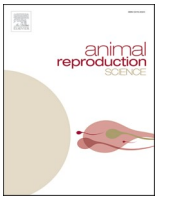
Animal Reproduction Science 253 (2023) 107261

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci



# Novel interpretation of sperm stress test and morphology for maturity assessment of young Norwegian Red bulls

Joanna Bremer [a,*,1], Bjørg Heringstad [b,2], Jane M. Morrell [a,c,3], Elisabeth Kommisrud [a,4]

[a] Department of Applied Ecology, Agricultural Sciences and Biotechnology, Inland Norway University of Applied Sciences, Hamar, Norway
[b] Department of Animal and Aquacultural Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, Ås, Norway
[c] Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden

ARTICLE INFO

*Keywords:*
Puberty
Sperm stress test
Computer-aided sperm analysis
Flow Cytometry
Scrotal circumference
Bull Breeding Soundness Evaluation

ABSTRACT

The use of genomic selection significantly reduces the age of dairy bulls entering semen production compared to progeny testing. The study aimed to identify early indicators that could be used for screening bulls during their performance testing period and could give us insight into their future semen production performance, acceptance for the AI station, and prediction of their future fertility. The study population consisted of 142 young Norwegian Red bulls enrolled at the performance test station, followed until we received semen production data, semen doses, and, subsequently, non-return rates (NR56) from the AI station. A range of semen quality parameters were measured with computer-assisted sperm analysis and flow cytometry from ejaculates collected from 65 bulls (9–13 months). The population morphometry of normal spermatozoa was examined, showing that Norwegian Red bulls at 10 months of age have homogenous sperm morphometry. Norwegian Red bulls could be separated into 3 clusters according to their sperm's reaction patterns to stress test and cryopreservation. Results of semi-automated morphology assessment of young Norwegian Red bulls showed that 42% of bulls rejected for the AI station and 18% of bulls accepted had ejaculates with abnormal morphology scores. For the youngest age group at 10 months, the mean (SD) proportion of spermatozoa with normal morphology was 77.5% (10.6). Using novel interpretation of sperm stress test combined with sperm morphology analysis and consecutive cryopreservation at a young age allowed identification of the candidate's sperm quality status. This could help breeding companies introduce young bulls earlier to the AI stations.

## 1. Introduction

The introduction of genomic selection is considered a game-changer in cattle breeding (Meuwissen et al., 2016). Bulls are being

* Correspondence to: Holsetgata 31, 2318 Hamar, Norway.
*E-mail address:* joanna.szlendak@inn.no (J. Bremer).
[1] https://orcid.org/0000-0001-7161-6230
[2] https://orcid.org/0000-0001-7388-3893
[3] https://orcid.org/0000-0002-5245-7331
[4] https://orcid.org/0000-0001-5867-4815

https://doi.org/10.1016/j.anireprosci.2023.107261
Received 8 March 2023; Received in revised form 16 May 2023; Accepted 17 May 2023
Available online 19 May 2023

used at the youngest possible age for semen collection for artificial insemination (AI) without any fertility records (Taylor et al., 2018). In Norway, the breeding company Geno genotypes 8000 Norwegian Red bull calves each year to identify the best candidates to become bulls for AI. The best calves are chosen for the performance test station (Geno (n.d.)). From around the age of 3–12 months, they are kept in the same environment and tested before further selection to the AI station. At approximately 10 months of age, the bulls are introduced to semen collection training, and their libido and several andrology traits are recorded (Olsen et al., 2020a, 2020b). Per annum, 50–60 Norwegian Red bulls are selected to become sires of the next generation (Geno (n.d.)). The performance testing period allows screening of the bulls during their pre- and peri-pubertal periods in search of indicators that can predict their future performance as AI bulls.

Each breeding company has its own semen acceptance thresholds, routines for age at collection, and logistics around the semen production process. Waite et al. (2019) proposed a system combining SC and body weight thresholds to increase juvenile bulls' likelihood of being ready for Bull Breeding Soundness Evaluation (BBSE). With SC $\geq$ 27 cm and bodyweight $\geq$ 349 kg, 98% of Holstein bulls had $\geq$ 70% morphologically normal sperm (Waite et al., 2019). Hurri et al. (2022a) showed that some ejaculates collected from 9 months old bulls already exceeded the breeding company threshold. Their results showed a strong correlation, r = 0.94, between the age at which the fresh ejaculate reached the quality threshold and the age at which the post-thaw ejaculate was considered acceptable. Several authors showed improvement in sperm quality in ejaculates over time and proposed alternative ways of making semen available from young genomic selected AI bulls by modifying semen handling protocols or collection of second consecutive ejaculates (Hurri et al., 2022b; Murphy et al., 2018; Taaffe et al., 2022).

The key research question of the present study was to identify novel early indicators in young bulls during their performance testing period, which could give insight into their future semen production performance, acceptance for the AI station, and predictions of their future fertility. The main objective was to investigate how a range of semen parameters from ejaculates of 10–13 month-old Norwegian Red bulls responded to sperm stress test and cryopreservation. Further aims were to examine the population morphometry of normal spermatozoa, perform a semi-automated morphology assessment, and measure the SC of young Norwegian Red bulls.

## 2. Materials and methods

### 2.1. Ethical approval

Ethical approval was not required in this study. We worked at the performance test station under the supervision of a qualified veterinarian employed at the breeding company Geno. The performance testing station is overseen by the Norwegian Food Safety Authority and meets EU requirements for the housing and care of bulls.

### 2.2. Animals

Yearly, 150 Norwegian Red bull calves are bought by Geno from all regions of Norway and transported to the performance test station in Øyer. Upon arrival at the station, at 3–5 months old, calves are quarantined for two weeks. After isolation, they are housed in groups of 10 and subsequently kept in the same group for the whole duration of the performance testing period. Temperament, conformation, and sperm quality testing are performed at the station. At around 12 months of age, they are approved or rejected for the AI station. Bulls are fed concentrate according to age and grass silage ad libitum. This study was performed during the period from September 2020 to December 2022. We followed 142 bulls enrolled in the performance testing program during this period and collected semen samples from 65 individuals which passed the sperm quality threshold and were available on the collection dates for this research.

### 2.3. Scrotal circumference measurements

The SC of 142 Norwegian Red bulls was measured manually on arrival at the performance test station (3–5 months) and at approximately 6, 9 and 12 months of age. A qualified veterinarian measured the SC of restrained bulls using scrotal tape.

### 2.4. Semen collection, sperm stress test and cryopreservation

Eighty-two ejaculates from 65 Norwegian Red bulls aged 10–13 months were collected using an artificial vagina. After collection, semen samples were kept at 35 °C for further examination. Measurement of sperm concentration (Bovine Photometer n°932, IMV technologies, L'Aigle, France), volume and subjective evaluation of motility were performed. Semen samples with suitable quality – volume > 2 mL, concentration > 200 $\times$ $10^6$/mL and motility > 60% - were selected for further analysis. An aliquot from each selected semen sample was diluted with Biladyl A extender (REF:13500/0004; Minitube GmbH, Tiefenbach, Germany) to a sperm concentration of 92 $\times$ $10^6$/mL and kept in an insulated box during the one-hour transportation to the university laboratory. From each sample, 250 µl of extended semen was aliquoted for flow cytometric analysis and automated motility analysis (Fresh - F and Stressed - S). Fresh samples were analysed on the day of the collection (F), and stressed samples on the consecutive day. The remaining samples were divided into two aliquots, one for cryopreservation on the same day and one for the sperm stress test. For the latter, aliquots were subjected to overnight storage at 4 °C, followed the next day by incubation for 3 h at 37 °C and cryopreservation. For freezing, the samples were cooled to 4 °C for 30 min. Next, Biladyl B glycerol-containing fraction (REF:13500/0006; Minitube GmbH) was added to a final sperm concentration of 48 $\times$ $10^6$/mL, and the mixture was incubated for 30 min at 4 °C. The semen was filled into 250 µl straws

(IMV technologies) and heat-sealed. The straws were placed on freezing racks and equilibrated at 4 °C for 2 h before freezing in an IceCube 14 S freezer (Minitube GmbH) using a standard freezing curve from 4 °C to − 150 C° in 7 min. The straws were transferred to liquid nitrogen and stored until further analysis (Thaw Fresh – TF and Thaw Stressed - TS).

### 2.5. Automated analysis of motility by computer-assisted sperm analysis (CASA)

Sperm motility was evaluated using a Sperm Class Analyzer®, version Evolution 6.5 (Microptic SL, Barcelona, Spain), equipped with a phase-contrast Eclipse Ci-S/Ci-L microscope (Nikon, Tokyo, Japan) and a Basler digital camera acA1300–200uc (Basler Vision Technologies, Ahrensburg, Germany) capturing 40 images at 169 fps. The proportion of motile sperm, progressive sperm motility, sperm subpopulations, sperm kinematics and hyperactivated sperm were assessed. The cut-off values for the subpopulations were set as curvilinear velocity VCL of 30–160, 161–300, and > 300 μm/s for slow, medium and rapid sperm, respectively. Progressive sperm motility was set to STR > 70 and the average path velocity points (VAP) to 9. To measure hyperactivation, breed-specific cut-off points were set up using Brackett and Oliphant sperm wash (BO) containing caffeine. Thresholds for sperm hyperactivation were created by visual selection of sperm with linear trajectory patterns and sperm with hyperactivated trajectory patterns, i.e. intermediate and starspin trajectories (Maree and Van Der Horst, 2013). Receiver operating characteristic (ROC) curve analysis for all kinematic parameters was performed; those with the highest sensitivity and specificity were chosen and added to the sort function in SCA software ALH > 3.5, LIN < 35, STR < 60, VLC > 300. Extended semen was diluted with PBS to a concentration of around 10–20 × $10^6$/mL, loaded into a pre-warmed 20 μm deep Leja-4 chamber slide (Leja products, Nieuw-Vennep, the Netherlands), and analysed as described by Maree and Van Der Horst (2013). A 10 X negative phase objective was used to capture at least 300 sperm from each bull. Each sample was analysed in triplicate. All motility files were subjected to quality control which consisted of visual inspection for correction of egg yolk particles marked as spermatozoa and immotile sperm classified as particles.

### 2.6. Flow cytometry – viability and acrosome integrity

Viability and the proportion of sperm with an intact or reacted acrosome were analysed with CytoFLEX Research Flow Cytometer (Beckman Coulter, Fullerton, CA, USA). Before each analysis, the protocol for quality control was followed. Propidium iodide (PI) (Invitrogen, Paisley, UK) was used to detect dead and live cells, binding only to the DNA of the dead sperm or those with a damaged membrane. To assess the acrosome integrity of spermatozoa, we used lectin peanut agglutinin (PNA) from Arachis hypogaea linked with Alexa Fluor™ 488 (Invitrogen), which binds to mannose and galactose of the acrosomal matrix (Hossain et al., 2011). Fluorescent nucleic acid stain SYTO60 (Invitrogen) was used for the detection of cells. For each sample, 1 mL of fresh staining solution was prepared with 980 μl of PBS, 0.2 μl of 2,4 mM of PI, 1 μl of 1:19 PNA and 1 μl of 1:100 5 mM SYTO60. One million sperm were added to 1 mL of staining solution and incubated in darkness for 10 min at room temperature. Each sample was analysed in triplicate. The CytExpert software (version 2.4, Beckman Coulter) was used to define subpopulations of interest: live spermatozoa with intact acrosome (AIL), live spermatozoa with reacted acrosome (ARL), dead spermatozoa with intact acrosome (AID) and dead spermatozoa with reacted acrosome (ARD).

### 2.7. Sperm morphology and morphometry

#### 2.7.1. Sperm staining

Raw semen (50 μl) from 79 ejaculates from 65 bulls was transferred to Eppendorf tubes and diluted with 1 mL PBS. Samples were transported at room temperature to the university laboratory. The samples were centrifuged (300 X g, 10 min) and resuspended in fresh PBS to a sperm concentration of 8 × $10^6$/mL. Two smears per ejaculate were made with 10 μl of semen and left to dry at room temperature, as described by van der Horst et al. (2018). Next, slides were stained for 2 min and 2 s in SpermBlue stain (Microptic SL) and gently submerged in distilled water for 2 s. Slides were dried at room temperature at an angle of 60 degrees. The next day slides were mounted with Eukitt® Quick-hardening mounting medium for microscopy (Sigma Aldrich, St. Louis, United States) and left for further analysis.

#### 2.7.2. Automated analysis of morphology and morphometry by Computer-aided sperm morphology analysis (CASMA)

The morphology module from CASA system Sperm Class Analyzer®, version Evolution 6.5 (Microptic SL), equipped with a phase-contrast Eclipse Ci-S/Ci-L microscope (Nikon) and a Basler digital camera acA1300–200uc (Basler Vision Technologies) was used for automated morphology and morphometry analysis. Images of 200 sperm from each sample were captured, applying a 40 X objective.

#### 2.7.3. Cut-off values for normal bull sperm morphology

Cut-off values for normal sperm morphology are species- and breed-specific (van der Horst et al., 2018). Cut-off values for normal sperm morphology in Norwegian Red bull were created based on 9 mature reference bulls from Geno's AI station. Sperm (n = 1182) were captured as described in the previous paragraph. We determined cut-off values of normal vs. abnormal bull sperm following the percentile method described by van der Horst et al. (2018). Next, we created an online survey to optimise the settings, where specialists were asked to perform a manual morphology assessment – normal vs. abnormal of 50 sperm. The settings were adjusted according to the responses. Final cut-off values were put into a configuration in the SCA Morphology module and used for subsequent analyses (Appendix 1). All morphology files were subjected to quality control and assessment of the tail defects. We decided to follow the standardisation threshold system for morphological abnormalities of bovine, published by Perry (2021), summarised in Table 1. The

individual is considered fertile, with at least 70% morphologically normal spermatozoa, but with additional thresholds for specific defects (Perry, 2021). Part of the quality control was a manual assessment of captured spermatozoa in the Edit function of the Morphology module. All incorrectly captured cells were removed from the analysis. Due to limitations in the edit function, the following group structure was created: amorphous – rolled head nuclear crest, teratoid forms; rolled tail – distal midpiece reflex, bent principal piece, coiled principal pieces; irregular – teratoid tail forms; abnormal tail – segmental aplasia of the mitochondrial sheath. We also counted separately the number of abaxial tails without accessory tail and distal cytoplasmic droplets, both of which are not considered to be an abnormality. The morphology score based on the described threshold was a two-level factor: normal or abnormal.

### 2.8. Thawing and analysis of sperm samples from the performance test station and AI station

Ejaculates (n = 82) from 65 bulls were collected at the performance test station and analysed fresh, stressed, and frozen-thawed. Among these bulls, 38 were accepted for the AI station based on genomic breeding value and andrology testing. Geno's AI station, Store Ree, provided approved frozen semen doses from the 38 bulls of interest. The cryopreserved semen doses were thawed for 1 min in a water bath at 37 °C. Three semen doses were pooled from each ejaculate. We performed the same sperm quality analysis as described in Sections 2.4 and 2.5 on thawed samples from the performance test station and ejaculates from the same bulls at the AI station.

### 2.9. Non-return rates

The NR56 were available from 25 bulls out of 38 accepted for the AI station. There are different reasons why semen from all bulls was not distributed in Norway: some bulls were used only for semen production for export, most were excluded based on their breeding value, and one had too low sperm quality. The NR56 were calculated based on the AI data extracted from the Norwegian Dairy Herd Recording System. We included AI records from 2021 and 2022. Only the first insemination after calving was used. Repeated insemination within 5 days after the first was defined as double insemination. The data were further restricted as follows: insemination date < July 2022 (to allow all females to have a second insemination 56 days after the first), breed of cow Norwegian Red, parity of cow < 8, insemination with Norwegian Red AI bull, sperm type conventional, and bulls with at least 100 AI in the dataset. The final dataset had 271,091 observations, and the overall mean NR56 was 0.74 ranging from 0.71 to 0.82. The General Linear Model used to analyse the NR56 data included the effects of double insemination, cow parity (0−7), month-year of insemination and bull. Least square means (LSmeans) of NR56 were calculated for bulls, including 25 of the bulls we followed. The LSmeans of NR56 for these 25 bulls ranged from 0.67 to 0.76.

### 2.10. Statistical analysis

Statistical analyses were performed using R Studio version 1.4.0 (https://www.r-project.org/). All ejaculates collected at the performance test station were analysed after the following 4 treatments (variable Type): fresh (F), stressed (S), post-thaw fresh (TF), and post-thaw stressed (TS). For bulls accepted for the AI station, one more ejaculate was analysed – post-thaw AI (TAI). We performed a principal component analysis (PCA) to characterize the variation in the morphometric, kinematic, motility, viability and acrosome integrity sperm parameters using function prcomp from package "stats". K-medoids clustering was performed with function pam from the package "cluster". Linear mixed-effects models were fitted and analysed with function lmer from R package "lme4″ (Bates et al.,

**Table 1**
Standardised threshold system for morphological abnormalities of bulls based on Perry (2021).

| Abnormality | Origin of abnormality | Threshold | Important information |
|---|---|---|---|
| proximal droplets | primary | < 20% | associated with poor pregnancy rates |
| distal droplets | secondary | - | not considered a defect |
| pyriform heads | primary | < 20% | caused by stress or scrotal insulation, can cause reduced cleavage |
| swollen acrosomes | primary | - | ageing of sperm, similar changes to capacitation acrosome is lifting |
| knobbed acrosomes | primary | < 25% | |
| rolled head nuclear creat syndrome | primary | < 20% | sperm can penetrate ZP but do not able to produce a viable embryo |
| teratoid sperm | primary | < 15% | severe disturbance to spermatogenesis and spermiogenesis |
| distal reflex midpieces | primary | < 30% | caused by hypotonic solution, cold shock, solution > pH7, stress first to appear, reverse motility, unable to penetrate ZP,16days recovery |
| dag-like defect | primary | < 30% | inherited, infertility if present in large number > 50%, disturbed motility, compensable trait, disturbance in testis or epididymis |
| segmental aplasia of mitochondrial sheath | primary | - | little effect on fertility can be permanent or transient. More significant gaps will cause fracture |
| abaxial tails | primary | | no decrease in fertility |
| abaxial tails with accessory tail | primary | < 20% | can cause a drop in fertility, disturbs separation of chromosomes during the first cleavage |
| loose heads | secondary | < 70% | cannot swim, compensable, testicular degeneration, hypoplasia, inflammation, heat stress, "rusty load" - "clean up" the accumulated sperm to get a representative sample |
| principal piece tail defects | secondary | < 30% | seldom seen in high numbers, caused by temperature shock or stress, decrease after 8–11days |

2015). For examination of possible significant fixed effects on the response variables, the proportions of live intact acrosome (AIL), motile, progressive, rapid and hyperactivated spermatozoa, we used the following mixed linear repeatability model:

$$Y_{ijkl} = Type_i + age\ in\ months_j + decision\ AI_k + bull_l + e_{ijkl}$$

where $Y_{ijkl}$ is the observation of AIL or other response variables of interest for bull *l,* accepted or rejected for the AI station (decision AI class *k, yes or no),* at the age in months *j* (4 groups: 10,11,12 and 13), under treatment Type *i* (4 groups F, S, TF, TS). The fixed effects of decision on starting AI or not were not significant for any of the variables and therefore excluded from further analyses. The mixed linear repeatability model with fixed effects of Type (4 treatments), Type progressivity (2 groups: motile and progressive) and random effect of bull was used for examination of possible significant fixed effects on the response variables VCL, VAP, VSL, ALH, WOB, LIN, STR and BCF for rapid subpopulation:

$$Y_{ijk} = Type_i + Type\ progressivity_j + bull_k + e_{ijk}$$

where $Y_{ijk}$ is the observation of VCL or other variables of interest for bull *k* classified as movement Type progressivity *j* under condition Type *i*. The data were tested for normality and differences with $p \leq 0.05$ were considered significant.

### *2.10.1. Clustering and PCA for identification of bulls' reaction to sperm stress test and freezing*

To identify possible differences between bulls in the reaction to the sperm stress test and freezing, and the development of sperm quality with age of bull, we used a combination of two analyses – k-medoids clustering and PCA (Bruni et al., 2022; Ikotun et al., 2023). First, we calculated the differences in mean values of motility, viability and acrosome integrity parameters of Types: Fresh - Stressed (F-S), Thawed Fresh -Thawed Stressed (TF-TS) and Thawed AI - Thawed Fresh (TAI-TF). First, with F-S and TF-TS, we aimed to see how the sperm of different bulls reacted to the sperm stress test. The second data set, TAI-TF, showed how variables of interest changed from the performance testing period to the AI station. Next, we scaled both data sets, performed k-medoids clustering, and saved the cluster number into the original data set (without subtracted values). The optimal cluster number k = 3 was chosen using the function fviz_nbclust from R-package „factoextra". Then for validation of k-medoids clustering, we performed PCA on the original data set and colored the PCA with clusters id. Finally, we returned to the original data set to visualize the data based on the clusters to observe trends.

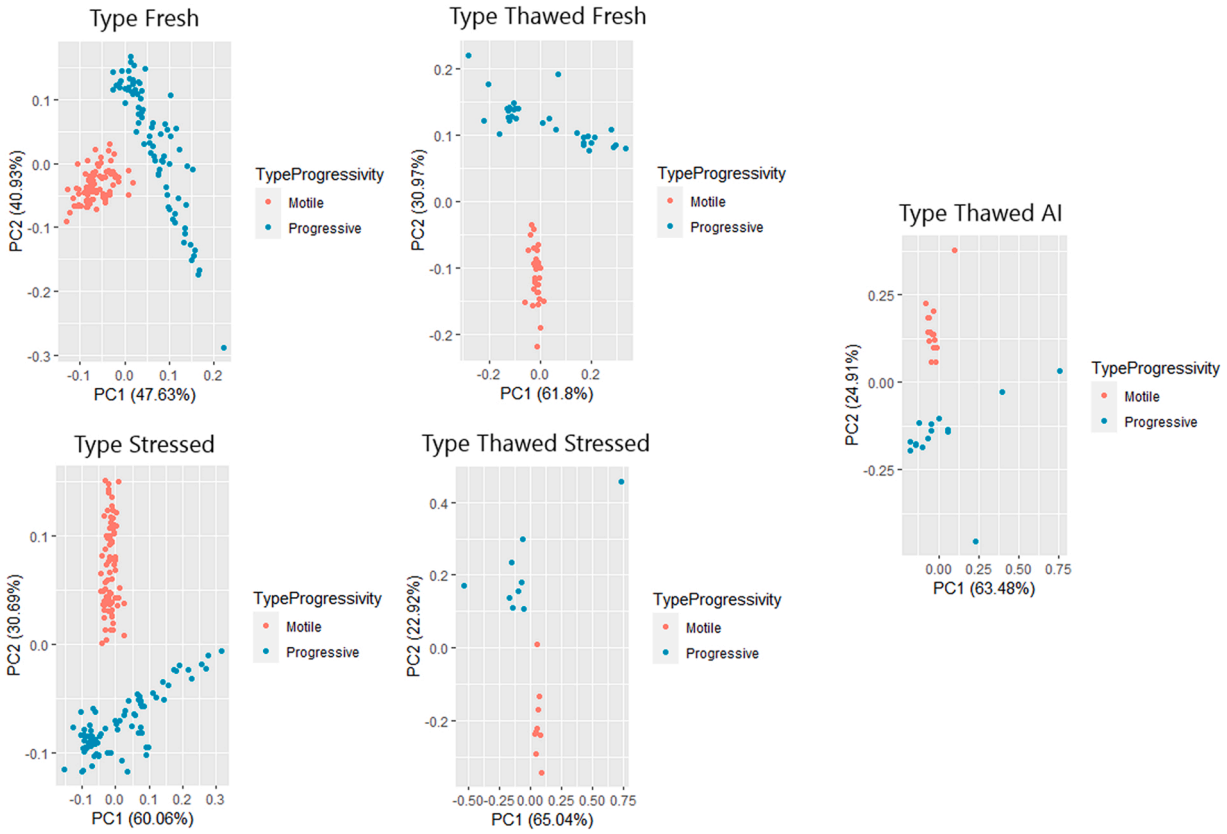



**Fig. 1.** Principal component analysis (PCA) in 2D. The first two Principal Components: Principal Component 1 and Principal Component 2 (PC1 and PC2) with the proportion of explained variance are presented. The plot of the kinematic parameters from rapid subpopulation coloured by Type Progressivity from the CASA for all Types.

*2.10.2. Adjustment of scrotal circumference to 12 months age and scrotal circumference threshold*

We assessed if Norwegian Red bulls from the performance test station would pass breeding soundness evaluation thresholds of different systems; SFT, WCABP and the system proposed for Spain by Garcia-Paloma (2015). We used the following parameters: SC (106 bulls), progressive motility (65 bulls) and morphology (63 bulls). The SC was adjusted to 365 days (12 months) using a regression coefficient from a linear regression of SC (cm) on age (days) (Garcia-Paloma, 2015). Data from 142 bulls measured at 4-time points were included ($p < 0.001$, adjusted $R^2 = 0.91$). The adjustment formula was SC12 = SC + 0.0765x(365 - age at measurement). To create the SC threshold for Norwegian Red bulls, we calculated the mean SC12 from the 106 bulls with SC measured at 10–13 months. The SC12 categories were the SFT threshold (30 cm), the SC12 mean minus one SD (31.6 cm), and the SC12 mean plus one SD (36 cm). Adjusted SC12 was used to compare Norwegian Red thresholds with Garcia-Paloma (2015) and unadjusted SC for comparison with SFT and WCABP. The bulls were classified either as unsatisfactory and satisfactory, or into four categories: unsatisfactory, questionable, satisfactory and superior.

## 3. Results

### 3.1. PCA analysis of kinematic parameters

The PCA analysis of kinematic parameters for the rapid subpopulation of all Types (F, TF, S, TS and TAI) confirmed two well-defined subpopulations from the CASA Type Progressivity factor: motile and progressive (Fig. 1). Two components from PCA analysis of kinematic parameters explained more than 75% of the variance for all Types. The kinematics VCL, VSL, VAP, ALH, LIN and WOB were different for Type and Type progressivity. For STR, the only difference was for Type progressivity ($p < 0.05$); for WOB, the only difference was for Type.

### 3.2. Treatment and age of bull influence sperm parameters

For both Type and age in months, AIL, the proportions of motile, progressive, and rapid spermatozoa were significantly different ($p < 0.05$). The proportion of hyperactivated spermatozoa was different only for Type ($p < 0.05$).

### 3.3. Response to the stress test of sperm at the performance test station and sperm quality development in time

Three clusters were formed from k-medoids clustering on differences between mean values of motility, viability and acrosome reaction parameters of Types F-S and TF-TS. Fig. 2 shows the distribution of individual AIL data points in % and population means ± SEM for Types F, S, TF and TS for four age groups. In this study, young Norwegian Red bulls from the performance test station were clustered into three patterns of response: cluster 1 – bulls with good initial sperm quality which reacted to the stress test before and after thawing; cluster 2 - bulls with good initial sperm quality which showed minimal reaction to the stress test before and after thawing but showed reaction to cryopreservation; cluster 3 - bulls with good initial sperm quality which showed minimal reaction to the stress test before thawing and reaction to stress test after thawing but their reaction to cryopreservation was the least (Appendix 5). For cluster 2, the population mean for F was 73%, and for S, 69%. After thawing, the population mean for TF was 29% and for TS 27%. Cluster 1 showed a change of population mean from F = 71% to S = 64%, and after thawing from TF = 22% to TS = 15%. Cluster 3 had a similar trend in the population mean of fresh samples from F = 79% and S = 73%. After thawing TF and TS were 41% and 19%, respectively. Similar trends were found for the proportion of motile, progressive, and rapid spermatozoa. The population mean of the

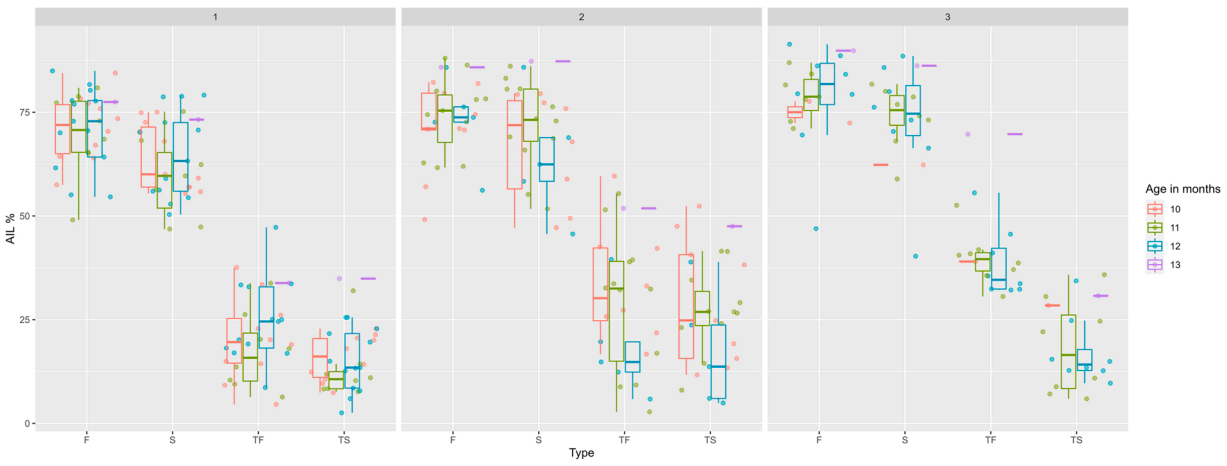



**Fig. 2.** Proportion of live spermatozoa with intact acrosome (AIL) presented as individual data points and population mean ± SEM for Types (Fresh - F, Stressed - S, Thaw Fresh - TF and Thaw Stressed – TS) for four age groups presented for three clusters.

proportion of hyperactivated spermatozoa increased in stressed samples for clusters 2 and 3 and decreased in cluster 1. In thawed samples, HA % decreased in TS for clusters 1 and 3 and stayed at a similar level for cluster 2. Appendix 5 shows the differences between clusters in the population mean for all motility, viability and acrosome reaction parameters. The k-medoids clustering on subtracted mean motility, viability and acrosome reaction parameters of Types TAI-TF showed 2 clusters. We can differentiate two trends between TF and TAI for all variables of interest, with cluster 2 showing a steeper slope of growth than cluster 1. Table 2 shows differences between clusters and Types TAI and TF for mean motility, viability and acrosome reaction parameters of interest.

### 3.4. Morphology and morphometry of spermatozoa in young Norwegian Red bulls

The population morphometry values of 10–13 months old Norwegian Red bulls are shown in Table 3. We found no significant differences in mean values of morphometry parameters between age groups 10, 11, 12, and 13 months (the number of cells analysed per age group was 4832, 5570, 5425 and 665, respectively). However, if we change the age interval to weeks 44–46, 47–49, 50–52, 53–55, and 56–58, we found a difference in mean head area between weeks 44–46 and 56–58 (43.2 $\mu m^2$ and 43.8 $\mu m^2$, respectively; $p < 0.05$. Two components from the PCA analysis of head variables explained 77.1% of the variance. Principal Component 1 (PC1) was represented by ellipticity, elongation and head width, and Principal Component 2 (PC2) referred to head area, perimeter, width and length. Regardless of age, the PCA results show one defined population (Fig. 3). There was a difference ($p < 0.01$) in all morphometry parameters, except ellipticity, between the population with micro heads and the population of spermatozoa classified as normal (Appendix 2). The mean ± SD proportion of spermatozoa with normal morphology for age groups 10, 11 and 12–13 months were 77.5 ± 10.6, 76.8 ± 10.0 and 78.9 ± 10.5, respectively. Table 4 shows the same parameter divided into bulls accepted and rejected for the AI station. Based on the standardisation threshold shown in Table 1, 16 out of 79 analysed ejaculates were classified as abnormal (Appendix 3). Of 16 ejaculates with abnormal morphology scores, the proportion of rejected and accepted bulls for the AI centre was 10 and 6, respectively. We can distinguish 10 ejaculates with more than 20% abaxial tails without accessory tail, and the proportion of acceptance or rejection by the AI centre is 1:1. There were no ejaculates with more than 20% distal droplets. In appendixes 3 and 4 we show detailed spermiograms of the 16 ejaculates with abnormal morphology. Of 16 abnormal ejaculates, 6 ejaculates had more than 20% micro heads. One ejaculate had > 20% of sperm with an abnormally sized midpiece. The remaining 9 ejaculates showed a spread of abnormalities across the head, midpiece and tail defects. We found no significant difference in teratozoospermic index (TZI) and multiple anomalies index (MAI) between ejaculates with a normal and abnormal score. The TZI was 1.62 for normal and 1.63 for abnormal score, and MAI was 2.06 and 2.03, respectively. All bulls with more than one ejaculate were either consistent in their morphology score (normal or abnormal) or showed an improvement. Two bulls with an abnormal first ejaculate had the same score for the second ejaculate, and one improved from abnormal to normal. The same was true for 11 bulls with ejaculates with normal scores whose second or third ejaculates were consistently normal.

### 3.5. Scrotal circumference of pre- and peri-pubertal Norwegian Red bulls

Scrotal circumference (cm) of pre- and peri-pubertal Norwegian Red bulls showed an increase in size over time. The mean ± SD SC for age groups Q, 6, 9, and 12 were 15.0 ± 1.60, 21.4 ± 2.51, 29.1 ± 2.45 and 34.0 ± 2.24 cm, respectively. We found no significant difference in mean SC between bulls accepted for the AI station and rejected ones for any of the age classes.

### 3.6. How Norwegian Red bulls pass different international BBSE systems

Our result showed that 98% of young Norwegian Red bulls between the age of 10 and 13 months pass the SFT SC threshold, and 76% pass the WCABP SC threshold (Table 5). After the adjustment of SC to 12 months, 70% of these young Norwegian Red bulls passed the satisfactory threshold proposed by Garcia-Paloma (2015). The proportions of bulls classified into the four categories of Norwegian Red proposed SC12, (unsatisfactory, questionable, satisfactory and superior), were 4.72%, 12.26%, 64.15% and 18.87%, respectively; 95.38% of Norwegian Red bulls passed the progressive sperm motility threshold in the STF system. When the same threshold was applied for the other systems, the distribution of bulls among the three categories used by WCABP was 13.85%, 41.54% and 44.62%, respectively. The normal morphology threshold for SFT was passed by 80.95% of Norwegian Red bulls. The WCABP and Garcia-Paloma systems had the same threshold, and the proportion of bulls classified into three categories was 0%, 19.05% and 80.95%. The Norwegian Red threshold was passed by 77.78% of bulls.

**Table 2**
Mean values for clusters and Types TF and TAI of motility, viability and acrosome reaction parameters.

| Cluster | Type | AIL % | Motile % | Progressive % | Rapid % | Hyperactive % (Motile) |
|---|---|---|---|---|---|---|
| 1 | TF | 49.45 | 30.87 | 15.66 | 12.46 | 29.62 |
| 1 | TAI | 46.53 | 43.45 | 27.76 | 23.78 | 42.91 |
| 2 | TF | 26.63 | 22.50 | 8.44 | 6.19 | 14.68 |
| 2 | TAI | 53.13 | 51.87 | 39.49 | 34.19 | 53.84 |

**Table 3**
Mean, median and standard deviation (SD) of population morphometry of (n = 79) 10–13-month-old Norwegian Red bulls calculated from cells classified as normal.

| Measurement | Mean | Median | SD |
|---|---|---|---|
| Acrosome % | 41.94 | 41.86 | 0.77 |
| Distance Midpiece µm | 0.25 | 0.24 | 0.01 |
| Ellipticity | 1.98 | 1.97 | 0.04 |
| Elongation | 0.33 | 0.33 | 0.01 |
| Gray Acrosome % | 152.21 | 151.84 | 3.17 |
| Head area µm$^2$ | 43.25 | 42.97 | 0.72 |
| Head length µm | 9.61 | 9.57 | 0.19 |
| Head perimeter µm | 20.93 | 20.93 | 0.40 |
| Head width µm | 4.85 | 4.87 | 0.10 |
| Midpiece angle ° | 2.69 | 2.66 | 0.15 |
| Midpiece area µm$^2$ | 9.82 | 9.47 | 0.32 |
| Midpiece width µm | 1.07 | 1.05 | 0.03 |
| Regularity | 0.85 | 0.85 | 0.02 |
| Roughness | 1.24 | 1.24 | 0.02 |

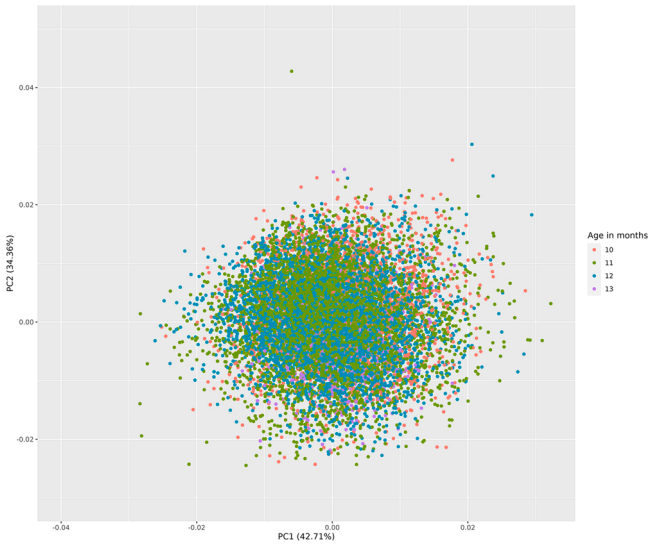



**Fig. 3.** Principal component analysis (PCA) in 2D. The first two Principal Components: Principal Component 1 and Principal Component 2 (PC1 and PC2) with the proportion of explained variance are presented. Head morphometry of the individual spermatozoa. The colour indicates the bull's age in months.

**Table 4**
The mean and standard deviation of percentage of spermatozoa with normal morphology for age groups 10, 11, 12–13 months from bulls accepted and rejected to AI station.

| Decision AI | Age in months | n | mean % | sd |
|---|---|---|---|---|
| AI_accepted | 10 | 15 | 81.29 | 6.03 |
| AI_accepted | 11 | 15 | 76.21 | 10.39 |
| AI_accepted | 12–13 | 17 | 79.63 | 7.23 |
| AI_rejected | 10 | 6 | 68.06 | 13.97 |
| AI_rejected | 11 | 13 | 77.41 | 9.88 |
| AI_rejected | 12–13 | 13 | 77.94 | 13.89 |

**Table 5**
Proportion of Norwegian Red bulls aged 10–13 months classified by category and system according to the thresholds.

| Category | BBSE system | trait | Unsatisfactory | Questionable | Satisfactory | Superior | n Bulls |
|---|---|---|---|---|---|---|---|
| **System**[a] | **SFT** | **SC t** | < 30 cm | | ≥ 30 cm | | |
| | | **SC** | 1.89% | | 98.11% | | 106 |
| | | **SM t** | < 30% | | ≥ 30% | | |
| | | **SM** | 4.62% | | 95.38% | | 65 |
| | | **NS t** | < 70% | | ≥ 70% | | |
| | | **NS** | 19.05% | | 80.95% | | 63 |
| | **WCABP** | **SC t** | < 32.83 cm | | ≥ 32.83 cm | | |
| | | **SC** | 23.58% | | 76.42% | | 106 |
| | | **SM t** | < 40% | 40–59% | ≥ 60% | | |
| | | **SM** | 13.85% | 41.54% | 44.62% | | 65 |
| | | **NS t** | < 50% | 50–69% | ≥ 70% | | |
| | | **NS** | 0% | 19.05% | 80.95% | | 63 |
| | **Proposed by**Garcia-Paloma (2015) | **SC15 t** | < 30 cm | 30.1–31.8 cm | 31.9–36.5 cm | ≥ 36.6 cm | |
| | | **SC12(days)** | 4.72% | 13.21% | 69.81% | 12.26% | 106 |
| | | **SM t** | < 40% | 40–59% | ≥ 60% | | |
| | | **SM** | 13.85% | 41.54% | 44.62% | | 65 |
| | | **NS t** | < 50% | 50–69% | ≥ 70% | | |
| | | **NS** | 0% | 19.05% | 80.95% | | 63 |
| | **NR Proposed** | **SC12 t** | < 30 cm | 30.1–31.59 cm | 31.6–35.9 cm | ≥ 36 cm | |
| | | **SC12(days)** | 4.72% | 12.26% | 64.15% | 18.87% | 106 |
| | | **SM t** | < 40% | 40–59% | ≥ 60% | | |
| | | **SM** | 13.85% | 41.54% | 44.62% | | 65 |
| | | **NS t** | < 70% +Table 1 | | ≥ 70% +Table 1 | | |
| | | **NS** | 22.22% | | 77.78% | | 63 |

Abbreviations: SC = Scrotal circumference. SC15 - SC adjusted to 15 months by Garcia-Paloma (2015). SC12 - SC adjusted to 12 months. SM - Progressive sperm motility. NS - Normal sperm. t – threshold.

[a] SFT - Society for Theriogenology. WCABP - Western Canadian Association of Bovine Practitioners. Proposed by Garcia-Paloma (2015) - Proposed system to promote consensus in Spain. NR Proposed – Proposed system for Norwegian Red cattle.

### *3.7. Non-return rate and the performance testing period*

Of 38 bulls accepted for the AI station, 25 had NR56 results, with values ranging between 0.66 and 0.76. Four of these bulls had a proportion of AIL below 40% for both TF and TAI treatments. The NR56 for these bulls were 0.68, 0.71, 0.72 and 0.74. The remaining 21 bulls with NR56 values had AIL above 40% for TAI; however, only 7 had AIL above 40% for the TF. Fig. 4 shows a change in the proportion of AIL for TF and TAI with time for the 25 bulls with NR56 values. Table 6 shows the distribution of bulls with normal and abnormal morphology scores for bulls accepted for the AI station that had NR56 values, bulls accepted for the AI station without NR56 values, and bulls rejected for the AI station. We observed that 42% of bulls rejected for the AI station and 18% of bulls accepted for the AI station had ejaculates with abnormal morphology scores. Five of the bulls accepted by the AI station with NR56 values had abnormal morphology scores (Appendix 3 and 4 bulls 1, 8, 9, 10, and 16). Two of these had 46.50% and 20.36% micro heads, which was the main reason for their abnormal score. The remaining three had abnormalities spread among head, midpiece and tail defects. We found no significant difference in mean SC at any of the 4 time points (Q, 6, 9, 12) between bulls accepted to the AI station with NR56 values, bulls accepted to the AI station without NR56 values, and bulls rejected by the AI station. There were no associations between the analysed parameters at the performance test station and the AI bulls with NR56.

## 4. Discussion

The present study aimed to evaluate young Norwegian Red bulls during their performance testing period to see if we can obtain insight into their future semen production, sperm quality and the potential for predicting future fertility. The results showed that sperm from young Norwegian Red bulls responded differently to sperm stress tests and subsequent cryopreservation. We also showed the importance of sperm morphology assessment of bulls before introduction to commercial semen production. Although improvements in semen quality with increasing age have been observed by Narud et al. (2022), and we observed improved cryo-survival with age, there were no associations between the analysed parameters at the performance test station and their NR56 results as an AI bull.

Since our study aimed to investigate the response of the range of semen parameters from ejaculates of 10–13 month-old Norwegian Red bulls to the sperm stress test and cryopreservation, we asked the question whether the settings we used in the CASA system to define the motile and progressive subpopulations were applicable for all types (F, S, TF, TS, TAI). Our PCA analysis of kinematic parameters for the rapid subpopulation confirmed that the settings could be used for both fresh and thawed samples. To our knowledge, no similar studies have been done. We acknowledge that the small number of rapid sperm in Types TS and TAI needs further investigation to confirm our result.

Hurri et al. (2022a) collected consecutive ejaculates from 10 to 11 months old dairy bulls. The findings of the current study show that mean post-thaw motility from cluster 3 with the best sperm quality was somewhere between the two groups presented by Hurri et al. (2022a). The possible explanation for lower progressive motility in our study might be a high proportion of hyperactivated

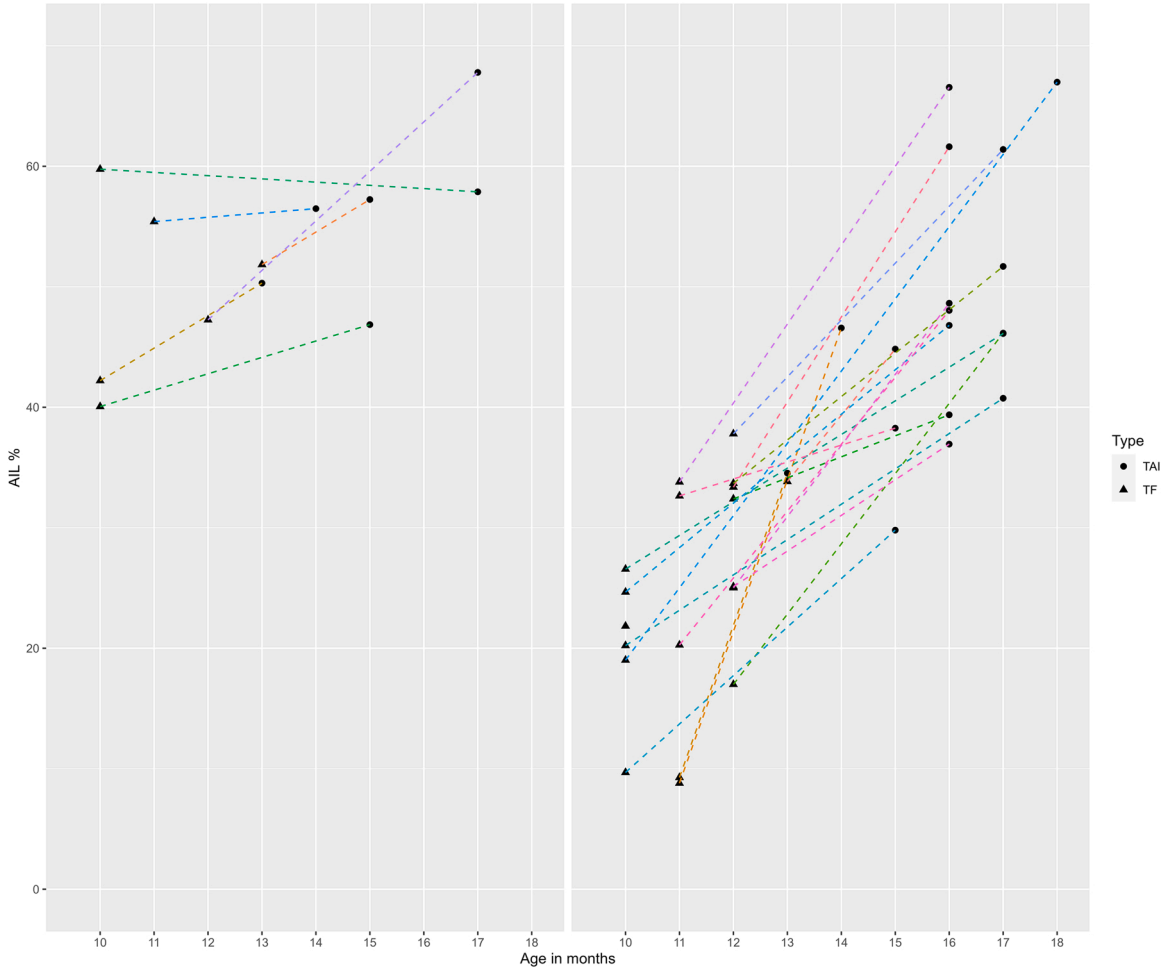



**Fig. 4.** Change in proportion of live spermatozoa with intact acrosome (AIL) for post-thaw fresh from the performance testing station (TF) and post-thaw from AI station (TAI) from 25 bulls with NR56 values. Data are presented as individual data points for each condition connected by a line to emphasise the change with age of bull. The figure is presented in two grids to separate bulls with post-thaw AIL > 40% (left) and AIL < 40% (right) for TF (arbitrary threshold).

**Table 6**
Number of bulls with abnormal and normal morphology scores for bulls accepted to the AI station with NR56 values, bulls accepted to the AI station without NR56 values, and bulls rejected to the AI station.

| Decision NR56 | Morphology score | n[a] |
|---|---|---|
| NR56 AI accepted[b] | normal[c] | 19 |
| NR56 AI accepted | abnormal | 5 |
| AI accepted[d] | normal | 15 |
| AI accepted | abnormal | 1 |
| Rejected[e] | normal | 19 |
| Rejected | abnormal | 8 |

[a] n - number of bulls
[b] NR56 AI accepted - bulls accepted to the AI station with NR56 values
[c] normal - ≥ 70% of normal spermatozoa + Table 1
[d] AI accepted - bulls accepted to the AI station without NR56 values
[e] Rejected - bulls rejected to the AI station

spermatozoa. This discrepancy could be attributed to age, breed, extender and differences in the CASA software and settings (Ntemka et al., 2016; O'Meara et al., 2022; Víquez et al., 2020). Clustering and PCA are established methods used for the identification of sperm subpopulations from motility and morphometry data (Martínez-Pastor, 2021). However, using these methods to group young bulls based on the patterns of their sperms´reaction to stress test and cryopreservation is novel. This approach has been applied to generate novel synthetic images of likeable drones for future social applications (Yamin and Cauchard, 2022). Our interpretation of the cluster analysis is that bulls from clusters 2 and 3 could produce sperm with good freezing ability. This finding could be used as a simple test to check the sperm "maturity status" of young bulls. Results of clustering of difference in sperm quality parameters between TF and TAI show that the rate of improvement in sperm quality of young bulls happens at different ages, which agrees with previous findings (Hurri et al., 2022a) and is an important topic for future research.

One of our aims was to perform automated morphometry and semi-automated morphology analysis of sperm from young Norwegian Red bulls. To increase the accuracy of the analysis, we created breed-specific cut-off values for normal sperm properties of Norwegian Red bulls (van der Horst et al., 2021). Although our study successfully demonstrated that young Norwegian Red bulls have a homogenous morphometry of spermatozoa at ten months of age, we revealed certain limitations. Our breed-specific cut-off values for normal sperm may have been too strict and influenced the PCA results since we did not include abnormal spermatozoa in the analysis. Valverde et al. (2016) used PCA combined with k-mean clustering to define the sperm subpopulations based on head morphometric parameters of thawed sperm of Holstein bulls. Their results of two components from PCA analysis of head variables explained 75.6% of the variance, which is comparable with our findings of 77.1%. However, we found that k-means clustering gave us false clusters resembling those of Valverde et al. (Valverde et al., 2016), which were defined in their paper as four subpopulations. The k-means clustering method is well-known and easy to use, but its main pitfall is that the user needs to pre-define the number of clusters used in the analysis, which means that the method will define clusters even on randomly distributed datasets (Ikotun et al., 2023). In comparison with other breeds, the mean head area of young Norwegian Red bulls was significantly bigger than in adult Holstein (Beletti et al., 2005; Vicente-Fiel et al., 2013) but comparable with Angus (van der Horst et al., 2021). Head length and width were comparable for all breeds. A possible explanation for differences in size might be different staining and measurement methods. Both Norwegian Red and Angus sperm morphometry was captured by the same CASA system and similarly stained (van Der Horst and Maree, 2009). In light of these breed differences, the significant difference in mean head area between weeks 44–46 and 56–58 in our results does not seem to be relevant. An elevated proximal droplet count is typically observed in pubertal bulls, but this decreases with age. In mature bulls, the presence of more than 10–15% proximal droplets is associated with lower fertility (Perry, 2021). In a comprehensive sperm morphology study, Felton-Taylor et al. (2020) showed that bulls younger than 20 months had a high proportion of proximal droplets, independently of breed, season and region, (number of bulls analysed = 7284). However, our findings show no bull aged 10–13 months with proximal droplets above 4%. These might be connected to differences between breeding goals and environment between Australia and Norway. The overall sperm quality of younger bulls is lower than that of older bulls. Hurri et al. (2022a) showed an increase in normal morphology during serial collection of ejaculates from young dairy bulls. Interestingly, in the current study. the normal morphology level for the youngest age group was higher than presented by Hurri et al. (2022a) for the last ejaculate.

The Society for Theriogenology recommends an SC of 30 cm for bulls $\geq$ 15 months (Armstrong and Act, 2022). In our study, 98% of young Norwegian Red bulls passed this threshold at 10–13 months, indicating that our study population was quite homogenous. Others have shown that mature bulls with an average or above average SC display more satisfactory semen quality than those with an SC below the minimum threshold (Barth, 2018). This finding illustrates how differences between thresholds could influence the BBSE results and the need for a BBSE system specific for age and breed. Scrotal circumference has been shown to be positively correlated with increased sperm output (Waite et al., 2019). Our previous research on the same population showed no association between SC and sperm volume and concentration of the last ejaculate from the performance test station or with these parameters for the first 10 ejaculates from the AI station (Bremer et al., 2023). On the other hand, the same study exhibited huge variation (50–100%) between the bulls concerning number of semen doses being accepted and discarded after sperm quality control (Bremer et al., 2023). This variation could be of economic importance to the breeding company. Young bulls exhibit higher variability in sperm volume and concentration between ejaculates than older bulls (Murphy et al., 2018). The current investigation was limited by the number of ejaculates per bull. Mostly we used single ejaculates from each bull and, in a few cases, two or three. The lack of association between SC and sperm volume and concentration of ejaculates from young Norwegian Red bulls might have been influenced by the bull's relatively young age during the study (Bremer et al., 2023). Such young bulls are inexperienced in semen collection routines, and as they were housed in free stalled groups, they could have been exposed to mounting and ejaculating outside the schedule of semen collection.

Our results show that both bulls accepted and rejected for the AI station had good initial fresh sperm quality; however, after thawing, we discovered individual variation in response to sperm cryopreservation, with a significant proportion of bulls with an AIL below 40%. Argov-Argaman et al. (2013) showed that bulls have altered semen lipid profiles with increasing age. The reduced proportions of major fatty acids found in mature bulls (mean age was 7 years) might reduce membrane fluidity, thereby affecting cryopreservation and/or sperm-oocyte fusion (Argov-Argaman et al., 2013). Deori et al. (2021) observed lower levels of heparin-binding proteins in ejaculates from 9 to 10 months old bulls compared with ejaculates from the same bulls at 14–16 months, indicating that seminal plasma composition changes with increasing age of bull. Heparin-binding proteins facilitate capacitation which is an important step towards successful fertilisation (Deori et al., 2021). The majority of semen doses on the market today come from bulls younger than 15 months (Schenk, 2018). With a lack of information on the lipid profiles of young pre-pubertal bulls, we can hypothesize that immature sperm membranes and lower level of some of the seminal plasma proteins might cause variability in the cryo-survival of spermatozoa of young bulls.

## 5. Conclusion

This study revealed that young Norwegian Red bulls reach their maturity in sperm quality at different ages, which was shown by the novel combination of sperm stress test and cryopreservation. Testing young bulls can indicate earlier which bulls are ready for sperm production at AI stations. This would result in more rapid incorporation of these bulls in the routine semen collection schedule, with the subsequent availability of their frozen semen for AI and hence an increase in the genetic gain for the population. We recommend using this novel interpretation of the sperm stress test and cryopreservation combined with early sperm morphology analysis to elucidate the sperm quality status of young bulls, thereby improving the selection criteria for AI at the youngest possible age.

## CRediT author statement

**Joanna Bremer:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization, Project administration. **Elisabeth Kommisrud:** Conceptualization, Methodology, Investigation, Funding acquisition, Data curation, Writing – review & editing, Supervision. **Bjørg Heringstad:** Validation, Formal analysis, Data curation, Writing – review & editing, Supervision. **Jane M. Morrell:** Conceptualization, Writing – review & editing, Supervision.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

The data presented in this study are available on request from the corresponding author.

## Acknowledgments

We would like to thank Hallstein Holen and his team at Geno's performance test station in Øyer: Jan Tore Rosingholm, Erik Skogli, Sigmund Høibakken and Stein Marius Brumoen for their time and indispensable help with data collection. The authors thank Michał Maj for his valuable ideas and discussions.

*Financial support statement*

This work was funded by an internal scholarship from the Inland Norway University of Applied Sciences.

## Appendix A. Cut-off values for normal sperm properties for Norwegian Red bulls

See Appendix A Section here.

| Measurement | Min | Max |
|---|---|---|
| Acrosome % | 36 | 46 |
| Ellipticity | 1.5 | 2.5 |
| Elongation | 0.2 | 0.5 |
| Head area μm$^2$ | 35 | 55 |
| Head length μm | 8.8 | 11 |
| Head perimeter μm | 19 | 25 |
| Head width μm | 4 | 6 |
| Regularity | 0.5 | 1 |
| Midpiece angle ° | 0 | 5 |
| Insertion distance ° | 0 | 0.5 |
| Midpiece width μm | 0.8 | 1.5 |

**Appendix B. Differences between population morphometry of 10–13-month-old Norwegian Red bulls calculated from cells classified as normal and micro heads populations. Significant differences are presented accordingly * * p < 0.01, * ** p < 0.001**

See Appendix B section here.

| Measurement | Micro heads mean | Population mean |
|---|---|---|
| **Acrosome %** | 41.50 * ** | 41.94 |
| **Distance Midpiece µm** | 0.32 * ** | 0.25 |
| **Ellipticity** | 1.99 | 1.98 |
| **Elongation** | 0.32 * ** | 0.33 |
| **Gray Acrosome %** | 149.17 * ** | 152.21 |
| **Head area µm$^2$** | 35.03 * ** | 43.25 |
| **Head length µm** | 8.43 * ** | 9.61 |
| **Head perimeter µm** | 18.64 * ** | 20.93 |
| **Head width µm** | 4.35 * ** | 4.85 |
| **Midpiece angle °** | 2.47 * ** | 2.69 |
| **Midpiece area µm$^2$** | 14.25 * * | 9.82 |
| **Midpiece width µm** | 1.21 * ** | 1.07 |
| **Regularity** | 0.82 * ** | 0.85 |
| **Roughness** | 1.27 * ** | 1.24 |

**Appendix C. Spermiograms of 16 ejaculates with abnormal morphology[5] All variables are presented as a proportion (%)**

See Appendix C Section here.

| Bull | Normal | Abnormal | Abaxial tails | Distal Droplets | Teratozoospermy index | Multiple anomalies index | Head defects | Midpiece defects | Tail defects | Proximal droplets |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 51.00 | 49.00 | 2.50 | 0.00 | 1.10 | 1.19 | 47.5 | 3.50 | 3.00 | 0.00 |
| 2 | 51.49 | 48.51 | 9.90 | 0.00 | 1.91 | 2.34 | 27.72 | 27.72 | 37.13 | 0.00 |
| 3 | 52.52 | 47.48 | 20.14 | 0.00 | 1.77 | 2.15 | 32.37 | 19.42 | 32.37 | 0.00 |
| 4 | 53.96 | 46.04 | 15.84 | 0.00 | 1.74 | 2.29 | 42.08 | 21.78 | 16.34 | 0.00 |
| 5 | 57.43 | 42.57 | 7.92 | 0.00 | 1.41 | 1.93 | 35.15 | 15.84 | 5.45 | 3.47 |
| 6 | 58.42 | 41.58 | 18.32 | 2.97 | 1.85 | 2.23 | 23.76 | 21.29 | 31.19 | 0.50 |
| 7 | 58.50 | 41.50 | 15.00 | 1.50 | 1.86 | 2.25 | 33 | 22.00 | 22.00 | 0.00 |
| **8** | 62.50 | 37.50 | 5.60 | 0.86 | 1.43 | 1.57 | 13.36 | 12.50 | 26.72 | 0.86 |
| **9** | 64.39 | 35.61 | 4.88 | 0.00 | 1.68 | 2.18 | 21.95 | 15.12 | 22.93 | 0.00 |
| **10** | 65.37 | 34.63 | 11.22 | 4.88 | 1.59 | 1.89 | 16.59 | 11.71 | 26.83 | 0.00 |
| 11 | 66.82 | 33.18 | 3.64 | 0.45 | 1.60 | 1.97 | 20.91 | 15.45 | 16.36 | 0.45 |
| 12 | 67.16 | 32.84 | 19.90 | 0.00 | 1.88 | 2.88 | 26.87 | 21.89 | 12.94 | 0.00 |
| 13 | 68.87 | 31.13 | 10.38 | 0.94 | 1.55 | 1.89 | 16.98 | 17.45 | 11.79 | 1.89 |
| 14 | 71.15 | 28.85 | 23.08 | 1.44 | 1.93 | 2.62 | 25 | 16.83 | 12.98 | 0.96 |
| 15 | 71.22 | 28.78 | 13.67 | 0.00 | 1.35 | 1.53 | 27.34 | 6.47 | 5.04 | 0.00 |
| **16** | 71.95 | 28.05 | 8.60 | 3.62 | 1.63 | 2.21 | 23.08 | 13.57 | 8.60 | 0.45 |

**Appendix D. Spermiograms of 16 ejaculates with abnormal morphology.[6] Detailed separation to specific abnormalities of head midpiece and tail. All variables are presented as a proportion (%)**

See Appendix D section here.

[5] Acceptance to AI station is based on genomic value and andrology testing.
[6] Acceptance to AI station is based on genomic value and andrology testing.

| | Head defects | | | | | | | | Midpiece defects | | | Tail defects | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bull | Micro | Macro | Tapered | Thin | Round | Pyriform | Amorphous | Abnormal Acrosome | Abnormal size | Abnormal Insertion | Abnormal Angle | Abnormal | Short | Without | Irregular | Rolled | Multiple | Age in months |
| **1** | 46.50 | 0.00 | 1.00 | 0.00 | 0.00 | 1.50 | 2.00 | 0.00 | 2.50 | 1.50 | 0.50 | 0.00 | 0.00 | 0.00 | 0.00 | 3.00 | 0.00 | 11 |
| 2 | 10.40 | 1.49 | 0.99 | 0.00 | 0.00 | 3.47 | 2.48 | 17.82 | 21.78 | 17.82 | 0.00 | 2.48 | 0.00 | 3.47 | 0.00 | 31.19 | 0.00 | 12 |
| 3 | 28.78 | 0.00 | 2.16 | 0.00 | 0.00 | 1.44 | 1.44 | 7.19 | 12.95 | 15.83 | 0.00 | 8.63 | 0.72 | 7.19 | 0.00 | 15.11 | 0.72 | 10 |
| 4 | 36.14 | 0.00 | 0.00 | 0.00 | 0.50 | 0.99 | 4.95 | 9.90 | 16.34 | 18.32 | 1.98 | 0.99 | 0.00 | 11.39 | 0.00 | 3.96 | 0.00 | 12 |
| 5 | 18.32 | 0.00 | 12.38 | 0.99 | 0.50 | 7.92 | 3.96 | 10.40 | 10.89 | 10.40 | 0.99 | 0.50 | 0.00 | 1.98 | 0.00 | 2.97 | 0.00 | 10 |
| 6 | 12.38 | 0.00 | 2.97 | 0.50 | 0.00 | 0.99 | 1.98 | 11.39 | 14.36 | 16.83 | 0.00 | 2.48 | 0.50 | 4.95 | 0.00 | 23.27 | 0.00 | 11 |
| 7 | 17.00 | 0.00 | 5.00 | 0.00 | 0.00 | 2.50 | 2.50 | 12.50 | 14.50 | 14.50 | 3.00 | 2.50 | 0.50 | 2.00 | 0.00 | 16.00 | 1.00 | 10 |
| **8** | 2.59 | 2.16 | 0.00 | 0.00 | 0.00 | 0.43 | 0.43 | 10.34 | 9.05 | 7.33 | 0.00 | 0.43 | 0.86 | 2.16 | 0.00 | 23.28 | 0.00 | 11 |
| **9** | 13.17 | 0.49 | 0.00 | 0.49 | 0.00 | 1.46 | 3.41 | 10.24 | 11.22 | 13.66 | 0.49 | 3.41 | 0.98 | 6.83 | 0.00 | 11.22 | 0.49 | 11 |
| **10** | 14.63 | 0.00 | 2.44 | 0.00 | 0.00 | 0.49 | 0.49 | 4.88 | 7.32 | 8.29 | 0.00 | 0.98 | 0.00 | 3.41 | 0.00 | 22.44 | 0.00 | 12 |
| 11 | 10.00 | 0.91 | 0.00 | 0.45 | 0.00 | 0.45 | 2.73 | 10.45 | 14.55 | 8.64 | 0.91 | 0.45 | 0.00 | 5.45 | 0.00 | 10.00 | 0.45 | 11 |
| 12 | 16.92 | 0.50 | 4.48 | 0.00 | 0.00 | 1.99 | 8.46 | 13.93 | 14.93 | 19.40 | 0.50 | 0.00 | 0.00 | 3.48 | 0.00 | 9.45 | 0.00 | 11 |
| 13 | 10.38 | 0.47 | 0.00 | 0.47 | 0.47 | 0.47 | 0.00 | 7.08 | 15.57 | 10.38 | 1.89 | 5.66 | 0.00 | 5.66 | 0.00 | 0.47 | 0.00 | 12 |
| 14 | 21.63 | 0.00 | 0.96 | 0.00 | 0.96 | 1.44 | 4.81 | 4.81 | 14.42 | 13.46 | 0.00 | 0.96 | 0.96 | 7.69 | 0.00 | 3.37 | 0.00 | 12 |
| 15 | 22.30 | 0.00 | 0.00 | 0.00 | 0.00 | 2.16 | 0.72 | 4.32 | 5.04 | 4.32 | 0.00 | 0.72 | 0.00 | 2.88 | 0.00 | 1.44 | 0.00 | 11 |
| **16** | 20.36 | 0.00 | 0.00 | 0.00 | 1.36 | 0.90 | 3.62 | 5.88 | 9.95 | 10.41 | 0.90 | 3.17 | 0.00 | 4.07 | 0.00 | 0.90 | 0.45 | 12 |

## Appendix E. Differences between 3 clusters in the population mean of motility, viability and acrosome reaction parameters for Types Fresh (F), Stressed (S), Thaw Fresh (TF) and Thaw Stressed (TS)

See Appendix E Section here.

| cluster | Type | AIL % | ARL % | Motile % | Progressive % | Rapid % | Medium % | Slow % | Immotile % | Rapid progressive % | Medium progressive % | Non progressive % | Immotile % | Hyperactive % (Motile) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | F | 71 | 0.15 | 67.92 | 54.73 | 46.32 | 14.29 | 7.31 | 32.08 | 13.25 | 41.47 | 13.19 | 32.08 | 55.16 |
| 1 | S | 64 | 0.22 | 54.78 | 40.12 | 34.52 | 13.60 | 6.66 | 45.22 | 7.45 | 32.68 | 14.66 | 45.22 | 50.99 |
| 1 | TF | 22 | 0.10 | 17.86 | 6.20 | 4.47 | 6.69 | 6.71 | 82.14 | 1.23 | 4.97 | 11.66 | 82.14 | 18.86 |
| 1 | TS | 15 | 0.11 | 10.42 | 1.92 | 1.42 | 3.22 | 5.79 | 89.58 | 0.26 | 1.66 | 8.50 | 89.58 | 9.24 |
| 2 | F | 73 | 0.15 | 71.50 | 54.57 | 41.00 | 19.96 | 10.53 | 28.50 | 15.41 | 39.16 | 16.92 | 28.50 | 41.51 |
| 2 | S | 70 | 0.17 | 65.23 | 53.72 | 47.92 | 11.63 | 5.68 | 34.77 | 11.86 | 41.86 | 11.51 | 34.77 | 58.53 |
| 2 | TF | 29 | 0.11 | 24.25 | 11.08 | 8.47 | 8.82 | 6.96 | 75.75 | 2.46 | 8.61 | 13.17 | 75.75 | 22.56 |
| 2 | TS | 27 | 0.10 | 21.07 | 8.54 | 7.28 | 7.39 | 6.40 | 78.93 | 1.04 | 7.49 | 12.53 | 78.93 | 22.62 |
| 3 | F | 79 | 0.13 | 76.68 | 61.99 | 53.15 | 15.20 | 8.34 | 23.32 | 11.68 | 50.31 | 14.69 | 23.32 | 57.47 |
| 3 | S | 73 | 0.22 | 68.34 | 56.93 | 51.93 | 11.64 | 4.77 | 31.66 | 8.34 | 48.58 | 11.41 | 31.66 | 66.83 |
| 3 | TF | 41 | 0.14 | 38.62 | 20.95 | 16.65 | 14.08 | 7.89 | 61.38 | 3.89 | 17.07 | 17.67 | 61.38 | 34.91 |
| 3 | TS | 19 | 0.11 | 12.24 | 2.55 | 1.80 | 4.63 | 5.81 | 87.76 | 0.32 | 2.23 | 9.69 | 87.76 | 14.01 |

## References


Argov-Argaman, N., Mahgrefthe, K., Zeron, Y., Roth, Z., 2013. Variation in lipid profiles within semen compartments-the bovine model of aging. Theriogenology 80, 712–721. https://doi.org/10.1016/j.theriogenology.2013.05.024.

Armstrong, C., Act, D., 2022. Bull breeding soundness and cryopreservation of semen. Am. Assoc. Bov. Pract. Conf. Proc. 55, 26–30. https://doi.org/10.21423/aabppro20228508.

Barth, A.D., 2018. Review: the use of bull breeding soundness evaluation to identify subfertile and infertile bulls. Animal 12, s158–s164. https://doi.org/10.1017/S1751731118000538.

Bates, D., Mächler, M., Bolker, B.M., Walker, S.C., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67, 1–48. https://doi.org/10.18637/jss.v067.i01.

Beletti, M.E., Costa, L.D.F., Viana, M.P., 2005. A comparison of morphometric characteristics of sperm from fertile Bos taurus and Bos indicus bulls in Brazil. Anim. Reprod. Sci. 85, 105–116. https://doi.org/10.1016/j.anireprosci.2004.04.019.

Bremer, J., Heringstad, B., Morrell, J.M., Kommisrud, E., 2023. Associations between insulin-like factor 3, scrotum circumference and semen characteristics in young Norwegian Red bulls. Animal 17, 100713. https://doi.org/10.1016/j.animal.2023.100713.

Bruni, V., Cardinali, M.L., Vitulano, D., 2022. A short review on minimum description length: an application to dimension reduction in PCA. Entropy 24, 1–16. https://doi.org/10.3390/e24020269.

Deori, S., Hurri, E., Karkehabadi, S., Morrell, J.M., 2021. Fast protein liquid chromatography profiles of seminal plasma proteins in young bulls: a biomarker of sperm maturity. Livest. Sci. 250, 104600 https://doi.org/10.1016/j.livsci.2021.104600.

Felton-Taylor, J., Prosser, K.A., Hernandez-Medrano, J.H., Gentili, S., Copping, K.J., Macrossan, P.E., Perry, V.E.A., 2020. Effect of breed, age, season and region on sperm morphology in 11,387 bulls submitted to breeding soundness evaluation in Australia. Theriogenology 142, 1–7. https://doi.org/10.1016/j.theriogenology.2019.09.001.

Garcia-Paloma, J.A., 2015. A bull breeding soundness evaluation system with greater emphasis on scrotal circumference. Pesqui. Vet. Bras. 35, 817–821. https://doi.org/10.1590/s0100-736×2015000900008.

Geno [WWW Document], n.d. URL ⟨https://www.norwegianred.com/Start/About-Geno/Geno-group-of-companies/⟩ (Accessed 6.29.20).

Hossain, M.S., Johannisson, A., Wallgren, M., Nagy, S., Siqueira, A.P., Rodriguez-Martinez, H., 2011. Flow cytometry for the assessment of animal sperm integrity and functionality: State of the art. Asian J. Androl. 13, 406–419. https://doi.org/10.1038/aja.2011.15.

Hurri, E., Lima-verde, I., Johannisson, A., Ntallaris, T., Morrell, J.M., Stålhammar, H., 2022a. Post-thaw semen quality in young bull ejaculates before being accepted for commercial semen doses. Vet. Rec. 191, 1–11. https://doi.org/10.1002/vetr.1386.

Hurri, E., Lima-Verde, I., Ntallaris, T., Johannisson, A., Stålhammar, H., Morrell, J.M., 2022b. Sperm quality in young bull semen can be improved by Single Layer Centrifugation. Animals 12, 1–14. https://doi.org/10.3390/ani12182435.

Ikotun, A.M., Ezugwu, A.E., Abualigah, L., Abuhaija, B., Heming, J., 2023. K-means clustering algorithms: a comprehensive review, variants analysis, and advances in the era of big data. Inf. Sci. 622, 178–210. https://doi.org/10.1016/j.ins.2022.11.139.

Maree, L., Van Der Horst, G., 2013. Quantification and identification of sperm subpopulations using computer-aided sperm analysis and species-specific cut-off values for swimming speed. Biotech. Histochem. 88, 181–193. https://doi.org/10.3109/10520295.2012.757366.

Martínez-Pastor, F., 2021. What is the importance of sperm subpopulations? Anim. Reprod. Sci. 246, 106844 https://doi.org/10.1016/j.anireprosci.2021.106844.

Meuwissen, T., Hayes, B., Goddard, M., 2016. Genomic selection: a paradigm shift in animal breeding. Anim. Front. 6, 6–14. https://doi.org/10.2527/af.2016-0002.

Murphy, E.M., Kelly, A.K., O'Meara, C., Eivers, B., Lonergan, P., Fair, S., 2018. Influence of bull age, ejaculate number, and season of collection on semen production and sperm motility parameters in holstein friesian bulls in a commercial artificial insemination centre. J. Anim. Sci. 96, 2408–2418. https://doi.org/10.1093/jas/sky130.

Narud, B., Khezri, A., Nordborg, A., Klinkenberg, G., Zeremichael, T.T., Stenseth, E.B., Heringstad, B., Kommisrud, E., Myromslien, F.D., 2022. Semen quality parameters including metabolites, sperm production traits and fertility in young Norwegian Red AI bulls. Livest. Sci. 255. https://doi.org/10.1016/j.livsci.2021.104803.

Ntemka, A., Tsousis, G., Brozos, C., Kiossis, E., Boscos, C.M., Tsakmakidis, I.A., 2016. Breed differences of bull frozen-thawed semen. Reprod. Domest. Anim. 51, 945–952. https://doi.org/10.1111/rda.12769.

O'Meara, C., Henrotte, E., Kupisiewicz, K., Latour, C., Broekhuijse, M., Camus, A., Gavin-Plagne, L., Sellem, E., 2022. The effect of adjusting settings within a Computer-Assisted Sperm Analysis (CASA) system on bovine sperm motility and morphology results. Anim. Reprod. 19, 1–14. https://doi.org/10.1590/1984-3143-AR2021-0077.

Olsen, H.B., Heringstad, B., Klemetsdal, G., 2020a. Genetic analysis of semen characteristic traits in young Norwegian Red bulls. J. Dairy Sci. 103, 545–555. https://doi.org/10.3168/jds.2019-17291.

Olsen, H.B., Heringstad, B., Klemetsdal, G., 2020b. Genetic correlations between body weight, daily weight gain, and semen characteristic traits in young Norwegian Red bulls. J. Dairy Sci. 103, 6311–6317. https://doi.org/10.3168/jds.2019-18116.

Perry, V.E.A., 2021. The role of sperm morphology standards in the laboratory assessment of bull fertility in Australia. Front. Vet. Sci. 8, 1–9. https://doi.org/10.3389/fvets.2021.672058.

Schenk, J.L., 2018. Review: Principles of maximizing bull semen production at genetic centers. Animal 12, s142–s147. https://doi.org/10.1017/S1751731118000472.

Taaffe, P., O'Meara, C.M., Stiavnicka, M., Byrne, C.J., Eivers, B., Lonergan, P., Fair, S., 2022. Increasing the frequency of ejaculate collection in young dairy bulls increases semen production and field fertility. Theriogenology 182, 45–52. https://doi.org/10.1016/j.theriogenology.2022.01.030.

Taylor, J.F., Schnabel, R.D., Sutovsky, P., 2018. Review: genomics of bull fertility. Animal 12, s172–s183. https://doi.org/10.1017/S1751731118000599.

Valverde, A., Arenán, H., Sancho, M., Contell, J., Yániz, J., Fernández, A., Soler, C., 2016. Morphometry and subpopulation structure of Holstein bull spermatozoa: variations in ejaculates and cryopreservation straws. Asian J. Androl. 18, 851–857. https://doi.org/10.4103/1008-682X.187579.

van der Horst, G., Skosana, B., Legendre, A., Oyeyipo, P., du Plessis, S.S., 2018. Cut-off values for normal sperm morphology and toxicology for automated analysis of rat sperm morphology and morphometry. Biotech. Histochem 93, 49–58. https://doi.org/10.1080/10520295.2017.1380842.

van der Horst, G., du Plessis, S.S., Maree, L., 2021. Have we conquered sperm morphology analysis in different Mammalian species as analysed by CASMA? XIIIth. Int. Symp. Spermatol. 287–301. https://doi.org/10.1007/978-3-030-66292-9_38.

van Der Horst, G., Maree, L., 2009. SpermBlue®: a new universal stain for human and animal sperm which is also amenable to automated sperm morphology analysis. Biotech. Histochem. 84, 299–308. https://doi.org/10.3109/10520290902984274.

Vicente-Fiel, S., Palacín, I., Santolaria, P., Hidalgo, C.O., Silvestre, M.A., Arrebola, F., Yániz, J.L., 2013. A comparative study of the sperm nuclear morphometry in cattle, goat, sheep, and pigs using a new computer-assisted method (CASMA-F). Theriogenology 79, 436–442. https://doi.org/10.1016/j.theriogenology.2012.10.015.

Víquez, L., Barquero, V., Soler, C., Roldan, E.R.S., Valverde, A., 2020. Kinematic sub-populations in bull spermatozoa: a comparison of classical and bayesian approaches. Biology 9, 1–16. ⟨https://doi.org/10.3390/biology9060138⟩.

Waite, R.K., Dwyer, C.J., Beggs, D.S., Mansell, P.D., Stevenson, M.A., Pyman, M.F., 2019. Scrotal circumference, bodyweight and semen characteristics in growing dairy-breed natural-service bulls in Tasmania, Australia. N. Z. Vet. J. 67, 109–116. https://doi.org/10.1080/00480169.2018.1563512.

Yamin, L.J., Cauchard, J.R., 2022. Generative adversarial networks and data clustering for likable drone design. Sensors 22, 6433 https://doi.org/doi: 10.3390/s22176433.