



## Sperm cellular and nuclear dynamics associated with bull fertility

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### ABSTRACT

The objective of this study was to ascertain cellular characteristics and the dynamics of the sperm chromatin proteins protamine 1 (PRM1) and protamine 2 (PRM2) in the sperm of Holstein bulls having a different fertility status. Important sperm variables were analyzed using computer-assisted sperm analysis (CASA). Sperm membrane, acrosome status, DNA integrity were also assessed using propidium iodide (PI), fluorescein isothiocyanate conjugated to *Arachis hypogaea* (FITC-PNA), and acridine orange (AO) followed by flow cytometry. In addition, abundances of PRM1 and PRM2 were analyzed using flow cytometry experiments. Differences in sperm decondensation capacity were assessed in bulls of varying fertility using a decondensation assay. As determined using CASA, average pathway velocity, amplitude of lateral head displacement and straightness were different ( $P < 0.05$ ) for sperm from high and low fertility bulls. There, however, were no differences between the high and low fertility bulls for characteristics of sperm plasma membrane, acrosome, and DNA integrity ( $P > 0.05$ ). Relative abundances of PRM1 and PRM2 in sperm from the high and low fertility bulls were inversely related ( $P < 0.0001$ ). Percentages of decondensed sperm were different between high and low fertility bulls ( $P < 0.0001$ ) and total numbers of decondensed sperm were greater in low fertility bulls than high fertility bulls ( $R^2 = 0.72$ ). Results of the present study are significant because molecular and morphological phenotypes of sperm that were detected affect fertility in livestock species.

### 1. Introduction

During spermiogenesis, spermatids differentiate into spermatozoa and most of the histones are replaced by protamines to protect the paternal genome. The chromosomal DNA of spermatozoa is compressed into a less than one nanometer space in the cell nucleus as

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a result of the actions of protamines and histones. Histones are basic proteins, whereas protamines are arginine rich proteins, highly basic than histones and are abundantly present in sperm (Takeda et al., 2016). Nuclear remodeling and condensation during spermiogenesis result in a controlled release of histones first by transition nuclear proteins and possibly primarily as a result of protamine actions (Balhorn et al., 1984; Kistler et al., 1996). The histone and protamine transition is essential for efficient chromatin compaction to inhibit sperm gene expression. In addition, for maintenance of sperm DNA integrity and for regulating the shape of the nucleus and thus the sperm head so these cells have greater hydrodynamic capacity (Braun, 2001).

Mammalian sperm chromatin is not entirely packed with protamines because in a 1%–15% portion of the sperm chromatin there is retention of the histones (Gatewood et al., 1987; Hammoud et al., 2009; Erkek et al., 2013; Samans et al., 2014). Among these retained histones few are specific to sperm such as TH2B, TH2A, H2AL1, H2AL2, H3.3A and H3.3B (Govin et al., 2007; Kutchy et al., 2017) and the linker histones H1T2 and H1LS1 (Martianov et al., 2005). Protamines are important for efficient compression of sperm DNA into a 10-fold greater compacted state compared to other spermatogenic cell types such as spermatogonia until there is differentiation into round spermatids as a result of protamine toroid actions (Ward, 2010). Protamines are positively charged and induce increased condensation with the negatively charged paternal genomic DNA (Oliva, 1995; Queralt and Oliva, 1995; Lewis et al., 2003). Because of cysteine amino acids in protamines, tight disulphide bridges are created between the protamine molecules resulting in a stabilized nucleoprotamine complex (Lewis et al., 2003; Vilfan et al., 2004).

Most primates have a single copy of PRM1 and PRM2 genes (Nelson and Krawetz, 1993; Queralt and Oliva, 1993; Nelson and Krawetz, 1994; Schlueter et al., 1996). The PRM1 and PRM2 genes create a loop domain in conjunction with the transition protein 2 (TNP2) plus a sequence termed as gene 4 (Wykes and Krawetz, 2003; Martins et al., 2004). This three-dimensional spatial organization may facilitate a coordinated expression of these genes during spermatogenesis. While protamines (PRM1 and PRM2) and transition proteins (TNP1 and TNP2) are present during specific spermatogenic stages the functions of these proteins having been extensively studied in humans and mice, but there is only limited information for cattle.

While protamines have important functions in bull sperm development and reproduction, the mechanisms regulating sperm physiology are unclear. Protamines function as a shield for the sperm genetic material and transport of the sperm DNA into the egg (Oliva and Dixon, 1991; Mengual et al., 2003). Compromised protamination leads to damage of sperm by endogenous or exogenous agents, such as nucleases, free radicals or mutagens (Irvine et al., 2000; Alvarez et al., 2002). As determined using a sperm chromatin structure assay (SCSA), abnormal protamination leads to DNA-fragmentation that in turn leads to less fertilization success when there is intracytoplasmic sperm injection (ICSI) and lesser *in vitro* fertilization (IVF) rates with use of these technologies (Evenson et al., 1980; Evenson and Wixon, 2005). Abundances of protamines in human sperm are directly related to sperm DNA integrity (Aoki et al., 2005) and developmental defects after fertilization have occurred as a result of decreased DNA integrity in mice (Tesarik et al., 2004; Lewis and Aitken, 2005; Sukanuma et al., 2005). Studies of protamines could lead to an enhanced understanding of epigenetic programming, and development of novel approaches for early diagnosis and treatment of infertility (Bao and Bedford, 2016). The objectives of the present study were to determine the associations between sperm kinetics, semen characteristics and bull fertility, and to ascertain the differential abundances of PRM1 and PRM2 in spermatozoa from bulls with contrasting fertility phenotypes.

## 2. Material and methods

### 2.1. Semen procedures and determination of sperm kinetics using computer-assisted sperm analysis system (CASA)

Semen samples were collected from ten mature Holstein bulls using an artificial vagina, and sperm were cryopreserved using standard procedures at Alta Genetics (Table 1; Alta Genetics, Inc., Watertown, WI, USA). Cryopreserved sperm stored in semen straws, from each bull, were used to analyze sperm motion characteristics. Methods for determination of bull fertility have been previously described (Peddinti et al., 2008). The calculation of bull fertility was similar to those used in previous studies (Killian et al., 1993; Moura et al., 2006, 2007). The conception rates were confirmed by either uterine palpation by a veterinarian or ultrasonic assessments in the field, and factors affecting fertility of sires such as environment and herd management were adjusted

**Table 1**  
Fertility data of bulls with sperm samples used in study.

Bulls	Fertility group	Number of breeding's	<i>In vivo</i> fertility scores	Std. dev.
High fertility bulls				
1	HF-1	748	3.0	1.406
2	HF-2	965	3.0	1.371
3	HF-3	682	2.9	1.339
4	HF-4	3647	2.8	1.311
5	HF-5	6051	2.6	1.198
Low fertility bulls				
6	LF-1	1020	−3.6	−1.658
7	LF-2	927	−3.6	−1.664
8	LF-3	901	−3.6	−1.649
9	LF-4	1105	−3.7	−1.5979
10	LF-5	987	−3.7	−1.73

(Zwald et al., 2004a, 2004b). The average conception based on breeding records and conception rates were determined using the Probit F90 software (Chang et al., 2004). There was selection of bulls for the study based on conception rates when there was use of their semen for AI. This occurred by selecting bulls that were two standard deviations greater and lesser than the conception rate average for bulls in the population for which there were available records. For this study, the fertility phenotypes were grouped into high fertility (HF;  $n = 5$ ) when bulls had percent differences in conception rates two standard deviations greater than the average and low fertility (LF;  $n = 5$ ) when sires had percent differences that were two standard deviations less than the average for conception rates.

For determination of cellular characteristics of the sperm, semen in straws (250  $\mu$ l) were thawed in a water bath at 37 °C for 30 s. Semen was then transferred to 2.0 ml centrifuge tubes and diluted to  $5 \times 10^6$  sperm/ml in 0.0067 M phosphate buffered saline (HyClone, GE Healthcare Life Sciences, Logan Utah). Motility and kinetic variables for bull sperm were assessed using HTM-IVOS (Hamilton-Thorne Biosciences; Beverly, MA, USA), adjusted for bull (SETUP “bovine species”). An aliquot of diluted semen (5  $\mu$ l) was placed on a pre-warmed standard count four chamber slide (depth 10  $\mu$ m) (Leja® Products B.V. Luzernestraat, Nieuw Vennep, Netherland), and at least 200 sperm were assessed using a phase contrast microscope at 100X magnification. Three aliquots from each bull semen sample were placed in a standard chamber and evaluated in randomly selected fields of view for each sample. The following variables were subsequently evaluated: total motility (%), progressive motility (%), average path velocity (VAP,  $\mu$ m/s), straight line velocity (VSL,  $\mu$ m/s), curvilinear velocity (VCL,  $\mu$ m/s), amplitude of lateral head displacement (ALH,  $\mu$ m), beat cross frequency (BCF, Hz), straightness (STR, %,  $VSL/VAP \times 100$ ), and linearity (LIN, %,  $VSL/VCL \times 100$ ). The spermatozoa were also classified as rapid (VAP > 80  $\mu$ m/s), medium (80  $\mu$ m/s > VAP > 25  $\mu$ m/s), slow (VAP > 25  $\mu$ m/s), and static (VAP = 0). Progressive motility cutoff values were VAP  $\geq$  80  $\mu$ m/s and STR  $\geq$  75%.

## 2.2. Evaluation of sperm membrane integrity and acrosome status using flow cytometry

The acrosome status of bull sperm was assessed using fluorescein isothiocyanate conjugated to *Arachis hypogaea* (peanut; L7381 FITC-PNA, Sigma-Aldrich Co., St. Louis, MO, USA) and using Propidium Iodide (PI) staining, as described previously (Nagy et al., 2003). Three semen straws ( $30 \times 10^6$ ) were thawed in a water bath at 37 °C for 30 s and washed with PBS three times using centrifugation at 700 g at 4 °C for 10 min. The supernatant was carefully discarded, and the pellet was re-suspended in 1 ml of PBS and were filtered (20  $\mu$ m syringe filter) through a flow cytometric tube using a cell strainer cap (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Sperm concentration was determined using a hemocytometer. The FITC-PNA working solution (Flesch et al., 1998) at a final concentration of 1  $\mu$ g/ml, 1 mg/ml of stock solution with DMSO (working solution further diluted five-fold with PBS) and 12  $\mu$ M PI solution (P4864, Aldrich Sigma-Aldrich Co., St. Louis, MO, USA) were added to 1 ml of diluted semen. Samples were mixed and incubated at 37 °C for 5 min. and remixed before analysis.

The processed semen samples were screened using a FACSCalibur flow cytometry (BD Bioscience San Jose, CA, USA), an automated benchtop flow cytometry system, and CellQuest Pro software (BD Biosciences, San Jose, CA, USA) were used to record a total of 10,000 events. The cytometer was used at the low flow rate of 12  $\mu$ l/min. The instrument was calibrated using BD Calibrite beads. The fluorescence from the sperm samples stained with FITC-PNA and PI was excited with a 488 nm laser and recorded with the use of FL1 (530/30 nm filter) and FL2 (584/42 nm filter). Compensation was used to minimize the spillover of fluorescence. Fluorescence parameters were displayed in log representation. The unstained sample was used as the negative control. The subpopulations of sperm were divided by quadrants, and the frequency of each subpopulation was quantified. Three replicates were performed for each bull sample.

## 2.3. Flow cytometric evaluation of DNA integrity in bull spermatozoa

The DNA integrity assay was used to measure the DNA fragmentation index and was conducted using the method previously described by Evenson et al. (Evenson et al., 2002). Briefly, frozen/thawed semen samples ( $10 \times 10^6$ ) from each bull were thawed at 37 °C for 30 s, washed three times with PBS (700 g; 4 °C; 10 min), and filtered through a flow cytometric tube. Following the last centrifugation, the supernatant was discarded, and the pellet was diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM disodium EDTA; pH 7.4) to a concentration of  $2 \times 10^6$  cells/ml and immediately mixed with 400  $\mu$ l of the acid-detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2, 4 °C). Then, cells were incubated for 30 s in 1.2 ml acridine orange solution (0.037 M citric acid, 0.126 M Na<sub>2</sub>HPO<sub>4</sub>, 0.0011 M disodium EDTA, 0.15 M NaCl, pH 6.0) containing 6  $\mu$ g/ml electrophoretically purified AO (24603-10 2% Acridine Orange, Polysciences, Inc, Warrington, PA) in a phosphate citrate buffer (0.037 M citric acid; 0.126 M Na<sub>2</sub>HPO<sub>4</sub>; 0.0011 M EDTA (disodium), 0.15 M NaCl, pH 6.0).

Sperm samples were analyzed using the BD-FACSCalibur flow cytometer (BD Bioscience, USA) as previously described (Section 2.2 of this manuscript). After calibration, 10,000 events were collected per sample. Values for green and red fluorescence intensities were collected with use of 530/30 nm and 584/42 nm filters, respectively utilizing BD CellQuest software. With these conditions, AO intercalated with double-stranded DNA emits a green fluorescence, and AO associated with single-stranded DNA emits a red fluorescence. Measurements were determined in triplicate, and unstained samples were used as negative controls. The DNA denaturation represents the ratio of red-to-red plus green fluorescence intensity.

## 2.4. Flow cytometric evaluation of PRM1 and PRM2 abundance in bull spermatozoa

To quantify the enrichment of PRM1 and PRM2 in bull spermatozoa, the signal intensities of these proteins were recorded using

flow cytometry. Semen straws from five HF and five LF Holstein bulls were removed from liquid nitrogen and thawed at 37 °C for 30 s. Samples were washed twice in PBS with 0.1% Bovine Serum Albumin (BSA) and centrifuged at 1,000 g at 4 °C for 5 min. Sperm were counted using a Neubauer-chamber and approximately 2–3 million cells were distributed in a separate centrifuge tube and then fixed in 1 ml of 4% formaldehyde at room temperature for 1 h. The samples were then centrifuged at 5000 g and 4 °C for 5 min. and the pellets were re-suspended in 250 µl of PBS and immediately permeabilized in 250 µl of 0.1% Triton X-100 in 0.1% of sodium citrate in PBS on ice for 2 min. The pellets were re-suspended in 500 µl of PBS, filtered through a flow cytometric tube using a cell strainer cap (Becton Dickinson Labware; catalogue no. 352235), and then incubated with the primary antibodies at 4 °C overnight. The primary antibodies used were rabbit polyclonal PRM1 (M-51) monoclonal (Santa Cruz Biotechnology, Dallas, TX, USA; catalog # sc30174; 1/250 dilution) and mouse monoclonal antibody PRM2 (Briar Patch Biosciences, Livermore, CA, USA; catalog # Hup 2B; 1/250 dilution). On the following day, samples were centrifuged at 3,000 g at 4 °C for 5 min, washed once in 500 µl washing buffer, centrifuged at 3,000 g and 4 °C for 5 min. and incubated with secondary antibodies at room temperature for 1.5 h. The secondary antibodies were Donkey anti rabbit IgG (FITC; Santa Cruz, Dallas, Texas, USA; catalog # sc2090; 1/250 dilution) and goat anti-mouse IgG H&L (DyLight® 650; Abcam, Cambridge, MA, USA; catalog # 96874; 1/250 dilution) Following the incubation, the samples were washed twice in PBS with 0.1% BSA (3,000 g at 4 °C for 5 min.). A total of  $6 \times 10^6$  sperm was used from each bull. The experiment was repeated five times, and sperm samples were then analyzed using the BD-FACSCalibur flow cytometer (BD Bioscience, USA) and data were collected as FL1 (530/30 nm filter) compared with FL4 (661/16 nm filter). There were 150,000 spermatozoa evaluated per sample in each replicate.

### 2.5. Sperm decondensation assay

The nuclear chromatin decondensation assay (NCD) was performed using a modified protocol as described by Gopalkrishnan (Gopalkrishnan et al., 1991) and Sreenivasa et al (Sreenivasa et al., 2012). Semen samples were thawed at 37 °C for 30 s, and centrifuged (700 g; 4 °C) for 10 min. Supernatant was then removed, and the pellet was washed thrice using 1 ml of TNE buffer. A sperm sample from each bull was collected with treatment which served as a control. The remaining sperm pellet from each bull was re-suspended with 1 ml of 5 mM of DTT and 40 µM heparin in double distilled water followed by incubation (37 °C for 60 min.) and centrifugation (10 min.). Washing was performed three times with 500 µl of TNE buffer at 700 g at 4 °C for 5 min. After removing the supernatant, 500 µl of 3% glutaraldehyde was added and mixed by vortexing. There was 500 µl of sample placed on clean glass slides and allowed to settle for 10 min., then 1 ml of DAPI in PBS was added for 10 min. Washing of the slide with 1 ml of PBS was performed twice. The slide was covered with a coverslip and sealed with vecta-shield.

### 2.6. Statistical analyses

Original flow cytometry data were analyzed using FlowJo (FlowJo LLC). Data collected using the CASA were analyzed using the GraphPad Prism software, version 5 (GraphPad, San Diego, CA, USA). Data were assessed for normality using the Shapiro-Wilk test and variables not normally distributed were arcsine transformed prior to the statistical analyses. The Levene's test was used to evaluate the homogeneity of variances. Statistical differences between the two groups (HF compared with LF bulls) were calculated using the t-test, and a  $P$ -value of  $P < 0.05$  was considered significant. Data were reported as the mean and standard deviation (SD). The relationship between PRM1 and PRM2 was determined using SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC). To meet the assumptions of the statistical tests, values for PRM1 and PRM2 were natural log transformed (LPRM1 and LPRM2). The linear mixed models were individually fit with PROC MIXED for LPRM1 and LPRM2 as outcomes. Grouping of bulls based on fertility score was included as a fixed effect with bull included as a random effect in each model, and individual fertility score was the intra-subject factor of the mixed model. In addition, separate linear mixed models were fit with LPRM1 and LPRM2 as response variables with LPRM2 and LPRM1 as fixed effects, respectively. Group as a fixed effect and bull as a random effect were also included in each of the models. Separate models with fertility score as the fixed effect were fit for both the HF and LF groups to improve the understanding of the association between fertility score and both LPRM1 and LPRM2. Regression line plots were developed using PROC SGPLOT. The regression line was determined using the model-predicted values of LPRM1 and LPRM2 for each value of fertility score, LPRM2, or LPRM1 as indicated using the mixed effects model. An alpha level of 0.05 was used to determine statistical significance. Differences between HF and LF bulls related to sperm decondensation were analyzed using the Kruskal-Wallis Test (SAS Institute, Inc., Cary, NC; alpha level = 0.05).

## 3. Results

### 3.1. Sperm kinetics, sperm plasma membrane, acrosome status and DNA chromatin damage

Based on analyses of semen samples using CASA, HF bulls had sperm with a greater average pathway velocity, lateral head displacement and straightness of sperm motility as indicated by its amplitude ( $P < 0.05$ ). Both the percentage of motile and rapidly progressive motile sperm, however, were similar ( $P > 0.05$ ) for the two groups of bulls (Table 2). Values obtained for sperm plasma membrane integrity, acrosome status, and DNA fragmentation assessments using flow cytometry were not different ( $P > 0.05$ ) between HF and LF bulls (Fig. 1).

**Table 2**

Sperm variables (Mean values  $\pm$  S) of frozen – thawed bull spermatozoa with different fertility phenotypes; Different superscript letters in the same row indicate differences ( $P < 0.05$ ).

Evaluations	Fertility phenotypes	
	High fertility	Low Fertility
Semen variables		
Motile spermatozoa (%)	55.6 $\pm$ 2.4	52.4 $\pm$ 3.6
Rapidly progressive motile spermatozoa (%)	40.5 $\pm$ 2.4	38.5 $\pm$ 3.0
Average path velocity ( $\mu\text{m/s}$ )	91.5 $\pm$ 1.7 <sup>a</sup>	82.61 $\pm$ 3.4 <sup>b</sup>
Amplitude of lateral head displacement ( $\mu\text{m/s}$ )	8.8 $\pm$ 0.4 <sup>a</sup>	7.69 $\pm$ 0.4 <sup>b</sup>
Path straightness (%)	70.4 $\pm$ 1.9 <sup>a</sup>	73.66 $\pm$ 1.6 <sup>b</sup>

### 3.2. PRM1 and PRM2 abundance in sperm from HF and LF bulls

Flow cytometric measurements of PRM1 and PRM2 sperm indicated there were different histogram profiles for sperm from the HF and LF bulls (Figs. 2A–C; 3 A&B). The abundance of LPRM1 and LPRM2 was not different between HF and LF bulls (Figs. 4A&B; 5 A&B). Considering all bulls (both HF and LF bulls), the relative abundances of LPRM1 and LPRM2 were inversely related to each other ( $P < 0.0001$ ; Fig. 6).

### 3.3. Sperm decondensation and bull fertility

The nuclear chromatin decondensation assay was performed to compare sperm nuclear decondensation between sperm from HF and LF bulls. The percentages of decondensed sperm cells were different between HF and LF bulls ( $P < 0.0001$ ; Fig. 7A–C). The percentage of decondensed sperm was greater in sperm from LF compared with HF bulls. The numbers of decondensed sperm were greater in sperm from LF than HF bulls ( $R^2 = 0.72$ ; Fig. 8).

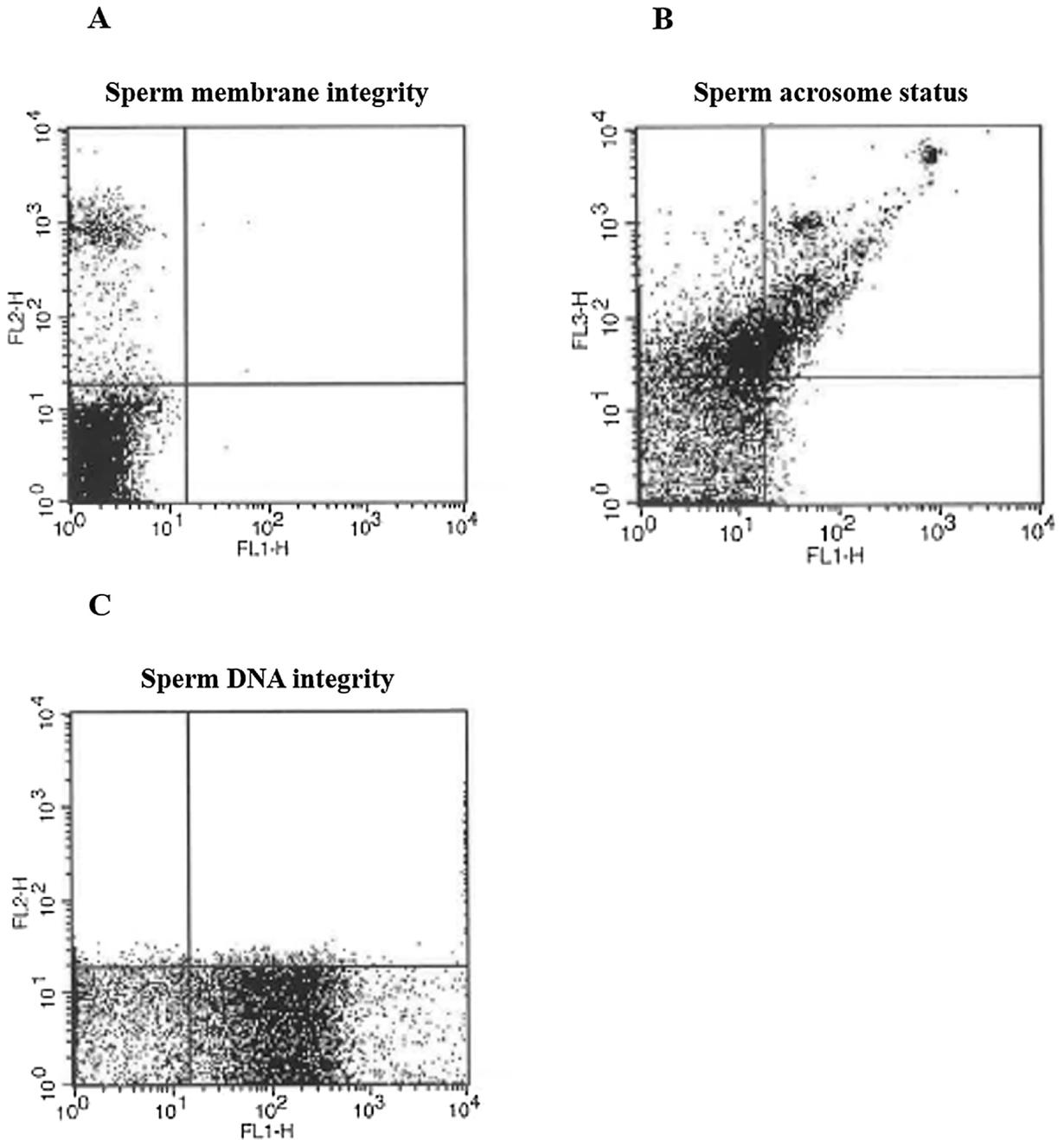
## 4. Discussion

Optimal rates of reproduction are important for efficient, profitable and sustainable production of cattle. The effects of sperm nuclear proteins on sperm head shape, size, motility and fertilizing capacity aid in determining male fertility of mammals. Although even with the production of ample sperm numbers having optimal motility and morphology, some bulls are infertile due to molecular defects in their sperm (Blaschek et al., 2011; Memili et al., 2012). The CASA system and flow cytometry analyses were used to test the hypothesis that sperm cellular phenotypes as well as PRM1 and PRM2 abundances are associated with bull fertility. There was a focus in this pioneering study on improving the knowledge base and bio-techniques that could be used for cattle reproduction.

The average pathway velocity, amplitude of lateral head displacement, and path straightness were different in sperm from HF and LF bulls. In the present study, proportions of rapidly progressive motility and lateral head displacement spermatozoa were less in sperm from LF compared with HF bulls which is consistent with findings in previous studies (Hoflack et al., 2007; Birck et al., 2010; Singh et al., 2016). Surprisingly, in the present study sperm plasma membrane integrity and acrosome status, as well as sperm DNA fragmentation status, were not different between semen samples from high and LF bulls. A functional plasma membrane is dependent on membrane ion channels (Petrunikina et al., 2001, 2004) and directly related to sperm fertilizing potential (Tartaglione and Ritta, 2004). The mechanisms underlying the alterations of membrane functionality and its effect on fertility are not fully understood. Results from previous studies also indicate there is a non-significant association between sperm characteristics and plasma membrane integrity among bulls (Mishra et al., 2013).

Sperm DNA is tightly packaged when replacement of histones by protamines occurs, which makes sperm chromatin highly condensed. This highly condensed state allows for protection of the integrity of paternal genetic material during the period of transiting through the female reproductive tract. Following fertilization, disulfide bonds between protamines are decreased, and highly condensed and transcriptionally inactive sperm chromatin is converted into the decondensed state by replacing protamines by maternal histones. Results from previous studies indicate incomplete protamine deposition during spermiogenesis is the major determinant for chromatin packaging (Sakkas et al., 1999). Furthermore, results from previous studies indicate abnormal sperm chromatin decondensation during pronucleus formation can cause fertility problems (Queralt and Oliva, 1995), and that variability in the PRM1 abundances within sperm are associated with bull fertility (Dogan et al., 2015). In the present study, LF bulls produced larger numbers of decondensed sperm compared with HF bulls (Figs. 7 & 8). It is known that PRM1 and PRM2 are important for sperm molecular morphology and chromatin condensation. Insufficient condensation of sperm chromatin limits fertility (Balhorn et al., 1988) by causing DNA denaturation, DNA strand breaks (Belokopytova et al., 1993; De Yebra et al., 1993), and is associated with an increase in the retention of histones by sperm chromatin (Foresta et al., 1992). As such, abnormal sperm decondensation makes the male genome more susceptible to external factors that lead to increases in sperm damage (Auger et al., 1990).

Relative abundances of PRM1 and 2 were inversely associated in the bulls in the present study. Results of the dynamic changes of PRM2 in sperm indicated that there is no change in abundance of this protein, thus, the functions of this protein remain unexplained. Possible reasons could be that both PRM1 and PRM2 protamines, if present together in sperm, have a synergistic effect, where PRM2 functions in the incorporation of PRM1. Another explanation would be that the absence of one protamine allows for augmentation of



**Fig. 1.** Assessment of plasma membrane integrity, acrosome status, and DNA integrity of bull spermatozoa; Fluorescence cytograms for samples stained with (A) Propidium iodide, (B) FITC-PNA, and (C) acridine orange stain followed by flow cytometry evaluation.

the other protamine. Alternatively, the presence of PRM2 in minute amounts could be an indicator of a viable sperm population in bulls. This assumption is well-supported by results from a previous study (Cho et al., 2001), where results indicated PRM1 requires PRM2 to for incorporation into sperm chromatin. Also, the lesser abundances of PRM2 in the majority of infertile bulls occurred because of an abnormal PRM1/PRM2 ratio (Aoki et al., 2005, 2006). This abnormal ratio is associated with lesser fertility, DNA fragmentation, and morphological changes in bull sperm (Carrell and Liu, 2001; Razavi et al., 2003; Nasr-Esfahani et al., 2004; Aoki et al., 2005, 2006). In other studies with different mammals, results indicate that abnormal quantities of protamines lead to conformational changes in sperm formation during spermatogenesis and hence a decrease in male fertility (Carrell and Liu, 2001; Mengual et al., 2003; Aoki et al., 2005).

In conclusion, average pathway velocity, amplitude of lateral head displacement and path straightness of frozen-thawed sperm evaluated using CASA, but not sperm plasma membrane, acrosome status or DNA integrity, were distinct between bulls with

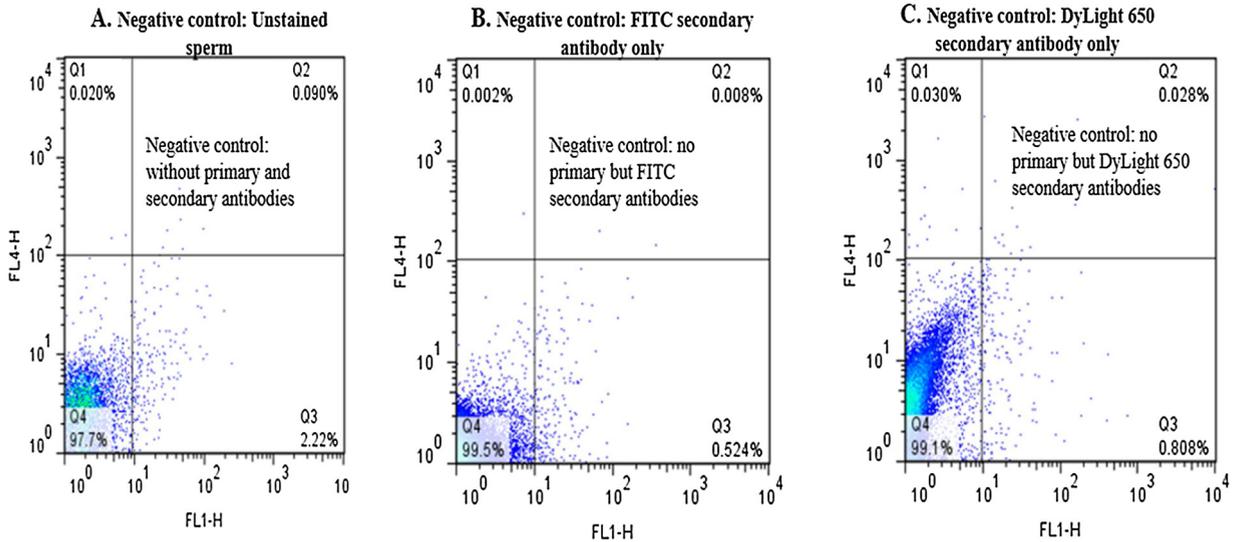


Fig. 2. Flow cytometry negative controls. There was an absence of fluorescence and unstained sperm in negative control samples (Q4; Fig. 2A); Negative control FITC as secondary antibody (Q4; Fig. 2B) to target PRM1; Negative control for DyLight 650 as secondary antibody (Q4; Fig. 2C) to target PRM2.

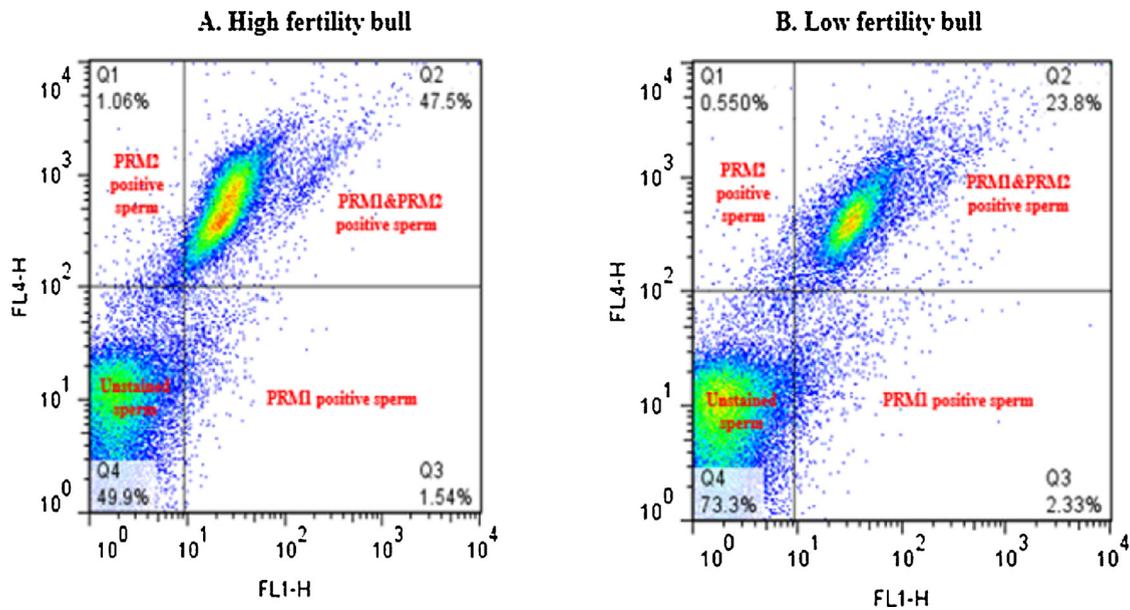


Fig. 3. Differential expression of PRM1 and PRM2 in bull spermatozoa; Percentage of spermatozoa with PRM2 (Q1), double positive (PRM1 and PRM2 together) (Q2), PRM1 (Q3), and unstained cells (Q4) in high fertility (Fig. 3A) and low fertility bulls (Fig. 3B).

contrasting *in vivo* fertility. Results from analysis of PRM1 and PRM2 abundances using flow cytometry indicated there were different histogram profiles in sperm from HF and LF bulls. Thus, flow cytometry and CASA analysis are valid approaches for evaluation of potential bull fertility. Furthermore, sperm nuclear proteins (PRM1 and PRM2) can also be important biomolecular markers of fertility. Results of the present study will advance the fundamental understanding of mammalian male gametes and provide information for reliable evaluation of semen quality and a more accurate prediction of bull fertility.

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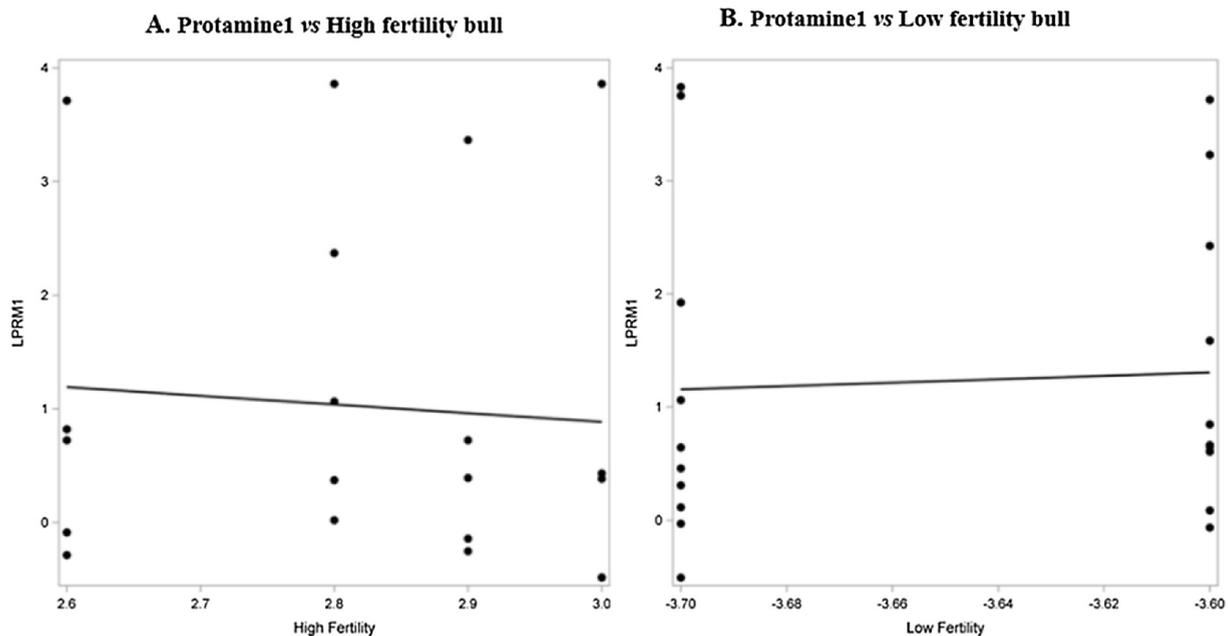


Fig. 4. Differential abundance of PRM1 in HF and LF bull sperm; Regression plot depicts the relationship of PRM1 with high (Fig. 4A) and low (Fig. 4B) bull fertility score.

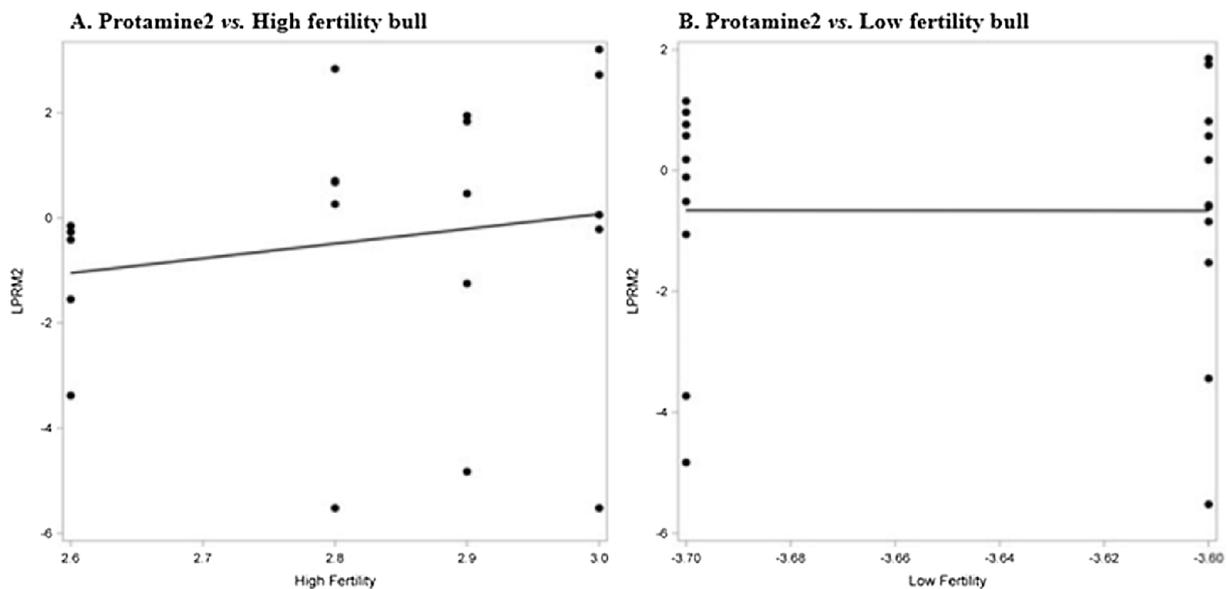
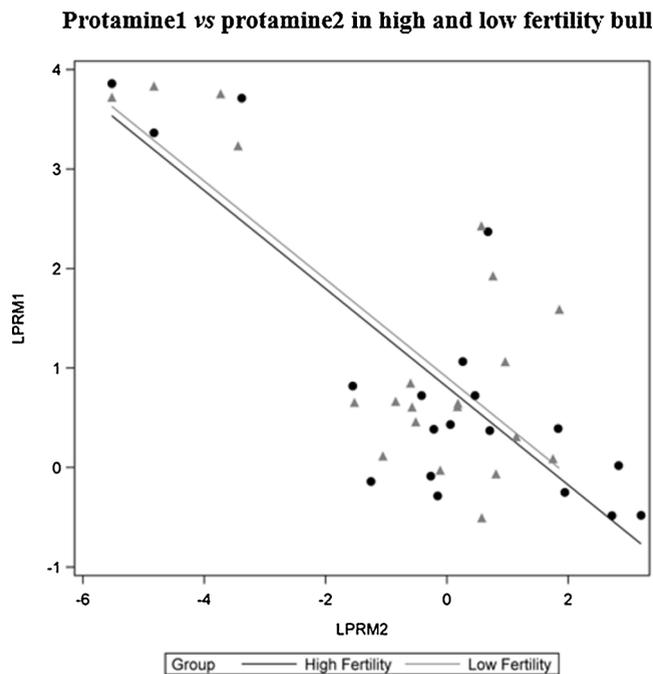


Fig. 5. Differential abundance of PRM2 in HF and LF bull sperm; Regression plot depicts the relationship of PRM2 with high (Fig. 5A) and low (Fig. 5B) bull fertility score.

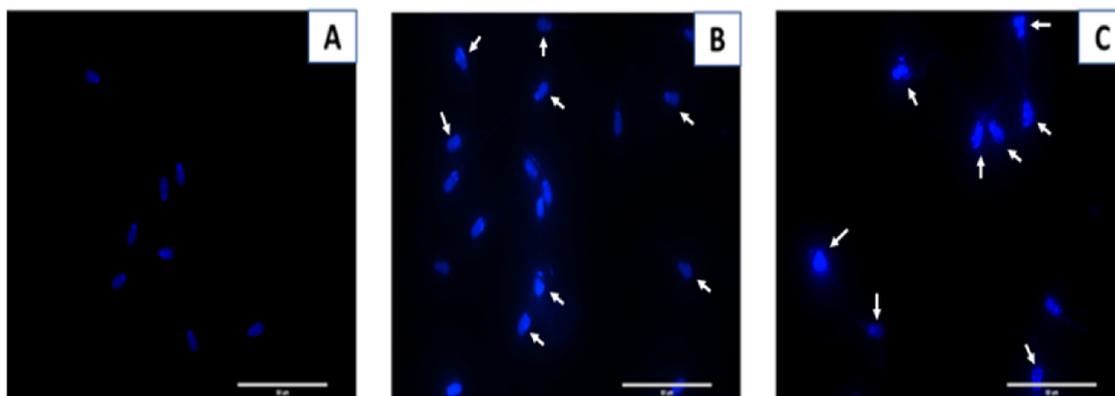
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**Author contributions**

This study was conceptualized by NAK, ESBM, EM, AK and AAM. Data were curated by NAK, MRU, RWW. The bull sperm samples and the fertility phenotypic data were provided by AK and ET. Investigations were conducted by NAK, ESBM, WT, MRU, AUH, HED, EB, HCE, FCS, and EM. The original manuscript draft was written by NAK, ESBM, MRU, EM. Reviews and editing were completed by



**Fig. 6.** Relationship between the abundances of PRM1 and PRM2 in bull sperm; Regression plot depicts the inverse relationship of log transformed PRM1 and PRM2 ( $P < 0.0001$ ).



**Fig. 7.** Fluorescent microscopy of decondensed sperm cells from high and low fertility bulls; Sperm samples from high fertility and low fertility bulls were treated with heparin glutaraldehyde, DTT, and then stained with DAPI; A) Negative Control; Sperm incubated for 1 h at room temperature in TNE buffer; B) Sperm from high fertility bull treated at 37 °C in heparin and glutaraldehyde, and DTT for 1 h; C) Sperm from low fertility bull treated at 37 °C in heparin and glutaraldehyde and DTT for 1 h; Arrows indicate de-condensed sperm.

NAK, ESBM, HED, EB, FS, AUH, HED, WT, RWW, ET, AK, AAM and EM. All authors read and approved the final version

#### Data statement

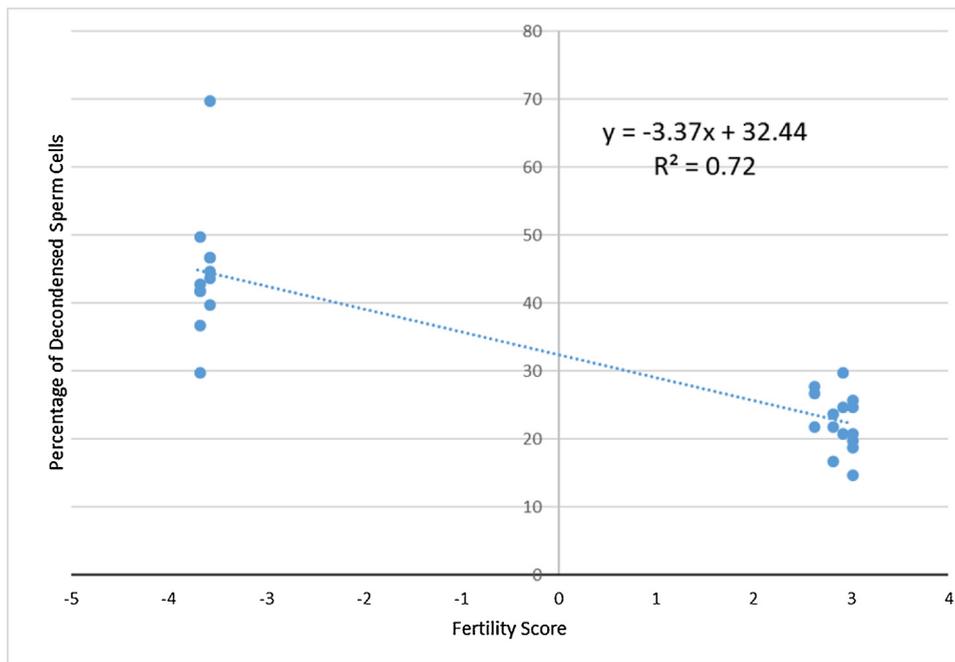
The data analyzed during the current study are available from the corresponding author on reasonable request.

#### Declaration of Competing Interest

The authors declare that there is no conflict of interest that could affect our objectivity in this study.

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**Fig. 8.** Differential decondensation rates in HF and LF bull sperm; Regression line represents percentage of decondensed sperm in high and low fertility bull sperm; 100 sperm cells/bull were evaluated using fluorescent microscopy to compare percentage of decondensed sperm cells in high and low fertility bulls; Difference between these two groups in terms of de-condensation tendencies ( $P < 0.0001$ ).

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