

The evaluation of sperm viability on fertilization outcomes using a heterospermic insemination
model in beef cattle

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Abstract

Predicted fertility of sires used in artificial insemination programs is based on two semen quality parameters: motility and morphology. While these characteristics are critical for assessing the potential of spermatozoa to reach the egg and complete fertilization, additional factors may further differentiate sire fertility. The longevity of sperm viability within the female reproductive tract could influence the likelihood of fertilization, especially when insemination and ovulation are not ideally synchronized in many timed AI protocols. Therefore, the objective of this study was to indirectly evaluate differences in the duration of semen viability within the female tract using heterospermic insemination composed of two bulls. Semen collected from two bulls in each trial was evaluated for morphology and motility before combining into straws containing equal proportions of motile sperm. Single-sire straws for each bull were stored for *in vitro* comparisons. All females across four field trials were set up for breeding using a 7-day CO-Synch+CIDR protocol to synchronize estrus. Insemination timing for each trial was as follows: Trial 1, heifers and cows were inseminated at 36 and 48 hours post-PGF/CIDR removal, respectively; Trials 2 and 3, heifers and cows were inseminated at 48 and 54 hours, respectively; and Trial 4, cows were inseminated at 60 hours post-PGF/CIDR removal. All females in Trials 2, 3, and 4 received a GnRH injection at the time of insemination. Blood samples were collected from calves for parentage verification. In Trials 2, 3, and 4, z-test analysis of confirmed AI-sired calves indicated a significant difference in sire contribution between the Hereford and Angus bulls within each respective trial ($p < 0.01$). Within Trial 4, single-sire ejaculate analysis revealed significant between-bull differences in both total capacitation ability via zinc signature analysis ($p = 0.01$) and progressive motility following 1 hour of *in vitro* capacitation ($p = 0.01$).

No other significant pairwise comparisons in zinc signature or progressive motility were observed across the remaining single-sire straws within trials.

Another objective of this study was to evaluate fertilization ability and early embryonic development between two bulls in an *in vitro* heterospermic fertilization assay. The two bulls were chosen because they demonstrated a skewed distribution of calves when used in heterospermic field trial inseminations. An *in vitro* fertilization (IVF) protocol from ivf Bioscience, Ltd., was performed, with minor modifications. Oocytes were obtained via follicular aspiration from abattoir-derived ovaries and matured under incubation at 38.5°C and 5.5% CO₂ for 21–24 hours before introduction into fertilization medium. The Angus and Hereford bulls from Trial 4 were selected for IVF, with semen distributed across 12 wells: 3 single-sire Angus, 3 single-sire Hereford, and 6 heterospermic mixtures of the two bulls. All presumptive zygotes were subsequently cultured for 5 days and evaluated for cell-stage development. Single-sire Hereford and the heterospermic semen resulted in a greater number of zygotes developing past the 2-cell stage ($p < 0.05$) compared to single-sire Angus semen.

Verification of progeny from heterospermic inseminations served as an indicator of relative fertility differences between bulls within this study. However, capacitation and progressive motility were not identified as primary contributors to these fertility differences. Collectively, these findings support heterospermic insemination as a viable tool for comparing relative sire fertility alongside other semen characteristics.

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Chapter 1 - Heterospermic insemination as a tool for evaluating bull fertility

Introduction

The ability to predict fertility in cattle is of great interest within the industry, with the most direct measurement of fertility being the outcome of pregnancy in field trial matings. The selection of sires plays a critical role in the success of both beef and dairy cattle programs, directly contributing to the genetic improvement of livestock herds. Bulls may be selected for use in either artificial insemination (AI) protocols or natural service matings, depending on the production system. In the dairy industry, AI is used for the majority of matings and is a key factor in the globally expanding artificial insemination market (Weiker, 2022). Although the beef industry still predominantly relies on natural service mating, bovines as a whole comprise the largest share of the global AI market (Weiker, 2022). The continued growth of AI reflects efforts to meet the increasing global demand for animal products by improving reproductive efficiency and genetic progress.

Reproductive technologies are continually advancing, and new methods are emerging for evaluating sire fertility. The selection of high fertility sires is essential for maximizing conception rates, particularly in systems utilizing timed-AI protocols. However, determining a bull's fertility potential is complex and is influenced by numerous factors, including sperm morphology and motility (Saacke, 2008; Oliveira et al., 2013), capacitation status (Kerns et al., 2018), spermatozoa DNA integrity (Dogan et al., 2015), and genetics (Taylor et al., 2018). Field matings may be necessary to accurately predict a sire's fertility, but are time-consuming and costly. Assessments done in vitro, while informative about the quality of semen from individual

bulls, may not accurately represent in vivo fertility. One possible approach to evaluate sire fertility is through heterospermic insemination, which is the deposition of semen into the female reproductive tract from more than one bull in a single insemination event. This method allows for direct comparison of relative fertility between bulls in a field setting and may reveal differences that are not apparent through standard semen quality assessments. Therefore, the purpose of this review is to investigate the use of heterospermic insemination as a tool for assessing sire fertility.

Current bull fertility assessments

Fertility assessment of bulls used in artificial insemination programs is critical for maximizing conception rates. However, fertility is a complex trait to estimate and cannot be accurately predicted using a single assessment method. Instead, a combination of multiple evaluations gives a more accurate estimate of fertility. Currently, methods used to assess fertility include breeding soundness exams (BSE), semen quality assessments, in vitro fertility assays, genetic predictions for fertility traits, and computer-assisted sperm analysis (CASA) technologies, among others.

Despite these approaches, the most reliable measure of fertility remains the conception rate derived from hundreds of single insemination events for an individual bull. Conception data from fewer than 100 inseminations is generally insufficient to accurately estimate a bull's true fertility (Flint et al., 2003).

An initial method for evaluating a bull's physical capability in breeding is the BSE, which assesses physical health, scrotal characteristics, and semen quality parameters. Structural soundness and body condition are essential for successful mating, as bulls must be capable of mounting and moving effectively within the herd. Scrotal evaluation includes the measurement

of scrotal circumference and palpation of the testes to identify abnormalities that may impair spermatogenesis. Following physical examinations, semen is collected and assessed for motility and morphology. Standard thresholds for a satisfactory potential breeder include a minimum of 30% progressive motility and 70% normal morphology (Armstrong and Koziol, 2022).

Sperm morphology plays a critical role in fertilization success. Abnormalities arise during spermatogenesis due to environmental or health factors. These abnormalities include detached heads, malformed acrosomes, midpiece and tail defects, and can each impair sperm function (Ghirardosi et al., 2018). Relationships between morphology and fertility are inconsistent. Ghirardosi and others (2018) reported no differences in abnormality percentage between low, medium, and high fertility bulls, which may be due to the small sample size in that study. Conversely, Bacinoglu et al. (2008) examined acrosome deformities between high and low fertility bulls and concluded that higher fertility bulls had lower acrosome deformities post-thaw, while the motility percent between high and low fertility bulls was not significantly different. This suggests that acrosome defects may be more predictive of reduced fertility. While a bull's potential fertility is still heavily assessed by motility and morphology parameters, its predictive value is highly variable. Reported correlations ($r = 0.15-0.83$) between sperm motility and fertility range widely (Kjøæstad et al., 1993; Bailey et al., 1994).

Deficiencies in sperm can be categorized as either compensable or uncompensable traits. Compensable traits reduce the likelihood that sperm reach the oocyte or complete fertilization, but can be offset by increasing the sperm concentration within an insemination dose. Examples of compensable traits include reduced motility or morphological defects. In contrast, uncompensable traits allow fertilization to occur, but impair embryo development and cannot be corrected through increased sperm numbers. An example of an uncompensable trait would be

reduced DNA integrity (Saacke et al., 2000). Saacke (2000) suggested that low fertility bulls with increased abnormalities may require a higher concentration of spermatozoa within an insemination straw to counteract their lower fertility rate, an example of correcting a compensable trait. Because fertility responses to increased sperm concentration may differ among bulls, this further emphasizes the complexity of male fertility (Sullivan and Elliott, 1968). Uncompensable traits are well explained by DNA damage in spermatozoa. DNA integrity plays a critical role in fertility, and studies have shown that sperm with normal morphology may still possess damaged DNA that is not detectable through standard evaluation methods (Fallon et al., 2023). Low-fertility bulls have been associated with increased DNA damage and reduced chromatin protamination, which can be visualized using a toluidine blue stain. A higher level of staining intensity reflects greater DNA damage and has been negatively correlated with in vivo fertility (Dogan et al., 2015). The distinction between compensable and uncompensable defects emphasizes the limitation of relying solely on traditional semen parameters to predict fertility.

Advanced in vitro techniques have been developed to improve evaluations of semen. Systems such as computer-assisted sperm analysis use phase-contrast microscopy and specialized software to quantify sperm motility, morphology, and assess DNA fragmentation. CASA-generated parameters, particularly when combined, have been shown to correlate with field fertility (Oliveira et al., 2013). However, technical factors such as sample handling, dilution ratios, and specific equipment differences can influence the results (Valverde et al., 2020). Within-system repeatability is very high for semen motility (Davis et al., 1992), which may be beneficial in objectively and consistently predicting a bull's motility for field fertility. Flow cytometry is another in vitro method in which sperm quality parameters like DNA content, acrosome integrity, and surface proteins can be evaluated. Using fluorescent probes, spermatozoa

are pushed through a flow cytometer, which reads the intensity of the fluorescence, and are then sorted or assessed based on the parameter of interest. The parameters that can be analyzed through the use of flow cytometry offer a more in-depth understanding of a bull's semen quality (Zuidema et al., 2021). Unfortunately, the longer handling and processing time of flow cytometry can be detrimental to semen and reduce its lifespan (Zuidema et al., 2021). The continued advancement of in vitro techniques to accurately and safely analyze semen traits is of great importance to the industry to improve fertility predictions.

Genetics can also contribute to bull fertility, although the heritability of fertility-related traits is generally low. Both loss-of-function (LOF) mutations and lethal haplotypes have been associated with increased embryonic loss and reduced reproductive success in cattle (Taylor et al., 2018). Genome-wide association studies (GWAS) in dairy cattle have uncovered several lethal haplotypes, many of which are associated with reduced fertility (Fritz et al., 2013). In the U.S. dairy genomic evaluation system, over 25 lethal haplotypes are currently tracked (Cole et al., 2013). The causal mutations responsible for embryonic lethality have been discovered for approximately half of these lethal dairy haplotypes (Fritz et al., 2013). For example, Flisikowski et al. (2010) identified a novel mutation in the maternally imprinted *PEG3* domain that results in a loss of *MIMT1* expression and causes abortions and stillbirths in *Bos taurus* cattle. Once identified, genomic testing for these lethal haplotypes and LOF mutations is routinely used to remove carriers (beef) or manage carrier matings (dairy) in breeding programs. Despite these advances, identifying additional fertility-associated mutations remains challenging because these variants are typically rare and relatively few bulls have been whole genome sequenced (WGS) at adequate depth. Furthermore, to date the majority of these genetic studies have focused on dairy cattle breeds, leaving a knowledge gap for beef breeds. Overall, beef genetic evaluations are still

limited by the current understanding of genes associated directly with fertility (Taylor et al., 2018).

Capacitation of spermatozoa

Capacitation involves a series of molecular changes to the plasma and acrosomal membranes of spermatozoa, enabling zona pellucida binding and priming the sperm for the acrosome reaction necessary for oocyte fertilization (Lefebvre and Suarez, 1996; Zimmerman and Sutovsky, 2009; Zigo et al., 2019). First, decapacitating factors must be removed from the surface of the spermatozoa, which causes downstream protein activation and spermatozoa surface remodeling to form the zona pellucida binding complexes (Zigo et al., 2019). Motile spermatozoa move upstream within the reproductive tract until they reach specific areas, known as sperm reservoirs. Within the oviduct, the isthmus serves as a reservoir where spermatozoa bind in a non-capacitated state before beginning capacitation (Lefebvre and Suarez, 1996), and most mammal spermatozoa can maintain their motility and fertilization potential for 3-4 days (Camara Pirez et al., 2020). Spermatozoa that have begun the capacitation process are released from the sperm reservoir and oviduct epithelium and will progress further upstream to the site of fertilization, the ampulla of the oviduct.

In *ex vivo* models of the oviduct epithelium, the spermatozoa's binding capabilities to the sperm reservoirs' epithelial membrane have been demonstrated. Non-capacitated sperm bind more readily to oviduct epithelium, compared to sperm incubated with heparin for 4 hours to induce a capacitated state before introduction to *ex vivo* isthmus and ampulla epithelium (Lefebvre and Suarez, 1996). In another *ex vivo* model utilizing oviduct epithelium, Camara Pirez and colleagues (2020) found that sperm that were motile or agile with actively beating tails bound to epithelial cells, while immotile sperm cells were flushed out due to the movement of

ciliary cells. This demonstrated that immotile spermatozoa may be unable to bind to the epithelium of sperm reservoirs and therefore cannot undergo capacitation before being flushed out of the oviduct.

The exact process of capacitation is complex, and there is still a large research interest in defining the process. It has been suggested that capacitation may occur at varying rates in individual males. Zigo and others (2019) showed boars had varying levels of capacitated sperm at similar time points during an *in vitro* capacitation study. Using fluorescence to stain lectin PNA found on the acrosome of spermatozoa, they were able to observe the remodeling of the proteins throughout the capacitation process by measuring the intensity of the fluorescence to understand the different stages of capacitation. Three boars had varying levels of intensity at the same time point, indicating differential capacitation rates (Zigo et al., 2019). Similarly, the usage of zinc signatures, measured by the use of epifluorescence, in spermatozoa has been used to describe their current stage of capacitation. Kerns and others (2018) demonstrated the stages of zinc signatures in boars, bulls, and human spermatozoa at varying stages of capacitation. The authors found that high fertility AI boars had increased numbers of spermatozoa that reached a capacitated state *in vitro* compared to low fertility boars. These results suggested varying capabilities of spermatozoa to capacitate within a designated time frame across different males. In artificial insemination, the effect of cryopreservation on spermatozoa post-thaw capacitation should be considered. Fresh bull semen reached a capacitated state *in vitro* in 4-9 hours when induced with heparin (Parrish et al., 1988), whereas cryopreserved semen reached a similar state in only 45 minutes (Fukui et al., 1990). Cryopreservation may initiate the beginning stages of capacitation, and once inseminated, frozen-thawed sperm may reach a fully capacitated and competent fertilizing stage earlier than fresh semen.

Although the entire process of capacitation may span several hours, the viable fertilization window of a fully capacitated spermatozoon is relatively short before dying. Keller and Kerns (2023) reported capacitated fresh boar semen to be viable for fertilization for 2-5 minutes and just 30-90 seconds for frozen-thawed bull semen. In another study utilizing zinc signatures on frozen-thawed bull semen, two key changes were observed by hour 24 after introduction to capacitation media: a decrease in zinc signatures 1 and 2, indicative of sperm in the early stages of capacitation still retaining high fertilization potential, and an increase in zinc signature 4, indicative of dead sperm cells. For zinc signature 3, which represents sperm that have completed capacitation and are dying, a significant effect of individual bull over time was found, as the percentage of sperm in this category decreased (Zoca et al., 2023a).

The onset and duration of sperm capacitation are important to consider for successful fertilization. Bulls with varying rates of capacitation and functional sperm lifespan may have fertility levels exacerbated under fixed-timed AI protocols. Nevertheless, most research regarding optimal AI timing has focused primarily on synchronizing insemination relative to estrus onset and ovulation in females, rather than considering potential male-specific differences.

Fixed-timed artificial insemination

The AM/PM rule, introduced by Trimberger in 1948, remains one of the most effective timing protocols for AI in cattle and is based on the observation that ovulation in females occurs 24-32 hours after the onset of estrus. Subsequent studies have demonstrated that optimal conception rates are achieved when AI is performed approximately 12 hours after estrus onset (Dorsey et al., 2011; Haadem et al., 2023). Despite this general recommendation, variation in conception rates is observed when insemination occurs earlier or later than this interval, which can be attributed to differences in ovulation timing among females (Trimberger, 1948). In fixed-

timed AI protocols, acceptable pregnancy rates can be achieved when females express estrus before insemination, and ovulation occurs after breeding (Larson et al., 2006; Malik et al., 2012; Whittier et al., 2013).

Synchronization of estrus and ovulation is an important management tool in production systems, and understanding the hormonal regulation and timing of key physiological events is essential for improving herd conception rates. In synchronization protocols such as the Ovsynch or CO-synch, the timing of insemination relative to estrus and ovulation is critical for successful fertilization (Colazo and Mapletoft, 2014). According to Saacke et al. (2000), AI success reflects a balance between insemination timing, oocyte age, and sperm viability. Insemination shortly after the onset of estrus results in reduced availability of viable sperm at ovulation, whereas insemination closer to 24 hours after estrus onset may decrease fertilization potential and expose the oocyte to aging. Macmillan and Curnow (1977) reported high variation in conception rates among sires of predetermined high or low fertility in cows inseminated earlier in estrus. High-fertility sires maintained conception rates in these females, whereas conception rates of low-fertility sires were reduced. The authors suggested that sperm from the low-fertility sires may have lost fertilizing capacity before ovulation occurred in some females. These observations indicate that differences among bull sperm survival within the female reproductive tract may contribute to fertility variation. In support of this hypothesis, Santos and Fair (2026) reported that the optimal insemination time may vary among bulls relative to the onset of estrus. Consequently, bulls whose spermatozoa maintain fertilizing ability for longer periods may have an advantage in fixed-timed AI programs when insemination occurs in females early in estrus.

Heterospermic insemination

Heterospermic insemination is the deposition of semen from two or more sires into the female reproductive tract during a single insemination event. In the dairy industry, the use of heterospermic beef semen has increased in the past few years, due to increased beef-on-dairy breeding programs (Weiker, 2023). In these beef-on-dairy programs, the paternity of the calves is not a primary concern because heterospermic insemination is meant to offset poor conception rates from sub-fertile bulls (Santos and Fair, 2026). This is not a common practice within the beef industry. Heterospermic insemination has been used in research settings to directly compare the fertility of two or more sires. The use of heterospermic insemination controls variation when comparing AI sire fertility within a herd. The variation induced by the female is minimized since sperm from all sires are exposed to the same uterine environment (Berger and Dally, 2001). Prior to semen being packaged and frozen, motility, normal morphology, and concentration can be adjusted to appropriate levels to mitigate the effects of compensable traits that may decrease chances of fertilization (Flint et al., 2003). However, only compensable traits can be accounted for during processing, whereas uncompensable traits are expressed following insemination and fertilization (Saacke, 2008).

When comparing the fertility of two or more bulls, heterospermic insemination has been used to amplify differences among bulls in fewer insemination events than would be required with normal homospermic insemination. In one of the earliest studies using heterospermic insemination in cattle, Beatty et al. (1969) demonstrated that heterospermic fertility was moderately to highly correlated with homospermic fertility rates of individual bulls. They inseminated cattle with an equal concentration of semen from one Friesian and one Hereford bull, and the calf parentage results were used to calculate a heterospermic fertility index. This

heterospermic index was compared to a previously established homospermic fertility index and was found to be positively and strongly correlated (Beatty et al., 1969). These findings suggested that heterospermic inseminations can be used to accentuate subtle fertility differences between bulls while using fewer inseminations required for homospermic breeding. It is important to note that since calf parentage is not verified until 9 months later, the fertility of semen from bulls used in the heterospermic trial could have changed, particularly if new ejaculates are used for subsequent inseminations. However, heterospermic insemination may be useful when comparing bulk semen intended for cryopreservation, whose fertility is not expected to change substantially over time (Beatty et al., 1969).

In multiple studies since the 1970's, heterospermic insemination has been valuable in determining which bulls sire more calves. It is thought that when bulls are placed in direct competition by heterospermic insemination, spermatozoa will compete to fertilize the oocyte. A multi-sire seminal treatment resulted in an increased conception rate compared to single sire inseminations (Nelson et al., 1975). In this study, there was no difference in conception rates across single-sire inseminations, but when progeny were determined in multi-sire inseminations, the bull effect varied. In another study conducted by the University of Nebraska, utilizing multi- and single-sire inseminations, conception rates did not differ between the types of insemination. One bull did sire fewer progeny compared to the two other bulls in a multi-sire insemination, but too few insemination events were conducted to determine significance (Christenson, 2024). In contrast, Stewart et al. (1974) explored differences in heterospermic insemination of fresh and frozen semen from four bulls. In fresh heterospermic insemination, all four bulls sired a similar number of calves, whereas in frozen heterospermic inseminations, one bull sired significantly more progeny compared to the other three sires. These authors interpreted these results as

indicating the four bulls had similar fertility with fresh semen, but semen from the bull producing more progeny after freeze-thaw was better able to survive cryopreservation. Collectively, these results suggest that heterospermic competition can detect a bull with a greater ability to sire more offspring in artificial insemination.

The use of heterospermic insemination has been debated in terms of its ability to increase AI single service conception rates compared to homospermic insemination. A study using heterospermic insemination reported no significant difference in non-return rates between heterospermic insemination (68.1%) and homospermic insemination (65.5%) (Beatty et al., 1969). In a follow-up study, they again found no difference in heterospermic or homospermic inseminations, with both reaching non-return rates greater than 66% at 16 weeks (Stewart et al., 1974). No pregnancy differences were observed between heterospermic and homospermic inseminations, although when the concentration of heterospermic doses was doubled, there was a numerical increase in pregnancy rates (Albrecht et al., 2004). Similar results were observed in Nelore cattle, where heterospermic insemination had similar pregnancy rates compared to homospermic inseminations (Almeida et al., 2017). In contrast, a study using heterospermic insemination found the non-return rate of mixed Holstein semen to be 11.4% greater compared to homospermic inseminations. An 8% increase in non-return rates was seen in Guernsey bulls in the same study (Hess et al., 1958). More recently, Santos and others (2026) found a significant increase in conception rates of heterospermic insemination compared to homospermic. Current literature on heterospermic insemination has not consistently demonstrated improvements in AI conception rates. This variability likely reflects differences in fertility among the sires included in each study. This highlights the need for further research to better understand the role of heterospermic insemination in sire fertility evaluation.

Typically, many inseminations are required to accurately estimate a bull's fertility, which is often after that bull has already been used extensively within breeding programs. By the time the data on calf crops is available, sub-fertile bulls may have contributed to reduced conception rates across many females. In contrast, heterospermic insemination allows bulls to be directly compared in fewer inseminations. This competitive environment can amplify fertility differences between bulls. As a result, heterospermic insemination may serve as an effective preliminary screening tool to identify superior fertility bulls before large-scale homospermic use, thereby improving efficiency in bull selection. While heterospermic insemination does not consistently increase AI conception rates relative to homospermic insemination, it may provide a buffer against sub-fertile sires by providing spermatozoa from multiple bulls capable of achieving fertilization. It is evident, though, that when progeny is verified in heterospermic inseminations, sire differences become prominent, suggesting a difference between bulls' individual fertility potential.

Conclusion

Bull fertility is a complex and multifactorial trait that remains difficult to predict accurately. Although breeding soundness exams and conventional semen evaluations provide valuable information regarding a bull's reproductive potential, substantial variation in fertility remains unexplained. Advanced technologies, such as CASA, flow cytometry, and DNA integrity assessments, have improved our understanding of sperm function, yet no single marker has consistently predicted fertility outcomes under field conditions.

One potential source of this unexplained variation may be differences in the duration of sperm capacitation among bulls. The timing of capacitation relative to ovulation is critical for successful fertilization, particularly in timed AI programs where variation exists in the interval

between insemination and ovulation. Bulls whose spermatozoa maintain fertilizing competence for longer periods may possess a fertility advantage that is not detected by conventional semen evaluations.

Heterospermic insemination provides a unique approach to directly compare the relative fertility of bulls under identical reproductive conditions. By placing spermatozoa from multiple sires in competition for fertilization, fertility differences that may otherwise remain undetected can become apparent. Collectively, the literature suggests that sperm capacitation dynamics may contribute to variation in sire fertility, and heterospermic insemination may serve as a valuable tool for evaluating these differences.

Chapter 2 - Evaluation of in-utero sperm viability using heterospermic insemination in beef cattle

Abstract

Standard semen evaluation methods for use in artificial insemination programs is based on two quality parameters: motility and morphology. While these characteristics are critical for assessing the potential of spermatozoa to reach the egg and complete fertilization, additional factors may further differentiate sire fertility. The longevity of sperm viability within the female reproductive tract could influence the likelihood of fertilization, especially when insemination and ovulation are not ideally synchronized in many timed AI protocols. Therefore, the objective of this study was to indirectly evaluate differences in the duration of semen viability within the female tract using heterospermic insemination composed of two bulls. Semen collected from an Angus and Hereford sire for each trial was evaluated for morphology and motility before combining in straws containing equal proportions of motile sperm. Single-sire straws for each bull were stored for *in vitro* comparisons. All females across four field trials were subjected to the 7-day CO-Synch+CIDR protocol for estrous synchronization. Insemination timing was adjusted by trial: in Trial 1, heifers and cows were inseminated at 36 and 48 hours post-PGF/CIDR removal, respectively; in Trials 2 and 3, at 48 and 54 hours, respectively; and in Trial 4, cows were inseminated at 60 hours post-PGF/CIDR removal. All females in Trials 2, 3, and 4 received a GnRH injection at the time of insemination. Blood samples were collected from calves for parentage verification. In Trials 2, 3, and 4, z-test analysis of confirmed AI-sired calves indicated a significant difference in sire contribution between the Hereford and Angus bulls within each respective trial ($p < 0.01$). For Trial 4, single-sire ejaculate analysis revealed significant between-bull differences in both total capacitation ability via zinc signature analysis

($p = 0.01$) and progressive motility following 1 hour of *in vitro* capacitation ($p = 0.01$). No other significant pairwise comparisons in zinc signature or progressive motility were observed across the remaining single-sire straws within trials. Zinc signature and progressive motility analysis did not result in differences linked to the disparity of calves born using heterospermic straws. Nonetheless, significant differences in sire contribution to AI success in heterospermic inseminations suggest a difference in in-utero sperm viability between bulls.

Introduction

Artificial insemination (AI) has been a tool used to accelerate genetic progress in cattle herds for decades. However, the beef industry's ability to accurately select high-fertility bulls has not substantially improved. Fertility is a complex trait to estimate, and additional factors continue to be identified that may affect sire fertility. The best estimate for AI fertility is the conception rates or non-return rates from hundreds or thousands of artificially inseminated females. Currently, the most common assessment for fertility in AI programs is the visual analysis of progressive motility and normal morphology of an ejaculate. However, the relationships between sperm quality factors and fertility are still variable and hard to determine (Kjøestad et al., 1993; Bailey et al., 1994; Bacinoglu et al., 2008; Ghirardosi et al., 2018). Compensable semen traits, such as motility, can be adjusted for within insemination straws by increasing the concentration of spermatozoa (Saacke, 2008). However, uncompensable traits, such as DNA damage, are more difficult to distinguish in visual analysis and cannot be compensated for by increasing the number of cells in an insemination dose. These traits must be detected using other analyses, like Computer Assisted Sperm Analysis (CASA) or DNA staining techniques (Saacke, 2008; Oliveira et al., 2013; Dogan et al., 2015).

Another possible factor for variation in male fertility in AI protocols is the duration of capacitation, which is variable between sires (Kerns et al., 2018; Zigo et al., 2019). Capacitation is a term used to explain the protein remodeling and start of hyperactivation of spermatozoa that aids in their advancement to the site of fertilization and subsequent ability to undergo the acrosome reaction, which is necessary for a spermatozoa to fertilize an oocyte (Puga Molina et al., 2018; Keller and Kerns, 2023). Kerns and colleagues (2018) described zinc signatures present in bull sperm, representative of capacitation status, and suggested their use in predicting the fertility of males. In addition, the effect of freezing semen should be taken into account for individual bulls, as it may initiate the process of capacitation, known as cryocapacitation, before spermatozoa are deposited in the female tract, potentially reducing their chances of reaching the oocyte within the optimal fertilization window (Fukui et al., 1990; Saacke et al., 2000). Fixed-timed AI (FTAI) protocols, in which imperfect synchronization of insemination and ovulation can occur, may affect a sire's fertility potential following cryopreservation due to the freeze/thaw process (Santos and Fair, 2026). However, whether these differences stem from uncompensable traits or variation in capacitation duration remains difficult to distinguish.

The use of a heterospermic insemination model was first introduced by Beatty et al. (1969) as a tool for reducing the number of insemination events needed to estimate a sire's fertility. Heterospermic insemination is the deposition of semen from two or more sires into the female reproductive tract during a single insemination event. In one of the first cattle studies utilizing heterospermic insemination, a moderate to high correlation was found with homospermic fertility rates of individual bulls (Beatty et al., 1969). These results suggested that heterospermic insemination could be used to accentuate present fertility differences between bulls, allowing for fewer inseminations required to detect relative fertility differences, and have

been supported by subsequent studies (Nelson et al., 1975; Christenson, 2024; Santos and Fair, 2026).

Current sire selection methods do not account for variation in capacitation rate or the duration of sperm viability within the female tract, which may significantly influence fertility outcomes in timed-AI protocols. A technique capable of detecting these differences could meaningfully improve AI sire selection. Therefore, this study was designed to indirectly evaluate the length of semen viability within the female reproductive tract using heterospermic insemination composed of two beef bulls.

Materials and Methods

The Kansas State University Animal Care and Use Committee approved the experimental procedures described in this study.

Trial 1 and Trial 2 (Southeast Research-Extension Center, Parsons, Kansas)

Semen Collection

Bulls were collected at the Kansas Artificial Breeding Service Unit (KABSU), a custom collection service unit that is certified by National Association of Animal Breeders. KABSU technicians evaluated all ejaculates used in the study for sperm motility and morphology. Semen collection and handling was performed in accordance with KABSU protocols. All ejaculates were maintained at 37°C during motility, morphology, and concentration assessments. Each ejaculate was assessed for minimum thresholds of 50% motile sperm and 70% normal morphology. Bull A and Bull B were selected for the preparation of heterospermic insemination straws. Bull A and Bull B were yearlings at the time of collection for Trial 1, and 2-year-olds in Trial 2. Each ejaculate was extended with a one-step non-animal extender (OptiXcell®, IMV Technologies) to a concentration of 20 million motile sperm per mL. The extended ejaculates

were then combined to create heterospermic 0.5 cc straws, with motility-adjusted contributions from each bull to ensure comparable sperm concentrations of 10 million motile sperm per bull per heterospermic straw. When possible, single-sire straws were also prepared from the same ejaculate used to make the heterospermic straws, for later *in vitro* assay analysis.

For freezing, straws were exposed to liquid nitrogen (LN₂) vapor for 10 minutes and then plunged into LN₂ for permanent storage. Post-thaw motility and morphology were assessed at 24 hours for the single-sire straws. Fresh ejaculates were collected each breeding year to prepare the heterospermic insemination straws. The semen characteristics from KABSU for the ejaculates are presented in Table 2.1.

Estrous Synchronization

Estrous synchronization was performed in 80 Angus cows and 25 heifers (Trial 1) and 75 Angus cows and 34 heifers (Trial 2) using the 7-day CO-Synch + CIDR protocol. Cows and heifers received a CIDR (Eazi-Breed CIDR®, 1.38 g, Zoetis) and an intramuscular injection of GnRH (Cystorelin®, 100 µg, Boehringer Ingelheim) on Day 0. On Day 7, CIDRs were removed, and all cattle received an intramuscular injection of prostaglandin F_{2α} (PGF_{2α}) (Lutalyse®, 5 mg/ml, Zoetis). Estrous detection patches were applied to the tailhead at the time of CIDR removal.

For fixed timed-AI, the following protocol was used for each breeding year. Semen straws were thawed in a thermostatic water bath for a minimum of 30 seconds at 37°C. All inseminations were performed by a single AI technician. Estrous patch color was recorded at the time of insemination. For Trial 1, heifers were inseminated 36 hours after CIDR removal, while cows were inseminated 48 hours after CIDR removal. No GnRH injection was administered at the time of AI. For Trial 2, heifers and cows were inseminated 48 and 54 hours after CIDR

removal, respectively. Unlike Trial 1, in Trial 2, all animals received an injection of GnRH at the time of AI. Clean-up sires were placed into the pasture one week after insemination in both trials. Transrectal ultrasonography was performed 50 days and 63 days after insemination for Trial 1 and Trial 2, respectively, to determine pregnancy status.

Blood samples for parentage determination were collected from calves after 9 -14 months of age via tail venipuncture into ACD-A anticoagulant vacutainer tubes. Tubes were stored at 4°C until DNA extraction.

Trial 3 (Southeast Research-Extension Center, Parsons, Kansas)

Semen Collection

Semen straws were made as previously described in Trials 1 and 2. For Trial 3, Bull C was used because Bull B from Trials 1 and 2 did not meet acceptable motility criteria at the time of collection. A new ejaculate from Bull A was combined to prepare the heterospermic insemination straws with Bull C. Single-sire semen straws were made for both bulls for *in vitro* comparisons (Table 2.1). Bull C was a 2-year-old, and Bull A was a 3-year-old at the time of collection.

Estrous Synchronization

For Trial 3, 84 Angus cows and 30 heifers were set up for breeding with the same estrous synchronization protocol (7-day CO-Synch + CIDR) used in Trials 1 and 2. Similar to Trial 2, heifers and cows were inseminated 48 and 54 hours after CIDR removal, respectively. For Trial 3, all animals received an injection of GnRH at the time of AI. Clean-up sires were placed in the pasture one week after insemination. Transrectal ultrasonography was performed to examine pregnancy status 105 days after insemination.

Blood samples for parentage determination were collected from calves after 9 -12 months of age via tail venipuncture into ACD-A anticoagulant vacutainer tubes. Tubes were stored at 4°C until DNA extraction.

Trial 4 (Private Kansas Ranch)

Semen Collection

The semen used in Trial 4 was from bulls provided by the ranch and was collected at KABSU. Single-sire and heterospermic semen straws were prepared as previously mentioned; the bulls for this trial are designated as Bull D and Bull E. Bull D was a 4-year-old and Bull E was a 3-year-old at the time of collection for this Trial. Semen characteristics of these bulls are presented in Table 2.1.

Estrous Synchronization

Synchronization of estrus was performed in 117 Angus cows utilizing the same 7-day CO-Synch + CIDR protocol as described for the previous trials. Cows were inseminated beginning 60 hours after CIDR removal, and all received an injection of GnRH. Clean-up sires were placed into the pasture immediately after the time of AI. Transrectal ultrasonography was performed on cows to examine pregnancy 34 days after insemination.

Blood collections on calves were conducted after 3 - 5 months of age via tail venipuncture into ACD-A anti-coagulant vacutainer tubes. Blood tubes were kept at 4°C until DNA extraction.

DNA Extraction

DNA for the Trial 1 steer calves was extracted using a phenol-chloroform isoamyl alcohol (25:24:1) protocol (Chomczynski and Sacchi, 1987). Whole blood samples were centrifuged at $2,100 \times g$ for 20 minutes at 4°C, and the buffy coat layer was transferred into

microcentrifuge tubes. Proteinase K was added to break down proteins, and the phenol-chloroform was added to remove protein contaminants from the DNA. Following this purification step, ammonium acetate and 100% ethanol were added to precipitate the DNA. The resulting DNA pellet was washed with 70% ethanol, briefly air-dried, and resuspended in Tris-EDTA buffer (Growcells, 1 mM EDTA, 10 mM Tris). Samples were stored at -80°C until parentage testing.

All other DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit, following the manufacturer's protocol with minor adjustments. Briefly, whole blood samples were centrifuged for 20 minutes at $2100 \times g$ and 4°C, and the buffy coat layer was transferred into a microcentrifuge tube containing Proteinase K and Gibco PBS. Buffer AL was added, and samples were incubated at 56°C for 10 minutes. Following incubation, 100% ethanol was added, and the mixture was transferred to a Qiagen spin column. Columns were centrifuged for 1 minute at 8500 rpm, and the flow-through was discarded. Buffer AW1 was added, and columns were centrifuged again for 1 minute at 8500 rpm. After discarding the flow-through, Buffer AW2 was added, and the columns were centrifuged for 3 minutes at 14,250 rpm. The flow-through was discarded. For elution of DNA, Buffer AE was added to each column positioned in a clean microcentrifuge tube and then centrifuged for 1 minute at 8500 rpm; this elution step was repeated to increase the final DNA yield. DNA concentration of the eluted samples was then measured using a NanoPhotometer® (Implen). Samples were stored in -80°C until the time of parentage testing.

All DNA samples were diluted to at least 30 ng/μl in 100 μl and sent to Neogen in Lincoln, NE, in a 96-well plate for SeekSire™ Parentage analysis.

Semen Analysis

Single-sire straws were frozen for each trial at a concentration of 20 million motile sperm per straw. The exception was for Trial 2, in which single-sire straws were not frozen and stored for *in vitro* comparisons. Heterospermic straws from each trial were also stored for *in vitro* comparisons. All semen straw formulations, including single-sire and heterospermic insemination straws, were assessed via *in vitro* comparisons.

Computer-Assisted Semen Analysis (CASA)

Semen straw formulations were evaluated using a CASA system as previously described by Weide et al. (2024). Semen samples were quickly thawed in a 37°C water bath for at least 30 seconds. After thaw, a 3.5 µL aliquot of each sample was loaded into a prewarmed Minitube counting chamber (Minitube GmbH, Tiefenbach, Germany) and assessed using a Zeiss Axioscope 5 microscope (Carl Zeiss Microscopy, LLC, Oberkochen, Germany) with a Basler ace ac2440-75uc camera (Basler AG, Ahrensburg, Germany) and a 10×/0.25 A-Plan objective lens using MinitubeAndroVision® software (Reference Module: 12,500/1000, Tiefenbach, Germany) (Weide et al., 2024). A second CASA assessment was taken after 1 hour of *in vitro* capacitation (IVC). The evaluated kinematic parameters included sperm concentration [10^6 /mL], total motility (TM), and progressive motility (PM).

Image-based Flow Cytometry

Reagents

All reagents were sourced from Fisher Biotech, unless otherwise noted. FluoZin™-3, AM (FZ3; zinc probe) from ThermoFisher (F24195) was reconstituted with DMSO to a stock solution of 500 µg/mL. Lectin PNA (*Arachis hypogaea*/peanut agglutinin) conjugated to Alexa Fluor™647 (PNA-AF647) was from Invitrogen™(L32460) with a stock solution of 1 mg/mL.

Hoechst 33342 (H33342) from Calbiochem (382065) was reconstituted with H₂O to a stock solution of 18 mM. Propidium Iodide (PI) from Across Organics (AC440300010) was reconstituted with H₂O to a stock solution of 1 mg/mL.

Multiplex Fluorescence Labeling for IBFC

Spermatozoa were washed of seminal fluids and extender media at $500 \times g$ for 4 minutes. Samples consisting of about 5 million spermatozoa were incubated for 30 minutes at room temperature with Lectin PNA-AF647 used at a concentration of 1:2000 from the stock concentration (1 mg/mL) for a final concentration of 0.5 μ g/mL, FZ3 used at a concentration of 1:500 from a stock solution (500 μ g/mL) for a final concentration of 1 μ g/mL, H33342 at a concentration of 1:1000 from a stock solution (18 mM) for a final concentration of 10 μ g/mL, and PI used at a concentration of 1:1000 from a stock solution (1 mg/mL) for a final concentration of 0.1 μ g/mL. After incubating with the fluorescent labels, samples were washed of the labels by centrifugation at $500 \times g$ for 4 minutes and resuspended with 100 μ L PBS and incubated for a second 30 minutes at 37°C. After the second incubation, samples were manually loaded into the ImageStream®X Mark II (AMNIS Cytex Biosciences, Fremont, CA, USA) image-based flow cytometer for IBFC image collection. There were three time points analyzed: 0 hours (non-capacitated, Non-Cap), 30 minutes (30 min IVC), and 1 hour (1 h IVC).

Image-Based Flow Cytometric Data Acquisition

IBFC was performed using the ImageStream®X Mark II (AMNIS Cytex Biosciences, Fremont, CA, USA) image-based flow cytometer, fitted with a dual camera, 12-channel system, and a 40x objective lens magnification. The following lasers were used: 405 nm laser for H33342 excitation at 10 mW, 488 nm laser for FZ3 excitation at 60 mW, 592 nm for PI excitation at 40 mW, 642 nm laser for PNA-AF647 excitation at 25 mW, and a 785 nm laser for

side scatter (SSC) at 10 mW. A minimum of 10,000 spermatozoa event images were collected per semen straw replicate, with brightfield images acquired in channel 1 and 9, FZ3 fluorescent images acquired in channel 2, PI fluorescent images acquired in channel 4, SSC images acquired in channel 6, H33342 fluorescent images acquired in channel 7, and PNA fluorescent images acquired in channel 11.

IBFC Data Analysis

First, raw image files were compensated. Next, images were analyzed in Amnis IDEAS[®] software, version 6.4.8.0 (AMNIS Cytex Biosciences, Fremont, CA, USA). Images were initially gated for those that were in focus, contained one sperm cell, and were aligned to the camera as similarly done in (Kerns et al., 2018). Gating boundaries for PNA and PI populations were determined by population segregations. Traditional flow cytometric analysis methods do not allow for easy distinction of zinc signatures based on whole-cell FZ3 intensity; therefore, creating masks that only analyze specific regions on the sperm proved to be key in distinguishing these populations. Zinc signature 1 was determined on whole FZ3 intensity, while the remainder of the signatures were determined based on the presence of FZ3 labeling in the sperm acrosome and tail. Zinc signatures 2, 3+5, and 5 were first gated based on FZ3 presence in the acrosome, followed by FZ3 intensity of the sperm tail. Zinc signatures 3 and 4 were first gated based on FZ3 absence in the acrosome, followed by FZ3 intensity of the sperm tail.

Digital masks used were previously described by Keller et al. (2026). Briefly, sperm heads were masked using a dilated threshold of channel 7, H33342 images to isolate the head (`head_only_mask = dilate (threshold (M07, ch07, 80), 1)`). Next, sperm tails were masked by creating a whole cell mask using channel 1 bright field images (`whole_cell_mask = object (M01, ch01, Tight)`) and subtracting the head mask (`tail_only_mask = object (M01, ch01, Tight)`) And

Not dilate (threshold (M07, ch07, 80), 1)). Features were made for each mask using channel 2 intensity. Acrosome localization was also determined using masks and features. The post-acrosomal region of the head mask was created first using the interface function on the head-only mask area of channel 1 brightfield images (bottom_head_mask = interface (head_only_mask, M01, 11)). The acrosome mask was created by subtracting the post-acrosomal region of the head mask from the head-only mask (top_head_mask = head_only_mask And Not bottom_head_mask). Features were made for each mask using channel 2 intensity.

Statistical Analysis

The proportion of calves sired by either bull in each trial originated from one group of cows, and the two bulls represent two possible categorical outcomes. Therefore, the data were treated as a single binomial sample. Differences between the two proportions of calves sired by the AI bulls were evaluated using a binomial test. The observed count for each bull was compared against the expected distribution under the null hypothesis of equal probability. Confidence intervals for each proportion were generated using Fisher's Exact Binomial method.

Estrous expression data were analyzed in R, Version 2026.04.0 Build 526, using logistic regression. Estrous patch score, animal type (heifer or cow), and trial year included as fixed effects. The model evaluated the association of estrous patch score with AI success.

CASA results were analyzed in R using a linear model. Three straws from all semen straw formulations (single-sire and heterospermic straws) were analyzed, and a mean value was generated for total sperm concentration and progressive motility. Fixed effects included in vitro capacitation (IVC) time (post-thaw or 1 h IVC) and semen straw formulation. Progressive motility was the response variable. Model effects were assessed using analysis of variance

(ANOVA), and pairwise comparisons among semen straw formulations were performed using estimated marginal means with Tukey-adjusted comparisons.

Zinc signature data were analyzed in R. First, replicate measurements within each straw were evaluated for repeatability. Replicates were subsequently averaged within the straw for all analyses. A composite capacitation metric (total capacitation) was calculated for each straw within semen straw formulation and time point by summing Zn Sig 3, 4, and 5. Capacitation difference was then calculated for each straw by subtracting the total capacitation value at 30 min IVC from the corresponding total capacitation value at 1 h IVC. Differences between select semen straw formulations for total capacitation at 1 h IVC and capacitation difference were assessed using Welch's two-sample t-tests.

To evaluate changes in zinc signatures across time points, an average proportion for each zinc signature across all semen straw formulations at each time point was calculated. Differences among time points were assessed using Welch's ANOVA. When significant effects were detected, pairwise comparisons for time points were conducted using Welch's two-sample t-tests with Bonferroni correction for multiple comparisons.

Welch's methods were used because it does not assume equal variances between groups.

Statistical significance was declared at $p < 0.05$.

Results

Conception to AI

Pregnancy to AI (P/AI) varied considerably across trials. In Trial 1, the overall P/AI was 17% (18/105; Figure 2.6). Heifers (n=25) achieved a P/AI rate of 12%, while cows (n=80) had a P/AI rate of 19% (Figure 2.5). In Trial 2, the overall P/AI was 40% (44/109). Heifers in Trial 2 had a P/AI of 53% (18/34), whereas cows achieved a P/AI rate of 36% (27/75). In Trial year 3,

the overall P/AI was 21 % (n=114). The heifers (n=30) and cows (n=84) in Trial year 3 had P/AI of 20 and 21 %, respectively. Lastly, Trial year 4 had a 43% P/AI with only cows being inseminated.

Estrous patch data were collected at the time of AI and used to evaluate the relationship between estrous expression and conception to AI. Only females with an estrous detection patch present at the time of AI were included in the analysis. Estrous patch activation was classified into five categories (Table 2.2). Females with a completely gray patch were assigned a score of 1, whereas scores of 2, 3, and 4 corresponded to approximately 25%, 50%, and 75% patch activation, respectively. A score of 5 indicated a complete patch activation (100% colored), which was classified as estrous expression. A significant difference in AI conception rate was observed between females that did not express estrus, score 1, and females that expressed estrus, score 5, before the time of AI ($p < 0.001$). There were no differences in AI conception between estrus scores 1, 2, 3, or 4, and no differences between 2, 3, 4, or 5. No differences in AI conception rate were detected between heifers and cows or among trial years. However, direct comparisons among trial years should be interpreted with caution because different sire combinations and insemination timings were utilized across Trials.

Calf Parentage

The phenotypic coat color of calves was compared with the parentage determination results (Figure 2.7). In general, calves sired by Angus bulls exhibited solid black coloration (Black), whereas calves sired by Hereford bulls displayed white facial markings (BWF). Coat color phenotype was largely consistent with genetic parentage results; however, several exceptions were observed. Three calves confirmed to be sired by Hereford bulls exhibited solid black coat coloration, while two calves sired by Angus bulls expressed white facial markings.

Parentage analysis of AI-sired calves revealed significant differences between sire contributions from heterospermic inseminations across the four trials (Figure 2.8, Table 2.3). In Trial 1, 18 calves were confirmed to be AI-sired. Of these, the 13 sired by Bull A tended to be greater ($p \leq 0.1$) than the 5 sired by Bull B. In Trial 2, the 36 Bull A sired calves were greater ($p \leq 0.01$) than the 8 calves sired by Bull B. Similarly, in Trial 3, the 22 Bull A sired calves were greater ($p \leq 0.01$) than the 2 Bull C sired calves. Lastly, in Trial 4, the 47 Bull E sired calves were greater ($p \leq 0.01$) than the 3 calves sired by Bull D.

CASA parameters

Descriptive CASA semen parameters for semen straw formulations from each trial are presented in Table 2.4. Three replicate straws were evaluated for each semen straw formulation, except for Bull A and Bull B single-sire straws from Trial 1, for which only 2 replicate straws were available for analysis. Single-sire straws were not prepared for Trial 2, therefore, only the heterospermic straw was analyzed. Bull A and Bull B used in Trial 1 were also used to prepare the heterospermic straw in Trial 2, with a different collected ejaculate. No significant differences in post-thaw sperm concentration were detected among single-sire straws within Trials.

Progressive motility (PM) was affected by IVC time ($p < 0.001$, Figure 2.9). A significant effect of semen straw formulation was also detected ($p = 0.008$, Figure 2.9). Pairwise comparisons revealed Bull E had a greater PM compared to Bull D ($p = 0.01$), with both semen straw formulations originating from Trial 4. Additionally, Bull C had significantly less PM compared to Bull E ($p = 0.048$). However, this comparison should be interpreted with caution because these bulls were not compared within the same trial. No other pairwise comparisons among semen straw formulations were significant ($p > 0.05$).

The interaction between semen straw formulation and IVC time was not significant ($p = 0.531$, Figure 2.9), indicating that the effect of IVC time on progressive motility was similar across all semen straw formulations.

Semen straw formulations were ranked according to their average progressive motility at the 1 h IVC time point (Figure 2.10). Bull E exhibited the highest progressive motility at 23.37%, whereas Bull D exhibited 10.47% progressive motility. Notably, both bulls were utilized in Trial 4. The heterospermic straw from Trial 4 had a progressive motility of 20.74% at 1 h IVC, ranking between the two single-sire straws from Trial 4 and more closely resembling the value observed for Bull E.

Zinc Signatures

All 5 previously identified zinc signatures found in the bull (Kerns et al., 2018) were present in all straw replicates analyzed across all semen straw formulations (Zinc Signatures 1, 2, 3, 4, and 5). Zinc Signatures are presented in Figure 2.11. Zinc Signature 3+5 is also presented because it combines the observations from Zn Sig 3 and Zn Sig 5, where zinc localization (as indicated by FZ3) is seen in the acrosomal region of the sperm head, absent in the post-acrosomal region of the sperm head, and present only in the midpiece of the sperm tail. This signature was observed in all straw replicates analyzed across all semen straw formulations.

Zinc Signature average frequencies were calculated across all straw replicates and semen straw formulations for each time point. There was a tendency for there to be a change in Zn Sig 1 distribution between Non-Cap and 1 h IVC ($p = 0.078$; Figure 2.13). There was a significant change in distribution between 30 min IVC and 1 h IVC for Zn Sig 2 ($p = 0.031$) and Zn Sig 3+5 ($p = 0.035$). Zn Sig 4 distribution was significantly different from Non-Cap to 1 h IVC ($p =$

0.013) and from 30 min IVC to 1 h IVC ($p = 0.005$). There were no differences across time points for Zn Sigs 3 or Zn Sig 5.

Total capacitation at 1 h IVC was compared between single-sire straws for bulls used in respective Trial inseminations (Figure 2.14). There was a tendency for Bull A and Bull C in Trial 3 to be different in total capacitation at 1 h IVC. Bull D and Bull E in Trial 4 were significantly different in total capacitation at 1 h IVC ($p = 0.012$). When the total capacitation was compared between single-sire straws and the heterospermic straw for Trial 4, Bull E had a lower total capacitation compared to the Heterospermic straw ($p = 0.004$), but no difference between Bull D and the Heterospermic straw. The total capacitation at 1 h IVC for the Trial 4 Heterospermic straw was more similar to Bull D. There was no difference in total capacitation for Bull A and Bull B in Trial 1. No significant differences were found between capacitation difference or total capacitation at 1 h IVC for the Bull A semen straw formulations in Trials 1 and 3.

The capacitation difference, as determined by the difference between total capacitation at 30 min IVC and 1 h IVC, was compared between single-sire straws for bulls used in respective Trial inseminations (Figure 2.14). There was no significant difference in capacitation difference for single-sire straws in the respective Trials (Bull A and Bull B Trial 1, Bull A and Bull C Trial 3, and Bull D and Bull E Trial 4).

Discussion

In this study, a heterospermic approach was used to compare the fertility of two sires in an insemination. Significant differences in sire contribution to AI success in heterospermic inseminations suggest a difference in in-utero sperm viability between bulls. Additionally, Zinc signature analysis was done in an attempt to find a molecular marker that was associated with in vivo fertility results.

In Vitro Semen Assessments

Individual zinc signature distributions changing by time points are indicative of the semen samples progressing through the capacitation stages (Kerns et al., 2018). The low frequency of Zn Sig 1 and 2 is typical of bovine spermatozoa (Zoca et al., 2023). The low frequency of Zn Sig 4 at the non-capacitated time point suggests that most sperm were thawed in a non-fully capacitated state and retained the potential to undergo capacitation and gain the ability to fertilize an oocyte.

The capacitation difference between total capacitation at 30 min and 1 h IVC provides insight into the rate at which spermatozoa progress through capacitation. Total capacitation measured at 1 h IVC may be considered an indicator of a bull's capacitation ability, representing the proportion of spermatozoa capable of reaching a capacitated state under the conditions evaluated. In contrast, the difference in total capacitation at 30 min and 1 h IVC may reflect the rate of the capacitation process for an individual bull. A small capacitation difference, where total capacitation at 30 minutes is similar to that observed at 1 hour, suggests that most spermatozoa capable of capacitation reach this state relatively quickly. Conversely, a large capacitation difference indicates that additional spermatozoa continue to undergo capacitation between 30 min and 1 h IVC, suggesting a slower progression toward maximal capacitation. Based on this concept, bulls may be characterized as relatively fast- or slow-capacitating. Fast-capacitating bulls would be expected to have a high proportion of capacitated spermatozoa early in the *in vitro* incubation period. In contrast, slow-capacitating bulls would exhibit a more gradual increase in capacitation over time. This concept has also been discussed by Santos and Fair (2026), who suggested that a heterospermic insemination containing semen from an early-

and late-capacitating bull would ensure capacitated spermatozoa present for longer periods and would maximize the potential for fertilization.

These differences may have biological relevance *in vivo*. Spermatozoa must complete capacitation to become hyperactivated and be able to undergo the acrosome reaction, which is necessary for successful fertilization (Puga Molina et al., 2018). After capacitation occurs, spermatozoa within oviduct sperm reservoirs unbind from oviduct epithelium and move to the site of fertilization (Lefebvre and Suarez, 1996). A slower progression of capacitation could extend the period during which spermatozoa become capable of fertilization, potentially increasing the likelihood that viable sperm are present at ovulation. Bulls with a similar capacitation difference may exhibit comparable capacitation durations, although they may still differ in overall capacitation ability based on the proportion of spermatozoa reaching total capacitation by 1 h IVC.

Of note, the bulls used in Trial 4 had different capacitation abilities, with Bull D having a higher percentage of spermatozoa reaching total capacitation compared to Bull E. Inversely, Bull E had a higher progressive motility value at 1 h IVC compared to Bull D. Ultimately, Bull E sired 94% of the calves in Trial 4. This would suggest that progressive motility was a better indicator of potential fertility for those 2 sires. Oliveira et al. (2013) found progressive motility to be an important predictor of fertility in an *in vitro* study comparing many sperm characteristics to conception rates. Furthermore, Sullivan and Elliott (1968) and Saacke (2008) have both described in detail the importance of progressively motile sperm in semen doses to reach maximum fertility levels. However, in a study using CASA motility parameters and zinc signature analysis, there was no significant difference in high vs low fertility bulls in progressive motility or zinc signatures 3 and 4 (Zoca et al., 2023).

While there was no difference in progressive motility between Bull A and both Bull B and Bull C, Bull A consistently sired the majority of calves in the first three trials. In Trial 2, Bull B's fresh ejaculate motility did not meet the established 50% motility or 70% normal morphology threshold. However, due to time limitations associated with semen collection and trial initiation, his ejaculate was retained for use because an alternative bull meeting the threshold criteria could not be identified within the required timeframe. Bull B's ejaculate characteristics from Trial 2 could explain his low sire contribution to heterospermic insemination. When comparing capacitation parameters, only Bull A and Bull C tended to be different in total capacitation at 1 h IVC. Interestingly, no differences were detected when Bull A semen straws from Trial 1 were directly compared with those from Trial 3. Altogether, these results imply that another underlying factor may be giving Bull A superior fertility over Bull B and C for mixed insemination straws.

The lack of differences between single-sire straws for capacitation variables suggests the rate of capacitation for each bull was not the factor influencing the fertility outcome of the heterospermic inseminations. Further investigation into acrosome and DNA integrity is warranted in an attempt to explain some fertility differences among sires post-cryopreservation.

Semen Morphology

Percentages for each abnormality type in Table 2.1 were only available for Bulls A and B in Trial 1, and Bulls D and E in Trial 4. In Trial 1, Bull A's ejaculate contained 3% detached heads (DH), 1% proximal droplets (PD), 6% bent tails (T), 11% abnormal heads (AH), 9% bent midpieces (MP), and 4% distal midpiece reflexes (DMR). Bull B's ejaculate contained 1% detached heads, 2% distal droplets (DD), 24% abnormal heads, 4% bent midpieces, and 5% distal midpiece reflexes. Bull A's collection for Trial 2 resulted in 77% normal morphology, and

Bull B's was 47% normal morphology, and the exact defect percentages were not recorded at the time of evaluation. In Trial 4, Bull D had 5% detached heads, 3% bent tails, 9% abnormal heads, 7% bent midpieces, and 2% distal midpiece reflexes, while Bull E had 4% detached heads, 3% bent tails, and 5% abnormal heads.

Elevated levels of abnormal spermatozoa have been shown to reduce bull fertility (Barth et al., 1992; Al Makhzoomi et al., 2008; Saacke, 2008; Ghirardosi et al., 2018). Saacke (2008) described physical barriers within the female reproductive tract that prevent abnormal spermatozoa from reaching the site of fertilization and further suggested that impaired motility may reduce an abnormal spermatozoon's ability to traverse the female tract. Spermatozoa that typically fail to reach the fertilization site due to physical barriers in the female tract include those with classically misshapen heads and abnormal tails (Saacke, 2008). The bulls in the present trials exhibited several such abnormalities, including abnormal heads, bent tails, bent midpieces, and distal midpiece reflexes, which could affect *in vivo* transport within the uterus and oviduct. Because these defects generally prevent the affected spermatozoa from reaching and fertilizing an oocyte, they are considered compensable deficiencies that could be offset by increasing a bull's sperm dosage (Saacke, 2008). In the present study, however, all bulls were standardized to an equal concentration of 10 million motile sperm per mL, so any compensatory effect of dosage was held constant. Bulls with a greater percentage of normal morphology would therefore be expected to achieve higher fertility. Consistent with this, Bull A had a higher percentage of normal spermatozoa than Bull B or Bull C across Trials 1-3 and consistently sired the most calves in each trial, while Bull B's higher proportion of abnormal heads in Trial 1 aligns with the known negative effect of this defect on fertility (Barth et al., 1992; Saacke et al., 1998; Al Makhzoomi et al., 2008). Likewise, in Trial 4, Bull E had fewer recorded abnormalities

compared to Bull D and sired the most calves. These patterns suggest that sperm morphology was a close predictor of the heterospermic fertility outcomes observed in this study.

Effect of Estrous Expression on P/AI

The heterospermic straws used for Trials 1 and 3 had similar progressive motility and capacitation parameters to those of the straws for Trials 2 and 4, and yet Trials 2 and 4 had lower AI conception rates. Collectively, the lower-than-industry-average of 60% AI conception rates for all trials may be due to other factors such as time of insemination post CIDR removal or female estrus expression. The timing of insemination relative to estrus and ovulation is a factor influencing conception rates in fixed-timed AI protocols. For the 7-day Co-Synch + CIDR protocol, recommended insemination times are 54 hours after CIDR removal for heifers and 60 hours after CIDR removal for cows (Beef Reproduction Task Force, 2022). The importance of insemination timing is supported by the well-established AM/PM rule, first described by Trimberger (1948), which recommends insemination approximately 12 hours after the onset of estrus. Subsequent studies have demonstrated that conception rates are optimized when spermatozoa are present within the female tract before ovulation, which generally occurs 24 to 32 hours after estrus onset (Dorsey et al., 2011; Haadem et al., 2023). Under these conditions, a sufficient population of spermatozoa is present and capable of fertilizing oocytes at ovulation (Saacke et al., 2000).

Trial 1 had the lowest conception rate among all four trials, which is most likely explained by the planned design to use earlier than optimum insemination times. Furthermore, low estrous patch activation at the time of insemination may have contributed to the low AI success rate, as expression of estrus has consistently been associated with increased pregnancy success in fixed-timed AI programs (Dorsey et al., 2011; Whittier et al., 2013). The adjustment

of insemination timing in Trial 2 coincided with an increase in AI conception rate. Similarly, Trial 4 achieved the highest AI conception rate observed in the study when insemination occurred at the recommended insemination time for cows. This improvement may be attributed to a better compromise between insemination time, sperm viability, and ovulation time (Saacke et al., 2000). In contrast, Trial 3 resulted in an AI conception rate of only 21%, despite utilizing insemination timing like that of Trial 2. Low estrus expression at the time of insemination may explain the decrease in AI conception rate. Another possible explanatory factor for the reduced AI conception rates observed in the present trials is that the earlier insemination times may have resulted in a mismatch between sperm capacitation and ovulation. Specifically, heterospermic semen formulations may have reached their maximum capacitation threshold too early, resulting in a reduced population of functionally viable sperm available at the time of ovulation.

Calf Coat Coloring

Each heterospermic insemination straw consisted of an Angus and a Hereford sire. Coat coloring phenotypes of the calves generally agreed with parentage assignments determined through DNA testing and followed expected inheritance patterns of Angus and Hereford crosses. Angus-sired calves from Angus dams predominantly exhibited solid black coat coloration, whereas Hereford-sired calves typically expressed white facial markings. In Angus × Hereford crosses, black coat color is dominant to the Hereford red coat color, while the white spotting pattern is commonly expressed on the face of heterozygous offspring (Rolf, 2017; Pitchford et al., 2021). Although most calves displayed expected coat color phenotypes, several exceptions were observed. It should be noted that the dams had various degrees of crossbreeding within their pedigrees. Therefore, these discrepancies in calf coat coloring likely reflect the complexity of coat color inheritance in crossbred cattle (Rolf, 2017).

Limitations and Future Research

A notable limitation to heterospermic insemination as an evaluation tool is that comparisons can only be made between bulls included within the same insemination, as results cannot be extrapolated to bulls evaluated under different conditions. This is largely because of differences in compensable and uncompensable traits within a bull's semen. Bulls may vary in the dosage of spermatozoa required per insemination straw to achieve maximum fertility, and this depends on the proportion of compensable traits in semen (Sullivan and Elliott, 1968; Saacke, 2008). Bulls carrying a high proportion of compensable defects would require an increased sperm concentration in a conventional straw to achieve an optimal fertilization rate. Therefore, if that concentration is reduced in the context of heterospermic insemination, there is a risk of artificially disadvantaging those bulls by preventing them from reaching their fertility ceiling. If a bull with a low compensable population of sperm were combined with a bull with a high compensable population, the former may be expected to sire a disproportionately higher number of calves, even if, under conventional insemination, both bulls achieve satisfactory conception rates. This disparity could be compounded further when uncompensable traits are considered, and no sperm concentration adjustment can compensate for their inherent reduction in fertility potential. This makes the heterospermic model complex when comparing bulls of pre-determined fertility levels based on conventional inseminations.

In the present study, historical AI fertility for the bulls used in each trial was unavailable for reference comparison, and no single-sire control inseminations were conducted concurrently due to the low numbers of females available. This restricts the ability to predict homospermic fertility for these bulls based on the results of the heterospermic inseminations conducted in this study.

Another limitation was the absence of a measurement for the time of ovulation relative to the time of insemination. Although GnRH administration at insemination was expected to induce a luteinizing hormone (LH) surge and synchronize ovulation, the timing of ovulation was not monitored by ultrasonography. Therefore, it was not possible to determine if differences in sire success were associated with variation in the time between insemination and ovulation. Studies incorporating serial ovarian ultrasonography could provide a more precise assessment of this relationship.

Future research should evaluate the effect of the interval between insemination and ovulation on fertility outcomes following heterospermic and homospermic inseminations. Coupled with *in vitro* zinc signature analysis, these investigations could help establish whether differences in capacitation rate contribute to variation in sperm viability during FTAI.

Conclusion

Heterospermic insemination provided a method for directly comparing the relative fertility of two sires within the same insemination event. When accounting for a bull's compensable traits within an insemination straw, this method may expose uncompensable deficiencies that would otherwise lower a bull's assessed fertility. While progressive motility and zinc signatures helped explain some of the variation in fertility observed between sires in the present study, neither emerged as a primary factor. Nonetheless, the results do validate the current understanding of zinc signatures present in bull spermatozoa. Moving forward, heterospermic insemination could be a valuable tool in reproductive research, particularly when comparing sire fertility alongside other semen variables, ultimately advancing our understanding of how those variables influence fertility outcomes.

Table 2.1. Summary of semen characteristics from individual bull ejaculates from KABSU

Bull	Age at Collection	Pre-freeze Motility %	% Normal Morphology	Abnormalities present ¹	Concentration of motile sperm/ml in the ejaculate	Post-thaw Motility %	Volume of ejaculate in straw (μl)
Trial 1							
Bull A	Yearling	65	66	DH, T, AH, PD, MP, DMR	551.9 mil	50	18
Bull B	Yearling	65	64	DH, DD, AH, MP, DMR	341.3 mil	35	29
Trial 2							
Bull A	2-year-old	60	77	- ²	490.2 mil	35	20
Bull B	2-year-old	35*	47	DH, MP, DMR	226.1 mil	30	44
Trial 3							
Bull A	3-year-old	50	71	DMR	962.5 mil	40	10
Bull C	2-year-old	50	62	DMR	434.5 mil	35	23
Trial 4							
Bull D	4-year-old	50	74	DH, T, AH, MP, DMR	228.5 mil	35	44
Bull E	3-year-old	50	88	DH, T, AH	632.5 mil	45	16

¹ Sperm Abnormalities: DH = detached heads, DD = distal droplet, T = bent tails, AH = abnormal heads, MP = bent midpieces, DMR = distal midpiece reflex

² Abnormalities were not available for this ejaculate

*Value did not meet KABSU minimum threshold of percent motile sperm, and the ejaculate dose was compensated for within the heterospermic insemination straw

Table 2.2. Count of estrous patch score for females with an estrous patch at the time of insemination for each trial

Trial	Estrous Patch Score					Total Females Inseminated
	1	2	3	4	5	
1	82	3	2	1	3	105
2	62	8	2	1	31	109
3	61	1	2	0	22	114
4	36	2	0	0	54	117

Table 2.3. Z-test analysis for calf parentage for each Trial.

Trial	Bull ID	P_O	z-score	p-value	95% L.C.I.¹	95% U.C.I.²
1	Bull B	0.28	-1.88	0.06	9.6	53.5
2	Bull B	0.18	-4.22	≤ 0.01	8.2	32.7
3	Bull C	0.08	-4.08	≤ 0.01	1.0	27.0
4	Bull E	0.94	6.20	≤ 0.01	83.5	98.7

A Z-test analysis was used to compare the proportion of expected ($P_E=0.5$) and the observed numbers of AI-sired calves (P_O) for one bull from each trial year from heterospermic inseminations. The critical value for a two-tailed Z-test with $\alpha = 0.05$ is ± 1.96 .

¹ Lower confidence interval

² Upper confidence interval

Table 2.4.CASA data for semen characteristics of semen straw formulations

Semen Straw Formulation	CONC post-thaw [10⁶/ml]*	PM post-thaw (%)**^a	CONC of PM post-thaw [10⁶/ml][^]	PM 1-hour IVC (%)**^b
Bull A – Trial 1	41.26 ± 3.39	40.48 ± 6.26	16.70	14.09 ± 6.93
Bull B - Trial 1	34.36 ± 1.17	31.29 ± 0.30	10.75	19.06 ± 4.03
Heterospermic Trial 1	37.43 ± 2.06	29.14 ± 7.72	10.91	20.22 ± 3.60
Heterospermic Trial 2	38.67 ± 0.86	30.87 ± 6.48	11.94	14.74 ± 2.13
Bull A - Trial 3	36.53 ± 2.56	25.62 ± 4.19	9.36	13.44 ± 1.23
Bull C	44.08 ± 0.81	20.37 ± 4.14	8.98	11.35 ± 2.79
Heterospermic Trial 3	40.56 ± 0.95	32.38 ± 1.50	13.13	18.50 ± 5.48
Bull D	43.29 ± 0.93	16.49 ± 0.84	7.14	10.47 ± 1.81
Bull E	45.21 ± 2.71	35.07 ± 1.96	15.86	23.37 ± 6.17
Heterospermic Trial 4	40.93 ± 2.07	26.28 ± 1.45	10.76	20.74 ± 1.56

CONC, Concentration of sperm; PM, Progressive motility; IVC, *in vitro* capacitation

*Data are presented as mean ± standard error (SE) (triplicates; except Bull A Trial 1 and Bull B Trial 1, in which 2 replicates were analyzed)

[^]An approximate concentration of progressively motile sperm was calculated by multiplying PM (%) post-thaw by the concentration of spermatozoa post-thaw.

^{a, b} Columns with different superscripts differ (p < 0.05)

Table 2.5. Summarization of heterospermic insemination trials

Trial	Bull IDs	Age & Breed of Bulls	Relative semen proportion in heterospermic straw[^]	Time of AI post-CIDR removal*	Conception Rate*	AI Calf Parentage Results⁺
1	Bull A	Yearling Angus	38%	Heifers @ 36 h	Heifers: 12% Cows: 19%	13/18
	Bull B	Yearling Hereford	62%	Cows @ 48 h		5/18
2	Bull A	2-year-old Angus	32%	Heifers @ 48 h	Heifers: 53% Cows: 36%	36/44
	Bull B	2-year-old Hereford	68%	Cows @ 54 h		8/44
3	Bull A	3-year-old Angus	31%	Heifers @ 48 h	Heifers: 20% Cows: 21%	22/24
	Bull C	2-year-old Hereford	69%	Cows @ 54 h		2/24
4	Bull D	4-year-old Angus	73%	Cows @ 60 h	Cows: 43%	3/50
	Bull E	3-year-old Hereford	27%			47/50

[^]The final relative proportion of each sire's ejaculate used in each heterospermic insemination straw after initial KABSU motility and morphology evaluations

*Time of AI post-CIDR removal and Conception Rate are representative for the respective Trial, not for individual sires used within each trial

⁺ Values are represented as the number of calves sired by each respective bull over the total AI sired calves for that respective Trial.

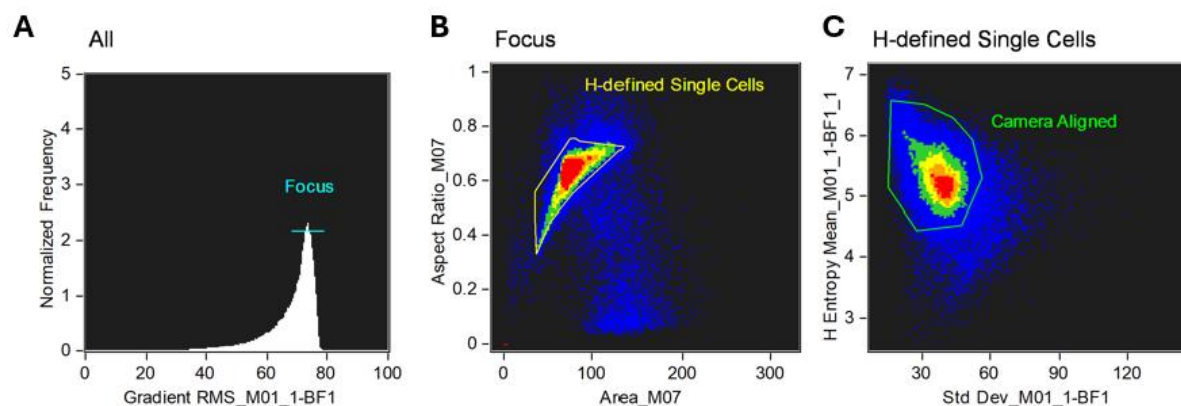


Figure 2.1. Initial gating strategy used for ImageStream analysis of bovine spermatozoa. (A) Acquired events were first gated based on image focus quality to retain sharply focused spermatozoa. **(B)** Focused events were subsequently gated according to object area and aspect ratio to identify and retain single sperm cells while excluding debris and cell aggregates. **(C)** The resulting population was further gated using Hoechst 33342 fluorescence intensity to select for camera-aligned cells with consistent nuclear staining. The final gated population was used for downstream ImageStream analysis.

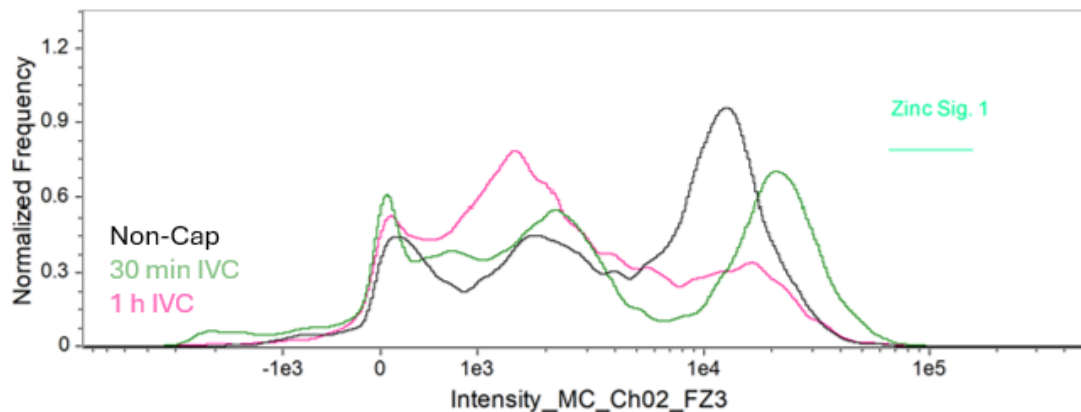


Figure 2.2. Representative time-course of total FZ3 intensity for spermatozoa at Non-Cap (black), 30 min IVC (green), and 1 h IVC (pink). FZ3 intensity is gated for Zn Sig 1. Spermatozoa exhibiting high FZ3 intensity across the sperm head and whole tail were considered Zn Sig 1. Corresponding histogram color code for time points in the figure.

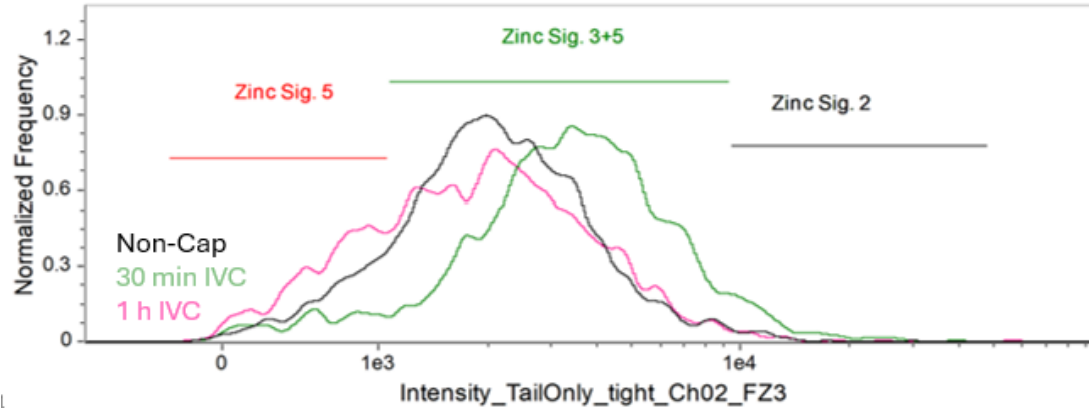


Figure 2.3. Representative acrosome FZ3 positive Zinc Signature time course (Non-Cap, 30 min IVC, and 1 h IVC). ZF3 intensities were gated for Zn Sig 5, Zn Sig 3+5, and Zn Sig 2. Spermatozoa with higher intensity of FZ3 in the sperm head and midpiece of the tail were considered Zn Sig 2. Spermatozoa with FZ3 labeling in the acrosomal region of the sperm head and midpiece of the tail were considered Zn Sig 3+5. Spermatozoa with FZ3 labeling in the acrosomal region of the sperm head were considered Zn Sig 5. Corresponding histogram color code for time points in the figure.

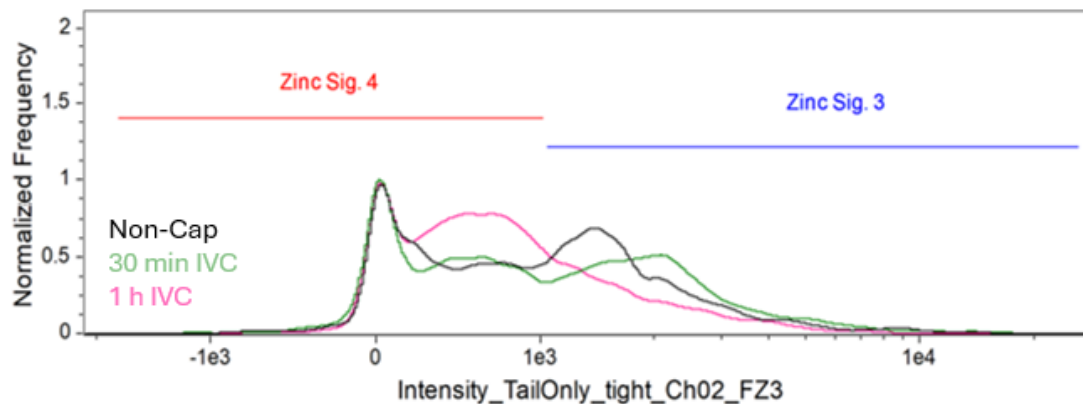


Figure 2.4. Representative acrosome FZ3 negative Zinc Signature time course (Non-Cap, 30 min IVC, and 1 h IVC). FZ3 intensities were gated for Zn Sig 3 and Zn Sig 4. Spermatozoa with FZ3 labeling in the midpiece of the tail only were considered Zn Sig 3. Zn Sig 4 was gated based on spermatozoa negative (<0 FZ3 intensity) for FZ3 in both the acrosomal region and the tail. Corresponding histogram color code for time points in the figure.

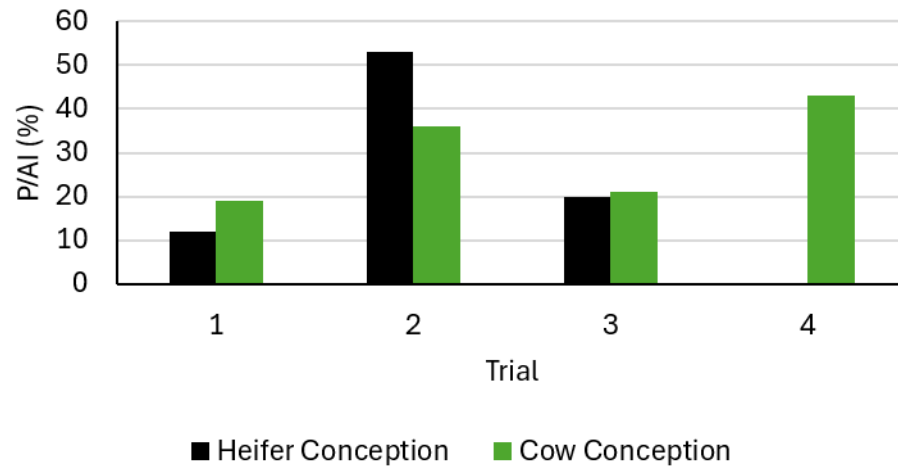


Figure 2.5. Pregnancy-to-AI conception by Trial for heifers and cows. The number of heifers inseminated in Trials 1, 2, 3, and 4 was 25, 34, 30, and 0, respectively. The number of cows inseminated in Trials 1, 2, 3, and 4 was 80, 75, 84, and 117, respectively.

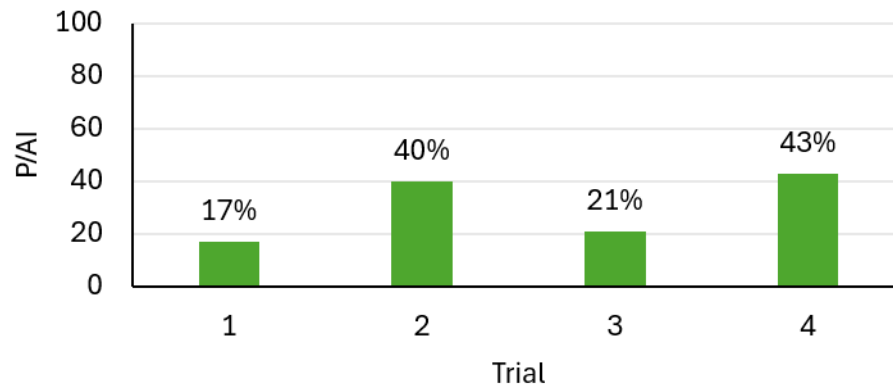


Figure 2.6. Overall Pregnancy-to-AI by Trial. The total number of females inseminated for Trials 1, 2, 3, and 4 was 105, 109, 114, and 117, respectively.

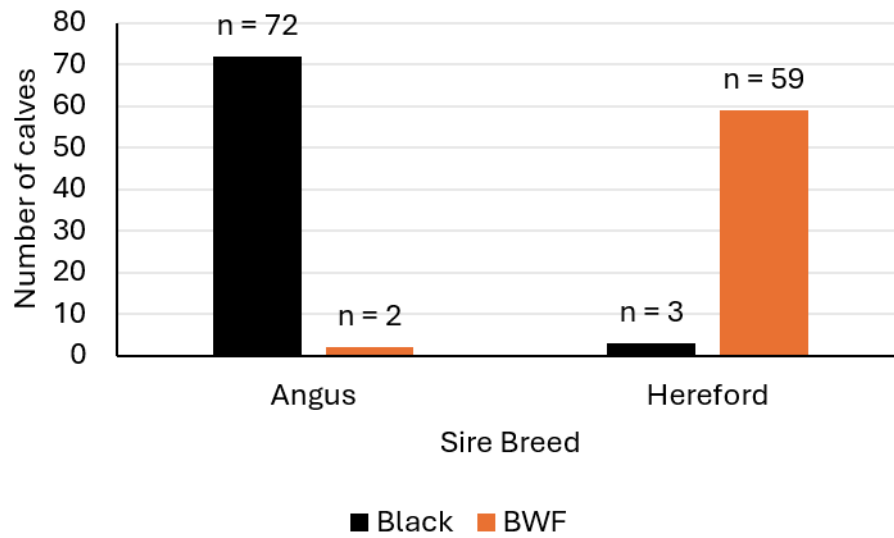


Figure 2.7. The number of AI-sired calves' phenotypic coat coloring by sire breed for all trials. Coat coloring Black indicated a calf with an entirely black coat color and no white facial markings. Coat color BWF represented a calf with any white facial markings present.

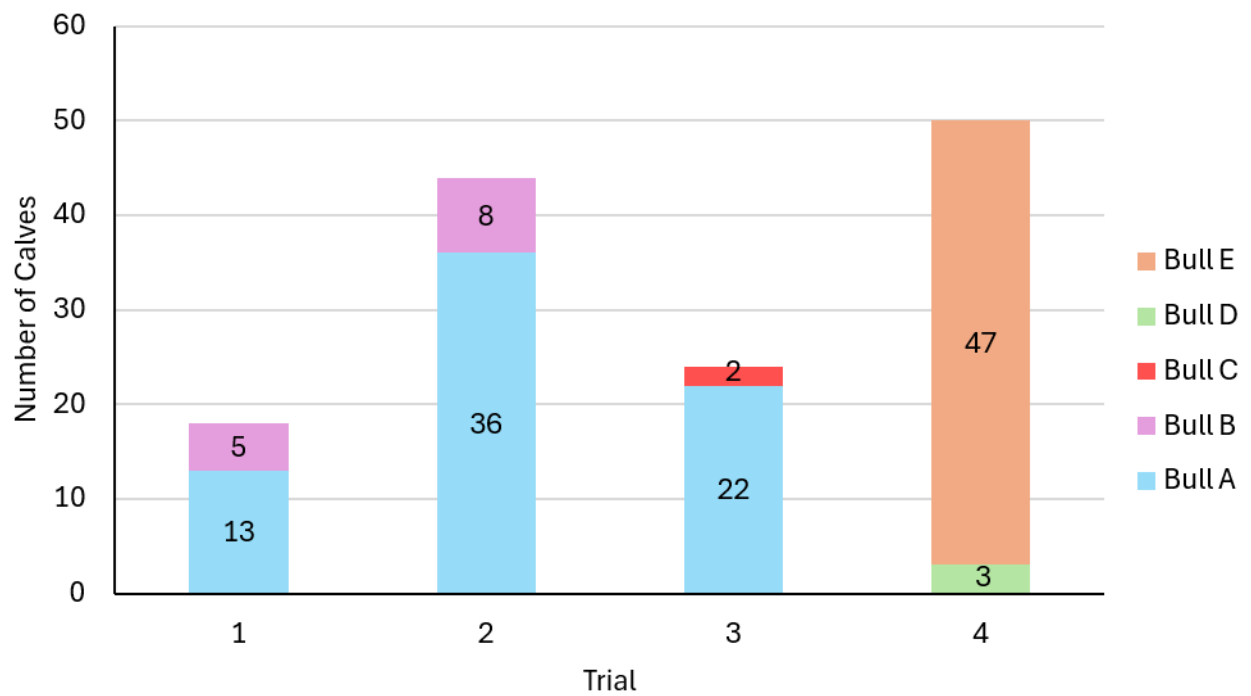


Figure 2.8. Parentage of AI calves from heterospermic inseminations by Trial.

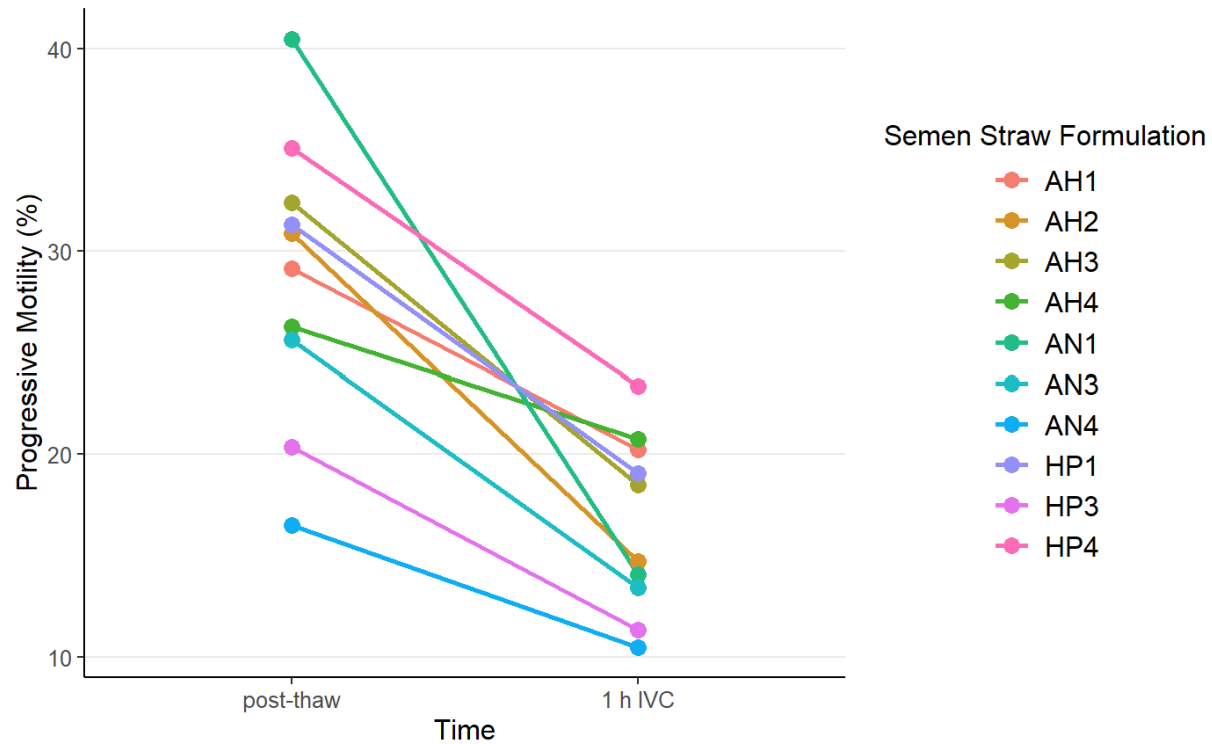


Figure 2.9. The comparison of progressive motility between time points for all semen straw formulations. Time points (X-axis): post-thaw and 1-hour *in vitro* capacitation. Progressive motility points are plotted as mean values from CASA analysis. Semen straw formulations are labeled as followed HP 4: Bull E; AN 3: Bull A Trial 3; AH 2: Heterospermic Trial 2; AH 3: Heterospermic Trial 3; HP 1: Bull B Trial 1; HP 3: Bull C; AH 4: Heterospermic Trial 4; AH 1: Heterospermic Trial 1; AN 1: Bull A Trial 1; AN 4: Bull D.

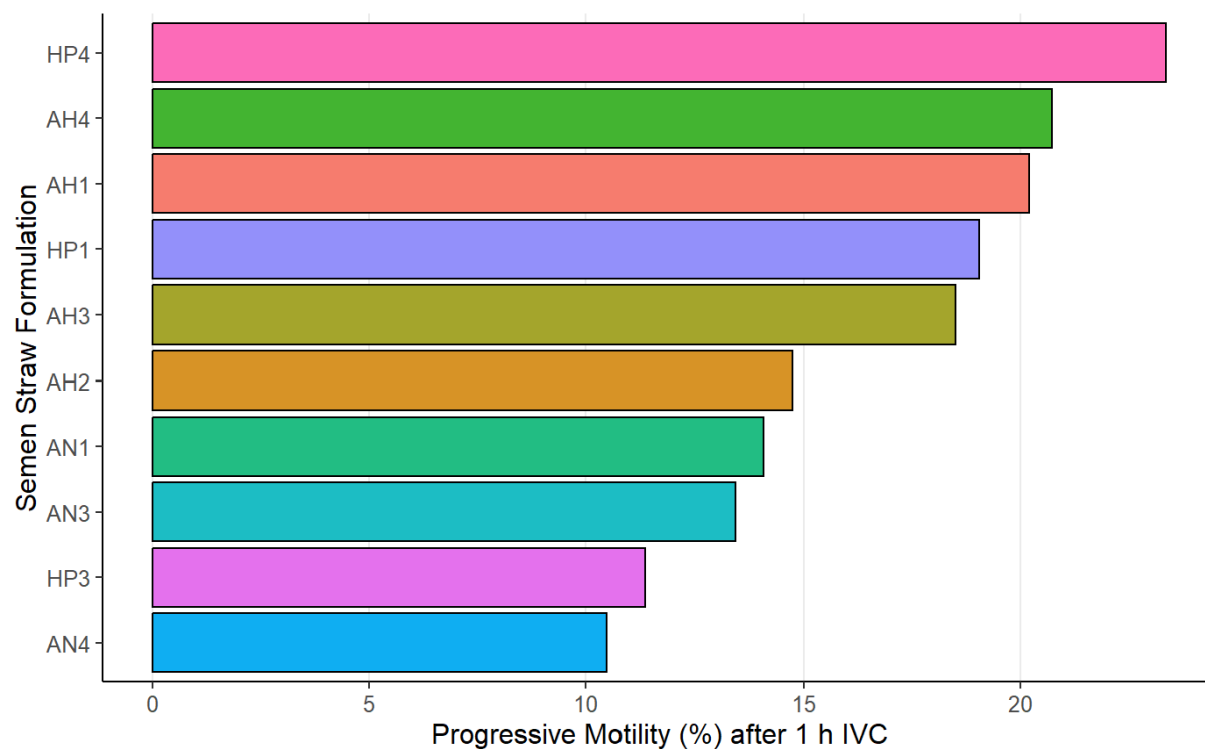


Figure 2.10. Semen straw formulations ranked, highest to lowest, by their 1 h IVC progressive motility. Progressive motility values are presented as means. Semen straw formulations are labeled as followed HP 4: Bull E; AN 3: Bull A Trial 3; AH 2: Heterospermic Trial 2; AH 3: Heterospermic Trial 3; HP 1: Bull B Trial 1; HP 3: Bull C; AH 4: Heterospermic Trial 4; AH 1: Heterospermic Trial 1; AN 1: Bull A Trial 1; AN 4: Bull D.

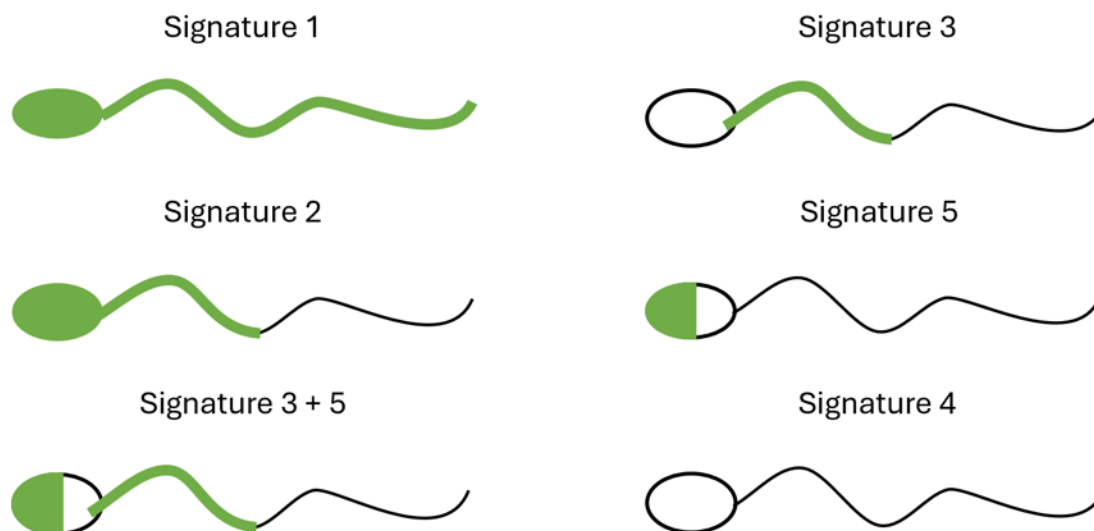


Figure 2.11. Pictogram showing Zinc signatures (Zn Sig; by FluoZinTM-3 AM, FZ3) observed in bulls. The Zinc signatures are arranged in order of capacitation progression, top to bottom and left to right. Zn Sig 1 is non-capacitated spermatozoa with uniform FZ3 labeling in the sperm head and tail. Zn Sig 2 is spermatozoa in early capacitation with uniform FZ3 labeling in the head and midpiece of the tail. Zn Sig 3+5 is a middle- to late-stage capacitated spermatozoa with FZ3 labeling in the acrosomal region of the head and the midpiece of the tail. Zn Sig 3 represents late-stage capacitation in which FZ3 labeling is only in the midpiece of the tail. Zn Sig 5 is also a middle-to-late-stage capacitation with FZ3 labeling only in the acrosomal region of the head. Zn Sig 4 are completely capacitated or dead spermatozoa with no FZ3 labeling (Adapted from Kerns et al., 2018).

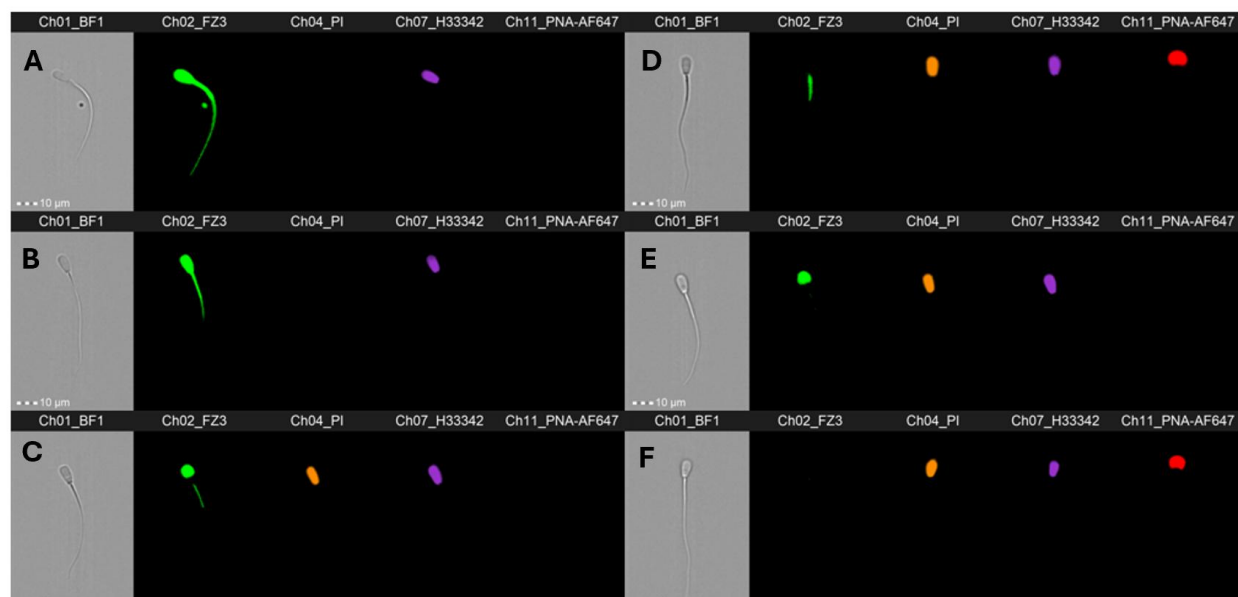


Figure 2.12. Compilation of channel images across the 6 Zinc Signatures (Ch02_FZ3) present in bulls. (A) Zn Sig 1, (B) Zn Sig 2, (C) Zn Sig 3+5, (D) Zn Sig 3, (E) Zn Sig 5, (F) Zn Sig 4. (Ch01_BF1) Bright field image of spermatozoa, (Ch04_PI) Propidium iodide stain for sperm viability, (Ch07_H33342) Hoechst 33342 stain for nuclear DNA visualization, (Ch11_PNA-AF647) Lectin PNA stain for acrosome integrity.

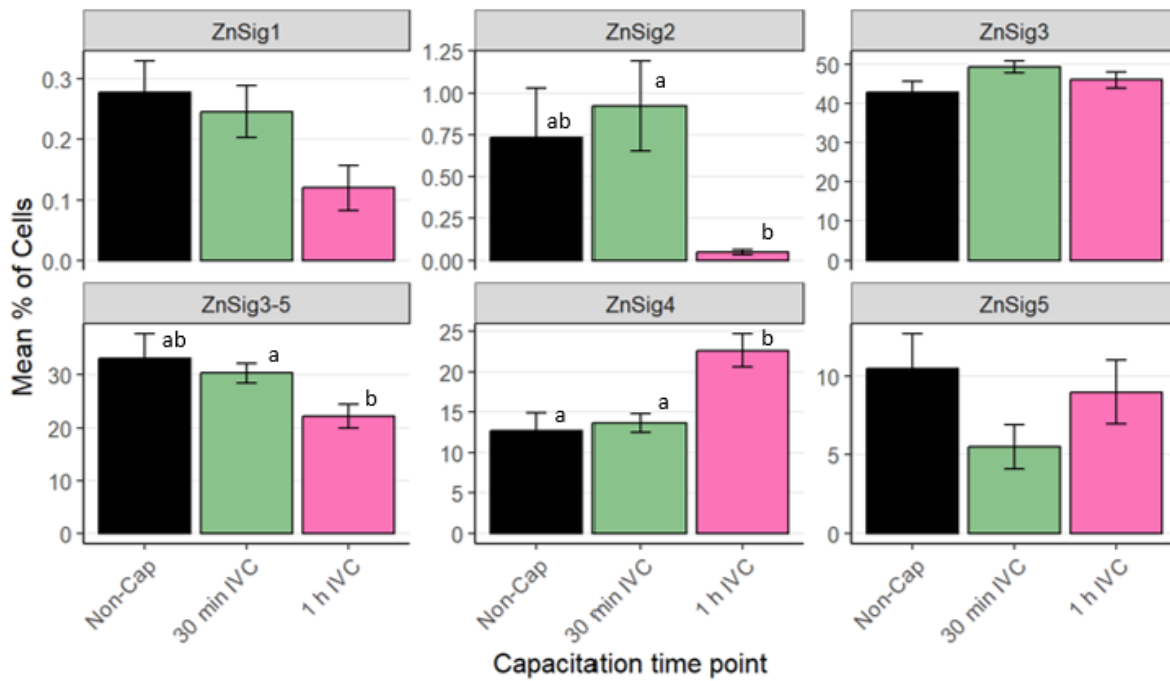


Figure 2.13. The distribution of zinc signatures across capacitation time points (Non-Cap, 30 min IVC, 1 h IVC). Values are presented as mean $\pm$ SE. Within Zn Sigs, values with different superscripts indicate significant differences between time points.

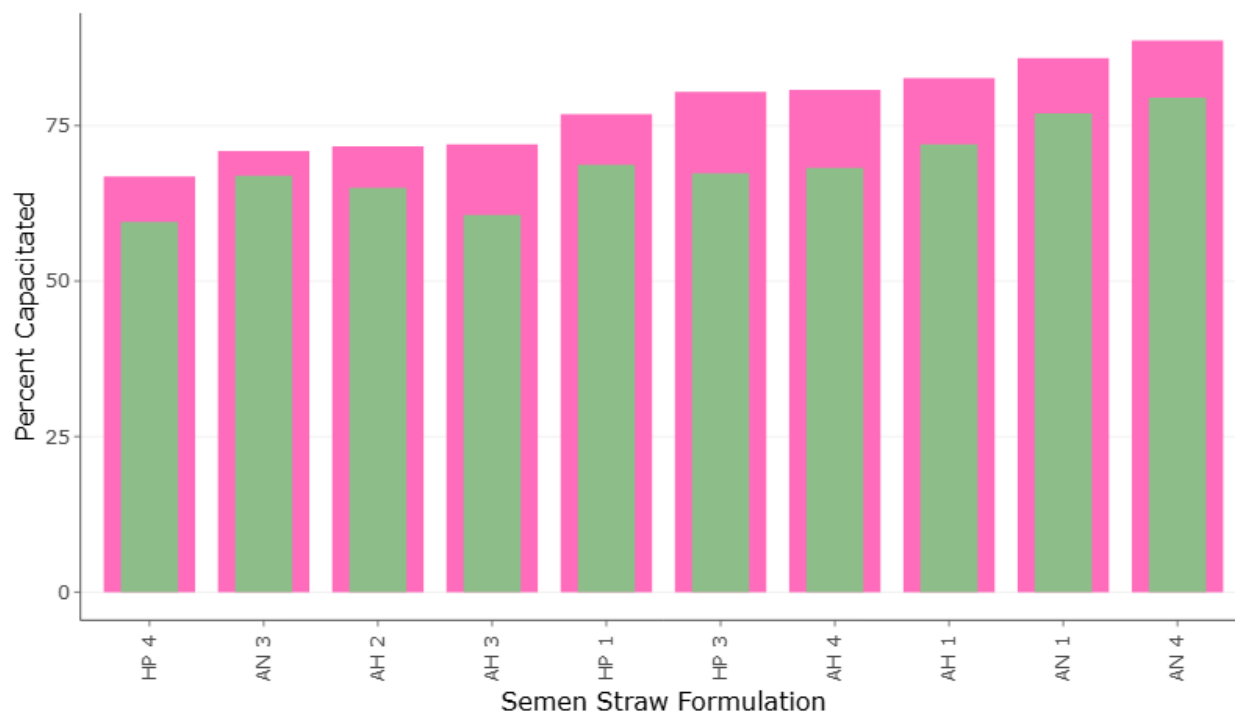


Figure 2.14. The total capacitation percentage of spermatozoa after 1 h IVC (pink) overlaid with the total capacitation percentage of spermatozoa at the 30 min IVC (green) time point per semen straw formulation. Total capacitation for this bar graph is calculated as a combination of Zn Sig 3, Zn Sig 5, and Zn Sig 4 and averaged across straw replicates for each semen straw formulation. Semen straw formulations are labeled as followed HP 4: Bull E; AN 3: Bull A Trial 3; AH 2: Heterospermic Trial 2; AH 3: Heterospermic Trial 3; HP 1: Bull B Trial 1; HP 3: Bull C; AH 4: Heterospermic Trial 4; AH 1: Heterospermic Trial 1; AN 1: Bull A Trial 1; AN 4: Bull D.

Chapter 3 - *In vitro* evaluation of heterospermic fertility following field-based sire comparisons

Abstract

Heterospermic fertilization assays provide a unique approach for evaluating fertility differences by placing spermatozoa from multiple bulls within the same fertilization environment. Therefore, the objective of this study was to evaluate fertilization ability and early embryonic development between two bulls in an *in vitro* heterospermic fertilization assay. The two bulls were chosen because they demonstrated a skewed distribution of calves when used in heterospermic field trial inseminations. An *in vitro* fertilization (IVF) protocol from ivf Bioscience was performed, with minor modifications. Oocytes were obtained via follicular aspiration from abattoir-derived ovaries and matured under incubation at 38.5°C and 5.5% CO₂ for 21–24 hours before introduction into fertilization medium. Three semen straw formulations were utilized across 12 wells: 3 single-sire Bull D, 3 single-sire Bull E, and 6 heterospermic (D+E). All presumptive zygotes were subsequently cultured for 5 days and evaluated for cell stage development. Bull E and D+E semen formulations resulted in a significant proportion of zygotes developing past the 2-cell stage ($p < 0.05$) compared to Bull D semen. Parentage analysis will be performed to assess whether the two bulls differ in relative contribution to fertilization outcomes in heterospermic IVF conditions. These findings suggest that sire-specific differences may influence early embryonic development in *in vitro* fertilization assays.

Introduction

Evaluation of bull fertility in artificial insemination (AI) programs is typically based on single-service conception rates obtained from many breedings. However, direct comparisons of fertility among sires can be difficult because sperm concentrations within AI doses are often adjusted to compensate for differences in semen quality. As a result, bulls may attain similar field conception rates despite differences in the functional capacity of spermatozoa post-insemination in the cow. On average, a single-service conception rate of approximately 60% is the industry goal. The fertility of individual AI sires can be challenged when used in fixed-time AI protocols, where the interval between time of insemination and ovulation is increased in females. Traditional semen evaluation methods rely heavily on motility and morphology assessments prior to and after freezing; however, these subjective measures are limited in their predictive accuracy, as no single trait can fully capture the multitude of factors that influence fertility. Sperm morphology and motility (Oliveira et al., 2013; Saacke, 2008), capacitation status (Kerns et al., 2018), sperm proteomics (Sutovsky, 2011), DNA integrity (Dogan et al., 2015), and genetics (Taylor et al., 2018) all contribute to a sire's fertility potential.

Beatty et al. (1969) introduced a heterospermic insemination model as a more efficient tool for comparing sire fertility. Heterospermic insemination involves the deposition of semen from two or more sires into the female reproductive tract during a single insemination event. Beatty et al. (1969) demonstrated that heterospermic fertility was moderately to highly correlated with homospermic field fertility rates, suggesting that heterospermic insemination could be used to accentuate fertility differences between bulls and thereby reduce the number of inseminations required to detect relative differences. These findings have been supported by subsequent studies (Nelson et al., 1975; Christenson, 2024; Santos and Fair, 2026).

The heterospermic model has been applied within *in vitro* fertilization (IVF) settings to further investigate the mechanisms underlying sire fertility. Some early applications focused on the competitive sperm binding ability to the zona pellucida, which is thought to play a role in the variability of sire fertility (Braundmeier et al., 2002; Puglisi et al., 2010). More recently, Puglisi et al (2012) utilized a heterospermic IVF approach and genotyped 8- to 16-cell embryos to determine parentage, finding a low to moderate correlation with field fertility estimates. These authors noted that *in vivo* fertility measures, such as non-return rates and AI conception rates, fail to account for fertilization failures or early embryonic loss, and thus, IVF success may more directly reflect a bull's fertilization potential. Supporting this, *in vitro* embryo developmental parameters have been correlated with AI field fertility and show promise as a selection tool for sires used in timed AI protocols (Sudano et al., 2011; Sun et al., 2021). Therefore, the objective of the present experiment was to evaluate fertilization ability and early embryo development between two bulls in an *in vitro* heterospermic fertilization assay. The two bulls were chosen because they demonstrated a skewed distribution of calves sired when used in heterospermic inseminations for a field trial.

Material & Methods

Media

All media were sourced from ivf Bioscience, unless otherwise stated. All media were used per the manufacturer's recommendations for their Bovine IVF protocol with minor modifications. A 0.9% (w/v) saline solution was prepared using NaCl (Fisher Scientific) and sterilized. Penicillin-Streptomycin (100X; Genesee Scientific, 10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin) was used at 1X for supplementation to IVF media.

Oocyte recovery and maturation

Ovaries were collected from an abattoir and transported to the laboratory within 4 hours in sterilized 0.9% saline supplemented with 0.1% (v/v) Penicillin-Streptomycin, maintained at approximately 30-35°C. Follicles measuring 2–8 mm in diameter were aspirated using an 18-gauge needle attached to a 6 mL syringe. Oocytes were collected in 60 mm dishes containing approximately 50:50 v/v of WASH medium and follicular fluid. Groups of 40-50 cumulus oocyte complexes (COCs) were transferred to wells containing 500 µl of BO-IVM medium. COCs were then incubated at 38.5°C and 5.5% CO₂ in a humidified atmosphere for 21-24 hours.

Semen collection

Semen from Bull D and Bull E was collected at the Kansas Artificial Breeding Service Unit (KABSU) following their established protocols. Each ejaculate was evaluated for a minimum motility threshold of 50% and extended using a one-step non-animal extender (OptiXcell®, IMV Technologies) to 20×10^6 sperm/mL. Single sire 0.5 cc straws were prepared and frozen for each bull. Extended ejaculates from both bulls were then combined, in equal motility-adjusted proportions, to produce heterospermic 0.5 cc straws, with each bull contributing 10×10^6 motile sperm per straw. All straws were frozen using KABSU's established protocols.

Semen preparation

In vitro fertilization was performed according to the Bovine IVF protocol from ivf Bioscience with minor modifications. Frozen semen straws were thawed in a 37°C water bath for a minimum of 30 seconds. Straws were processed separately by transferring semen into 15 mL centrifuge tubes containing 2 mL of BO-SemenPrep medium and centrifuged at $328 \times g$ for 5 minutes. The supernatant was pipetted off, an additional 2 mL of BO-SemenPrep was added, and

the sample was centrifuged again under the same conditions. The supernatant was then pipetted to the 1 mL mark, and a concentration calculation was performed to determine the final concentration of motile sperm per mL of sperm solution.

Oocyte fertilization

Following the maturation period, COCs were removed from the incubator and evaluated for cumulus expansion. All COCs were then washed through three 100 μ L droplets of BO-IVF medium and categorized into “Good”, “Fair”, or “Poor”. All categories of COCs were approximately evenly distributed within groups of 30-45 COCs into wells containing 500 μ L of BO-IVF medium supplemented with 5 μ L of Penicillin-Streptomycin. COCs were returned to the incubator until semen addition. Three semen straw formulations were used: Bull D single-sire, Bull E single-sire, and heterospermic (D+E). A concentration of 2×10^6 sperm/mL was added to mature oocytes. Fertilization was carried out at 38.5°C and 5.5% CO₂ in a humidified atmosphere for 18-20 hours.

In vitro culture

All presumptive zygotes were removed from IVF medium in 150 μ L volumes and transferred to 1.5 mL microcentrifuge tubes: each well was processed separately. Tubes were vortexed for 2 minutes to mechanically remove cumulus cells. The zygotes were subsequently washed through two 50 μ L BO-WASH medium droplets and one 50 μ L BO-IVC medium droplet, then transferred to wells containing 500 μ L BO-IVC medium overlaid with 400 μ L BO-OIL. No mixing of presumptive zygotes and developing embryos between semen straw formulations occurred. Embryos were cultured at 38.5°C and 5.5% CO₂ for 5 days, after which cleavage rates were assessed.

DNA extraction

Embryos were transferred to 0.2 mL PCR tubes containing 10 µL of QuickExtract™ DNA Extraction solution (LGC, Biosearch Technologies™) and underwent a thermocycle at 65°C for 6 minutes, followed by 98°C for 2 minutes. Embryo samples were then stored at -80°C until DNA extraction and amplification were performed.

Statistical Analysis

Embryo data were analyzed using RStudio, Version 2026.04.0 Build 526. Embryo cell stage was analyzed using a mixed effects model to evaluate the effect of semen straw formulation on cell stage development. The model included semen straw formulation as a fixed effect and IVF well as a random effect.

Results

An average of 33 oocytes per group was placed in IVF wells, and an average of 16 embryos was collected from each well after 5 days in culture. Embryo development appeared to stop at an 8-cell stage. A total of 183 embryos were saved for DNA extraction. Pairwise comparisons indicated semen from Bull E and heterospermic (D+E) resulted in a significantly greater ($p < 0.05$) number of zygotes developing past the 2-cell stage compared to semen from Bull D (Figure 3.1). There was no difference in cell stage development between Bull E and heterospermic semen straws.

Discussion

This study aimed to evaluate *in vitro* fertilization outcomes of two bulls previously used in heterospermic field trial inseminations. The results demonstrated that embryos produced from Bull E sperm had approximately greater odds of reaching a more advanced cell stage compared to embryos fertilized with Bull D sperm. These findings suggest that sire-specific differences

may influence early embryonic development. O'Callaghan et al. (2021) investigated the early embryonic development from dairy bulls exhibiting high and low AI fertility. The authors found that high fertility bulls had a greater proportion of embryos reaching a more advanced cell-stage of development compared to low fertility bulls. Interestingly, in previous heterospermic field trials, Bull E sired the majority of calves compared with Bull D (Chapter 2). Collectively, these results suggest that Bull D may possess an underlying sperm trait that negatively affects fertility outcomes both *in vitro* and *in vivo*. The similarity in embryo development progression between heterospermic (D+E) and Bull E semen suggests that spermatozoa from Bull E may have contributed positively to the fertilization rates and further embryo development within the heterospermic semen formulation. However, future parentage analysis will be conducted to confirm fertilization outcomes from the heterospermic semen formulation.

Table 3.1. *In vitro* fertilization wells and semen treatment assignment.

Well ID	Semen Straw Formulation	# Oocytes/Well¹	# Embryos Yielded²	Fertilization Rate (%)
A1	Bull D	34	14	41
A2	Bull E	34	14	41
A3	D+E	31	18	58
A4	D+E	37	19	51
B1	D+E	31	15	48
B2	D+E	34	15	44
B3	Bull E	42	19	45
B4	Bull D	32	12	37
C1	D+E	37	16	43
C2	Bull E	38	14	36
C3	Bull D	27	12	44
C4	D+E	29	15	51

¹ The number of oocytes placed in each IVF well before the addition of semen.

² The number of embryos produced, from zygote to 8-cell, placed in QuickExtract media for DNA extraction after 5 days of IVC.

Table 3.2. Quality of embryos stored for DNA extraction by semen straw formulation.

Semen Straw Formulation	Poor¹	Fair¹	Good¹	Total
Bull D	10	23	5	38
Bull E	7	31	9	47
D+E	27	53	18	98
Total	44	107	32	183

¹A visual classification of embryo quality was determined before embryos were placed in QuickExtract media and stored at -80°C until DNA extraction.

Table 3.3. Number of embryos at each developmental stage before storage for DNA extraction.

Semen Straw Formulation*	2 cells	4 cells	8 cells	Total
Bull D^b	14	14	10	38
Bull E^a	6	19	31	47
D+E^a	19	23	56	98
Total	39	47	97	183

*Semen straw formulations with different superscripts differed significantly in the progression beyond the 2-cell stage ($p < 0.05$)

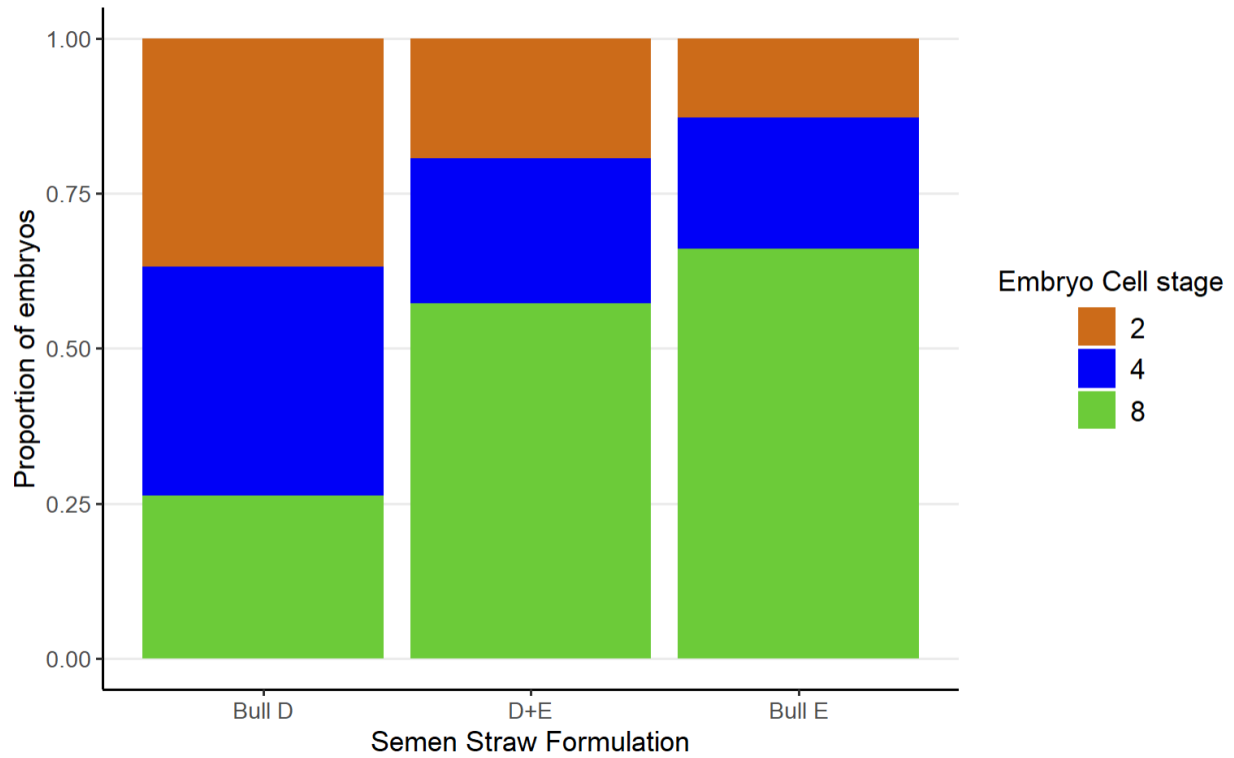


Figure 3.1. The proportion of embryo cell stage development by semen treatment. Semen straw formulations with different superscripts differed significantly in the ability to develop past the 2-cell stage ($p < 0.05$).

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