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ORIGINAL RESEARCH

Post-thaw semen quality in young bull ejaculates before being accepted for commercial semen doses

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Abstract

Background: Genomic selection enables bulls with desirable characteristics to be identified at a young age, but sperm quality can be poor in the ejaculates of young bulls. Few studies have been done on post-thaw sperm quality in bulls less than 10 months old. The objective of this study was to determine the age at which post-thaw sperm quality was acceptable for artificial insemination.

Methods: Semen was collected by artificial vagina; samples containing 100–500 million spermatozoa/ml were frozen for this study. Post-thaw analyses of membrane integrity (MI), mitochondrial membrane potential (MMP), chromatin integrity, morphology, production of reactive oxygen species and sperm kinematics were made.

Results: The age at which ejaculates exceeded the breeding company's thresholds of acceptance varied considerably among individuals, with 285 days being the earliest. Morphology ($p < 0.003$), MI ($p = 0.0096$), high MMP ($p = 0.043$) and superoxide production ($p = 0.0084$) increased between the first and last ejaculates but reached acceptable levels at different ages for individual bulls.

Conclusions: It was possible to obtain acceptable post-thaw sperm quality from samples even though sperm concentration was lower than the breeding company's threshold. Therefore, it might be feasible to use ejaculates earlier than is currently considered possible, by modifying semen handling protocols.

KEYWORDS
Artificial insemination industry, first ejaculates, proximal cytoplasmic droplets, sperm fertility

INTRODUCTION

Genomic selection of cattle[1] enables future breeding sires to be identified at a young age but does not guarantee good ejaculate quality. There is considerable interest in being able to use genetically selected young bulls as breeding sires for artificial insemination (AI) as young as possible, to take advantage of their superior genetics.[2] Genomic selection has been well adopted by the AI industry, with the result that, currently, most of the commercially available semen doses are from young bulls without progeny testing. Young bulls are also promoted as sires of sons.[3] However, the sperm quality of young bulls is considered to be inferior to older bulls: for example, sperm morphology can be abnormal, with many retained cytoplasmic droplets and other morphological abnormalities.[4] Little work has been done to define other sperm characteristics, such as membrane integrity (MI), chromatin integrity of young bulls or to determine at what age these bulls produce ejaculates that give an acceptable result on thawing.

Each breeding company has its own quality control standards for frozen semen doses for artificial insemination. To achieve these standards, the raw ejaculate is evaluated for sperm concentration and parameters such as sperm motility, MI and morphology, depending on the company's internal quality standards. Fresh semen samples exceeding these thresholds are frozen as semen doses for insemination; further evaluation of sperm quality is performed after thawing. Only samples reaching acceptable

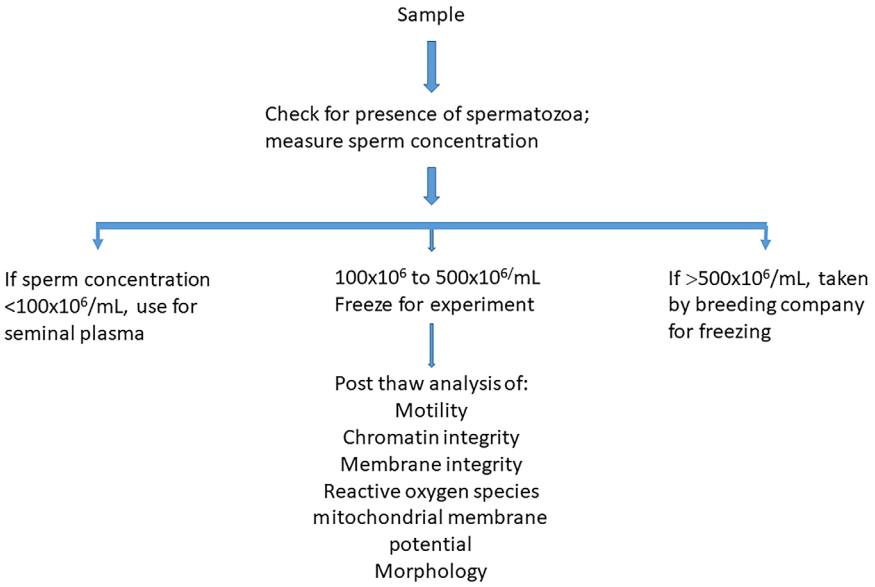

FIGURE 1 Experimental design. Note: seminal plasma collected and frozen for a different study [5]

thresholds at this stage are released for sale after quarantine.

The purpose of this study was two-fold: i) to define the post-thaw characteristics of semen from young bulls, and ii) to determine the age at which they could produce ejaculates that survive freezing.

METHODS

Nineteen 8-month-old bulls at a commercial semen collection centre (VikingGenetics, Örnsro, Sweden) were made available. These were the bulls that entered the centre over a 6-month period in 2017. They were 11 Swedish Red, seven Holstein and one Fell breed. Bulls were weighed at monthly intervals. A breeding soundness evaluation was carried out when the bulls arrived. The age and weight when entering and leaving the study (i.e., when the samples were taken for commercial freezing) were recorded.

The bulls were brought into the semen collection barn once a week from the age of 8 months and were allowed to mount teaser animals. Once erection occurred, the penis was stimulated using an artificial vagina. Any samples obtained were checked for the presence of spermatozoa; samples with less than 100×10^6 spermatozoa/ml were used to prepare seminal plasma (SP) for a different study.[5] Samples with a sperm concentration of 100–500 $\times$ 10^6/ml were used for the present study (Figure 1). Samples with a sperm concentration of more than 500×10^6/ml and acceptable subjective motility were needed for freezing for commercial purposes and thus were not available for the study. The number of samples that did not reach our threshold sperm concentration of 100×10^6/ml varied between bulls, from 0 to 10 (mean $\pm$ SD: 4.5 $\pm$ 3.4). Therefore, the number of samples available for the study varied between bulls, from 2 to 10 (mean $\pm$ SD: 5 $\pm$ 2) (Table S1). The freezing protocol was performed according to the routine procedure at VikingGenetics; the semen was extended in Andromed (Minitüb International; Tiefenbach, Germany) at a concentration of 69×10^6 spermatozoa/ml, before packing in 0.25 ml straws. The semen was cooled overnight before freezing using an automated freezing machine (DigitCool 5300; IMV Technologies, l'Aigle, France).

Sperm analyses

Straws were transported to the laboratory at the Swedish University of Agricultural Sciences (SLU), Uppsala, in liquid nitrogen and were thawed at 37°C for 12 seconds for the following analyses.

Sperm concentration was measured using the Nucleocounter SP100, as described by Goodla et al.[6] Briefly, 50 μl of semen were mixed with 5 ml detergent reagent S100 and the mixture was loaded into cassettes containing propidium iodide (PI). Fluorescence from the stained sperm nuclei was measured using the fluorescence meter and converted to sperm concentration.

Flow cytometry

Sperm plasma MI, mitochondrial membrane potential (MMP), the DNA fragmentation index (%DFI), and production of reactive oxygen species (ROS) were evaluated by flow cytometry after staining with fluorescent probes,[6] as described below. All analyses were performed using a FACSVerse flow cytometer (BD Biosciences; Franklin Lakes, NJ, USA). It was equipped with a solid-state 488 nm/20 mW blue laser, a solid-state 640 nm/40 mW red laser and a solid-state 405 nm/40 mW violet laser. The area of the collected signals was used in all analyses. List-mode data were collected using FACSuite software (BD Biosciences). In all assays, forward scatter (FSC) and side scatter (SSC) were collected with linear amplification and used for gating spermatozoa. Fluorescence parameters were collected using logarithmic amplification, with the exception of the chromatin integrity experiments, where they were collected using linear amplification. For MI and ROS, the sperm concentration

was first adjusted to 2×10^6/ml while for MMP it was adjusted to 2.5×10^6/ml using a buffered salt solution (buffer B; patent applied for).

Membrane integrity

Aliquots of all samples were stained with 0.08 μM SYBR 14 and 24 μM PI (Live-Dead Sperm Viability Kit L-7011; Invitrogen, Eugene, OR, USA). After incubating in the dark at 37°C for 10 minutes, the samples were evaluated using a FACSVerse flow cytometer (BD Biosciences; Franklin Lakes, NJ, USA). Excitation was induced by a blue laser (488 nm). The threshold was set at FSC at the level 10,000, with a voltage of 323.4; SSC was collected using a voltage of 375.4. Green fluorescence (FL1) from SYBR 14 was detected with a 507 nm long-pass dichroic mirror, followed by a band-pass filter (527/32 nm) with a voltage setting of 264.7, and red fluorescence (FL3) from PI was measured using a 665 nm long-pass dichroic mirror, followed by a band-pass filter (700/54 nm) with a voltage setting of 403.2. A compensation of 24.12% was used for FL3-FL1, while no compensation was used for FL1-FL3. A total of 30,000 events was evaluated for each sample. After gating to identify spermatozoa, the cells were classified as membrane intact (SYBR14-positive/PI-negative), or membrane damaged (SYBR14-negative/PI-positive or SYBR14-positive/PI-positive). For the purposes of this study, only proportions of membrane intact spermatozoa are reported.

Mitochondrial membrane potential

Aliquots of all samples were stained with 12 μM of the lipophilic cationic probe 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes) according to Cossarizza et al.,[7] and incubated at 37°C for 30 minutes in the dark. Excitation of stained cells was obtained with a blue laser (488 nm). Threshold was set at FSC at the level 10,000, with a voltage of 361.8; SSC was collected using a voltage of 383. Emitted fluorescence was detected using FL1 (527/32 nm; green) collected through a 507 nm long-pass dichroic mirror, followed by a band-pass filter (527/32 nm) at a voltage of 280.2, and FL2 (586/42 nm; orange) collected through a 560 nm long-pass filter followed by a band-pass filter, at a voltage of 355.9. Compensation was applied between channels (for FL2-FL1 78.88%, for FL1-FL2 19.69%). Evaluation of 30,000 cells was followed by gating to identify spermatozoa and classifying them into two groups: spermatozoa with high MMP (orange fluorescence) and those with low MMP (FL1). Only high MMP results are reported here.

Chromatin integrity

The method is a modification of Evenson et al.[8] Aliquots of sperm samples were mixed 1:1 with TNE buffer (tris-sodium chloride-EDTA; 0.15 mol/L NaCl, 0.01 mol/L tris-HCl, 1 mmol/L EDTA, pH 7.4), snap-frozen in liquid nitrogen and stored at −80°C until required for analysis. After thawing the samples on ice, aliquots (10 μl) were extended with TNE buffer (90 μl) and subjected to partial DNA denaturation in situ with 0.2 ml detergent solution (0.17% Triton X-100, 0.15 mol/L NaCl, and 0.08 mol/L HCl; pH 1.2). The samples were stained with acridine orange (0.6 ml; 6 μg/ml in 0.1 mol/L citric acid, 0.2 mol/L Na_2HPO_4, 1 mmol/L EDTA, 0.15 mol/L NaCl; pH 6.0). Within 3–5 minutes, the samples were analysed using a flow cytometer (FACSVerse; BD Biosciences). For each sample, a minimum of 10,000 events was analyzed at a speed of 200 cells/second after excitation with a blue laser (488 nm). The FSC was used as a threshold with a setting of 10,000, and a voltage of 267. The voltage for SSC was 376.5. The FL1 was collected through a 507 nm long-pass dichroic mirror, followed by a band-pass filter (527/32 nm) at a voltage of 339.2 and FL3 was collected using a 665 nm long-pass dichroic mirror, followed by a band-pass filter (700/54 nm) with a voltage setting of 479.9. The %DFI (the ratio of cells with denatured, single-stranded DNA to total cells acquired), the standard deviation of DFI and the mean of the DFI distribution were calculated for each sample using FCS Express version 5 (De Novo Software, Pasadena, CA, USA).

Production of ROS

The procedure of Guthrie and Welch,[9] modified slightly by Goodla et al.[6] was used. The modification was that the volume of the reagents was halved. Aliquots were stained with Hoechst 33258 to distinguish live from dead spermatozoa at a concentration of at 1.2 μM (HO; Sigma, Stockholm), 1.2 μM hydroethidine (HE; Invitrogen Molecular Probes, Eugene, OR, USA) was used to detect superoxide (SO), and 60 μM dichlorodihydrofluorescein diacetate (DCFDA; Invitrogen Molecular Probes) was used to detect hydrogen peroxide (H_2O_2). The samples were incubated at 37°C for 30 minutes in the dark. Excitation was with a blue laser (488 nm) and a violet laser (405 nm). FSC, with a threshold of 10,000 and voltage of 223.9 as well as SSC with a voltage of 379.1 was collected. Detection of FL1 from DCFDA, excited by the blue laser, was collected through a 507 nm long-pass dichroic mirror, followed by a band-pass filter (527/32 nm) at a voltage of 339.2; FL3 from HE, excited by the blue laser, was collected using a 665 nm long-pass dichroic mirror, followed by a band-pass filter (700/54 nm) with a voltage setting of 433.8; and blue fluorescence (FL5) from HO, excited by the violet laser, was detected via a band-pass filter (528/45 nm) with a voltage of 352.4. Compensation values used were for FL3-FL1 30.03%, for FL5-FL1 26.36%, for FL1-FL3 0%, for FL5-FL3 0%, for FL1-FL5 0% and for FL3-FL5 0%. In total, 30,000 sperm specific-events were evaluated. After gating for spermatozoa in the FSC-SCC dot-plot, they were classified as live, SO-negative; live, SO-

positive; dead, SO-positive; live, H_2O_2-negative; live, H_2O_2-positive; dead, H_2O_2-negative; and dead, H_2O_2-positive.

Morphology

Smears were air dried and stained with carbolfuchsin-eosin.[10] Five hundred spermatozoa were evaluated using 1000x magnification with oil immersion, recording morphological abnormalities such as proximal cytoplasmic droplets, detached heads, acrosome defects, nuclear pouches and tail defects. Each individual spermatozoon was classified as having normal morphology if no abnormality was detected. Further aliquots of semen were fixed in formol-saline and used to make wet mounts for evaluation of 200 spermatozoa at 1000x magnification. The proportion of normal spermatozoa in the sample was calculated from these two sets of data. The morphology evaluation was carried out by skilled personnel in the Swedish Sperm Reference Laboratory at SLU.

Sperm motility

Computer-assisted sperm analysis (CASA) was carried out using SpermVision (Minitűb International, Tiefenbach, Germany). An aliquot (5μl) was placed on a glass slide on the warm stage (38°C) of an Olympus BX 51 microscope. The kinematics were measured in eight fields, containing at least 1000 spermatozoa, using the settings for bull spermatozoa recommended by the manufacturer at a frame rate of 60/second. Particles with an area ranging from 20 to 100 μm^2 were included in the analysis. The following kinematics were measured: total motility (TM, %), progressive motility (%), average path velocity (VAP; μm/s), curvilinear velocity (VCL; μm/s straight-line velocity (VSL; μm/s), the ratios straightness (STR; VSL/VAP), linearity (LIN; VSL/VCL) and wobble (WOB; VAP/VCL), the amplitude of lateral head displacement (ALH; μm), and beat cross frequency (BCF; Hz). Spermatozoa were identified as immotile (BCF < 0.2; VSL < 0.2), hypermotile (VCL > 80; LIN < 0.65; ALH > 6.5), and progressively motile (STR > 0.5 and LIN > 0.35).

Statistical analysis

Data were analyzed by the MIXED procedure for linear mixed models using SAS software (version 9.4; SAS Institute Inc., Cary, NC). The model included the fixed effect of sampling occasion (two classes: the first ejaculate in the sampling series and the last ejaculate before acceptance for commercial freezing), season (two classes: autumn and winter), and their interaction. The random effects of bull and age were included in the model. The residuals from the observations generated from the mixed models were tested for normal distribution. Data on DFI and live H_2O_2+ concentrations deviated from a normal distribution and were log transformed. However, to improve clarity, avoid redundancy and facilitate interpretation, the respective log-transformed values are referred to and presented as untransformed values throughout the paper. The least-squares means (LSM ± SEM) estimated by the models were adjusted using the Scheffé adjustment for multiple post-ANOVA comparisons and compared. The contrast statement was used to test an individual hypothesis. A repeated effect was tested with the correlations between bulls by specifying a correlation structure (UN) among residuals. Differences with $p \leq 0.05$ were considered significant. Differences with $0.05 < p \leq 0.10$ were considered as tendencies.

Pearson correlations between age and various sperm quality parameters for all ejaculates within each bull were performed using the formula in Excel (Microsoft Office package).

RESULTS

Number of attempts before sperm concentration reached 100×10^6/ml, and number of ejaculates with a sperm concentration between 100 and 500×10^6/ml

The number of weekly attempts before sperm concentration reached 100×10^6/ml varied from 0 to 10 (mean ± SD: 4.4 ± 3.4), that is some bulls achieved this sperm concentration in the first sample they produced whereas others took several weeks. The number of ejaculates with a sperm concentration between 100 and 500×10^6/ml (and therefore available for this study) varied between two and 10 for different bulls (Table S1).

Age and weight at which post-thaw ejaculates were approved, according to the company's internal standards

The mean (±SD) age at which the MI of the frozen sperm samples was greater than 40% (the main quality standard used by this breeding company) was 354 ± 39 days for Swedish Red and 318 ± 33 days for Holstein (range: 285–415 days); the Fell bull was 358 days old. (Figure 2). There was a strong correlation between the age at which the fresh ejaculate was considered acceptable and the age at which the post-thaw ejaculate was considered acceptable ($r = 0.94$; $p < 0.001$). The interval between acceptance before freezing and acceptance after freezing varied from 0 in eight bulls to 35 days in four bulls (mean ± SD: 25 ± 11).

The mean body weight of the bulls when the frozen ejaculates were first approved by the breeding company was higher for Swedish Red bulls than for Holstein bulls (424 ± 48 and 364 ± 33 kg, respectively). The Fell bull was 380 kg when the frozen samples were approved. The distribution of body weight when the ejaculate achieved the breeding company's standards for acceptable pre-freeze and post-thaw quality is shown in Figure 3. There was a poor association between the weight of the bull when the fresh

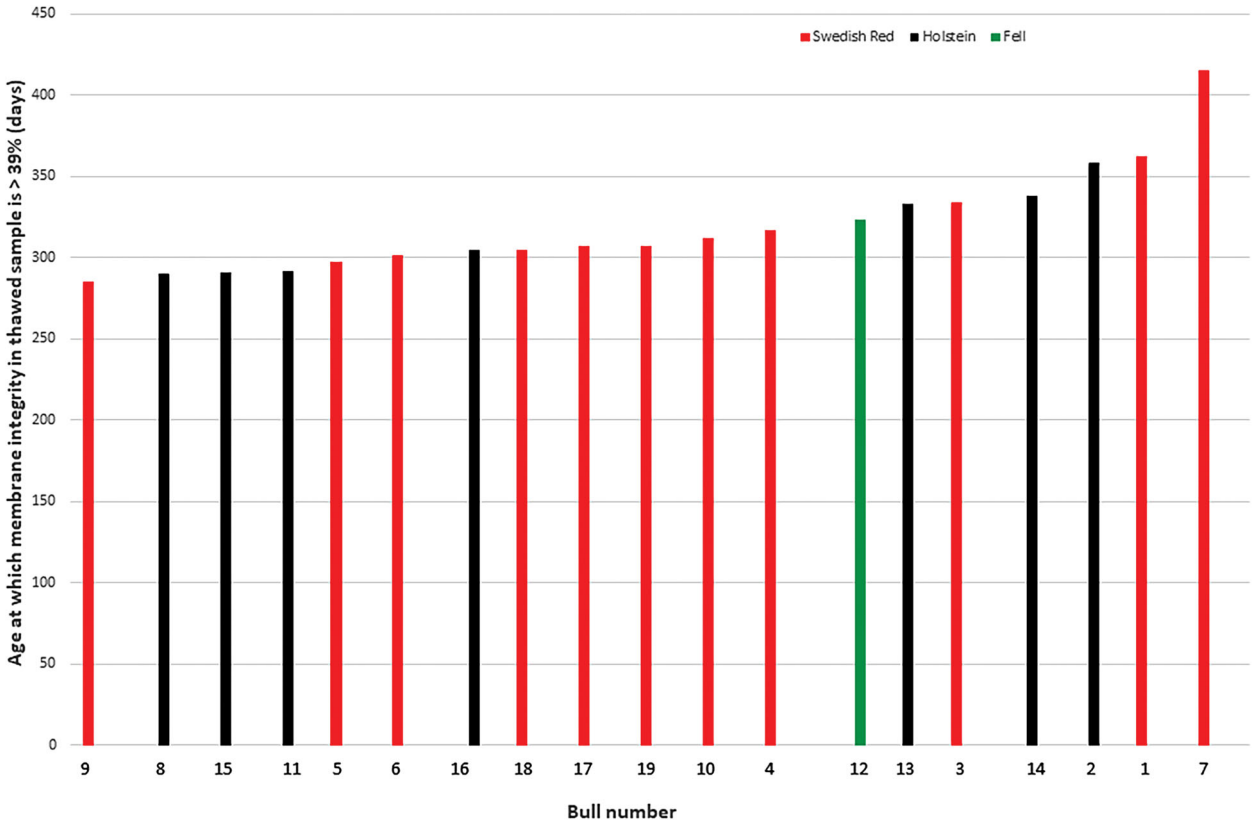

FIGURE 2 Age at which sperm quality of fresh and frozen ejaculates reached the breeding company's internal standards for acceptability ($n = 19$). Note: black bars represent Holstein bulls, red bars represent Swedish Red bulls and the green bar represents Fell bulls

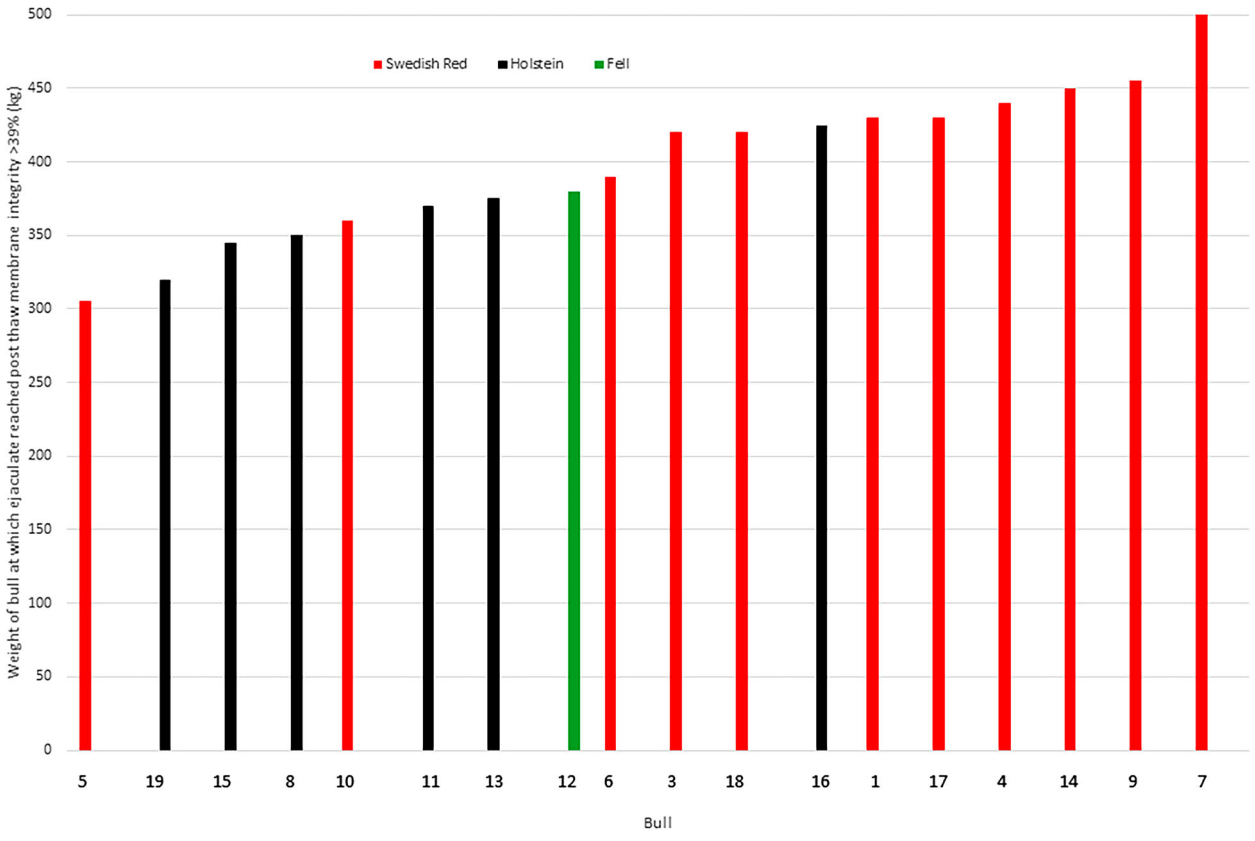

FIGURE 3 Weight of bull at which sperm quality of fresh and frozen ejaculates reached the breeding company's internal standards for acceptability ($n = 19$). Note: black bars represent Holstein bulls, red bars represent Swedish Red bulls and the green bar represents Fell bulls. No weight was recorded for Bull 2 when the ejaculates had satisfactory post-thaw sperm quality

TABLE 1 Mean values of membrane integrity, high mitochondrial membrane potential, DNA fragmentation index and sub-populations of spermatozoa producing reactive oxygen species in first and last ejaculates from 19 young bulls

	MI (%)	High MMP (%)	%DFI	Live H_2O_2- (%)	Live H_2O_2+ (%)	Live SO– (%)	Live SO+ (%)
First	37.3 ± 8.8	26.2 ± 2.8	25.1 ± 7.6	36.9 ± 2.9	0.17 ± 0.08	40.36 ± 11.56	−12.5 ± 7.6
Last	50.0 ± 9.8	34.4 ± 3.6	22.5 ± 8.3	46.8 ± 2.6	0.16 ± 0.08	44.73 ± 12.99	−8.6 ± 8.5
p	= 0.0096	=0.043	<0.04	<0.002	NS	NS	<0.001

Note: first = the first ejaculate that contained more than 100×10^6 spermatozoa/ml; last = the last ejaculate before the sample was considered to be acceptable for freezing by the commercial company.
Abbreviations: %DFI, DNA fragmentation index; H_2O_2, hydrogen peroxide; MI, membrane integrity; MMP, mitochondrial membrane potential; NS, not significant; SO, superoxide.

ejaculate was considered to be acceptable for freezing and the weight when the post-thaw sperm quality was considered to be acceptable ($r = 0.29$; NS).

Sperm quality in the non-approved samples

There was considerable variation among bulls in all the semen quality traits measured. In general, sperm quality improved with increasing age for any individual bull, but the various traits improved at different ages in different individuals (Tables S1 and 2).

Membrane integrity, chromatin integrity, and MMP

Mean sperm MI and proportion of spermatozoa with high mitochondrial potential were higher in the last ejaculate than in the first ($p < 0.001$, $p < 0.0096$ and $p = 0.043$, respectively) whereas mean fragmented DNA (%DFI) was lower in the last ejaculate than in the first ($p < 0.04$) (Table 1). There was no effect of season of semen collection on these parameters, although there was a sample × season interaction for %DFI ($p < 0.03$)

Reactive oxygen species

Mean live SO+ and live H_2O_2– (Table 1 and Table S1) were increased in the last ejaculate compared to the first ($p = 0.0084$ and $p = 0.0128$, respectively), although there were no differences in live SO- or live H_2O_{2+} between the first and last ejaculates. There was no effect of season on these parameters, although there was a sample × season interaction ($p = 0.039$) for live H_2O_2-.

Sperm morphology

There was an effect of age on sperm morphology (Table 2): the mean proportion of spermatozoa with normal morphology was 50 ± 26% for the first sample, rising to 71 ± 15% for the second sample ($p < 0.001$). The mean proportion of spermatozoa with a retained proximal cytoplasmic drop was 59 ± 49% in the first sample and 25 ± 32% in the second sample ($p < 0.001$). Similarly, the mean proportion of spermatozoa with abnormal heads was 12 ± 7% in the first sample and 7 ± 3% in the second sample ($p < 0.001$). Overall, four bulls had an acceptable spermiogram (>70% normal spermatozoa) for the first sample and seven for the second sample. For eight of the bulls, the non-sperm cell content of the sample (epithelial cells, pyknotic cells, boat cells and spermatogenic cells) was lower in the second sample compared to the first sample (subjective assessment).

Kinematics

The proportion of motile spermatozoa post-thaw varied from 0% to 79% but was not related to bull age. Total and progressive motilities (Table 3 and Table S2) were higher in the last ejaculate than in the first ($p = 0.003$). Mean sperm kinematics varied among bulls (Table 2), and were higher in the last ejaculate than in the first except for STR, LIN, WOB and BCF, which were not different between the first and last ejaculates. Season affected BCF ($p = 0.044$), STR ($P = 0.015$), LIN ($p = 0.0002$) and WOB ($p < 0.03$). There was a sample × season interaction for STR ($p = 0.01$) and LIN ($p = 0.0011$).

Trends in sperm quality with age for individual bulls

Each of the 19 bulls showed a different pattern of development in sperm quality. Trends in MI, high MMP, TM and %DFI with age are shown for a Holstein bull (Figure 4) and for a Swedish Red bull (Figure 5). For the Holstein bull, post-thaw MI improved with age and was at the breeding company's acceptable level at 283 days, and the %DFI decreased throughout the whole period. In contrast, for the Swedish Red bull, post-thaw MI reached the desired level only at 400 days of age, and both high MMP and TM increased throughout the collection period, whereas %DFI was still elevated in the last ejaculate. The %DFI at day 414 (approximately 15%) was approximately the same level as measured in the Holstein bull ejaculate at 283 days.

DISCUSSION

This study was carried out to define the semen characteristics of young bulls and to determine the age

TABLE 2 Spermiogram for 19 young bulls at different ages

Bull	Age (days)	Normal (%)	Pathological heads (%)	Proximal drops (%)	Non-sperm cells
1a	306	43	29.4	47	++; pyknotic cells
1b	355	72	11.4	8	++
2a	337	60	14.8	7	+++; boat cells
2b	366	78	10	4	++
3a	396	65	6.8	28.5	++
3b	410	75	44	17.5	+
4a	366	88	6.6	1.5	++; boat cells
4b	381	89	7.2	2.5	+; boat cells
5a	276	29	16.8	45	Boat cells
5b	325	81	6.2	4.5	Boat cells
6a	271	13	13	70	++: boat cells
6b	320	75	4	12	+++; boat cells
7a	331	33	13	37.5	+; boat cells
7b	408	64	5.6	11.5	+; spermatogenetic cells
8a	290	72	6.4	13	++; boat cells
8b	304	78	6.4	6.5	++
9a	306	63	8	23.5	+; boat cells
9b	341	67	8.6	23	+++
10a	286	78	3.2	2.5	+; boat cells
10b	314	84	4.6	0	+; boat cells
11a	278	26	10	53	+
11b	285	45	10	37.5	+
12a	358	89	5.2	1	+
12b	372	85	4.4	0	0
13a	312	79	3.6	5	boat cells
14a	338	73	4.8	3	+; boat cells
14b	352	80	4.4	1	++; boat cells
15a	291	8	9.4	64	+
15b	311	52	7.2	16.5	0; boat cells
16a	284	57	18	5	+
16b	347	70	12.6	2.5	++; Boat cells
17a	298	22	9.4	64	+++
17b	354	33	4	61.5	+
18a	326	79	5.6	5	Boat cells
19a	293	31	22.8	41	–
19b	307	77	6	7.5	–

Non-sperm cells refers to epithelial cells (+ individual; ++ several; +++ many), pyknotic cells, spermatogenetic cells and boat cells. *N* = bull identity; a, b refer to the sampling occasion for morphology, with a being the first and b the second.

TABLE 3 Mean kinematics of first and last ejaculates from 19 young bulls

	TM (%)	PM (%)	VAP (μm/second)	VCL (μm/second)	VSL (μm/second)	STR	LIN	WOB	ALH (μm)	BCF (Hz)
First	30.5 ± 4.8	28.0 ± 4.49	49.4 ± 2.1	82.2 ± 4.46	36.9 ± 1.5	0.93 ± 0.08	0.58 ± 0.05	0.590.03	2.2 ± 1.9	28.8 ± 3.4
Last	51.2 ± 4.5	47.6 ± 4.4	58.3 ± 2.4	109.2 ± 5.4	41.7 ± 2.1	0.93 ± 0.09	0.57 ± 0.05	0.58 ± 0.04	3.08 ± 1.1	28.1 ± 3.8
p	<0.003	<0.003	<0.008	=0.01	=0.06	NS	NS	NS	=0.011	NS

Note: first = the first ejaculate that contained more than 100×10^6 spermatozoa/ml; last ejaculate = the last ejaculate before the sample was considered to be acceptable for freezing by the commercial company.

Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; PM, progressive motility; STR, straightness (VSL/VAP); TM, total motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; LIN, linearity (VSL/VCL); WOB, wobble (VAP/VCL).

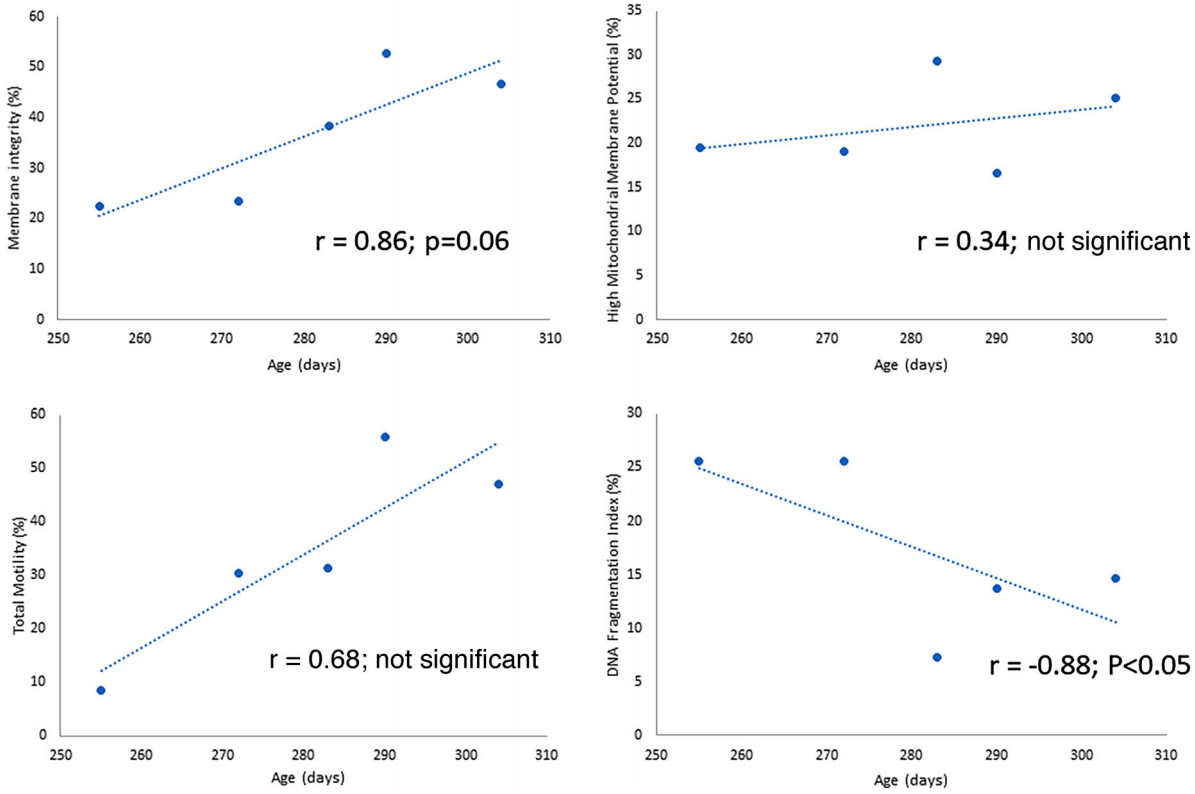

FIGURE 4 Relationship between age and sperm quality for a Holstein bull; upper left, membrane integrity; upper right, high mitochondrial membrane integrity; lower left, total motility, lower right, DNA fragmentation index. Note: the DNA fragmentation index showed a significant improvement with age ($p < 0.05$) and there was a trend towards significance for an improvement in membrane integrity with age ($p = 0.06$)

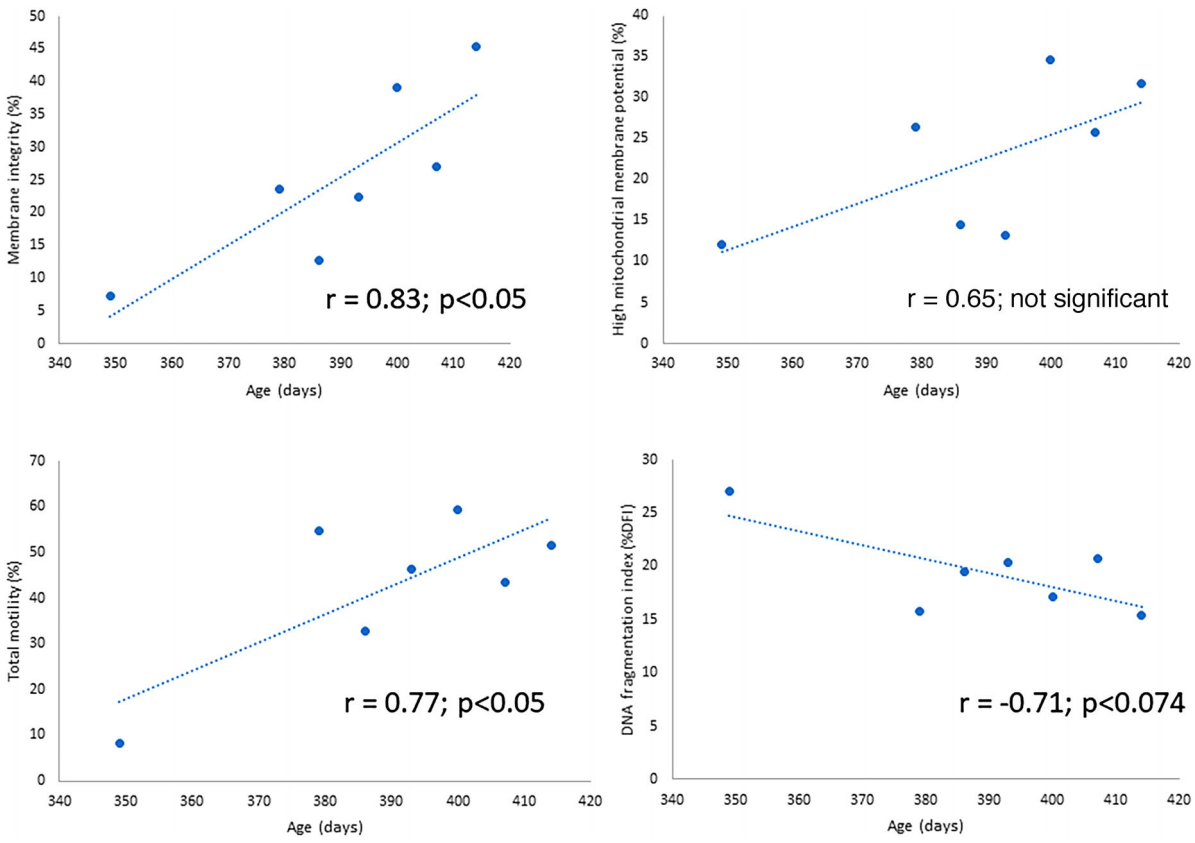

FIGURE 5 Relationship between age and sperm quality for a Swedish Red bull; upper left, membrane integrity; upper right, high mitochondrial membrane integrity; lower left, total motility, lower right, DNA fragmentation index. Note: there was a significant improvement in membrane integrity and total motility with age ($p < 0.05$ for both) and a trend towards significance for an improvement in DNA fragmentation index with age ($p < 0.074$)

at which these bulls could produce ejaculates that survived freezing with acceptable post-thaw sperm quality. The results showed that although sperm quality tended to improve and stabilize with increasing age, the age at which acceptable post-thaw sperm quality could be obtained varied considerably among bulls, with 285 days being the earliest. Individual traits reached acceptable levels at different ages. These results are in agreement with other recent studies in older bulls, for example, that increasing age was associated with improved sperm quality in Norwegian Red bulls of 10.5–15.5 months old[11] and Holstein-Friesian bulls less than 1 year old had lower sperm quality than bulls over 1 year old.[2]

Flow cytometry

MI, chromatin integrity and MMP did not show a direct relationship to the age of bull in our study, although MI and MMP tended to be higher within bull as the bulls matured. However, the %DFI showed a more variable pattern. Other studies on chromatin integrity have investigated the levels of DNA fragmentation in older bulls, reporting levels of 2%–10% in Swedish Red and Holstein bulls[6,12] and 2%–22% in Holstein bulls.[13] Thus, some of the sperm samples in this study had an elevated level of chromatin damage compared with levels in older bulls.

Sperm morphology

Fertility is strongly related to the proportion of morphologically normal spermatozoa in the sample.[14,15] Amann et al.[4] found that in vitro fertilization (IVF) was severely hindered if more than 30% of the sperm samples had proximal cytoplasmic droplets. In our study, sperm morphology improved with the age of the bull, with an increase in normal spermatozoa and a decrease in retained cytoplasmic droplets as well as in the non-sperm cellular content of the ejaculate. These results are in accordance with other published data in older bulls. Proximal droplets were seen to occur more frequently in bulls of several *Bos indicus* or *Bos taurus* breeds that were younger than 20 months old compared to older bulls,[16] and a negative correlation was found between age of dairy bull and proportion of abnormal spermatozoa.[17] However, in another study, the proportions of abnormal spermatozoa were not different in beef bulls of 11–18 months compared to older bulls.[18] Opinion remains divided concerning whether the proximal cytoplasmic droplet is a compensable defect or not.[19] Certainly, an increased proportion of cytoplasmic droplets is associated with decreased fertility in AI,[20] and fertility in IVF is reduced if >30% spermatozoa have proximal cytoplasmic droplets. However, it is possible that young bulls with 30% cytoplasmic droplets may be fertile if the droplet retention is merely a factor of their youth and not an indication of a fundamental problem with spermatogenesis.[4]

Kinematics

Mean values of sperm kinematics were generally higher in the last ejaculates than in the first. To our knowledge, the kinematics of young bull semen has not been reported in detail, apart from the study on Norwegian Red bulls.[21] However, some studies have found associations between kinematics and fertility for older bulls, for example, Gliozzi et al.,[22] whereas others have not found such an association, for example, Kumaresan et al.[23] These contrasting results may be due to factors such as differences in the type of instrument used, chambers type and temperature of analysis.[24]

Future possibilities

The choice of a threshold sperm concentration of $>500 \times 10^6$/ml for commercial freezing is due to the concept that ejaculates with a lower sperm concentration would be mixed with a lower volume of cryomedium than ejaculates with a higher sperm concentration to achieve the desired number of spermatozoa per insemination dose. As a result, the amount of antibiotics present in the final sample might not be sufficient to inhibit microbial growth. However, in some cases in the present study it was possible to obtain acceptable post-thaw sperm quality from samples even though they did not reach the breeding company's acceptable sperm concentration for freezing (500×10^6spermatozoa/ml). This observation implies that it should be possible to freeze some ejaculates from young bulls and obtain acceptable sperm quality when the sperm concentration is between 100 and 500×10^6/ml, that is a lower sperm concentration than is current praxis, if the volume of SP could be reduced, for example, by gentle centrifugation through a low-density colloid.[25] It might be possible to use these ejaculates by changing the sperm handling protocols, particularly with regard to the proportion of cryoprotectant, and using hand-filling of straws instead of machine filling because of the low volume of extended semen. These possibilities should be considered for young bulls of high genetic merit, to advance the availability of their semen for artificial insemination.

CONCLUSION

In this study, the age at which young bulls could produce an ejaculate with acceptable post-thaw quality varied considerably among individuals, with 285 days being the earliest age. Morphology, MI, MMP and ROS production all improved with age but reached acceptable levels at different ages for different bulls. Although these ejaculates did not meet the production criteria of the breeding company in terms of sperm concentration in the original sample, it was possible to obtain post-thaw samples with sperm quality that reached quality control thresholds. Additional trials are needed

to determine the optimal sperm number per insemination dose of young bull semen.

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CONFLICT OF INTEREST

Emma Hurri was employed by VikingGenetics at the time of the study.

AUTHOR CONTRIBUTIONS

EH collected the samples and did some of the analyses. ILV did the rest of the analyses under the supervision of AJ. HS arranged for the samples to be collected and helped negotiate the funding. JM applied for the funding, designed the experiment, organised the project and wrote the first draft. All authors read and approved the final draft.

ETHICS APPROVAL

This study did not require official or institutional ethical approval. The animals were owned by the commercial company VikingGenetics for the purpose of semen collection by an artificial vagina. The animals were handled according to high ethical standards and national legislation. No additional procedures were carried out on the animals themselves.

DATA AVAILABILITY STATEMENT

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request, in relation to the data protection act.

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SUPPORTING INFORMATION

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