Semen quality parameters including metabolites, sperm production traits and fertility in young Norwegian Red AI bulls

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ABSTRACT

Genomic selection in cattle breeding has gradually allowed younger bulls to be recruited for semen production. In this study, sperm quality parameters, seminal plasma and sperm metabolites, semen production capacity and fertility in young Norwegian Red bulls were analysed. For *in vitro* analyses of sperm quality and metabolites, ejaculates were collected

from the same 25 bulls at both 14 and 17 months of age. Semen production and fertility data were collected for all Norwegian Red bulls in production from December 2017 throughout 2019. Bull fertility was measured as 56 days non-return rate (NR56), for both age groups. In both fresh and frozen-thawed semen samples, the proportion of hyperactive spermatozoa, average path velocity, curvilinear velocity and amplitude of lateral head displacement were higher in samples collected at 17 months of age compared to 14 months (P<0.05). In addition, several amino acids including arginine, glutamine, cysteine and proline, were affected by age (P<0.05). The concentrations of K and Ba increased significantly (P<0.05) with age in both seminal plasma and sperm cells. Sperm concentration and volume of the ejaculate increased significantly with increasing age, while the percentage of discarded batches decreased. The bulls' NR56 decreased significantly with increasing age (75% vs. 74%). However, the difference of 1% is unlikely to be of biological importance. In conclusion, even small age differences in young bulls may significantly affect several sperm quality parameters, metabolite levels and semen production traits.

Keywords:

Bull age; Sperm motility; Metabolites; Trace elements; Semen production; Non-return rate

1. Introduction

The introduction of genomic selection (GS) has allowed the dairy cattle industry to select bulls for artificial insemination (AI) at a younger age, thus reducing the generational interval and increasing the genetic gain (Meuwissen et al., 2001; Murphy et al., 2018). However, GS has also created challenges as increased market demands for semen doses from young bulls conflicts with onset of puberty and sexual maturity. Young bulls have

lower semen production capacity compared to mature bulls, due to the ongoing development of the testis and accessory glands during sexual maturation (Almquist et al., 1976; Brito et al., 2002; Schenk, 2018). During spermatogenesis, a cascade of complex and hormone-dependent events take place to ensure proper development of germ cells. Peri-pubertal bulls usually have poor semen quality that gradually improves and eventually fulfils quality criteria around 16 months of age (Brito et al., 2012; Lambert et al., 2018). Performing AI with semen produced before that age may affect bull reproductive performance.

Reproductive performance of bulls may be measured by the non-return (NR) rate, which means the proportion of inseminated females that do not return to estrus within a specific interval after the first AI (Foote, 2003), e.g. NR56 for 56 days. The fertility outcome of AI is affected by several factors, including age of the bull and the sperm quality of frozen-thawed semen (Zhang et al., 1999; Oliveira et al., 2012; Kumaresan et al., 2017).To succeed with fertilization, sperm cells should possess traits that enhance their ability to reach and fertilize the oocyte, such as intact plasma membranes and acrosomes (Christensen et al., 2011; Kumaresan et al., 2017), metabolism for energy production (Garrett et al., 2008), progressive sperm motility (Farrell et al., 1998; Puglisi et al., 2012; Gliozzi et al., 2017), and capacity for hyperactive motility (Suarez et al., 1991). Moreover, DNA integrity is important for fertilization and embryonic development (Waterhouse et al., 2006; Gliozzi et al., 2017). Although studies have shown associations between field fertility and sperm quality parameters, there is still no single *in vitro* analysis that reliably can predict the true fertilization potential of a semen sample.

New technologies, such as genomics, proteomics and metabolomics have encouraged the search for novel male fertility and infertility biomarkers. Metabolites, which are low molecular-weight components (<1500 Da) including organic acids, amino acids, amines,

lipids, nucleosides, vitamins and minerals (Xiao et al., 2012; Zhao et al., 2018), are present both in seminal plasma and in sperm. As metabolites are the end products of biochemical pathways, they are considered representative of phenotypic traits (Kumar et al., 2015; Guijas et al., 2018). Metabolites with the potential to serve as fertility biomarkers have been identified by untargeted approaches in bovine seminal plasma and sperm (Kumar et al., 2015; Velho et al., 2018). However, to the authors' best knowledge, there are no publications focusing on the association between bull age and metabolites in seminal plasma and sperm.

Based on this background, we hypothesised that even small differences in age might affect the reproductive performance of young peri-pubertal bulls. Therefore, the aim of the present study was to assess sperm quality of fresh and frozen-thawed semen, metabolites in seminal plasma and sperm cells, semen production traits and fertility in Norwegian Red AI bulls of 14 and 17 months of age.

2. Materials and methods

2.1. Animals and semen processing

Semen samples for *in vitro* sperm analyses were provided by the breeding company Geno (Geno Breeding and AI Association, Hamar, Norway). Ejaculates were collected from 25 young Norwegian Red bulls being in regular semen production at Geno's AI station, Store Ree (Stange, Norway). The bulls were raised and fed uniformly, and cared for according to the Norwegian Animal Welfare Act (LOV 2009-06-19 no. 97). All semen production procedures were in compliance with European Union Directive 88/407. Semen for the *in vitro* analyses was collected from each bull at the age 14 and 17 months, respectively. In general, bulls were collected once a week upon arrival, with two ejaculates with approximately 15 minutes interval. This procedure continued for 5-6 weeks, thereafter the bulls were collected twice a week. Only ejaculates with sperm

concentration > 390 x 10^6/mL, subjective total motility > 70%, and normal morphology > 85% were further processed. Each ejaculate was diluted to a final concentration of 12 x 10^6 spermatozoa per AI dose in French mini straws (IMV, L'Aigle, France), using a two-step dilution procedure with Biladyl extender (Minitübe, GmbH, Tiefenbach, Germany, 13500/0004-0006). First-dilution was performed at 35 °C, while the second glycerol containing extender was added at 5 °C. Cryopreservation was performed according to standard procedures (Standerholen et al., 2014). Semen with post-thaw motility < 50% were discarded. For the analyses of metabolites, 2 mL of neat semen was centrifuged (110 x *g*, 10 min) to separate the seminal plasma (supernatant) from the sperm cells (pellet). Thereafter, both sperm and seminal plasma samples were snap-frozen in liquid nitrogen and shipped to SINTEF (SINTEF Industry, Trondheim, Norway) for analyses of metabolites and trace elements. Furthermore, sperm quality parameters were analysed in first-diluted semen on the day of collection (hereafter referred to as fresh semen samples). For analyses of sperm quality in frozen-thawed samples, the cryopreserved semen doses were thawed for 1 min in a 37 °C water-bath. Two semen doses from each ejaculate were thawed and mixed together.

2.2. Assessment of sperm motility by CASA

Sperm motility analysis was performed using a CASA system (Sperm Class Analyzer®, version 6.1, Microptic SL, Spain) equipped with a phase contrast Eclipse Ci-S/Ci-L microscope (Nikon, Japan) and a Basler digital camera (Basler Vision Technologies, Ahrensburg, Germany), as described by Narud et al. (2020). Briefly, semen samples were incubated for 15 min at 37 °C, and directly diluted (1:2) with pre-warmed PBS to a final concentration of 26 x 10^6 cells/mL. A volume of 3 µL of the diluted sample was loaded into the chamber of a 20 µm depth Leja® 4 slide (Leja products, Nieuw-Vennep, the Netherlands). A minimum of eight microscope fields and at least 800 cells

were analysed per sample. Each sample was analysed twice at a frame rate of 45 frames per second. Sperm cells were identified by sperm head area of 20 μm^2- 80 μm^2. The kinematic parameters recorded 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 the ratio of VSL/VAP (%), linearity (LIN) of the curvilinear path defined as the ratio of VSL/VCL (%), and amplitude of lateral head displacement (ALH, µm). Total motility (TM) was defined as sperm cells with VCL > 15 µm/s, progressive motility (PM) was defined as sperm cells with STR > 70 %. Sperm cells with VCL > 80 µm/s, ALH> 6.5 µm and LIN < 65 % were defined as having hyperactive motility (HYP).

2.3. Flow cytometry

Flow cytometry analyses were performed using a Cell Lab Quanta TM SC MPL flow cytometer (Beckman Coulter, Fullerton, CA, USA). The flow cytometer was checked daily for optical alignment by Flow-check™ beads (6605359, Beckman Coulter) and for each assessment, an unstained semen sample was included as negative control. An argon laser with 488 nm illumination was used as the excitation light source. The Cell Lab Quanta flow cytometer uses Electronic Volume (EV) for calculating cell size, which has shown to successfully remove non-sperm events without the inclusion of a sperm identification marker (Standerholen et al., 2014).

2.3.1. Sperm plasma membrane integrity

For the analysis of sperm plasma membrane integrity, the semen samples were stained with propidium iodide (PI, Sigma-Aldrich), which only stain sperm cells with damaged plasma membrane (non-viable cells). The semen samples were diluted in SP-Talp media (105 mM NaCl, 3.1 mM KCl, 0.4 mM $MgCl_2$, 2.0 mM $CaCl_2 \cdot 2H_2O$, 0.3 mM $NaH_2PO_4 \cdot H_2O$, 1 mM sodium pyruvate, 21.6 mM sodium lactate, 20 mM Hepes, 20 mM

Hepes salt, 5 mM glucose, 50 µg/mL gentamycin) to a concentration of 1 x 10^6 sperm cells per mL, stained with 0.48 µM PI and incubated for 10 min in room temperature (RT) prior to flow cytometry analyses. Samples were analysed in triplicate, where 10,000 events were collected for each sample at a flow rate of ~200 events/sec. The histograms for EV and Side Scatter- signals were used to define gates for excluding debris and to identify spermatozoa as previously described by Standerholen et al. (2014). PI fluorescence was detected using a 670 nm long pass filter, and gating was performed to reveal percentages of spermatozoa with intact plasma membranes (viable spermatozoa). The data generated was further analysed by Kaluza® Analysis software, Version 2.1 (Beckman Coulter Ltd).

2.3.2. Sperm chromatin integrity

The chromatin integrity of the sperm cells was analysed by the Sperm Chromatin Structure Assay (SCSA), as previously described by Evenson and Jost (2001) and Narud et al. (2020). In brief, semen samples were diluted to 2 x 10^6 sperm cells/mL in TNE buffer (10 mM Tris-HCL, 0.1 M NaCl, 1 mM EDTA, pH 7.4) in a final 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 incubation at RT for 30 sec. Then, 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. Data acquisition started exactly at the end of 3 min setup mode, in which 5000 events were collected for each sample at a flow rate of ~200 events/sec. The signals were separated by a 550 nm dichroic long pass mirror, before green fluorescence was detected by a 525 nm band pass filter and red fluorescence was detected by a 670 nm long pass filter. Prior to sample analysis the flow cytometry instrument 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. 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 analysing every fifth sample. This was performed using reference semen 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 the cytogram, both on a linear scale. The percentage of red (ssDNA) and green (dsDNA) fluorescence was determined using FCS Express 6 Flow cytometry Software (Denovo Software, Los Angeles, CA, USA). Based on a histogram of the fluorescence ratio red / (red + green), the percentage of spermatozoa with fragmented DNA (DFI, %) was calculated. The bivariate cytogram was used to determine high DNA stainability (HDS, %), which correspond to the spermatozoa with the most intensive green fluorescence, recognized as immature spermatozoa (Evenson et al., 2002).

2.4. Intracellular ATP content

The intracellular ATP content was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) and FLUOstar OPTIMA® luminometer (BMG LABTECH, San Diego, CA, USA) with MARS data analysis software (Version 1.10, BMG LABTECH, Germany), as previously described by Narud et al. (2020). For each sample, three replicates of 3 x 10^5 sperm cells were analysed. The data recorded for each sample, measured in relative luminescence units (RLU), was converted to corresponding ATP values in nM according to a prepared standard curve, and the results are shown as µM ATP per million motile cells.

2.5. Analyses of metabolites

Analyses of amino acids, amines and trace elements were performed in both seminal plasma and sperm samples for all 25 bulls, whose semen was collected at the two different

ages. However, due to problems with the preparation of one sperm sample collected at 14 months of age, only 24 sperm cell samples were analysed for this group. Furthermore, some metabolites measured were below the limit of detection. The samples below the detection limit were excluded from further analysis, resulting in a variation in the sample number. For the study of metabolites in seminal plasma, 25 samples were analysed from 14 months old bulls while 21 samples were analysed from 17 months old bulls. For the study of metabolites in sperm cells, 24 samples were analysed from 14 months old bulls while 25 samples were analysed from 17 months old bulls.

2.5.1. Quantitative analysis of amino acids and amines

Analysis was performed on an Agilent 1290 Infinity II LC system (Agilent, Santa Clara, USA) coupled to an Agilent 6495 QqQ mass spectrometer, using one method for amino acids and one for amines. The QqQ-MS was equipped with a jet-stream ESI source operated in positive mode. The QqQ-MS was operated in dynamic multiple-reaction monitoring (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 Supplementary Table S1.

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 propyl chloroformate (PCF) derivatisation, as described by Narud et al. (2020). The chromatographic separation was performed in reversed phase mode employing an Ascentis Express C8 (2.1 x 150mm, 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. Sample preparation consisted of protein precipitation and PCF-derivatisation. Protein precipitation was achieved by addition of four volumes of ice-cold methanol. Following centrifugation, a 50 µL aliquot of the supernatant was collected, and dried using a speed-vac. 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 derivatized 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 evaporated to dryness. The derivatized amino acids were dissolved in methanol prior to analysis.

For the amines (Choline, Creatine, and L-Carnitine), the chromatographic separation was performed in HILIC mode employing a BEH Amide (2.1 x 150 mm, 2.7 µM) column (Waters, Milford, USA) and gradient elution using a 80:20 mixture of 25 mM Formic acid and 50 mM ammonium acetate as eluent A, and acetonitrile as eluent B, at a flow rate of 0.3 mL/min. The gradient used started at 90% B and was decreased stepwise to 5% B at 6.5 min. The column thermostat was maintained at 35 °C and the autosampler at 8°C. The injection volume was 1 µL. Mixed standards at 0, 0.5, 1, 10, 50, 100, 500, 1000, 5000 nM were used for calibration and quantitation. Sample preparation consisted of protein precipitation and dilution. Protein precipitation was achieved by addition of four volumes of ice-cold methanol. Following centrifugation, a 100 µL aliquot of the supernatant was

collected, and dried using a speed-vac. The amines were dissolved in aqueous internal standard-mix prior to analysis.

2.5.2. Analysis of trace elements by ICP-MS

ICP-MS analysis of Na, Mg, Al, P, S, K, Ca, Fe, Cu, Zn, Se, Sr, Cr, Mn, Co, Ni, As, Ag, Cd, Ba, and Pb was performed on an Agilent 8800 Triple Quadrupole ICP-MS (G3663A) mass spectrometer connected to a SPS4 autosampler, as described by Narud et al. (2020). A Peltier-cooled (2 °C) spray chamber with a MicroMist 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 -195 V. Sample preparation prior to ICP-MS analysis involved digestion of the sperm samples in HNO_3 in a microwave digestion unit. A 100 µL sample aliquot was used and mixed with 2.5 mL of 50% HNO_3 and digestion was performed in a Milestone Ultraclave (Sorisole, Italy) using a pre-set 8 step digestion program at 160 bar and increasing temperature stepwise from 50 °C to 245 °C.

2.5.3. Scaling of data

In order to adjust for differences in sperm cell numbers, the data for the sperm samples was scaled prior to statistical analysis. A scaling factor was determined based on the quantified amounts of a selection of amino acids in the samples. Seven amino acids (Aspartic acid, 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. The average concentrations for these seven amino acids were calculated and the ratio of observed concentration in each sperm sample to this average was determined, giving each sample its own scaling factor. This scaling factor

was used to compensate for differences in sample material by dividing the observed concentrations on the values of the corresponding scaling factors for the different sperm samples. The scaling was performed for both the amino acid, amine and trace element concentrations.

2.6. Semen production traits and fertility records

For the assessment of possible age effect on semen production traits and fertility, data was captured from all Norwegian Red bulls in semen production from December 2017 throughout 2019. Semen production data were obtained from Geno and included information on semen collections, where ejaculates were assigned consecutive and unique batch numbers for each bull. Only bulls introduced in semen production from December 1st 2017 and having batches used for AI before the end of 2019 were included in the analyses, resulting in data on a total of 1,800 batches from 56 bulls. The number of batches per bull varied from 6 to 80.

Data on AIs were obtained from the Norwegian Dairy Herd Recording System to calculate the bulls' field fertility, expressed as NR56. The dataset was edited prior to further statistical analyses. Repeated AIs within 5 days after the first inseminations were excluded from the analysis. If a second AI was reported in the interval from 5 to 56 days after the first insemination NR56=0 (female returned to estrus), otherwise NR56=1 (female did not return to estrus). Records where AI doses from the 1,800 batches were used for first insemination of Norwegian Red heifers or cows in parity < 7 were included. Batches with less than 25 reported AIs were excluded. After edits the final dataset had 91,948 NR56 records and included 40 bulls. The number of AIs per bull varied from 503 to 7370, with a mean of 2299.

2.7. Statistical analyses

Statistical analyses were performed using SAS® (version 9.4, SAS Institute Inc., Cary, NC, USA) for Windows. All data was tested for normal distribution by Shapiro-Wilk test, and parameters that did not show a normal distribution were log transformed prior to further statistical analysis. The homogeneity of variances was tested before the mixed procedure in SAS was used to perform least square means analyses. For the sperm quality parameters, the effect of the explanatory variables on the outcome variables; motility parameters, viability, DFI, HDS and ATP, were estimated by the following mixed linear model:

$$Y_{ijkl} = \mu + A_i + S_j + G_k + bull_l + e_{ijkl}$$

where:

Y_{ijkl} = observation of *in vitro* sperm parameter per semen sample;

μ = overall mean of the *in vitro* sperm parameter;

A_i = fixed effect of bulls age, i =1 (14 months) or 2 (17 months);

S_j = fixed effect of semen state, j = 1 (fresh) or 2 (frozen-thawed);

G_k = fixed effect of analysis group, k = 1, 2 or 3 (groups of bulls analysed at the same day);

$bull_l$ = Random effect of bull, l= 1 to 25;

e_{ijkl} = random error.

The mixed procedure in SAS was further used to perform a least square means analysis for the metabolites and trace elements in sperm and seminal plasma, using the

311 same model, but without the effect of semen state, because these were analysed only in
312 fresh samples.

313 The General Linear Model procedure in SAS was used to perform a least square
314 means analysis for the semen production data. Two age groups were defined, based on
315 the number of collected batches for each bull. Age group 1 (batch 1-5) included young
316 bulls of 14-15 months of age, while age group 2 (batches collected more than 100 days
317 after the first collection) represented bulls of approximately 17 months and older. The
318 semen production traits ejaculate volume, sperm concentration and discarded batches
319 (pre-freeze and post-thaw) were analysed using the following model:

320 $Y_{ijkl} = \mu + B_i + S_j + D_k + A_l + e_{ijkl}$

321 where:

322 Y_{ijkl} = observation of ejaculate volume, sperm concentration or discarded doses, per batch
323 per bull;

324 μ = overall mean ejaculate volume, sperm concentration or discarded doses;

325 B_i = effect of bull, i = 1-56;

326 S_j = season of semen collection, j = 4 classes (1: Dec-Feb, 2: Mar-May, 3: Jun-Aug, 4:
327 Sep-Nov);

328 D_k = days since last semen collection, k = 5 classes (1: < 3 days, 2: 3-4 days, 3: 5-7 days,
329 4: 8-14 days, 5: >14 days);

330 A_l = effect of age group, l=1 (batch 1-5) or 2 (batches collected more than 100 days after
331 the first collection);

e_{ijkl} = random error.

The General Linear Model procedure in SAS was further used to perform a least square means analysis for NR56 using the following model:

$Y_{ijkl} = \mu + C_i + I_j + B_k + A_l + e_{ijkl}$

where:

Y_{ijkl} = observation of NR56;

μ = overall mean NR56;

C_i = effect of female age and parity, i = 48 classes (for parity < 3 classes were a combination of parity and female age in months, for parity > 2 there were one class per parity);

I_j = effect of insemination month and year, j= 18 classes (1: May 2018, 2: June 2018…18: Oct 2019);

B_k = effect of bull, k = 1-40;

A_l = effect of age group, l=1 (batch 1-5) or 2 (batches collected more than 100 days after the first collection);

e_{ijkl} = random error.

The Tukey test was applied for pairwise comparisons between means.

3. Results

3.1. Effects of bull age on sperm quality in fresh and frozen-thawed semen

In vitro sperm quality analyses showed that several parameters were significantly different between the two age groups (Table 1). In both fresh and frozen-thawed samples, there was an effect of age on sperm HYP, VAP, VCL and ALH, with higher levels found in samples collected at 17 months compared to 14 months ($P<0.05$). Further, HDS, VSL, STR and LIN increased with age in sperm from frozen-thawed samples, while the ATP level increased with age in fresh samples ($P<0.05$).

3.2. Effects of bull age on metabolites in seminal plasma and spermatozoa

The effects of bull age on amino acid and amine concentrations were assessed in seminal plasma and sperm cells of fresh semen samples. There was a significant effect ($P<0.05$) of age on 17 of the 22 amino acids studied in seminal plasma, and 10 of the amino acids studied in sperm (Figure 1). In seminal plasma (Figure 1A), the level of arginine, asparagine, aspartic acid, GABA, glutamic acid, isoleucine, leucine, lysine, methionine, proline, threonine and tryptophan decreased with increasing age. In contrast, the level of cysteine and glutamine were higher in the samples collected at 17 months compared to 14 months ($P<0.05$). In spermatozoa (Figure 1B), glutamic acid, leucine and proline decreased with age, while an increase in concentration of alanine, arginine, cysteine, glutamine, serine, threonine and valine was observed with increasing age ($P<0.05$). No significant effect of age was found for any of the amines analysed.

A total of 21 trace elements were studied in both seminal plasma and sperm cells. Due to problems with levels being under the limit of detection, As, Al, Mn, Cr, Co, Ni, Ag, Cd and Pb were excluded from further analysis. The trace elements with concentration

differences between the two age groups and the most essential elements with possible influence on fertility are presented in Figure 2. The results show that levels of K and Ba in seminal plasma (Figure 2A) and sperm cells (Figure 2B), are affected by age ($P<0.05$), with higher levels found in samples collected at 17 months compared to 14 months.

3.3. Effects of bull age on NR56 and semen production traits

The effect of bull age on ejaculate volume, sperm concentration, number of discarded batches and NR56 are presented in Table 2. The ejaculate volume and sperm concentration were higher in age group 2 than in age group 1 ($P<0.05$). The percentage of discarded batches, pre-freeze and post-thaw, decreased with increasing age ($P<0.05$). The bulls' NR56 was higher in age group 1 compared to age group 2 ($P<0.05$), however, the difference was only 1% unit.

4. Discussion

The aim of the present study was to evaluate sperm quality, seminal plasma and sperm metabolites, semen production efficiency and fertility in young peri-pubertal bulls at 14 and 17 months of age. The results show that sperm hyperactivity and the kinematic motility parameters VAP, VCL and ALH, increased significantly with age in both fresh and frozen-thawed samples. Sperm motility and hyperactivity are important for sperm transport and penetration of the zona pellucida (Yanagimachi, 1969; Stauss et al., 1995; Suarez, 2002; Suarez and Ho, 2003). Thus, it was expected that sperm from 17 months old bulls in our study were superior in reaching and fertilizing the oocyte compared to younger bulls. However, this was not reflected by the NR56 data. A possible explanation can be that the motility parameters are characterized as compensable sperm traits and that sperm abnormalities can be overcome by increasing the amount of sperm in the AI dose (Kastelic, 2013). Thus, it is possible that the observed differences in sperm motility

parameters between the two age groups, are camouflaged by the relatively high number of spermatozoa (~12 x 10^6) used per AI dose in the present study.

Chromatin integrity is crucial for successful fertilization and consecutive embryo development (Sadeghi et al., 2009). During spermatogenesis, the majority of the core histones are replaced by protamines, resulting in chromatin hyper-compaction of the sperm nucleus. Improper chromatin packaging is one of the underlying factors of sperm DNA damage and contributes to male infertility (Dogan et al., 2015; Boe-Hansen et al., 2018). It is reported that young bulls have higher levels of DNA fragmentation and deficient protamination, which indicate a state of immaturity compared to adult bulls (Carreira et al., 2017). In the present study, no difference in chromatin integrity expressed as DFI was found between the two age groups. This is in agreement with Fortes et al. (2012), who also studied the chromatin integrity of young bulls with the mean ages of 13, 18 and 24 months (Fortes et al., 2012). However, in frozen-thawed samples, our results showed a small negative effect of age on chromatin integrity expressed as HDS, which indicate more immature spermatozoa in 17 months samples (Evenson et al., 2002).

In mammalian sperm, amino acids have been shown to play an important role in metabolic processes involved in sperm motility, capacitation and acrosome reaction (Cheah and Yang, 2011). Furthermore, free amino acids of seminal plasma are involved in protecting sperm cells against oxidative stress and denaturation, and are associated with the freezability of bull semen (Ugur et al., 2019). As bulls mature, the composition of the seminal plasma changes (Argov-Argaman et al., 2013; Holden et al., 2017; Vince et al., 2018), thus it is possible that age of young bulls affect the amino acid composition of semen samples. Our results showed that the concentration of 14 amino acids in seminal plasma and 10 amino acids in sperm cells were significantly different between the two age groups, including arginine, glutamine, cysteine and proline. In mammals, these amino

acids are reported to be involved in processes such as protection against lipid peroxidation and oxidative stress, sperm motility and glycolysis (Rudolph et al., 1986; Patel et al., 1998; Trimeche et al., 1999; Srivastava et al., 2000; Srivastava et al., 2006; Krishnan et al., 2008). The involvement of these amino acids in protection against oxidative stress was not assessed in the present study. However, our results may indicate that differences in the level of amino acids in both seminal plasma and sperm from bulls of the two different age groups, affects important processes such as sperm motility and the ability to withstand the negative consequences of reactive oxygen species. A recent study on metabolites in frozen-thawed bull semen showed that several amino acids correlated with fertility. Furthermore, the sperm intracellular amino acids were associated with parameters such as chromatin integrity, viability, acrosome integrity, ATP level and motility (Narud et al., 2020).

There was a significant effect of age on the level of tryptophan in seminal plasma, with decreased levels in samples collected at 17 months. Studies of hamster and human sperm have shown that L-tryptophan is used in the synthesis of 5-hydroxytryptamine, which promotes the acrosomal reaction and regulate sperm motility and tyrosine phosphorylation activity (Meizel and Turner, 1983; Jiménez-Trejo et al., 2012). The concentration of 5-hydroxytryptamine increases in the epididymis during sexual maturation (Jiménez-Trejo et al., 2007). It can be speculated that the decreased level of tryptophan at 17 months is due to a higher utilization of tryptophan for 5-hydroxytryptamine synthesis in the more mature sperm. Leucine and isoleucine in seminal plasma have previously been reported as potential biomarkers associated with bull fertility (Kumar et al., 2015). Our results showed that the level of both these amino acids in seminal plasma decreased in samples from 17 months old bulls compared to samples collected at 14 months.

While some trace elements (e.g. Ca, Cu, Fe, Se and Zn) are important for mammalian sperm cell function and protection against oxidative stress (Hong et al., 1984; Kantola et al., 1988; Chia et al., 2000; Aydemir et al., 2006; Tvrdá et al., 2013), other elements (e.g. Pb, Cd and As) may have a toxic effect on spermatozoa (Wang et al., 2017; Li et al., 2018). In the present study, K and Ba levels were significantly affected by age in seminal plasma and sperm cells, with higher levels in samples collected at 17 months compared to 14 months. Together with Na, K is reported to be responsible for the maintenance of seminal osmolarity and activity (Massanyi et al., 2008). Additionally, K-ion channels play vital roles in volume regulation of spermatozoa, motility and the acrosome reaction (Darszon et al., 1999; Barfield et al., 2005). However, others have reported that the K level in bull seminal plasma and sperm correlates negatively with total motility and progressive motility (Tvrdá et al., 2013). This does not corroborate our results, where the samples collected at 17 months had higher levels of K and increased sperm motility.

There is little available information about the effect of Ba on male reproductive health. One study in humans found that men with low-quality semen had significantly higher Ba concentrations in the seminal fluid than participants with normal-quality semen, and that Ba was negatively correlated to sperm viability (Sukhn et al., 2018). Our results, however, showed that several sperm parameters were improved for the samples collected at 17 months even though the level of Ba in seminal plasma and sperm was significantly increased. Despite the known importance for trace elements in reproductive performance and fertility, there are only a few studies focusing on trace elements in bovine semen (Aguiar et al., 2012; Tvrdá et al., 2013). Further investigations are therefore required to fully understand the mechanism and role of trace elements in maintaining semen quality and bull reproductive performance.

Young bulls have shown to have lower semen production compared to older, mature bulls (Karabinus et al., 1990; Devkota et al., 2008; Al-Kanaan et al., 2015), which corroborates our results, where ejaculate volume and sperm concentration significantly increased with increasing bull age. These findings may be linked to testicular size, as mature bulls with larger testes are shown to produce more sperm than young bulls with smaller testes (Amann and DeJarnette, 2012; Schenk, 2018). Semen quality improves during puberty, and sperm cells fulfil the quality criteria for normal motility and morphology around 16 months of age (Lambert et al., 2018). These seminal traits are likely associated with the normalization of spermatogenesis and epididymal function (Schenk, 2018). In agreement with this, the number of discarded batches significantly decreased with age, indicating that the semen quality improved during these few months of the bulls' life. The overall percentage of discarded doses were high, which may have been influenced by the fact that the breeding company introduced new semen collection and processing facilities prior to the study period. However, age, genetics and the time of fulfilled puberty are likely to be important for the observed number of discarded doses in this study. The fertility results, expressed as NR56, did not show the same tendency as the semen production traits and decreased with increasing age. However, the NR56 was considered high for all bulls and the difference between the age groups (approximately 1% unit) is considered to have no biological importance. These results indicate that young peri-pubertal bulls are mature enough for their semen to fulfil the fertilization process. However, the reduced semen production capacity of approved AI doses from the youngest animals, influence semen production efficiency, and could be improved by extensive and flexible pre-production andrology testing.

In Norway today, young GS bulls are introduced as breeding bulls at the age of 14 months. Most AI companies start semen collection even earlier when young sires are 11-

12 months old, and this will likely be the case in Norway in the future. As the onset of puberty and sexual maturation in bulls are affected by breed and individual bull differences, it would be beneficial for the AI industry to find biomarkers that can predict the maturity and subsequent reproductive performance of individual bulls. Our results showed that several sperm attributes, including amino acids and trace elements have the potential to differentiate between young bulls, even though the age difference was only three months. However, further investigations, using a larger number of bulls are necessary to identify if any of these parameters can be used as maturity biomarkers in the future.

5. Conclusion

In conclusion, reduced semen production efficiency in the youngest bulls is a challenge, and it would be beneficial to identify biomarkers in semen that can predict bull maturity and subsequent reproductive performance. This study has revealed that even small differences in age significantly affects sperm quality parameters and level of metabolites in semen from young Norwegian Red bulls. However, the results further suggest that 14-15 months old bulls are mature enough for their semen to fulfil successful fertilization.

CRediT authorship contribution statement

Birgitte Narud: Data curation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Abdolrahman Khezri:** Data curation, Formal analysis, Writing - review & editing. **Anna Nordborg:** Data curation, Formal analysis, Writing - review & editing. **Geir Klinkenberg:** Methodology, Writing - review & editing. **Teklu Tewoldebrhan Zeremichael:** Data curation, Formal analysis, Writing - review & editing. **Else-Berit Stenseth:** Data curation, Formal analysis, Writing - review & editing. **Bjørg Heringstad:** Formal analysis, Writing - review & editing. **Elisabeth Kommisrud:**

Conceptualization, Data curation, Formal analysis, Writing - review & editing. **Frøydis Deinboll Myromslien:** Conceptualization, Data curation, Formal analysis, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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750 Sperm quality parameters of fresh and frozen-thawed semen samples collected from 25 bulls at 14 and

751 17 months of age. Results are presented as mean ± SD.

	Fresh semen		Frozen-thawed semen	
Sperm parameter	14 months	17 months	14 months	17 months
TM (%)	83.7 ± 9.8	85.9 ± 6.3	51.15 ± 13.2	53.89 ±10.2
PM (%)	76.4 ± 11.3	82.9 ± 7.2	45.9 ± 13.1	48.5 ± 10.4
HYP (%)	22.3 ± 8.56[a]	27.3 ± 9.7 [b]	10.3 ± 6.1 [a]	16.8 ± 7.1 [b]
VAP(µM/s)	80.4 ± 10.2 [a]	87.9 ± 8.3 [b]	71.9 ± 10.2 [a]	87.0 ± 10.2 [b]
VCL (µM/s)	159.2 ± 19.9 [a]	177.8 ± 19.8 [b]	144.5 ± 24.0 [a]	180.2 ± 26.7 [b]
VSL (µM/s)	56.7 ± 9.2	60.6 ± 9.2	55.0 ± 9.1 [a]	67.6 ± 9.7 [b]
STR (%)	67.5 ± 4.6	66.7 ± 4.8	70.5 ± 3.8 [a]	75.3 ± 4.0 [b]
LIN (%)	36.8 ± 5.1	35.5 ± 4.2	37.4 ± 4.3 [a]	39.7 ± 4.6 [b]
ALH (µM)	4.8 ± 0.7 [a]	5.3 ± 0.7 [b]	4.4 ± 0.7 [a]	5.3 ± 0.8 [b]
Viability (%)	80.3 ± 10.3	81.6 ± 7.0	52.6 ± 10.5	57.7 ± 8.5
DFI (%)	2.6 ± 1.9	3.0 ± 1.7	3.0 ± 1.6	2.6 ± 2.1
HDS (%)	0.7 ± 0.3	0.9 ± 0.4	0.6 ± 0.3 [a]	1.4 ± 0.8 [b]
ATP (nM)	2.5 ± 0.8 [a]	3.5 ± 0.5 [b]	1.3 ± 0.5	1.6 ± 0.4

752 Different superscripts represents significant differences between age groups within fresh and frozen-

753 thawed samples based on linear mixed model (P<0.05). TM = total motile, PM = progressive motile,

754 HYP = hyperactive, VAP = velocity average path, VCL = velocity curvilinear, VSL = velocity straight-

755 line, STR = straightness, LIN = linearity, ALH = amplitude of lateral head displacement, DFI = DNA

756 fragmentation index, HDS = High DNA stainable.

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Table 2

Effect of age on non-return rate after 56 days (NR56) (n = 40) and semen production capacity (n = 56) in young Norwegian Red bulls. Results are presented as Least Squares mean ± SE.

	Age group 1	Age group 2
NR56 (%)	75 ± 0.6	74 ± 0.7*
Volume (mL)	5.3 ± 0.2	7.0 ± 0.1 ***
Concentration (x10^6/mL)	915.4 ± 28.4	1129.1 ± 18.6 ***
Discarded batches pre-freeze (%)	24.2 ± 2.3	8.5 ± 1.5 ***
Discarded batches post-thaw (%)	14.9 ± 2.2	7.1 ± 1.4 **

Age group 1 represents bulls of approximately 14-15 months of age (batch number 1-5), while Age group 2 represents bulls of approximately 17 months and older (batches collected more than 100 days after the first collection). Significant differences between the age groups based on general linear model: * P<0.05, ** P<0.01, ***P<0.0001.

Figure legends

Figure 1. The mean level of targeted amino acids studied in the seminal plasma (A) and sperm cells (B) in semen samples collected from 25 bulls at 14 and 17 months. 14 months = black bar, 17 months = grey bar. Whiskers represents SD. Significant differences between the age groups based on linear mixed model: * P<0.05, ** P<0.01, *** P<0.001.

Figure 2. The mean level of trace elements studied in the seminal plasma (A) and sperm cells (B) in semen samples collected from 25 bulls at 14 and 17 months of age. 14 months = black bar, 17 months = grey bar. Whiskers represents SD. Significant differences between the age groups based on linear mixed model: * P<0.05, ** P<0.01, *** P<0.001.

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782 Supplementary material

783 **Table S1**

784 Method parameters and instrument settings for targeted LC-MS/MS method used for analysis of
785 amino acids and amines in fresh semen samples. Precursor masses, product masses, specific
786 collision energies and retention time (RT) for each analyte. Analysis performed in multiple-
787 reaction monitoring (MRM) mode using electrospray ionisation.

	Precursor ion	Product ion	CV (V)	RT (min)	Internal standard
Amino acids					
Alanine	218.0	130.0	8	4.3	D3-Alanine
Arginine	303.2	70.1	44	1.1	13C6-Arginine
Asparagine	243.1	157.0	5	3.9	D3-Asparagine
Aspartic acid	304.4	216.0	10	6.6	
Cysteine	336.0	248.0	8	9.2	D2-Cysteine
GABA	232.0	85.9	16	4.3	D6-GABA
Glutamic acid	318.0	84.0	25	6.9	D5-Glutamic acid
Glutamine	275.0	84.2	28	2.2	13C5-Glutamine
Glycine	204.0	75.9	12	3.6	13C2-Glycine
Histidine	370.0	109.9	40	5.6	13C6-Histidine
Isoleucine	260.0	172.0	8	8.3	
Leucine	260.0	172.1	8	8.3	D10-Leucine
Lysine	361.0	301.1	4	5.9	13C6-Lysine
Methionine	278.0	189.9	4	5.8	13CD3-Methionine
Ornithine	347.2	287.2	5	5.2	D6-Ornithine
Phenylalanine	294.3	206.1	8	7.9	D5-Phenylalanine
Proline	244.2	156.2	10	5.1	D3-Proline
Serine	234.2	60.1	24	2.7	D3-Serine
Threonine	248.0	74.1	15	3.2	
Tryptophan	333.0	245.1	20	6.8	D5-Trypthophan
Tyrosine	396.0	222.2	20	10.3	13C9-Tyrosine
Valine	246.0	158.2	5	6.5	D8-Valine
Amines					
Choline	104.1	60.0	18	4.2	D13-Choline
Creatine	132.1	44.0	25	6.0	

L-Carnitine	162.2	103.0	15	5.9	D3-L-Carnitine

Additional instrument settings, analysis of amino acids: capillary voltage (CV) = 4 kV, nebulizer pressure (NP) = 40 psi, drying gas flow (DGF) = 20 L/min, gas temperature (T) = 210 °C, fragmentor voltage (FV) = 380 V, sheat gas temperature (SGT) = 400 °C, sheath gas flow (SGF) = 11 L/min, iFunnel positive high/low pressure RF = 150/60, and negative high/low pressure RF = 90/60. Analysis of amines: CV = 2 kV, NP = 30 psi, DGF = 14 l/min, T = 250 °C, FV = 380 V, SGT = 400 °C, SGF = 10 L/min, iFunnel positive high/low pressure RF = 120/60, and negative high/low pressure RF = 90/60.