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Bull field fertility differences can be estimated with *in vitro* sperm capacitation and flow cytometry

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Introduction: This study evaluated whether post in vitro capacitation changes in sperm could be used to estimate field fertility differences between bulls.

Methods: Frozen-thawed semen from five bulls (two to four ejaculates per bull) previously identified as high (48.1% and 47.7%), intermediary (45.5%) or low (40.7% and 43.1%) fertility, based on pregnancy per AI (P/AI), were evaluated for total and progressive motility, sperm plasma membrane integrity (viability), acrosome integrity (viable sperm with an intact or disrupted acrosome), reactive oxygen species (ROS; viable sperm ROS+ or ROS-), mitochondrial membrane energy potential, zinc signatures (signatures 1-to-4) and CD9 protein populations at pre-wash and post-wash (only total and progressive motility), h0 (diluted with non-capacitation media), and at h0, h0 CM, h3, h6, and h24 after dilution with capacitation media (CM) and incubation at 37ºC. Data were analyzed using the GLIMMIX procedure as repeated measures in SAS with bull, time and the interaction as fixed effects.

Results: Bull by time interaction was significant ($P \leq 0.03$) for total motility, viability, viable sperm with disrupted acrosome, and zinc signature 3. There tended ($P=0.06$) to be a bull by time interaction for zinc signatures 1+2 combined. Time was significant ($P \leq 0.003$) in all analyses, except viable ROS- ($P=0.12$). There was a significant effect of bull ($P \leq 0.03$) for viability, viable sperm with disrupted acrosome, zinc signatures 1, 2 and 1+2, viable CD9- and dead CD9+. High and intermediary fertility bulls had greater ($P \leq 0.04$) percentages of viable sperm, zinc signature 2 and zinc signature 1+2 compared to low fertility bulls. High and intermediary fertility bulls had decreased ($P \leq 0.05$) percentage of dead CD9+ compared to low fertility bulls. Viable CD9+ differed ($P=0.02$) and viable sperm with an intact acrosome and viable CD9+ tended to differ ($P=0.06$) amongst bulls; however, association with field fertility was not observed. There was a positive correlation between P/AI and zinc signature 2 ($P=0.04$), and there tended to be a positive correlation between P/AI and viability ($P=0.10$), and zinc signature 1+2 ($P=0.10$).

Discussion: In summary, incubation of sperm in CM and flow cytometry analyses for viability, zinc signatures 2 and 1+2, and dead CD9+ seems promising to estimate in vivo fertility differences amongst bulls.

KEYWORDS

bull fertility, capacitation, CD9 protein, flow cytometry, sperm, zinc signature

1 Introduction

An ejaculate is a heterogeneous population of sperm; consequently, some sperm will likely display undesirable characteristics. For a bull to be categorized as "high fertility", it is important that a large proportion of its ejaculate has desirable characteristics (normal morphology, progressive motility, intact acrosome and plasma membranes, stable DNA, the ability to undergo capacitation) (Rodriguez-Martinez, 2003; Saacke, 2008; Vincent et al., 2012; Garner, 2014). The concentrations and types of undesirable characteristics of a bull's ejaculate will determine, to some extent, the bull's fertility rating. Some insemination problems (i.e. caused by "compensable" sperm characteristics) can be overcome by increasing the insemination dose; however, increasing the insemination dose cannot overcome other problems (i.e. caused by "uncompensable" sperm characteristics). In brief, defects that prevent the sperm from reaching the site of fertilization are considered to be compensable and defects that hinder embryo development after fertilization are considered to be uncompensable. The concept of compensable and uncompensable sperm characteristics was originally described by Saacke et al. (1994).

According to the Society for Theriogenology, a bull breeding soundness exam (BSE) focuses on the potential quantity of sperm produced (measure of scrotal circumference) and evaluates the quality of sperm ejaculated and physical soundness of the sperm on the day of the exam (Koziol and Armstrong, 2018). Conventional BSEs can detect differences in fertility levels with a high degree of accuracy; however, animals with a fertility level that is below average or low may still be incorrectly classified as satisfactory potential breeders. Even among artificial insemination (AI) sires that pass semen quality control analysis, it is impossible to guarantee a high level of fertility because of unknown or unmeasured semen characteristics (DeJarnette, 2005). Thus, the development of new methods to estimate bull field fertility is still necessary.

Sperm need to reside in the oviduct for approximately 6 h to acquire fertilization capacity. During this time, sperm undergo a series of biochemical transformations that are collectively called capacitation (Austin, 1951; Chang, 1951). Capacitation can be induced *in vitro* and has been reported to affect *in vitro* oocyte fertilization (Parrish et al., 1986; Parrish et al., 1988). Several methods of measuring sperm capacitation have been developed (reviewed by Gillan et al., 2005). Recently, intracellular zinc was used to determine sperm capacitation status through changes in zinc signatures, and this method has been used to improve boar fertility (Kerns et al., 2018). The ability of individual sperm to undergo capacitation varies within an ejaculate and across bulls and may affect fertility; thus, evaluation of sperm capacitation could be a suitable new method to estimate bull field fertility.

It has been demonstrated that proteins present in the sperm head are associated with sperm adhesion to or fusion with the oocyte plasma membrane in mice. These proteins are equatorin (or MN9 antigen), CD9, and IZUMO1 (Toshimori et al., 1998; Manandhar and Toshimori, 2001; Inoue et al., 2005; Ito et al., 2010; Satouh et al., 2012). In addition, oocyte JUNO (IZUMO1 receptor) and tetraspanins CD9 and CD81 have been demonstrated to be required for fertilization in mice (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Rubinstein et al., 2006; Bianchi et al., 2014). The proteins CD9, JUNO, and IZUMO1 have been reported to be present in bovine gametes (Zhou et al., 2009; Antalíková et al., 2015; Fukuda et al., 2016; Zhao et al., 2018). When zona-free oocytes were incubated with anti-CD9 antibodies, oocyte fertilization rates significantly decreased (41.6% vs. 81.3%; Zhou et al., 2009); however, the requirement for JUNO and IZUMO1 in bovine fertilization has not been demonstrated. The protein CD9 has been well characterized in oocytes (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Rubinstein et al., 2006; Sutovsky, 2009; Zhou et al., 2009). In relation to sperm, the characterization and function of CD9 is not fully understood; however, it has been reported that CD9 is present in the sperm of mice (Rubinstein et al., 2006; Barraud-Lange et al., 2007; Ito et al., 2010; Barraud-Lange et al., 2012), boars (Kaewmala et al., 2011), and bulls (Antalíková et al., 2015; Zoca et al., 2022a; Zoca et al., 2022b). The objective of this study was to evaluate whether *in vitro* capacitation of sperm coupled with flow cytometric analysis can be used to estimate fertility differences among bulls. A secondary objective was to identify the presence of CD9 in bovine sperm and its possible role as a fertility biomarker.

2 Materials and methods

2.1 Experimental design

Semen from five Angus bulls with known field fertility as evaluated in two research trials (Richardson et al., 2017; Zoca et al., 2020) were used in this study. Bull identification was the same as in Zoca et al. (2020) for bulls A through E (bull age at the time of semen collection was 2, 4, 3, 2, and 2 years for bulls A through E, respectively). Bulls A and D were used in both research

projects (Zoca et al., 2020 and Richardson et al., 2017) and were bulls 1 and 2, respectively, from Richardson et al. (2017). Samples from a total of 15 collection dates were evaluated, with a 55-d range between first and last collection. For bulls A to E, pregnancy per artificial insemination (P/AI) rate, number of breedings per research project per bull, collection dates per bull evaluated, range of days between first and last semen collection, and P/AI rate in relation to estrus expression as described by Richardson et al. (2017) are described in Table 1. Semen straws were thawed and evaluated at pre-wash, at post-wash, and at h0, h3, h6, and h24 of incubation at 37°C for total motility (TMOT) and progressive motility (PROG). Plasma membrane integrity (viability), acrosome integrity, reactive oxygen species (ROS), zinc signatures, mitochondrial membrane energy potential (mito-potential), CD9 protein populations, and CD9 fluorescence intensity (i.e., relative concentration) were measured with flow cytometry as described below. Samples were also used to characterize the localization of CD9 in sperm by fluorescence microscopy. At h0 samples were diluted in a capacitation medium (CM) and in a bovine non-capacitation medium (bNCM) for baseline assessment of each measurement.

2.2 Semen handling

Two or three straws of semen from the same bull and collection date were thawed at 37°C for 60 s and combined in a single tube. An aliquot was removed for computer-assisted TMOT and PROG analysis (CASA; IVOS II; Hamilton Thorne, Beverly, MA, USA). The remaining semen was pipetted into two or three (according to number of straws thawed) 15-mL conical tubes filled with 10 mL of bNCM pre warmed to 37°C; tubes were centrifuged at 500 × g for 10 min, the supernatant was removed, and pellets were combined in a 2-mL tube and resuspended with approximately 200 µL of bNCM. bNCM was composed of NaCl (100 mM), NaH_2PO_4 (0.3 mM), KCl (3.1 mM), $MgC_{l2}{\cdot}6H_2O$ (0.4 mM), polyvinyl alcohol (PVA; 0.01 mM, FW 10,000 with unknown % hydrolyzed), Na-pyruvate (1 mM), Na-lactate (22 mM, 60% w/w), HEPES (40 mM), Gentamicin 10 mg/mL stock (21 mM), and penicillin G (0.174 mM); pH 7.20. The medium was sterile filtered and stored at 4°C for no more than 14 d. Bovine CM was composed of bNCM with the following reagents added (final concentration): $CaC_{l2}{\cdot}2H_2O$ (2.1 mM), $NaHCO_3$ (2 mM), heparin (10 µg/mL), and fatty acid-free bovine serum albumin (BSA; 6 g/mL); pH 7.40. The CM was prepared daily.

Samples were evaluated for post-wash TMOT, PROG, and sperm concentration. The samples were then diluted in bNCM to a concentration of 40 million sperm per mL, followed by dilution in CM to 17 million sperm per mL. At baseline (h0) an aliquot was diluted to 17 million sperm per mL in bNCM. Thus, the final volume and concentration of semen in each tube used for incubation was the same for all samples. A preliminary study conducted in our laboratory determined that samples diluted in CM and samples diluted only in bNCM, and incubated for up to 24 h at 37°C, reacted differently. For sperm diluted in CM compared with sperm diluted in bNCM only, there was a significant ($P < 0.0001$) decrease in viability (14.6 vs. 21.8), zinc signature 2 (10.5 vs. 18.2), and zinc signature 1+2 (12.0 vs. 20.9) percentages. Because of semen extender mixed with semen in straws and the sperm concentration was unknown, evaluation of pre- and post-wash sperm by flow cytometry was not possible. Semen from a control bull was thawed and washed as described in this section at each time point (h0, h0 CM, h3, h6, and h24). Semen from the control bull was diluted in bNCM at h0 and used to ensure proper machine accuracy for all analyses; therefore, the control results were used as a covariate adjustment for all analyses. Semen was always maintained

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

Bull	P/AI[1], %	Breeding, *n*		Collection dates[4], *n*	Range, *d*	Fertility
	Zoca[2]	Zoca[2]	Richardson[3]			
A[5]	48.1[a]	1,050	200	4	55	High
B	47.7[a]	1,058		2	3	High
C	40.7[c]	1,206		2	3	Low
D[5]	45.5[ab]	747	189	3	45	Intermediate
E	43.1[bc]	805		4	10	Low

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

[1] P/AI = pregnancy per artificial insemination.
[2] Zoca = numbers in the column adapted from Zoca et al. (2020).
[3] Richardson = numbers in the column adapted from Richardson et al. (2017).
[4] Collection date = number of collection dates evaluated per bull in study II.
[5] Bulls A and D were represented in both Richardson et al. (2017) and Zoca et al. (2020); bulls A and D represent bulls 1 and 2 from Richardson et al. (2017), respectively.
[a–c] P/AI with different superscripts; P ≤ 0.05 (Zoca et al., 2020).

at 37°C except when at centrifugation- and assay-specific temperatures. Aliquots of semen were removed at each time point for analysis. All samples were analyzed in duplicate, and the average of the duplicates were used for statistical analyses. *In vitro* capacitation was induced as described previously (Kerns et al., 2018).

2.3 Semen analyses

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

All flow cytometric assays were performed in flat-bottom polystyrene 96-well plates and evaluated with a Guava EasyCyte 5HT (IMV Technologies, France) flow cytometer; data acquisition and analyses were performed using the GuavaSoft software (version 1.0; IMV Technologies). A total of 10,000 cells were analyzed per sample (5,000 cells per duplicate). The flow cytometer was cleaned and EasyCheck calibration beads were used to assure proper machine performance daily.

Plasma membrane integrity was evaluated with SYBR-14 and propidium iodide (PI) (adapted from Garner et al., 1994; 1997). In brief, samples were incubated for 10 min with SYBR-14 (900 nM working solution) and PI (1 mg/mL). Results for viability were expressed as percentage of sperm with intact plasma membrane (viable; SYBR-14 positive and PI negative). Sperm acrosome integrity was determined by fluorescein isothiocyanate-conjugated peanut agglutinin (PNA) as previously described (Purvis et al., 1990; Tao et al., 1993). In brief, samples were incubated for 10 min with a stain mix (1 µL of PI, 0.5 µL of PNA, and 48.5 µL of bNCM, and filtered in a 0.22-nm filter). Results for acrosome status were expressed as percentage of viable sperm with intact acrosome (viable intact; PI negative and PNA negative) or disrupted acrosome (viable disrupted; PI negative and PNA positive) and disrupted sperm plasma membrane (dead) with intact acrosome (dead intact; PI positive and PNA negative) or disrupted acrosome (dead disrupted; PI positive and PNA positive).

Reactive oxygen species in sperm were measured using EasyKit 3 (IMV Technologies) following the manufacturer's procedures. In this assay, sperm are challenged with H_2O_2; sperm that react to this challenge are considered ROS positive [ROS+; green dye (proprietary information) positive] and sperm that do not react to this challenge are considered ROS negative (ROS–; green dye negative). Results for ROS were expressed as percentage of viable ROS+, viable ROS–, dead ROS+, and dead ROS–. The main population of interest in this assay was the viable and ROS+ sperm, and it is worth noting that, as this was a 3-hour assay, modifications in sperm physiology were expected and a true 0 h was not possible. Mitochondrial membrane potential (mito-potential) was evaluated with JC-1 (8 µM), diluted in ethanol (200 proof) and bNCM, and incubated for 30 min (adapted from Garner et al., 1997; Guthrie and Welch, 2008). Results were expressed as percentage of high mito-potential.

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

For CD9 evaluation, anti-CD9 antibody (mouse anti-bovine, IVA50, monoclonal; Invitrogen, Waltham, MA, USA) was conjugated to fluorescein isothiocyanate [FITC conjugation kit (fast) – lightning-link, ab188285, ABCAM, United Kingdom], final concentration 0.83 µg/µL. Samples (15 µL; ~250,000 sperm) were diluted in bNCM (35 µL) and incubated with 1 µL of anti-CD9/FITC and 1 µL of PI for 1 h at 37°C (adapted from Antalíková et al., 2015). Flow cytometric CD9 and PI evaluation included the following populations: viable CD9+, dead CD9+, viable CD9–, and dead CD9–. Assays were performed using 250 µL of bNCM and 5 µL of incubated sample per well. In addition, CD9 concentrations in viable and dead populations were evaluated. The localization of CD9 in sperm was characterized by fluorescence microscopy (BZ-X710; Keyence) at 600× magnification under oil immersion.

2.4 Statistical procedures

Flow cytometry and CASA results were evaluated with the GLIMMIX procedure of SAS (9.4). For all analyses, data were assumed to be beta distributed and the link function logit was used. The degrees of freedom method used was the Kenward–Roger method. Bull (A to E), time (pre wash, post wash, h0, h0 CM, h3, h6, and h24), and their interaction were used as fixed effects. Bulls A and B were "high-fertility" bulls, bulls C and E were "low-fertility" bulls, and bull D was an "intermediate-fertility" bull. Three random statements were used. The first random statement was used to model the R-side of residuals to analyze the data as repeated measures. The subject was collection date per bull, with covariate structures selected based on the smaller -2 Res Log Pseudo-Likelihood. The covariate structures selected for each variable were first-order ante-dependence (ANTE(1); dead intact, dead

disrupted, dead ROS+, zinc signature 3, zinc signature 4, viable CD9–, and dead CD9–), first-order autoregressive (AR(1); viable disrupted), heterogeneous first-order autoregressive (ARH(1); viable intact, viable ROS– and ROS+, dead ROS–, mito-potential, zinc signature 1+2), heterogeneous compound symmetry (CSH; viability and zinc signature 2), Toeplitz (TOEP; TMOT, zinc signature 1, viable CD9+, and dead CD9+), and variance components (VC; PROG). The second random statement was the intercept and the third was the residual. Least square means were compared using the PDIFF option, and the ilink function was used to inverse transform least square means. CD9 concentration was evaluated with the MIXED procedure of SAS for repeated measures with bull, time, and their interaction as fixed effects. Collection date per bull was used as subject, and ANTE(1) was selected as the covariate structure for both live and dead sperm CD9 concentration based on the smaller BIC value. CD9 localization in sperm was characterized; however, no statistical analysis was performed. Both the correlation between overall bull effect least square mean and P/AI reported by Zoca et al. (2020) and the correlation of CD9 population and CD9 concentration with all sperm parameters were evaluated using the CORR procedure in SAS. Results are presented as mean ± SE. The level of significance was $P \leq 0.05$, and a P-value > 0.05 and ≤ 0.10 was considered to indicate a tendency.

3 Results

There was no interaction between bull and time for PROG (P = 0.36; Figure 1A), dead disrupted (P = 0.33; Supplementary Figure 1A), viable intact (P = 0.82; Supplementary Figure 1B), dead intact (P = 0.20; Supplementary Figure 1C), viable ROS+ (P = 0.21; Supplementary Figure 2A), dead ROS+ (P = 0.47; Supplementary Figure 2B), viable ROS– (P = 0.93; Supplementary Figure 2C), mito-potential (P = 0.88; Supplementary Figure 3A), zinc signatures 1, 2, and 4 ($P \geq 0.16$; Figures 2A, C, D, respectively), and CD9 populations ($P \geq 0.18$; Figures 3A–D). Nevertheless, the effect of bull by time interaction was significant for TMOT (P = 0.0002; Figure 1B). All bulls had a decrease in percentage of TMOT by time ($P \leq 0.05$); however, bulls C and E had a greater TMOT at h0 CM than in bNCM at h0. At pre wash, high-fertility (bulls A and B) and intermediate-fertility (bull D) bulls had a greater percentage of TMOT ($P \leq 0.05$) than low-fertility bulls (bulls C and E). After washing (post wash), bull E's TMOT was decreased compared with that of bulls A and D ($P \leq 0.03$) but was not different from that of bull B (P = 0.48); however, bull C's TMOT tended to be decreased compared with that of bulls A and D (P = 0.07) and was not different from that of bull B (P = 0.55; Figure 1B). At h0, bull A had greater TMOT than bulls C and E ($P \leq 0.03$) and at time h0 CM, h3, h6, and h24 no differences ($P > 0.10$) were detected among bulls. Thus, total motility measured by CASA after thawing (pre-wash time point) of multiple ejaculates was able to estimate fertility differences between these five Angus bulls, with increased percentages of TMOT for high- and intermediate-fertility bulls compared with those of low-fertility bulls.

The percentage of sperm undergoing spontaneous acrosome reaction (viable disrupted) was significant for the interaction effect between bull and time (P = 0.03; Figure 4A). The viable disrupted percentage increased over time for bulls A, B, D, and E ($P \leq 0.05$; Figure 4A), and bull C did not differ between time points. Nevertheless, there was no association between viable disrupted differences and bull fertility. There was also a significant interaction between bull and time for dead ROS– (P = 0.03; Figure 4B). There was an increase in the percentage of dead ROS– over time for all bulls ($P \leq 0.05$). At h0, high-fertility (bulls A and B) and intermediate-fertility (bull D) bulls had a decreased percentage of dead ROS– compared with bull E ($P \leq 0.005$). Bull A had a decreased percentage of dead ROS– compared with bull C (P = 0.05), and bulls B and D tended to have a decreased percentage of dead ROS– compared with bull C ($P \leq 0.08$). At h0 CM, bulls A and D had a decreased percentage of dead ROS– compared with bulls C and E ($P \leq 0.04$); however, bull B's percentage of dead ROS– did not differ from that of bulls C and E ($P \geq 0.16$). Thus, dead ROS– at 0 h

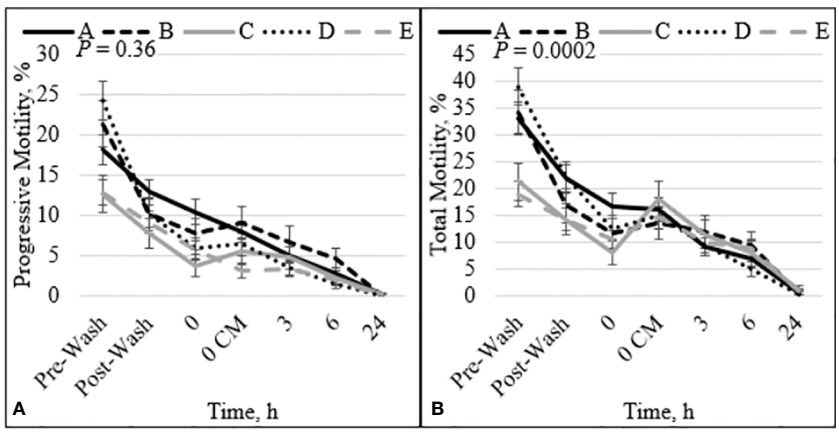

FIGURE 1
Effect of bull by time interaction on sperm progressive **(A)** and total **(B)** motility. Bulls that were previously classified as high fertility (A, B), low fertility (C, E), and intermediate fertility (D) were evaluated for total and progressive motility by computer-assisted sperm analysis. Sperm were evaluated after thawing (pre wash), after being washed (post wash), and after 0 (diluted in non-capacitation medium), 0 CM (diluted in capacitation medium), 3, 6, and 24 h of incubation at 37°C.

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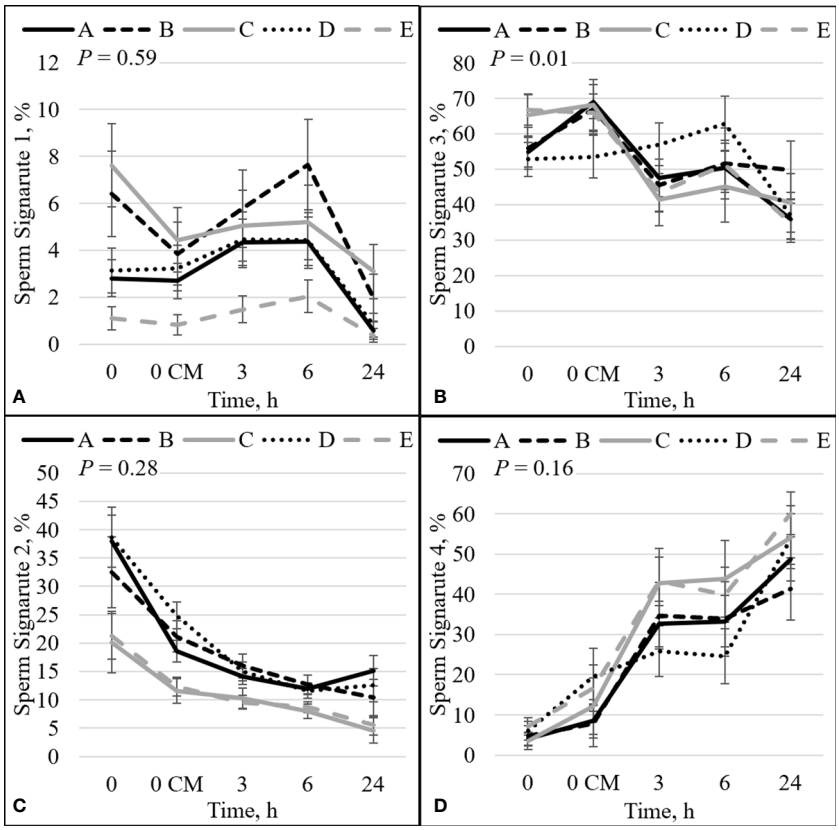

FIGURE 2
Effect of bull by time interaction on sperm plasma membrane integrity (viability) and zinc concentration. The percentage of sperm with intact plasma membrane (viable) and high zinc concentration (signature 1; sperm not capacitated; **A**). The percentage of sperm with disrupted plasma membrane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

could be used to estimate differences in fertility, with high- and intermediate-fertility bulls having a decreased or a tendency to have a decreased percentage of dead ROS– compared with low-fertility bulls.

There was an interaction between bull and time for zinc signature 3 (P = 0.01; Figure 2B). In high-fertility bulls, zinc signature 3 increased statistically (bull A; P = 0.02) or numerically (bull B; P = 0.19) between 0 h and 0 CM, followed by a decrease in the percentage of zinc signature 3; however, low-fertility bulls (bulls C and E) had no changes in zinc signature 3 between 0 h and 0 CM ($P \geq 0.73$), followed by a decrease in the percentage of zinc signature 3. Interestingly, bull D (the intermediate-fertility bull) had no change in the percentage of zinc signature 3 from 0 to 6 h incubation, despite a numerical increase at 6 h incubation ($P \geq 0.26$); however, there was a decrease in the percentage of zinc signature 3 at h24.

There was a significant interaction between bull and time on the percentage of viable sperm (P = 0.0004; Figure 4C). There was a decrease over time for all bulls ($P < 0.05$). Even though an increase in the percentage of viable sperm appeared between h0 and h0 CM for bull B (32.5% vs 36.4%), no statistical differences were detected (P = 0.38). At h0, high- and intermediate-fertility bulls had a greater ($P \leq 0.03$) or tended to have a greater (P = 0.09) percentage of viable sperm than low-fertility bulls. A greater percentage of viable sperm was observed in high- and intermediate-fertility bulls than in low-fertility bulls at h0; however, sperm diluted with CM could not be used to estimate fertility differences between high- and low-fertility bulls at any single time point. Thus, viability at h0 in bNCM could be used to estimate fertility differences, with differences (or tendencies) between high- and intermediate-fertility and low-fertility bulls.

The combination of zinc signatures 1+2 represents the percentage of viable cells measured by zinc signature assay (≈1 h difference from viability assay). There tended to be an effect of the interaction between bull and time for zinc signature 1+2 (P = 0.06; Figure 4D). All bulls had a decrease in the percentage of zinc signature 1+2 over time ($P \leq 0.05$). At h0, high-fertility (bulls A and B) and intermediate-fertility (bull D) bulls had a greater percentage of signature 1+2 ($P \leq 0.02$) than bull E. In addition, bulls A and D tended ($P \leq 0.10$) to be different than C; however, bull B did not differ (P = 0.16) from bull C. At h0 CM, high- and intermediate-fertility bulls had a greater ($P \leq 0.003$) percentage of zinc signature 1 +2 than bull E. In addition, bulls B and D were different ($P \leq 0.02$)

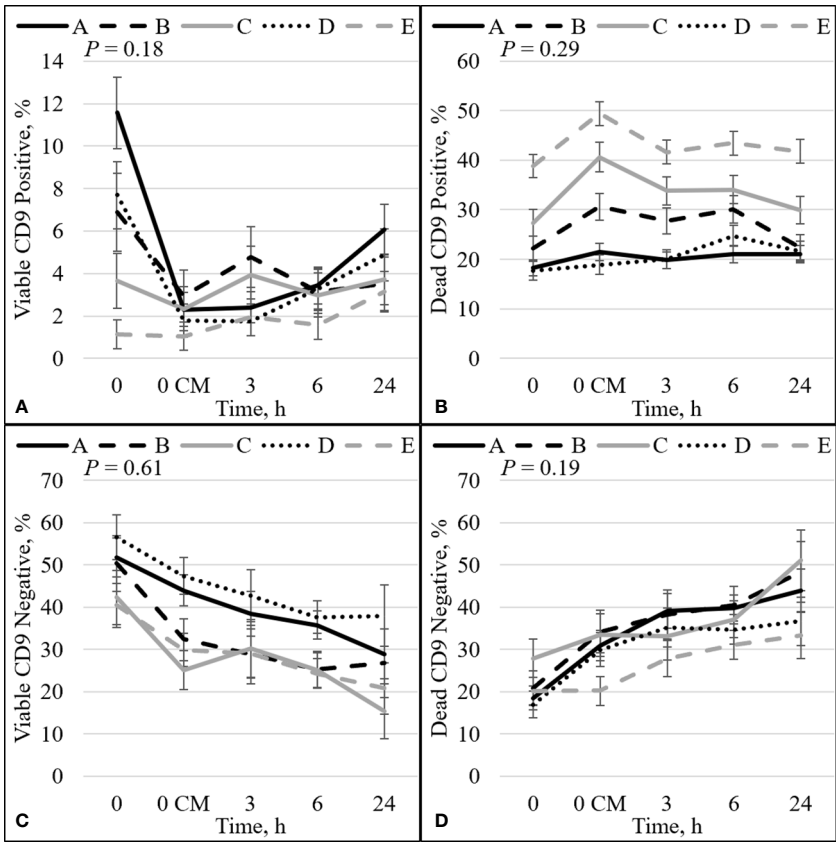

FIGURE 3
Effect of bull by time interaction on sperm plasma membrane integrity (viability) and CD9. The percentage of sperm with intact plasma membrane (viable) and CD9 positive **(A)**. The percentage of sperm with disrupted plasma membrane (dead) and CD9 positive **(B)**. The percentage of viable sperm and CD9 negative **(C)**. The percentage of dead sperm and CD9 negative **(D)**. Bulls that were previously classified as high fertility (A, B), low fertility (C, E), and intermediate fertility (D) were evaluated for viability and CD9 protein (IVA50; Invitrogen) by flow cytometry. Sperm were evaluated at 0 (diluted in non-capacitation medium), 0 CM (diluted in capacitation medium; CM), 3, 6, and 24 h of incubation at 37°C.

and bull A tended ($P = 0.10$) to be different than bull C. Thus, zinc signature 1+2 at 0 CM can be used to estimate fertility differences, with all high- and intermediate-fertility bulls having a greater or tending to have a greater percentage of zinc signature 1+2 than low-fertility bulls. No other individual time point could be used to successfully estimate fertility among bulls.

There was no overall effect of bull on the percentage of TMOT, PROG, dead intact, viable ROS+, dead ROS+, viable ROS–, mito-potential, zinc signatures 3 and 4, and dead CD9– (Table 2). The overall effect of bull was significant; however, it could not be used to estimate fertility differences between bulls for viable and dead disrupted, dead ROS–, zinc signature 1, and viable CD9–. In addition, it tended to be significant for viable intact and viable CD9+ (Table 2). The overall effect of bull that was significant and estimated fertility differences between bulls were viability, zinc signature 2, zinc signature 1+2, and dead CD9+ (Table 2), of which high- and intermediate-fertility bulls had a greater overall percentage of viable, zinc signature 2, zinc signature 1+2, and a decreased percentage of dead CD9+ than low-fertility bulls. There was a positive correlation between field fertility and zinc signature 2 ($r = 0.89$; $P = 0.04$) and there tended to be a positive correlation between field fertility and viability ($r = 0.81$; $P = 0.10$) field fertility and zinc signature 1+2 ($r = 0.80$; $P = 0.10$); however, dead CD9+ did not correlate with field fertility ($r = -0.68$; $P = 0.20$). Although the percentage of dead ROS– did not estimate fertility differences between bulls, dead ROS– was negatively correlated with field fertility ($r = -0.91$; $P = 0.03$). There was no correlation between field fertility and the other sperm parameters evaluated ($P > 0.10$).

The overall effect of time was significant for all analyses except for viable ROS– ($P = 0.12$; Supplementary Figure 4C). There was a decrease ($P \leq 0.001$) over time of the percentage of TMOT, PROG, mito-potential, viability, zinc signature 1+2, viable ROS+, viable intact, zinc signature 2 (Supplementary Figures 3–9), and viable CD9– ($P < 0.0001$; Figure 5C). There was an increase ($P \leq 0.0001$) over time in the percentage of dead ROS–, viable disrupted, dead intact, zinc signature 4 (Supplementary Figures 4, 8, 9), and dead CD9– ($P < 0.0001$; Figure 5D). Other significant ($P \leq 0.001$) effects of time were on dead ROS+, dead disrupted acrosome, zinc signatures 1 and 3 (Supplementary Figures 4, 8, 9), and viable CD9+ and dead CD9+ ($P \leq 0.0002$; Figures 5A, B).

The protein CD9 was present in the acrosomal region in both viable and dead sperm (Figures 6–8). Staining varied from all acrosomal region stained to partial acrosomal region stained;

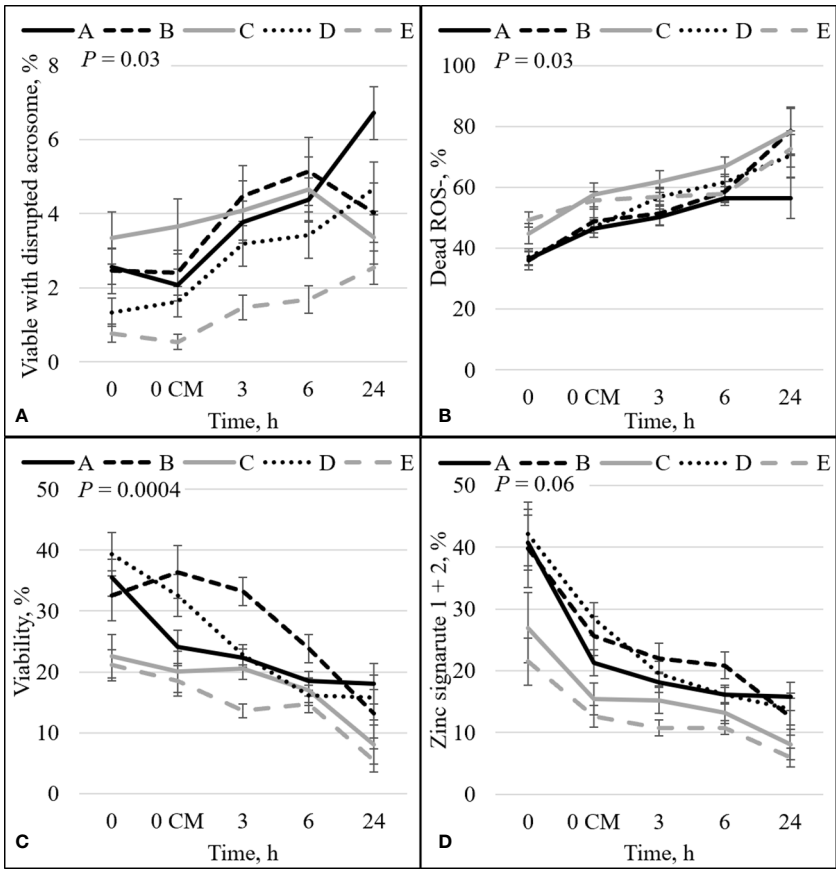

FIGURE 4
Effect of bull by time on the percentage of: sperm with intact plasma membrane (viable) and disrupted acrosome **(A)**, dead and ROS negative (ROS−) sperm **(B)**, sperm plasma membrane integrity (viability; **C**), and sperm with an intact plasma membrane (viable) with high (signature 1) and low (signature 2) zinc concentration combined (signature 1+2; **D**). Bulls that were previously classified as high fertility (A, B), low fertility (C, E), and intermediate-fertility (D) were evaluated by flow cytometry. Sperm were evaluated at 0 (diluted in non-capacitation medium), 0 CM (diluted in capacitation medium), 3, 6, and 24 h of incubation at 37°C.

however, there was no change in localization of CD9 before and after capacitation (data not shown). Nevertheless, there were changes in population percentage (Figure 5) and concentration (Figure 9) of viable and dead populations. There was no effect of bull ($P = 0.12$) or bull by time interaction ($P = 0.55$) on viable CD9 concentration. There was a significant interaction of bull by time on dead CD9 concentration ($P = 0.03$; Figure 9F); bull E had the greatest concentration at all time points except at 0 h, where bull E was not different than bull B ($P = 0.26$), and tended to be different than bulls A, C, and D ($P \leq 0.10$). There was a bull effect for dead CD9 concentration (Figure 9B); bull E had the greatest concentration among all bulls ($P \leq 0.002$). There was an effect of time for both viable and dead CD9 concentration ($P \leq 0.0004$; Figures 9C, D, respectively). Concentration for viable sperm decreased when sperm were diluted with CM and increased during the incubation period; however, dead sperm CD9 concentration decreased over time (Figures 9C, D).

The correlation between the CD9 population and CD9 concentration was evaluated. Not surprisingly, there were positive correlations ($P < 0.01$) between viable CD9+ and viable CD9 concentration ($r = 0.61$) and between dead CD9+ and dead CD9 concentration ($r = 0.54$; Table 3). In addition, there was a positive correlation ($P < 0.01$) between viable CD9− and dead CD9 concentration ($r = 0.21$; Table 3). There were negative correlations ($P < 0.01$) between viable CD9+ and dead CD9+ ($r = -0.30$), dead CD9+ and viable CD9− ($r = -0.57$), viable CD9− and dead CD9− ($r = -0.68$), and dead CD9− and dead CD9 ($r = -0.72$; Table 3) concentrations. Viable CD9+ was correlated ($P < 0.05$) with viable disrupted, dead disrupted, zinc signature 2, zinc signature 3, and zinc signature 1+2, and tended ($P \leq 0.10$) to be correlated with dead ROS+ (Table 4). Dead CD9+ was correlated ($P < 0.05$) with viability, viable intact, viable disrupted, dead disrupted, dead ROS−, mito-potential, and zinc signatures 2 and 1+2, and tended ($P \leq 0.10$) to be correlated ($P < 0.05$) with PROG, viable ROS+, and zinc signatures 1 and 4 (Table 4). Viable CD9− was correlated ($P < 0.05$) or tended ($P \leq 0.10$) to be correlated with all sperm parameters except zinc signatures 1 and 3 (Table 4). Dead CD9− was correlated ($P < 0.05$) or tended ($P \leq 0.10$) to be correlated with all sperm parameters except dead disrupted, viable ROS−, and zinc signatures 1 and 3 (Table 4). Viable sperm CD9 concentration was correlated ($P < 0.05$) with viable ROS+, viable ROS−, and zinc signatures 2, 4, and 1+2 (Table 4). In addition, dead CD9 concentration was correlated ($P < 0.05$) with all sperm parameters except for viable intact, viable ROS−, dead ROS +, and zinc signature 1 (Table 4).

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

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

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

4 Discussion

It is well established that cows must conceive in the first 21 d of the breeding season to achieve maximum fertility potential and maximize profitability. A delay in conception will lead to a decrease in the longevity of the cows and will hinder calf weaning weight and overall productivity (Cushman et al., 2013). To conceive early in the breeding season and maintain a pregnancy, cows must have resumed regular estrous cycle, in good physical condition and on a positive plane of nutrition; however, bull fertility also plays an important role. A BSE is essential for the selection of bulls with potential satisfactory fertility levels that will contribute to early conception in a breeding season (Barth, 2018); however, passing a BSE does not guarantee a high level of fertility. In the beef and dairy industries, frozen semen is used for AI. It is expected that differences in fertility between AI sires are not statistically significant and that more than 90% of semen from these bulls is within ± 3% of the average fertility rate (Clay and McDaniel, 2001; DeJarnette, 2005).

An ejaculate is composed of a heterogeneous population of sperm, and fertility is multifactorial (Rodriguez-Martinez, 2003). Amann and Hammerstedt (1993) suggest that an ejaculate or inseminate must have "enough" of all necessary sperm characteristics to reach a high level of fertility. In the present study, most of the sperm characteristics measured were not associated or correlated with field fertility. It has been reported that acrosome integrity, ROS, and mito-potential are associated or correlated with bull fertility (Oliveira et al., 2014; Kumaresan et al.,

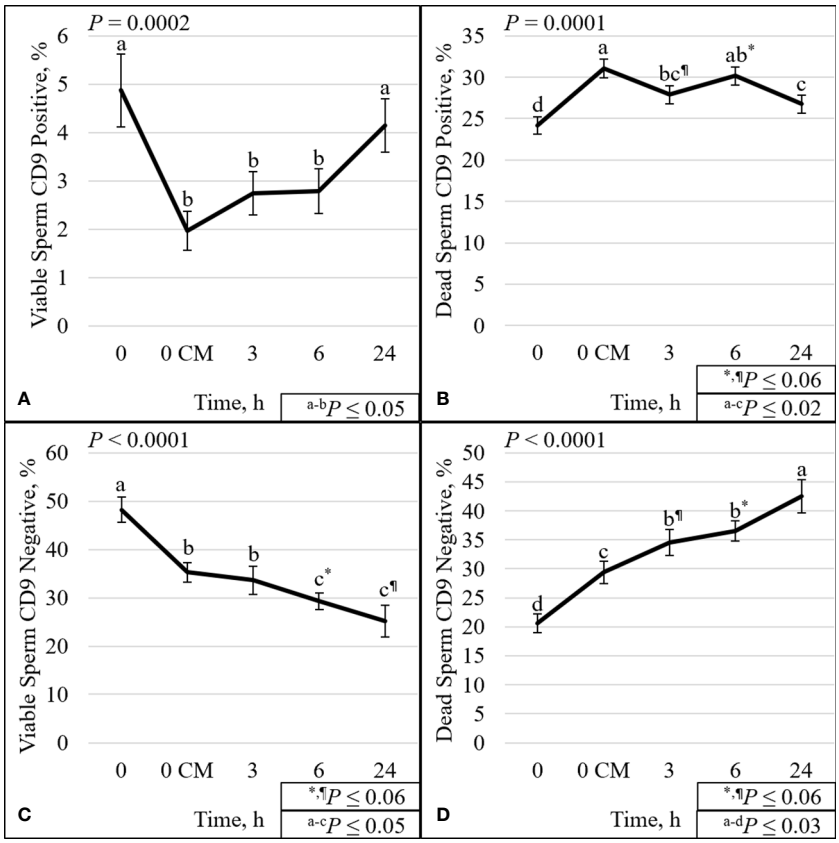

FIGURE 5
Effect of time on sperm plasma membrane integrity (viability) and CD9 protein. The percentage of sperm with intact plasma membrane (viable) and CD9 positive **(A)**. The percentage of sperm with disrupted plasma membrane (dead) and CD9 positive **(B)**. The percentage of viable sperm and CD9 negative **(C)**. The percentage of dead sperm and CD9 negative **(D)**. Sperm were evaluated for viability and CD9 protein (IVA50; Invitrogen) by flow cytometry at 0 (diluted in non-capacitation medium), 0 CM (diluted in capacitation medium; CM), 3, 6, and 24 h of incubation at 37°C.

2017; Bernecic et al., 2021), but in the present study these traits were not associated with the field fertility of the evaluated bulls. One difference between the studies is the range in fertility levels among the tested bulls. There was on average difference of 11 to 28 percentages points in pregnancy rates between high- and low-fertility bulls (Oliveira et al., 2014; Kumaresan et al., 2017; Bernecic et al., 2021); however, bulls in the present study varied by only 4.6 to 7.4 percentage points in their pregnancy rates. Thus,

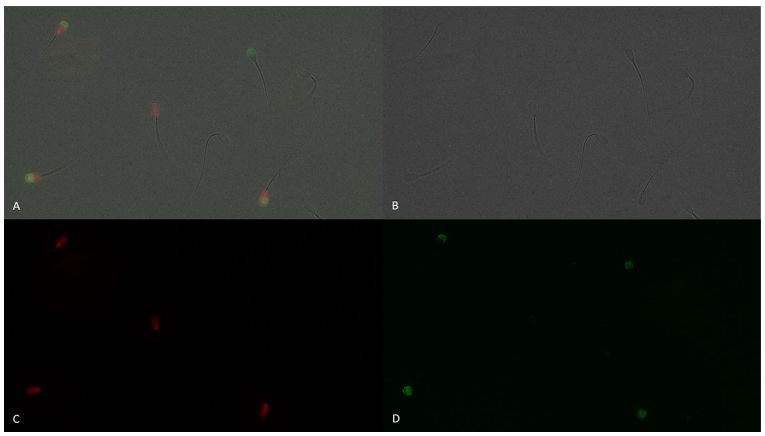

FIGURE 6
Sperm plasma membrane integrity [dead, propidium iodide positive (red), and viable, propidium iodide negative] and positive (green fluorescence) or negative for CD9 protein (IVA50; Invitrogen). **(A)**: merged view of fields **(B, C,** and **D) (B)**: bright field. **(C)**: red fluorescence = propidium iodide. **(D)**: green fluorescence = anti-CD9-FITC labeling. 600× magnification under oil immersion.

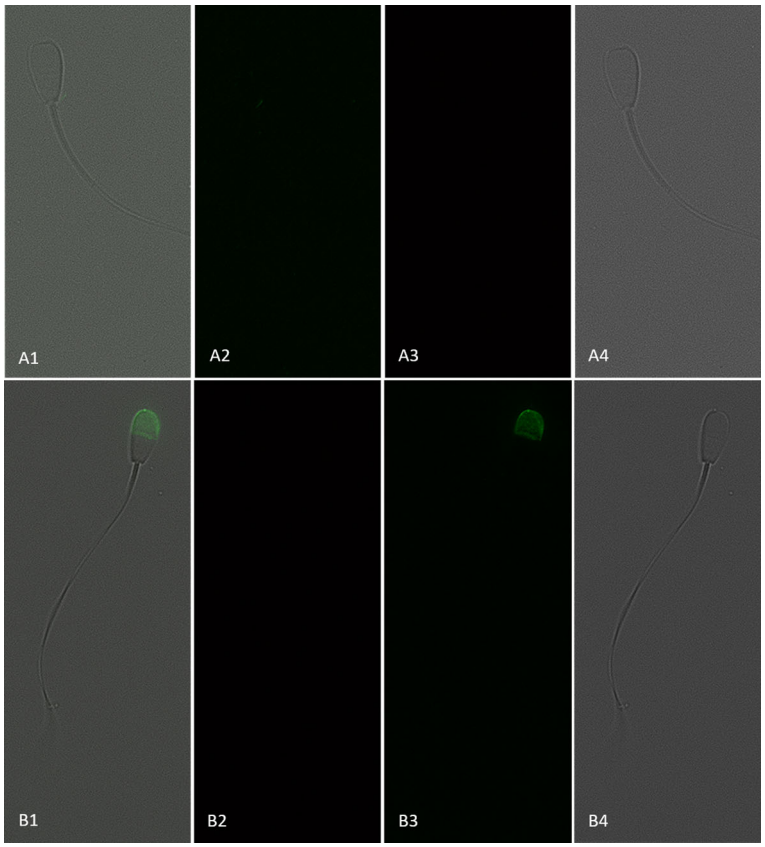

FIGURE 7
Sperm with an intact plasma membrane (viable; propidium iodide negative) and negative **(A1–4)** or positive **(B1–4)** for CD9 protein (IVA50; Invitrogen). (1) Merged view of fields 2, 3, and 4. (2) Red fluorescence = propidium iodide. (3) Green fluorescence = anti-CD9-FITC labeling. (4) Bright field. 600× magnification under oil immersion.

it is possible to conclude that the fertility of bulls in this study was not associated with only acrosome integrity, ROS, or mito-potential.

Fertility variation is not expected to be significant in a large sample of bulls (Clay and McDaniel, 2001; DeJarnette, 2005). Nevertheless, Richardson et al. (2017) and Zoca et al. (2020) demonstrated fertility differences between bulls. Thus, the study of semen characteristics that can better estimate bull fertility is necessary. In the present study, semen from two studies (Richardson et al., 2017; Zoca et al., 2020) was analyzed to evaluate the effect of inducing capacitation *in vitro* and the ability to estimate differences between different fertility levels by CASA and flow cytometry analyses. The interaction between bull and time for TMOT was associated with differences in the field fertility of bulls, but only pre wash. Zoca et al. (2020) reported differences in TMOT between bulls, but TMOT did not differ between bulls A (high fertility) and C (low fertility) (31.8% vs 26.5%, respectively). In the present study, more ejaculates were evaluated (bull A) and differences in TMOT at pre-wash between high- and low-fertility bulls were detected; however, the present study failed to detect differences between high-fertility bulls and the intermediate-fertility bull. Farrell et al. (1998) reported a moderate correlation ($r = 0.58$) between TMOT and bull fertility, which agrees with the lack of relationship between TMOT and field fertility observed in the overall bull effect and by Zoca et al. (2020); however, when more ejaculates were added to the analysis in the present study the relationship between TMOT and field fertility was observed at pre-wash. In addition, it is important to highlight that the intermediate-fertility bull (bull D) had the greatest TMOT in the present study (38.9%) and in Zoca et al. [(2020); 51.6%] but not in Richardson et al. [(2017); bull A (1) 51%; bull D (2) 38.5%], which agrees with the moderate correlation between TMOT and fertility reported previously (Farrell et al., 1998). Thus, it is possible to infer that TMOT is a useful tool in identifying poor-quality ejaculates (samples); however, prediction of fertility level beyond that is questionable.

Sperm viability (defined as plasma membrane integrity; SYBR-14 positive and PI negative) was associated with the field fertility of bulls. High-fertility bulls had a greater overall percentage of viable sperm than low-fertility bulls. Again, the intermediate-fertility bull was not different from the high-fertility bulls. There was also a positive correlation (tendency) between field fertility and sperm viability (overall bull effect). The interaction of bull by time could be used to detect differences related to fertility at time h0 in bNCM; however, no other time point could be used to estimate differences associated with field fertility. It is possible that high- and intermediate-fertility bulls required a smaller concentration of capacitating agents (e.g., heparin and bicarbonate) than low-fertility bulls, since only small changes in sperm viability were

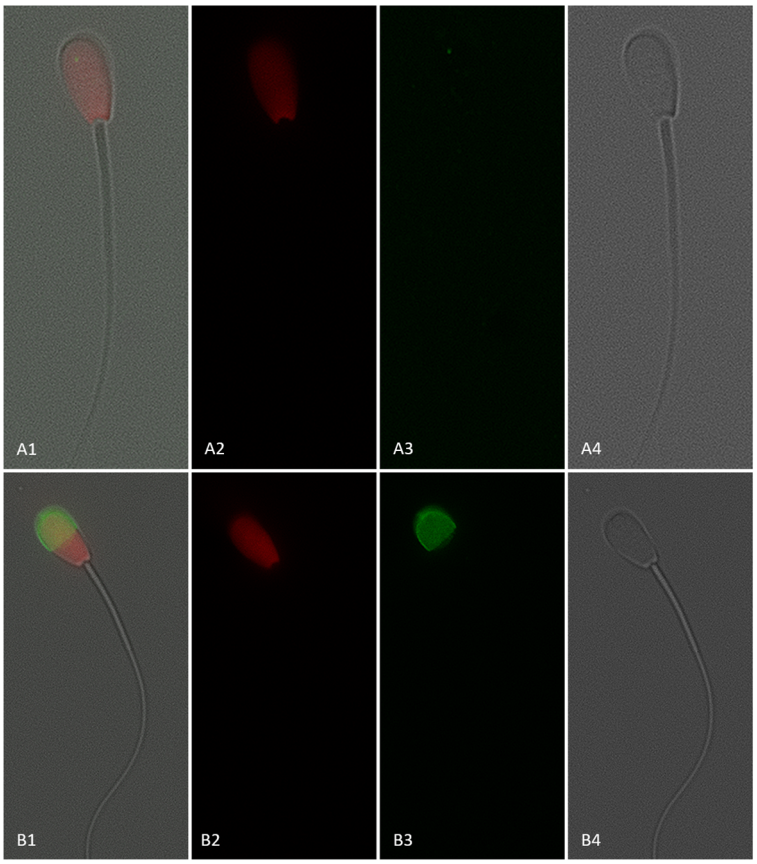

FIGURE 8
Sperm with a disrupted plasma membrane (dead; propidium iodide positive) and negative **(A1–4)** or positive **(B1–4)** for CD9 protein (IVA50; Invitrogen). (1) Merged view of fields 2, 3, and 4. (2) Red fluorescence = propidium iodide. (3) Green fluorescence = anti-CD9-FITC labeling. (4) Bright field. 600× magnification under oil immersion.

observed in low-fertility bulls compared with high- and intermediate-bulls. This hypothesis is supported by an increase (statistically or numerically) in zinc signature 3 at h0 CM for high-fertility bulls and at h6 for intermediate-fertility bulls, whereas low-fertility bulls had decreased zinc signature 3. Nevertheless, the induction of capacitation and incubation of sperm allowed for better separation between high and low fertility as observed in the overall effect of bull, because high- and intermediate-fertility bulls maintained a greater (numerically or statistically) percentage of viable sperm at all time points. Differences in sperm viability between bulls were detected by Zoca et al. (2020), and their results were similar to what was observed at 0 h. Interestingly, the intermediate-fertility bull had the greatest value for viability in both studies at 0 h, but this was not the case in Richardson et al. (2017), where no differences were detected between high- and intermediate-fertility bulls when overall viability was evaluated. The correlation between sperm plasma membrane integrity and field fertility has been widely studied; however, results vary, with a weak correlation (r = 0.05 to 0.20; Alm et al., 2001; DeJarnette et al., 2021), moderate correlation (r = 0.41 to 0.68; Januskauskas et al., 2001; Anzar et al., 2002; Januskauskas et al., 2003; Christensen et al., 2005), and strong correlation (r = 0.85 and 0.87; Anzar et al., 2002; Kumaresan et al., 2017) having been reported. In the present study, the correlation between field fertility and viability was strong. In addition, both low-fertility bulls had decreased viability either statistically or numerically at all time points and overall, which suggests that the inclusion of a viability assay as a quality control analysis for AI studs and possibly at a BSE test would assist in the identification of high-fertility bulls.

A new marker of sperm capacitation has been recently reported. This marker uses zinc ion efflux to determine the capacitation status of the sperm and classifies sperm into four signatures (Kerns et al., 2018; 2020). It has been reported that non-capacitated sperm, usually found in fresh ejaculates, has elevated intracellular zinc (signature 1; Michailov et al., 2014; Kerns et al., 2018). The active removal of zinc (i.e., the transition from signature 1 to signature 2) has been reported to be a prerequisite of sperm capacitation (Andrews et al., 1994); however, complete removal of zinc has been reported to abolish sperm motility (Michailov et al., 2014; Kerns et al., 2018). Thus, sperm zinc signatures 1 and 2 represent the population of sperm with a high degree of fertility potential, while signature 3 represents sperm that have completed capacitation and are dead or dying. Signature 4 represents sperm that are dead and may or may not have gone through capacitation before dying (Kerns et al., 2018). In boars, zinc signature 3 was increased from pre-capacitated to post-capacitated sperm among high-fertility boars, but no change was observed in low-fertility boars (Kerns et al., 2018). In

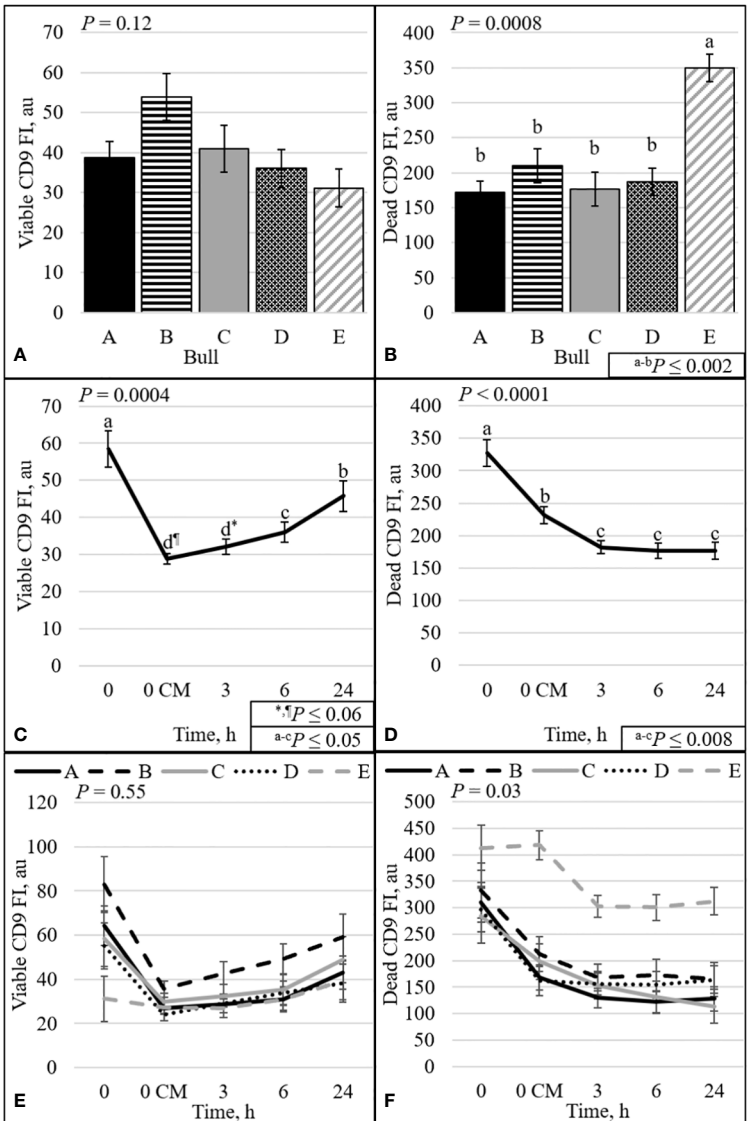

FIGURE 9
Effect of bull **(A, B)**, time **(C, D)**, and bull by time interaction **(E, F)** on fluorescence intensity (FI) of sperm with intact plasma membrane (viable; **A**, **C**, and **E**) or disrupted plasma membrane (dead; **B**, **D**, and **F**). Bulls that were previously classified as high- fertility (A, B), low- fertility (C, E), and intermediate fertility (D) were evaluated for viability and CD9 protein (IVA50; Invitrogen) by flow cytometry. Sperm were evaluated at 0 (diluted in non-capacitation medium), 0 CM (diluted in capacitation medium; CM), 3, 6, and 24 h of incubation at 37°C.

TABLE 3 Pearson's correlation coefficient (shaded area above diagonal) and significance level (below diagonal) between CD9 populations [intact (viable) or disrupted (dead) sperm plasma membrane and CD9 positive (+) or negative (-)] and concentration (FI; *n* = 70).

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

Bolded values represent statistically significant correlations and associated P-value.

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

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

[1] Ejaculates of five bulls evaluated at 0 h in non-capacitation medium and at 0, 3, 6, and 24 h in capacitation medium (n = 70).
**P < 0.05.
*P ≤ 0.10.

the present study, the percentage of zinc signature 3 in bull sperm decreased over time and did not seem to follow the same trend as was reported for boars (Kerns et al., 2018). Nonetheless, zinc signature 2 and zinc signature 1+2 were associated with the field fertility of bulls. As for viability, the zinc signature 1+2 of the intermediate-fertility bull was not different to that of the high-fertility bulls, but low- and high-fertility bulls were different.

Richardson et al. (2017) reported differences in fertility by time between bulls A and D when cows were inseminated prior to estrus but not after the onset of estrus; however, bull D (intermediate fertility) had a similar level of viability and zinc signatures 2 and 1+2 to those of bull A (high fertility). Thus, the mechanism for decreased fertility of bull D compared with bull A is likely associated with other factors not related to sperm capacitation ability as measured in this study. Nevertheless, bull A and D had a similar reduction in the percentage of zinc signature 2 between h0 and h0 CM. However, bull A had an increase in zinc signature 3 while bull D maintained similar percentages; thus, it is likely that bull A's sperm was undergoing capacitation and bull D's sperm was dying, which can be observed by a numerical increase in sperm zinc signature 4 for bull D at h0 CM compared to bull A.

In the present study, the presence of, localization, and quantity of CD9 in relationship with bull fertility was evaluated. The protein CD9 has been well characterized in oocytes (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Rubinstein et al., 2006; Sutovsky, 2009; Zhou et al., 2009); however, in sperm, the characterization and function of CD9 is not fully understood. The localization of CD9 described here was similar and agrees with what has been previously described for bull sperm (Antalíková et al., 2015). Antalíková et al. (2015) reported that 75% to 85% of sperm were positive for CD9, with minimal change during capacitation. The proportion of CD9+ sperm observed in the present study was lower than that reported elsewhere (only 20% to 50% of sperm were positive for CD9), with the lowest percentage and the greatest percentage identified at h0 CM (bulls D and E, respectively). Differences between the two studies could be related to breed (Holstein vs. Angus), method of analysis (fixed samples vs. "fresh" samples; primary and secondary antibodies vs. primary antibody), or simply animal-to-animal variation; however, it was observed that dead sperm with a disrupted acrosome were strongly positively correlated with dead CD9+ sperm. This finding may indicate that CD9 is present in the inner portion of the acrosome and may be externalized during capacitation, or that CD9 can be detected only in sperm with a disrupted acrosome. This may explain the differences in CD9+ percentage identified between the results of the present study and Antalíková et al.'s (2015) results since fixation of sperm can cause membrane permeabilization. In the present study, however, acrosome status and CD9 were evaluated in separate assays. It was observed that one low-fertility bull (bull E) had elevated concentrations of CD9 compared with other bulls among the dead sperm population;

however, no differences were observed in the viable population. The fluorescence intensity of CD9 on dead sperm decreased over time, which might be related to the release of this protein. Interestingly, viable sperm CD9 concentration greatly decreased when sperm was diluted with CM and slowly increased with incubation. Antalíková et al. (2015) reported a decrease of *in vitro* fertilization rates when sperm were treated with anti-CD9 antibodies compared with untreated sperm (64.4% vs. 89.4%, respectively). Interestingly, low-fertility bulls had a greater proportion of dead CD9+ sperm than high- and intermediate-fertility bulls. Thus, CD9 protein assay, more specifically dead CD9+, could be a negative marker of fertility.

In conclusion, multiple analyses over time in capacitation medium of viability, zinc signature 2, zinc signature 1+2, and dead CD9+ were associated with the field fertility of bulls. In addition, TMOT at pre-wash, viability at h0, and zinc signature 1 2 at h0 CM could be used to estimate fertility differences between bulls. The inclusion of viability, a zinc signature, or a CD9 protein assay in quality control measurements may have the potential to better predict bull fertility; however, a larger number of bulls with known fertility and different breeds need to be evaluated to validate these results.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The study was conducted on straws of semen that were collected from a commercial bull stud. These samples were provided by the bull stud; thus, no actual animals were used in this study, only the straws of semen from the bulls.

Author contributions

SZ, TG, JD, and GP: experimental design and conceptualization. SZ, TG, and AZ: data collection. SZ: data management. SZ, RC, and GP: statistical analyses. SZ, JD, BH, MU, and GP: provided original samples used for research. TG, JW, and GP: funding acquisition. SZ: wrote the original draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors BH and MU were employed by the company Select Sires, Inc., which provided samples for the project.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fanim.2023.1180975/full#supplementary-material

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