Proceedings, Applied Reproductive Strategies in Beef Cattle
September 4-5, 2024; Athens, GA

NOVEL STRATEGIES TO IDENTIFY BEEF BULLS WITH SUPERIOR FERTILITY

S. M. Zoca, and G. A. Perry

Introduction

Bull fertility is affected by several factors; in a Breeding Soundness Evaluation (BSE), physical soundness, scrotal circumference and sperm quality are evaluated to predict bull fertility (Barth and Oko, 1989; Barth and Waldner, 2002; Barth, 2007; Barth, 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; Saacke, 2008; Caballero et al., 2011; Sullivan and Saez, 2013; Sullivan, 2016; Dalton et al., 2017; Sullivan and Belleannée, 2017; Amann et al., 2018; Barth, 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; Januskauskas et al., 2003; Rodriguez-Martinez, 2003; DeJarnette, 2005; Dalton et al., 2017; Harstine et al., 2018; Dalton, 2019; DeJarnette et al., 2021). In the female, vaginal, cervical, uterine, and oviductal environment affect sperm transport, hyperactivation, capacitation and sperm-egg interaction (Austin, 1951; Chang, 1951; Austin, 1952; Austin, 1975; Yanagimachi, 1994; Suarez, 2006; Suarez and Pacey, 2006; Suarez, 2008; Sutovsky, 2009; Florman and Fissore, 2015; Suarez, 2015, 2016; Sutovsky, 2018). 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 the livestock 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). Nevertheless, several advances in understanding sperm biology and its impact on field fertility (i.e. pregnancy success) has been accomplished. In this proceeding paper, we will discuss some of the work that has been done in semen preparation, handling, and sperm biology, and how that may influence our ability to identify bulls with superior fertility.

Abbreviations

AI = Artificial insemination; **AKAP4** = A-kinase anchor protein-4; **ALH** = Amplitude of lateral head displacement; **BSE** = Breeding Soundness Exam; **BSP1** = Binder of sperm protein 1; **CALM1** = Calmodulin; **CASA** = Computer Assisted Sperm Analysis; **DAG1** = Dystroglycan; **FAA** = Fertility-associated antigen; **FTAI** = Fixed-time Artificial Insemination; **NAAB-CSS** = National Association of Animal Breeders-Certified Semen Services; **PEBP4** = Phosphatidylethanolamine-binding protein 4; **SERPINA5** = Plasma serine protease inhibitor; **sncRNA** = Small non-coding RNAs; **SNP** = Single nucleotides polymorphism; **SPADH2** = Spermadhesin Z13.

Sperm Preservation and Evaluation

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; Almquist et al., 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 ~50 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) (DeJarnette, 2005; DeJarnette, 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; DeJarnette, 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).

Insemination dose

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 (Fig 1.) (Salisbury and VanDemark, 1961; Sullivan and Elliott, 1968; 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; Amann and DeJarnette, 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 (Fig. 2) (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). In fig. 1, bulls A and B are a good example of compensable sperm traits, where it is overcome by increasing insemination dose until they reached the optimum fertility of

the female population. 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). Although an increase in sperm dosage increased the fertility level of bulls C and D (Fig. 1), they never reached the optimum female fertility, thus these bulls are a good example of uncompensable sperm traits.

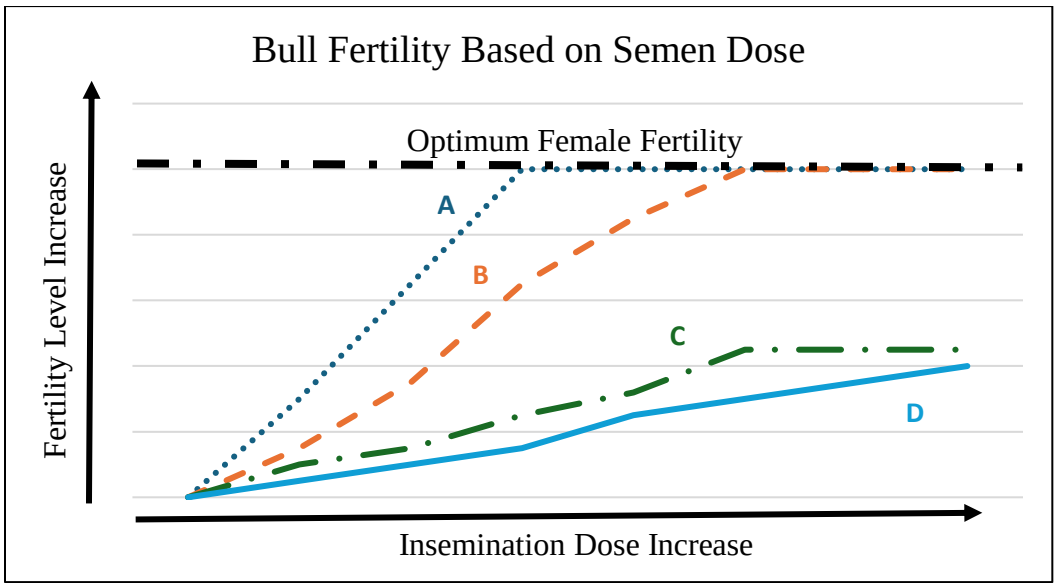

Figure 1. Illustration of change in bull fertility relative to insemination dose and optimum female fertility. Bull fertility increases as the number of viable sperm inseminated increases until it reaches the optimum female fertility or male maximum fertility (Adapted from Salisbury and VanDemark, 1961; Sullivan and Elliott, 1968; Den Daas et al., 1998).

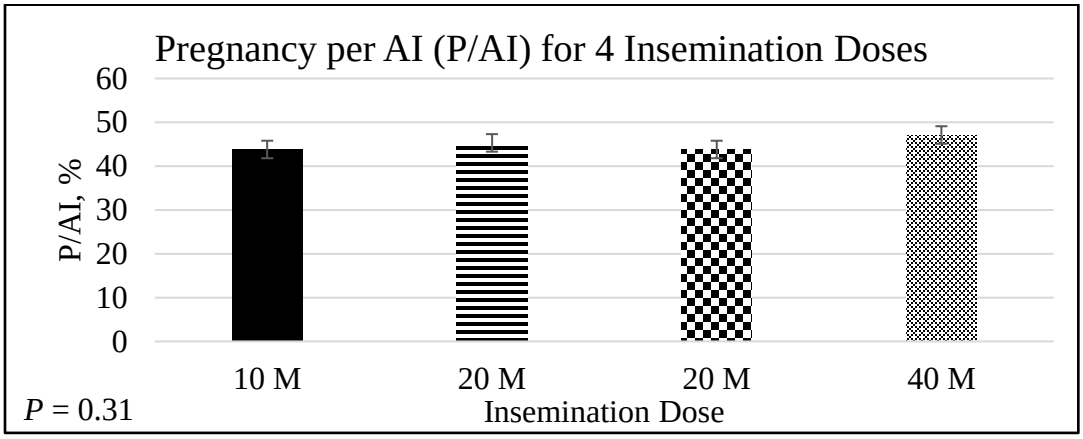

Figure 2. Pregnancy rates for insemination doses ranging from 10 to 40 million sperm per ml. Difference between the two 20 M was only the color of the semen straw. No difference was detected between all four doses (P = 0.31) (adapted from (Zoca et al., 2020).

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, be able to fertilize, activate the

oocyte and block polyspermy, and support early embryonic development (Rodriguez-Martinez, 2003; Saacke, 2008; Vincent et al., 2012; Garner, 2014; Lockhart et al., 2023; Costes et al., 2024). 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 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, 2008; 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.

Prediction of Fertility

The delivery of a high-quality sperm dose, one that meets or exceeds industry standards is the responsibility of AI centers and is accomplished by meeting or exceeding the guidelines established by the NAAB-CSS (DeJarnette, 2012; Mitchell, 2012). Sperm analysis is improved greatly when more objective methods are used, such as CASA and flow cytometry, compared to previous subjective methods (visual 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 (Barth and Oko, 1989; Rodriguez-Martinez, 2003; Barth, 2007; Vincent et al., 2012; Barth, 2018). A semen quality control was added to the NAAB-CSS audit program in 2011; however, quality control minimum standards and procedures are held privately (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 (≥ 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). Also, it has been reported that most bulls from AI centers will have fertility within 3% of the average fertility, either above or below (Clay and McDaniel, 2001)

The search for a method to predict 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; Costes et al., 2024). Male fertility depends on multiple factors as discussed previously. Each ejaculate/insemination dose must contain sperm with "enough" of the 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 reported 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 a BSE and at AI centers to predict bull fertility. In an unselected population, 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 previously stated, 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 exceedingly 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 decreases 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 will improve the ability to detect lowly fertile animals through new tests compared to predicting highly fertile ones (DeJarnette, 2005; Dalton, 2019).

Fertility Markers

Flow cytometry:

Initially, only fluorescent microscopy would be used to make inference on a few hundred sperm and that would be extrapolated to the whole population; however, flow cytometry can increase the number of cells evaluated for a specific sperm trait of interest from a few hundred to several thousands which gives us more confidence that the true value of the population is being evaluated. Flow cytometry has been used to measure several characteristics in sperm, such as plasma membrane integrity (viability), acrosomal membrane integrity, the energy potential present in the mitochondria of the sperm, the changes in the fluidity of the plasma membrane associated with sperm capacitation, intracellular ions (e.g.: calcium and zinc), reactive oxygen species concentration, ability to respond to an oxidative stress, and many others. As previously mentioned, the sperm must have 'enough' of all desired characteristics in order to fertilize the oocyte. Further, the ejaculate is composed of a heterogeneous population of sperm, thus, a large proportion of this population must display enough of all desired characteristics for a bull to be of high fertility. Nevertheless, we don't know how much is enough for several characteristics.

The relationship of intact plasma membrane (viable), intact acrosome, concentration of reactive oxygen species, ability to react to oxidative stressors, mitochondrial energy potential and DNA integrity and bull fertility have been demonstrated in several studies (Oliveira et al., 2014; Kumaresan et al., 2017; Bernecic et al., 2021). Bulls with greater proportion of viable sperm with intact acrosome, greater mitochondrial energy potential, lower reactive oxygen species, lower DNA fragmentation would be the desired population; however, how much is necessary to be of high fertility is unknown. Therefore, other studies have failed to identify a relationship between some of these characteristics and field fertility (Zoca et al., 2020; Zoca et al., 2023a), likely, bulls in these studies had enough of these characteristics, even the lower fertility bulls. Fertility difference between high and low fertility bulls in Zoca's study ranged from 4.6 to 7.4 percentage points (pregnancy rates difference) whereas the fertility variation for the other studies mentioned was 11 to 28 percentages points (pregnancy rates). Thus, the greater the fertility difference, the more likely more sperm characteristics will be associated with fertility as a bigger disruption to sperm biology would be expected.

In our recent study, however, we were able to identify a relationship between viability, and zinc signature (a measure of sperm capacitation) and field fertility (Zoca et al., 2023a). The zinc signature assay used in that study classified sperm in four populations: 1) sperm with high intracellular zinc that is considered non-capacitated and with high fertilization ability; 2) sperm with moderate intracellular concentration of zinc that have started the process of capacitation but has high fertilization ability; 3) intracellular zinc is limited to the acrosomal region and/or midpiece, sperm have undergone capacitation and is in the process of cell death, and have low or no fertilization capacity; 4) no intracellular zinc, sperm is dead and may or may not have gone

through capacitation before dying. Thus, in the referred study, high fertility bulls had greater viability and greater proportion of sperm in the signatures 1 and 2 combined. Flow cytometry can be used to measure several sperm characteristics as mentioned above. Another use of flow cytometry would be to identify proteins in the sperm, either presence and absence or even concentration of those proteins. Some of the studies in the protein section below have used flow cytometry to study sperm proteins and their association with bull fertility (Odhiambo et al., 2014; Zoca et al., 2023a).

Proteins:

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; Florman and Fissore, 2015; Suarez, 2015, 2016; Sutovsky, 2018; Saint-Dizier et al., 2020; Mahé et al., 2021). Sperm has a very limited metabolic function, and it is dependent on the media/environment surrounding them, such as the testicular and epididymal fluids, and seminal plasma in the male, semen extenders when used in assisted reproductive technologies, and the fluid in the female's vagina, cervix, uterus, and oviduct. In the female tract, viable sperm reaches the oviduct 6 to 12 hours after insemination and populates the isthmus where they form the sperm reservoir (Hunter and Wilmut, 1984; Wilmut and Hunter, 1984; Lefebvre et al., 1995), these interactions are dependent on proteins (glycoproteins). Thus, cell-to-cell interactions (i.e. sperm to oviduct and sperm to oocyte) are essential processes during fertilization and may be drivers for fertility variation between bulls. 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) reported 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 compared to low 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 binder of sperm protein 1 (BSP1) was more abundant in low fertility bulls (Somashekar et al., 2015; Somashekar et al., 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; Bellin et al., 1996; Bellin et al., 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 negative for FAA (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; Sutovsky et al., 2002; Sutovsky et al., 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).

Recently, we worked with three sperm proteins, plasma serine protease inhibitor (SERPINA5), dystroglycan (DAG1), and CD9, and their potential as bull sperm fertility markers. We reported those proteins to be attached to the sperm (Zoca et al., 2022) and confirmed their presence in bull sperm (Fig. 3 and 4). However, there was no relationship between SERPINA5 or DAG1 and sire conception rate (dairy bulls), total and progressive motility, plasma membrane integrity (viability), or in vitro embryo production outcomes (cleavage and blastocyst rate, and their ratio; (Zoca et al., 2023b). Nevertheless, when we investigated CD9, fertility differences between 5 angus bulls were explained by dead CD9+ populations (Fig 4A), where bulls with superior fertility had lower proportion of sperm categorized as dead CD9+ compared to lower fertility bulls. However, these results need to be validated in a larger population of bulls with known fertility.

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 into a literature (https://pubmed.ncbi.nlm.nih.gov/ search, since 1946, 9,491 results are found and 7,151 of those publications happened in the past 20 yr, 4,655 in past 10 yr and 2,824 in the past 5 years (searched on August 21, 2024). In combination, these studies demonstrated 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 is 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.

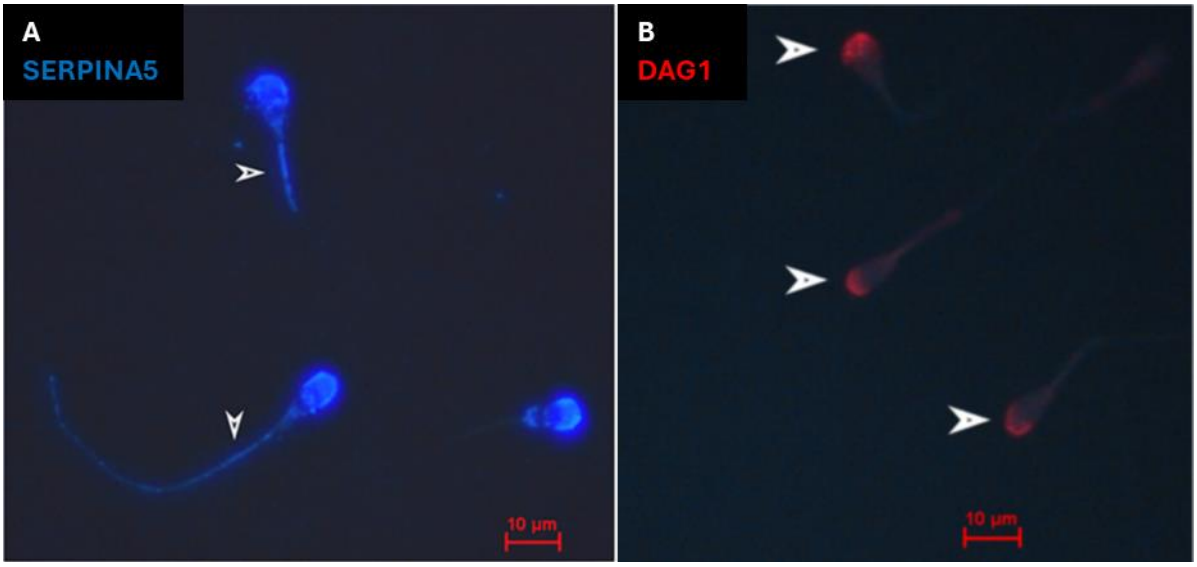

Figure 3. Immunofluorescence picture of sperm labeled with anti-SERPINA5 antibody (A) and anti-DAG1 (B; adapted from (Zoca et al., 2023b).

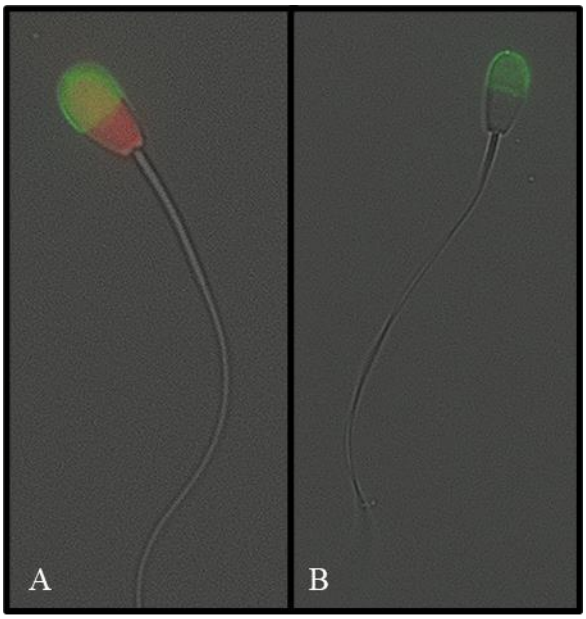

Figure 4. Immunofluorescence picture of two sperm labeled with anti-CD9 antibody (green) where one has the plasma membrane disrupted (dead; A) and the other is a viable sperm (B; adapted from (Zoca et al., 2023a).

Omic's

More recently we have started to investigate the relationship of "Omic's" in sperm biology and bull fertility. The omic's world involves analyses of DNA (genomics), RNA and miRNA (transcriptomics), proteins (proteomics), metabolites (metabolomics), DNA-methylation and histone acetylation (epigenomics), lipids (lipidomic) and others. Previously, it was believed that sperm only delivered the paternal genome to the oocyte; however, research has provided evidence that in addition to the sperm's centrosome for reactivation of meiosis II (Albertson, 1984) and sperm specific phospholipase C-ζ which plays a role in activation of embryonic development

(Saunders et al., 2007), approximately 5 to 10 fg of paternal RNA can be delivered to the oocyte (Boerke et al., 2007). Recently, some studies have used multiple of these omics together in a multiomics approach to investigate bull fertility (Talluri et al., 2022; Costes et al., 2024). Very briefly, Talluri et al. (2022) used transcriptomic, proteomic, and metabolomic analyses of sperm from high and low fertility dairy bulls. They reported that in several levels (transcript, protein, and metabolite), sperm metabolism of high fertility bulls was different than low fertility bulls where energy utilization/production was one of the major differences. More specifically, low fertility bulls had genes involved in oxidative phosphorylation, and proteins involved in metabolic pathways downregulated compared to high fertility bulls. Further, in the mutiomic's approach, major metabolic pathways that were dysregulated in individual approaches were revealed to be interactive between transcriptomic, proteomic, and metabolomic that included Butanoate metabolism, Glycolysis and gluconeogenesis, and Methionine and cysteine metabolism dysregulated in low fertility bulls which are molecules associated with energy productions in the sperm. Other dysregulated pathways were Phosphatidyl inositol phosphate, pyrimidine metabolism and saturated fatty acid beta oxidation. The authors concluded that sperm metabolism is likely involved in bull fertility regulation.

In the other multiomic's approach to understand bull fertility, Costes et al. (2024) used genotypes, sperm DNA methylation at CpGs and sperm small non-coding RNAs (sncRNA) and semen parameters from 98 dairy bulls with contrasting fertility. All semen used for their study was from commercially available bulls in France, no surprising semen parameter included in the analyses (sperm motility, velocities, and viability) had little to no correlation to fertility as a rigorous quality control was implicated before semen was approved for breeding. As previously mentioned, semen quality as commonly measure (motility, morphology, and viability) are associated to fertility to a certain level; however, above a certain threshold, little to no correlation is observed. Nevertheless, the authors observed a great relationship between genotypes (measured by single nucleotides polymorphism; SNP), DNA methylation, and sncRNA and fertility. More specifically, 170 regions were more methylated and 83 sncRNA were greatly expressed in low-fertility bulls while only 3 regions were more methylated and 120 sncRNA were greatly expressed in high fertility. Most of these sncRNA were miRNA and ribosomal RNAs derived fragments.

Roughly 20% of miRNA present in sperm are contributed to the oocyte during fertilization (Amanai et al., 2006), and miRNA families that have had predictive ability of bull fertility were associated with developmental process (cell differentiation, and embryonic and fetal development) (Yuan et al., 2016; Wang et al., 2017; Alves et al., 2019). Many research groups have identified miRNA that are differentially expressed between different fertility classifications (Fagerlind et al., 2015; Alves et al., 2019; Alves et al., 2020; Menezes et al., 2020; Keles et al., 2021; Werry et al., 2022; Salas-Huetos et al., 2023), but the lists of differentially expressed miRNA varies among the studies conducted. These differences are likely due to how fertility classifications were made and the fact that they play a larger role post fertilization compared to other factors that would play a role in sperm transport, formation of the sperm reservoir or fertilization.

In summary, multi-omic's studies, have demonstrated a potential in improving our predictive models of bull fertility. The use of omic's is likely the next step in identifying superior sires and potentially predicting the fertility of bulls. Further, not only using omic's but a multiomic approach to combine two or more of these high-throughput methods of analyzing sperm will increase our predictive ability.

Summary

Methods of bull fertility evaluation have not changed significantly in the past several years. 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. Thus, evaluation of bull fertility will begin with a through 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.

As new tests of fertility are developed, they need to be easy to perform by AI industry personnel and veterinarians in the field. Identification of new fertility markers may improve the ability to detect lower fertility bulls that need to be culled before they enter the breeding season or predict which bulls will have high fertility. Genotypes (SNP), sperm DNA methylation at CpGs, sncRNA, proteins, metabolites, and lipids are all potential targets for new fertility markers that may influence fertilization and early embryonic development. Since sperm population is heterogeneous, studies should focus on methods to identify sperm population within an ejaculate that could generate a viable embryo and bulls that would possess a greater proportion of their ejaculate with these characteristics. Thus, the understanding of what is 'enough' of each characteristic is a crucial step in prediction of bull fertility.

Literature Cited

Albertson, D. G. 1984. Formation of the first cleavage spindle in nematode embryos. Developmental biology 101(1):61-72. doi: 10.1016/0012-1606(84)90117-9

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

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.

Alves, M. B. R., E. C. C. Celeghini, and C. Belleannée. 2020. From sperm motility to sperm-borne microRNA signatures: new approaches to predict male fertility potential. Frontiers in Cell and Developmental Biology 8:791.

Alves, M. B. R., R. P. de Arruda, T. H. C. De Bem, S. A. Florez-Rodriguez, M. F. d. Sá Filho, C. Belleannée, F. V. Meirelles, J. C. da Silveira, F. Perecin, and E. C. C. Celeghini. 2019. Sperm-borne miR-216b modulates cell proliferation during early embryo development via K-RAS. Scientific Reports 9(1):10358.

Amanai, M., M. Brahmajosyula, and A. C. Perry. 2006. A restricted role for sperm-borne microRNAs in mammalian fertilization. Biology of reproduction 75(6):877-884.

Amann, R., and J. DeJarnette. 2012. Impact of genomic selection of AI dairy sires on their likely utilization and methods to estimate fertility: a paradigm shift. Theriogenology 77(5):795-817.

Amann, R., and R. Hammerstedt. 1993. In vitro evaluation of sperm quality: an opinion. Journal of andrology 14(6):397-406. doi:10.1002/j.1939-4640.1993.tb03247.x

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. doi: 10.1146/annurev-animal-030117-014829

Austin, C. 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.

Barth, A. 2018. The use of bull breeding soundness evaluation to identify subfertile and infertile bulls. animal 12(s1):s158-s164. doi:10.1017/S1751731118000538

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., 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.

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.

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. doi:0.3168/jds.2021-20319

Boerke, A., S. J. Dieleman, and B. M. Gadella. 2007. A possible role for sperm RNA in early embryo development. Theriogenology 68:S147-S155. doi:10.1016/j.theriogenology.2007.05.058

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 2011:13. doi:10.4061/2011/757194

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. doi:10.1095/biolreprod57.6.1293

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

Clay, J., and B. McDaniel. 2001. Computing mating bull fertility from DHI nonreturn data. Journal of dairy science 84(5):1238-1245. doi:10.3168/jds.S0022-0302(01)74585-7

Costes, V., E. Sellem, S. Marthey, C. Hoze, A. Bonnet, L. Schibler, H. Kiefer, and F. Jaffrezic. 2024. Multi-omics data integration for the identification of biomarkers for bull fertility. PLoS One 19(2):e0298623. doi: 10.1371/journal.pone.0298623

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

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., 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. doi:10.1016/j.theriogenology.2011.08.011

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. doi:10.3168/jds.S0022-0302(01)74690-5

DeJarnette, J., B. Harstine, K. McDonald, and C. Marshall. 2021. Commercial application of flow cytometry for evaluating bull sperm. Animal Reproduction Science:106838. doi:10.1016/j.anireprosci.2021.106838

DeJarnette, J. M. 2005. The effect of semen quality on reproductive efficiency. Veterinary Clinics: Food Animal Practice 21(2):409-418. doi: 10.1016/j.cvfa.2005.02.011

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.

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.

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.

Fagerlind, M., H. Stålhammar, B. Olsson, and K. Klinga-Levan. 2015. Expression of mi RNA s in bull spermatozoa correlates with fertility rates. Reproduction in Domestic Animals 50(4):587-594.

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. doi:10.1016/S0093-691X(98)00036-3

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.

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.

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.

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.

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. doi:10.1017/S1751731118000319

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

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. doi:10.1051/rnd:19840508

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.

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. doi:10.1016/S0093-691X(01)00456-3

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. doi:10.1016/S0093-691X(03)00050-5

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.

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.

Keles, E., E. Malama, S. Bozukova, M. Siuda, S. Wyck, U. Witschi, S. Bauersachs, and H. Bollwein. 2021. The micro-RNA content of unsorted cryopreserved bovine sperm and its relation to the fertility of sperm after sex-sorting. BMC genomics 22:1-19.

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.

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.

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.

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

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. doi:10.3168/jds.2016-12484

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.

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. doi:10.1095/biolreprod53.5.1066

Lockhart, K. N., L. C. Fallon, and M. S. Ortega. 2023. Paternal determinants of early embryo development. Reproduction, Fertility and Development

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.

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:10.1093/biolre/ioab105

Menezes, E. S., P. R. Badial, H. El Debaky, A. U. Husna, M. R. Ugur, A. Kaya, E. Topper, C. Bulla, K. E. Grant, and O. Bolden-Tiller. 2020. Sperm miR-15a and miR-29b are associated with bull fertility. Andrologia 52(1):e13412.

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.

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.

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.

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. doi:10.1016/j.theriogenology.2014.06.007

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, M. Leibfried-Rutledge, E. Critser, W. Eyestone, and N. First. 1986. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 25(4):591-600. doi:10.1016/0093-691X(86)90143-3

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. 2008. 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. doi:10.2527/jas.2008-1181

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.

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

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.

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. doi:10.1016/j.anireprosci.2016.01.013

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. 2003. Laboratory semen assessment and prediction of fertility: still utopia? Reproduction in Domestic Animals 38(4):312-318. doi:10.1046/j.1439-0531.2003.00436.x

Saacke, R. 2008. Sperm morphology: Its relevance to compensable and uncompensable traits in semen. Theriogenology 70(3):473-478. doi: 10.1016/j.theriogenology.2008.04.012

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. doi:10.1016/S0378-4320(00)00137-8

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. doi:10.1016/S0093-691X(05)80047-0

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

Salas-Huetos, A., J. Ribas-Maynou, Y. Mateo-Otero, C. Tamargo, M. Llavanera, and M. Yeste. 2023. Expression of miR-138 in cryopreserved bovine sperm is related to their fertility potential. Journal of Animal Science and Biotechnology 14(1):129.

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.

Saunders, C. M., K. Swann, and F. A. Lai. 2007. PLCζ, a sperm-specific PLC and its potential role in fertilization. In: Biochemical Society Symposia. p 23-36.

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. Dairy Vet. Sci. J 11:555803.

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.

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

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.

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.

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

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. 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. doi:10.1007/s00441-015-2244-2

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

Sullivan, J., and F. Elliott. 1968. Bull fertility as affected by an interaction between motile spermatozoa concentration and fertility level in artificial insemination. In: VI Int Congr Anim Reprod Artif Insemination. p 1307-1310.

Sullivan, R. 2016. Epididymosomes: role of extracellular microvesicles in sperm maturation. Front Biosci (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.

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

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 Mol Dis 35(1):1-10. doi:10.1016/j.bcmd.2005.03.005

Sutovsky, P. 2009. Sperm-egg adhesion and fusion in mammals. Expert Rev Mol Med 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., 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.

Talluri, T. R., A. Kumaresan, M. K. Sinha, N. Paul, J. P. Ebenezer Samuel King, and T. K. Datta. 2022. Integrated multi-omics analyses reveals molecules governing sperm metabolism potentially influence bull fertility. Scientific Reports 12(1):10692. doi:10.1038/s41598-022-14589-w

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

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.

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

Wang, M., Y. Gao, P. Qu, S. Qing, F. Qiao, Y. Zhang, J. Mager, and Y. Wang. 2017. Sperm-borne miR-449b influences cleavage, epigenetic reprogramming and apoptosis of SCNT embryos in bovine. Scientific Reports 7(1):13403.

Werry, N., S. J. Russell, D. J. Gillis, S. Miller, K. Hickey, S. Larmer, M. Lohuis, C. Librach, and J. LaMarre. 2022. Characteristics of miRNAs present in bovine sperm and associations with differences in fertility. Frontiers in Endocrinology 13:874371.

Wilmut, I., and R. Hunter. 1984. Sperm transport into the oviducts of heifers mated early in oestrus. Reproduction Nutrition Développement 24(4):461-468. doi:10.1051/rnd:19840411

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.

Yuan, S., A. Schuster, C. Tang, T. Yu, N. Ortogero, J. Bao, H. Zheng, and W. Yan. 2016. Sperm-borne miRNAs and endo-siRNAs are important for fertilization and preimplantation embryonic development. Development 143(4):635-647.

Zoca, S. M., T. W. Geary, A. L. Zezeski, K. C. Kerns, J. C. Dalton, B. R. Harstine, M. D. Utt, R. A. Cushman, J. A. Walker, and G. A. Perry. 2023a. Bull field fertility differences can be estimated with in vitro sperm capacitation and flow cytometry. Frontiers in Animal Science 4(Original Research) doi:10.3389/fanim.2023.1180975

Zoca, S. M., E. J. Northrop-Albrecht, J. A. Walker, R. A. Cushman, and G. A. Perry. 2022. Proteomic analyses identify differences between bovine epididymal and ejaculated spermatozoa that contribute to longevity. Theriogenology 184:51-60. doi:10.1016/j.theriogenology.2022.02.021

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. doi:10.1016/j.theriogenology.2019.11.021

Zoca, S. M., J. A. Walker, A. C. Kline, T. N. Andrews, J. J. J. Rich, K. M. Epperson, J. N. Drum, M. S. Ortega, R. A. Cushman, and G. A. Perry. 2023b. Relationship of field and in vitro fertility of dairy bulls with sperm parameters, including DAG1 and SERPINA5 proteins. Frontiers in Animal Science 4(Original Research) doi:10.3389/fanim.2023.1180967