1-22-2022

Use of Sperm In Vitro Capacitation and Flow Cytometry to Estimate Bull Fertility

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Beef Day 2022

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Objective

The objectives of this study were to evaluate whether motility or flow cytometric analyses after induced capacitation was related to field fertility, and to characterize whether CD9 on sperm could be used as a fertility biomarker.

Study Description

Frozen-thawed semen from five bulls previously identified as high (48.1% and 47.7%, bulls A and B, respectively), intermediary (45.5%, bull C) or low (43.1% and 40.7%, bulls D and E, respectively) fertility, based on pregnancy per AI, were evaluated with several laboratory measures. Measures included total motility, sperm plasma membrane integrity (viability), acrosome integrity, reactive oxygen species (ROS), mitochondrial membrane energy potential (mito-potential), zinc signatures (signatures 1 to 4), and CD9 protein populations at pre-wash, post-wash, h 0 (diluted with non-capacitation media), and at 0, 3, 6, and 24 h after dilution with capacitation media and incubation at 37 ºC. Data were analyzed using the GLIMMIX procedure of SAS for repeated measures with bull, time, and the interaction as fixed effects. Bull by time interaction was significant ($P \leq 0.01$) for total motility and viability. There tended ($P = 0.06$) to be a bull by time interaction for zinc signatures 1 + 2 combined. 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- (CD9 negative), and dead CD9+ (CD9 positive). High and intermediary field 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. There was or tended to be a positive correlation between pregnancy per AI and viability ($P = 0.10$; $r = 0.81$), zinc signature 2 ($P = 0.04$; $r = 0.89$), and zinc signature 1 + 2 ($P = 0.10$; $r = 0.80$).

Take Home Points

In summary, these measures of zinc signatures 2 and 1 + 2 combined, and dead CD9+ provide promising measures to estimate field fertility differences amongst bulls. These results may help the AI industry improve bull selection and improve overall bull fertility which has the potential to improve overall beef cattle reproductive performance. The value of these measures has not yet been assessed in fresh semen ejaculates from bulls.

Introduction

Bull fertility is an important factor in herd fertility and can impact overall pregnancy rates. Bull fertility is commonly evaluated through a breeding soundness exam (BSE); however, even among bulls that pass a BSE and/or AI quality control analysis in commercial AI semen service centers, it is not possible to guarantee that bulls will have high fertility (DeJarnette, 2005). In order to complete fertilization, sperm must have normal morphology, progressive motility, intact membranes (e.g., acrosome and plasma membrane), stable DNA, and the ability to undergo capacitation (Rodriguez-Martinez, 2003; Saacke, 2008; Vincent et al., 2012; Garner,

2014). An ejaculate is a heterogeneous population of sperm; thus, it is normal for some sperm to display undesirable characteristics, but for a bull to have high fertility, it is important that a great proportion of the ejaculate has these desirable characteristics. Concentration and type of undesirable characteristics will determine, to some extent, the fertility of the ejaculate. Overcoming some inseminate problems can occur by increasing the insemination dose (number of viable sperm in a straw; Saacke et al., 1994) which is controlled by AI companies.

In order to undergo capacitation, sperm must reside in the female reproductive tract for approximately 6 h; however, it can be induced *in vitro* (Austin, 1951; Chang, 1951; Parrish et al., 1986; Parrish et al., 1988). Several methods of measuring sperm capacitation have been developed (reviewed by Gillan et al., 2005). More recently intracellular zinc ions were utilized to determine sperm capacitation status through changes in zinc signatures and were also associated with boar fertility (Kerns et al., 2018). The ability of sperm to undergo capacitation may vary among bulls and may also affect fertility. Several proteins have also been demonstrated to be involved with sperm/egg adhesion or fusion in mice, including the protein CD9 (Toshimori et al., 1998; Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Manandhar and Toshimori, 2001; Inoue et al., 2005; Rubinstein et al., 2006; Ito et al., 2010; Satouh et al., 2012; Bianchi et al., 2014). Bovine gametes also contain CD9 (Zhou et al., 2009; Antalíková et al., 2015) and it is possible that bovine sperm CD9 is involved with bull fertility. The objectives of this study were to evaluate whether motility or flow cytometric analyses after induced capacitation was related to field fertility, and to characterize whether CD9 on sperm could be used as a fertility biomarker.

Experimental Procedures

Experimental design

Semen from five bulls with known field fertility as evaluated in two research trials (Richardson et al., 2017; Zoca et al., 2020) were used in this study. Bulls were previously identified as high (48.1% and 47.7%, bulls A and B, respectively), intermediary (45.5%, bull C), or low (43.1% and 40.7%, bulls D and E, respectively) fertility, based on pregnancy per AI (P/AI; approximately 1,000 AI per bull) previously reported. Semen was thawed in a water bath (37 ºC), an aliquot was removed for pre-wash analysis, and the remaining sample was diluted with a non-capacitation media and centrifuged (500 x g for 10 min). After centrifugation supernatant was removed and the sperm pellet was resuspended in approximately 200 µL of non-capacitation media, an aliquot was removed for post-wash analysis. Sperm samples were then diluted to a constant 17 million sperm per mL in capacitation media, except for a small aliquot diluted to 17 million sperm per mL in non-capacitation media (used for a time 0 h baseline analysis). Sperm samples were evaluated for motility and flow cytometry at pre- and post-wash (sperm motility only), 0 h (non-capacitation media), 0, 3, 6, and 24 h (capacitation media).

In vitro Capacitation

In vitro capacitation was induced as previously described by Kerns et al. (2018). Semen was always maintained at 37 °C except during centrifugation and assay specific temperatures. Aliquots of semen were removed at each time point (pre-wash, post-wash, 0 in non-capacitation media, 0 in capacitation media, 3, 6, 24 h) for analyses. All samples were analyzed in duplicate, and the average of the duplicates was used for statistical analyses.

Semen Analyses

Analysis of sperm motility was performed using a computer assisted sperm analysis (CASA) system; samples were evaluated for sperm concentration (post-wash only for sperm dilution) and total motility. All flow cytometric assays were performed with a Guava EasyCyte 5HT (IMV Technologies, France) flow cytometer; data acquisition and analyses were performed using the GuavaSoft software (version 1.0; IMV Technologies). A total of 5,000 cells per duplicate were analyzed. Samples were evaluated for plasma membrane integrity (viability) and acrosome integrity [percentage of viable sperm with intact acrosome (viable intact) or disrupted acrosome (viable disrupted) and disrupted sperm plasma membrane (dead) with intact acrosome (dead intact) or disrupted acrosome (dead disrupted)]. Reactive oxygen species (ROS) in sperm were measured to

determine the ability of sperm to withstand oxidative stress with EasyKit 3 (IMV Technologies) following manufacturer's procedures. Results for ROS were expressed as a percentage of viable ROS+, viable ROS-, dead ROS+, and dead ROS-. The main population of interest in this assay was the viable sperm with the ability to withstand oxidative stress (viable ROS+), and it is worth noting that this was a 3-hour assay. Mitochondrial membrane potential (mito-potential) was evaluated as a measure of energy potential of sperm and results were expressed as percentage of sperm with high mito-potential.

Sperm zinc signatures are a measure of sperm capacitation and have been characterized in human, boar, and bovine by Kerns et al. (2018). The zinc signature assay used here was adapted from Kerns et al. (2018). Zinc signature results were expressed as percentage of sperm with signature 1 (viable non-capacitated sperm with high intracellular zinc and high fertility potential), signature 2 (viable sperm in the process of capacitation with low intracellular zinc and high fertility potential), signature 3 (dead and capacitated sperm with high intracellular zinc in the mitochondrial sheath or the acrosome region or both, and no fertility potential), and signature 4 (dead sperm without zinc and no fertility potential). For CD9 evaluation, anti-CD9 antibody (mouse anti-bovine, IVA50, monoclonal, Invitrogen, Waltham, MA) was conjugated to fluorescein isothiocyanate [FITC conjugation kit (fast) – lightning-link, ab188285, ABCAM, United Kingdom]. Samples were diluted with bNCM, and incubated with anti-CD9/FITC and propidium iodide (PI) for 1 h at 37 °C (adapted from Antalíková et al., 2015). The results for CD9 evaluation included the following populations viable CD9+, dead CD9+, viable CD9-, dead CD9-.

Statistical Procedures

Total motility and flow cytometry measures [viability, acrosome integrity, ROS, mito-potential, zinc signatures and combination of signature 1 and 2 (signature 1 + 2), and CD9 protein] were evaluated with the GLIMMIX procedure of SAS (9.4) for repeated measures with bull, time and the interaction as fixed effects. The correlation between overall bull effect least square mean and P/AI reported by Zoca et al. (2020) were evaluated using the CORR procedure of SAS. Results are presented as mean ± SE. Statistical difference was defined as $P \leq 0.05$ and when $P > 0.05$ but $P \leq 0.10$ the results were considered as tendency.

Results and Discussion

It is well established that females must conceive in the first 21-d of the breeding season to achieve their maximum fertility potential and maximize profitability. A delay in conception will lead to decreased longevity in the herd, will hinder calf weaning weight, and overall production (Cushman et al., 2013). To conceive early in the breeding season and maintain a pregnancy, females must be cyclic, in good body condition, and on a positive plane of nutrition; however, bull fertility also plays an important role. A BSE is essential for selection of highly fertile bulls that will contribute to early conception in a breeding season (Barth, 2018); however, passing a BSE does not guarantee high fertility. Thus, the study of semen characteristics that can better predict bull fertility is necessary. In the present study, semen from two studies (Richardson et al., 2017; Zoca et al., 2020) were analyzed to evaluate the effect of inducing capacitation *in vitro* and the ability to estimate differences between bulls with different fertility classifications.

An ejaculate is composed of a heterogeneous population of sperm, and fertility is multifactorial (Rodriguez-Martinez, 2003). Amann and Hammerstedt (1993) suggested that an ejaculate or inseminate must have "enough" of all necessary sperm characteristics to reach a high level of fertility. In the present study, the difference between bulls (overall effect of bull) for acrosome integrity results were significant ($P < 0.01$) for viable disrupted and dead disrupted, tended to be significant for viable intact ($P = 0.06$), and were not significant ($P = 0.12$) for dead intact. Reactive oxygen species results were not different between bulls ($P \geq 0.25$) for viable ROS+, viable ROS-, and dead ROS+, and it was significantly different between bulls for dead ROS- ($P < 0.01$). There were also no differences between bulls for mito-potential. Even though some of the results for acrosome integrity, ROS, and mito-potential were different between bulls, they did not have an association with field fertility of the bulls evaluated. Similarly, bull by time interaction for those sperm characteristics was not able to estimate bull fertility differences. It has been reported that acrosome integrity, ROS, and mito-potential were associated or correlated with bull fertility (Oliveira et al., 2014; Kumaresan et al.,

2017; Bernecic et al., 2021). One difference between studies is the range in fertility among bulls tested. Thus, it is possible to conclude that fertility of bulls in this study were not limited by acrosome integrity, ROS, or mito-potential.

The present study was able to detect differences ($P \leq 0.05$) between bulls at pre-wash with total motility analysis (Fig. 1), at 0 h when diluted in a non-capacitation media with viability analysis (Fig. 2), and at 0 h when diluted in a capacitation media with zinc analysis, more specifically zinc signature 1 + 2 combined (Fig. 3). These analyses and time points were statistically different and were able to estimate differences in fertility between bulls. In these analyses and time points, respectively, bulls classified as high fertility (A and B) and bulls classified as intermediary fertility (C) had a greater proportion of total motility, viability, and zinc signature 1 + 2 combined compared to bulls classified as low fertility (D and E). When all time points were evaluated (overall bull effect; table 1), high and intermediary fertility bulls had a greater proportion of viable sperm ($P < 0.01$; viability assay), zinc signature 2 ($P < 0.01$), and zinc signature 1 + 2 ($P < 0.01$) compared to low fertility bulls. Also, the proportion of dead CD9+ was lower ($P < 0.01$) in high and intermediary fertility bulls compared to low fertility bulls (Table 1). The correlation between sperm parameters and fertility has been extensively studied, motility has been reported to have a moderate correlation (r = 0.58; Farrell et al., 1998), while viability has been reported to have a weak correlation ($r \leq 0.20$; Alm et al., 2001; DeJarnette et al., 2021) to a moderate correlation ($0.40 < r < 0.70$; Januskauskas et al., 2001; 2003; Anzar et al., 2002; Christensen et al., 2005) to a strong correlation ($r \geq 0.85$; Anzar et al., 2002; Kumaresan et al., 2017). We observed an association between field fertility and viability, zinc signature 2, zinc signature 1 + 2, and dead CD9+ among bulls. When correlations were evaluated, there was or tended to be a strong positive correlation between field fertility and viability ($P = 0.10$; r = 0.81), zinc signature 2 ($P = 0.04$; r = 0.89) and zinc signature 1 + 2 ($P = 0.10$; r = 0.80); however, dead CD9+ did not correlate with fertility ($P = 0.20$; r = -0.68). Although percent dead ROS- did not estimate fertility differences between bulls, dead ROS- was negatively correlated with field fertility ($P = 0.03$; r = -0.91). There was no correlation between field fertility and other sperm parameters evaluated ($P > 0.10$).

In conclusion, measures of viability, zinc signature 2, zinc signature 1 + 2 and dead CD9+ in capacitation media provided estimates of bull fertility. Also, total motility at pre-wash, viability at 0 h in non-capacitation media, and zinc signature 1 + 2 at 0 h in capacitation media were able to estimate field fertility differences between bulls.

Implications

The inclusion of a viability, a zinc signature, or a CD9 protein assay in AI studs' quality control measurements may have the potential to predict bull fertility; however, a larger number of bulls with known fertility need to be evaluated to validate these results. CD9 protein appears to be a promising biomarker of bull fertility; however, more research is necessary to confirm these results.

Acknowledgements

This research was funded through the donation of semen by Select Sires, Inc. and through the Support Hatch Funds. Dr. Thomas W. Geary and Abigail L. Zezeski are located at USDA-ARS, Fort Keogh Livestock and Range Research Laboratory, Miles City, MT; Dr. Karl C. Kerns is located at University of Iowa, Ames, IA; Dr. Joseph C. Dalton is located at University of Idaho, Caldwell, ID; Dr. Bo R. Harstine and Dr. Matthew D. Utt are located at Select Sires Inc., Plain City, OH; Dr. Robert A. Cushman, USDA-ARS, Roman L. Hruska US Meat Animal Research Center, Clay Center NE; and Dr. George A. Perry current location is Texas A&M University AgriLife Research and Extension Center, Overton, TX. South Dakota State University and USDA are a equal opportunity providers and employers.

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Tables

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

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

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

Figures

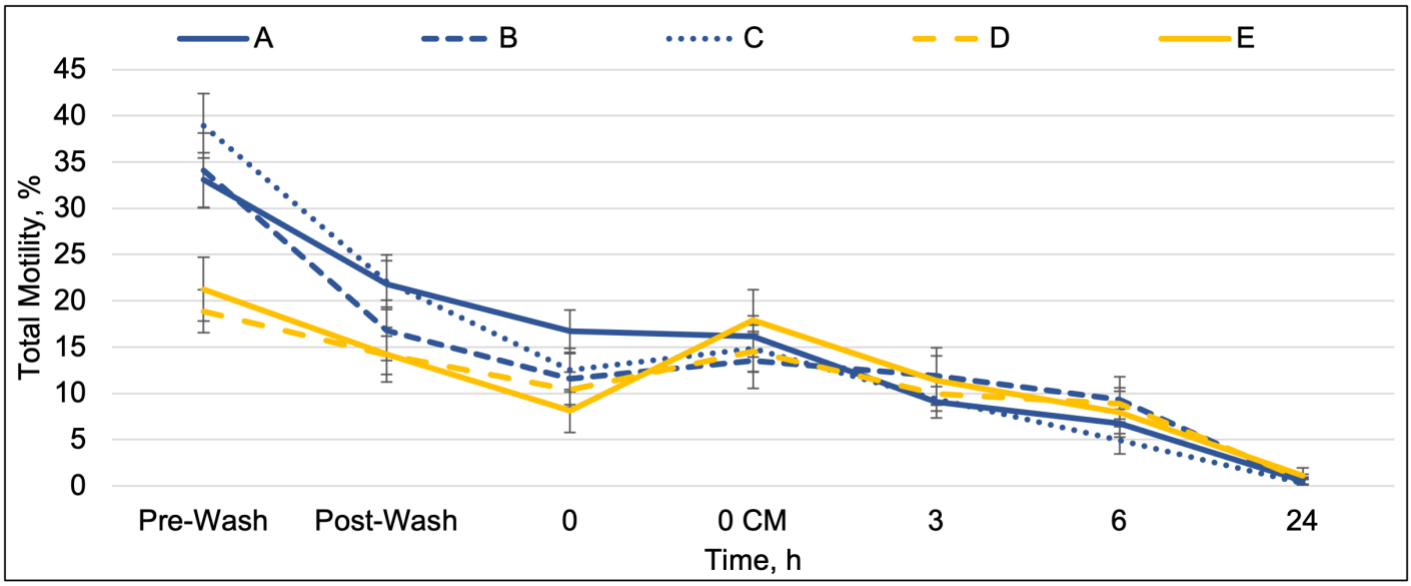

Figure 1. Percentage of total motility per bull at each time point incubated in capacitation media. There was a bull by time interaction ($P < 0.01$). Bulls A and B were classified as high fertility, bull C as intermediary fertility, and bulls D and E were classified as low fertility. At 0 h samples were evaluated in a non-capacitation media (0 h) and in a capacitation media (0 CM).

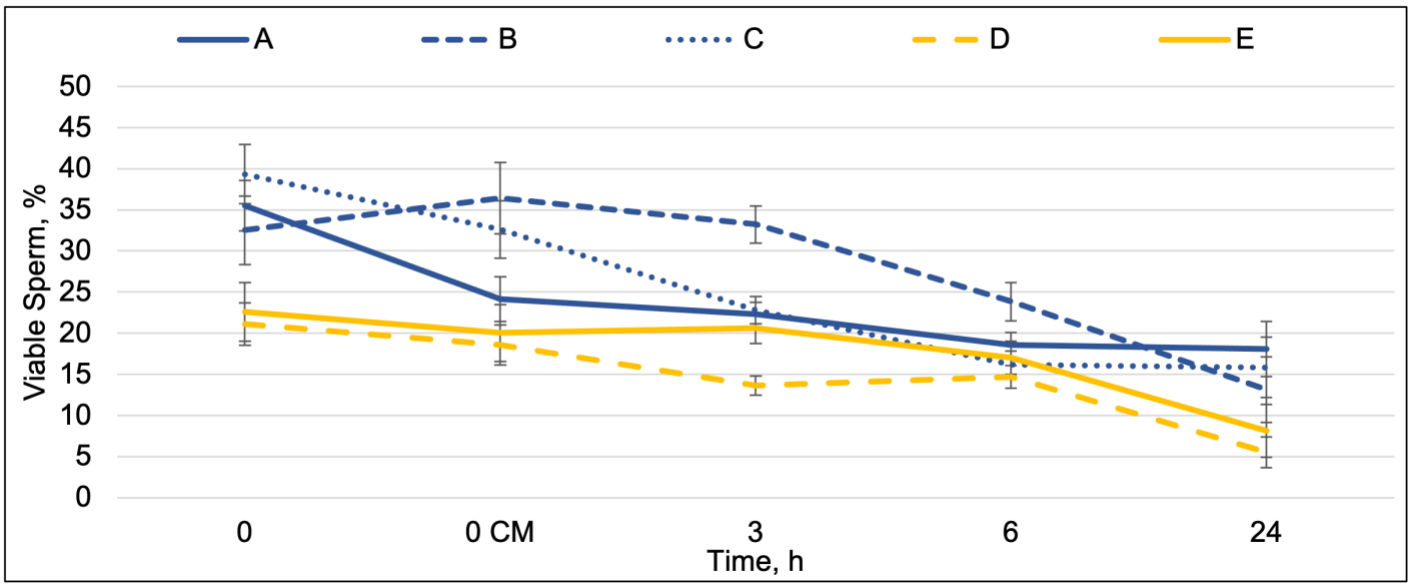

Figure 2. Percentage of viable sperm from the viability assay per bull at each time point incubated in capacitation media. There was a bull by time interaction ($P < 0.01$). Bulls A and B were classified as high fertility, bull C as intermediary fertility, and bulls D and E were classified as low fertility. At 0 h samples were evaluated in a non-capacitation media (0 h) and in a capacitation media (0 CM).

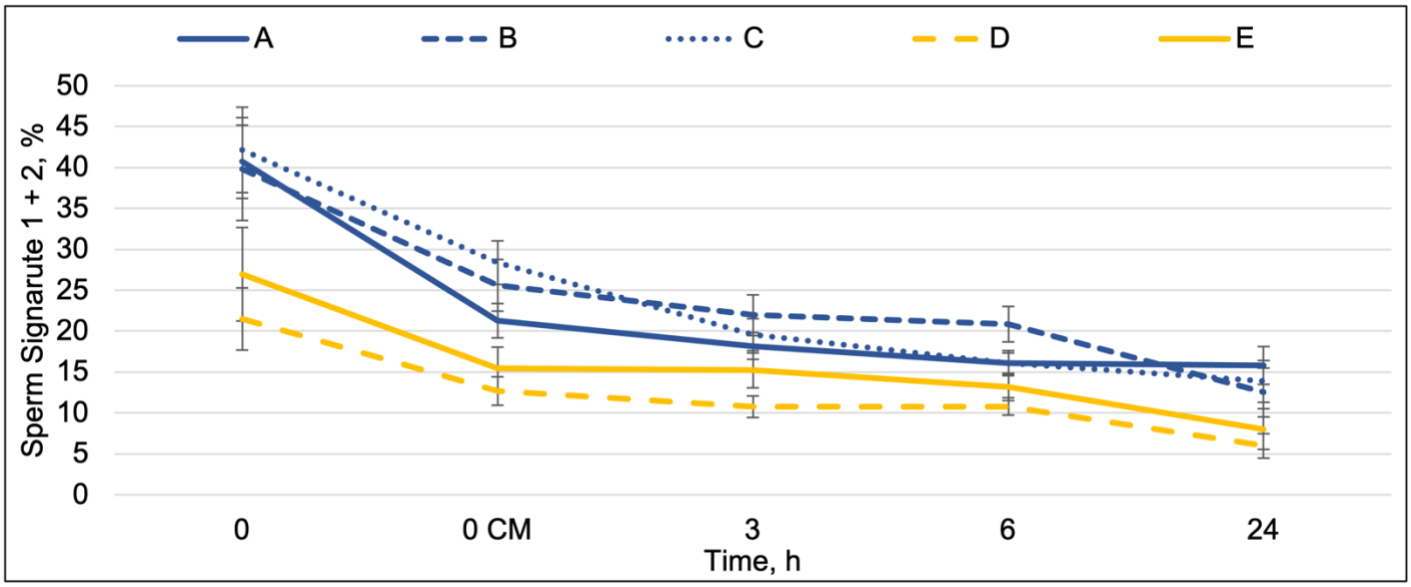

Figure 3. Percentage of zinc signature 1 + 2 sperm from the zinc signature assay per bull at each time point incubated in capacitation media. There tended to be a bull by time interaction (*P* = 0.06). Bulls A and B were classified as high fertility, bull C as intermediary fertility, and bulls D and E were classified as low fertility. At 0 h samples were evaluated in a non-capacitation media (0 h) and in a capacitation media (0 CM).