Characterization of Phosphatidylserine Expression in Bovine Sperm

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ABSTRACT

Many factors influence male fertility, and conventional fertility evaluations are not able to reliably identify sub-fertile animals. The overall goal of this work was to explore the expression of phosphatidylserine (PS) on bovine sperm and investigate what factors may impact it, as previous research demonstrated that phosphatidylserine (PS) plays a role in murine fertilization. Despite conventionally being an apoptotic marker, it is present on viable and fertilization-competent murine sperm, however, less is known of the possible role of PS in bovine fertilization. In experiment 1, viable bovine sperm cells expressing PS were identified and PS levels in fresh and frozen semen were compared. Phosphatidylserine levels in frozen samples were significantly less than in fresh samples. We conclude that the cryopreservation process has an impact on PS expression in sperm by altering the proportion of sperm cells which are capable of fertilization. Experiment 2 examined PS levels in bulls with varying fertility levels based on sire conception rate (SCR). There was no difference in PS levels between high and low fertility bulls. There was a significant difference in PS levels of uncapacitated samples and those capacitated for one hour. These results warrant further investigation into the role of phosphatidylserine in bovine fertilization.

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GENERAL AUDIENCE ABSTRACT

Improving the fertility of cattle is incredibly important to meet ever-growing consumer demands for animal protein. Researchers and producers can utilize a variety of reproductive technologies to improve their herds' reproductive efficiency. Phosphatidylserine (PS) is a glycerophospholipid which makes up a part of all cellular plasma membranes. Typically, it is used as a marker for cell death or apoptosis, however, some cells expose it on their surface temporarily while still viable, including sperm. Phosphatidylserine was found to be exposed on sperm from mice that were still viable and able to fertilize oocytes. Following that, the expression of PS in bovine sperm was investigated. Using bulls as a model, fresh semen was collected and analyzed for the level of PS expression then frozen and reanalyzed. We saw that there was a significant decrease in the level of PS expression in sperm that had been previously frozen, possibly due to damage to their membranes during the freezing process. Frozen semen from beef bulls with either high or low fertility was also analyzed. No difference was observed between bulls with varying levels of fertility. Addressing fertility issues in bulls is a complicated and multi-faceted issue which requires the use of many technologies and fertility markers. Further developing the knowledge of PS exposure in bulls and its relation to fertility and fertilization is worthwhile to attempt to improve the reproductive efficiency of cattle herds.

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List of Abbreviations

AI: Artificial insemination

ART: Assisted reproductive technology

CASA: Computer-assisted sperm analysis

EEJ: Electroejaculation

ER: Endoplasmic reticulum

ET: Embryo transfer

IVF: *In vitro* fertilization

PLC ζ: Phospholipase C zeta

PC: Phosphatidylcholine

PE: Phosphatidylethanolamine

PI: Propidium iodide

PS: Phosphatidylserine

UTJ: Utero-tubal junction

ZP: Zona pellucida

Chapter 1

Introduction

Increasing fertilization rates in cattle herds around the world is important to improving production efficiency and meet the ever-growing global demand for food, as the world population is predicted to increase by around 83 million people per year and reaching approximately 11.2 billion in 2100 (Nations, 2017). Along with this increasing population is an increase in consumers desires for animal protein (FAO, 2017) and decreasing amounts of land for animal production to be based on. From 1990 to 2009, global meat consumption increased by approximately 60% (Henchion et al., 2014) and is expected to increase to 76% by 2050 from consumption in 1997 (WRAP, 2015). The beef and dairy cattle industries would benefit from adopting new assisted reproductive technologies (ART) focused on fertility to aid in improving their efficiency to meet this ever-growing demand for animal protein on limited land.

A primary focus of cow-calf producers is to produce one calf per cow every year; however, infertility is a significant barrier to this. Infertility in the U.S. can cost the beef industry $3 billion per year and for every 1% decrease in pregnancy rate, there is an economic loss of $6.25 for every exposed cow for producers (Lamb et al., 2014). Improving the reproductive rate in American beef cattle herds would result in fewer females being culled due to reproductive issues (Engelken et al., 2007). Due to this, fertility is regarded as more significant than some production traits despite it being more difficult to genetically select as fertility traits have low heritability. Focusing on improving male fertility in cattle is especially advantageous as one bull can be used to breed many females every year, especially if their semen is frozen and distributed across many farms.

The majority of reproductive failure in cattle can be classified as embryonic loss, which occurs in the first 35 days of gestation (Chebel et al., 2004). For beef cattle, the greatest losses occur during the first 7 days, mostly attributed to early cleavage and development, along with days 16 to 32, due to errors in the maternal recognition of pregnancy (Reese et al., 2020), further, these periods of greatest loss are also seen in dairy cattle. For beef cattle, only approximately 50% will maintain their pregnancy by day 100 (Reese et al., 2020) and dairy cattle usually experience pregnancy loss of greater than 50%, especially in high-producing animals (Wiltbank et al., 2016).

There is a tremendous amount of research focused on fertilization, especially in mammals. This research has been used in a variety of ways, but it has been the most impactful in its aid in the development of assisted reproductive technologies for livestock. Understanding of the events leading up to fertilization and beyond has led to the creation of technologies such as *in vitro* fertilization (IVF), and artificial insemination (AI), among others. Adoption of assisted reproductive technologies is greater in the dairy industry than in the beef industry; however, the development of new and refined fertility measures could aid in increasing adoption of technologies and improving the efficiency of animal production systems. Many factors impact fertility but the majority of the focus on improving fertility is directed towards female animals, with male fertility typically being regarded as satisfactory even though improvements can be made.

Review of the literature related to mammalian fertilization reveals there are many factors involved in oocytes, spermatozoa, or both that need to be investigated. This thesis will demonstrate how the understanding of mammalian fertilization has aided in the development of

new fertility technologies and markers. Further, analysis of a new factor that may influence the fertility of male animals will be explored and the impacts of its investigation will be discussed.

Chapter 2

Literature Review

Overview of Bovine Assisted Reproductive Technologies

Assisted reproductive technologies (ARTs) are techniques that allow for the establishment and maintenance of pregnancy through at least partially artificial practices. Bovine ARTs include embryo transfer (ET), *in vitro* embryo production (IVP), cloning, gene editing, artificial insemination (AI), gamete retrieval, and gamete cryopreservation. Infertility is not only relevant to cattle production and research but also other species, such as humans wanting to start or grow their families. In both dairy and beef cattle, infertility can contribute to significant economic losses. The cause of bovine infertility cannot be narrowed down to just one issue as it is a complex and often multifactorial problem consisting of decreased fertilization rates, increased early embryonic mortality, and decreased conception rates amongst other factors (Lucy, 2001; Diskin and Morris, 2008; Diskin et al., 2011). Fertilization rates in beef and dairy cows often meet or exceed 90%, however, average pregnancy rates decrease to around 50% by day 30 of gestation due to early embryonic loss (Reese et al., 2020). In the state of Virginia alone, the benefit of combating infertility is over $4.5 million annually for every 1% increase in pregnancy rate of beef females.

Breeding Soundness Examination

Breeding Soundness Examinations (BSEs) were developed to serve as a quick and relatively inexpensive procedure to evaluate the fertility of bulls. They can be used to improve herd fertility as breeding soundness relates to the ability of a bull to impregnate females. There are five general categories of factors which impact bull fertility - structural soundness, capability

of reproductive organs, semen quality, libido, and nutritional status (Koziol and Armstrong, 2018). Although BSEs can be used to identify bulls with significant fertility issues, some bulls pass but are sub-fertile, resulting in pregnancy rates that differ by 20-25 percent (Larson and Miller, 2000). These differences in fertility of bulls which pass is due to limitations of the BSE as the evaluations for certain parameters are subjective, such as sperm motility, and do not provide a certainty that the sperm analyzed are capable of fertilization (Kastelic and Thundathil, 2008).

The procedures and assessments used are determined by the Society of Theriogenology and are updated periodically in accordance with new advances in fertility evaluations. They include evaluations over four categories in which the bull must meet the minimum requirements to be considered a satisfactory breeder and pass (Koziol and Armstrong, 2018). These four categories consist of a - general and reproductive physical examination, scrotal circumference measurement, sperm motility evaluation, and morphology evaluation (Koziol and Armstrong, 2018). The general and reproductive physical examination includes observations of the overall body condition of bulls for abnormalities and injuries that may impact their ability to successfully breed. Scrotal circumference is important as testicular volume is highly correlated with sperm output. Motility is evaluated using microscopy and bulls must have at least 30% motility to pass (Koziol and Armstrong, 2018). Morphology is evaluated using Eosin-Nigrosin staining and microscopy and bulls must have >70% normal sperm, with defects classified as primary or secondary abnormalities (Koziol and Armstrong, 2018). Breeding soundness examinations have been found to provide a benefit/cost ratio of 17:1 for beef cattle (Chenoweth, 2000) and 14:1 in dairy cattle (Dwyer, 2013) when eliminating bulls used in natural breeding.

Overall, BSEs serve as a tool to standardize selection of breeding bulls and can aid in improving herd fertility and reproductive management.

Gamete Cryopreservation

Cryopreservation is a process which allows the indefinite storage of various cells and involves utilizing cryoprotective agents to prevent damage to cells that can occur during the freezing process (McGann, 1978). There are various methods of freezing and concentrations of the extender components depending on the species and technical preference. It involves the use of extenders which usually consist of a combination of cryoprotectants, buffers, salts, sugars, and antibiotics (Evans and Maxwell, 1987). Cryoprotectants are either non-permeating or permeating and are used together to help ensure survival through the freezing process (Evans and Maxwell, 1987).

Slow freezing and vitrification are the two primary cryopreservation methods. Slow freezing consists of cooling the sperm slowly to encourage dehydration in an attempt to prevent various cellular damage that can occur during freezing due to intracellular ice formation (Arav et al., 2002). Vitrification involves freezing samples quickly in an attempt to limit cold shock, however, sperm have slower heat transfer compared to other cells and vitrification can cause excess crystallization (Arav et al., 2002). Decreases in motility, viability, and the ability for sperm to traverse the female reproductive tract can also be observed in cryopreserved semen that has been thawed (Salamon and Maxwell, 2000).

Cryopreserved gametes can be kept alive indefinitely if the proper storage conditions are maintained; however, the long-term impacts, if any, are not fully understood on samples that are frozen for prolonged periods of time. Long term storage is aided by cessation of cellular activity, which is reacquired following thawing (Mazur, 1984). It has been reported that ram sperm frozen

for 27 years did not impact fertility, so many operate under the assumption that under standard storage conditions fertility will not be impacted (Salamon and Maxwell, 2000). The invention and implementation of cryopreservation of especially male gametes has greatly assisted in the expansion of some ARTs such as AI and IVF (Medeiros et al., 2002). The main focuses of germplasm cryopreservation in agriculture are using it for more rapid genetic improvement, preserving rare breeds, controlling inbreeding, transgenic animal production, research, and decreasing labor and production costs (Holt, 1997). Any decreases in fertility rates due to cellular and DNA damage during cryopreservation can be ameliorated with increased concentrations of sperm in straws of semen (Watson, 2000).

Embryo Transfer

Embryo transfers (ETs) are another popular ART in cattle. The first embryo transfer for any species was conducted by Walter Heape in rabbits in the 1890s (Biggers, 1991). The first calf born through ET was in 1951 at the University of Wisconsin by E.L. Willett (Willett et al., 1951). Procedures for bovine ET started being written in the 1940s and continued to be refined through the 1960s. The modern commercial ET industry was developed in the 1970s through a joint effort of researchers and commercial ET technicians. ETs in cattle were initially done surgically through a mid-ventral surgical approach, but the economic cost and risk of infertility for approximately 10% of cattle caused by the surgery (Elsden, 1977) helped to drive the development of a non-surgical approach. A highly cited paper published in 1976 outlines the non-surgical approach that uses modified Foley catheters and it is still used today (Elsden et al., 1976).

In Vitro Fertilization

Bovine *in vitro* fertilization (IVF) originally began as a tool to treat infertility problems (Sirard, 2018). The first report of a normal, healthy calf born from IVF was in 1982 (Brackett et al., 1982). Over time, however, it became apparent that bovine IVF and IVP overall were powerful technologies that could greatly enhance genetic selection of livestock and aid in decreasing the generation interval (Sirard, 2018). This focus on utilizing bovine IVF to drive genomic selection is currently the most prevalent in the dairy industry (Sirard, 2018). From its origins and up to modern times, bovine IVF is the most successful out of all the species it has currently been developed for (Brackett et al., 1982; Hasler, 1998). Bovine IVP consists of several steps including retrieval of oocytes, *in vitro* maturation, IVF, and *in vitro* culture. *In vitro* maturation supports meiotic maturation of follicles with the goal of achieving high fertilization rates. *In vitro* fertilization consists of thawing and washing cryopreserved sperm followed by co-incubation with oocytes in a media that encourages capacitation while also maintaining oocyte viability. *In vitro* culture encourages early embryonic growth and development up to the blastocyst stage at day 7-8. Each stage of IVP has unique media which supports the growth and development of oocytes into zygotes and beyond, with new research focusing primarily on optimizing media conditions to increase development rates beyond their current levels.

Sexed Semen

More recently, the development of a technique to sort sperm by their sex has been developed for multiple species. It operates based on the principle that there is a 3-4% difference in the mass of DNA in the X and Y chromosomes that can be identified using a flow cytometric method which is up to 90% accurate (Johnson et al., 1999). This technology is limited by its lower productivity, as only approximately 5-6 million sperm can be sorted per hour because each sperm must be examined individually (Johnson and Welch, 1999). Bovine genetic companies

utilize semen sexing technology and offer sexed semen from bulls with greater genetic value, a practice which is more common in the dairy industry (Seidel, 2009). Reports have predicted that using sex-sorted semen can result in 9% (Dematawewa and Berger, 1998) or 15% (Van Vleck, 1981) increases in annual genetic progress for dairy herds when compared to using conventional semen. Beef producers could utilize sex-sorted semen in several different manners depending on their production goals - those who rely on bull sales could use more Y-bearing semen, while other producers with more valuable heifers could use X-bearing semen.

Some disadvantages of using sex-sorted semen include decreased sperm viability and quality after the sorting procedure (Seidel Jr and Garner, 2002; Seidel Jr, 2007), in addition semen straws only contain 2 million sperm (Seidel Jr, 2007) compared to the conventional dose of 12-15 million. Due to these factors, it is recommended that producers only use sexed-semen to breed females exhibiting estrus (Sá Filho et al., 2012).

Intracytoplasmic Sperm Injection

Intracytoplasmic sperm injection (ICSI) was first developed in the hamster in 1977 (Uehara and Yanagimachi, 1977) and consists of microinjecting one sperm into the cytoplasm of a mature oocyte. The first bovine birth as a result of ICSI occurred in 1990 (Goto et al., 1990). Its use is not as popular in cattle as bovine ICSI has a success rate of 14%, which is significantly less than other domestic species (Salamone et al., 2017). There are several reasons for this lower success rate including, nuclear decondensation and pronuclei formation failure (Águila et al., 2017), lack of calcium oscillations (Malcuit et al., 2005), and microtubule organization dysfunction (Hara et al., 2011). However, several methods of pre-treating sperm intended for ICSI have helped to overcome some of these issues. These include immobilizing sperm with tail scoring (Katayose et al., 1999; Wei and Fukui, 1999) and exposure to chemicals which

encourage nuclear decondensation and pronuclei formation (Wei and Fukui, 1999). It has also been shown to produce more embryos when using sex-sorted semen compared to conventional IVF methods (Jo et al., 2014) and can be used in livestock to help increase productivity, produce transgenic livestock, and increase biodiversity in rare breeds.

Gene Editing

Some of the first successful transgenic livestock production occurred in rabbits, sheep, and pigs (Hammer et al., 1985). These livestock were produced using pronuclear injection, which involves injecting one of the pronuclei of a zygote with DNA (Simons et al., 1988; Nottle et al., 2001). The first report of successful production of transgenic cattle occurred a few years later. This was accomplished through microinjection of IVP embryos followed by a non-surgical ET (Krimpenfort et al., 1991). The technology associated with producing transgenic livestock has advanced tremendously since its inception. More recently, use of the CRISPR-Cas9 system has resulted in the generation many animals of different species with genetic modifications. The first report of successful disruption of endogenous genes with the CRISPR-Cas9 system in cattle involved the generation of *NRAMP1* knock-in cattle to aid in increasing resistance to tuberculosis (Gao et al., 2017). The production of transgenic livestock has many purposes ranging from improving animal health, decreasing negative environmental impacts, producing animal models for biomedicine, and increasing the efficiency of food production (Menchaca et al., 2020).

Steps of Fertilization

Spermatogenesis and sperm morphology

Prior to developing fertilization capacity, spermatozoa undergo spermatogenesis and maturation in the male reproductive system. Spermatogenesis entails the entire process through

which germ cells multiply and differentiate to ultimately generate spermatozoa. Sperm are made up of a large head, a midpiece, and long flagellum or tail. They are haploid, contain a large nucleus with tightly packaged DNA, an acrosome to aid in penetration of the zona pellucida (ZP), and mitochondria within the anterior portion of the flagellum along with some other minor cellular machinery (Eddy et al., 1994). Mitochondria produce ATP that fuels spermatozoa motility by enabling the flagellar beating to ensure they reach the oocyte to initiate fertilization (Medeiros et al., 2002).

Sperm shape and size varies widely by species. Bovine sperm have paddle-shaped heads similar to humans. Anatomical defects can be in the head, midpiece, and tail and can negatively impact sperm function and viability to varying degrees (Barth and Oko, 1989). Sperm can also have primary or secondary defects. Primary defects originate during sperm development in the seminiferous tubules and are typically regarded as being more severe, while secondary defects originate as the sperm move through the epididymis and following ejaculation (Menon et al., 2011). Some examples of morphological abnormalities include double headed sperm, misshapen heads, pyriform heads, detached heads, bent tail or midpiece, coiled tail, and distal droplets.

Spermatogenesis takes place within the seminiferous tubules of the testis and is made up of three parts - spermatocytogenesis, meiosis, and spermiogenesis (Staub and Johnson, 2018a). Spermatocytogenesis consists of germ cell mitosis to produce spermatogonia and primary spermatocytes. The first mitotic division generates spermatogonia, which continue to undergo mitosis a total of six times (Hochereau, 1967a) to produce preleptotene spermatocytes. These preleptotene spermatocytes then move across the blood-testis barrier and enter meiotic prophase. They will then undergo several stages of differentiation and two meiotic divisions to ultimately produce spermatozoa (Staub and Johnson, 2018a). Meiosis is the duplication and exchange of

genetic material that results in the generation of haploid round spermatids. Spermiogenesis consists of the differentiation and maturation of round spermatids into spermatozoa. Lastly, spermiation is when sperm enter the lumen of the seminiferous tubules.

Spermatogenesis in bulls occurs over a 61-day time period and consists of 4.5 consecutive cycles that are each 13.5 days long within the seminiferous epithelium (Barth and Oko, 1989; Staub and Johnson, 2018b). These cycles are made possible as the epithelium of the seminiferous tubules contains different generations of stem cells that follow each other in a particular order of time (Regaud, 1901). The bull has eight stages of the cycle and the three parts that make up each cycle – spermatocytogenesis, meiosis, and spermiogenesis (Roosen-Runge; Ortavant, 1959; Cupps and Laben, 1960; Amann, 1962; Hochereau, 1967b; Hochereau-de Reviers, 1970; Curtis and Amann, 1981). Along with spermatogenesis, there is also the spermatogenic wave within the seminiferous tubule. The spermatogenic wave describes the spatial arrangement of cells along the seminiferous tubules. Each wave contains every stage of the cycle and does not change. The efficiency of bulls spermatogenesis is approximately 12×10^6/g and is determined by the daily sperm production per gram of decapsulated testis (Amann, 1981). Bulls experience two periods of significant loss in germ cells during spermatocytogenesis (Hochereau-de Reviers, 1970; Berndston and Desjardins, 1974) but not during spermiogenesis (Johnson, 1986).

Sperm in the Female Reproductive System

After sperm reach the female reproductive tract, several changes occur which prepare them for fertilization. During conventional breeding, semen is deposited in the fornix vagina and faces more obstacles in the female reproductive tract compared to during AI, where semen is deposited directly into the uterine body. During both conventional breeding and AI, there is an

immediate retrograde loss of semen where it travels outwards through the female reproductive system towards the vulva (Gallagher and Senger, 1989).

Throughout the reproductive tract there are various barriers which attempt to ensure that only high-quality spermatozoa have a chance to fertilize the oocyte. The cervix is the next site of significant loss after the vagina and only a small percentage of sperm deposited in the vagina enter the cervix (Hawk, 1983). Cattle have an average of three to four cervical rings or annular folds in their cervix made up of a series of grooves and folds that serve as an extra barrier to entering the uterus (el-Banna and Hafez, 1972).

Once sperm reach the uterine cavity, they must then enter the oviduct at the utero-tubal junction (UTJ), the next significant barrier during their journey to the oocyte. There are 13 genes that have been identified that are integral to sperm passing through the UTJ, however, the molecular mechanism associated with sperm passing through the UTJ is currently unknown (Bianchi and Wright, 2016). Twelve of the genes (*Ace*, *Calr3*, *Clgn*, *Pdilt*, *Pmis2*, *Rnase10*, *Tpst2*, *Adam1a*, *Adam2*, *Adam3*, *Tex101*, and *Prss37*) are involved with the cell surface receptor ADAM3 which appears to have a role in ZP binding (Bianchi and Wright, 2016). The thirteenth gene (*Ly6k*) impacts sperm migration (Bianchi and Wright, 2016). Following successful advancement through the UTJ, sperm attach to the mucosal epithelium of the oviduct in the isthmus or ampulla where they stay for up to several days waiting for an oocyte (Hunter and Wilmut, 1984). Oviductal secretions during this period are thought to interact with sperm during this time and may alter their interaction with the ZP and fertilization ability (Rodriguez-Martinez, 2007).

Capacitation

Even though spermatozoa undergo maturation in the epididymis and are morphologically mature, they are not fertilization competent until they have been artificially induced or interact with the female reproductive tract. This process of becoming fertilization competent begins with capacitation. Capacitation consists of biochemical and physical changes which function to prepare sperm for fertilization. These changes include decreases in cholesterol (Salicioni et al., 2007), calcium and intracellular pH increase, membrane hyperpolarization (Martínez-López et al., 2009), and protein modifications through phosphorylation at various amino acid residues (Yanagimachi, 1994; Grasa et al., 2009; Alvau et al., 2016; Jin and Yang, 2017; Ramió-Lluch et al., 2019; Zhao and Kan, 2019). Capacitation is required for fertilization and is unique to mammalian reproduction (Bavister, 2002). Although capacitation is required for *in vivo* fertilization to be successful, it can be skipped *in vitro* and produce healthy offspring with the addition of calcium ionophores to cause the acrosome reaction (Triana et al., 1980; Tateno et al., 2013).

While within the epididymis, the plasma membranes of sperm have a variety of surface molecules which consist of proteins and carbohydrates. During ejaculation, these surface molecules are coated with seminal plasma proteins that remain in place until they within the female reproductive tract as it causes the removal of the seminal plasma and some of the surface molecules (Rodríguez-Martínez et al., 2011). As the plasma membrane changes, the sperm is able to bind to the ZP of the oocyte and at the same time sperm motility progressively increases, leading to hyperactivation and the ability to undergo the acrosome reaction (Suarez, 2008). Hyperactivation consists of sperm engaging in a vigorous swimming pattern driven by an asymmetrical beat of the flagellum which aids in traversing the female reproductive tract and helps to eliminate defective sperm which cannot move as effectively (Yanagimachi and Usui,

1974). Bovine capacitation can be stimulated *in vitro* with the addition of heparin along with serum albumin, which plays an important role in removing cholesterol from sperm membranes (Davis, 1976; Davis et al., 1979).

In cattle and most other mammals, oocytes are arrested during meiosis at Metaphase-II (Wassarman and Albertini, 1988) and resumption of meiosis is triggered following oocyte activation. Sperm penetration of the oocyte triggers calcium oscillations in the oocyte (Nomikos et al., 2012) which ultimately leads to activation of the oocyte (Anifandis et al., 2016) and the resumption of meiosis. Phospholipase C zeta (PLC ζ) acts as an oocyte activating sperm-specific factor (Saunders et al., 2002; Knott et al., 2005; Saunders et al., 2007; Yoon et al., 2008; Heytens et al., 2009; Kashir et al., 2012) that has been identified in various species, including cattle (Ross et al., 2008; Felmer and Arias, 2015). It is a phosphoinositide-phospholipase protein located in the perinuclear theca region of the sperm and is released into the oocyte via small vesicles following sperm engulfment (Fujimoto et al., 2004; Escoffier et al., 2015). Another sperm specific protein, Na/K-ATPase α4 has been found to co-localize with PLC ζ in the post-acrosomal region of capacitated bovine sperm (Thundathil et al., 2018) and was immunolocalized to the entire head of fresh sperm (Unnikrishnan et al., 2021). It is also known to be involved in capacitation (Thundathil et al., 2006; Newton et al., 2010) and sperm motility (Jimenez et al., 2010; Jimenez et al., 2012) and is another protein of interest for fertilization. Lack of PLC ζ release is thought to be one cause of fertilization failure, as it results in the absence of calcium oscillations and ultimately oocyte activation (Malcuit et al., 2005).

Acrosome Reaction and Zona Pellucida Penetration

Interestingly, although capacitation is unique to mammals, the acrosome reaction can be observed across most multicellular animals (Okabe, 2018). The function of the acrosome

reaction is to allow sperm to penetrate the ZP and it aids in the future fusion of the oocyte plasma membrane and the sperm. It was originally hypothesized that the interaction of ZP3 with sperm initiated the acrosome reaction (Florman and Wassarman, 1985), however, following the invention of gene editing technologies, it was discovered that ZP2 regulates sperm binding the ZP (Rankin et al., 1998; Rankin et al., 2003). Current research into the molecular mechanisms of induction of the acrosome reaction is still unclear on the exact manner the acrosome reaction is induced.

The acrosome reaction begins with vesiculation, which involves portions of the sperm plasma membrane fusing with the outer acrosomal membrane to generate pores that allow for the passage of acrosomal enzymes (Yanagimachi, 1994). These acrosomal enzymes aid in the penetration of the ZP. The acrosomal enzyme acrosin helps to degrade the ZP, aiding the sperm in entering the perivitelline space (Cesari et al., 2004). Bovine acrosin is primarily found in its inactive form proacrosin (NagDas, 1992). Acrosomal exocytosis refers to the release of many enzymes during the acrosomal reaction, however, the purpose of all the enzymes have not been determined yet.

Sperm Zona Pellucida Binding

After reaching the oocyte, sperm must bind to the ZP to enter the oocyte and initiate the next stages of fertilization. The ZP is a membrane that surrounds the oocyte which serves as a protective barrier along with having roles in oocyte maturation, fertilization, and embryo preimplantation development. The thickness varies by species, with cattle having a thickness of ~ 10μm (Boccaccio et al., 2012). Its development begins during early secondary follicle growth and consists of a network of glycoprotein microfilaments named ZP2, ZP3, and ZP4 (Harris et al., 1994; Noguchi et al., 1994). Along with protecting the oocyte, the ZP serves as a species-

specific barrier to fertilization, preventing different species from penetrating it and fertilizing the oocyte of others (Yanagimachi, 1978).

Sperm-Oocyte Membrane Fusion

Currently, there are three cell surface proteins present on sperm or oocytes that have been identified as necessary for fertilization - *CD9*, IZUMO1, and JUNO. The gene *CD9* encodes a cell surface protein located on the plasma membrane of the oocyte and it is required for fertilization. It belongs to the tetraspanin family of multipass receptors (Maecker et al., 1997) which is made up of over 30 members known for their involvement in cell proliferation, adhesion, motility, and signaling (Ikeyama et al., 1993; Anton et al., 1995; Hadjiargyrou and Patterson, 1995; Shaw et al., 1995). The specific molecular mechanisms involved in the negative impacts that the absence of *CD9* has on fertilization are currently unknown. It is hypothesized to aid in organizing different proteins on the cell surface involved with sperm adhesion and fusion. Knockout genotypes of *CD9* have been found to produce strong infertility phenotypes in female mice (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000).

The protein IZUMO1 is currently the only identified cell surface protein on sperm that is essential for fertilization and it is a type I cell surface protein. During the acrosome reaction it is located on the plasma membrane and afterwards it is localized in the equatorial region of the sperm head (Inoue et al., 2005). This region is also where the sperm and oocyte membranes fuse (Satouh et al., 2012). It is found on both the inner and outer acrosomal membrane (Satouh et al., 2012). Although IZUMO1 deficient male mice produce morphologically normal sperm, they are infertile as their sperm is unable to bind or fuse with oocytes (Inoue et al., 2005). It interacts with JUNO on the oocyte membrane and play a role in gamete recognition or adhesion.

The protein JUNO is found on the oocyte surface and is a GPI-anchored protein that acts as a receptor for IZUMO1. It was identified as a member of the folic acid receptor family and was originally named *Folate Receptor 4* (Spiegelstein et al., 2000). Following studies into the identity of the IZUMO1 receptor, it was renamed JUNO after the roman goddess because it was discovered that it could not bind folate (Bianchi et al., 2014). Mice deficient in JUNO are infertile because their oocytes cannot bind or fuse with sperm despite producing morphologically normal oocytes (Bianchi et al., 2014). Following discoveries involving IZUMO1 and JUNO and their interaction, they were labeled as the first required receptor-ligand interaction essential for gamete recognition and this interaction is hypothesized to be unique to mammals (Bianchi et al., 2014). Research has suggested that the IZUMO1-JUNO interaction performs the adhesion step of gamete recognition and other molecules have a role in fusion. The interaction between IZUMO1 on the sperm side and JUNO on the oocyte side is hypothesized to make up the first of two distinct fusion processes (Okabe, 2018). The second step involves engulfment of the sperm and breakdown of the sperm plasma membrane (Austin and Bishop, 1958).

For fusion to occur, the equatorial segment of the sperm must line up with the oocyte plasma membrane in the perivitelline space. Zona protein 2 is activated after the acrosome reaction helping to promote fusion (Bleil and Wassarman, 1986; Bleil et al., 1988; Mortillo and Wassarman, 1991). During fusion, the oocyte engulfs the sperm and cortical granule exocytosis is initiated. This process begins when the cortical granule membrane fuses with the oocyte plasma membrane and helps to form the zona block to prevent polyspermy or when multiple sperm fertilize one oocyte (Austin; Braden et al., 1954; Austin, 1961). Some species including cattle also have a vitelline block (Hunter et al., 1998). Ultimately, polyspermy blocks operate through two methods and involve three locations – preventing more sperm from reaching the

oocyte and restricting the acrosome reaction at the ZP, cell membrane of the oocyte (oolemma), and the cytoplasm of the oocyte (ooplasm).

Decondensation of Sperm Nucleus and Formation of Male Pronuclei

Following sperm-oocyte fusion, the sperm nucleus must decondense as it is bound by protamines (Conover and Gwatkin, 1988; Ohnami et al., 2012; Ravaux et al., 2016) which are formed during spermatogenesis and are highly compacted by disulfide bonds (Fuentes-Mascorro et al., 2000; Kierszenbaum, 2001; Hao et al., 2019). Protamines are proteins rich in arginine that are smaller than histones – which normally compact nuclear DNA (Balhorn, 1982). While two types of protamines have been identified in mammals, cattle only have protamine 1, which can also be found in any species' sperm (Maier et al., 1990; Queralt et al., 1995; Corzett et al., 2002). Protamines act to do more than simply compact DNA, they are also hypothesized to protect it (Zini and Libman, 2006), as damage to sperm DNA can cause fertilization and embryo development failures as well as contribute to offspring developing health issues (Makker et al., 2009; Lewis and Simon, 2010).

Decondensation of the sperm nucleus is achieved primarily through glutathione from the oocyte which breaks down the disulfide bonds formed during sperm maturation in the epididymis. Sperm decondensation can be induced *in vitro* using dithiothreitol (DTT) (Galli et al., 2003; Watanabe and Fukui, 2006; Watanabe et al., 2010; Shirazi et al., 2011). Once the disulfide bonds are reduced, the nucleus decondenses and the male and female pronuclei are formed. Following this, protamines are replaced by histones as sperm chromatin is transcriptionally silent while so highly compacted (Ajduk et al., 2006; Ward, 2009). Although different species have the same sperm chromatin structure, the decondensation time of sperm DNA when exposed to a lysis solution varies by species, with cattle taking longer due to a higher

degree of DNA condensation (Ribas-Maynou et al., 2021). Over 12 hours, parental DNA migrates and forms chromosomes that will fuse to produce a zygote or one cell embryo that develops to produce the offspring (Gilbert, 2000).

Methods to Increase Fertilization

Increasing the utilization of several ARTs can also greatly aid in improving fertilization and ultimately pregnancy rates of cattle herds. One of the most accessible technologies is AI, which enables producers to advance the genetic merit of their herd and reduce their calving interval more rapidly. Artificial insemination can also be used to overcome some fertility issues as fertility increases with sperm dose up to a certain level (Salisbury and VanDemark, 1961; Saacke et al., 1994; Den Daas et al., 1998; Saacke et al., 2000; Braundmeier and Miller, 2001; Dalton, 2015), so commercial AI companies work to determine optimal insemination doses for various bulls based on compensable and non-compensable traits (Dalton, 2015). Some newer and developing technologies will be addressed below that have potential to aid in increasing fertilization and pregnancy rates.

More recent research has shown that sperm have a greater genetic role in fertility than previously hypothesized. Rather than just delivering the paternal portion of DNA, sperm also provide a variety of other genetic contents (Krawetz, 2005). RNA transcripts from sperm are thought to have a role in early embryo development (Card et al., 2013) and the transcriptomes of bulls with varying fertility levels have been found to be substantially different (Feugang et al., 2010). Currently, there have not been any significant differences in embryonic development rates and morphology observed amongst bulls with transcriptomic differences, but sires of varying fertility levels were observed to have significant differences in the transcriptomic profiles of the embryos their sperm produced (Kropp et al., 2017). Also, there is the possibility RNA within

sperm could be analyzed through genomics to determine fertility capabilities (Ostermeier et al., 2002; Kasimanickam et al., 2012). These studies warrant further investigation into associations between male fertility and embryonic development.

Utilization of genomics is another powerful tool which can aid in improving genetics of breeding herds and beyond. Genomics allows for producers to estimate direct genomic breeding values, improve genetic selection, and increase genetic advancement (Meuwissen et al., 2001; Hayes et al., 2009). This technology requires using data from large reference populations to quantify impacts of single nucleotide polymorphisms on phenotype and involves validation of quantitative trait loci – genomic regions which correspond with various quantitative traits (Calus et al., 2013; Berry et al., 2014). Recent genomic studies have led to the hypothesis that semen quality is genetically controlled as genes that may impact sperm concentration, semen volume, sperm number, and motility have been identified but require further investigation (Suchocki and Szyda, 2015). The use of genomic selection for young bulls would aid in improving reproductive performance of herds, however, more research is required to refine the technique and knowledge of genes involved in various reproductive processes.

Some other advanced approaches for evaluating fertility and improving genetic selection for fertility include computer-assisted sperm analysis (CASA), sperm DNA evaluation, sperm epigenomics, and sperm proteomics. Sperm can be evaluated with CASA to determine motility, velocity, linearity, and lateral displacement of the head and uses these factors to predict fertility. Sperm chromatin structure assays can be used to assess the sperm DNA quality and is highly correlated with fertility (Ballachey et al., 1988; Karabinus et al., 1990). High quality DNA is desirable within sperm because irregularities in the structure can have tremendous impact on the development and survival of offspring (Johnson, 2011). Epigenomics consists of modifications

made to the DNA and histone structure that do not change the DNA sequence but alter its accessibility and expression (Jenkins et al., 2015). Changes to the epigenome of a bull may impact fertility, for example increases in testicular temperature can change the sperm protein expression (Newton et al., 2009). Evaluation of a bull's epigenome could aid in the selection for increased fertility and highlight environmental hazards that may negatively impact reproductive performance. Lastly, proteomics could be used to identify new fertility markers as it allows for the investigation of expression profiles of various proteins in an organism. Some proteins have already been identified as sperm functional proteins and can be used as fertility markers in bulls, such as a testis-specific isoform of Na^+/K^+ATPase (Newton et al., 2009; Newton et al., 2010). Ultimately, utilization of these technologies along with the traditional bull BSE would aid in improving not only fertilization but also overall fertility and productivity of both beef and dairy herds.

Role of Phosphatidylserine in Fertilization

Structure, Synthesis, and Function of Phosphatidylserine

Phosphatidylserine (PS) is a glycerophospholipid that is an integral part of mammalian cellular membranes. Within mammalian cells, glycerophospholipids constitute around 70% of the total lipid content (Leventis and Grinstein, 2010). The other glycerophospholipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol, and phosphatidic acids and they interact to form the lipid bilayer of plasma membranes along with other lipids (Huang et al., 2017). This bilayer formation is the typical orientation of plasma membranes, however, during certain cellular processes it is disrupted, and various components of the membrane are translocated, including PS.

Phosphatidylserine can be detected through various methods despite being unevenly distributed throughout the cellular membrane. Observations of red blood cell lipid distributions demonstrated that PS tends to be present in the inner leaflet of plasma membranes (Zachowski, 1993). More recent research demonstrated that almost all of PS in membranes is present in the inner leaflet (Murate et al., 2015). Within intracellular organelles, PS appears to be present at higher concentrations in the cytoplasmic leaflet of the endoplasmic reticulum (ER) (Tsuji et al., 2019). Interestingly, PS exposure varies greatly between platelets involved in blood clotting and cells undergoing apoptosis – while PS is exposed within minutes in platelets (Bevers and Williamson, 2016), it takes hours for PS to be exposed on apoptotic cells (Nagata et al., 2010).

Mammalian cells have two pathways for PS biosynthesis. One pathway involves the placement of a serine at the headgroup of PC or PE by phosphatidylserine synthases (PSS) (Vance, 2008). PSS1 places the serine for PC and PSS2 places the serine for PE (Vance, 2008). PSS1 and PSS2 are present within mitochondrial-associated membranes (Rusiñol et al., 1994; Stone and Vance, 2000). The production of PS occurs in the ER before it moves to the Golgi apparatus, where it proceeds along a secretory pathway that ultimately results in its placement in the plasma membrane (Leventis and Grinstein, 2010).

These biosynthesis pathways were found to have some redundancy through the generation of PSS-deficient (Kuge et al., 1986; Voelker and Frazier, 1986; Kuge et al., 1991) cells and gene knockout experiments in mice (Bergo et al., 2002; Arikketh et al., 2008). Knocking out only one of the two enzymes resulted in mice with reduced fertility, while double knockout mice were infertile (Arikketh et al., 2008). PS degradation is conducted via decarboxylation, with decarboxylation of PS producing PE. Mammalian cells have one PS decarboxylase – Psd1p – located in the outer portion of the inner mitochondrial membrane

(Leventis and Grinstein, 2010). Two PS phospholipases A1 and A2 can hydrolyze PS within the cell plasma membrane to signal different cellular pathways (Leventis and Grinstein, 2010). Recognition of PS is completed by multiple receptors with redundant pathways due to the head group of PS being charged in a manner which permits recognition and specificity to multiple receptors (Ravichandran and Lorenz, 2007; Gregory and Pound, 2011; Rothlin et al., 2015).

Phosphatidylserine makes up a minor portion of the total lipid content in cellular membranes, however, it still plays an important role. It is involved in cell signaling cascades and the recruitment of various enzymes and other components of cellular membranes. One significant area of PS regulation is in coagulation cascades, where PS serves as a key signal (Bevers et al., 1982). It is also associated with apoptosis signaling pathways, as PS is exposed on the outer portion of plasma membranes from cells undergoing apoptosis or programmed cell death (Fadok et al., 1992; Martin et al., 1995; Koopman et al., 2011) because it serves as a signal for phagocytes which engulf dying cells to prevent harmful enzymes from being released (Fadok et al., 1992; Miyanishi et al., 2007; Park et al., 2009). This translocation of PS from the inner to outer leaflet to signal phagocytes breaks the asymmetry of the membrane which is a main marker for apoptosis (Martin et al., 1995; Vermes et al., 1995).

Movement of Phosphatidylserine in Cellular Membranes

Flippases, floppases, and scramblases are three types of lipid transporters that may be involved in the translocation of PS in the cellular membrane. Flippases move phospholipids from the outer to inner side of a membrane or organelle. They are ATP-dependent, and a phospholipid-translocating ATPase is thought to be involved in this process (Seigneuret and Devaux, 1984). Floppases move phospholipids in the opposite direction of flippases - from the inner to outer side of membranes. Floppases are thought to be proteins from the ATP-binding

cassette transporter superfamily and are ATP-dependent (Pohl et al., 2005). One member of the family, ABCA1, is hypothesized to be linked to the movement of PS (Oram and Vaughan, 2000). Lastly, scramblases operate in an ATP-independent manner and can move phospholipids bidirectionally (Kato et al., 2002).

More recent research has suggested that PS movement and exposure is regulated by combination of flippases and scramblases (Nagata et al., 2020). Flippases that aid in the movement of PS were identified (Seigneuret and Devaux, 1984; Tilley et al., 1986) and determined to have PS-dependent ATPase activity (Devaux, 1988). These flippases, ATP11A and ATP11C, are members of the P4-superfamily of P-type ATPases that were investigated due to their common expression and redundancy in various cell types (Nagata et al., 2020). It is hypothesized that these two flippases internalize PS after it is exposed via scramblases, as PS is not exposed rapidly after inactivating the flippases (Kornberg and McConnell, 1971) and PS remains exposed on cells that lack ATP11A and ATP11C when PS exposure is completed by a Ca^{2+}-dependent scramblase (Segawa et al., 2018).

Two families of proteins were identified to serve as possible scramblases for PS – TMEM16F and XKR. TMEM16F was identified as a Ca^{2+}-dependent scramblase (Suzuki et al., 2010). Xkr8 is a member of the XK-family (Suzuki et al., 2013a) and is localized in plasma membranes (Basu et al., 2002). Apoptosis activates Xkr8 which exposes PS to serve as part of the ‘eat me’ signal to phagocytes (Nagata et al., 2020). Xkr8 can also be activated by phosphorylation, causing PS to be exposed via a kinase-dependent manner (Sakuragi et al., 2019). These scramblases are hypothesized to translocate PS for different reasons, as $TMEMF16^{-/-}$ cell were found to expose PS when stimulated to undergo apoptosis but not when exposed to a

Ca^{2+} ionophore (Suzuki et al., 2013b) and $Xkr^{-/-}$ cells were found to be unresponsive to apoptotic stimuli, however, translocated PS following exposure to a Ca^{2+} ionophore (Suzuki et al., 2013a).

Phosphatidylserine and Mammalian Fertilization

Although typically associated with apoptosis, recent research has suggested that PS may play a role in fertilization and be expressed transiently in viable cells (Rival et al., 2019). Other cells have been previously found to temporarily express PS including activated B (Dillon et al., 2000; Elliott et al., 2006) and T cells (Fischer et al., 2006), myoblasts (Sessions and Horwitz, 1981; van den Eijnde et al., 2001), trophoblasts (Zhang et al., 2020), and macrophages (Marguet et al., 1999). Sperm PS exposure has been known about for years (Gadella and Harrison, 2002; De Vries et al., 2003; Martin et al., 2005), however, it was historically thought to be related to apoptosis (Hichri et al., 2018). This is because there are various stressors which can lead to apoptosis in male germ cells, including oxidative stress (Aitken et al., 1998), scrotal insulation (Karabinus et al., 1997), cryopreservation, and more.

Exposure of PS on sperm can be observed with Annexin V staining, as Annexin V is a protein which binds to phospholipids with a high PS affinity. When conjugated with the fluorescein isothiocyanate fluorochrome, its PS affinity is still high, allowing it to be analyzed with flow cytometry. In many studies looking at PS as a marker of apoptosis, propidium iodide (PI), a common viability marker, is used along with Annexin V to isolate apoptotic and necrotic cells from viable cells. However, 7AAD, another viability marker, is preferable as PI can bind to the pannexin channels leading to an artificial increase in the percentage of necrotic cells (Torres et al., 2017). Pannexin channels are proteins which serve as transmembrane channels that aid in the movement of ions and molecules of various sizes, including ATP, ethidium bromide, and PI (Bao et al., 2004; Dourado et al., 2014).

Transient exposure of PS on sperm was found to be required for fertilization and is present on viable and motile spermatozoa in mice (Rival et al., 2019). Exposure of PS has been observed on sperm within the epididymis, with concentrations of exposure increasing as sperm progress from the caput to the cauda and following capacitation and the acrosome reaction (Rival et al., 2019). It localizes to head and midpiece of murine sperm – with the greatest concentration present in the head (Rival et al., 2019). This corresponds with the localization of Izumo1, a factor involved in sperm:oocyte fusion (Bianchi and Wright, 2016). The hypothesized importance of PS in fertilization was supported by a study that used PS-targeting reagents which resulted in significantly decreased *in vitro* murine fertilization (Rival et al., 2019).

Five possible PS receptors on oocytes have been identified- CD36, BAI1, BAI3, Tim-4, and Mer-TK – based on their historical interactions with PS on different cells (Rival et al., 2019). CD36 and BAI1/3 were observed on murine oocytes and blocking them resulted in a significant decrease in fertilization rates (Rival et al., 2019). BAI1/3 are members of the type II adhesion family of G protein-coupled receptors previously reported to bind to PS (Nishimori et al., 1997; Park et al., 2007; Bolliger et al., 2011; Hochreiter-Hufford et al., 2013; Hamann et al., 2015; Sigoillot et al., 2015; Zhu et al., 2015). CD36 is a member of the scavenger receptor family previously identified to bind to PS (Fadok et al., 1998; Tait and Smith, 1999; Greenberg et al., 2006; Silverstein and Febbraio, 2009). *In* vitro fertilization was also significantly decreased in Tim-4, BAI1, and Mer-TK knock-out mice (Rival et al., 2019).

Bovine PS Expression

Phosphatidylserine has traditionally been used as a marker of apoptosis in studies involving cattle (Anzar et al., 2002; Dogan et al., 2012; Song et al., 2012; Wu et al., 2014; Erickson et al., 2016). It is known that apoptosis plays an important role in balancing germ and

Sertoli cells during spermatogenesis and irregularities in that balance can lead to infertility (Aitken et al., 2011). Apoptosis functions to maintain an optimal ratio of germ cells to Sertoli cells to ensure proper development and genetic quality (Aitken et al., 2011). Greater percentages of apoptotic sperm in semen have been linked to poor fertility in bulls as they are associated with decreases in viability of sperm cells (Anzar et al., 2002; Martin et al., 2004; Martin et al., 2007).

Initial studies investigating relationships between PS expression and fertility have been inconclusive or found no significant correlation between PS translocation and fertility. It is important to note that the following studies used Annexin V/PI assays rather than the Annexin V/7AAD assay used in the experiments that will be described in later chapters. One study found that the live sperm population is positively correlated to fertility but no correlation between PS exposure and fertility was observed (Erickson et al., 2016), attributing the hypothesized negative impact of PS positive sperm being overcome by a greater number of 'healthy sperm' because of insemination doses (Saacke, 2008). PS exposure has also been used as a measure of sperm injury along with mitochondrial membrane potential in relation to cryopreservation (Wu et al., 2014). Other studies reported that PS expression significantly increases on bovine sperm following cryopreservation (Anzar et al., 2002; Januskauskas et al., 2003; Wu et al., 2014), possibly because of destabilization and breakage of the plasma membrane. The percentage of PS expression in sperm from different bulls has been found to vary significantly and fertility appears to be associated with the percentage of viable sperm in bulls (Anzar et al., 2002; Januskauskas et al., 2003). Exposure of PS in bull sperm has also been found to be positively correlated with abnormal chromatin condensation, which may cause issues in sperm DNA packaging, and inversely correlated with viability (Januskauskas et al., 2003).

Summary and Research Goals

This review has provided an overview of current and past research regarding bovine fertilization and focused on the roles of phosphatidylserine in fertilization. Not all research involving mammalian and more specifically bovine fertilization has been discussed, however, the most appropriate studies have been mentioned, as there are centuries of research that have investigated fertilization in a wide variety of species.

Cattle are an excellent model for human reproduction and research involving them has led to the development and advancement of various ARTs that are utilized worldwide every day. Mammalian fertilization is comprised of numerous interconnected pathways which impact whether fertilization is successful. Phosphatidylserine has been identified as being crucial to mammalian fertilization and its role in bovine fertilization was briefly addressed. It is expressed on bovine sperm prior to and during capacitation, with levels increasing as capacitation occurs and is expressed at varying levels in different bulls. Interest in using PS as a possible *in vitro* fertility marker in cattle is attractive to researchers and producers as it could greatly aid in fertility prediction as *in vivo* fertility evaluations are time consuming and costly.

The research that follows will examine PS expression in bulls as there is currently little published research into the role of PS expression in bovine fertilization. The first experiment examines the question of whether PS is expressed in fresh bull semen and how that expression changes following cryopreservation. The second experiment examines PS expression in high and low fertility bulls along with how PS expression changes with capacitation.

Chapter 3

Estimation of Phosphatidylserine Surface Expression in Fresh and Frozen-thawed Bull Semen

Introduction

Male fertility in cattle can be evaluated using a variety of means, but one of the most common evaluations is the BSE. A BSE is used to evaluate fertility based on the structural soundness, capability of reproductive organs, semen quality, libido, and nutritional status of individual bulls (Koziol and Armstrong, 2018). However, it has been demonstrated that bulls labeled as satisfactory potential breeders can have varying fertility levels of 20-25% (Larson and Miller, 2000) due to subjective measurements such as sperm motility (Kastelic and Thundathil, 2008). The current bovine male fertility evaluations require further development, including the necessity for developing new technologies which can aid in selecting more fertile bulls.

Phosphatidylserine surface expression is required for murine fertilization and is hypothesized to play a role in sperm:oocyte fusion (Rival et al., 2019). However, there is little research determining the role of phosphatidylserine surface expression on sperm in bovine fertilization and what impacts varying expression levels have on bull fertility. It is unclear how cryopreservation impacts phosphatidylserine surface expression in *Bos taurus* bull. It is also unclear how changes in phosphatidylserine surface expression in bovine sperm impacts bull fertility. This study aimed to evaluate how phosphatidylserine surface expression on bovine sperm was impacted by cryopreservation. We evaluated how phosphatidylserine surface expression changes between fresh and frozen-thawed bull semen.

Materials and Methods

All experimental procedures were approved by the Virginia Polytechnic Institute and State University Animal Care and Use Committee (Protocol 21-090).

Experimental Design

Mature beef bulls (n = 4) from the Virginia Tech beef herd were involved in this experiment. Bull breeds included Angus, Simmental, and Charolais. Semen was collected from bulls via electroejaculation (EEJ) during a conventional BSE performed by a licensed veterinarian. Semen was immediately evaluated for ejaculate density and gross motility through visual evaluation. Semen smears of semen mixed with an eosin-nigrosin strain were prepared on glass microscope slides for later evaluation. Semen was then pre-extended and processed for Annexin V/7AAD staining as semen samples were diluted and equilibrated for cryopreservation. Data from the BSE was included in the analysis and the results are listed in Table 3.1.

Semen Freezing

Semen was frozen according to the OptiXcell™ protocol. OptiXcell™ and milliQ water was placed in a water bath at 34°C for 15 minutes. OptiXcell™ media was then mixed with the water and maintained at 34°C. Following semen collection, raw semen was pre-extended with extension media using a 1:1 volume ratio slowly and incubated at 34°C until final dilution. A single-step dilution was completed with extender warmed to 34°C. Diluted semen was maintained at room temperature for 20 minutes prior to packaging in straws at room temperature. Packaged straws were cooled off to 4°C for 90 minutes. Semen was then maintained at 4°C for 4 hours prior to freezing. Semen was frozen by placing straws above liquid nitrogen vapors for 10 minutes prior to their immersion in liquid nitrogen. Frozen straws were stored at -196°C.

Sperm Staining

Fresh semen was pre-extended with OptiXcell™ semen extender. Samples were washed with non-capacitating media (HEPES-TALP) and then resuspended in capacitating (IVF-TALP) media prior to incubation at 37.5°C 5% CO2 for 60 minutes. Samples were washed with non-capacitating media and resuspended in binding buffer. Sample aliquots were stained with FITC Annexin V for 15 minutes at room temperature followed by 7AAD for 15 minutes at room temperature. Stained samples were tested within 30 minutes using the Attune NxT Flow Cytometer.

FACS Analysis

Samples were analyzed using Attune NxT Acoustic Focusing Cytometer and gating was completed with the FlowJo Software Package. Each bull was analyzed for the percentage of Annexin V and 7AAD positive and negative cells in fresh and frozen semen samples that were capacitated for 1 hour. Capacitated samples were mixed with IVF-TALP and placed in an incubator at 37.5°C 5% CO2. Sperm gated through FSC-A vs SSC-A plot were further refined in forward scatter along with their ability to uptake 7AAD to gate out cell aggregates and early apoptotic cells.

Statistical Analysis

SAS (version 9.4; SAS/STAT, SAS Inst. Inc. Cary, NC, USA) statistical package was used for all statistical analyses. All data was analyzed using the MIXED procedure. Average of cell populations were analyzed as repeated measures with bull as the subject and time as repeated. Statistical differences were considered significant at $P \leq 0.05$ and tendencies considered at $0.05 < P \leq 0.10$.

Results and Discussion

Previous studies have reported that PS is expressed transiently on viable and fertilization-competent murine sperm (Rival et al., 2019), despite its conventional use as a marker for apoptosis as it is exposed as part of the apoptosis pathways in cells. The objective of this study was to identify Annexin $V^+/7AAD^-$ sperm in fresh bull semen and investigate how cryopreservation changes the percentage of Annexin $V^+/7AAD^-$ sperm.

All sampled bulls successfully passed the BSE and were classified as satisfactory potential breeders based on parameters of scrotal circumference, ejaculate density, gross motility, and morphology.

The percentage of Annexin V^+ $7AAD^-$, Annexin V^+ $7AAD^+$, Annexin V^- $7AAD^-$ spermatozoa are summarized in Table 3.2 and 3.3. Overall, the sampled bulls had an average of 67.14% (Table 3.2) of Annexin V^+ $7AAD^-$ sperm in fresh semen and 47.35% (Table 3.3) in semen that was frozen. There was an average of 0.97% and 2.22% (Tables 3.2 and 3.3) of Annexin V^+ $7AAD^+$ sperm cells in fresh and frozen samples, respectively. Lastly, there was an average of 31.69% and 49.8% (Tables 3.2 and 3.3) of Annexin V^- $7AAD^-$ sperm in fresh and frozen samples, respectively.

In the present study, there was a significant difference ($P < 0.05$) in the percentage of Annexin V^+ $7AAD^-$ sperm cells, which are viable cells expressing PS, among fresh and frozen semen samples (Table 3.2. and 3.3.). There was also a significant difference ($P < 0.05$) among sperm cells which were Annexin V^+ $7AAD^+$ among fresh and frozen samples (Table 3.2. and 3.3.), which are those that are expressing PS and apoptotic, along with sperm cells that are Annexin V^-7AAD^-, or those not undergoing apoptosis.

Our findings indicate that cryopreservation alters the proportion of Annexin V^+7AAD^- sperm cells, possibly due to damage that occurs to the plasma membrane during the freezing

process (Anzar et al., 2002; Januskauskas et al., 2003; Wu et al., 2014). Previous studies have reported increases in PS levels following cryopreservation as well; however, PI was used as the cell viability marker rather than 7AAD and its use can lead to an increase in the observed number of apoptotic cells that are actually viable (Torres et al., 2017).

Further investigation into how freezing alters the PS expression level in bulls from varying levels of fertility could aid in refining insemination doses for breeding bulls. Insemination doses for bulls with higher fertility can consist of reduced sperm concentrations compared to conventional semen doses (Saacke, 2008). Bulls with lower fertility can also be used for breeding through AI as the negative impacts of their lesser fertility can be ameliorated by increasing the sperm concentration of each insemination dose (Saacke, 2008). It is possible bulls with greater PS expression could be bred using inseminations doses less than the average. This could be due to these bulls having more fertilization-competent sperm present in their semen. Another study investigating the PS levels of bulls with varying insemination doses could aid in identifying the impacts of varying PS levels on bovine fertilization and bull fertility.

Breed is another factor which can influence male fertility and the impacts of breed on PS expression on viable sperm cells requires further investigation. In this experiment, bull 3 had the smallest percentage of sperm with normal morphology (Table 3.1) and the least Annexin V^+7AAD^- sperm cells in the fresh semen sample (Table 3.2). Bull 3 is a Charolais, which is one of the breeds with the worst fertility of those analyzed in a study evaluating hundreds of bulls from various bovine genetic companies (Berry et al., 2011). Although no conclusions can be drawn from this individual bull, there is a possibility that certain abnormalities in the sperm from this bull could impact the level of Annexin V^+7AAD^- sperm present in the semen. This alteration

in viable sperm cells expressing PS could then ultimately impact the fertility of the bull despite it being labeled as a satisfactory potential breeder by the BSE.

Here we identified bovine PS positive sperm cells in fresh and frozen semen which are not apoptotic (Annexin $V^+/7AAD^-$). We also observed that freezing alters the proportion of PS positive sperm cells capable of fertilization. Further investigation into the role of PS in bovine fertilization and what impacts different PS levels have on fertilization is warranted.

Table 3.1. Breeding Soundness Exam results from four beef bulls of different breeds.

Bull	Breed	Scrotal Circumference (cm)	Ejaculate Density (million/mL)	Gross Motility	Normal Morphology (%)	Head Defects (%)	Midpiece Defects (%)	Tail Defects (%)
1	Angus	37.5	400-750	Very good - rapid swirls	78	0	2	20
2	Simmental	37	400-750	Very good - rapid swirls	84	1	0	15
3	Charolais	39.5	250-400	Fair - no swirls, motion present	48	20	2	30
4	Angus	38	400-750	Good - slower swirls	68	0	2	30

Table 3.2. Overview of Annexin V and 7AAD Dot Plot results in Fresh Semen from Beef Bulls of Different Breeds[1]

Bull	Annexin V^+7AAD^- (%)	Annexin V^+7AAD^+ (%)	Annexin V^-7AAD^- (%)
1	72	1.11	26.5
2	69.2	0.84	29.95
3	62.9	0.51	36.6
4	64.45	1.41	33.7
Mean	67.1375	0.9675	31.6875

[1] Results from FACS analysis of fresh semen samples. Annexin V^+7AAD^- are viable cells expressing PS. Annexin V^+7AAD^+ are early apoptotic cells expressing PS. Annexin V^-7AAD^- are viable cells not expressing PS.

Table 3.3. Overview of Annexin V and 7AAD Dot Plot results in Frozen Semen from Beef Bulls of Different Breeds[1]

Bull	Annexin V^+7AAD^- (%)	Annexin V^+7AAD^+ (%)	Annexin V^-7AAD^- (%)
1	44	0.35	55.6
2	55.2	0.55	44.2
3	44.6	4.56	49.7
4	45.6	3.42	49.7
Mean	47.35	2.22	49.8

[1] Results from FACS analysis of frozen semen samples. Annexin V^+7AAD^- are viable cells expressing PS. Annexin V^+7AAD^+ are early apoptotic cells expressing PS. Annexin V^-7AAD^- are viable cells not expressing PS.

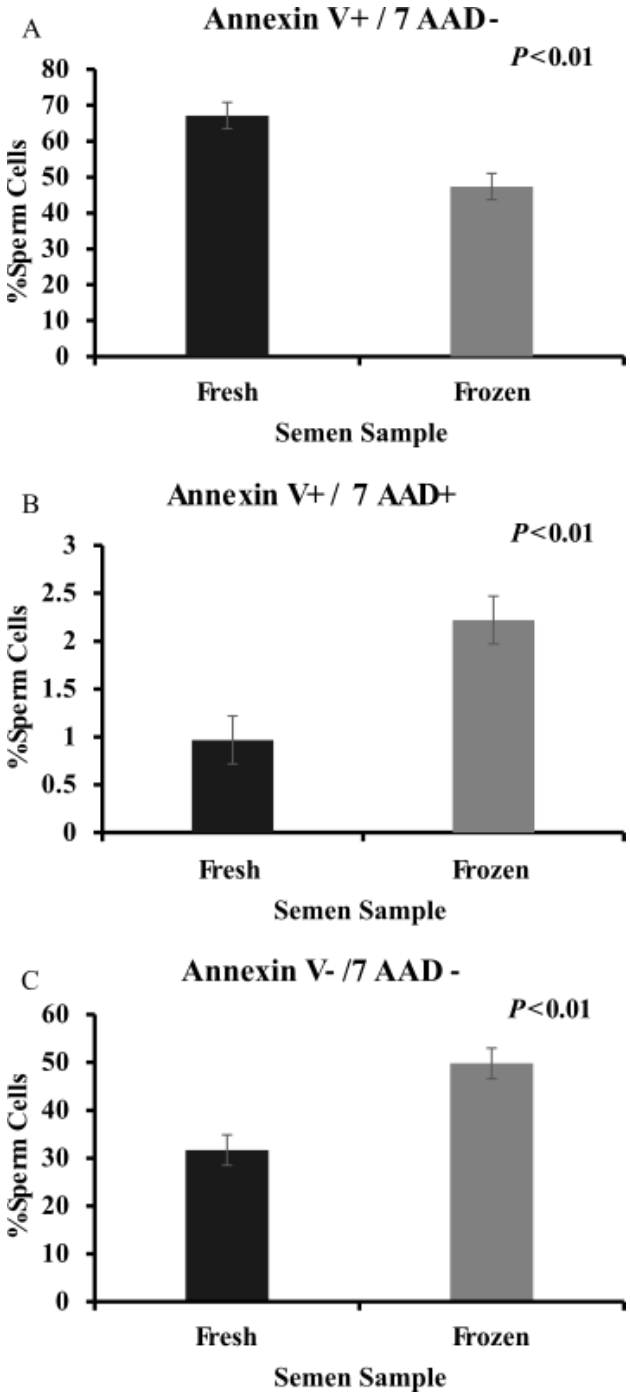

Figure 3.1. Changes in Annexin V and 7AAD levels in fresh and frozen semen samples from beef bulls of different breeds (n=4) (A) Annexin $V^{+}7AAD^{-}$ viable phosphatidylserine (PS) positive cells (B) Annexin $V^{+}7AAD^{+}$ early apoptotic cells (C) Annexin $V^{+}7AAD^{-}$ viable cells not expressing PS.

Chapter 4

Estimation of Phosphatidylserine Expression in Sperm from High and Low Fertility Bulls

Introduction

One measure of bull fertility for dairy bulls is sire conception rate (SCR). This evaluation communicates the probability that semen from a specific bull will produce a pregnancy compared to the average of all other bulls that could be used and includes the results from the first 7 breedings completed through AI (Kuhn and Hutchison, 2008). It was developed in 2008 (Kuhn and Hutchison, 2008) following research focused on evaluating bull fertility by artificial inseminations with conventional semen and is intended to be used as a conception rate evaluation (Kuhn et al., 2006; Kuhn et al., 2008).

Surface expression of PS is hypothesized to play a role in sperm:oocyte fusion after the discovery that is required for murine fertilization (Rival et al., 2019). However, there is little research determining the role of PS surface expression on sperm in bovine fertilization and what impacts varying expression levels have on bull fertility. It is unclear how fertility determined by SCR impacts phosphatidylserine surface expression in *Bos taurus* bull. It is also unclear how changes in phosphatidylserine surface expression in bovine sperm impacts bull fertility. This study aimed to evaluate how phosphatidylserine surface expression on bovine sperm was impacted by fertility and capacitation status. We evaluated how phosphatidylserine surface expression changes between bulls labeled as having high or low fertility and sperm of different capacitation status.

Materials and Methods

Animals

Cryopreserved semen samples from Holstein (n=18) and Jersey bulls (n=5) classified as having high (n=10) and low (n=13) fertility based on SCR were analyzed for PS expression. Data on number of services, age, and evaluation date were also evaluated.

Semen Staining

Uncapacitated samples were washed twice with non-capacitating media then resuspended in binding buffer. Sample aliquots were stained with FITC Annexin V for 15 minutes at room temperature followed by 7AAD for 15 minutes at room temperature. Capacitated samples were washed with non-capacitating media (HEPES-TALP) and then resuspended in capacitating media containing heparin (IVF-TALP) prior to incubation at 37.5°C 5% CO2 for 60 minutes. Capacitated samples were washed with non-capacitating media and resuspended in binding buffer. Sample aliquots were stained with FITC Annexin V for 15 minutes at room temperature followed by 7AAD for 15 minutes at room temperature. Stained samples were tested within 30 minutes using the Attune NxT Flow Cytometer.

FACS Analysis

Samples were analyzed using Attune NxT Acoustic Focusing Cytometer and gating was completed with the FlowJo Software Package. Each bull was analyzed for the percentage of Annexin V and 7AAD positive and negative cells in samples that were non-capacitated and capacitated for 1 hour. Sperm gated through FSC-A vs SSC-A plot were further refined in forward scatter along with their ability to uptake 7AAD to gate out cell aggregates and early apoptotic cells.

Statistical Analysis

SAS (version 9.4; SAS/STAT, SAS Inst. Inc. Cary, NC, USA) statistical package was used for all statistical analyses. All data was analyzed using the MIXED procedure. Average of cell

populations were analyzed as repeated measures with bull as the subject and time as repeated. Statistical differences were considered significant at $P \leq 0.05$ and tendencies considered at $0.05 < P \leq 0.10$.

Results and Discussion

In the present study, there was a significant difference in Annexin V positive cells ($P < 0.05$) among uncapacitated and capacitated sperm (Figure 4.1). There was no difference ($P > 0.05$) among high and low fertility bulls for Annexin V^+ $7AAD^-$, Annexin V^+ $7AAD^+$, or Annexin V^- $7AAD^-$ sperm cells (Table 4.1). There was a significant difference ($P < 0.01$) in the percentage of viable sperm cells with PS exposed for uncapacitated and capacitated samples (Figure 4.1a). In sperm cells exposing PS that were apoptotic, there was a significant difference ($P < 0.01$) in PS exposure for uncapacitated and capacitated samples (Figure 4.1b). SCR, age, and number of services for all bulls ranged from -6.5 to 4.1, 1.91 to 15.17 years, and 514 to 30,198 services, respectively. Semen was collected from bulls in either December or August of the same year.

Our findings indicate capacitation alters the proportion of bovine sperm cells which expose PS, possibly due to PS having a role in bovine fertilization. This possible role of PS in bovine fertilization requires further investigation as PS has been demonstrated to have a role in murine fertilization (Rival et al., 2019). Previous studies have also reported increases in PS exposure of cells during capacitation and the acrosome reaction (Rival et al., 2019). This increase in PS exposure on viable bovine sperm during capacitation and possibly the acrosome reaction could function to improve the chances of sperm:oocyte fusion, as receptors for PS have been identified on murine oocytes (Rival et al., 2019), but have not yet been identified on bovine oocytes. Other studies have not reported investigating PS expression for use as a marker of

fertility, but rather focused on its role in apoptosis. However, the results from these studies have shown that individual bulls have varying levels of PS expression which may have an impact on fertility (Anzar et al., 2002; Januskauskas et al., 2003) which was also observed in this experiment. Studies have also shown that PS exposure in bovine sperm is positively correlated with abnormal chromatin condensation, so perhaps bulls with increased levels of Annexin V^+ $7AAD^+$ sperm have decreased sperm viability due to errors in sperm nuclear DNA packaging which can lead to fertilization or early embryo development failures (Januskauskas et al., 2003). Further investigation on what impacts different proportions of Annexin V^+ $7AAD^+$ sperm cells have on fertility could aid in developing a threshold for fertility decisions and help in calculating semen doses for individual bulls.

Breed is another factor which can impact male fertility in bulls. One study that analyzed bull fertility across multiple breeding companies for bulls found that there was a significant difference in service bull fertility among different breeds for both beef and dairy cattle (Berry et al., 2011). The breeds with the worst fertility in this study included Charolais, Limousin, Simmental, and Holstein while the breeds with the best fertility included Belgian Blue, Hereford, and Montebelliarde (Berry et al., 2011). This experiment analyzed Holstein and Jersey bulls with similar percentages of Annexin V^+ $7AAD^-$ sperm cells but analysis of more bulls from each breed is required before determining what impacts breed may have on PS expression.

In this experiment bulls were classified as either having high or low fertility based on SCR. If PS exposure on viable bovine sperm cells is found to be involved in fertilization and thus impact fertility, higher SCRs may correlate with greater PS expression in bulls. This would require evaluations of a greater number of bulls than completed in this experiment, however, if

SCR and PS expression were positively correlated, this would aid in conducting bull fertility evaluations and bull selection.

Here we identified PS positive sperm cells in high and low fertility bulls which are not apoptotic but failed to demonstrate a relationship between PS expression and fertility (measured as SCR). We also observed that capacitation alters the proportion of PS positive sperm cells capable of fertilization. Further investigation into the role of PS in bovine fertilization and what impacts different PS levels have on fertilization is warranted. Future studies should examine greater numbers of bulls to investigate what impacts fertility has on the proportion of Annexin V^+ $7AAD^-$ sperm cells. Also, investigating the impacts season, nutrition, breed have on PS exposure is also warranted.

Table 4.1. Summary of FACS Analysis Results from Annexin V/7AAD Dot Plots for High and Low Fertility Semen[1]

Semen Type	Capacitation Status	N	SCR range	Annexin $V^+/7AAD^-$	Annexin $V^+/7AAD^+$	Annexin $V^-/7AAD^-$
High Fertility		10	3.4 to 4.6			
	Capacitated	-		59.34	0.46	45.43
	Uncapacitated	-		17.78	0.09	82.10
Low Fertility		13	-6.5 to -3.0			
	Capacitated	-		62.24	0.82	36.79
	Uncapacitated	-		19.68	0.12	80.12

[1]Averages for high and low fertility semen based on SCR from a total of 23 Holstein and Jersey bulls.

Table 4.2. Comparison of FACS Analysis from Annexin V/7AAD Dot Plots for Different Breeds of Dairy Cattle[1]

Breed	**Capacitation Status**	**N**	**Annexin $V^+/7AAD^-$**	**Annexin $V^+/7AAD^+$**	**Annexin $V^-/7AAD$-**
Holstein		18			
	Capacitated	-	60.03	0.79	42.00
	Uncapacitated	-	18.12	0.12	81.69
Jersey		5			
	Capacitated	-	64.40	0.21	35.3
	Uncapacitated	-	21.50	0.05	78.42

[1]Frozen semen from Holstein and Jersey bulls was analyzed. Jersey bulls were all in low fertility group (n=5) while Holstein bulls made up the entirety of the high fertility group (n=10) and a part of the low fertility group (n=8).

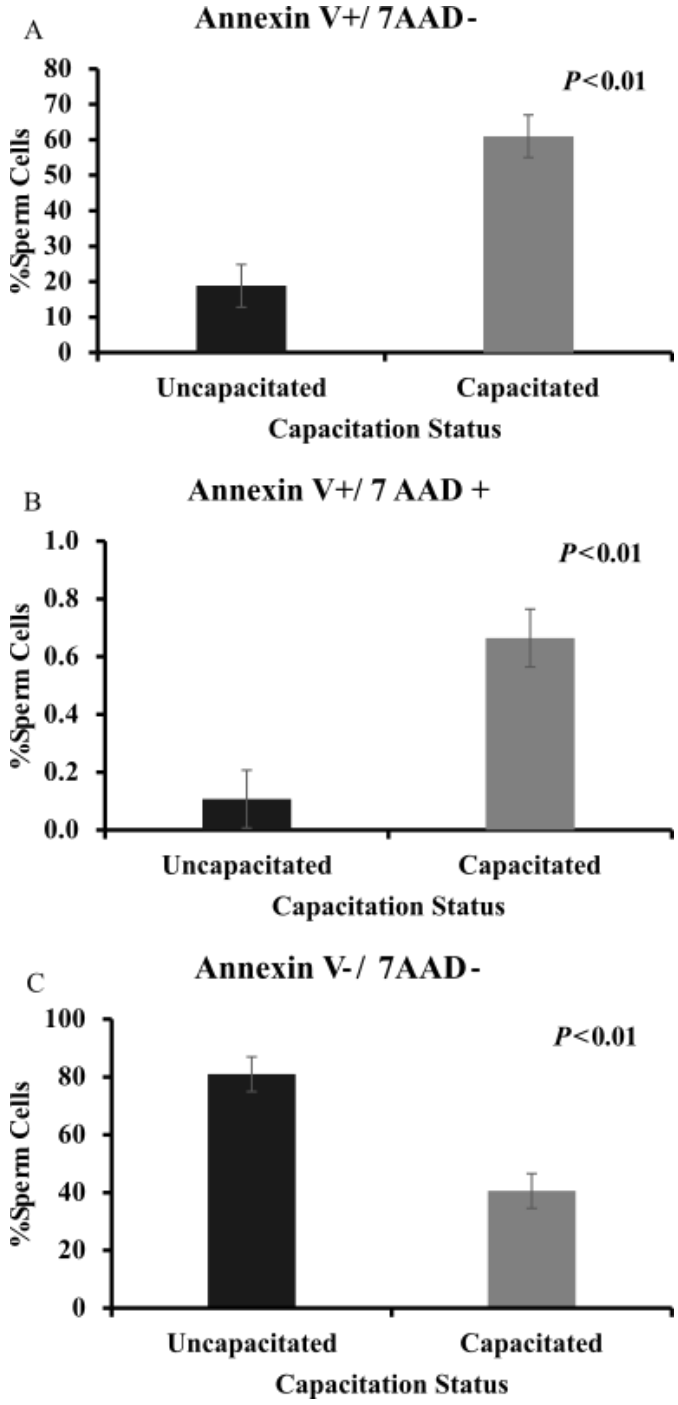

Figure 4.1. Comparison of Annexin V and 7AAD levels in uncapacitated and capacitated semen samples for different breeds of dairy cattle (n=23) (A) Annexin V+ 7AAD- viable phosphatidylserine (PS) positive cells (B) Annexin V+ 7AAD+ early apoptotic cells (C) Annexin V7AAD- viable cells not expressing PS.

Chapter 5

Summary and Conclusions

Fertility is one of the primary issues that cattle producers face to maximize efficiency and profit. While there are numerous factors that impact fertility, male fertility is typically regarded as simpler and more straightforward than female fertility. However, even when using conventional fertility evaluations for bulls, there is a significant difference in fertility of males that pass these evaluations, warranting the development of new fertility assays and technologies along with refinement of current ones. Ultimately, the development and use of more detailed male fertility evaluations could greatly benefit the efficiency and profitability of cattle production in the United States and beyond.

Global demand for animal protein is expected to double by 2050 (United Nations 2017) due to the rapidly growing world population and socio-economic changes resulting in the need for more efficient cattle production systems (Delgado 2003). Although the demand for beef and dairy products is increasing, there is also an increase in consumer concerns about the environmental impacts of animal production, as 12% of greenhouse gas emission and 30% of the human-driven biodiversity losses are a result of livestock production (Westhoek et al 2011). By increasing the fertility of their cattle, producers can work to ensure food security while sustainably producing animals to meet the growing demand for animal protein. This work provides an exploration of factors that influence male fertility along with a new factor that may be able to be utilized as a fertility marker in bulls.

Recently, PS has been hypothesized to play a vital role in mammalian fertilization, possibly during sperm:egg fusion (Rival et al 2019). Externalization of PS on bovine sperm may

play a role in fertilization and fertility of animals and quantification of its expression has potential to serve as a fertility marker. The present work has shown differences in PS expression levels between individual bulls and explored the impacts cryopreservation has on PS expression. Further studies are required explore the possible link between PS expression and fertility in bulls and the impacts of semen handling and freezing.

Phosphatidylserine expression was found to be greater in fresh semen of bulls compared to semen that has been cryopreserved and was shown to increase with capacitation. It is possible that increased levels of PS equate to increased fertility as PS is hypothesized to play a role in sperm:oocyte fusion, thus the expression level will be related to the chance of successful fusion and ultimately pregnancy. Although it is hypothesized that increased PS levels are associated with increased fertility, there was no difference in PS expression found in bulls classified with high and low fertility based on conventional measures. Further studies with a greater number of bulls are required to determine the link between PS expression and fertility in bulls

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Appendix A

Annexin V Staining of Bull Sperm

Based off the protocols developed by Rival et al,, 2019

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Reagents

HEPES-TALP and IVF-TALP (refer to Hansen Lab IVP protocol for recipes)

10X Binding Buffer

Annexin V FITC (BD Pharmingen 556419)

7AAD Viability Marker (Biolegend 420403)

Reagent Preparation

1. 10x Binding Buffer
    a. 0.1M Hepes (pH:7.4), 1.4M NaCl, 25 mM $CaCl_2$
        i. To make 100mL: Dissolve 2.603g Hepes, 8.182g NaCl, 0.3675g $CaCl_2$ in milliQ water

General Preparation

1. Aliquot the necessary amount of IVF-TALP and HEPES-TALP into 50 mL conical tubes
    a. Place IVF-TALP in 38.5°C 5% CO2 incubator overnight
        i. Each sample takes ~3 mL of IVF-TALP
    b. Place HEPES-TALP in 38.5°C water bath at least 2 hours prior to washing
        i. Each sample takes ~25 mL of HEPES-TALP

Protocol:

1. Prepare 10-20 mL 1X binding buffer (BB)
    a. 10 mL: 1 mL 10X BB and 9 mL DI H_2O
2. Prepare tubes with 1 mL of non-capacitating (NC) media (HEPES-TALP)
3. Thaw straws for ~1 min at 37°C in water (Transport straws in dry ice if needed)
4. Cut crimped end and let sperm diffuse into tubes with 1 mL of NC media
5. Wash with 15 mL of NC media for 6 min at 300 rcf
6. Aspirate supernatant (leave less than 1 ml)
7. Resuspend cells in ~3 mL of capacitation media (IVF-TALP)

8. Incubate for 1 hour at 38.5°C 5% CO_2
9. Wash with 10 mL NC media for 6 min at 300 rcf
10. Aspirate supernatant
11. Add 1 mL of 1X BB and count cells
    a. Dilute to $1x10^6$ cells
12. Pipette 100 uL of $1x10^6$ cells and into FACS tube
13. Annexin V staining: add 5 uL to FACS tube and vortex briefly
    a. Incubate for 15 min at room temp and in dark
14. 7AAD Staining: add 5 uL to FACS tube and vortex briefly
    a. Incubate for 15 min at room temp and in dark
15. Add 500 uL 1X BB
16. Vortex FACS tubes briefly
17. Analyze samples with Attune NxT Acoustic Focusing Cytometer