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Differences in sperm functionality and intracellular metabolites in Norwegian Red bulls of contrasting fertility

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ABSTRACT

In the dairy breeding industry, prediction of bull fertility in artificial insemination (AI) is important for efficient and economically sustainable production. However, it is challenging to identify bulls with superior fertility applying conventional *in vitro* sperm assays. In the present study, sperm functionality was investigated to identify a multivariate model that could predict fertility. Two groups of young Norwegian Red bulls were selected, one with inferior fertility (18 bulls) and one with superior fertility (19 bulls) based on non-return rate after 56 days (NR56). Frozen-thawed semen doses were analysed for sperm chromatin integrity, viability, acrosome integrity, motility, and ATP content. A targeted approach was used to study intracellular concentrations of amino acids and trace elements in viable sperm cells. Significant differences between the two groups of bulls were observed, both for sperm functional attributes and intracellular concentrations of metabolites. Pearson correlation analyses indicated a negative relationship between NR56 and chromatin integrity parameters, DNA fragmentation index (DFI) and high DNA stainability (HDS). Several motility parameters correlated positively with NR56. The concentrations of cysteine and glutamic acid in sperm cells correlated negatively with NR56, while the concentrations of aspartic acid, leucine and serine showed a positive NR56-correlation. The sperm intracellular concentrations of the trace elements Fe, Al and Zn, correlated negatively with NR56. Correlations were observed between several sperm parameters and metabolites. Stepwise multiple regression analysis indicated that the best predictor of NR56 was a model containing %DFI, together with the intracellular sperm concentration of aspartic acid, Fe and Zn. This model explained 59% of the variability in NR56.

1. Introduction

Artificial insemination (AI) revolutionized cattle breeding in the 1950s. With the introduction of genomic selection (GS), breeding of dairy cattle has undergone a new paradigm shift allowing faster genetic progress [1]. This has increased the demand for using young bulls for semen production and AI. The bulls are typically in production only for a short and intensive period of time before they are replaced by younger, genetically superior bulls [2]. This shortens the available time to assess the fertility status of a bull before its semen is widely distributed. A common measure of the reproductive performance of AI bulls is non-return (NR) rate: the percentage of inseminated females that do not return to estrus within a specific time after the first AI [3]. The reliability of NR data

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depends on the number of AIs per bull and how the AI and its outcome is reported, being considered highly reliable in Norway [4].

Before Norwegian Red bulls are selected as AI bulls, they are subjected to a breeding soundness evaluation, including conventional semen analysis to determine sperm concentration, morphology, and motility. Infertile bulls are normally identified and excluded by this method. However, it is challenging to predict and distinguish bulls with superior fertility from those of lower performance [5,6]. It would benefit the AI industry to have a reliable *in vitro* method, which, at an early stage, could distinguish superior bulls from the rest. It has been demonstrated that parameters such as sperm motility [7–9], viability [10,11] acrosome integrity [12] and DNA integrity [7,13] correlate with field fertility. However, it is unlikely that the fertility of a bull can be predicted by a single sperm attribute alone. The fertilisation process is complex and it is expected that a multifactorial approach is needed [11,14,15].

Recently, metabolomics has been introduced to identify potential fertility and infertility biomarkers in seminal plasma and sperm from both men [16,17] and bulls [18–20]. Metabolomics is the study of small, low molecular weight metabolites. Metabolites are defined as final products of metabolic pathways and play significant roles in sperm physiology such as energy metabolism, motility and regulation of metabolic activities [16]. In a recent study, 63 metabolites were identified in bull seminal plasma [19]. Later, the same group reported 22 distinct metabolites in spermatozoa from bulls with either high or low fertility [20]. A common approach for studying metabolites in semen has been to separate seminal plasma from the sperm pellet by centrifugation, allowing for analysis of metabolites in both seminal plasma and spermatozoa. This does not, however, take into account differences between viable and non-viable cells, the viable cells having capacity to fertilize the egg. To the best of our knowledge, there is no study yet focusing on intracellular metabolites of frozen-thawed, viable sperm cells.

The aim of this study was to identify parameters that, in combination, could be used to discriminate bulls with superior fertility from bulls of lower performance. This was conducted by assessing several sperm attributes associated to fertility, including sperm metabolites, in frozen-thawed semen from bulls with contrasting NR rates after 56 days (NR56).

2. Material and methods

2.1. Animals and semen processing

Cryopreserved semen samples from 37 Norwegian Red bulls of known field fertility were provided by the breeding company Geno (Geno Breeding and AI Association, Hamar, Norway). NR56 was used as a measure of bull fertility. In Norway, the insemination technicians are employed by the breeding company and paid according to reported AIs, thus the NR data are considered highly reliable [4]. Breedings were reported to the Norwegian Dairy Herd Recording System (NDHRS), where data on AI were made available. Least square mean (LSmean) for NR56 was calculated for 507 Norwegian Red bulls used in AI from 2013 to 2018. The General Linear Model (PROC GLM in SAS®) included effects of bull, AI month and year, parity of the female and repeated AI within 1–4 days. Based on these results, a group of 19 bulls with high NR56 (hereafter referred to as HF) and a group of 18 bulls with low NR56 (hereafter referred to as LF) were selected for the study. LSmean for NR56 ranged from 0.76 to 0.78 for HF and from 0.46 to 0.65 for LF. The total number of first AIs were 29 240 for HF (range: 496 to 8542) and 12 636 for LF (range: 204 to 976). All bulls were in regular semen production, with a mean (±SD) age corresponding to the collection date of ejaculates analysed *in vitro* being 517 (±162) days for HF and 459 (±35) days for LF. For details on numbers of AIs and bull age, see Table 1. Post collection, the semen samples were diluted to a final concentration of 12×10^6 spermatozoa per AI dose in French mini straws (IMV, L'Aigle, France). This was performed in a two-step dilution procedure in Biladyl® extender containing glycerol (Minitube, Verona, WI, USA, 13 500/0004–0006) and fresh egg yolk. Only ejaculates with sperm concentration >390 million/mL, subjective total motility >70% pre-freeze, > 50% post-thaw and normal morphology >85% were used for AI. Cryopreservation was performed according to standard procedures [21] and the semen straws were stored in liquid nitrogen (−196 °C) until used. For *in vitro* analyses, the semen doses were thawed for 1 min in a water bath at 37 °C. For sperm functionality analyses, two semen doses from each bull were thawed and mixed together. For analysis of metabolites, seven semen straws from each bull were mixed.

2.2. Motility measurements by CASA

Analysis of sperm motility characteristics was performed using the SCA evolution CASA system (Sperm Class Analyzer® version 6.1, Microptic SL, Barcelona, Spain), equipped with a phase contrast Eclipse Ci-S/Ci-L microscope (Nikon, Japan), and a Basler digital camera (Basler Vision Technologies, Ahrensburg, Germany). The semen samples were incubated at 37 °C for 15 min and diluted (1:2) with pre-warmed PBS to a final concentration of 26×10^6 cells/mL. Then, 3 μL of the diluted sample was loaded into a pre-warmed 20 μm depth Leja-4 chamber slide (Leja products, Nieuw-Vennep, the Netherlands). A minimum of 8 microscope fields and at least 800 cells were analysed per sample and all samples were analysed in duplicate. The instrument settings were 45 Hz frame rate and 30 frames captured per sample, with sperm cell detection based on head area of 20 μm^2 to 80 μm^2. The kinematic parameters measured were: average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), straightness (STR) of the average path defined as VSL/VAP (%), linearity (LIN) of the curvilinear path defined as VSL/VCL (%), Wobble (WOB) defined as VAP/VCL (%), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz). Total motility (MOT) was defined as sperm cells with VCL > 15 μm/s, progressive motility (PROG) was defined as sperm cells with STR > 70 μm/s. Sperm cells with VCL >80 μm/s, ALH >6.5 μm and LIN <65% were defined as hyperactive (HYP).

2.3. Flow cytometry

All sperm functionality analyses by flow cytometry were performed with a Cell Lab Quanta TM SC MPL flow cytometer (Beckman Coulter, Fullerton, CA, USA). For each day of analysis, the flow cytometer was checked for optical alignment by running Flowcheck™ beads (6605359, Beckman Coulter), and a negative control (unstained semen sample) was included for each run. A 488 nm argon laser was used as light source for excitation.

2.3.1. Analysis of sperm plasma membrane and acrosome integrity

For the analysis of sperm plasma membrane integrity, the semen samples were stained with propidium iodide (PI, LIVE/DEAD® kit, L7011, Invitrogen) which discriminates between live and dead spermatozoa by binding only to DNA of sperm cells with damaged membranes. Lectin peanut agglutinin (PNA) from *Arachis hypogaea* (peanut) conjugated with Alexa Fluor 488 (PNA– Alexa 488, L21409; Invitrogen, Paisley, UK) was used to identify acrosome-reacted spermatozoa. A SP-Talp (105 mM NaCl, 3.1 mM KCl, 0.4 mM $MgCl_2$, 2.0 mM $CaCl_2 \cdot 2H_2O$, 0.3 mM $NaH_2PO_4 \cdot H_2O$,

Table 1
Details of fertility, age at semen collection for *in vitro* analyses and numbers of inseminations for the bulls used in the study. The age is expressed as days and NR56 is expressed as LSmean.

Low NR56				High NR56			
Bull	Age	NR56	No. AIs	Bull	Age	NR56	No. AIs
LF1	481	0.46	689	HF1	447	0.76	831
LF2	439	0.53	803	HF2	478	0.76	821
LF3	542	0.55	805	HF3	474	0.76	842
LF4	443	0.58	695	HF4	449	0.76	651
LF5	500	0.61	713	HF5	1083	0.76	8542
LF6	413	0.62	840	HF6	428	0.76	686
LF7	432	0.63	837	HF7	458	0.76	922
LF8	460	0.63	976	HF8	449	0.76	986
LF9	510	0.63	791	HF9	467	0.76	857
LF10	454	0.64	922	HF10	787	0.76	4156
LF11	489	0.64	318	HF11	422	0.76	611
LF12	421	0.64	652	HF12	422	0.76	825
LF13	453	0.64	333	HF13	423	0.77	764
LF14	487	0.65	204	HF14	553	0.77	710
LF15	414	0.65	751	HF15	571	0.77	1069
LF16	434	0.65	708	HF16	419	0.77	2149
LF17	440	0.65	700	HF17	453	0.78	767
LF18	457	0.65	899	HF18	485	0.78	496
				HF19	556	0.78	2555

1 mM sodium pyruvate, 21.6 mM sodium lactate, 20 mM Hepes, 20 mM Hepes salt, 5 mM glucose, 50 μg/mL gentamycin) staining solution of fluorochromes PNA-Alexa 488 and PI was prepared with stock concentrations of 0.05 μg/mL and 0.48 μM, respectively. Sperm samples, at a concentration of 1×10^6 sperm cells/mL, were stained and incubated for 10 min at room temperature (RT) prior to flow cytometric analysis. All samples were analysed in triplicate. Identification of spermatozoa and exclusion of debris particles was performed by Electronic Volume- (EV) and Side Scatter- (SS) signals, as previously described by Standerholen et al. [21]. PI fluorescence was detected using a 670 nm long pass filter, while PNA-Alexa 488 fluorescence was detected using a 510-to-540 nm band pass filter. Gating was performed to reveal four sub-populations: acrosome-intact and live spermatozoa (AIL), acrosome-intact and dead spermatozoa (AID), acrosome-reacted and live spermatozoa (ARL) and acrosome-reacted and dead spermatozoa (ARD). The data generated was further analysed by Kaluza® Analysis software, Version 2.1 (Beckman Coulter Ltd).

2.3.2. Analysis of sperm chromatin integrity

Sperm chromatin integrity was analysed using the Sperm Chromatin Structure Assay (SCSA), as previously described by Waterhouse et al. and Evenson and Jost [13,22]. The semen samples were diluted in TNE buffer (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4) to a final concentration of 2×10^6 sperm cells/mL in a volume of 200 μL. Next, 400 μL acid detergent solution (0.38 M NaCl, 80 mM HCl, 0.1% (w/v) Triton X-100, pH 1.2) was added, followed by exactly 30 s of incubation at RT. Further, 1.2 mL acridine orange (AO) staining solution (6 μg/mL AO (A3568, Life Technologies, OR, USA)) in a buffer containing 37 mM citric acid, 0.126 M Na_2HPO_4, 1.1 μM EDTA, and 0.15 M NaCl (pH 6) was added. After 3 min setup mode, data acquisition started, in which 5000 events were captured for each sample at a rate of ~200 events/sec. Signals were separated by a 550 nm dichroic long pass mirror, a 525 nm band pass filter detected the green fluorescence while a 670 nm long pass filter detected the red fluorescence. The flow cytometer was AO-saturated by running AO equilibration solution (1.2 mL AO staining solution and 400 μL acid detergent solution) through the system for 5 min prior to sample analysis. To control the stability of the laser, the mean green and red fluorescence signals were set to 425 ± 5 and 125 ± 5, respectively, first at the start of analysis and later after every fifth sample was analysed. This was performed using semen samples from a bull of known DNA fragmentation index (DFI) in a bivariate cytogram. The FL1 (green) was presented on the x-axis and FL3 (red) on the y-axis of cytogram, both on a linear scale. Analysis in FCS Express 6 Flow cytometry Software (Denovo Software, Los Angeles, CA, USA) revealed the percentage of red (ssDNA) and green (dsDNA) fluorescence. Based on a histogram of the fluorescence ratio red/(red + green), the spermatozoa with fragmented DNA (%DFI), average extent of DNA fragmentation (mean DFI), and variation in extent of DNA fragmentation (SD DFI) were calculated. The bivariate cytogram was used to determine the percentage of spermatozoa with high DNA stainability (%HDS).

2.4. ATP content by luminescence assay

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) was used for measuring ATP levels in bull semen. The semen samples (60 μL, 3×10^5 sperm cells) were added to a white 96-well microtiter plate (NUNC™, Denmark) before the addition of 60 μL CellTiter-Glo® reagent. The mixture was gently shaken for 2 min on a rotary shaker (IKA® MS 3 digital, USA) prior to 15 min incubation at RT. A FLUOstar OPTIMA multiwell plate reader (BMG LABTECH GmbH, Offenburg, Germany) with MARS data analysis software (Version 1.10, BMG LABTECH, Germany) was used for measuring the bioluminescence. Samples were analysed in triplicate, and the data recorded was measured in relative luminescence units (RLU). Using a prepared standard curve, RLU values were converted to corresponding ATP values in nM. Results are presented as μM ATP per million cells, and corrected for the percentage of viable spermatozoa, as measured by flow cytometry.

2.5. Analysis of intracellular sperm metabolites

2.5.1. Chemicals and reagents

All solvents, acids and salts used for LC-MS mobile phases and preparation of extracts were purchased from Sigma-Aldrich (Darmstadt, Germany) and were of LC-MS or pro analysis quality. Water was of MilliQ quality. All standards were obtained from Sigma Aldrich and internal standards were obtained from either Sigma Aldrich or TRC (North York, Canada). Standards for ICP-MS analysis were obtained from Inorganic Ventures (Christiansburg, USA).

2.5.2. Isolation of viable sperm cells by single layer colloid centrifugation

Single layer centrifugation (SLC) was conducted with a species-specific colloid, Bovicoll (patent application submitted, J. M. Morrell), according to Nongbua et al. [23] with modifications. The frozen-thawed semen (190 μL) was carefully layered on top of 1 mL Bovicoll prewarmed to room temperature in a 15 mL Falcon tube. The samples were centrifuged at 300×*g* for 20 min using a swing out rotor before the supernatant (seminal plasma, semen extender and most of the colloid) was removed. The pellets containing viable sperm cells were aspirated to sterile 2 mL tubes. To wash and remove remaining colloid material, the sperm pellets were carefully re-suspended in 200 μL PBS and centrifuged at 300×*g* for 1 min in two repeating steps. After washing, the sperm pellets were pipetted into 15 mL 50% MeOH and snap frozen in liquid nitrogen to lyse the sperm cells before metabolomic analysis. The samples were thawed and frozen in three cycles before they were freeze-dried. Before the last cycle of snap freezing, 15 mL of Milli-Q water was added to prevent thawing of the sample during the freeze-dry procedure. The samples were re-suspended in water and divided into aliquots for quantitative analysis of amino acids and elemental analysis. Due to a problem during the sample preparation for one of the semen samples, the following analyses were conducted for 36 bulls (19 samples from HF and 17 samples from LF bulls).

2.5.3. Quantitative analysis of amino acids

Samples were derivatised by propyl chloroformate (PCF) prior to analysis. The freeze-dried sample aliquot was re-suspended in methanol and the dissolved material was collected and dried using a speed-vac. A mix of deuterated amino acids (internal standard-mix) was added followed by 1 M NaOH (390 μL), 1-propanol (335 μL) and pyridine (65 μL), followed by addition of the derivatisation reagent PCF (80 μL). For extraction of the derivatised amino acids, a 400 μL volume of chloroform was added followed by 50 mM $NaHCO_3$ (400 μL). Vortex mixing was performed after each addition of solvent and reagent. A 200 μL aliquot of the chloroform phase was thereafter transferred to a clean vial and air-dried. The derivatised amino acids were dissolved in methanol prior to analysis.

Analysis was performed on an Agilent 1290 Infinity II LC system (Agilent, Santa Clara, USA) coupled to an Agilent 6495 QqQ mass spectrometer. The QqQ-MS was equipped with a jet-stream ESI source operated in positive mode. The QqQ-MS was operated in dynamic MRM mode (delta Rt = 1 min) with unit mass resolution for both mass filters. The MRM transitions for standards and internal standards and the employed collision energies, gas temperatures and flows, are given in Table 2. Amino acids (Alanine, Arginine, Asparagine, Aspartic acid, Cysteine, ɣ-Aminobutyric acid (GABA), Glutamic acid, Glutamine, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Ornithine, Phenylalanine, Proline, Serine, Threonine, Tryptophan, Tyrosine, and Valine) were analysed by LC-MS/MS following PCF derivatisation. The chromatographic separation was performed in reversed phase mode employing an Ascentis Express C8 (2.1 × 150 mm, 2.7 μM) column (Sigma-Aldrich), and gradient elution using 25 mM Formic acid as eluent A, and acetonitrile as eluent B, at a flow rate of 0.3 mL/min. The gradient used started at 35% B and was increased stepwise to 90% B at 14 min. Complete washout was performed by increasing to 100% B before the column was reconditioned with the starting conditions. The column thermostat was maintained at 35 °C and the autosampler at 6 °C. The injection volume was 2 μL. Mixed standards at 0-0.1-1-10-50-100-500-1000-4000 μM were used for calibration and quantitation. Internal standards were used in the analysis.

2.5.4. Analysis of trace elements by ICP-MS

Sample preparation prior to ICP-MS analysis involved digestion of the sperm samples in HNO_3 in a microwave digestion unit. The freeze-dried sample aliquot was mixed with 2.5 mL of 50% HNO_3 and digestion was performed in a Milestone Ultraclave (Sorisole, Italy) using a preset 8-step digestion program at 160 bar and increasing temperature stepwise from 50 °C to 245 °C. ICP-MS analysis of Mg, Al, P, S, K, Ca, Cr, Mn, Fe, Zn, As, and Ba was performed on an Agilent 8800 Triple Quadrupole ICP-MS (G3663A) (Agilent, Santa Clara, USA) mass spectrometer connected to a SPS4 autosampler. A Peltier-cooled (2 °C) spray chamber with a Micro-Mist nebulizer was used as the introduction system. The RF Power was set to 1550 W and the RF Matching to 1.80 V, and the nebulizer gas was set at 1.05 L/min. The cell gases used were He and O_2 at 4.3 and 1 mL/min, respectively. Analysis was performed in MS/MS mode. Extract voltage 1 and 2 were set at 0 and -195V, respectively.

2.5.5. Scaling of data

The number of viable sperm cells in a semen sample varied between individual bulls (49.57 ± 12.83 (LF) and 58.87 ± 8.41 (HF)). After Bovicoll centrifugation, there are mostly only viable spermatozoa in the pellet, and therefore differences in sperm number between samples may be observed. Thus, it was necessary to scale the data in order to adjust for differences in cell number between the samples. A scaling factor was determined based on the quantified amounts of a selection of amino acids in the samples after Bovicoll isolation of viable sperm cells. Eight amino acids (Aspartic acid, Glycine, Leucine, Lysine, Methionine, Proline, Threonine and Tyrosine) were included in the scaling factor, chosen based on their co-variance in the sample series, and their similar profiles in all samples (supplementary material, Fig. S1). The average concentrations for these eight amino acids were calculated and the ratio of observed concentration in each bull sample to this average was determined, giving each bull sample its own scaling factor. This scaling factor was used to compensate for different sperm numbers in sample material by dividing the observed concentrations by the values of corresponding scaling factors for the different bull samples. The scaling was performed for both the amino acid and trace element concentrations.

2.6. Statistics

The statistical analyses were performed using SAS Version 9.4 for Microsoft Windows (SAS Institute, Cary, NC, USA). The data was tested for normal distribution by the Shapiro-Wilk test. The parameters that did not show a normal distribution were log-transformed prior to further statistical analysis. Differences in sperm parameters and metabolites between HF and LF bulls was assessed by unpaired *t*-test. In cases where normal distribution was not achieved after log transformation, the non-parametric Wilcoxon test was applied. Correlation coefficients (Pearson) were calculated and considered statistically significant when $p < 0.05$. The correlation between sperm parameters/metabolites and NR56 was assessed, followed by the correlations between sperm functionality parameters and metabolites. To determine which combination of parameters that best could explain the bulls NR56, a forward stepwise multivariate regression analysis was conducted (PROC REG). The NR56 LSmean was the dependent variable and the sperm functional parameters and metabolites shown to correlate with NR56 were the predictive independent variables. Forward variable selection was applied where the selection started with only the intercept term in the model. For each of the independent variables the F statistic was calculated to determine each variable's

Table 2
Method parameter (multiple-reaction monitoring and retention time (RT)) and instrument settings for LS-MS determination of amino acids.

	Precursor ion	Product ion	CV (V)	RT (min)	Internal standard
Alanine	218	130	8	4.3	D3-Alanine
Arginine	303.2	70.1	44	1.1	13C6-Arginine
Asparagine	243.1	157	5	3.9	D3-Asparagine
Aspartic acid	304.4	216	10	6.6	
Cysteine	336	248	8	9.2	D2-Cysteine
GABA	232	85.9	16	4.3	D6-GABA
Glutamic acid	318	84	25	6.9	D5-Glutamic acid
Glutamine	275	84.2	28	2.2	13C5-Glutamine
Glycine	204	75.9	12	3.6	13C2Glycine
Histidine	370	109.9	40	5.6	13C6-Histidine
Isoleucine	260	172	8	8.3	
Leucine	260	172.1	8	8.3	D10Leucine
Lysine	361	301.1	4	5.9	13C6Lysine
Methionine	278	189.9	4	5.8	13CD3Methionine
Ornithine	347.2	287.2	5	5.2	D6Ornithine
Phenylalanine	294.3	206.1	8	7.9	D5Phenylalanine
Proline	244.2	156.2	10	5.1	D3Proline
Serine	234.2	60.1	24	2.7	D3Serine
Threonine	248	74.1	15	3.2	
Tryptophan	333	245.1	20	6.8	D5Trypthophan
Tyrosine	396	222.2	20	10.3	13C9-Tyrosine
Valine	246	158.2	5	6.5	D8Valine

Instrument settings; Capillary voltage (CV) = 4 kV, Nebulizer pressure = 40 psi, drying gas flow = 20 L/min, gas temperature = 210 °C, Fragmentor voltage = 380 V, Sheath gas temperature = 400 °C, sheath gas flow = 11 L/min, iFunnel positive high/low pressure RF = 150/60, and negative high/low pressure RF = 90/60.

contribution to the model. The variable with the smallest p-value below the cut-off value 0.1, indicating statistical significance, was kept in the model.

3. Results

3.1. Sperm functionality

Differences in sperm attributes between HF and LF bulls are presented in Table 3. For the chromatin integrity parameters, lower %DFI ($p < 0.001$) and HDS ($p < 0.01$) were observed in sperm cells from HF bulls compared to LF bulls. ATP levels, as well as the sperm motility parameters MOT, PROG, HYP, VAP, VCL, VSL, STR and ALH, were higher in semen from HF vs. LF bulls ($p < 0.01$). For sperm viability and acrosome integrity, higher proportions of live and AIL spermatozoa and lower proportions of ARD spermatozoa were observed in the HF group compared to the LF group ($p < 0.05$).

Table 3
Mean and standard deviation for sperm functionality parameters of bulls with low (n = 18) and high (n = 19) NR56 rates. Significant differences between the groups are marked by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Sperm parameter	Low NR56	High NR56
DFI (%)	3.50 ± 1.44	1.84 ± 0.80***
HDS (%)	4.31 ± 1.65	2.93 ± 1.15**
Live (%)	49.57 ± 12.83	58.87 ± 8.41**
AIL (%)	46.15 ± 13.16	55.82 ± 8.92*
ARL (%)	3.40 ± 1.73	3.07 ± 1.60
AID (%)	17.37 ± 3.88	16.13 ± 4.01
ARD (%)	33.08 ± 12.41	24.93 ± 6.48*
ATP (nM)	1.12 ± 0.47	1.52 ± 0.36**
MOT (%)	40.31 ± 14.32	51.92 ± 11.17**
PROG (%)	34.45 ± 14.64	48.28 ± 10.87**
HYP (%)	8.95 ± 4.51	16.69 ± 5.98**
VAP (μM/s)	82.76 ± 14.29	94.03 ± 4.94**
VCL (μM/s)	161.34 ± 28.39	184.26 ± 11.91**
VSL (μM/s)	64.83 ± 12.13	75.77 ± 5.35**
STR (%)	75.01 ± 4.85	79.45 ± 2.87**
LIN (%)	41.65 ± 4.17	44.14 ± 3.89
WOB (%)	53.50 ± 2.77	54.03 ± 3.19
ALH (μM)	4.75 ± 0.78	5.36 ± 0.49**
BCF (Hz)	20.81 ± 1.69	21.29 ± 1.53

DFI = DNA fragmentation index, HDS = high DNA stainable, AIL = acrosome intact live, ARL = acrosome reacted live, AID = acrosome intact dead, ARD = acrosome reacted dead, MOT = motile, PROG = progressive, HYP = hyperactive, VAP = velocity average path, VCL = velocity curvilinear, VSL = velocity straight-line, STR = straightness, LIN = linearity, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat-cross frequency.

Sperm functionality parameters were further assessed by Pearson correlation analysis to identify possible relationships to NR56. The results (Table 4) indicate that %DFI had the strongest (negative) correlation to NR56. DNA stainability (%HDS) was also correlated negatively with NR56, whereas the sperm motility parameters MOT, PROG, HYP, VAP, VCL, VSL, STR, and ALH correlated positively with NR56. The remaining sperm parameters did not show any significant correlations with NR56 ($p > 0.05$).

3.2. Concentration of sperm intracellular metabolites

The intracellular sperm concentrations of cysteine and glutamic acid were lower in semen doses from HF than from LF bulls ($p < 0.01$), while the concentrations of threonine, serine, leucine ($p < 0.05$) and aspartic acid ($p < 0.01$) were higher in HF compared to LF bulls (Table 5). Pearson correlations between amino acid concentration and NR56 were significantly different from zero ($p < 0.05$) for cysteine ($r = -0.37$), glutamic acid ($r = -0.39$), aspartic acid ($r = 0.46$), leucine ($r = 0.34$), and serine ($r = 0.36$).

The sperm intracellular concentrations of Al, Fe and Zn were significantly lower in sperm cells from HF bulls compared to LF bulls (Table 5). Pearson correlation coefficient values between trace element concentrations and NR56 were significantly different from zero ($p < 0.05$) for Fe ($r = -0.61$), Al ($r = -0.38$) and Zn ($r = -0.39$).

3.3. Correlation of sperm parameters and intracellular metabolites

Pearson correlation analyses indicated that there were correlations ($p < 0.05$) between several of the sperm functionality parameters and intracellular amino acid concentrations (Table 6). Arginine, glutamine, cysteine and threonine were correlated to most sperm attributes, with correlations to parameters such as chromatin integrity, viability, acrosome integrity, ATP level and

Table 4
The Pearson correlation coefficient (corr) and p-values for the correlations between NR56 and sperm parameters assessed for the bulls (n = 37). Only parameters showing significant correlation ($p < 0.05$) with NR56 are listed in the table.

Sperm parameter	Corr	p-value
DFI (%)	−0.57	0.0003
HDS (%)	−0.37	0.026
HYP (%)	0.42	0.010
VAP (μM/s)	0.42	0.010
VCL (μM/s)	0.44	0.007
VSL (μM/s)	0.44	0.006
STR (%)	0.40	0.013
ALH (μM)	0.41	0.012

DFI = DNA fragmentation index, HDS = high DNA stainable, HYP = hyperactive, VAP = velocity average path, VCL = velocity curvilinear, VSL = velocity straight-line, STR = straightness, ALH = amplitude of lateral head displacement.

several different sperm motility characteristics. Arginine, threonine, aspartic acid, glutamic acid and serine correlated with chromatin integrity parameters. Furthermore, correlations were observed between several of the trace elements and sperm functionality parameters (Table 7). The trace elements, whose concentrations correlated with most sperm attributes, were S, Ca, Mn, Fe and Ba. They correlated with i.e. chromatin integrity, acrosome integrity, viability and motility parameters. The intracellular concentration of Mg correlated positively with several kinematic motility parameters. The sperm concentrations of Al, S, K, Ca, Fe and Ba correlated positively with %DFI.

3.4. Identification of sperm parameters and metabolites associated with NR56

Stepwise multiple regression analysis with a variable selection criteria of $p < 0.1$, indicated that the best predictor of bulls' NR56 was a model containing %DFI and the intracellular sperm concentration of aspartic acid, Fe and Zn:

NR56 = 0.051 + (0.004 x aspartic acid) - (0.076 x Fe) - (0.067 x Zn) - (0.052 x DFI).

This model explained 59% of the variability in NR56.

4. Discussion

In the present study, a retrospective approach was used to study sperm attributes in selected groups of bulls with high and low NR56. Significant relationships between sperm functional parameters, metabolites and NR56 rates were revealed. To our knowledge this is the first study focusing on metabolomics of the viable fraction of frozen-thawed semen samples. The multiple correlations between metabolites and sperm parameters, as well as the correlation to NR56 indicate that metabolomics can be a useful tool in the identification of biomarkers for male fertility.

Table 5
Mean and standard deviation for amino acid and trace element concentrations (μM) of frozen-thawed viable sperm cells from bulls (n = 36) of low and high NR56. Only metabolites with significant differences between the fertility groups are presented: *p < 0.05, **p < 0.01, ***p < 0.001.

Metabolite	Low NR56	High NR56
Aspartic acid	74.65 ± 3.96	78.75 ± 3.82**
Cysteine	18.05 ± 3.83	16.01 ± 2.46**
Glutamic acid	252.39 ± 9.46	242.77 ± 8.21**
Leucine	134.29 ± 13.78	142.57 ± 7.24*
Serine	124.70 ± 9.12	131.08 ± 5.68*
Threonine	92.77 ± 8.42	87.78 ± 5.12*
Al	0.13 ± 0.03	0.10 ± 0.04**
Fe	0.105 ± 0.046	0.066 ± 0.018***
Zn	0.080 ± 0.020	0.066 ± 0.024*

Several sperm attributes are presumed important for fertilising potential [5,24]. In the present study, differences between two contrasting groups of bulls were found for sperm motility, viability, acrosome integrity and ATP level. However, only motility parameters were significantly correlated to NR56. Motility is commonly believed to be one of the most important sperm attributes associated with fertility [25]. However, there are conflicting results regarding correlation between sperm motility and field fertility [8,26–29]. Based on motility kinematics, sperm from HF and LF bulls appeared to be in different stages concerning the presence of a hyperactive motility pattern. The HF bulls had sperm cells with increased levels of VCL and ALH, which was reflected in the corresponding observed increase in hyperactive motility. These results corroborate those from a previous study [30], where it was suggested that sperm cells from HF bulls are in transition to a hyperactivated motility pattern. This transition phase, which is termed a progressive hyperactivated motility phase, has been described also by others [31,32]. It has been suggested that the transitional phase plays an important role in sperm transport [31]. Thus, it may be hypothesised that the increase in hyperactivity observed for HF bulls in the present study reflects their sperm being in a progressive hyperactivated motility phase with enhanced ability to reach and fertilize the oocyte.

For the SCSA-derived chromatin integrity parameters, significantly lower %DFI and %HDS were observed in sperm from HF bulls compared to LF bulls. While DFI represents the spermatozoa with DNA damage, HDS is thought to represent immature spermatozoa with incomplete chromatin condensation due to lack of full protamination [33,34]. Our results may indicate that LF bulls have spermatozoa with less compact chromatin and more DNA strand breaks compared to HF bulls. Sperm %DFI was the attribute correlating strongest with NR56 and was also the only sperm parameter with a significant contribution to the model predicting NR56. This corroborates results from previous studies in bulls, where %DFI was found to correlate significantly with field fertility [7,11,13,28,35]. In agreement with our findings, Waterhouse et al. [13] tested the relationship between multiple sperm parameters and field fertility, and reported that only variables related to sperm DNA damage contribute significantly to the model. The main causes of DNA fragmentation are failure in the replacement of histones by protamines during sperm maturation, apoptosis and insufficient protection against reactive oxygen species (ROS) [36,37].

While DNA fragmentation is considered an uncompensable sperm attribute, motility, viability, acrosome integrity and ATP level are considered compensable traits [38,39]. It is possible that the observed differences between HF and LF bulls for the compensable sperm traits are camouflaged by the relatively high number of spermatozoa used per AI dose in the present study. This may explain why the regression model demonstrated the uncompensable trait %DFI to be the best indicator of fertility among the sperm parameters analysed. Given that most sperm quality traits are correlated to some extent, the results of regression studies can be influenced by collinearity problems [40]. To avoid this problem, we tested the correlation among the sperm functional attributes and avoided combining highly correlated parameters in the model.

Metabolomics of bovine seminal plasma and sperm cells has to our knowledge only been conducted using fresh semen samples [18–20]. Recently, 22 distinct metabolites were identified in spermatozoa from Holstein bulls [20]. In the present study, we used frozen-thawed semen samples, which also are used for AI and thus correspond better to the NR56 data. Cryopreservation is known to have a detrimental effect on sperm viability [41]. We hypothesised that the dead spermatozoa may leak out some of the intracellular metabolites due to damaged sperm membranes. This may affect the

Table 6
Pearson correlation coefficient values between sperm functional parameters and amino acid concentrations in bovine sperm cells that were significantly ($p < 0.05$) different from zero. Only amino acids and sperm attributes showing significant correlation are listed.

	Arg	Asp	Cys	GABA	Glu	Gln	His	Ile	Lys	Ser	Thr
DFI	−0.34		0.53		0.40					−0.33	0.35
HDS		−0.39			0.35					−0.41	
AIL	0.46		−0.45			0.45					−0.48
ARL	−0.34		−0.22			−0.38	0.36				
ARD	−0.42		0.46			−0.34					0.52
ATP	0.52		−0.21			0.56					−0.40
MOT	0.55		−0.37			0.44					−0.52
PROG	0.63		−0.36			0.49					−0.58
HYP	0.58		−0.33					−0.34	0.34		−0.59
VAP	0.65					0.56					−0.47
VCL	0.67					0.45			0.34		−0.57
VSL	0.55					0.55					−0.36
STR				0.34		0.40					
LIN						0.33					
ALH	0.55								0.35		−0.54
BCF	0.43					0.46					

DFI = DNA fragmentation index, HDS = high DNA stainable, AIL = acrosome intact live, ARL = acrosome reacted live, ARD = acrosome reacted dead, MOT = motile, PROG = progressive, HYP = hyperactive, VAP = velocity average path, VCL = velocity curvilinear, VSL = velocity straight-line, STR = straightness, LIN = linearity, ALH = amplitude of lateral head displacement, BCF = beat-cross frequency, Arg = arginine, Asp = aspartic acid, Cys = cysteine, GABA = gamma aminobutyric acid, Glu = glutamic acid, Gln = glutamine, His = histidine, Ile = isoleucine, Lys = lysine, Ser = serine, Thr = threonine.

results, depending on the proportion of live/dead spermatozoa in the different samples. Thus, we emphasized to select the viable sperm population in the present study. We found the concentrations of several amino acids to correlate significantly with NR56 as well as with different sperm parameters. Arginine and glutamine in human sperm cells have previously been demonstrated to correlate with sperm progressivity [42], which is in agreement with our findings. In a study by Zhao et al. (2018) asthenozoospermic patients (men with poor sperm motility) had reduced levels of several amino acids including leucine, glutamic acid, cysteine and tryptophan [17]. Our results for bull spermatozoa also showed a significantly lower concentration of leucine in sperm cells from LF bulls. However, the group of LF bulls also showed higher concentrations of glutamic acid and cysteine compared to HF bulls. Glutamic acid is the key component of glutathione, which has been demonstrated to protect cells from damage caused by ROS and lipid peroxidation [43]. Furthermore, glutamic acid has been identified as the most abundant amino acid of bull seminal plasma [44], and was also the most abundant amino acid in sperm cells in our study. A derivative of L-cysteine, N-acetyl-cysteine, is an antioxidant and contributes to glutathione synthesis [45,46]. It can be speculated that the observed decrease in glutamic acid and cysteine concentrations in HF bull sperm cells is a consequence of their consumption of glutamic acid and cysteine for protection against oxidative stress. Oxidative stress is linked to DNA damage and male infertility [47], and may contribute to the explanation of lower observed %DFI in sperm cells from HF bulls. Both glutamic acid and cysteine correlated significantly with %DFI. Aspartic acid of bull seminal plasma has previously been shown to correlate with fertility [48]. We found a significant positive correlation between aspartic acid and bulls' NR56, with aspartate being the only amino acid exhibiting a significant contribution to the model predicting NR56. Furthermore, we found aspartic acid to correlate negatively with %HDS. This may indicate that aspartic acid is involved in the packaging of chromatin and maturation of spermatozoa. Interestingly, a study performed on human seminal plasma and sperm cells found D-aspartic acid (D-Asp) to be related to the quality of semen, with higher levels of D-Asp in men with normal semen quality compared to oligoasthenoteratospermic donors. Based on these results, they hypothesised that D-Asp could have a specific role in spermatogenesis and that it is involved in sperm maturation and fertility [49].

Both seminal plasma and sperm cells are known to contain a variety of trace elements; some are essential for proper sperm cell function and fertility, while others may have an adverse effect on reproduction [50,51]. In the present study, concentrations of Al, Fe and Zn in spermatozoa were found to be significantly higher in bulls of low vs. high NR56, and they were correlated significantly with NR56. A high level of Fe in human seminal plasma was shown to have a negative impact on sperm motility [52] and it has been reported that Fe can cause an increase in sperm DNA damage [53]. This corroborates our findings, where the level of Fe correlated positively with %DFI and exhibited negative correlation with several of the sperm motility parameters. Zinc is an essential element for male fertility and works as an antioxidant protecting sperm cells from ROS [54,55]. The lower levels of Zn in HF bulls may be explained by the same hypothesis as stated above, that sperm cells from HF bulls exhaust more Zn in their protection against oxidative damage. Metals such as Al have a toxic effect on sperm

Table 7
Pearson correlation coefficient values between sperm functional parameters and trace element concentrations in bovine sperm cells that were significant ($p < 0.05$) different from zero. Only elements and sperm attributes showing significant correlation are listed.

	Mg	Al	S	K	Ca	Mn	Fe	As	Ba
DFI		0.39	0.35	0.33	0.39		0.46		0.52
AIL			−0.39		−0.45	−0.38	−0.33		−0.47
ARL								−0.37	
ARD			0.48	0.39	0.50	0.42	0.34		0.49
ATP			−0.28		−0.35	−0.25	−0.34		−0.44
MOT			−0.38		−0.44	−0.35	−0.43		−0.44
PROG			−0.36		−0.43	−0.40	−0.45		−0.44
HYP			−0.42		−0.45	−0.39	−0.38		−0.45
VAP	0.34					−0.35	−0.35		−0.32
VCL						−0.40	−0.34		−0.35
VSL	0.39						−0.35		
STR	0.39							0.38	
LIN	0.40								
WOB	0.36								
ALH						−0.39			−0.35
BCF	0.39								

DFI = DNA fragmentation index, AIL = acrosome intact live, ARL = acrosome reacted live, ARD = acrosome reacted dead, MOT = motile, PROG = progressive, HYP = hyperactive, VAP = velocity average path, VCL = velocity curvilinear, VSL = velocity straight-line, STR = straightness, LIN = linearity, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat-cross frequency.

cells [56]. In a study in rats, exposure to Al was shown to impair spermatogenesis, sperm quality and to increase oxidative stress and inflammation [57]. Our results showed a significant correlation between Al levels and %DFI, with LF sperm cells displaying significantly higher levels of both Al and %DFI.

In the regression model based on a variable selection criteria of $p < 0.1$, the sperm functionality parameter, %DFI, together with the sperm intracellular concentrations of aspartic acid, Fe and Zn, were identified as predictors of bulls' NR56. Together, these parameters explained 59% of the variation in NR56. Although this is a notable regression, the model does not explain the total variation in fertility. This could be partially due to the fact that the bulls used in the study were preselected based on breeding soundness evaluation, as is customary at the AI station. If subfertile bulls had been included in the study, the prediction model might have been even stronger. The fertility data used in the present study were based on AIs with a sperm number per AI dose of ~12×10^6. A reduction in the sperm number per semen dose might have yielded other results regarding compensable sperm parameters. Before recommending this prediction model to AI companies, it needs to be validated using a larger number of bulls with known fertility. As this was the first time that metabolites were included as a method of analysis for Norwegian Red bulls, we did not have data available to perform such a validation. However, our results show strong correlations between fertility and %DFI, which corroborates findings of several others [7,11,13,28,35], and may indicate that conventional semen analysis at the bull station could benefit from augmentation with analysis of DNA integrity. In this study, we presented correlations identified between sperm functional parameters and metabolite concentrations. However, sperm functionality traits also correlate with each other and this is important for the implementation of the quality control program at AI stations. Due to the high degree of correlation among sperm traits, it is debatable whether a new analytical method gives novel information or simply provides an alternative technique to measure what is already known [58]. Nonetheless, the multiple correlations between metabolites/trace elements and sperm parameters found in the present study may suggest that a convergence of these different technologies can increase the knowledge of factors influencing and predicting bull fertility.

In this study, there was greater variation in LSmean NR56 for LF bulls (0.46–0.65) than for HF bulls (0.76–0.78). This may explain why larger SDs were observed for several of the sperm parameters and metabolite concentrations for LF bulls. It would be preferable to have a homogenous groups representing both HF and LF bulls. However, the numbers of bulls with inferior fertility are scarce and to obtain enough bulls in the LF group, a larger variation in NR56 had to be accepted. The number of AIs underlying the NR56 data affects the reliability of the data [58]. Three LF bulls had less than 500 inseminations recorded, and their NR56 data might have changed if the number of AIs had been higher.

In conclusion, bulls of high and low NR56 rates differed in several aspects of sperm functionality and metabolome characteristics. In particular, the combination of %DFI, and sperm concentrations of aspartic acid, Fe and Zn seem to predict bulls' NR56 rates.

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CRediT authorship contribution statement

Birgitte Narud: Data curation, Formal analysis, Visualization, Writing - original draft. **Geir Klinkenberg:** Conceptualization, Methodology, Writing - review & editing. **Abdolrahman Khezri:** Data curation, Formal analysis, Writing - review & editing. **Teklu Tewoldebrhan Zeremichael:** Data curation, Formal analysis, Writing - review & editing. **Else-Berit Stenseth:** Data curation, Formal analysis, Writing - review & editing. **Anna Nordborg:** Data curation, Formal analysis, Writing - original draft. **Tonje Husby Haukaas:** Data curation, Writing - original draft. **Jane M. Morrell:** Resources, Writing - review & editing. **Bjørg Heringstad:** Formal analysis, Writing - review & editing. **Frøydis Deinboll Myromslien:** Conceptualization, Writing - review & editing. **Elisabeth Kommisrud:** Project administration, Conceptualization, Data curation, Writing - original draft.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

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