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

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16 **ABSTRACT**

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

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

36 *Keywords:*

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

## 39 **1. Introduction**

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

45 lower semen production capacity compared to mature bulls, due to the ongoing  
46 development of the testis and accessory glands during sexual maturation (Almquist et al.,  
47 1976; Brito et al., 2002; Schenk, 2018). During spermatogenesis, a cascade of complex  
48 and hormone-dependent events take place to ensure proper development of germ cells.  
49 Peri-pubertal bulls usually have poor semen quality that gradually improves and  
50 eventually fulfils quality criteria around 16 months of age (Bruto et al., 2012; Lambert et  
51 al., 2018). Performing AI with semen produced before that age may affect bull  
52 reproductive performance.

53 Reproductive performance of bulls may be measured by the non-return (NR) rate,  
54 which means the proportion of inseminated females that do not return to estrus within a  
55 specific interval after the first AI (Foote, 2003), e.g. NR56 for 56 days. The fertility  
56 outcome of AI is affected by several factors, including age of the bull and the sperm  
57 quality of frozen-thawed semen (Zhang et al., 1999; Oliveira et al., 2012; Kumaresan et  
58 al., 2017). To succeed with fertilization, sperm cells should possess traits that enhance  
59 their ability to reach and fertilize the oocyte, such as intact plasma membranes and  
60 acrosomes (Christensen et al., 2011; Kumaresan et al., 2017), metabolism for energy  
61 production (Garrett et al., 2008), progressive sperm motility (Farrell et al., 1998; Puglisi  
62 et al., 2012; Gliozzi et al., 2017), and capacity for hyperactive motility (Suarez et al.,  
63 1991). Moreover, DNA integrity is important for fertilization and embryonic development  
64 (Waterhouse et al., 2006; Gliozzi et al., 2017). Although studies have shown associations  
65 between field fertility and sperm quality parameters, there is still no single *in vitro* analysis  
66 that reliably can predict the true fertilization potential of a semen sample.

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

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

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

## 83 **2. Materials and methods**

### 84 *2.1. Animals and semen processing*

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

95 concentration  $> 390 \times 10^6/\text{mL}$ , subjective total motility  $> 70\%$ , and normal morphology  
96  $> 85\%$  were further processed. Each ejaculate was diluted to a final concentration of  $12 \times$   
97  $10^6$  spermatozoa per AI dose in French mini straws (IMV, L'Aigle, France), using a two-  
98 step dilution procedure with Biladyl extender (Minitübe, GmbH, Tiefenbach, Germany,  
99 13500/0004-0006). First-dilution was performed at  $35^\circ\text{C}$ , while the second glycerol  
100 containing extender was added at  $5^\circ\text{C}$ . Cryopreservation was performed according to  
101 standard procedures (Standerholen et al., 2014). Semen with post-thaw motility  $< 50\%$   
102 were discarded. For the analyses of metabolites, 2 mL of neat semen was centrifuged ( $110$   
103  $\times g$ , 10 min) to separate the seminal plasma (supernatant) from the sperm cells (pellet).  
104 Thereafter, both sperm and seminal plasma samples were snap-frozen in liquid nitrogen  
105 and shipped to SINTEF (SINTEF Industry, Trondheim, Norway) for analyses of  
106 metabolites and trace elements. Furthermore, sperm quality parameters were analysed in  
107 first-diluted semen on the day of collection (hereafter referred to as fresh semen samples).  
108 For analyses of sperm quality in frozen-thawed samples, the cryopreserved semen doses  
109 were thawed for 1 min in a  $37^\circ\text{C}$  water-bath. Two semen doses from each ejaculate were  
110 thawed and mixed together.

## 111 *2.2. Assessment of sperm motility by CASA*

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

120 were analysed per sample. Each sample was analysed twice at a frame rate of 45 frames  
121 per second. Sperm cells were identified by sperm head area of  $20\ \mu\text{m}^2$ -  $80\ \mu\text{m}^2$ . The  
122 kinematic parameters recorded were: average path velocity (VAP,  $\mu\text{m/s}$ ), curvilinear  
123 velocity (VCL,  $\mu\text{m/s}$ ), straight line velocity (VSL,  $\mu\text{m/s}$ ), straightness (STR) of the  
124 average path defined as the ratio of VSL/VAP (%), linearity (LIN) of the curvilinear path  
125 defined as the ratio of VSL/VCL (%), and amplitude of lateral head displacement (ALH,  
126  $\mu\text{m}$ ). Total motility (TM) was defined as sperm cells with  $\text{VCL} > 15\ \mu\text{m/s}$ , progressive  
127 motility (PM) was defined as sperm cells with  $\text{STR} > 70\ \%$ . Sperm cells with  $\text{VCL} > 80$   
128  $\mu\text{m/s}$ ,  $\text{ALH} > 6.5\ \mu\text{m}$  and  $\text{LIN} < 65\ \%$  were defined as having hyperactive motility (HYP).

### 129 2.3. Flow cytometry

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

#### 138 2.3.1. Sperm plasma membrane integrity

139 For the analysis of sperm plasma membrane integrity, the semen samples were  
140 stained with propidium iodide (PI, Sigma-Aldrich), which only stain sperm cells with  
141 damaged plasma membrane (non-viable cells). The semen samples were diluted in SP-  
142 Talp media (105 mM NaCl, 3.1 mM KCl, 0.4 mM  $\text{MgCl}_2$ , 2.0 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.3 mM  
143  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 1 mM sodium pyruvate, 21.6 mM sodium lactate, 20 mM Hepes, 20 mM

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

#### 154 2.3.2. *Sperm chromatin integrity*

155 The chromatin integrity of the sperm cells was analysed by the Sperm Chromatin  
156 Structure Assay (SCSA), as previously described by Evenson and Jost (2001) and Narud  
157 et al. (2020). In brief, semen samples were diluted to  $2 \times 10^6$  sperm cells/mL in TNE  
158 buffer (10 mM Tris-HCL, 0.1 M NaCl, 1 mM EDTA, pH 7.4) in a final volume of 200  
159 µL. Next, 400 µL acid detergent solution (0.38 M NaCl, 80 mM HCL, 0.1 % (w/v) Triton  
160 X-100, pH 1.2) was added, followed by incubation at RT for 30 sec. Then, 1.2 mL acridine  
161 orange (AO) staining solution (6 µg/mL AO (A3568, Life Technologies, OR, USA)) in a  
162 buffer containing 37 mM citric acid, 0.126 M  $\text{Na}_2\text{HPO}_4$ , 1.1 µM EDTA, and 0.15 M NaCl  
163 (pH 6) was added. Data acquisition started exactly at the end of 3 min setup mode, in  
164 which 5000 events were collected for each sample at a flow rate of ~200 events/sec. The  
165 signals were separated by a 550 nm dichroic long pass mirror, before green fluorescence  
166 was detected by a 525 nm band pass filter and red fluorescence was detected by a 670 nm  
167 long pass filter. Prior to sample analysis the flow cytometry instrument was AO-saturated,  
168 by running AO equilibration solution (1.2 mL AO staining solution and 400 µL acid



169 detergent solution) through the system for 5 min. To control the stability of the laser, the  
170 mean green and red fluorescence signals were set to  $425 \pm 5$  and  $125 \pm 5$ , respectively,  
171 first at the start of analysis and later after analysing every fifth sample. This was performed  
172 using reference semen from a bull of known DNA fragmentation index (DFI) in a  
173 bivariate cytogram. The FL1 (green) was presented on the x-axis and FL3 (red) on the y-  
174 axis of the cytogram, both on a linear scale. The percentage of red (ssDNA) and green  
175 (dsDNA) fluorescence was determined using FCS Express 6 Flow cytometry Software  
176 (Denovo Software, Los Angeles, CA, USA). Based on a histogram of the fluorescence  
177 ratio red / (red + green), the percentage of spermatozoa with fragmented DNA (DFI, %)  
178 was calculated. The bivariate cytogram was used to determine high DNA stainability  
179 (HDS, %), which correspond to the spermatozoa with the most intensive green  
180 fluorescence, recognized as immature spermatozoa (Evenson et al., 2002).

#### 181 *2.4. Intracellular ATP content*

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

#### 190 *2.5. Analyses of metabolites*

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

193 ages. However, due to problems with the preparation of one sperm sample collected at 14  
194 months of age, only 24 sperm cell samples were analysed for this group. Furthermore,  
195 some metabolites measured were below the limit of detection. The samples below the  
196 detection limit were excluded from further analysis, resulting in a variation in the sample  
197 number. For the study of metabolites in seminal plasma, 25 samples were analysed from  
198 14 months old bulls while 21 samples were analysed from 17 months old bulls. For the  
199 study of metabolites in sperm cells, 24 samples were analysed from 14 months old bulls  
200 while 25 samples were analysed from 17 months old bulls.

#### 201 *2.5.1. Quantitative analysis of amino acids and amines*

202 Analysis was performed on an Agilent 1290 Infinity II LC system (Agilent, Santa  
203 Clara, USA) coupled to an Agilent 6495 QqQ mass spectrometer, using one method for  
204 amino acids and one for amines. The QqQ-MS was equipped with a jet-stream ESI source  
205 operated in positive mode. The QqQ-MS was operated in dynamic multiple-reaction  
206 monitoring (MRM) mode ( $\Delta Rt = 1$  min) with unit mass resolution for both mass  
207 filters. The MRM transitions for standards and internal standards and the employed  
208 collision energies, gas temperatures and flows, are given in Supplementary Table S1.

209 Amino acids (Alanine, Arginine, Asparagine, Aspartic acid, Cysteine,  $\gamma$ -  
210 Aminobutyric acid (GABA), Glutamic acid, Glutamine, Glycine, Histidine, Isoleucine,  
211 Leucine, Lysine, Methionine, Ornithine, Phenylalanine, Proline, Serine, Threonine,  
212 Tryptophan, Tyrosine, and Valine) were analysed by LC-MS/MS following propyl  
213 chloroformate (PCF) derivatisation, as described by Narud et al. (2020). The  
214 chromatographic separation was performed in reversed phase mode employing an  
215 Ascentis Express C8 (2.1 x 150mm, 2.7  $\mu$ M) column (Sigma-Aldrich), and gradient  
216 elution using 25 mM Formic acid as eluent A, and acetonitrile as eluent B, at a flow rate  
217 of 0.3 mL/min. The gradient used started at 35% B and was increased stepwise to 90% B

218 at 14 min. Complete washout was performed by increasing to 100% B before the column  
219 was reconditioned with the starting conditions. The column thermostat was maintained at  
220 35 °C and the autosampler at 6 °C. The injection volume was 2 µL. Mixed standards at 0,  
221 0.1, 1, 10, 50, 100, 500, 1000, 4000 µM were used for calibration and quantitation.  
222 Internal standards were used in the analysis. Sample preparation consisted of protein  
223 precipitation and PCF-derivatisation. Protein precipitation was achieved by addition of  
224 four volumes of ice-cold methanol. Following centrifugation, a 50 µL aliquot of the  
225 supernatant was collected, and dried using a speed-vac. Internal standard-mix was added  
226 followed by 1 M NaOH (390 µL), 1-propanol (335 µL) and pyridine (65 µL), followed  
227 by addition of the derivatisation reagent PCF (80 µL). For extraction of the derivatized  
228 amino acids, a 400 µL volume of chloroform was added followed by 50 mM NaHCO<sub>3</sub>  
229 (400 µL). Vortex mixing was performed after each addition of solvent and reagent. A 200  
230 -µL aliquot of the chloroform phase was thereafter transferred to a clean vial and  
231 evaporated to dryness. The derivatized amino acids were dissolved in methanol prior to  
232 analysis.

233 For the amines (Choline, Creatine, and L-Carnitine), the chromatographic separation  
234 was performed in HILIC mode employing a BEH Amide (2.1 x 150 mm, 2.7 µM) column  
235 (Waters, Milford, USA) and gradient elution using a 80:20 mixture of 25 mM Formic acid  
236 and 50 mM ammonium acetate as eluent A, and acetonitrile as eluent B, at a flow rate of  
237 0.3 mL/min. The gradient used started at 90% B and was decreased stepwise to 5% B at  
238 6.5 min. The column thermostat was maintained at 35 °C and the autosampler at 8°C. The  
239 injection volume was 1 µL. Mixed standards at 0, 0.5, 1, 10, 50, 100, 500, 1000, 5000 nM  
240 were used for calibration and quantitation. Sample preparation consisted of protein  
241 precipitation and dilution. Protein precipitation was achieved by addition of four volumes  
242 of ice-cold methanol. Following centrifugation, a 100 µL aliquot of the supernatant was

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

#### 245 *2.5.2. Analysis of trace elements by ICP-MS*

246 ICP-MS analysis of Na, Mg, Al, P, S, K, Ca, Fe, Cu, Zn, Se, Sr, Cr, Mn, Co, Ni, As,  
247 Ag, Cd, Ba, and Pb was performed on an Agilent 8800 Triple Quadrupole ICP-MS  
248 (G3663A) mass spectrometer connected to a SPS4 autosampler, as described by Narud et  
249 al. (2020). A Peltier-cooled (2 °C) spray chamber with a MicroMist nebulizer was used  
250 as the introduction system. The RF Power was set to 1550 W and the RF Matching to 1.80  
251 V, and the nebulizer gas was set at 1.05 L/min. The cell gases used were He and O<sub>2</sub> at 4.3  
252 and 1 mL/min, respectively. Analysis was performed in MS/MS mode. Extract voltage 1  
253 and 2 were set at 0 and -195 V. Sample preparation prior to ICP-MS analysis involved  
254 digestion of the sperm samples in HNO<sub>3</sub> in a microwave digestion unit. A 100 µL sample  
255 aliquot was used and mixed with 2.5 mL of 50% HNO<sub>3</sub> and digestion was performed in a  
256 Milestone Ultraclave (Sorisole, Italy) using a pre-set 8 step digestion program at 160 bar  
257 and increasing temperature stepwise from 50 °C to 245 °C.

#### 258 *2.5.3. Scaling of data*

259 In order to adjust for differences in sperm cell numbers, the data for the sperm  
260 samples was scaled prior to statistical analysis. A scaling factor was determined based on  
261 the quantified amounts of a selection of amino acids in the samples. Seven amino acids  
262 (Aspartic acid, Leucine, Lysine, Methionine, Proline, Threonine and Tyrosine) were  
263 included in the scaling factor, chosen based on their co-variance in the sample series, and  
264 their similar profiles in all samples. The average concentrations for these seven amino  
265 acids were calculated and the ratio of observed concentration in each sperm sample to this  
266 average was determined, giving each sample its own scaling factor. This scaling factor

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

## 271 *2.6. Semen production traits and fertility records*

272 For the assessment of possible age effect on semen production traits and fertility, data  
273 was captured from all Norwegian Red bulls in semen production from December 2017  
274 throughout 2019. Semen production data were obtained from Geno and included  
275 information on semen collections, where ejaculates were assigned consecutive and unique  
276 batch numbers for each bull. Only bulls introduced in semen production from December  
277 1<sup>st</sup> 2017 and having batches used for AI before the end of 2019 were included in the  
278 analyses, resulting in data on a total of 1,800 batches from 56 bulls. The number of batches  
279 per bull varied from 6 to 80.

280 Data on AIs were obtained from the Norwegian Dairy Herd Recording System to  
281 calculate the bulls' field fertility, expressed as NR56. The dataset was edited prior to  
282 further statistical analyses. Repeated AIs within 5 days after the first inseminations were  
283 excluded from the analysis. If a second AI was reported in the interval from 5 to 56 days  
284 after the first insemination NR56=0 (female returned to estrus), otherwise NR56=1  
285 (female did not return to estrus). Records where AI doses from the 1,800 batches were  
286 used for first insemination of Norwegian Red heifers or cows in parity < 7 were included.  
287 Batches with less than 25 reported AIs were excluded. After edits the final dataset had  
288 91,948 NR56 records and included 40 bulls. The number of AIs per bull varied from 503  
289 to 7370, with a mean of 2299.

## 290 *2.7. Statistical analyses*

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

$$299 Y_{ijkl} = \mu + A_i + S_j + G_k + \text{bull}_l + e_{ijkl}$$

300 where:

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

302  $\mu$  = overall mean of the *in vitro* sperm parameter;

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

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

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

307  $\text{bull}_l$  = Random effect of bull,  $l = 1$  to 25;

308  $e_{ijkl}$  = random error.

309 The mixed procedure in SAS was further used to perform a least square means  
310 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  $\mu$  = 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);

332  $e_{ijkl}$  = random error.

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

335 
$$Y_{ijkl} = \mu + C_i + I_j + B_k + A_l + e_{ijkl}$$

336 where:

337  $Y_{ijkl}$  = observation of NR56;

338  $\mu$  = overall mean NR56;

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

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

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

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

347  $e_{ijkl}$  = random error.

348 The Tukey test was applied for pairwise comparisons between means.



349 **3. Results**

350 *3.1. Effects of bull age on sperm quality in fresh and frozen-thawed semen*

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

357 *3.2. Effects of bull age on metabolites in seminal plasma and spermatozoa*

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

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

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

### 376 3.3. *Effects of bull age on NR56 and semen production traits*

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

## 383 4. Discussion

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

396 parameters between the two age groups, are camouflaged by the relatively high number  
397 of spermatozoa ( $\sim 12 \times 10^6$ ) used per AI dose in the present study.

398 Chromatin integrity is crucial for successful fertilization and consecutive embryo  
399 development (Sadeghi et al., 2009). During spermatogenesis, the majority of the core  
400 histones are replaced by protamines, resulting in chromatin hyper-compaction of the  
401 sperm nucleus. Improper chromatin packaging is one of the underlying factors of sperm  
402 DNA damage and contributes to male infertility (Dogan et al., 2015; Boe-Hansen et al.,  
403 2018). It is reported that young bulls have higher levels of DNA fragmentation and  
404 deficient protamination, which indicate a state of immaturity compared to adult bulls  
405 (Carreira et al., 2017). In the present study, no difference in chromatin integrity expressed  
406 as DFI was found between the two age groups. This is in agreement with Fortes et al.  
407 (2012), who also studied the chromatin integrity of young bulls with the mean ages of 13,  
408 18 and 24 months (Fortes et al., 2012). However, in frozen-thawed samples, our results  
409 showed a small negative effect of age on chromatin integrity expressed as HDS, which  
410 indicate more immature spermatozoa in 17 months samples (Evenson et al., 2002).

411 In mammalian sperm, amino acids have been shown to play an important role in  
412 metabolic processes involved in sperm motility, capacitation and acrosome reaction  
413 (Cheah and Yang, 2011). Furthermore, free amino acids of seminal plasma are involved  
414 in protecting sperm cells against oxidative stress and denaturation, and are associated with  
415 the freezability of bull semen (Ugur et al., 2019). As bulls mature, the composition of the  
416 seminal plasma changes (Argov-Argaman et al., 2013; Holden et al., 2017; Vince et al.,  
417 2018), thus it is possible that age of young bulls affect the amino acid composition of  
418 semen samples. Our results showed that the concentration of 14 amino acids in seminal  
419 plasma and 10 amino acids in sperm cells were significantly different between the two  
420 age groups, including arginine, glutamine, cysteine and proline. In mammals, these amino

421 acids are reported to be involved in processes such as protection against lipid peroxidation  
422 and oxidative stress, sperm motility and glycolysis (Rudolph et al., 1986; Patel et al.,  
423 1998; Trimeche et al., 1999; Srivastava et al., 2000; Srivastava et al., 2006; Krishnan et  
424 al., 2008). The involvement of these amino acids in protection against oxidative stress  
425 was not assessed in the present study. However, our results may indicate that differences  
426 in the level of amino acids in both seminal plasma and sperm from bulls of the two  
427 different age groups, affects important processes such as sperm motility and the ability to  
428 withstand the negative consequences of reactive oxygen species. A recent study on  
429 metabolites in frozen-thawed bull semen showed that several amino acids correlated with  
430 fertility. Furthermore, the sperm intracellular amino acids were associated with  
431 parameters such as chromatin integrity, viability, acrosome integrity, ATP level and  
432 motility (Narud et al., 2020).

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

446 While some trace elements (e.g. Ca, Cu, Fe, Se and Zn) are important for mammalian  
447 sperm cell function and protection against oxidative stress (Hong et al., 1984; Kantola et  
448 al., 1988; Chia et al., 2000; Aydemir et al., 2006; Tvrdá et al., 2013), other elements (e.g.  
449 Pb, Cd and As) may have a toxic effect on spermatozoa (Wang et al., 2017; Li et al.,  
450 2018). In the present study, K and Ba levels were significantly affected by age in seminal  
451 plasma and sperm cells, with higher levels in samples collected at 17 months compared  
452 to 14 months. Together with Na, K is reported to be responsible for the maintenance of  
453 seminal osmolarity and activity (Massanyi et al., 2008). Additionally, K-ion channels play  
454 vital roles in volume regulation of spermatozoa, motility and the acrosome reaction  
455 (Darszon et al., 1999; Barfield et al., 2005). However, others have reported that the K  
456 level in bull seminal plasma and sperm correlates negatively with total motility and  
457 progressive motility (Tvrdá et al., 2013). This does not corroborate our results, where the  
458 samples collected at 17 months had higher levels of K and increased sperm motility.

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

470 Young bulls have shown to have lower semen production compared to older, mature  
471 bulls (Karabinus et al., 1990; Devkota et al., 2008; Al-Kanaan et al., 2015), which  
472 corroborates our results, where ejaculate volume and sperm concentration significantly  
473 increased with increasing bull age. These findings may be linked to testicular size, as  
474 mature bulls with larger testes are shown to produce more sperm than young bulls with  
475 smaller testes (Amann and DeJarnette, 2012; Schenk, 2018). Semen quality improves  
476 during puberty, and sperm cells fulfil the quality criteria for normal motility and  
477 morphology around 16 months of age (Lambert et al., 2018). These seminal traits are  
478 likely associated with the normalization of spermatogenesis and epididymal function  
479 (Schenk, 2018). In agreement with this, the number of discarded batches significantly  
480 decreased with age, indicating that the semen quality improved during these few months  
481 of the bulls' life. The overall percentage of discarded doses were high, which may have  
482 been influenced by the fact that the breeding company introduced new semen collection  
483 and processing facilities prior to the study period. However, age, genetics and the time of  
484 fulfilled puberty are likely to be important for the observed number of discarded doses in  
485 this study. The fertility results, expressed as NR56, did not show the same tendency as the  
486 semen production traits and decreased with increasing age. However, the NR56 was  
487 considered high for all bulls and the difference between the age groups (approximately  
488 1% unit) is considered to have no biological importance. These results indicate that young  
489 peri-pubertal bulls are mature enough for their semen to fulfil the fertilization process.  
490 However, the reduced semen production capacity of approved AI doses from the youngest  
491 animals, influence semen production efficiency, and could be improved by extensive and  
492 flexible pre-production andrology testing.

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

495 12 months old, and this will likely be the case in Norway in the future. As the onset of  
496 puberty and sexual maturation in bulls are affected by breed and individual bull  
497 differences, it would be beneficial for the AI industry to find biomarkers that can predict  
498 the maturity and subsequent reproductive performance of individual bulls. Our results  
499 showed that several sperm attributes, including amino acids and trace elements have the  
500 potential to differentiate between young bulls, even though the age difference was only  
501 three months. However, further investigations, using a larger number of bulls are  
502 necessary to identify if any of these parameters can be used as maturity biomarkers in the  
503 future.

504

## 505 **5. Conclusion**

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

## 513 **CRedit authorship contribution statement**

514 **Birgitte Narud:** Data curation, Formal analysis, Visualization, Writing - original  
515 draft, Writing - review & editing. **Abdolrahman Khezri:** Data curation, Formal analysis,  
516 Writing - review & editing. **Anna Nordborg:** Data curation, Formal analysis, Writing -  
517 review & editing. **Geir Klinkenberg:** Methodology, Writing - review & editing. **Teklu**  
518 **Tewoldebrhan Zeremichael:** Data curation, Formal analysis, Writing - review & editing.  
519 **Else-Berit Stenseth:** Data curation, Formal analysis, Writing - review & editing. **Björg**  
520 **Heringstad:** Formal analysis, Writing - review & editing. **Elisabeth Kommisrud:**

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

#### 524 **Declaration of Competing Interest**

525 The authors have no conflicts of interest to declare.

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**Table 1**

Sperm quality parameters of fresh and frozen-thawed semen samples collected from 25 bulls at 14 and 17 months of age. Results are presented as mean  $\pm$  SD.

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

Different superscripts represents significant differences between age groups within fresh and frozen-thawed samples based on linear mixed model ( $P < 0.05$ ). TM = total motile, PM = progressive motile, HYP = hyperactive, VAP = velocity average path, VCL = velocity curvilinear, VSL = velocity straight-line, STR = straightness, LIN = linearity, ALH = amplitude of lateral head displacement, DFI = DNA fragmentation index, HDS = High DNA stainable.

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760 **Table 2**

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

	Age group 1	Age group 2
NR56 (%)	75 $\pm$ 0.6	74 $\pm$ 0.7*
Volume (mL)	5.3 $\pm$ 0.2	7.0 $\pm$ 0.1 ***
Concentration ( $\times 10^6$ /mL)	915.4 $\pm$ 28.4	1129.1 $\pm$ 18.6 ***
Discarded batches pre-freeze (%)	24.2 $\pm$ 2.3	8.5 $\pm$ 1.5 ***
Discarded batches post-thaw (%)	14.9 $\pm$ 2.2	7.1 $\pm$ 1.4 **

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

767 **Figure legends**

768 **Figure 1.** The mean level of targeted amino acids studied in the seminal plasma (A) and  
 769 sperm cells (B) in semen samples collected from 25 bulls at 14 and 17 months. 14 months  
 770 = black bar, 17 months = grey bar. Whiskers represents SD. Significant differences  
 771 between the age groups based on linear mixed model: \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .  
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773 **Figure 2.** The mean level of trace elements studied in the seminal plasma (A) and sperm  
 774 cells (B) in semen samples collected from 25 bulls at 14 and 17 months of age. 14 months  
 775 = black bar, 17 months = grey bar. Whiskers represents SD. Significant differences  
 776 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
<i>Amino acids</i>					
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-Tryptophan
Tyrosine	396.0	222.2	20	10.3	13C9-Tyrosine
Valine	246.0	158.2	5	6.5	D8-Valine
<i>Amines</i>					
Choline	104.1	60.0	18	4.2	D13-Choline
Creatine	132.1	44.0	25	6.0	

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	L-Carnitine	162.2	103.0	15	5.9	D3-L-Carnitine
788	Additional instrument settings, analysis of amino acids: capillary voltage (CV) = 4 kV, nebulizer					
789	pressure (NP) = 40 psi, drying gas flow (DGF) = 20 L/min, gas temperature (T) = 210 °C,					
790	fragmentor voltage (FV) = 380 V, sheath gas temperature (SGT) = 400 °C, sheath gas flow (SGF)					
791	= 11 L/min, iFunnel positive high/low pressure RF = 150/60, and negative high/low pressure RF					
792	= 90/60. Analysis of amines: CV = 2 kV, NP = 30 psi, DGF = 14 l/min, T = 250 °C, FV = 380 V,					
793	SGT = 400 °C, SGF = 10 L/min, iFunnel positive high/low pressure RF = 120/60, and negative					
794	high/low pressure RF = 90/60.					