sperm indicates fertility potential. Journal of Animal Science 76(8):2032-2039.

Bennett, G. L., J. W. Keele, L. A. Kuehn, W. M. Snelling, A. M. Dickey, D. Light, R. A. Cushman, and T. G. McDaneld. 2021. Using genomics to measure phenomics: Repeatability of bull prolificacy in multiple-bull pastures. Agriculture 11(7):603.

Bergqvist, A. S., J. Ballester, A. Johannisson, M. Hernandez, N. Lundeheim, and H. Rodriguez-Martinez. 2006. *In vitro* capacitation of bull spermatozoa by oviductal fluid and its components. Zygote 14(3):259-273.

Bergqvist, A. S., J. Ballester, A. Johannisson, N. Lundeheim, and H. Rodríguez-Martínez. 2007. Heparin and dermatan sulphate induced capacitation of frozen-thawed bull spermatozoa measured by merocyanine-540. Zygote 15(3):225-232.

Berndston, W., and C. Desjardins. 1974. The cycle of the seminiferous epithelium and spermatogenesis in the bovine testis. American Journal of Anatomy 140(2):167-179.

Bernecic, N. C., E. Donnellan, E. O'Callaghan, K. Kupisiewicz, C. O'Meara, K. Weldon, P. Lonergan, D. A. Kenny, and S. Fair. 2021. Comprehensive functional analysis reveals that acrosome integrity and viability are key variables distinguishing artificial insemination bulls of varying fertility. Journal of Dairy Science 104(10):11226-11241.

Bianchi, E., B. Doe, D. Goulding, and G. J. Wright. 2014. Juno is the egg Izumo receptor and is essential for mammalian fertilization. Nature 508(7497):483.

Bianchi, E., and G. J. Wright. 2020. Find and fuse: Unsolved mysteries in sperm–egg recognition. PLoS Biology 18(11):e3000953.

Blockey, M. A. de B. 1980. Sheep and cattle mating behaviour. In: Behaviour in relation to reproduction, management and welfare of farm animals. Proceedings of a symposium held at the University of New England, Armidale, NSW, Australia, September 1979. p 53-61.

Boerke, A., P. Tsai, N. Garcia-Gil, I. Brewis, and B. Gadella. 2008. Capacitation-dependent reorganization of microdomains in the apical sperm head plasma membrane: functional relationship with zona binding and the zona-induced acrosome reaction. Theriogenology 70(8):1188-1196.

Brackett, B. G., and G. Oliphant. 1975. Capacitation of rabbit spermatozoa *in vitro*. Biology of Reproduction 12(2):260-274.

Caballero, J., G. Frenette, and R. Sullivan. 2011. Post testicular sperm maturational changes in the bull: important role of the epididymosomes and prostasomes. Veterinary Medicine International 2011(Article ID 757194):13. doi: 10.4061/2011/757194.

Caballero, J., G. Frenette, O. D'Amours, C. Belleannée, N. Lacroix-Pepin, C. Robert, and R. Sullivan. 2012. Bovine sperm raft membrane associated Glioma Pathogenesis-Related 1-like protein 1 (GliPr1L1) is modified during the epididymal transit and is potentially involved in sperm binding to the zona pellucida. Journal of Cellular Physiology 227(12):3876-3886.

Caballero, J. N., G. Frenette, C. Belleannée, and R. Sullivan. 2013. CD9-positive microvesicles mediate the transfer of molecules to bovine spermatozoa during epididymal maturation. PloS One 8(6):e65364.

Cancel, A. M., D. A. Chapman, and G. J. Killian. 1997. Osteopontin is the 55-kilodalton fertility-associated protein in Holstein bull seminal plasma. Biology of Reproduction 57(6):1293-1301.

Carafoli, E., and M. Crompton. 1978. The regulation of intracellular calcium, Current topics in membranes and transport No. 10. Elsevier. p. 151-216.

Carlsson, L., G. Ronquist, M. Stridsberg, and L. Johansson. 1997. Motility stimulant effects of prostasome inclusion in swim-up medium on cryopreserved human spermatozoa. Archives of Andrology 38(3):215-221.

Carr, D. W., and T. S. Acott. 1984. Inhibition of bovine spermatozoa by caudal epididymal fluid: I. Studies of a sperm motility quiescence factor. Biology of Reproduction 30(4):913-925.

Carr, D. W., M. C. Usselman, and T. S. Acott. 1985. Effects of pH, lactate, and viscoelastic drag on sperm motility: a species comparison. Biology of Reproduction 33(3):588-595.

Carr, D. W., and T. S. Acott. 1989. Intracellular pH regulates bovine sperm motility and protein phosphorylation. Biology of Reproduction 41(5):907-920.

Carroll, E. J., L. Ball, and J. A. Scott. 1963. Breeding soundness in bulls -- a summary of 10,940 examinations. Journal of the American Veterinary Medical Association 142:1105-1111.

Chang, H., and S. S. Suarez. 2012. Unexpected flagellar movement patterns and epithelial binding behavior of mouse sperm in the oviduct. Biology of Reproduction 86(5):140, 141-148.

Chang, M. 1951. Fertilizing Capacity of Spermatozoa deposited into the Fallopian Tubes. Nature 168(4277):697-698.

Cheng, P., L. Casida, and G. Barrett. 1947. Effects of dilution on motility of bull spermatozoa. Journal of Animal Science 6(4):496.

Cheng, P., L. E. Casida, and G. R. Barrett. 1949. Effects of Dilution on Motility of Bull Spermatozoa and the Relation between Motility in High Dilution and Fertility. Journal of Animal Science 8(1):81-88. doi: 10.1093/ansci/8.1.81.

Chenoweth, P. 2000. The breeding soundness evaluation of bulls: a rationale. Compendium on Continuing Education for the Practising Veterinarian 22(2):S48-S55.

Chenoweth, P., and J. P. Kastelic. 2007. Clinical reproductive physiology and endocrinology of bulls. In: R. S. Youngquist and W. R. Threlfall, editors, Current

Therapy in Large Animal Theriogenology, 2. Saunders/Elsevier, St. Louis, MO. p. 221-228.

Chian, R. C., and M. A. Sirard. 1995. Fertilizing ability of bovine spermatozoa cocultured with oviduct epithelial cells. Biology of Reproduction 52(1):156-162.

Cho, C., D. O. D. Bunch, J. E. Faure, E. H. Goulding, E. M. Eddy, P. Primakoff, and D. G. Myles. 1998. Fertilization defects in sperm from mice lacking fertilin β. Science 281(5384):1857-1859.

Christensson, A., and H. Lilja. 1994. Complex formation between protein C inhibitor and prostate-specific antigen *in vitro* and in human semen. European Journal of Biochemistry 220(1):45-53.

Christensen, P., D. Boelling, K. M. Pedersen, I. R. Korsgaard, and J. Jensen. 2005. Relationship between sperm viability as determined by flow cytometry and nonreturn rate of dairy bulls. Journal of Andrology 26(1):98-106.

Clapper, D. L., J. A. Davis, P. J. Lamothe, C. Patton, and D. Epel. 1985. Involvement of zinc in the regulation of pHi, motility, and acrosome reactions in sea urchin sperm. The Journal of Cell Biology 100(6):1817-1824.

Clay, J., and B. McDaniel. 2001. Computing mating bull fertility from DHI nonreturn data. Journal of Dairy Science 84(5):1238-1245.

Cooper, T., G. Waites, and E. Nieschlag. 1986. The epididymis and male fertility. A symposium report. International Journal of Andrology 9(2):81-90.

Cooper, T. 1998. Interactions between epididymal secretions and spermatozoa. Journal of Reproduction and Fertility Supplement 53:119-136.

Coulter, G., and R. Foote. 1979. Bovine testicular measurements as indicators of reproductive performance and their relationship to productive traits in cattle: a review. Theriogenology 11(4):297-311.

Coulter, G. 1986. Puberty and postpuberal development of beef bulls. In: D. Morrow, editor, Current therapy in Theriogenology. Saunders, Philadelphia. p. 142-148.

Coulter, G., and G. Kozub. 1989. Efficacy of methods used to test fertility of beef bulls used for multiple-sire breeding under range conditions. Journal of Animal Science 67(7):1757-1766.

Coulter, G., R. Cook, and J. Kastelic. 1997. Effects of dietary energy on scrotal surface temperature, seminal quality, and sperm production in young beef bulls. Journal of Animal Science 75(4):1048-1052.

Cross, N., and P. Mahasreshti. 1997. Prostasome fraction of human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist progesterone. Archives of Andrology 39(1):39-44.

Cuasnicú, P. S., V. G. Da Ros, M. W. Munoz, and D. J. Cohen. 2016. Acrosome reaction as a preparation for gamete fusion. In: M. G. Buffone, editor, Sperm Acrosome Biogenesis and Function During Fertilization. Springer International Publishing, Cham, Switzerland. p. 159-172. doi: 10.1007/978-3-319-30567-79.

Curtis, S. K., and R. Amann. 1981. Testicular development and establishment of spermatogenesis in Holstein bulls. Journal of Animal Science 53(6):1645-1657.

Cushman, R., L. Kill, R. N. Funston, E. Mousel, and G. Perry. 2013. Heifer calving date positively influences calf weaning weights through six parturitions. Journal of Animal Science 91(9):4486-4491.

Cyr, D. G. 2011. Connexins and pannexins: coordinating cellular communication in the testis and epididymis. Spermatogenesis 1(4):325-338.

Dacheux, J.-L., C. Belleannée, R. Jones, V. Labas, M. Belghazi, B. Guyonnet, X. Druart, J. L. Gatti, and F. Dacheux. 2009. Mammalian epididymal proteome. Molecular and Cellular Endocrinology 306(1-2):45-50.

Dalton, J., S. Nadir, J. Bame, M. Noftsinger, R. Nebel, and R. Saacke. 2001. Effect of time of insemination on number of accessory sperm, fertilization rate, and embryo quality in nonlactating dairy cattle. Journal of Dairy Science 84(11):2413-2418.

Dalton, J., L. Deragon, J. L. M. Vasconcelos, C. Lopes, R. Peres, and A. Ahmadzadeh. 2012. Fertility-associated antigen on Nelore bull sperm and reproductive outcomes following first-service fixed-time AI of Nelore cows and heifers. Theriogenology 77(2):389-394.

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

Dalton, J. 2019. Predicting and promoting fertility in bulls. In: Applied Reproductive Strategies in Beef Cattle, Knoxville, TN. p 250-260.

Dandekar, P., J. Aggeler, and P. Talbot. 1992. Structure, distribution and composition of the extracellular matrix of human oocytes and cumulus masses. Human Reproduction 7(3):391-398.

Davis, B. K. 1981. Timing of fertilization in mammals: sperm cholesterol/phospholipid ratio as a determinant of the capacitation interval. Proceedings of the National Academy of Sciences 78(12):7560-7564.

DeJarnette, J. M. 2005. The effect of semen quality on reproductive efficiency. Veterinary Clinics: Food Animal Practice 21(2):409-418.

DeJarnette, J. M. 2012. Semen quality control and quality assurance in AI centers. In: Proceedings for 8th Association for Applied Animal Andrology Conference, Vancouver, BC, Canada. p 7-18.

DeJarnette, J., B. Harstine, K. McDonald, and C. Marshall. 2021. Commercial application of flow cytometry for evaluating bull sperm. Animal Reproduction Science:106838.

Demott, R. P., and S. S. Suarez. 1992. Hyperactivated sperm progress in the mouse oviduct. Biology of Reproduction 46(5):779-785.

Den Daas, J., G. De Jong, L. Lansbergen, and A. Van Wagtendonk-De Leeuw. 1998. The relationship between the number of spermatozoa inseminated and the reproductive efficiency of individual dairy bulls. Journal of Dairy Science 81(6):1714-1723.

Desnoyers, L., and P. Manjunath. 1992. Major proteins of bovine seminal plasma exhibit novel interactions with phospholipid. Journal of Biological Chemistry 267(14):10149-10155.

Dobrowolski, W., and E. Hafez. 1970. Transport and distribution of spermatozoa in the reproductive tract of the cow. Journal of Animal Science 31(5):940-943.

Dym, M., and D. W. Fawcett. 1970. The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. Biology of Reproduction 3(3):308-326.

Ecke, S., M. Geiger, I. Resch, I. Jerabek, L. Sting, M. Maier, and B. R. Binder. 1992. Inhibition of tissue kallikrein by protein C inhibitor. Evidence for identity of protein C inhibitor with the kallikrein binding protein. Journal of Biological Chemistry 267(10):7048-7052.

Ecke, S., M. Geiger, and B. R. Binder. 1997. Heparin Binding of Protein-C Inhibitor Analysis of the Effect of Heparin on the Interaction of Protein-C Inhibitor with Tissue Kallikrein. European Journal of Biochemistry 248(2):475-480.

Eickhoff, R., C. Baldauf, H. W. Koyro, G. Wennemuth, Y. Suga, J. Seitz, R. Henkel, and A. Meinhardt. 2004. Influence of macrophage migration inhibitory factor (MIF) on the zinc content and redox state of protein-bound sulphydryl groups in rat sperm: indications for a new role of MIF in sperm maturation. Molecular Human Reproduction 10(8):605-611. doi: 10.1093/molehr/gah075.

Eid, L., S. Lorton, and J. Parrish. 1994. Paternal influence on S-phase in the first cell cycle of the bovine embryo. Biology of Reproduction 51(6):1232-1237.

Elisen, M. G., R. J. van Kooij, M. A. Nolte, J. Arnoud Marquart, T. M. Lock, B. N. Bouma, and J. C. Meijers. 1998. Protein C inhibitor may modulate human sperm-oocyte interactions. Biology of Reproduction 58(3):670-677.

Elmore, R., C. Bierschwal, C. Martin, and R. Youngquist. 1975. A summary of 1127 breeding soundness examinations in beef bulls. Theriogenology 3(6):209-218.

Elrod, C. C., and W. R. Butler. 1993. Reduction of fertility and alteration of uterine pH in heifers fed excess ruminally degradable protein. Journal of Animal Science 71(3):694-701.

Ericsson, S., D. Garner, C. Thomas, T. Downing, and C. Marshall. 1993. Interrelationships among fluorometric analyses of spermatozoal function, classical semen quality parameters and the fertility of frozen-thawed bovine spermatozoa. Theriogenology 39(5):1009-1024.

Erikson, D. W., A. L. Way, D. A. Chapman, and G. J. Killian. 2007. Detection of osteopontin on Holstein bull spermatozoa, in cauda epididymal fluid and testis homogenates, and its potential role in bovine fertilization. Reproduction 133(5):909-917. doi: 10.1530/REP-06-0228.

España, F., M. Berrettini, and J. H. Griffin. 1989. Purification and characterization of plasma protein C inhibitor. Thrombosis Research 55(3):369-384.

Etkovitz, N., Y. Tirosh, R. Chazan, Y. Jaldety, L. Daniel, S. Rubinstein, and H. Breitbart. 2009. Bovine sperm acrosome reaction induced by G protein-coupled receptor agonists is mediated by epidermal growth factor receptor transactivation. Developmental Biology 334(2):447-457.

Farrell, P., G. Presicce, C. Brockett, and R. Foote. 1998. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenology 49(4):871-879.

Fitzpatrick, L., G. Fordyce, M. McGowan, J. Bertram, V. Doogan, J. De Faveri, R. Miller, and R. Holroyd. 2002. Bull selection and use in northern Australia: Part 2. Semen traits. Animal Reproduction Science 71(1-2):39-49.

Florman, H. M., and N. L. First. 1988a. The regulation of acrosomal exocytosis: I. Sperm capacitation is required for the induction of acrosome reactions by the bovine zona pellucida *in vitro*. Developmental Biology 128(2):453-463.

Florman, H. M., and N. L. First. 1988b. Regulation of acrosomal exocytosis: II. The zona pellucida-induced acrosome reaction of bovine spermatozoa is controlled by extrinsic positive regulatory elements. Developmental Biology 128(2):464-473.

Florman, H. M., R. M. Tombes, N. L. First, and D. F. Babcock. 1989. An adhesion-associated agonist from the zona pellucida activates G protein-promoted elevations of internal Ca2+ and pH that mediate mammalian sperm acrosomal exocytosis. Developmental Biology 135(1):133-146.

Florman, H. M., M. K. Jungnickel, and K. A. Sutton. 2004. Regulating the acrosome reaction. International Journal of Developmental Biology 52(5-6):503-510.

Florman, H. M., and R. A. Fissore. 2015. Fertilization in Mammals. In: T. M. Plant and A. J. Zeleznik, editors, Knobil and Neill's Physiology of Reproduction No. 1. Academic Press, San Diego, CA. p. 149-196.

Fournier-Delpech, S., G. Colas, M. Courot, R. Ortavant, G. Brice, C. Cornu, Y. Guérin, and Y. Lebreton. 1979. Epididymal sperm maturation in the ram: motility, fertilizing ability and embryonic survival after uterine artificial insemination in the ewe. In: Annales de Biologie Animale, Biochimie, Biophysique. p 597-605.

Fraser, L. R., L. Abeydeera, and K. Niwa. 1995. Ca2+-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. Molecular Reproduction and Development 40(2):233-241.

Frenette, G., and R. Sullivan. 2001. Prostasome-like particles are involved in the transfer of P25b from the bovine epididymal fluid to the sperm surface. Molecular Reproduction and Development: Incorporating Gamete Research 59(1):115-121.

Frenette, G., C. Lessard, and R. Sullivan. 2002. Selected proteins of “prostasome-like particles” from epididymal cauda fluid are transferred to epididymal caput spermatozoa in bull. Biology of Reproduction 67(1):308-313.

Frenette, G., C. Lessard, E. Madore, M. A. Fortier, and R. Sullivan. 2003. Aldose reductase and macrophage migration inhibitory factor are associated with epididymosomes and spermatozoa in the bovine epididymis. Biology of Reproduction 69(5):1586-1592. doi: 10.1095/biolreprod.103.019216.

Frenette, G., C. Lessard, and R. Sullivan. 2004. Polyol pathway along the bovine epididymis. Molecular Reproduction and Development 69(4):448-456. doi: 10.1002/mrd.20170.

Frenette, G., C. Legare, F. Saez, and R. Sullivan. 2005. Macrophage migration inhibitory factor in the human epididymis and semen. Molecular Human Reproduction 11(8):575-582. doi: 10.1093/molehr/gah197.

Fujihara, Y., M. Okabe, and M. Ikawa. 2014. GPI-anchored protein complex, LY6K/TEX101, is required for sperm migration into the oviduct and male fertility in mice. Biology of Reproduction 90(3):60, 61-66.

Fukuda, M., M. Sakase, M. Fukushima, and H. Harayama. 2016. Changes of IZUMO1 in bull spermatozoa during the maturation, acrosome reaction, and cryopreservation. Theriogenology 86(9):2179-2188.e3.

Gadella, B. 2008. Sperm membrane physiology and relevance for fertilization. Animal Reproduction Science 107(3-4):229-236.

Gadella, B. 2011. Interaction of sperm with the zona pellucida during fertilization. Reproduction in Domestic Ruminants VII:267.

Galantino-Homer, H. L., P. E. Visconti, and G. S. Kopf. 1997. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3', 5'-monophosphate-dependent pathway. Biology of Reproduction 56(3):707-719.

García-Ruiz, A., J. B. Cole, P. M. VanRaden, G. R. Wiggans, F. J. Ruiz-López, and C. P. Van Tassell. 2016. Changes in genetic selection differentials and generation intervals in US Holstein dairy cattle as a result of genomic selection. Proceedings of the National Academy of Sciences 113(28):E3995-E4004.

Garner, D., L. Johnson, S. Yue, B. Roth, and R. Haugland. 1994. Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. Journal of Andrology 15(6):620-629.

Garner, D. L., C. A. Thomas, H. W. Joerg, J. M. DeJarnette, and C. E. Marshall. 1997. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biology of Reproduction 57(6):1401-1406.

Garner, D. 2014. Use of Flow Cytometry for Semen Evaluation. In: 25th Technical Conference on Artificial Insemination and Reproduction, Green Bay, WI. p 114-121.

Garty, N. B., and Y. Salomon. 1987. Stimulation of partially purified adenylate cyclase from bull sperm by bicarbonate. FEBS Letters 218(1):148-152.

Geary, T., and J. Reeves. 1992. Relative importance of vision and olfaction for detection of estrus by bulls. Journal of Animal Science 70(9):2726-2731.

Geussova, G., J. Peknicova, J. Capkova, P. Kalab, J. Moos, V. V. Philimonenko, and P. Hozak. 1997. Monoclonal antibodies to canine intra-acrosomal sperm proteins recognizing acrosomal status during capacitation and acrosome reaction. Andrologia 29(5):261-268.

Gibb, Z., S. R. Lambourne, B. J. Curry, S. E. Hall, and R. J. Aitken. 2016. Aldehyde dehydrogenase plays a pivotal role in the maintenance of stallion sperm motility. Biology of Reproduction 94(6):133, 131-111.

Gibb, Z., R. A. Griffin, R. J. Aitken, and G. N. De Iuliis. 2020. Functions and effects of reactive oxygen species in male fertility. Animal Reproduction Science 220:106456.

Gillan, L., G. Evans, and W. Maxwell. 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology 63(2):445-457.

Gillan, L., T. Kroetsch, W. C. Maxwell, and G. Evans. 2008. Assessment of *in vitro* sperm characteristics in relation to fertility in dairy bulls. Animal Reproduction Science 103(3-4):201-214.

Girouard, J., G. Frenette, and R. Sullivan. 2008. Seminal plasma proteins regulate the association of lipids and proteins within detergent-resistant membrane domains of bovine spermatozoa. Biology of Reproduction 78(5):921-931.

Girouard, J., G. Frenette, and R. Sullivan. 2011. Comparative proteome and lipid profiles of bovine epididymosomes collected in the intraluminal compartment of the caput and cauda epididymidis. International Journal of Andrology 34(5 Pt 2):e475-486. doi: 10.1111/j.1365-2605.2011.01203.x.

Goossens, E., and H. Tournaye. 2017. Spermatogenesis: Clinical and Experimental Considerations. In: C. J. De Jonge and C. L. R. Barratt, editors, The Sperm Cell: Production, Maturation, Fertilization, Regeneration. Cambridge University Press, Cambridge, UK. p. 1-20.

Goyal, H. O. 1985. Morphology of the bovine epididymis. American Journal of Anatomy 172(2):155-172.

Grant, J. K., F. M. Abreu, N. L. Hojer, S. D. Fields, B. L. Perry, and G. A. Perry. 2011. Influence of inducing luteal regression prior to a modified controlled internal drug releasing device treatment on control of follicular development. Journal Animal Science 89(11):3531-3541. doi: 10.2527/jas.2011-3852.

Greube, A., K. Müller, E. Töpfer-Petersen, A. Herrmann, and P. Müller. 2001. Influence of the bovine seminal plasma protein PDC-109 on the physical state of membranes. Biochemistry 40(28):8326-8334.

Gunasena, K. T., and J. K. Critser. 1997. Utility of viable tissues ex vivo: banking of reproductive cells and tissues, Reproductive Tissue Banking. Elsevier. p. 1-21.

Guthrie, H. D., and G. R. Welch. 2008. Determination of high mitochondrial membrane potential in spermatozoa loaded with the mitochondrial probe 5, 5′, 6, 6′-

tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) by using fluorescence-activated flow cytometry, Advanced Protocols in Oxidative Stress I. Springer. p. 89-97.

Gwathmey, T. M., G. G. Ignotz, and S. S. Suarez. 2003. PDC-109 (BSP-A1/A2) promotes bull sperm binding to oviductal epithelium *in vitro* and may be involved in forming the oviductal sperm reservoir. Biology of Reproduction 69(3):809-815.

Gwathmey, T. M., G. G. Ignotz, J. L. Mueller, P. Manjunath, and S. S. Suarez. 2006. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. Biology of Reproduction 75(4):501-507.

Hagaman, J. R., J. S. Moyer, E. S. Bachman, M. Sibony, P. L. Magyar, J. E. Welch, O. Smithies, J. H. Krege, and D. A. O'Brien. 1998. Angiotensin-converting enzyme and male fertility. Proceedings of the National Academy of Sciences 95(5):2552-2557.

Hall, P. F. 1994. Testicular steroid synthesis: organization and regulation. In: E. Knobil and J. Neill, editors, The Physiology of Reproduction No. 1. Ravens Press, New York, NY. p. 1335-1362.

Hall, S. E., R. J. Aitken, B. Nixon, N. D. Smith, and Z. Gibb. 2017. Electrophilic aldehyde products of lipid peroxidation selectively adduct to heat shock protein 90 and arylsulfatase A in stallion spermatozoa. Biology of Reproduction 96(1):107-121.

Hammerstedt, R. H. 1993. Maintenance of bioenergetic balance in sperm and prevention of lipid peroxidation: a review of the effect on design of storage preservation systems. Reproduction, Fertility and Development 5(6):675-690.

Handrow, R., R. Lenz, and R. Ax. 1982. Structural comparisons among glycosaminoglycans to promote an acrosome reaction in bovine spermatozoa. Biochemical and Biophysical Research Communications 107(4):1326-1332.

Harstine, B., M. Maquivar, L. Helser, M. Utt, C. Premanandan, J. DeJarnette, and M. Day. 2015. Effects of dietary energy on sexual maturation and sperm production in Holstein bulls. Journal of Animal Science 93(6):2759-2766.

Harstine, B., M. Utt, and J. DeJarnette. 2018. Integrating a semen quality control program and sire fertility at a large artificial insemination organization. Animal 12(s1):s63-s74.

Hawk, H. 1983. Sperm survival and transport in the female reproductive tract. Journal of Dairy Science 66(12):2645-2660.

Hermans, J. M., R. Jones, and S. R. Stone. 1994. Rapid inhibition of the sperm protease acrosin by protein C inhibitor. Biochemistry 33(18):5440-5444.

Hermo, L., J. Wright, R. Oko, and C. Morales. 1991. Role of epithelial cells of the male excurrent duct system of the rat in the endocytosis or secretion of sulfated glycoprotein-2 (clusterin). Biology of Reproduction 44(6):1113-1131.

Hernández-González, E. O., D. Martínez-Rojas, D. Mornet, A. Rendon, and A. Mújica. 2001. Comparative distribution of short dystrophin superfamily products in various guinea pig spermatozoa domains. European Journal of Cell Biology 80(12):792-798.

Hernández-González, E. O., D. Mornet, A. Rendon, and D. Martínez-Rojas. 2005. Absence of Dp71 in mdx3cv mouse spermatozoa alters flagellar morphology and the distribution of ion channels and nNOS. Journal of Cell Science 118(1):137-145.

Hess, K. C., B. H. Jones, B. Marquez, Y. Chen, T. S. Ord, M. Kamenetsky, C. Miyamoto, J. H. Zippin, G. S. Kopf, and S. S. Suarez. 2005. The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. Developmental Cell 9(2):249-259.

Ho, H.-C., and S. S. Suarez. 2001a. Hyperactivation of mammalian spermatozoa: function and regulation. Reproduction 122(4):519-526.

Ho, H.-C., and S. S. Suarez. 2001b. An inositol 1, 4, 5-trisphosphate receptor-gated intracellular Ca2+ store is involved in regulating sperm hyperactivated motility. Biology of Reproduction 65(5):1606-1615.

Ho, H.-C., K. A. Granish, and S. S. Suarez. 2002. Hyperactivated motility of bull sperm is triggered at the axoneme by Ca2+ and not cAMP. Developmental Biology 250(1):208-217.

Ho, H.-C., and S. S. Suarez. 2003. Characterization of the intracellular calcium store at the base of the sperm flagellum that regulates hyperactivated motility. Biology of Reproduction 68(5):1590-1596.

Hochereau-de Reviers, M., C. Monet-Kuntz, and M. Courot. 1987. Spermatogenesis and Sertoli cell numbers and function in rams and bulls. Journal of Reproduction and Fertility Supplement 34(10):1-114.

Holt, W. 2000. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology 53(1):47-58.

Hu, D., D. Ansari, K. Pawlowski, Q. Zhou, A. Sasor, C. Welinder, T. Kristl, M. Bauden, M. Rezeli, Y. Jiang, G. Marko-Varga, and R. Andersson. 2018. Proteomic

analyses identify prognostic biomarkers for pancreatic ductal adenocarcinoma. Oncotarget 9(11):9789-9807. doi: 10.18632/oncotarget.23929.

Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2008. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature Protocols 4(1):44-57.

Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2009. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37(1):1-13.

Hung, P. H., and S. S. Suarez. 2012. Alterations to the bull sperm surface proteins that bind sperm to oviductal epithelium. Biology of Reproduction 87(4):88. doi: 10.1095/biolreprod.112.099721.

Hunter, R., and I. Wilmut. 1984. Sperm transport in the cow: peri-ovulatory redistribution of viable cells within the oviduct. Reproduction Nutrition Developpement 24(5A):597-608.

Ibraghimov-Beskrovnaya, O., J. M. Ervasti, C. J. Leveille, C. A. Slaughter, S. W. Sernett, and K. P. Campbell. 1992. Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature 355(6362):696-702.

Ibrahim, N. M., G. R. Gilbert, K. J. Loseth, and B. G. Crabo. 2000. Correlation between clusterin-positive spermatozoa determined by flow cytometry in bull semen and fertility. Journal of Andrology 21(6):887-894.

Igboeli, G., and R. Foote. 1968. Maturation changes in bull epididymal spermatozoa. Journal of Dairy Science 51(10):1703-1705.

Ignotz, G. G., M. C. Lo, C. L. Perez, T. M. Gwathmey, and S. S. Suarez. 2001. Characterization of a fucose-binding protein from bull sperm and seminal plasma that may be responsible for formation of the oviductal sperm reservoir. Biology of Reproduction 64(6):1806-1811.

Ignotz, G. G., and S. S. Suarez. 2005. Calcium/calmodulin and calmodulin kinase II stimulate hyperactivation in demembranated bovine sperm. Biology of Reproduction 73(3):519-526.

Ignotz, G. G., M. Y. Cho, and S. S. Suarez. 2007. Annexins are candidate oviductal receptors for bovine sperm surface proteins and thus may serve to hold bovine sperm in the oviductal reservoir. Biology of Reproduction 77(6):906-913.

Ikawa, M., I. Wada, K. Kominami, D. Watanabe, K. Toshimori, Y. Nishimune, and M. Okabe. 1997. The putative chaperone calmegin is required for sperm fertility. Nature 387(6633):607-611.

Ikawa, M., K. Tokuhiro, R. Yamaguchi, A. M. Benham, T. Tamura, I. Wada, Y. Satouh, N. Inoue, and M. Okabe. 2011. Calsperin is a testis-specific chaperone required for sperm fertility. Journal of Biological Chemistry 286(7):5639-5646.

Inoue, N., M. Ikawa, A. Isotani, and M. Okabe. 2005. The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature 434(7030):234.

Ito, C., K. Yamatoya, K. Yoshida, M. Maekawa, K. Miyado, and K. Toshimori. 2010. Tetraspanin family protein CD9 in the mouse sperm: unique localization, appearance, behavior and fate during fertilization. Cell and Tissue Research 340(3):583-594.

Januskauskas, A., A. Johannisson, and H. Rodriguez-Martinez. 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(4):947-961.

Januskauskas, A., A. Johannisson, and H. Rodriguez-Martinez. 2003. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. Theriogenology 60(4):743-758.

Jodar, M., E. Sendler, and S. A. Krawetz. 2016. The protein and transcript profiles of human semen. Cell and Tissue Research 363(1):85-96. doi: 10.1007/s00441-015-2237-1.

Johnson, G. P., A.-M. English, S. Cronin, D. A. Hoey, K. G. Meade, and S. Fair. 2017. Genomic identification, expression profiling, and functional characterization of CatSper channels in the bovine. Biology of Reproduction 97(2):302-312.

Johnson, L., D. Varner, M. Roberts, T. Smith, G. Keillor, and W. Scrutchfield. 2000. Efficiency of spermatogenesis: a comparative approach. Animal Reproduction Science 60:471-480.

Jones, R., and R. Murdoch. 1996. Regulation of the motility and metabolism of spermatozoa for storage in the epididymis of eutherian and marsupial mammals. Reproduction, Fertility and Development 8(4):553-568.

Kaewmala, K., M. J. Uddin, M. U. Cinar, C. Große-Brinkhaus, E. Jonas, D. Tesfaye, C. Phatsara, E. Tholen, C. Looft, and K. Schellander. 2011. Association study and expression analysis of CD9 as candidate gene for boar sperm quality and fertility traits. Animal Reproduction Science 125(1-4):170-179.

Kaji, K., S. Oda, T. Shikano, T. Ohnuki, Y. Uematsu, J. Sakagami, N. Tada, S. Miyazaki, and A. Kudo. 2000. The gamete fusion process is defective in eggs of Cd9-deficient mice. Nature Genetics 24(3):279.

Kantsler, V., J. Dunkel, M. Blayney, and R. E. Goldstein. 2014. Rheotaxis facilitates upstream navigation of mammalian sperm cells. Elife 3:e02403.

Kaproth, M. T., J. E. Parks, G. C. Grambo, H. E. Rycroft, J. A. Hertl, and Y. T. Gröhn. 2002. Effect of preparing and loading multiple insemination guns on conception rate in two large commercial dairy herds. Theriogenology 57(2):909-921.

Kasimanickam, R., V. Kasimanickam, C. Thatcher, R. Nebel, and B. Cassell. 2007. Relationships among lipid peroxidation, glutathione peroxidase, superoxide dismutase, sperm parameters, and competitive index in dairy bulls. Theriogenology 67(5):1004-1012.

Kastelic, J., R. Cook, G. Coulter, and R. Saacke. 1996. Insulating the scrotal neck affects semen quality and scrotal/testicular temperatures in the bull. Theriogenology 45(5):935-942.

Kastelic, J., R. Cook, R. Pierson, and G. Coulter. 2001. Relationships among scrotal and testicular characteristics, sperm production, and seminal quality in 129 beef bulls. Canadian Journal of Veterinary Research 65(2):111.

Kathiravan, P., J. Kalatharan, M. J. Edwin, and C. Veerapandian. 2008. Computer automated motion analysis of crossbred bull spermatozoa and its relationship with *in vitro* fertility in zona-free hamster oocytes. Animal Reproduction Science 104(1):9-17.

Kennedy, C. E., K. B. Krieger, M. Sutovsky, W. Xu, P. Vargovič, B. A. Didion, M. R. Ellersieck, M. E. Hennessy, J. Verstegen, and R. Oko. 2014. Protein expression pattern of PAWP in bull spermatozoa is associated with sperm quality and fertility following artificial insemination. Molecular Reproduction and Development 81(5):436-449.

Kennedy, S., J. Spitzer, F. Hopkins, H. Higdon Iii, and W. Bridges Jr. 2002. Breeding soundness evaluations of 3648 yearling beef bulls using the 1993 Society for Theriogenology guidelines. Theriogenology 58(5):947-961.

Kerns, K., M. Zigo, E. Z. Drobnis, M. Sutovsky, and P. Sutovsky. 2018. Zinc ion flux during mammalian sperm capacitation. Nature Communications 9(1):1-10.

Kerns, K., M. Sharif, M. Zigo, W. Xu, L. E. Hamilton, M. Sutovsky, M. Ellersieck, E. Z. Drobnis, N. Bovin, and R. Oko. 2020. Sperm cohort-specific zinc signature acquisition and capacitation-induced zinc flux regulate sperm-oviduct and sperm-

zona pellucida interactions. International Journal of Molecular Sciences 21(6):2121.

Killian, G. J., D. A. Chapman, and L. A. Rogowski. 1993. Fertility-associated proteins in Holstein bull seminal plasma. Biology of Reproduction 49(6):1202-1207.

Kirchhoff, C. 1994. A major messenger ribonucleic acid of the rodent epididymis encodes a small glycosylphosphatidylinositol-anchored lymphocyte surface antigen. Biology of Reproduction 50(4):896-902.

Kirchhoff, C., and G. Hale. 1996. Cell-to-cell transfer of glycosylphosphatidylinositol-anchored membrane proteins during sperm maturation. Molecular Human Reproduction 2(3):177-184.

Kirichok, Y., B. Navarro, and D. E. Clapham. 2006. Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca 2+ channel. Nature 439(7077):737-740.

Kjoestad, H., E. Ropstad, and K. A. Berg. 1993. Evaluation of spermatological parameters used to predict the fertility of frozen bull semen. Acta Veterinaria Scandinavica 34(3):299-303.

Koefoed-Johnsen, H. 1960. Influence of ejaculation frequency on the time required for sperm formation and epididymal passage in the bull. Nature 185(4705):49-50.

Koziol, J. H., and C. L. Armstrong. 2018. Manual for Breeding Soundness Examination of Bulls. Second ed. Society for Theriogenology.

Krege, J. H., S. W. John, L. L. Langenbach, J. B. Hodgin, J. R. Hagaman, E. S. Bachman, J. C. Jennette, D. A. O'Brien, and O. Smithies. 1995. Male–female differences in fertility and blood pressure in ACE-deficient mice. Nature 375(6527):146-148.

Krzyzosiak, J., P. Molan, and R. Vishwanath. 1999. Measurements of bovine sperm velocities under true anaerobic and aerobic conditions. Animal Reproduction Science 55(3-4):163-173.

Kuhn, L. A., J. H. Griffin, C. L. Fisher, J. S. Greengard, B. N. Bouma, F. España, and J. A. Tainer. 1990. Elucidating the structural chemistry of glycosaminoglycan recognition by protein C inhibitor. Proceedings of the National Academy of Sciences 87(21):8506-8510.

Kuhn, M., J. Hutchison, and G. Wiggans. 2006. Characterization of Holstein heifer fertility in the United States. Journal of Dairy Science 89(12):4907-4920.

Kumaresan, A., A. Johannisson, E. M. Al-Essawe, and J. M. Morrell. 2017. Sperm viability, reactive oxygen species, and DNA fragmentation index combined can

discriminate between above-and below-average fertility bulls. Journal of Dairy Science 100(7):5824-5836.

La Spina, F. A., L. C. P. Molina, A. Romarowski, A. M. Vitale, T. L. Falzone, D. Krapf, N. Hirohashi, and M. G. Buffone. 2016. Mouse sperm begin to undergo acrosomal exocytosis in the upper isthmus of the oviduct. Developmental Biology 411(2):172-182.

Larimore, E., O. Amundson, S. Bird, B. Funnell, S. Kruse, G. Bridges, and G. Perry. 2015. Influence of estrus at fixed-time artificial insemination on early embryonic development in beef cattle. Journal of Animal Science 93(6):2806-2812.

Larsson, B., and K. Larsson. 1985. Distribution of spermatozoa in the genital tract of artificially inseminated heifers. Acta Veterinaria Scandinavica 26(3):385-395.

Laurell, M., A. Christensson, P.-A. Abrahamsson, J. Stenflo, and H. Lilja. 1992. Protein C inhibitor in human body fluids. Seminal plasma is rich in inhibitor antigen deriving from cells throughout the male reproductive system. The Journal of Clinical Investigation 89(4):1094-1101.

Le Naour, F., E. Rubinstein, C. Jasmin, M. Prenant, and C. Boucheix. 2000. Severely reduced female fertility in CD9-deficient mice. Science 287(5451):319-321.

Lee, C., and R. Ax. 1984. Concentrations and composition of glycosaminoglycans in the female bovine reproductive tract. Journal of Dairy Science 67(9):2006-2009.

Lefebvre, R., P. J. Chenoweth, M. Drost, C. T. LeClear, M. MacCubbin, J. T. Dutton, and S. S. Suarez. 1995. Characterization of the oviductal sperm reservoir in cattle. Biology of Reproduction 53(5):1066-1074.

Lefebvre, R., and S. S. Suarez. 1996. Effect of capacitation on bull sperm binding to homologous oviductal epithelium. Biology of Reproduction 54(3):575-582.

Lefebvre, R., M. C. Lo, and S. S. Suarez. 1997. Bovine sperm binding to oviductal epithelium involves fucose recognition. Biology of Reproduction 56(5):1198-1204.

Lin-Moshier, Y., P. J. Sebastian, L. Higgins, N. D. Sampson, J. E. Hewitt, and J. S. Marchant. 2013. Re-evaluation of the role of calcium homeostasis endoplasmic reticulum protein (CHERP) in cellular calcium signaling. Journal of Biological Chemistry 288(1):355-367.

Lishko, P. V., Y. Kirichok, D. Ren, B. Navarro, J.-J. Chung, and D. E. Clapham. 2012. The control of male fertility by spermatozoan ion channels. Annual Review of Physiology 74:453-475.

Lone, S., A. Paray, S. Mir, B. Ganaie, R. Sinha, and P. Singh. 2017. Breeding soundness evaluation in bulls: a review. Biomedical Journal of Scientific & Technical Research 1(5):1267-1270.

Lonergan, P. 2018. Historical and futuristic developments in bovine semen technology. Animal 12(s1):s4-s18.

Lu, C.-H., R. K.-K. Lee, Y.-M. Hwu, S.-L. Chu, Y.-J. Chen, W.-C. Chang, S.-P. Lin, and S.-H. Li. 2011. SERPINE2, a serine protease inhibitor extensively expressed in adult male mouse reproductive tissues, may serve as a murine sperm decapacitation factor. Biology of Reproduction 84(3):514-525.

Lunstra, D., J. Ford, and S. Echternkamp. 1978. Puberty in beef bulls: hormone concentrations, growth, testicular development, sperm production and sexual aggressiveness in bulls of different breeds. Journal of Animal Science 46(4):1054-1062.

Lyons, E. A., P. J. Taylor, X. H. Zheng, G. Ballard, C. S. Levi, and J. V. Kredentser. 1991. Characterization of subendometrial myometrial contractions throughout the menstrual cycle in normal fertile women. Fertility and Sterility 55(4):771-774.

Magargee, S. F., E. Kunze, and R. H. Hammerstedt. 1988. Changes in lectin-binding features of ram sperm surfaces associated with epididymal maturation and ejaculation. Biology of Reproduction 38(3):667-685.

Mahé, C., A. M. Zlotkowska, K. Reynaud, G. Tsikis, P. Mermillod, X. Druart, J. Schoen, and M. Saint-Dizier. 2021. Sperm migration, selection, survival and fertilizing ability in the mammalian oviduct. Biology of Reproduction 105(2):317-331. doi: doi.org/10.1093/biolre/ioab105.

Mahfouz, R., N. Aziz, R. Sharma, H. Abdelrazek, E. Sabanegh, and A. Agarwal. 2008. Comparing flowcytometry and chemoluminescense in assessing human sperm production of superoxide and hydrogen peroxide in different sperm fractions. Fertility and Sterility 90:S337.

Mahmoud, A. I., and J. J. Parrish. 1996. Oviduct fluid and heparin induce similar surface changes in bovine sperm during capacitation: a flow cytometric study using lectins. Molecular Reproduction and Development 43(4):554-560. doi: 10.1002/(SICI)1098-2795(199604)43:4<554::AID-MRD19>3.0.CO;2-Z.

Manandhar, G., and K. Toshimori. 2001. Exposure of sperm head equatorin after acrosome reaction and its fate after fertilization in mice. Biology of Reproduction 65(5):1425-1436.

Manjunath, P., and I. Thérien. 2002. Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. Journal of Reproductive Immunology 53(1-2):109-119.

Marcello, M. R., W. Jia, J. A. Leary, K. L. Moore, and J. P. Evans. 2011. Lack of tyrosylprotein sulfotransferase-2 activity results in altered sperm-egg interactions and loss of ADAM3 and ADAM6 in epididymal sperm. Journal of Biological Chemistry 286(15):13060-13070.

Marquez, B., and S. S. Suarez. 2004. Different Signaling Pathways in Bovine Sperm Regulate Capacitation and Hyperactivation1. Biology of Reproduction 70(6):1626-1633. doi: 10.1095/biolreprod.103.026476.

Marquez, B., and S. S. Suarez. 2007. Bovine Sperm Hyperactivation Is Promoted by Alkaline-Stimulated Ca2+ Influx1. Biology of Reproduction 76(4):660-665. doi: 10.1095/biolreprod.106.055038.

Martin-DeLeon, P. A. 2006. Epididymal SPAM1 and its impact on sperm function. Molecular and Cellular Endocrinology 250(1-2):114-121.

Mattner, P. 1968. The distribution of spermatozoa and leucocytes in the female genital tract in goats and cattle. Reproduction (Cambridge, England) 17(2):253-261.

McGowan, M., M. Holland, and G. Boe-Hansen. 2018. Ontology and endocrinology of the reproductive system of bulls from fetus to maturity. Animal 12(s1):s19-s26.

Menegassi, S. R. O., J. O. J. Barcellos, V. d. N. Lampert, J. B. S. Borges, and V. Peripolli. 2011. Bioeconomic impact of bull breeding soundness examination in cow-calf systems. Revista Brasileira de Zootecnia 40:441-447.

Michailov, Y., D. Ickowicz, and H. Breitbart. 2014. Zn2+-stimulation of sperm capacitation and of the acrosome reaction is mediated by EGFR activation. Developmental Biology 396(2):246-255.

Miki, K., and D. E. Clapham. 2013. Rheotaxis guides mammalian sperm. Current Biology 23(6):443-452.

Mitchell, J., P. Senger, and J. L. Rosenberger. 1985. Distribution and retention of spermatozoa with acrosomal and nuclear abnormalities in the cow genital tract. Journal of Animal Science 61(4):956-967.

Mitchell, J. R. 2012. Developing an NAAB/CSS semen quality control program. In: Proceedings for 8th Association for Applied Animal Andrology Conference, Vancouver, BC, Canada. p 1-6.

Miyado, K., G. Yamada, S. Yamada, H. Hasuwa, Y. Nakamura, F. Ryu, K. Suzuki, K. Kosai, K. Inoue, and A. Ogura. 2000. Requirement of CD9 on the egg plasma membrane for fertilization. Science 287(5451):321-324.

Moeller, A., and N. VanDemark. 1951. The relationship of the interval between inseminations to bovine fertility. Journal of Animal Science 10(4):988-992.

Monaco, E., B. Gasparrini, L. Boccia, A. De Rosa, L. Attanasio, L. Zicarelli, and G. Killian. 2009. Effect of osteopontin (OPN) on *in vitro* embryo development in cattle. Theriogenology 71(3):450-457.

Morin, G., C. Lalancette, R. Sullivan, and P. Leclerc. 2005. Identification of the bull sperm p80 protein as a PH-20 ortholog and its modification during the epididymal transit. Molecular Reproduction and Development 71(4):523-534.

Morin, G., R. Sullivan, I. Laflamme, C. Robert, and P. Leclerc. 2010. SPAM1 isoforms from two tissue origins are differentially localized within ejaculated bull sperm membranes and have different roles during fertilization. Biology of Reproduction 82(2):271-281.

Moss, S. B., R. M. Turner, K. L. Burkert, H. VanScoy Butt, and G. L. Gerton. 1999. Conservation and function of a bovine sperm A-kinase anchor protein homologous to mouse AKAP82. Biology of Reproduction 61(2):335-342.

Moura, A. A., H. Koc, D. A. Chapman, and G. J. Killian. 2006. Identification of proteins in the accessory sex gland fluid associated with fertility indexes of dairy bulls: a proteomic approach. Journal of Andrology 27(2):201-211.

Müller, P., K.-R. Erlemann, K. Müller, J. J. Calvete, E. Töpfer-Petersen, K. Marienfeld, and A. Herrmann. 1998. Biophysical characterization of the interaction of bovine seminal plasma protein PDC-109 with phospholipid vesicles. European Biophysics Journal 27(1):33-41.

Müller, P., A. Greube, E. Töpfer-Petersen, and A. Herrmann. 2002. Influence of the bovine seminal plasma protein PDC-109 on cholesterol in the presence of phospholipids. European Biophysics Journal 31(6):438-447.

Mullins, K., and R. Saacke. 1989. Study of the functional anatomy of bovine cervical mucosa with special reference to mucus secretion and sperm transport. The Anatomical Record 225(2):106-117.

Nauc, V., and P. Manjunath. 2000. Radioimmunoassays for bull seminal plasma proteins (BSP-A1/-A2, BSP-A3, and BSP-30-Kilodaltons), and their quantification in seminal plasma and sperm. Biology of Reproduction 63(4):1058-1066.

Nesvizhskii, A. I., A. Keller, E. Kolker, and R. Aebersold. 2003. A statistical model for identifying proteins by tandem mass spectrometry. Analytical Chemistry 75(17):4646-4658.

Nishimura, H., E. Kim, T. Nakanishi, and T. Baba. 2004. Possible function of the ADAM1a/ADAM2 Fertilin complex in the appearance of ADAM3 on the sperm surface. Journal of Biological Chemistry 279(33):34957-34962.

Niswender, G., T. Nett, and A. Akbar. 1974. The hormones of reproduction. In: E. S. E. Hafez, editor, Reproduction in Farm Animals. Lea & Fabiger, Philadelphia, PA. p. 57-81.

Nixon, B., and R. J. Aitken. 2009. The biological significance of detergent-resistant membranes in spermatozoa. Journal of Reproductive Immunology 83(1-2):8-13.

Norman, H., J. Hutchison, and P. VanRaden. 2011. Evaluations for service-sire conception rate for heifer and cow inseminations with conventional and sexed semen. Journal of Dairy Science 94(12):6135-6142.

Northrop-Albrecht, E. J., J. J. J. Rich, R. A. Cushman, R. Yao, X. Ge, and G. A. Perry. 2021. Influence of estradiol on bovine trophectoderm and uterine gene transcripts around maternal recognition of pregnancy. Biology of Reproduction 105(2):381-392. doi: doi.org/10.1093/biolre/ioab091.

Northrop, E. J., J. J. J. Rich, R. A. Cushman, A. K. McNeel, É. M. Soares, K. Brooks, T. E. Spencer, and G. A. Perry. 2018. Effects of preovulatory estradiol on uterine environment and conceptus survival from fertilization to maternal recognition of pregnancy. Biology of Reproduction 99(3):629-638.

O'Flaherty, C. 2019. Orchestrating the antioxidant defenses in the epididymis. Andrology 7(5):662-668.

Odhiambo, J., M. Sutovsky, J. 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(6):1168-1176.

Odhiambo, J. F., J. M. DeJarnette, T. W. Geary, C. E. Kennedy, S. S. Suarez, M. Sutovsky, and P. Sutovsky. 2014. Increased Conception Rates in Beef Cattle Inseminated with Nanopurified Bull Semen1. Biology of Reproduction 91(4)doi: 10.1095/biolreprod.114.121897.

Oliveira, B. M., R. P. Arruda, H. E. Thomé, M. Maturana Filho, G. Oliveira, C. Guimarães, M. Nichi, L. A. Silva, and E. C. C. Celeghini. 2014. Fertility and uterine hemodynamic in cows after artificial insemination with semen assessed by fluorescent probes. Theriogenology 82(5):767-772.

Oliveira, L. Z., R. P. Arruda, A. F. de Andrade, R. M. Santos, M. E. Beletti, R. F. Peres, J. P. Martins, and V. F. de Lima. 2012. Effect of sequence of insemination after simultaneous thawing of multiple semen straws on conception rate to timed AI in suckled multiparous Nelore cows. Theriogenology 78(8):1800-1813. doi: 10.1016/j.theriogenology.2012.07.007.

Oliveira, L. Z., R. P. de Arruda, A. F. de Andrade, E. C. Celeghini, P. D. Reeb, J. P. Martins, R. M. dos Santos, M. E. Beletti, R. F. Peres, F. M. Monteiro, and V. F. Hossepian de Lima. 2013. Assessment of *in vitro* sperm characteristics and their importance in the prediction of conception rate in a bovine timed-AI program. Animal Reproduction Science 137(3-4):145-155. doi: 10.1016/j.anireprosci.2013.01.010.

Ortavant, R. 1959. Déroulement et durée du cycle spermatogénétique chez le bélier. Première partie. In: Annales de Zootechnie. p 183-244.

Ortega, M. S., N. A. Rocha-Frigoni, G. Z. Mingoti, Z. Roth, and P. J. Hansen. 2016. Modification of embryonic resistance to heat shock in cattle by melatonin and genetic variation in HSPA1L. Journal of Dairy Science 99(11):9152-9164.

Ortega, M. S., J. G. Moraes, D. J. Patterson, M. F. Smith, S. K. Behura, S. Poock, and T. E. Spencer. 2018. Influences of sire conception rate on pregnancy establishment in dairy cattle. Biology of Reproduction 99(6):1244-1254.

Overstreet, J., and G. Cooper. 1978. Sperm transport in the reproductive tract of the female rabbit: I. The rapid transit phase of transport. Biology of Reproduction 19(1):101-114.

Overstreet, J., G. Cooper, and D. Katz. 1978. Sperm transport in the reproductive tract of the female rabbit: II. The sustained phase of transport. Biology of Reproduction 19(1):115-132.

Palmer, C., L. Brito, A. Arteaga, L. Söderquist, Y. Persson, and A. Barth. 2005. Comparison of electroejaculation and transrectal massage for semen collection in range and yearling feedlot beef bulls. Animal Reproduction Science 87(1-2):25-31.

Panner Selvam, M. K., A. Agarwal, P. N. Pushparaj, S. Baskaran, and H. Bendou. 2019. Sperm proteome analysis and identification of fertility-associated biomarkers in unexplained male infertility. Genes 10(7):522.

Parent, S., L. Lefièvre, Y. Brindle, and R. Sullivan. 1999. Bull subfertility is associated with low levels of a sperm membrane antigen. Molecular Reproduction and Development 52(1):57-65.

Parrish, J., J. Susko-Parrish, and N. First. 1985. Effect of heparin and chondroitin sulfate on the acrosome reaction and fertility of bovine sperm *in vitro*. Theriogenology 24(5):537-549.

Parrish, J., J. Susko-Parrish, M. Leibfried-Rutledge, E. Critser, W. Eyestone, and N. First. 1986. Bovine *in vitro* fertilization with frozen-thawed semen. Theriogenology 25(4):591-600.

Parrish, J., J. Susko-Parrish, M. Winer, and N. First. 1988. Capacitation of bovine sperm by heparin. Biology of Reproduction 38(5):1171-1180.

Parrish, J. 1989. Capacitation of spermatozoa *in vivo*: physiochemical and molecular mechanisms. Journal of Molecular Andrology 1:87-111.

Parrish, J., J. Susko-Parrish, and N. First. 1989. Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of intracellular pH. Biology of Reproduction 41(4):683-699.

Parrish, J. J. 2014. Bovine *in vitro* fertilization: *in vitro* oocyte maturation and sperm capacitation with heparin. Theriogenology 81(1):67-73.

Peddinti, D., B. Nanduri, A. Kaya, J. M. Feugang, S. C. Burgess, and E. Memili. 2008. Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility. BMC Systems Biology 2(1):1-13.

Perry, G., and B. Perry. 2008a. Effect of preovulatory concentrations of estradiol and initiation of standing estrus on uterine pH in beef cows. Domestic Animal Endocrinology 34(3):333-338.

Perry, G., and B. Perry. 2008b. Effects of standing estrus and supplemental estradiol on changes in uterine pH during a fixed-time artificial insemination protocol. Journal of Animal Science 86(11):2928-2935.

Perry, G. A., R. A. Cushman, B. L. Perry, A. K. Schiefelbein, E. J. Northrop, J. J. Rich, and S. D. Perkins. 2020. Role of preovulatory concentrations of estradiol on timing of conception and regulation of the uterine environment in beef cattle. Systems Biology in Reproductive Medicine 66(1):12-25.

Phillips, P. H., and H. A. Lardy. 1940. A yolk-buffer pabulum for the preservation of bull sperm. Journal of Dairy Science 23:399-404.

Pholpramool, C., J. Zupp, and B. Setchell. 1985. Motility of undiluted bull epididymal spermatozoa collected by micropuncture. Reproduction (Cambridge, England) 75(2):413-420.

Pisanu, S., S. Ghisaura, D. Pagnozzi, G. Falchi, G. Biosa, A. Tanca, T. Roggio, S. Uzzau, and M. F. Addis. 2012. Characterization of sheep milk fat globule proteins by two-dimensional polyacrylamide gel electrophoresis/mass spectrometry and generation of a reference map. International Dairy Journal 24(2):78-86. doi: 10.1016/j.idairyj.2011.05.009.

Pluta, K., P. A. McGettigan, C. J. Reid, J. A. Browne, J. A. Irwin, T. Tharmalingam, A. Corfield, A. Baird, B. J. Loftus, and A. C. Evans. 2012. Molecular aspects of mucin biosynthesis and mucus formation in the bovine cervix during the periestrous period. Physiological Genomics 44(24):1165-1178.

Polge, C., A. U. Smith, and A. S. Parkes. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164(4172):666-666.

Polge, C., and L. Rowson. 1952. Fertilizing capacity of bull spermatozoa after freezing at −79° C. Nature 169(4302):626-627.

Pollard, J. W., C. Plante, W. Allan King, P. J. Hansen, K. J. Betteridge, and S. S. Suarez. 1991. Fertilizing capacity of bovine sperm may be maintained by binding to oviductal epithelial cells. Biology of Reproduction 44(1):102-107.

Pratt, C., and F. Church. 1992. Heparin binding to protein C inhibitor. Journal of Biological Chemistry 267(13):8789-8794.

Purvis, K., H. Rui, A. Schølberg, S. Hesla, and O. P. F. Clausen. 1990. Application of flow cytometry to studies on the human acrosome. Journal of Andrology 11(4):361-366.

Que, E. L., R. Bleher, F. E. Duncan, B. Y. Kong, S. C. Gleber, S. Vogt, S. Chen, S. A. Garwin, A. R. Bayer, and V. P. Dravid. 2015. Quantitative mapping of zinc fluxes in the mammalian egg reveals the origin of fertilization-induced zinc sparks. Nature Chemistry 7(2):130-139.

Ren, D., B. Navarro, G. Perez, A. C. Jackson, S. Hsu, Q. Shi, J. L. Tilly, and D. E. Clapham. 2001. A sperm ion channel required for sperm motility and male fertility. Nature 413(6856):603-609.

Richardson, B. N., S. L. Hill, J. S. Stevenson, G. D. Djira, and G. A. Perry. 2016. Expression of estrus before fixed-time AI affects conception rates and factors that impact expression of estrus and the repeatability of expression of estrus in sequential breeding seasons. Animal Reproduction Science 166:133-140.

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. Animal Reproduction Science 178:61-66.

Riffo, M., S. Leiva, and J. Astudillo. 1992. Effect of zinc on human sperm motility and the acrosome reaction. International Journal of Andrology 15(3):229-237.

Rifkin, J. M., and G. E. Olson. 1985. Characterization of maturation-dependent extrinsic proteins of the rat sperm surface. The Journal of Cell Biology 100(5):1582-1591.

Robaire, B., and R. S. Viger. 1995. Regulation of epididymal epithelial cell functions. Biology of Reproduction 52(2):226-236.

Robaire, B., and B. T. Hinton. 2015. The Epididymis. In: T. M. Plant and A. J. Zeleznik, editors, Knobil and Neill's Physiology of Reproduction No. 1. Academic Press, San Diego, CA. p. 691-771.

Robbins, R., R. G. Saacke, and P. Chandler. 1976. Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in French straws. Journal of Animal Science 42(1):145-154.

Rodriguez-Martinez, H., L. Nicander, S. Viring, S. Einarsson, and K. Larsson. 1990. Ultrastructure of the uterotubal junction in preovulatory pigs. Anatomia, Histologia, Embryologia 19(1):16-36.

Rodriguez-Martinez, H. 2003. Laboratory semen assessment and prediction of fertility: still utopia? Reproduction in Domestic Animals 38(4):312-318.

Ronquist, G., I. Brody, A. Gottfries, and B. Stegmayr. 1978a. An Mg2+ and Ca2+-stimulated adenosine triphosphatase in human prostatic fluid: part I. Andrologia 10(4):261-272.

Ronquist, G., I. Brody, A. Gottfries, and B. Stegmayr. 1978b. An Mg2+ and Ca2+-stimulated adenosine triphosphatase in human prostatic fluid-part II. Andrologia 10(6):427-433.

Rooney, I. A., J. P. Atkinson, E. S. Krul, G. Schonfeld, K. Polakoski, J. E. Saffitz, and B. P. Morgan. 1993. Physiologic relevance of the membrane attack complex inhibitory protein CD59 in human seminal plasma: CD59 is present on extracellular organelles (prostasomes), binds cell membranes, and inhibits complement-mediated lysis. The Journal of Experimental Medicine 177(5):1409-1420.

Rubinstein, E., A. Ziyyat, M. Prenant, E. Wrobel, J.-P. Wolf, S. Levy, F. Le Naour, and C. Boucheix. 2006. Reduced fertility of female mice lacking CD81. Developmental Biology 290(2):351-358.

Russell, L. D., and M. D. Griswold. 1993. The Sertoli Cell. Cache River Press, St Louis, MO.

Saacke, R., S. Nadir, and R. Nebel. 1994. Relationship of semen quality to sperm transport, fertilization, and embryo quality in ruminants. Theriogenology 41(1):45-50.

Saacke, R., J. Dalton, S. Nadir, R. Nebel, and J. Bame. 2000. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. Animal Reproduction Science 60:663-677
.
Saacke, R. 2008. Sperm morphology: Its relevance to compensable and uncompensable traits in semen. Theriogenology 70(3):473-478.

Saez, F., G. Frenette, and R. Sullivan. 2003. Epididymosomes and prostasomes: their roles in posttesticular maturation of the sperm cells. Journal of Andrology 24(2):149-154.

Saint-Dizier, M., C. Mahé, K. Reynaud, G. Tsikis, P. Mermillod, and X. Druart. 2020. Sperm interactions with the female reproductive tract: A key for successful fertilization in mammals. Molecular and Cellular Endocrinology:110956. doi: 10.1016/j.mce.2020.110956.

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

Satouh, Y., N. Inoue, M. Ikawa, and M. Okabe. 2012. Visualization of the moment of mouse sperm–egg fusion and dynamic localization of IZUMO1. Journal of Cell Science 125(21):4985-4990.

Seidel Jr, G., and R. Foote. 1969a. Influence of semen collection interval and tactile stimuli on semen quality and sperm output in bulls. Journal of Dairy Science 52(7):1074-1079.

Seidel Jr, G., and R. Foote. 1969b. Influence of semen collection techniques on composition of bull seminal plasma. Journal of Dairy Science 52(7):1080-1084.

Sergeant, N., L. Briand-Amirat, D. Bencharif, and M. Delehedde. 2019. The sperm specific protein proAKAP4 as an innovative marker to evaluate sperm quality and fertility. Journal of Dairy and Veterinary Science 11(1):JDVS.MS.ID.555803.

Shalgi, R., T. T. Smith, and R. Yanagimachi. 1992. A quantitative comparison of the passage of capacitated and uncapacitated hamster spermatozoa through the uterotubal junction. Biology of Reproduction 46(3):419-424.

Sharpe, R. 1994. Regulation of spermatogenesis. In: E. Knobil and J. Neill, editors, The Physiology of Reproduction No. 1. Raven Press, New York, NY. p. 1363-1434.

Silva, M., V. Pedrosa, J. Borges-Silva, J. Eler, J. Guimarães, and L. Albuquerque. 2013. Genetic parameters for scrotal circumference, breeding soundness examination and sperm defects in young Nellore bulls. Journal of Animal Science 91(10):4611-4616.

Singh, A. P., and S. Rajender. 2015. CatSper channel, sperm function and male fertility. Reproductive Biomedicine Online 30(1):28-38.

Smith, L. B., and W. H. Walker. 2015. Hormone signaling in the testis. In: T. M. Plant and A. J. Zeleznik, editors, Knobil and Neill's Physiology of Reproduction No. 1. Elsevier North-Holland, Inc., San Diego, CA. p. 637-690.

Smith, M., D. Morris, M. Amoss, N. Parish, J. Williams, and J. Wiltbank. 1981. Relationships among fertility, scrotal circumference, seminal quality, and libido in Santa Gertrudis bulls. Theriogenology 16(4):379-397.

Smith, M. F., R. D. Geisert, and J. J. Parrish. 2018. Reproduction in domestic ruminants during the past 50 yr: discovery to application. Journal of Animal Science 96(7):2952-2970.

Smith, T., and R. Yanagimachi. 1991. Attachment and release of spermatozoa from the caudal isthmus of the hamster oviduct. Reproduction (Cambridge, England) 91(2):567-573.

Somashekar, L., S. Selvaraju, S. Parthipan, and J. P. Ravindra. 2015. Profiling of sperm proteins and association of sperm PDC-109 with bull fertility. Systems Biology in Reproductive Medicine 61(6):376-387.

Somashekar, L., S. Selvaraju, S. Parthipan, S. K. Patil, B. K. Binsila, M. M. Venkataswamy, S. Karthik Bhat, and J. P. Ravindra. 2017. Comparative sperm protein profiling in bulls differing in fertility and identification of phosphatidylethanolamine-binding protein 4, a potential fertility marker. Andrology 5(5):1032-1051. doi: 10.1111/andr.12404.

Sprott, L., M. Harris, D. Forrest, J. Young, H. Zhang, J. Oyarzo, M. Bellin, and R. Ax. 2000. Artificial insemination outcomes in beef females using bovine sperm with a detectable fertility-associated antigen. Journal of Animal Science 78(4):795-798.

Stålhammar, E., L. Janson, and J. Philipsson. 1994. The impact of sperm motility on non-return rate in preselected dairy bulls. Reproduction Nutrition Development 34(1):37-45.

Starbuck, M. J., R. A. Dailey, and E. K. Inskeep. 2004. Factors affecting retention of early pregnancy in dairy cattle. Animal Reproduction Science 84(1-2):27-39.

Staub, C., and L. Johnson. 2018. Spermatogenesis in the bull. Animal 12(s1):s27-s35.

Stauss, C. R., T. J. Votta, and S. S. Suarez. 1995. Sperm motility hyperactivation facilitates penetration of the hamster zona pellucida. Biology of Reproduction 53(6):1280-1285.

Stegmayr, B., and G. Ronquist. 1982. Promotive effect on human sperm progressive motility by prostasomes. Urological Research 10(5):253-257.

Stoecklein, K. S., M. S. Ortega, L. D. Spate, C. N. Murphy, and R. S. Prather. 2021. Improved cryopreservation of *in vitro* produced bovine embryos using FGF2, LIF, and IGF1. PloS One 16(2):e0243727.

Suarez, S. S., and R. A. Osman. 1987. Initiation of hyperactivated flagellar bending in mouse sperm within the female reproductive tract. Biology of Reproduction 36(5):1191-1198.

Suarez, S. S., L. Vincenti, and M. W. Ceglia. 1987. Hyperactivated motility induced in mouse sperm by calcium ionophore A23187 is reversible. Journal of Experimental Zoology 244(2):331-336.

Suarez, S. S., K. Brockman, and R. Lefebvre. 1997. Distribution of mucus and sperm in bovine oviducts after artificial insemination: the physical environment of the oviductal sperm reservoir. Biology of Reproduction 56(2):447-453.

Suarez, S. S., I. Revah, M. Lo, and S. Kölle. 1998. Bull sperm binding to oviductal epithelium is mediated by a Ca2+-dependent lectin on sperm that recognizes Lewis-a trisaccharide. Biology of Reproduction 59(1):39-44.

Suarez, S. S. 2006. Gamete and zygote transport. In: J. D. Neill, editor, Knobil and Neill's Physiology of Reproduction No. 1. Academic Press, San Diego, CA. p. 113-145.

Suarez, S. S., and A. Pacey. 2006. Sperm transport in the female reproductive tract. Human Reproduction Update 12(1):23-37.

Suarez, S. S. 2008. Control of hyperactivation in sperm. Human Reproduction Update 14(6):647-657.

Suarez, S. S. 2015. Gamete and Zygote Transport. In: T. M. Plant and A. J. Zeleznik, editors, Knobil and Neill's Physiology of Reproduction No. 1. Academic Press, San Diego, CA. p. 197-232.

Suarez, S. S. 2016. Mammalian sperm interactions with the female reproductive tract. Cell and Tissue Research 363(1):185-194.

Suga, T., and S. Higaki. 1971. Studies on the uterine secretion of the cow. II. Distribution of spermatozoa and seminal plasma after intra-uterine insemination in the

reproductive tract of cow during estrous stage. Chiba city National Institute of Animal Industry Bulletin (Japan) 24:41.

Sullivan, R., F. Saez, J. Girouard, and G. Frenette. 2005. Role of exosomes in sperm maturation during the transit along the male reproductive tract. Blood Cells, Molecules & Diseases 35(1):1-10. doi: 10.1016/j.bcmd.2005.03.005.

Sullivan, R., G. Frenette, and J. Girouard. 2007. Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. Asian Journal of Andrology 9(4):483-491.

Sullivan, R., and F. Saez. 2013. Epididymosomes, prostasomes, and liposomes: their roles in mammalian male reproductive physiology. Reproduction (Cambridge, England) 146(1):R21-R35.

Sullivan, R. 2016. Epididymosomes: role of extracellular microvesicles in sperm maturation. Frontiers in Bioscience (Schol Ed) 8:106-114.

Sullivan, R., and C. Belleannée. 2017. Role of epididymis in sperm maturation. In: C. J. De Jonge and C. L. R. Barratt, editors, The Sperm Cell: Production, Maturation, Fertilization, Regeneration. Cambridge University Press, Cambridge, UK. p. 73-87.

Sutovsky, P., R. Moreno, J. Ramalho-Santos, T. Dominko, W. E. Thompson, and G. Schatten. 2001. A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. Journal of Cell Science 114(9):1665-1675.

Sutovsky, P., E. Neuber, and G. Schatten. 2002. Ubiquitin-dependent sperm quality control mechanism recognizes spermatozoa with DNA defects as revealed by dual ubiquitin-TUNEL assay. Molecular Reproduction and Development 61(3):406-413.

Sutovsky, P., W. Plummer, K. Baska, K. Peterman, J. Diehl, and M. Sutovsky. 2007. Relative Levels of Semen Platelet Activating Factor-Receptor (PAFr) and Ubiquitin in Yearling Bulls With High Content of Semen White Blood Cells: Implications for Breeding Soundness Evaluation. Journal of Andrology 28(1):92-108.

Sutovsky, P. 2009. Sperm-egg adhesion and fusion in mammals. Expert Reviews in Molecular Medicine 11:e11. doi: 10.1017/s1462399409001045.

Sutovsky, P. 2018. Sperm–oocyte interactions and their implications for bull fertility, with emphasis on the ubiquitin–proteasome system. Animal 12(s1):s121-s132.

Sutovsky, P., K. Kerns, M. Zigo, and D. Zuidema. 2019. Boar semen improvement through sperm capacitation management, with emphasis on zinc ion homeostasis. Theriogenology 137:50-55.

Szklarczyk, D., A. Franceschini, S. Wyder, K. Forslund, D. Heller, J. Huerta-Cepas, M. Simonovic, A. Roth, A. Santos, K. P. Tsafou, M. Kuhn, P. Bork, L. J. Jensen, and C. von Mering. 2015. STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Research 43(Database issue):D447-452. doi: 10.1093/nar/gku1003.

Tampion, D., and R. Gibbons. 1962. Orientation of spermatozoa in mucus of the cervix uteri. Nature 194(4826):381-381.

Tao, J., E. S. Critser, and J. K. Critser. 1993. Evaluation of mouse sperm acrosomal status and viability by flow cytometry. Molecular Reproduction and Development 36(2):183-194.

Thérien, I., D. Bousquet, and P. Manjunath. 2001. Effect of seminal phospholipid-binding proteins and follicular fluid on bovine sperm capacitation. Biology of Reproduction 65(1):41-51.

Thundathil, J. C., A. L. Dance, and J. P. Kastelic. 2016. Fertility management of bulls to improve beef cattle productivity. Theriogenology 86(1):397-405. doi: 10.1016/j.theriogenology.2016.04.054.

Toelle, V., and O. Robison. 1985. Estimates of genetic correlations between testicular measurements and female reproductive traits in cattle. Journal of Animal Science 60(1):89-100.

Tokuhiro, K., M. Ikawa, A. M. Benham, and M. Okabe. 2012. Protein disulfide isomerase homolog PDILT is required for quality control of sperm membrane protein ADAM3 and male fertility. Proceedings of the National Academy of Sciences 109(10):3850-3855.

Toshimori, K., D. Saxena, I. Tanii, and K. Yoshinaga. 1998. An MN9 antigenic molecule, equatorin, is required for successful sperm-oocyte fusion in mice. Biology of Reproduction 59(1):22-29.

Tríbulo, P., R. M. Rivera, M. S. O. Obando, E. A. Jannaman, and P. J. Hansen. 2019. Production and culture of the bovine embryo, Comparative Embryo Culture. Springer. p. 115-129.

Tung, C.-k., F. Ardon, A. G. Fiore, S. S. Suarez, and M. Wu. 2014. Cooperative roles of biological flow and surface topography in guiding sperm migration revealed by a microfluidic model. Lab on a Chip 14(7):1348-1356.

Tung, C.-k., L. Hu, A. G. Fiore, F. Ardon, D. G. Hickman, R. O. Gilbert, S. S. Suarez, and M. Wu. 2015. Microgrooves and fluid flows provide preferential passageways for sperm over pathogen Tritrichomonas foetus. Proceedings of the National Academy of Sciences 112(17):5431-5436.

Uhrin, P., M. Dewerchin, M. Hilpert, P. Chrenek, C. Schöfer, M. Zechmeister-Machhart, G. Krönke, A. Vales, P. Carmeliet, and B. R. Binder. 2000. Disruption of the protein C inhibitor gene results in impaired spermatogenesis and male infertility. The Journal of Clinical Investigation 106(12):1531-1539.

USDA. 2018. Dairy 2014, “Health and Management Practices on U.S. Dairy Operations, 2014”, USDA–APHIS–VS–CEAH–NAHMS. Fort Collins, CO.

USDA. 2020. Beef 2017, "Beef Cow-calf Management Practices in the United States, 2017, report 1.", USDA–APHIS–VS–CEAH–NAHMS. Fort Collins, CO.

Utt, M. D. 2016. Prediction of bull fertility. Animal Reproduction Science 169:37-44.

Valour, D., P. Michot, C. Eozenou, R. Lefebvre, A. Bonnet, A. Capitan, S. Uzbekova, E. Sellem, C. Ponsart, and L. Schibler. 2015. Dairy cattle reproduction is a tightly regulated genetic process: Highlights on genes, pathways, and biological processes. Animal Frontiers 5(1):32-41.

VanDemark, N., and A. Moeller. 1951. Speed of spermatozoan transport in reproductive tract of estrous cow. American Journal of Physiology-Legacy Content 165(3):674-679.

VanDemark, N., and R. Hays. 1952. Uterine motility responses to mating. American Journal of Physiology-Legacy Content 170(3):518-521.

Vijayaraghavan, S., K. D. Trautman, S. A. Goueli, and D. W. Carr. 1997. A tyrosine-phosphorylated 55-kilodalton motility-associated bovine sperm protein is regulated by cyclic adenosine 3',5'-monophosphates and calcium. Biology of Reproduction 56(6):1450-1457.

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

Vredenburgh-Wilberg, W., and J. Parrish. 1995. Intracellular pH of bovine sperm increases during capacitation. Molecular Reproduction and Development 40(4):490-502.

Wales, R., J. Wallace, and I. White. 1966. Composition of bull epididymal and testicular fluid. Reproduction (Cambridge, England) 12(1):139-144.

Wallis, M., P. Waters, and J. Graves. 2008. Sex determination in mammals—before and after the evolution of SRY. Cellular and Molecular Life Sciences 65(20):3182-3195.

Wang, J., J. Wang, H.-R. Zhang, H.-J. Shi, D. Ma, H.-X. Zhao, B. Lin, and R.-S. Li. 2009. Proteomic analysis of seminal plasma from asthenozoospermia patients reveals proteins that affect oxidative stress responses and semen quality. Asian Journal of Andrology 11(4):484.

Wassarman, P. M. 1990. Profile of a mammalian sperm receptor. Development 108(1):1-17.

White, I. 1974. Mammalian semen. In: E. S. E. Hafez, editor, Reproduction in Farm Animals. Lea & Febiger Philadelphia, PA. p. 101-122.

Whitman, R. W. 1975. Weight change, body condition and beef-cow reproduction, Colorado State University.

Wiggans, G. R., J. B. Cole, S. M. Hubbard, and T. S. Sonstegard. 2017. Genomic selection in dairy cattle: the USDA experience. Annual Review of Animal Biosciences 5:309-327.

Williams, A., and W. Ford. 2004. Functional significance of the pentose phosphate pathway and glutathione reductase in the antioxidant defenses of human sperm. Biology of Reproduction 71(4):1309-1316.

Wilmut, I., and R. Hunter. 1984. Sperm transport into the oviducts of heifers mated early in oestrus. Reproduction Nutrition Développement 24(4):461-468.

Wiltbank, J., and N. Parish. 1986. Pregnancy rate in cows and heifers bred to bulls selected for semen quality. Theriogenology 25(6):779-783.

Wolf, D. E., S. S. Hagopian, and S. Ishijima. 1986. Changes in sperm plasma membrane lipid diffusibility after hyperactivation during *in vitro* capacitation in the mouse. The Journal of Cell Biology 102(4):1372-1377.

Wolf, F., J. Almquist, and E. Hale. 1965. Prepuberal behavior and puberal characteristics of beef bulls on high nutrient allowance. Journal of Animal Science 24(3):761-765.

Wrobel, K.-H., R. Kujat, and G. Fehle. 1993. The bovine tubouterine junction: general organization and surface morphology. Cell and Tissue Research 271(2):227-239.

Yamagata, K., T. Nakanishi, M. Ikawa, R. Yamaguchi, S. B. Moss, and M. Okabe. 2002. Sperm from the calmegin-deficient mouse have normal abilities for binding and fusion to the egg plasma membrane. Developmental Biology 250(2):348-357.

Yamaguchi, R., Y. Muro, A. Isotani, K. Tokuhiro, K. Takumi, I. Adham, M. Ikawa, and M. Okabe. 2009. Disruption of ADAM3 impairs the migration of sperm into oviduct in mouse. Biology of Reproduction 81(1):142-146.

Yanagimachi, R., and M. Chang. 1963. Sperm ascent through the oviduct of the hamster and rabbit in relation to the time of ovulation. Reproduction 6(3):413-420.

Yanagimachi, R., and N. Usui. 1974. Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. Experimental Cell Research 89(1):161-174.

Yanagimachi, R., Y. Kamiguchi, K. Mikamo, F. Suzuki, and H. Yanagimachi. 1985. Maturation of spermatozoa in the epididymis of the Chinese hamster. American Journal of Anatomy 172(4):317-330.

Yanagimachi, R. 1994. Mammalian fertilization. In: E. Knobil and J. D. Neill, editors, The Physiology of Reproduction No. 1. Raven Press, New York. p. 189-317.

Yániz, J. L., F. Lopez-Gatius, P. Santolaria, and K. J. Mullins. 2000. Study of the functional anatomy of bovine oviductal mucosa. The Anatomical Record: An Official Publication of the American Association of Anatomists 260(3):268-278.

Yonezawa, N. 2014. Posttranslational modifications of zona pellucida proteins. Posttranslational Protein Modifications in the Reproductive System:111-140.

Yuan, Y., W. Chen, Q. Shi, L. Mao, S. Yu, X. Fang, and E. Roldan. 2003. Zona pellucida induces activation of phospholipase A2 during acrosomal exocytosis in guinea pig spermatozoa. Biology of Reproduction 68(3):904-913.

Zeng, Y., E. N. Clark, and H. M. Florman. 1995. Sperm membrane potential: hyperpolarization during capacitation regulates zona pellucida-dependent acrosomal secretion. Developmental Biology 171(2):554-563.

Zhang, N., F. E. Duncan, E. L. Que, T. V. O'Halloran, and T. K. Woodruff. 2016. The fertilization-induced zinc spark is a novel biomarker of mouse embryo quality and early development. Scientific Reports 6(1):1-9.

Zhao, X. M., N. Wang, H. S. Hao, C. Y. Li, Y. H. Zhao, C. L. Yan, H. Y. Wang, W. H. Du, D. Wang, and Y. Liu. 2018. Melatonin improves the fertilization capacity and developmental ability of bovine oocytes by regulating cytoplasmic maturation events. Journal of Pineal Research 64(1):e12445.

Zheng, X., M. Geiger, S. Ecke, E. Bielek, P. Donner, U. Eberspacher, W. D. Schleuning, and B. R. Binder. 1994. Inhibition of acrosin by protein C inhibitor and localization of protein C inhibitor to spermatozoa. The American Journal of Physiology 267(2 Pt 1):C466-472. doi: 10.1152/ajpcell.1994.267.2.C466.

Zhou, G.-B., G.-S. Liu, Q.-G. Meng, Y. Liu, Y.-P. Hou, X.-X. Wang, N. Li, and S.-E. Zhu. 2009. Tetraspanin CD9 in Bovine Oocytes and Its Role in Fertilization. Journal of Reproduction and Development 55(3):305-308. doi: 10.1262/jrd.20099.

Zoca, S. M., B. Shafii, W. Price, M. Utt, B. Harstine, K. McDonald, L. Cruppe, M. DeJarnette, L. Peters, J. L. M. Vasconcelos, and J. Dalton. 2020. Angus sire field fertility and *in vitro* sperm characteristics following use of different sperm insemination doses in Brazilian beef cattle. Theriogenology 147:146-153.

APPENDIX

Appendix 1. Pearson's correlation coefficient of bull field fertility[1] and *in vitro* embryo production [cleavage rate (CL), blastocyst rate (BL), and BL/CL ration] with bull effect least square means (n = 5) for sperm total (TMOT) and progressive (PROG) motility, plasma membrane integrity (viability), acrosome integrity (viable intact, viable disrupted, dead intact, dead disrupted), reactive oxygen species (ROS; viable ROS+, viable ROS-, dead ROS-, dead ROS-), mitochondrial membrane potential (mito-potential), zinc signatures (signature 1, signature 2, signature 3, signature 4, and signature 1 + 2), and CD9 populations [viable or dead sperm and CD9 positive (+) or negative (-)].

Variables	Field Fertility	CL	BL	BL/CL
TMOT	0.48	0.17	-0.43	-0.49
PROG	0.21	-0.04	-0.60	-0.55
Viability	0.81*	0.33	0.62	0.44
Viable intact	0.48	0.63	0.19	-0.15
Viable disrupted	0.23	-0.38	-0.10	0.13
Dead intact	0.34	-0.36	0.29	0.49
Dead disrupted	-0.59	-0.35	-0.26	-0.07
Viable ROS+	0.65	0.00	0.23	0.24
Viable ROS-	0.16	0.66	-0.05	-0.42
Dead ROS+	0.68	0.04	0.22	0.21
Dead ROS-	-0.91**	-0.84*	-0.17	0.29
Mito-potential	0.52	0.01	0.56	0.55
Signature 1	-0.08	-0.71	0.11	0.51
Signature 2	0.89**	0.65	0.62	0.26
Signature 3	0.20	-0.14	0.53	0.60
Signature 4	-0.75	-0.18	-0.29	-0.20
Signature 1 + 2	0.80*	0.36	0.63	0.43
Viable CD9+	0.64	0.07	0.15	0.12
Dead CD9+	-0.68	-0.34	-0.46	-0.28
Viable CD9-	0.63	0.65	0.61	0.24
Dead CD9-	0.14	-0.51	-0.12	0.18
Viable CD9 FI	0.41	-0.26	0.22	0.37
Dead CD9 FI	-0.27	0.21	-0.17	-0.30

[1]Field fertility values were retrieved from (Zoca et al., 2020)

**$P < 0.05$

*$P \leq 0.10$

Appendix 2. Bovine non-capacitation media and bovine capacitation media recipes.

Bovine Non-Capacitation Medium (bNCM)

Reagent	Formula Weight	Concentration, mM	500 mL	1000 mL
NaCl	58.44	100	2.922 g	5.844g
NaH_2PO_4	119.98	0.3	0.0180 g	0.036g
KCl	74.55	3.1	0.1156 g	0.2312g
$MgCl_2$ $6H_20$	203.30	0.4	0.0407 g	0.0814g
PVA	10K Unknow % hydrolyzed	0.01	0.0500 g	0.1000g
Na-pyruvate °	110.04	1	0.0550 g	0.1100g
Na-lactate °	112.06; 60% w/w	22	2.06 mL	4.12mL
HEPES	238.30	40	4.766 g	9.5320g
Gentamycin (10 mg/mL stock) °	477.60	21	1.25 mL	2.50mL
Penicillin G	372.5	0.174	0.0325 g	0.0650g

pH = 7.20

Bovine Capacitation Medium (bCM)
Use bNCM and add the following ingredients:

Reagent	Formula Weight	Concentration (mM)	50 mL
$CaCl_2$ 2 H_20	147.00	2.1 mM	0.0250 g
$NaHCO_3$	84.00	2 mM**	0.0084 g
		10 mM	0.0420 g
		25 mM	0.1050 g
Heparin	-	10 µg/mL	1 mL Stk A
BSA °*		0.6%	0.3 g

pH = 7.4
° Stored in 4 °C
* Add last and only make with the final amount of CM needed; very expensive! (E.g. most times less than 5 mL of final CM (5 mL is minimum to make for pH'ing reason), in such case would only use 0.03 g BSA); Bovine relies on heparin, not BSA for cholesterol efflux.
**Prefer using 2 mM $NaHCO_3$ -> more sequential-like, does not capacitate too quickly.

COMMENTS:
PVA can be added to bNCM to better dissolve it – add 0.0500 g of PVA in 500 mL of bNCM to maintain 0.01 mM – usually first ingredient added to the water

bNCM can be sterile filtered and stored for up to 3 weeks at 4 °C

Make CM fresh every day because sodium bicarbonate ($NaHCO_3$) evaporates

Add BSA to the volume needed for the daily assays.

Heparin Stock A: 0.05 g / 100 mL bNCM ->
- ➔ Aliquot into 1 mL each for -20 °C storage until use

Summary:

Make 500 mL bNCM -> make 49 mL CM without BSA ->
1 mL Heparin Stock A + 49 mL bCM without BSA (bCM - BSA) ->
Exact amount bCM + BSA needed
➔ Adjust pH to 7.4