Inland Norway
University of
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Faculty of Applied Ecology, Agricultural Sciences and Biotechnology

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Sperm quality, semen production, and fertility in young Norwegian Red bulls

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Abstract

With the application of genomic selection in dairy cattle breeding, the choice of elite sires is based on their estimated genomic breeding values instead of progeny testing. Consequently, bulls are introduced into semen production at a younger age than previously. The main aim of this thesis was to identify novel early indicators of sperm production onset and maturity status of young Norwegian Red bulls during their performance test period, to provide insight into their potential future semen production, acceptance for the AI station, and field fertility. In Paper 1, flow cytometry and computer-aided sperm analysis were used to analyse various sperm quality parameters in ejaculates collected from 65 bulls aged 9-13 months. Semen samples were subjected to stress tests and cryopreservation. The bulls were classified into three clusters with different responses to sperm stress tests. By incorporating sperm stress tests, cryopreservation, and early morphology analysis, valuable insights into the maturity of bulls for sperm production could be gained. This approach would allow for the integration of younger bulls into semen collection, facilitating reduced generation interval and increased genetic gain. The focus in Paper 2 is on investigating the potential of insulin-like factor 3 as a biomarker for predicting the onset of sperm production in young Norwegian Red bulls. Blood samples and scrotal circumference measurements were collected from 142 bulls at four time-points between 2 and 12 months of age. The aim of the study was to determine the relationship between insulin-like factor 3, scrotal circumference, and semen characteristics. While a positive correlation was found between insulin-like factor 3 and scrotal circumference, no significant correlations were observed between scrotal circumference and semen characteristics. Due to the substantial inter-individual variability in the Norwegian Red bull population, insulin-like factor 3 is currently not a reliable biomarker for predicting the onset of sperm production in this breed. In Paper 3 an automated method for measuring scrotal circumference of Norwegian Red bulls using 3D images and convolutional neural networks is presented. 3D images were captured, and manual scrotal circumference measurements made of bulls at different ages. The study compared the manual and automated measurements obtained through semantic segmentation. The results showed that the automated scrotal circumference measurements were similar to manual measurements. Mean prediction error varied depending on bull age and image quality. This novel measurement method has the potential to be implemented in bull breeding soundness evaluations at performance test stations and semen collection centers, providing a fast and efficient approach for assessing scrotal circumference.

Sammendrag

Ved bruk av genomisk seleksjon i storfeavlen blir eliteokser selektert basert på deres estimerte genomiske avlsverdier i stedet for ved avkomsgransking. Oksene er derfor yngre når de blir tatt i bruk i sædproduksjon enn tidligere. Hovedmålet med denne avhandlingen var å identifisere nye indikatorer for når sædproduksjonen er i gang hos unge Norsk Rødt Fe okser, og som kan måles i løpet av testperioden og gi informasjon om oksenes potensielle fremtidige sædproduksjon, aksept for semin-stasjonen samt fruktbarhet i felt. I Artikkel 1 ble flowcytometri og Computer-Aided Sperm Analysis brukt til å analysere ulike spermiekvalitetsparametere i ejakulater fra 65 okser i alderen 9-13 måneder. Sædprøver ble utsatt for stresstester og kryokonservering. Oksene ble klassifisert i tre grupper med ulik respons på spermie-stresstester. Ved å benytte spermie-stresstester, kryokonservering og morfologianalyse tidlig i testperioden, kan en få verdifull innsikt i når oksene er tilstrekkelig utviklet for sædproduksjon. Med denne tilnærmingen vil en kunne ta i bruk yngre okser i sæduttak og -produksjon, og dermed bidra til redusert generasjonsintervall og økt genetisk framgang. I Artikkel 2 ble det fokusert på å undersøke potensialet til insulin-like factor 3 som en biomarkør for å predikere når sædproduksjonen starter hos unge Norsk Rødt Fe okser. Det ble tatt blodprøver og samtidig utført målinger av skrotumomkrets på 142 okser på fire tidspunkt mellom 2 og 12 måneders alder. Studien hadde som mål å belyse sammenhenger mellom nivået av insulin-like factor 3, skrotumomkrets og ulike sædparametere. Det ble funnet en positiv korrelasjon mellom insulin-like factor 3 og skrotumomkretsen, men det ble ikke funnet signifikante sammenhenger mellom skrotumomkretsen og sædparametere. På grunn av betydelige individuelle variasjoner i den undersøkte norske okse-populasjonen, er insulin-like factor 3 foreløpig ikke en egnet biomarkør til å kunne predikere når sædproduksjonen starter hos denne rasen. I Artikkel 3 presenteres en automatisert metode for å måle skrotumomkretsen hos Norsk Rødt Fe okser ved hjelp av 3D-bilder og konvolusjonelle nevrale nettverk. 3D-bilder ble tatt samtidig som manuelle målinger av skrotumomkretsen ble utført på oksene, noe som ble gjentatt ved ulike aldere. Studien sammenlignet de manuelle og automatiserte målingene oppnådd ved semantisk segmentering. Det ble vist at de automatiserte målingene av skrotumomkretsen ga tilsvarende resultater som de manuelle målingene. Gjennomsnittlig prediksjonsfeil varierte med oksenes alder og kvaliteten på 3D-bildene. Denne nye målemetoden har potensiale til å kunne implementeres i breeding soundness evaluation ved testings- og seminstasjoner, og kan gi en rask og effektiv vurdering av skrotumomkretsen.

Preface

All research included in this thesis was performed at the Department of Biotechnology, Inland Norway University of Applied Sciences in Hamar, and the Øyer performance testing station of breeding company Geno. This work was funded by the internal scholarship of Inland Norway University of Applied Sciences sponsored by Sparebankstiftelsen Hedmark. This study used a 3D camera from the project supported by the Research Council of Norway under the BIONÆR program, project number 282252, "New traits in pigs and cattle based on 3D imaging technology".

Thank you,

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List of papers

1. Bremer J, Heringstad B, Morrell JM, Kommisrud E. Novel interpretation of sperm stress test and morphology for maturity assessment of young Norwegian Red bulls. Anim Reprod Sci. 2023 Jun;253

2. Bremer J, Heringstad B, Morrell JM, Kommisrud E. Associations between insulin-like factor 3, scrotal circumference and semen characteristics in young Norwegian Red bulls. Animal. 2023 Mar;17(3)

3. Bremer J, Maj M, Nordbø Ø, Kommisrud E. Deep learning–based automated measurements of the scrotal circumference of Norwegian Red bulls from 3D images. Smart Agric. Technol. 2023. Mar; 3(10)

List of abbreviations

AI - artificial insemination

AIL - live spermatozoa with intact acrosome

ALH - amplitude of lateral head displacement

ATP - adenosine triphosphate

BBSE - Bull Breeding Soundness Evaluation

BCF - beat cross frequency (Hz)

CASA - Computer-aided sperm analysis

CASA-Mot - Computer-aided sperm motility analysis

CASMA - Computer-aided sperm morphology analysis

Ca2+ - calcium ions

CNNs - Convolutional Neural Networks

DNA - deoxyribonucleic acid

FC - flow cytometry

FSH - follicle-stimulating hormone

GnRH - gonadotropin hormone-releasing hormone

GPS - global positioning system

INSL3 - insulin-like factor 3

LH - luteinizing hormone

LIN - linearity VSL/VCL x 100 (%)

MAI - multiple anomalies index

MPE - mean prediction error

MSPE - mean squared prediction error

mRNA - messenger ribonucleic acid

NDHRS - The Norwegian Dairy Herd Recording System

NRR56 - non-return rates

PUFA - polyunsaturated fatty acids

Q - quarantine

ROS - reactive oxygen species

S - Stressed

SC - scrotum circumference

SFT - Society for Theriogenology

STR - straightness VSL/VAP x 100 (%)

TAI – Thawed AI

TF - Thawed Fresh

TMI - total merit index

TS - Thawed Stressed

TZI - teratozoospermic index

VAP - average path velocity (μm/s)

VCL - curvilinear velocity (μm/s)

VSL - straight line velocity (μm/s)

WCABP - Western Canadian Association of Bovine Practitioners

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1. Introduction

1.1. General background

Prediction of bull fertility is an objective of many studies. There have been two main shifts in the dairy breeding industry in recent years: the introduction of genomic selection and, as its consequence, the use of younger bulls in semen production (Fair and Lonergan, 2018). The growing world population, globalisation, economic growth in developing countries, climate changes, higher ecological awareness, and changing standards towards food quality affect animal production. For these reasons, the world requires innovative solutions in animal reproduction, health, nutrition, and welfare leading to balanced production. Genomics research aims to use genomic information for better results in animal selection (Rexroad et al., 2019). Genomic selection has become cheaper and more accessible for artificial insemination (AI) breeding companies, and it is used in dairy cattle breeding worldwide. It is possible because of available data from progeny-tested bulls with phenotypes from their daughters´ performances over many herds. International collaborations such as Eurogenomics, American Consortium, and others created a big reference population. International projects, such as the 1000 Bull Genomes Project, aimed to improve the genomic selection accuracy in diverse cattle populations (Boichard et al., 2016; Hayes and Daetwyler, 2019; Meuwissen et al., 2016). The introduction of younger bulls into semen production is a worldwide trend due to genomic selection. It allows progeny testing programs to be eliminated, thus shortening the time between generations (Fair and Lonergan, 2018; Matthews et al., 2019; Rexroad et al., 2019).

1.2. Norwegian Red

Geno is a breeding organisation for the dairy breed, Norwegian Red. It is the leading breed covering more than 90% of the dairy cattle population in Norway. Norwegian Red is a dual-purpose medium-sized cattle breed known for its health and fertility traits. Progeny testing of sires was performed until 2016 when Geno implemented genomic selection. Historically, the breeding program included health and fertility traits. Since 1972, fertility has been incorporated in the total merit index (TMI) for Norwegian Red (Garmo et al., 2008). With a broad and balanced breeding goal, Geno demonstrated that genetic gain for production traits simultaneously with health and fertility traits is possible despite unfavourable genetic correlations. Thanks to the long-term progeny testing program, a single-step genomic selection program could be implemented. Breeding values for both genotyped and non-genotyped bulls

and cows are estimated in the same procedure. Phenotype, genotype, and pedigree are used to predict breeding value. The Norwegian Dairy Herd Recording System (NDHRS) is a national database with information on milk recordings, health recordings, and fertility for every individual cow in Norway (e.g., age of heifers at first AI, days in milk at first AI) (Geno, 2023b). More than 90% of Norwegian Red dairy cattle are enrolled in NDHRS. The data from the NDHRS, collected from many sources over many years, allows the calculation of good-quality breeding values (Geno, 2023b).

1.3. Sexual development and puberty onset in bulls

Time from infancy to puberty is crucial for the sexual development of the bull, which will affect sperm production throughout his life. Puberty marks the beginning of the bull's reproductive ability. To reach puberty, a bull needs to go through complex maturation mechanisms of the hypothalamic-pituitary-testicular axis, the development of gonads, and secondary sexual organs. According to changes in gonadotrophins and testosterone levels, sexual development in bulls can be divided into three stages: infantile, prepubertal, and pubertal (Brito, 2014; Rawlings et al., 2008). Figure 1 gives an overview of the development of some parts of the reproductive system and patterns of serum concentrations of reproductive hormones from birth to puberty.

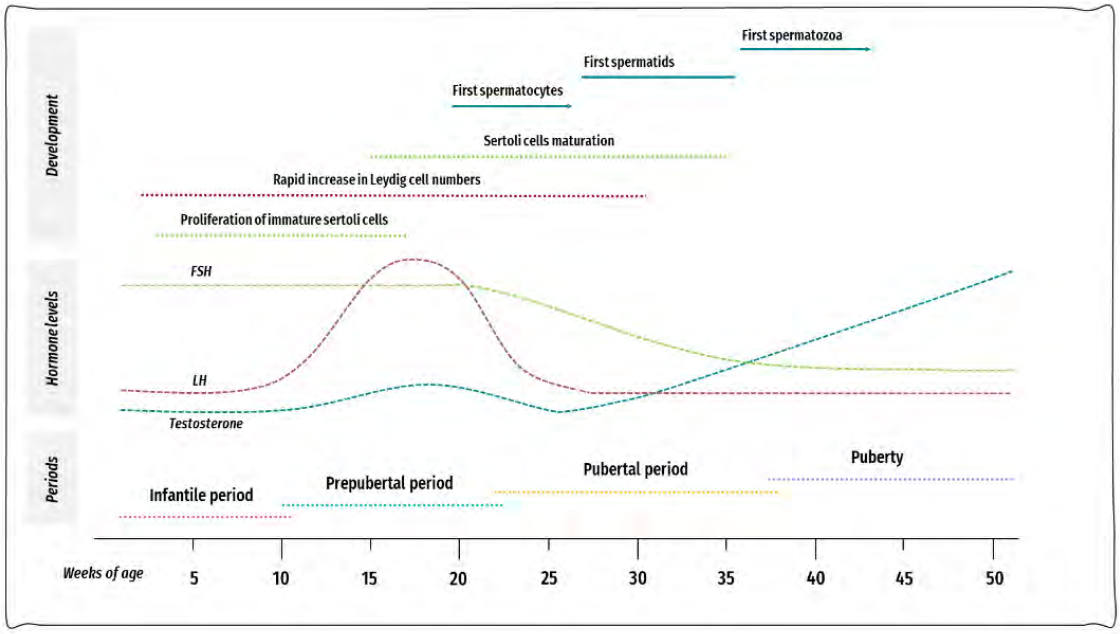

Fig. 1. Growth and development of some segments of the reproductive system and patterns of serum concentrations of reproductive hormones of the bull calf from birth to puberty. Figure based on Bollwein et al., 2016 and Rawlings et al., 2008.

The infantile period, which lasts until approximately two months after birth, is characterised by low gonadotrophin and testosterone levels due to reduced gonadotropin hormone-releasing hormone (GnRH) secretion. With the maturation of the hypothalamus, GnRH pulse secretion increases and marks the transition to the prepubertal period. From 2 to 6 months of age, bulls go through an early gonadotrophin rise which is characterised by a transient increase in luteinizing hormone (LH) and follicle-stimulating hormone (FSH) concentrations. The frequency of LH pulses increases from less than one per day at 1 month of age to approximately 12 per day at 4 months of age. This increase is crucial for Leydig cell proliferation, differentiation and, consequently, testosterone production. Increased levels of FSH concentrations stimulate the proliferation of undifferentiated Sertoli cells. It is suggested that this process is completed under the influence of testosterone secreted by Leydig cells, which leads to the differentiation of gonocytes into spermatogonia (Bollwein et al., 2016; Brito, 2014; Rawlings et al., 2008). By the end of the prepubertal period, the blood-testis barrier and tubular lumen are in place, and germ cell meiosis begins. Nutrition has a significant effect on development in this period, and a high plane of nutrition pre-6 months of age may hasten puberty onset, regardless of the plane of nutrition post-6 months of age (Brito, 2014; Byrne et al., 2018; Dance et al., 2015; Sethi et al., 2022). In addition, Perrier et al. (2020) found that enhanced nutrition before 6 months of age results in changes in sperm deoxyribonucleic acid (DNA) methylation profiles after puberty. Recent studies show the importance of maternal nutrition during the periconception period and early gestation, which may alter male testis development and delay the puberty onset of the offspring (Sethi et al., 2022).

With the decrease in gonadotrophin secretion and rise in testosterone concentration, the pubertal period occurs after the age of 6 months until puberty. At the age of 8-10 months, mature sperm appear in the seminiferous tubules. Generally, in test stations, puberty is defined by the production of ejaculates with > 50 million (M) sperm with > 10% of motile sperm (Kastelic, 2014). To properly define the time of puberty, we need to consider how management, nutrition, genetics, breed and climate differences can affect it. Bulls enter puberty, on average, at 42 weeks and 28-30 cm scrotum circumference (SC), (Kastelic, 2014). Pubertal sperm quality is associated with lower motility and an increased number of morphological defects, especially proximal cytoplasmic droplets and abnormal head shape, compared to older bulls. It should be noted, however, that sperm quality and production increase for some time following puberty (Brito, 2014; Byrne et al., 2018; Staub and Johnson, 2018). There is a high variability in the onset of puberty in bull calves. Based on previous research, the puberty onset range for dairy bulls was shown to be between 283-369 days, with high variability within and across breeds

(Kenny et al., 2018). To maximise the use of genomic selection and successfully shorten generation intervals, we need to learn about the possible reasons for this range and ways to identify individuals with early puberty onset and sexual maturation (Bollwein et al., 2016; Rawlings et al., 2008).

1.4. Insulin-like factor 3 – a possible marker of sperm production

The main function of mature mammalian Leydig cells is to produce testosterone which is essential for spermatogenesis (Anand-Ivell et al., 2021). Leydig cells also secrete a hormone called insulin-like factor 3 (INSL3) (Ivell et al., 2013). This hormone is a small peptide that is capable of passing through the blood-testis barrier, thereby being present in the luminal fluid of various structures such as seminiferous tubules, rete testis, epididymis, and blood serum (Ivell et al., 2013). It was previously shown that INSL3 mRNA is exclusively expressed in the testis. As illustrated in Figure 2 INSL3 production is dependent on the number and developmental stage of Leydig cells (Sansone et al., 2019). The differentiation of Leydig cells is influenced by the hormone LH, which in turn influences the production of INSL3 (Anand-Ivell et al., 2019). RXFP2 is a receptor specific to the INSL3 hormone. This hormone/receptor system can be found in some of the oldest vertebrate order genomes. It has been lost in reptiles and birds but is preserved in all mammals and amphibians (Ivell et al., 2020). The INSL3/RXFP2 system belongs to the so-called neohormone group. Neohormones are essential to the major evolutionary changes in reproductive physiology, lactation, and internal fertilisation (Ivell et al., 2020). In female mammals, it is often observed that the levels of INSL3 in the peripheral blood are typically negligible or not detectable. However, a female mammal carrying a male fetus presents an exception to this trend. INSL3 was detected in bovine maternal blood and amniotic or allantoic fluid in pigs, rats, and humans (Anand-Ivell et al., 2011; Ivell et al., 2020). In contrast to testosterone and LH, INSL3 has a constitutive expression pattern, low intraindividual biological variance, and low technical variance (Anand-Ivell et al., 2019). The first phase of testicular descent is induced by large amounts of INSL3 secreted by Leydig cells of the fetal testis (Ivell and Anand-ivell, 2009). Studies have shown that INSL3 in various mammalian species, such as bovine, can be used as a metric for the development of Leydig cells during puberty (Anand-Ivell et al., 2019; Johansen et al., 2014). Increased LH pulsatility during prepuberty causes Leydig cell differentiation and a burst of INSL3 in blood (Ivell et al., 2013). Thus, INSL3 has been proposed as a potential biomarker to evaluate puberty in dairy bulls. A study conducted by Anand-Ivell et al. (2019) examined the impact of high-plane of nutrition during the first six months of life on spermatogenesis.

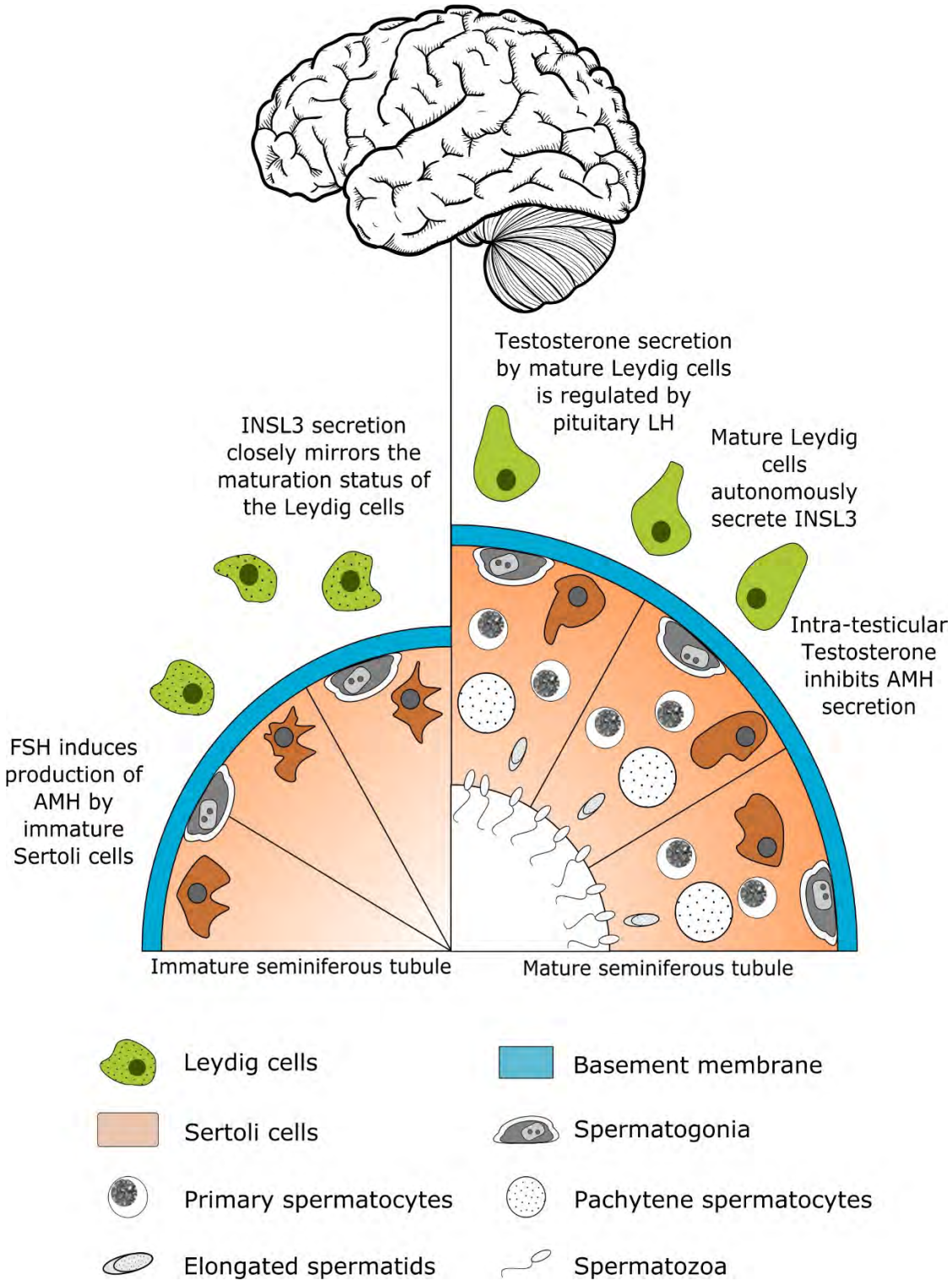

Fig. 2. Taken from - AMH and INSL3 in testicular and extragonadal pathophysiology: what do we know? by (Sansone et al., 2019). "Testicular endocrine function before and after testicular maturity (Sansone et al., 2019)."

The findings demonstrated that INSL3 exhibited a negative correlation with the timing of puberty while displaying a positive correlation with total testis weight at 18 months (Anand-Ivell et al., 2019).

1.5. The journey from bull calf to breeding bull – comparison of management across breeding companies

All our studies were done in collaboration with Geno, the breeding organisation for Norwegian Red. The study designs were adjusted to the management and routines at the performance test and AI stations. The overview of Geno's selection process and performance test station management is described by Geno (2023b) and Olsen et al. (2020). Our research for this chapter revealed that little information is published concerning the practiced management of breeding companies. To give an overview of the journey from bull calf to breeding bull and point out possible similarities and differences, we sent out questionnaires to nine international breeding companies. We received answers from the following four companies: Swissgenetics (Switzerland), Synetics (Germany and France), VikingGenetics (Denmark, Sweden, and Finland), and Semex (Canada). Like Geno, all four companies use genomic selection in their breeding programs. The same applies to genotyping bull calves to select the bulls for performance test or AI stations. The results of our questionnaires highlight one big difference between Geno's system and other companies, namely not all companies have performance test stations, although some have other facilities which serve different functions. Only one company informed us that they perform semen collection training before moving bulls to the AI station, as is done by Geno. All companies which answered our questionnaire buy bulls of multiple breeds for their AI station, but the proportion of each breed was not given. Swissgenetics buys 160 bull calves yearly and places them in a rearing station. 60% of these are selected for semen production. At the rearing station, they collect data about the growth rate of the young bulls and use them to optimise feeding rations and management. Swissgenetics performs semen collection training at the rearing station, so bulls are familiar with all processes and are ready for production when arriving at the AI station. They monitor mounting behaviour and semen quality from 7-8 months of age. Synetics buys 340 bull calves per annum (4-10 weeks old), which are moved to a rearing station after four weeks of quarantine. Based on the growth rates and behaviour observed in the barn, the company selects bulls ready for semen collection, the earliest at 9-10 months. VikingGenetics does not have a performance test station, and we did not receive any information about another facility they use before the AI station. They select 220 bulls yearly for semen production. At Semex, approximately 6 months old bull calves are kept in an isolation facility where a good physical foundation is ensured before the transition to the AI station. After arrival, the company-specific Bull Breeding Soundness Evaluation (BBSE) is performed, including scrotal measure, body condition score, size, weight, general

conformation, and physical health. The BBSE is done again in the middle of the bull's stay and a few weeks before they leave for the AI production facility, altogether at three different timepoints. Around 12 months of age, bulls that are in good health are moved to the AI station and tested for their semen quality and production capacity.

The questionnaires revealed differences in semen quality thresholds between companies. We were interested in semen quality thresholds at the performance test station, which, based on our experience with Geno, differ from those at the AI station. However, only one company performs an analysis of semen quality before bulls enter the AI station, and because they work with a range of different breeds which reach puberty at different ages, they do not have a strict semen quality threshold. The quality threshold of Synetics at the AI station includes <25% abnormal sperm morphology and >50% post-thaw progressive motility. For VikingGenetics, raw semen with vitality >70%, motility >65%, concentration 0.5 x 10^9 per ml, and volume >0.5 ml pass the quality assessment. Interestingly, SC measurements are part of the breeding soundness evaluation at Swissgenetics, VikingGenetics, and Semex but not at Synetics or Geno. Semex measure SC several times during the stay at the isolation facility. The company analysed scrotal growth patterns across their bull population and developed internal target growths for SC based on breed that allows them to identify outliers that are above or below expected growth. This overview highlights the company-specific management practices and lack of standardisation in the selection process of bulls for the AI station (Table 1).

Table 1. Overview of the journey from bull calf to breeding bull to point out possible similarities and differences between breeding companies based on the answers from the questionnaires.

Company name	**Geno**	**Swissgenetics**	**Synetics**	**VikingGenetics**	**Semex**
Country / Countries	Norway	Switzerland	Germany, France	Denmark, Sweden, Finland	Canada
Use of genomic selection in breeding programs	Yes	Yes	Yes	Yes	Yes
Genotyping bull calves to select the bulls for performance test or AI stations	Yes	Yes	Yes	Yes	Yes
Type of facility before selection to AI station	Performance test station	Rearing station	Rearing station	N/A [1]	Isolation facility
Numbers of bulls bought and (selected to AI station) per year	150 (50-60)	160 (90-100)	340 (N/A)	N/A (220)	N/A
Semen collection training and sperm quality assessment before selection to the AI station	Yes	Yes	No	N/A	No
Scrotal circumference measurements as part of BBSE [2]	No	Yes	Yes	Yes	Yes
Semen quality threshold at AI station	fresh motility >70%, post-thaw motility >50%, concentration 390 x 10^6/ml	N/A	abnormal sperm morphology <25%, post-thaw progressive motility >50%	vitality >70%, motility >65%, concentration 0.5 x 10^9/ml, and volume >0.5 ml	N/A

[1] N/A - No answer

[2] BBSE - Bull Breeding Soundness Evaluation

1.6. Bull Breeding Soundness Evaluation and importance of scrotal circumference measurements

Bull Breeding Soundness Evaluation is a low-cost tool that reduces the risk of using sub-fertile bulls (Barth, 2018). The BBSE systems are well-defined and implemented in some countries, such as the United States of America (the Society for Theriogenology [SFT]), Canada (the Western Canadian Association of Bovine Practitioners [WCABP]), and the United Kingdom (British Cattle Veterinary Association), while in other countries BBSE depends on the breeding associations (Felton-Taylor et al., 2020; Fordyce et al., 2006; Garcia-Paloma, 2015). The BBSE is used for different purposes in dairy and beef bulls. For the majority of dairy bulls, BBSE is part of the selection process at the AI stations. For beef bulls, the examination is performed before sale to confirm fertility before the bull is used for natural mating (Chenoweth and McPherson, 2016; Fordyce et al., 2006). The common traits assessed by BBSE are SC, libido, sperm motility, and morphology (Barth, 2018).

Scrotal circumference is the most common and easily measurable method to assess the testes size in field conditions, and it has been shown to be an accurate predictor of the paired testis weight (Kastelic, 2014). Early gonadotrophin rise plays an important role in testicular development. The LH concentrations in serum were shown to be significantly higher in early maturing beef bulls compared with late maturing ones (Kastelic, 2014). Scrotal circumference is influenced by e.g. age, body weight, diet (especially before the age of 6 months), and breed. A larger SC is associated with an earlier onset of puberty, greater sperm production, and improved fertility outcomes (Bollwein et al., 2016; Kastelic, 2014; Penitente-Filho et al., 2018; Rawlings et al., 2008; Waite et al., 2019). Sperm production is related to testicular size, and a high correlation of 0.81 was reported between sperm output and SC (Rawlings et al., 2008). Brito et al. (2012) studied the effect of growth rate from 6 to 16 months of age on sexual development in beef bulls. The SC and paired-testes volume were one of the predictors of pubertal and mature status suggested by the authors (Brito et al., 2012). In bulls, SC exhibits a sigmoidal growth pattern, beginning with an increase before six months of age and increasing rapidly during the peripubertal phase. The SC is an important reproductive trait with moderate to high heritability (from 0.36 to 0.69) (Ferreira et al., 2021; Kastelic, 2014; Penitente-Filho et al., 2018). A genome-wide association study conducted by Ferreira et al. (2021) confirmed the value of SC as a selection criterion in herds. Results showed that additive gene effects were responsible for 39% and 48% of the differences in SC adjusted for age and SC adjusted for age and weight between bulls, respectively (Ferreira et al., 2021).

The BBSE thresholds and way of adjusting for age and breed for different parameters differ significantly between systems. For example, WCABP adjusts the SC for age and breed, while SFT does not make any adjustments; their threshold for acceptance is fixed at 30 cm. The age of the bull influences their BBSE score since puberty onset can vary between breeds and populations (Garcia-Paloma, 2015). In Australia, a lot of work was done to standardise the BBSE across the continent. The Bull Reporter tool created by Fordyce et al. (2006) can be used regardless of type (beef or dairy), breed, level of management, genotypes, and environments, which presents a potential for the standardisation of BBSE across the world. This standardisation enabled large-scale research across breed, age, season, and regions. The distribution of morphological abnormalities was studied on 500 Australian and Pacific Islands herds during the yearly BBSE of 11,387 bulls (Felton-Taylor et al., 2020).

1.7. Objective sperm quality assessment

Sperm are one of the most diverse cell types known to science, with differences in size and shape between species (Birkhead et al., 2008). All semen analyses aim to assess the fertilisation ability of the ejaculate/individual (World Health Organization, 2021). Sperm morphology is an established part of sperm quality assessment and is applied in the field of taxonomy, sperm competition, and toxicology. Many studies showed that subjective sperm morphology assessment is imprecise and lacks repeatability due to differences in classification and stains used, human error, and technician variability (van der Horst et al., 2021; Yániz et al., 2015). In the review of morphology analysis of different species, van der Horst et al. (2021) explain how the human brain takes shortcuts and selects the most likely interpretation of what we see, which could impact the manual analysis of morphology. Electron, light, phase-contrast (positive and negative), and fluorescence microscopy can be used to examine sperm morphology (Yániz et al., 2015). For several decades researchers have been developing automatic systems for semen analysis. Computer-aided sperm morphology analysis (CASMA) method combines brightfield microscopy with software that detects sperm components from smears of stained sperm (van der Horst et al., 2021; Yániz et al., 2015). One of the CASMA software programs, Sperm Class Analyzer, measures 16 different morphometric parameters of the sperm head, midpiece, and tail. The CASMA reduces human bias, gives higher objectivity than manual assessment, and is not prone to errors or incorrect usage (van der Horst et al., 2021). To correctly classify sperm as normal versus abnormal, we need to define what is normal by setting species/breed-specific cut off points (van der Horst et al., 2021, 2018). Van der Horst et al. (2018) grouped morphometric measurements into a range of percentiles and used minimum and maximum

values as the normal range of cut-off points for rat sperm in the CASMA system. Other useful parameters measured by CASMA are the teratozoospermic index (TZI) and the multiple anomalies index (MAI), which give us information on more than one abnormality per sperm cell in the context of the population (Morselli et al., 2019). Currently, the range of studies aims to automate and improve the quality of objective sperm selection using, for example, deep learning-based image recognition (Riordon et al., 2019; Shahzad et al., 2023; You et al., 2021). Shahzad et al. (2023) used a sequential deep-learning method that labelled head, acrosome, and vacuole with high accuracy of 90%, 92%, and 89%, respectively.

As the ultimate goal of spermatozoa is to fertilise an ovum, they must be motile and be able to travel relatively long distances in internal fertilisers, such as cattle (Maree and van der Horst, 2013). To reduce the subjectivity of sperm motility analysis, Computer-aided sperm motility analysis (CASA-Mot) systems were developed (Yániz et al., 2018). With the use of CASA-Mot, more than 50 motility and kinematic parameters and sperm functional tests, such as hyperactivation and mucus penetration, can be assessed (van der Horst, 2021). Computer-aided sperm analysis (CASA) is used in commercial and research settings for sperm quality assessment of a wide range of species. This is why species and breed-specific settings are essential (van der Horst, 2020). Many factors can affect the CASA analysis: methods of collection, sampling vials, semen handling, time from sampling to analysis, type of pipette and media used, temperature control, microscope settings, type of chamber, field selection, sperm concentration, number of sperm analysed and settings of the software (e.g., frame rate and subpopulation cut-off values) (Maree and van der Horst, 2013; van der Horst, 2021, 2020; Yániz et al., 2018). When comparing the kinematic values between studies, we need to pay attention to the frame rate and media used during analysis. It is established that curvilinear velocity (VCL) and beat cross frequency (BCF) increase with increasing frame rate while linearity (LIN) decreases (Bompart et al., 2018; van der Horst, 2020; Yániz et al., 2018). O'Meara et al. (2022) studied the effect of adjusting settings within a CASA-Mot system on bovine sperm motility and morphology results. One of the results was a significant reduction in the proportion of progressive spermatozoa as a consequence of increasing the cut-off values for straightness (STR) and average path velocity (VAP) (O'Meara et al., 2022). Those results show the importance of setting standardisation to compare data. Many authors highlight the importance of kinematic sub-populations and rapid, medium progressive, or non-progressive percentage groupings (Maree and van der Horst, 2013; Martínez-Pastor, 2021; van der Horst, 2021; Yániz et al., 2018). In a review, Martínez-Pastor, (2021) states that sperm subpopulations are an outcome of evolution, linked to the species' reproductive strategies, genital tract

structures, and copulatory and fertilisation processes. One of the methods of defining sperm with similar motility characteristics consists of principal component analysis combined with clustering (Víquez et al., 2020; Yániz et al., 2018). Maree and van der Horst, (2013) proposed a method of defining sperm subpopulations using the swimming speed of individual spermatozoa adjusted for different species. There has been progress in the automation of motility analysis with CASA-Mot systems and its implementation in clinical practice, AI stations, and research. The development of a new generation of CASA and mobile CASA systems is expected to be stimulated by current advances in deep learning algorithms, optomechanics, microchips, and open-source programs (Waberski et al., 2022; Zhao et al., 2022).

Another gold standard method for the evaluation of heterogeneous sperm populations is flow cytometry (FC). In FC, fluorophores are used to assess a specific aspect of an individual cell's physiology or morphology (de Lima Rosa et al., 2023; Purdy et al., 2022). With FC, we can evaluate several sperm traits, such as sperm plasma membrane measures, acrosome integrity, mitochondrial membrane potential, reactive oxygen species (ROS), DNA damage, and intracellular calcium ions (Ca^{2+}) levels (Bucher et al., 2019; Quirino et al., 2022). This technique was shown to be objective, sensitive, and highly repeatable across semen samples (straws) and evaluation days (DeJarnette et al., 2022). Both CASA and FC are used in commercial settings in the assessment of bull semen quality (DeJarnette et al., 2022). With the availability of new fluorescent probes and multiple lasers, user-friendly flow cytometers multicolour flow cytometric assays have been developed for a range of species (Bucher et al., 2019; Quirino et al., 2022). Using multicolour analysis eliminates one of the major problems in sperm evaluation: the inability to test multiple parameters simultaneously in a single cell (de Lima Rosa et al., 2023). Quirino et al. (2022) successfully implemented a five-colour assay for simultaneous analysis of mitochondrial activity, lipid ordering of plasma membranes as well as acrosomal status, and plasma membrane integrity of boar and stallion sperm. Another study combined five fluorescent probes to analyse viability, acrosomal status, intracellular calcium levels, and mitochondrial function of cryopreserved bovine sperm from low and high-fertility bulls. As a result, they could successfully cluster 75% of bulls into low and high-fertility groups (Bucher et al., 2019). Bulkeley et al. (2023) studied the relationship between stallion sperm morphologies and ROS production using image flow cytometry. In both fresh and cooled ejaculates, significantly higher ROS levels were found in cells with abnormal heads, midpieces, and proximal droplets (Bulkeley et al., 2023).

1.8. Consequences of sperm cryopreservation

Cryopreservation of sperm transformed the dairy cattle industry, allowing for long-term storage, export, and distribution of sperm doses worldwide, the optimal time of insemination at the farm, and the use of genetically superior sires. It also allowed to reduce the risk of disease transmission in contrast to natural mating (Hitit and Memili, 2022). A range of studies showed that there is individual variability in sperm cryo-tolerance regardless of good results with natural mating and insemination with fresh semen (Catal and Yeste, 2022; Hitit et al., 2020; Loomis and Graham, 2008). Currently, research focuses on identifying markers of so-called "good and bad freezers", optimizing freezing protocols and cryoprotectants, and adding substances that improve the low-temperature environment (Catal and Yeste, 2022). Cooling, freezing, and thawing result in major changes in the sperm, causing, for example, damage to the sperm membrane due to alterations in lipid-protein complexes, thereby decreasing sperm quality. Roughly 50% of thawed sperm are dead or impaired due to cryopreservation. (Catal and Yeste, 2022; Hitit et al., 2020). Figure 3 gives an overview of structural and molecular alterations in mammalian sperm due to cryopreservation (Catal and Yeste, 2022). As a result of damage to the plasma membrane, sperm can have a different movement pattern, which can alter the categorisation of sperm cells into subpopulations (Ibanescu et al., 2020). The sperm membrane is a flexible structure due to polyunsaturated fatty acids (PUFA) residues in membrane lipids (Jakop et al., 2022). The important processes leading to successful fertilisation, such as the acrosome reaction, require membrane fluidity. Cryopreservation increases the level of oxidative stress, which is an important challenge to membrane lipids (Catal and Yeste, 2022; Jakop et al., 2022). Based on the findings of Jakop et al. (2022), bull sperm membranes are typically high in plasmalogens (alkenyl ether lipids), which are susceptible to oxidative stress. Semen quality characteristics such as sperm concentration, motility, and morphology tend to be inferior in older bulls (Murphy et al., 2018). Trevizan et al. (2018) found a negative effect of age on sperm quality in Nellore bulls due to higher susceptibility to oxidative damage. However, this study group included mature "young" 1.8-2 and adult 3.5-7.0 years old bulls (Trevizan et al., 2018). This raises the question of whether the sperm of pubertal bulls have different cryo-tolerance abilities due to their potentially immature membranes and are even more susceptible to oxidative stress than mature bulls.

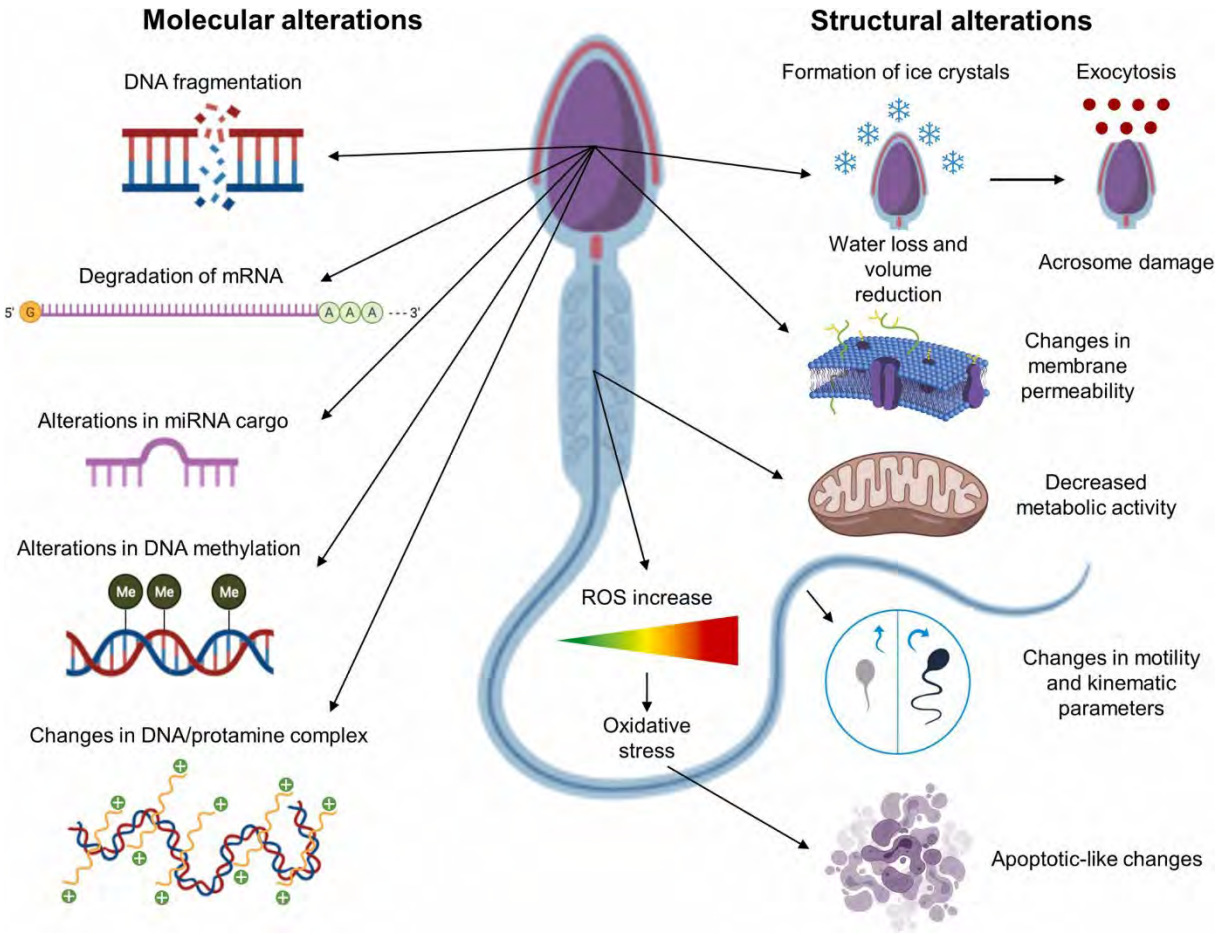

Fig. 3. Taken from - Advances in sperm cryopreservation in farm animals: cattle , horse , pig and sheep by Catal and Yeste, (2022). "Structural and molecular alterations in mammalian sperm following cryopreservation. Freeze-thawing decreases plasma membrane and acrosome integrity, motility, metabolic and mitochondrial activity, and increases ROS production. In addition, cryopreservation augments DNA fragmentation, may affect the DNA methylation signature, leads to degradation of mRNAs and miRNAs, and induces alterations in the integrity of the nucleoprotein structure (Catal and Yeste, 2022)."

1.9. Migration of sperm through the female reproductive tract and sperm functional assays

In mammals, to reach and fertilise the oocyte, sperm migrate through the female reproductive tract, interacting with luminal epithelial cells and secreted components of oviductal cells (Mahé et al., 2021; Rickard and de Graaf, 2020; Waberski et al., 2022). They also undergo a chain of processes influenced by the female reproductive tract: capacitate, acrosome react, penetrate the zona pellucida, enter the oocyte, and activate it (Pitnick et al., 2020). Physiological factors such as ions, signalling molecules, and receptors influence sperm movement and flagellar beating patterns throughout the tract. Physical factors, such as the anatomy of different parts of the tract, the flow of fluids, and its viscoelasticity, also influence

the speed and trajectory of the sperm (Waberski et al., 2022). The female reproductive tract was shown to support the fertilisation process and select sperm based on their morphology and motility (Lüpold and Pitnick, 2018; Waberski et al., 2022). Physiological and physical factors ensure that successful fertilisation occurs at the right place and time. To achieve this objective only a small number of capacitated spermatozoa are needed in the oviductal ampulla (Rickard and de Graaf, 2020; Waberski et al., 2022). Sperm tend to swim along the walls, avoiding stronger fluid flows. The architecture of the reproductive tract, such as microgrooves (around 20 µm wide) in the inner surfaces of the walls of the bovine cervix, has been shown to "guide" spermatozoa along the way to the uterine lumen (Waberski et al., 2022). Ligands of the sperm head and receptors on the epithelium form binding interactions which enable the spermatozoa to remain viable in the oviduct until ovulation (Ardon et al., 2016; Waberski et al., 2022). Heparin has been shown to release sperm from oviductal epithelial cells by interfering with the binding interaction. In some cases, hyperactivation was shown to assist the process. Ardon et al. (2016) demonstrated an *in vitro* system where sperm attached to ampullar epithelium required both heparin and hyperactivation to detach from the epithelium, sperm that had bound to isthmic epithelium were removed by heparin alone.

The main goal of quality control at AI stations is to detect and discard semen samples that would result in sub-optimal fertility if utilised for AI, thereby preventing economic losses for both companies and customers. Motility and morphology are fundamental aspects of sperm quality, and measures of these are used routinely in AI centers (Waberski et al., 2022). The physiology of sperm motility in the female tract is mostly overlooked during sperm quality assessment by technicians in andrology laboratories, except for sperm activation at body temperature via pre-incubation and warming on the microscopic stage. As a result, the diagnosis of "idiopathic subfertility" may be limited by the inability to detect an altered flagellar activity in sperm samples with high apparent motility due to the discrepancy between *in vitro* assessment and *in vivo* conditions (Waberski et al., 2022). Specialised chambers and microfluidics models which mimic the *in vivo* conditions, including biochemical components of the female tract, could be the future of testing at AI stations (Waberski et al., 2022). Evaluating the capacity of sperm to undergo hyperactivation is inexpensive and possible to perform with the use of CASA systems, which are now commonly used for sperm quality assessment at semen collection centers (van der Horst, 2020). However, the sperm tests that are routinely performed at AI stations do not assess the ability of sperm to reach and populate the sperm reservoirs at the uterotubule junction, or sperm functionality after the release of sperm from the oviductal epithelium. Bulls that pass the sperm quality threshold at the AI station can

differ in non-return rates by up to 20–25% (Hamze et al., 2020). Therefore, an indirect means of assessing sperm function is still needed. Methods to examine the functional capability of sperm through *in vitro* fertilisation are available, but the procedure is time-consuming and costly. It also requires trained personnel, specialised equipment, and ovaries from the slaughterhouse or oocytes collected by ovum pick-up (Hamze et al., 2020). A recent study tested a new sperm-binding assay based on magnetic sepharose beads coated with bovine recombinant JUNO protein (oocyte plasma membrane receptor described as involved in gamete binding) (Hamze et al., 2020). The results showed that bull sperm attach to JUNO, confirming that the JUNO-IZUMO1 interaction, essential in gamete recognition and fusion, is conserved in cattle. The proposed binding assay was able to distinguish between epididymal and ejaculated sperm and discriminate between sperm from bulls with varying levels of fertility (Hamze et al., 2020). This model is currently being tested to be commercialised for human IVF clinics by Spermosens (https://spermosens.com/technology/?lang=sv) and could be a potential easy-to-apply functional test at AI stations. However, success in IVF does not guarantee success in AI and therefore additional tests for sperm function after AI are still needed.

1.10. Relationship between sperm quality parameters and field fertility

Several studies have shown correlation between sperm quality parameters and non-return rates after 56 days (NRR56) in different dairy cattle breeds (Morrell et al., 2017; Nagy et al., 2015; Puglisi et al., 2012; Sellem et al., 2015; Shojaei et al., 2012) while others showed weak or no correlation (Farrell et al., 1998; Januskauskas et al., 2003). However, results between studies are not necessarily comparable due to differences in sperm number per AI dose (Kastelic, 2013). It has been demonstrated that there are breed differences concerning sperm quality parameters and their role as fertility predictors (Morrell et al., 2017). A significant positive correlation between NRR56 and viability measured by FC was identified (Christensen et al., 2005; Shojaei et al., 2012). Correlations between sperm quality in frozen-thawed semen and fertility were demonstrated to be higher than in raw samples (Christensen et al., 2005). A positive correlation between the proportion of morphologically normal spermatozoa with field fertility was observed in Swedish dairy bulls. The authors showed a negative correlation with fertility among sperm abnormalities such as pear-shaped sperm heads. Sperm morphology assessment is of importance both for bulls in performance test stations and proven bulls in AI stations (Al-Makhzoomi et al., 2008). Morphometric dimension measurements showed a significant positive correlation of pre-freeze width of sperm head with NRR56. This finding confirms that cryopreservation-induced membrane changes in bull spermatozoa can cause a

difference in fertility outcomes (Gravance et al., 2009). Bull field fertility outcome was positively correlated with motility parameters measured by CASA. One study reported a positive correlation between the proportion of bull spermatozoa with fast and nonlinear movements before freezing and NRR56 following AI with frozen sperm (Waberski et al., 2022). In various studies, velocity parameters such as VCL and VSL were considered to be good predictors of fertility (Farrell et al., 1998; Kathiravan et al., 2011; Nagy et al., 2015; Shojaei et al., 2012). The highest correlations were obtained by combining the results from different assays (Farrell et al., 1998; Gillan et al., 2008; Morrell et al., 2017; Puglisi et al., 2012). One of the research teams used stepwise regression analysis to create mixed models of *in vitro* diagnostic tests which in combination were highly correlated with field fertility (Gillan et al., 2008). Sellem et al. (2015) showed that CASA and FC may provide a reasonable prediction of bull fertility. Sperm subpopulations are considered by some investigators to be a better way of quantification of sperm quality than mean values from the whole population which oversimplify the analysis. Sperm subpopulations have been associated with fertility potential (Ibanescu et al., 2020; Maree and van der Horst, 2013).

1.11. Automation of physiological and behavioural traits in the cattle industry

With increasing pressure on making cattle industry sustainable and, at the same time, meeting increasing food demand, the management of farming must change. Precision agriculture, both in crop and livestock farming, uses advanced technologies to optimise production, emphasizing resource efficiency, sustainability, and profitability (Monteiro et al., 2021). Precision livestock farming allows farmers to make decisions based on automated measurements of different variables for each individual in real time. The methods used for data acquisition are sensor technology cameras, microphones, wireless communication tools, internet connections, and cloud storage (Monteiro et al., 2021). The new technologies in smart farming are used for monitoring a wide range of traits, health, welfare, and feeding automation across groups of livestock species. Many physiological and behavioural traits are monitored via automated herd control systems in dairy farms, including estrus detection, calving time, lameness, and rumen pH (Grodkowski et al., 2018; Kang et al., 2022). For body condition scoring of various species, 3D camera scanning has shown to be successful in conjunction with Convolutional Neural Networks (CNNs) or other machine learning algorithms (Kojima et al., 2022; Pallottino et al., 2015; Shigeta et al., 2018; Shuai et al., 2020; Tao et al., 2022; Yang et al., 2022). A widespread example of precision farming is the use of automatic milking systems

(Grodkowski et al., 2018; Monteiro et al., 2021), which have also enabled using depth images and point-cloud data for teat detection and initiation of automatic milking (Monteiro et al., 2021; Zheng et al., 2023). Computer vision methods based on deep learning are increasingly used in the field of reproduction (Butola et al., 2020; Creasy et al., 2021; Riordon et al., 2019). In the coming years, precision agriculture could revolutionise the animal breeding industry through multidisciplinary collaborations (Fu and Yuna, 2022; Monteiro et al., 2021; Neethirajan, 2022).

2. Aims of the thesis

The main aim of this PhD project was to identify novel early indicators of sperm production onset and maturity status of young Norwegian Red bulls during their performance test period. We hypothesised that early indicators could provide insight into the bull's future semen production performance, acceptance for the AI station, and future field fertility. We aimed to develop methods and use tests that could be easily adapted to existing routines at performance test and AI stations. To achieve these objectives, the following sub-goals were addressed:

- To evaluate the effect of sperm stress tests and cryopreservation on semen parameters from ejaculates of 10–13 months old Norwegian Red bulls and to examine the population morphometry of normal spermatozoa, perform semi-automated morphology assessment and measure SC of the young bulls (Paper 1).
- To investigate INSL3 as a potential biomarker of sperm production onset in Norwegian Red bulls (Paper 2).
- To develop a method for automated SC measurements using 3D images of the scrotum and convolutional neural networks (Paper 3).

3. Results: summary of individual papers

Paper 1: Novel interpretation of sperm stress test and morphology for maturity assessment of young Norwegian Red bulls

The age of dairy bulls in semen production significantly decreased due to the introduction of genomic selection as a selection tool. The research questions addressed by the present study included identifying novel early indicators of the young bulls´ performance at the performance test station that could provide insight into their future semen production, acceptance for the AI station, and fertility prediction. We followed 142 young Norwegian Red bulls enrolled at the performance test station. The semen production data, semen doses, and NR56 from the AI station were used. Flow cytometry and CASA were used to measure a range of sperm quality parameters from ejaculates collected from 65 bulls at age 9-13 months and further semen doses from the bulls accepted by the AI station. We subjected aliquots of fresh semen samples to stress test and cryopreservation to obtain four groups for comparison: Fresh (F), Stressed (S), Thawed Fresh (TF) and Thawed Stressed (TS). According to the study results, young Norwegian Red bulls could be separated into 3 clusters based on the different responses to sperm stress tests and subsequent cryopreservation. The population morphometry of normal spermatozoa showed homogenous sperm morphometry at 10 months of age. A semi-automated morphology assessment of young Norwegian Red bulls revealed that 42% of bulls rejected and 18% accepted for the AI station had ejaculates with abnormal morphology scores. Interestingly, the mean (SD) proportion of spermatozoa with normal morphology was 77.5% (10.6) for the youngest group at 10 months. Based on the novel interpretation of sperm stress test and cryopreservation, we demonstrated that Norwegian Red bulls reach maturity in sperm quality at various ages. An early indication of bull sperm production maturity can be achieved by incorporating the sperm stress test and cryopreservation combined with early sperm morphology analysis at the performance test station. Consequently, these bulls would be integrated more rapidly into routine semen collection schedules, resulting in their frozen semen becoming available for AI and increasing the population's genetic gain.

Paper 2: Associations between insulin-like factor 3, scrotal circumference and semen characteristics in young Norwegian Red bulls

When Norwegian Red bull calves are selected as potential AI bulls based on their genomic breeding value, they are kept in performance test stations from 3 to 12 months of age, allowing samples to be collected and analysed during their pre- and peripubertal periods. We hypothesised that there is potential for insulin-like factor 3, produced by Leydig cells in the testes, to serve as a biomarker of the onset of sperm production. This study investigated the level and variation of INSL3 in peripheral serum in young Norwegian Red bulls during pre- and peripubertal periods and evaluated the relationship between INSL3, SC, and semen characteristics. In addition, we explored the factors that might influence individual variability in INSL3 concentration in Norwegian Red bulls, such as the season and place of birth. The analysis of INSL3 was performed for 142 Norwegian Red bulls, for the first time in this population. Blood samples and SC were collected on the same day at four time-points: upon arrival at the performance test station (quarantine (Q): 2–5 months) and later at approximately 6, 9, and 12 months of age. The breeding company Geno provided data on date and place of birth as well as semen characteristics from the test station and the AI station. For age groups Q, 6, 9, and 12, the median SCs were 15, 21.5, 29, and 34 cm, respectively. A positive correlation was found between INSL3 and SC ($R = 0.4$) but not with any of the semen characteristics. There were no significant correlations between SC and sperm characteristics from data on ejaculates analysed at the performance test and AI stations. The findings of this study indicate that INSL3 displays substantial inter-individual variability in the Norwegian Red bull population. This variability cannot be attributed to the season or place of birth, but it may be related to the large effective population size of Norwegian Red cattle. We conclude that, due to high inter-individual variability, INSL3 cannot be used as a biomarker of sperm production onset in this breed at present.

Paper 3: Deep learning–based automated measurements of the scrotal circumference of Norwegian Red bulls from 3D images

The main goal was to create an innovative approach for automating SC measurements of Norwegian Red bulls using 3D images of the scrotum based on convolutional neural networks. The automated SC measurement solution could be implemented into feeding stations at the performance test stations and bovine semen collection centers. In this study, we measured manual SC and captured 3D images of bull calves recruited for performance test station at four different time points: upon arrival in Q and thereafter at approximately 6, 9, and 12 months of age. The results show that the SC measurements obtained by semantic segmentation are comparable to those obtained by manual measurement. The mean prediction error significantly differed between age groups Q, 6, 9, and 12, being -3.07 cm, -3.02 cm, -1.79 cm, and -1.11 cm, respectively. This study found that the image quality, bull age, and the intensity and quantity of natural and artificial light are important variables affecting the prediction accuracy of SC based on 3D images. When images were categorised into three quality groups, significantly lower mean prediction error (MPE) and mean squared prediction error (MSPE) were achieved for image quality 2 in age groups 6, 9, and 12. The values of the Dice coefficient for the training, validation, and test set were 99.8%, 99.5%, and 99.3%. To our knowledge, this is the first time a method for automation of SC measurement was developed. In combination with a user-friendly application, it allows a fast integration into the breeding soundness evaluation of Norwegian Red bulls at performance test and bovine semen collection centers. For the future, to obtain higher prediction accuracy, we recommend analysing individuals older than 6 months, paying attention to light conditions, and capturing 3D images of quality 2 only.

4. Discussion

4.1. Methodological considerations

4.1.1. Sampling and management at the performance test station

Norwegian Red bulls bought by the breeding company Geno were placed at the performance test station where they undergo training and selection to become breeding bulls. Here they learn the process of ejaculation into an artificial vagina, and their sperm quality is assessed (Olsen et al., 2020). Individual bulls have different libido levels, which directly affect their ability to become sexually aroused, and their sexual response time during semen collection (Schenk, 2018). It was shown that mounting procedure, collection frequency, sexual stimulation, and preparation (restraint and false mounts) influence sperm output (Brito, 2014). Those factors are even more critical in young inexperienced bulls who are learning the procedure. Our study population consisted of young Norwegian Red bulls from the performance test station housed in groups of 10. Since young bulls are inexperienced in semen collection routines and housed in groups, they could have been exposed to mounting behaviours and ejaculation outside the schedule of semen collection. We acknowledge that this could be the cause of lack of ejaculation at some collections, potentially adding bias to our study. Another limitation of our study was the number of ejaculates collected per bull. Semen collections at the performance test station were performed twice a week in a group of around 20 individuals. We had no way of knowing which bulls would successfully mount, ejaculate, nor which semen samples would pass the quality threshold of our study (volume > 2 ml, concentration > 200 x 10^6/ml, and motility > 60%). Thus, the only available information was which bulls were available on the collection dates. To reduce the risk of empty collections and lower quality samples we excluded the first collection of each group, thus avoiding bulls which had never provided a sample previously. From 142 bulls, we obtained 82 ejaculates from 65 individuals, which means that for most, we had only one ejaculate per bull. Once a bull had produced a satisfactory number of ejaculates (~3) with good quality, his semen is no longer collected at the performance test station and could no longer be used in the study. Due to the on-site management at the performance test station, as described above, we collected semen from as many bulls as was feasible with as many repeated measures as possible. The above-mentioned limitations are recognised and were considered in the statistical analysis. For the linear mixed-effects models, the random effect of the bull was used.

Scrotal circumference measurements and blood collection for analysis of the INSL3 were done at four time points: upon arrival at the performance test station Q: 2–5 months) and later at approximately 6, 9, and 12 months of age. The INSL3 levels increase during pubertal development with Leydig cell proliferation and differentiation as a result of increasing LH pulses (Anand-Ivell et al., 2019). The considerable age range in the quarantine group could influence our picture of the INSL3 patterns for some bulls which entered quarantine at 4-5 months. The age range in the quarantine group was determined by the transport schedule of bull calves bought by the breeding company. This limitation also applies to measurement of SC, which was done at the same time points. Automating the SC measurements using 3D images will allow for fast collection of multiple data points, thus generating individual SC growth curves for each bull at the station. To avoid introducing handler bias, SC and blood collection were performed by two qualified veterinarians, and 3D images were performed by the same person during the study period.

4.1.2. Sperm stress test, freezing and thawing

All the semen samples collected at the performance test station were treated to mimic the production process at the AI station. We used the same concentration of 12 x 10^6 sperm per straw, extender and freezing protocol, including the automatic freezer. There were the following differences between our protocols and those used at the AI station: the time from the first to the second dilution was longer, and the filling and sealing of straws were done manually. The performance test station is 75 km from the laboratory, where all analyses were performed, which was why the period between the first and second dilution varied between ejaculates. We could not access an automatic filling and sealing machine commonly used at the AI station. Manual filling and sealing could potentially cause straw variation (E. Kommisrud; personal communication). However, we ensured that the same two people performed the filling, sealing, freezing, and thawing of all samples. At the performance test station, a sperm stress test at 4 C° for up to 3 days is routinely done to evaluate the sperm quality of young bulls. We know that most of the AI stations analyse the post-thaw motility directly after thawing. However, in the production of sexed semen, standard procedure is to analyse motility after 3 h at 37 °C. DeJarnette and Marshall, (2005) showed that incubation of straws for 3 h at 37 °C magnified differences in sperm motility between tested groups of interest. Alm-Kristiansen et al. (2018) used 3h post-thaw incubation at 38 C° to measure the effect on sperm adenosine triphosphate (ATP) content, motility, and viability, between two preservation methods. In another study, 3, 24, and 48 hr incubation intervals in 38 C° were used to assess the differences between

Norwegian Red bull semen processed by SpermVital and conventional procedure with Biladyl extender (Alm-Kristiansen et al., 2018). In our study, we decided to use overnight storage at 4 °C, followed the next day by incubation for 3 h at 37 °C as a sperm stress test. We aimed to assess the individual variation in reaction to sperm stress test before and after cryopreservation.

4.1.3. *In vitro* sperm quality analysis

In our study, a range of sperm quality parameters was measured with FC and CASA motility and morphology modules. Ejaculates from 65 bulls at ages 9-13 months and semen doses from bulls accepted by the AI station and processed there were analysed. For this study, aliquots of fresh semen samples obtained at the performance test station were subjected to stress tests and cryopreservation to obtain four groups for comparison. All analyses were performed by the same person. Before performing morphology analysis on study group samples, we tested and adjusted Sperm Blue staining and mounting protocol to our laboratory conditions, as suggested by the manufacturer (https://www.micropticsl.com/wp-content/uploads/2013/09/spermblue_protocol.pdf). Next, we defined breed-specific cut-off values for normal sperm morphology based on 9 mature reference bulls from Geno's AI station (number of sperm analysed = 1182). Using the percentile method described by van der Horst et al. (2018), we determined cut-off values for normal and abnormal bull sperm. Based on SD and variance values of all morphometric parameters, we decided that 9 reference bulls would be representative of the mature Norwegian Red population. Cut-off values for normal Head area (μm^2) ranged from 35 μm^2 to 55 μm^2, and SD for Head area for both fresh and thawed samples was 3.76 μm^2 and 3.078 μm^2, respectively. To validate the settings, we asked a group of specialists to complete the online manual morphology survey of 50 sperm cells. The limitations of this approach could be too few cells in the survey and additional subjectivity of analysis from the images where a change of focal point is not possible, unlike when using a microscope. We compared the results with the classification from CASMA and adjusted cut-off values accordingly to provide the final setting used throughout the study. Additional quality control assessments of tail defects were performed on all files based on the standardisation threshold system for morphological abnormalities of bovids, published by (Perry, 2021).

Based on the work of Maree and van der Horst, (2013), we created breed-specific cut-off points for hyperactivated spermatozoa and sperm subpopulations. In our study, the progressive sperm motility was set to STR > 70, and the VAP points to 9. O'Meara et al. (2022) showed a significant effect of cut-off values change from low STR = 40 to high STR = 80 on the proportion of progressive motility. Too strict a cut-off point for STR in our study could be a

reason for the lower proportion of progressive motility compared to other studies. Because of the differences between CASA systems used in our study and by O'Meara et al. (2022) we cannot compare the VAP cut-off values used for progressive motility. Sperm motility, viability, and acrosome status analysis for all groups (F, S, TF, TS, TAI) were performed simultaneously on the same ejaculate subsample or three pooled straws. To control the instrument and our thawing procedure, semen doses from a reference bull were tested for viability and acrosome status on each thawing day. The negative control for flow cytometric analysis was an unstained sperm sample. Three technical replicates were used for both CASA and flow cytometry. As an additional quality control test, we compared viability and motility results and repeated the thawing of samples with significant discrepancies. Many authors address the importance of species and breed-specific settings (van der Horst et al., 2021, 2018; Yániz et al., 2015). A recent collaborative study focused on the standardisation of CASA settings for the assessment of bovine samples according to the extender type (O'Meara et al., 2022). In our study, we defined a range of breed-specific settings for both CASMA and CASA-Mot modules. Those settings are new and instrument-specific, limiting direct comparison with other studies (Mortimer et al., 2015). We diluted all semen samples with egg-yolk-based extender Biladyl (Minitube GmbH, Tiefenbach, Germany). The egg particles and debris were commonly present in all the samples analysed by CASA and flow cytometry and required extra steps to eliminate them for both techniques. In the case of CASA motility analysis, we adjusted the minimum particle size as suggested by Maree and van der Horst (2013). In addition, we subjected all motility files to quality control to correct egg yolk particles marked as spermatozoa and immotile sperm classified as particles. To differentiate between cells and debris or egg yolk particles in the flow cytometric analysis, SYTO60 was used to stain the DNA of both live and dead sperm cells. Berg et al. (2018) successfully used the same method on bull semen extended with Biladyl.

4.1.4. Experimental design for study 1

The aim of study 1 was to identify early indicators in young Norwegian Red 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. Semen aliquots for this research were collected during routine andrology testing at the performance test station from 10 to 13 months old Norwegian Red bulls. We could not influence the age of the first collection or know how long it would take from the first jump to the first successful ejaculation, which would pass our quality threshold. Previous study on 14 and 17 months old

Norwegian Red bulls showed that sperm quality parameters and the level of metabolites in their semen were significantly affected by even small differences in age (Narud et al., 2022). Therefore, we hypothesised that an age difference of 4 months during such a crucial period (10-13 months) would reveal individual differences in sperm quality and give a better overview of the general change in the population. Age at collection was included as a variable in our statistical analysis. The results showed the influence of age on several of the examined parameters. One of the aims of study 1 was to predict future fertility with parameters analysed at the performance test station. The Norwegian Red breed is known for its high field fertility. This proved to be a challenge in our study. Of 38 bulls accepted for the AI station, 25 had NR56 results with values ranging between 0.66 and 0.76. One of the reasons we only had NR56 from 25 bulls was that NR56 are not known for bulls whose semen is used only for export. The correlation analysis revealed no association between the parameters analysed at the performance test station and NR56 of AI bulls. The lack of association might be explained by too few ejaculates analysed per bull and possible intra-individual differences in ejaculate quality at a young age.

4.1.5. Experimental design for studies 2 and 3

Our experimental design required that data collection for studies 1, 2, and 3 happened simultaneously. We followed 142 Norwegian Red bulls during their performance test period until the selected ones were accepted by the AI station. With such a design, we were restricted to the bull's time at the performance test station, and we could always lose some individuals from the study due to different reasons, such as injury or unsuitable temperament. The PhD project duration is three years, and we knew that from the moment we started collecting data from a 3-month-old bull, we might receive his NR56 within 1.5 years. In study 2, we hypothesised that INSL3 could be a potential biomarker of sperm production onset in Norwegian Red bulls. One of the limitations of the study 2 design is the frequency of blood and SC collection. Anand-Ivell et al. (2019) collected monthly blood samples from 1 to 12 months to analyse INSL3 levels in young dairy bulls fed four different nutritional regimens. In another study that measured INSL3 concentrations in young Japanese Black beef bulls; blood samples were obtained monthly from the bulls from 4 to 24 months of age. As mentioned in section 4.1.1, we collected blood samples at four time-points, and Norwegian Red bull calves entered performance test stations at ages 2 to 5 months. That meant our first data points for blood and SC collection had an extended age range. In study 3, we aimed to automate the SC measurements using convolutional neural networks. Some of the decisions about methods used

in study 3 had to be made during the collection period. We had a vision and idea of tools needed for the best possible outcome to happen, but we acquired them along the way. We started with a semi-automated method, but the persistent idea of full automation and a user-friendly app led to multidisciplinary collaboration. During image collection, we observed that light conditions and age impact 3D SC image quality. We included those variables in the analysis and successfully established the best light conditions and image quality for future research. Looking back, we could have performed more repeated measures on each individual. The 3D SC images were captured at the same time points as we measured SC and collected blood samples for INSL3 analysis. We acknowledge that these 4 time-points might not give us an optimal age cut-off point for good prediction accuracy. To perform semantic segmentation of a scrotum, we decided to use CNNs, specifically the U–Net architecture. The Dice, IoU, and Tversky coefficients are the most commonly used compatibility measures representing coverage between ground truth and predicted segmentation masks (Küçükdemirci et al., 2022; Montazerolghaem et al., 2023). Our model's Dice, IoU, and Tversky coefficients for the training, validation, and test sets were above 99%. Such high performance of our model did not require us to use more advanced models, such as UNet++ or Res-UNet.

4.2. Prediction of bull sperm production onset and future performance at the AI station

Most published studies on different aspects of sperm quality, functionality, and its relation to fertility were performed on AI bulls older than a year (Gillan et al., 2008; Nagy et al., 2015; Narud et al., 2022). Many standards, such as the average SC cut-off point of 28 cm for puberty onset, were established when older bulls were used for semen collection (Wolf et al., 1965). Those standards should be adjusted according to the current changes in the management at AI stations. With the introduction of genomic selection, a significant shift in age at semen production stations was accomplished, with most of the currently producing bulls being below 15 months of age (Schenk, 2018). Breeding companies aim to decrease the age at semen collection, and some, like Viking Genetics, have successfully done so, as revealed in the study of Gebreyesus et al. (2021). The performance test station of Geno gave us an opportunity to study young Norwegian Red bulls from 3 to 13 months of age to derive early indicators of future semen production capacity and potential fertility for the effective selection of future breeding sires. Since AI is performed in the majority of dairy herds (Cojkic and Morrell, 2023), post-thaw sperm quality control is standard across AI stations. To be able to produce semen from the youngest possible sires, we need to know when bulls start producing semen of good

enough quality to pass post-thaw quality controls thresholds (Schenk, 2018). In study 2, we tested if INSL3 blood serum levels could be a potential biomarker of sperm production onset in Norwegian Red bulls. Our findings showed that INSL3 in the Norwegian Red bull population exhibits high inter-individual variability, which was not explained by season and location of birth. Since SC is an essential part of BBSE and larger SC was associated with an early onset of puberty (Rawlings et al., 2008), we included it in the screening of young bulls during their performance testing period. However, our results showed no relationship between INSL3 and SC and selected semen characteristics from performance test and AI station. This agrees with the results of Penitente-Filho et al. (2018), which showed that young Nellore bulls with different testicular sizes reach similar SC values in adult life. We believe that the automated measurements of SC with 3D imaging we propose in study 3 could produce an accurate growth curve of SC and allow for more accurate analysis.

In study 1, one of the key objectives was to investigate how a range of semen parameters from ejaculates of 10-13 month-old Norwegian Red bulls responded to the sperm stress test and cryopreservation. Our results revealed that a novel interpretation of the sperm stress test paired with cryopreservation could reveal the intra-individual age differences at which bulls reach maturity in sperm quality. To collect semen samples into an artificial vagina, young bulls need to go through training (Schenk, 2018). The time the bulls start training depends on the management at the performance test or AI stations. Study 1 revealed that some Norwegian Red bulls at 10 months had post-thaw sperm quality superior to the oldest bulls at 13 months (Article 1, Figure 4). We propose to start the collection training of bulls at the performance test station earlier to identify the early-maturing bulls and move them to the AI station. Another change in the management which allows for implementing our results in the performance test station of Geno has already been made. The quarantine period between stations was removed thanks to the new facility built for the quarantine of young calves arriving at the station. Consequently, individually selected bulls can be moved directly from the performance test station to the AI station enabling semen to be collected routinely, without an enforced break due to moving bulls from one premises to another. An extensive dataset gathered over a 4-year period was analysed to study the effect of bull age, ejaculate number, and season of collection on semen production data from Holstein Friesian bulls aged between 9 months and 8 years (Murphy et al., 2018). Their study (Murphy et al., 2018) demonstrates the difficulties in obtaining sufficient volume and quality of ejaculates from bulls less than one year of age. To our knowledge, only one other study group has published work on sperm quality and identification of early maturing dairy bulls (Deori et al., 2021; Hurri et al., 2022; Lima-Verde et al., 2022). Results of Hurri et al.

(2022a) and our study 1 showed a considerable variation in the age at which ejaculates exceed the breeding company's acceptance thresholds. Both studies showed an improvement in post-thaw sperm quality over time (Hurri et al., 2022). The above-mentioned similarities between the two studies strengthen the argument that young bulls should be selected individually when mature enough for their post-thaw semen quality. The main conclusion of study 1 is that a novel interpretation of the sperm stress test and cryopreservation combined with early sperm morphology analysis can elucidate the sperm quality status of young bulls. It thereby improves the selection criteria for AI at the youngest possible age. Another solution was proposed by Deori et al. (2021), who suggested that the proportion of heparin-binding proteins in seminal plasma could indicate maturity in young bulls.

Since our project was done in collaboration with the breeding company Geno, we aimed to choose tests and create methods that could be easily implemented into existing routines at the performance test station without much additional workload and costs. It has become a standard practice in cattle and pig AI stations to automate the evaluation of sperm motility using CASA systems (O'Meara et al., 2022; Waberski et al., 2022). Sperm morphology and morphometry analysis can be done using CASMA systems. As mentioned before, a version of the sperm stress test is already performed at the performance test station of Geno. Pairing it with cryopreservation, an established routine done by the company, could be easily applied. The automated 3D SC method could be incorporated into the feeding station, generating reports and SC growth curves. The only analyses requiring outsourcing or investment are viability and acrosome integrity performed with a flow cytometer. DeJarnette et al. (2022) suggested that flow cytometric assessments of novel sperm quality attributes are promising for the detection of subfertile sires in the near future. If those assays will prove to be useful for the selection of sperm maturity status of young bulls, such investment would be of interest to the breeding companies.

The cost of genomic technologies, including whole genome sequencing, has decreased and become more available across various species. Consequently, genetic improvement of important traits has shifted from conventional breeding to markers-assisted and genomic selection. Despite these advances, phenotyping has become a bottleneck that hinders genetic improvement. Phenomics is a field that aims to take advantage of new technologies to achieve cost-efficient, high-quality, and repeatable automated measurements of multiple traits, which could be used in breeding programs (Fu and Yuna, 2022); it is in this respect that scrotal imaging could play an important role in the future, as a safer and more accurate method of evaluating scrotal size than making physical measurements.

4.3. Tested variables at the performance test station in relation to field fertility

One of the aims of the thesis was to identify a relationship between measured parameters at the performance test station and non-return rates at 56 days for young Norwegian Red bulls accepted to the AI station. A previous study performed on young peri-pubertal Norwegian Red bulls at 14 and 17 months of age showed a significant increase with age in sperm hyperactivity and the kinematic parameters VCL and amplitude of lateral head displacement (ALH) in both fresh and frozen-thawed samples (Narud et al., 2022). Authors expected that older bulls at 17 months would have higher fertilising ability; however, the NR56 data did not reflect this hypothesis (Narud et al., 2022). This observation agrees with the results of our study 1, which fail to show the relationship between semen quality parameters and NR56. An increased number of cells in the AI dose can compensate for motility and some morphological abnormalities, which could be a reason for the lack of correlations with NR56 (Kastelic, 2013; Perry, 2021). Narud et al. (2022) showed a significant decrease in the number of discarded batches between 14 and 17 months of age. In a commercial setting, semen doses that do not pass quality control are not distributed into the field, masking the possible impact on NR56. Consequently, the capacity to correlate values of semen quality and *in vivo* fertility data could be reduced (DeJarnette et al., 2022). With decreasing age of bulls introduced into AI stations, the number of discarded doses will likely be higher than for older bulls. We hypothesise that in the future, parameters measured at the performance test station could be correlated with both NR56 and the proportion of discarded doses. Compared to older bulls, young bulls exhibit higher variation in sperm volume and concentration between ejaculates (Murphy et al., 2018). Additionally, as mentioned before, one of the limitations of study 1 was the number of ejaculates analysed per bull. Those could be reasons for the lack of correlations between the parameters we measured and field fertility. A high degree of precision must be applied when collecting fertility data in the field to establish a reliable relationship between *in vitro* tests and NR56 (Rodríguez-Martínez, 2003). In Norway, AI technicians are paid for the reported number of AIs performed, which increases the quality of NR56 ("Norwegian Dairy Herd Recording System | Norwegian Red," 2023). Using CASA and parameters related to DNA integrity, Gliozzi et al. (2017) developed a prediction model that explained nearly half of the variance in field fertility using only 18 bulls separated into high and low fertility groups. DeJarnette et al. (2022) discuss that it is more likely that correlations will be detected when a small sample size is combined with predetermined ranges in fertility variation. In our studies, we followed young Norwegian Red

bulls from calves at the performance test station to AI bulls. We did not know the number of bulls and the range of NR56 we would receive. The small range of NR56 (from 0.66 to 0.76) could be another reason we could not explain the variance in field fertility.

5. Conclusions

Our work demonstrated that young Norwegian Red bulls reach maturity in sperm quality at different ages, which will require early andrology testing and individualised selection at the performance test station. To improve the selection criteria for AI station at the youngest possible age, we propose to use the novel interpretation of the sperm stress test and cryopreservation combined with early sperm morphology analysis to elucidate the sperm quality status of young bulls. We conclude that using our novel automated scrotal circumference measurement method at the performance test station could be of interest to the breeding company.

Specific conclusions:

- Despite young Norwegian Red bulls having good initial fresh sperm quality, not all ejaculates had acceptable post-thaw sperm quality. The sperm stress test could aid identification of bulls with good freezing ability and would be a practical test for breeding companies to carry out in the laboratory. However, our study was based only on Norwegian Red bulls; similar studies are needed with other breeds to assess breed differences as well as individual differences.
- Already at 10 months of age some bulls exceeded the sperm quality thresholds for post-thaw samples, the proportion of spermatozoa with normal morphology and SC threshold of SFT, which shows that the introduction of younger individuals to semen production can be achieved with individualised selection using proposed methods.
- Although we found a moderate correlation between insulin-like factor 3 levels and scrotal circumference in young Norwegian Red bull calves, there was high individual variation, rendering insulin-like factor 3 problematic as a biomarker of sperm production onset, and thus spermatogenesis, in this breed at present. More work is needed to understand factors influencing the inter-individual variability in insulin-like factor 3 in young bulls of this breed.
- Using convolutional neural networks, we developed a novel automated method of SC measurements from 3D images. Our findings indicate that the bull's age and image quality play significant roles in determining how well SC can be predicted from 3D images. Dice, IoU, and Tversky coefficients used in our study were above 99% for all training, validation, and test sets, which indicates high performance of the U-net model. We recommend analysing individuals older than 6 months, paying attention to light conditions, and capturing only 3D images of quality 2.

6. Future perspectives

Based on the individual age differences between young Norwegian Red bulls in reaching semen maturity (Paper 1), future research should focus on determining the suitable time for the start of the semen collection training. The implementation of automated 3D SC measurements could be used for defining the SC cut-off point, which would maximise the number of young bulls ready for the first sperm collection. After defining the arbitrary cut-off point with SC, the sperm stress test and cryopreservation combined with early sperm morphology analysis could be applied to identify the suitable age when a significant number of young bulls pass the quality threshold. If this study is performed in close collaboration with the breeding companies, the intra-individual variability in semen concentration, volume, and sperm quality can be researched. Further, if the study continues for an extended period, more NR56 data can be collected, and models for predicting future semen production capacity at the AI station and field fertility could be developed.

The machine learning approach for predicting bull sperm quality could be used as in the study of Hürland et al. (2023). For the analysis of sperm morphology and morphometry, we used CASMA combined with a standardised threshold system for morphological abnormalities of bulls based on the article by Perry (2021). In the following study, deep learning-based image classification of bull sperm morphology could be developed and compared with existing results from paper 1. Such an automated method could allow for faster morphology evaluation of young Norwegian Red bulls at the performance test station.

The developed method of automated 3D SC measurements using convolutional neural networks should be validated. The results of study 3 conclude that if recommended image quality and light conditions are used during 3D image measurements, the mean prediction error and mean percentage prediction error will not increase. An additional test of more bulls is required to confirm our results and define accurate cut-off age for good prediction. Further development of the method is needed to introduce the automated method into the feeding station at the performance test station. The camera will need to be able to communicate with the feeding station to recognise the bull ID tag. Automated sorting of images of bad quality will have to be developed. The full automation of the SC measurements would allow for testing as a potential trait that could be included in the TMI for the selection of sires (Geno, 2023c).

The breeding program for Norwegian Red has through decades included traits of low heritability resulting in good fertility, something which the breed is now internationally recognised for. Our research was performed on Norwegian Red bulls, providing Geno with

potential points for improvements. Other breeds, now facing challenges concerning fertility, might have even bigger benefits from implementing methods suggested in our work. Thus, international cattle breeding could benefit from corresponding research performed on other breeds.

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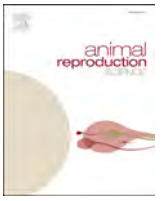

Novel interpretation of sperm stress test and morphology for maturity assessment of young Norwegian Red bulls

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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

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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 > 200×10^6/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 92×10^6/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 48×10^6/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.

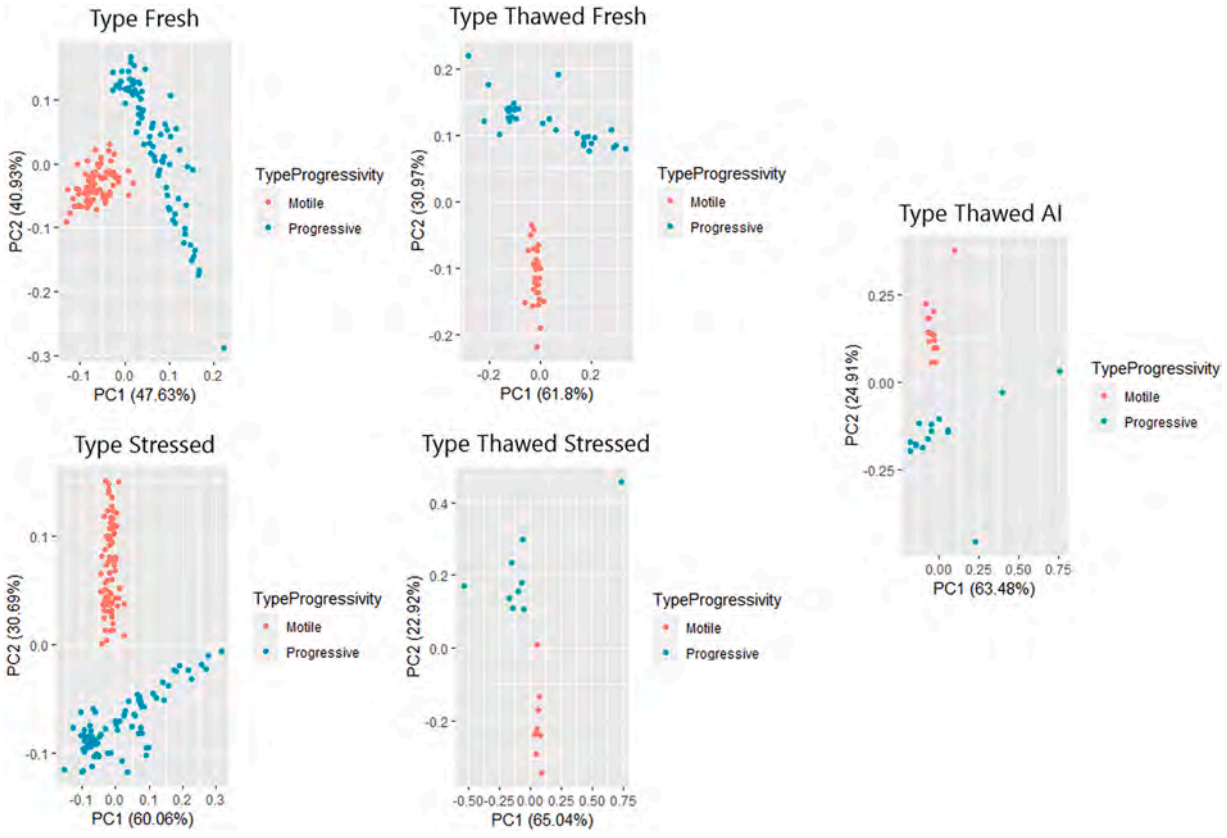

Fig. 1. Principal component analysis (PCA) in 2D. The first two Principal Components: Principal Component 1 and Principal Component 2 (PC1 and PC2) with the proportion of explained variance are presented. The plot of the kinematic parameters from rapid subpopulation coloured by Type Progressivity from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

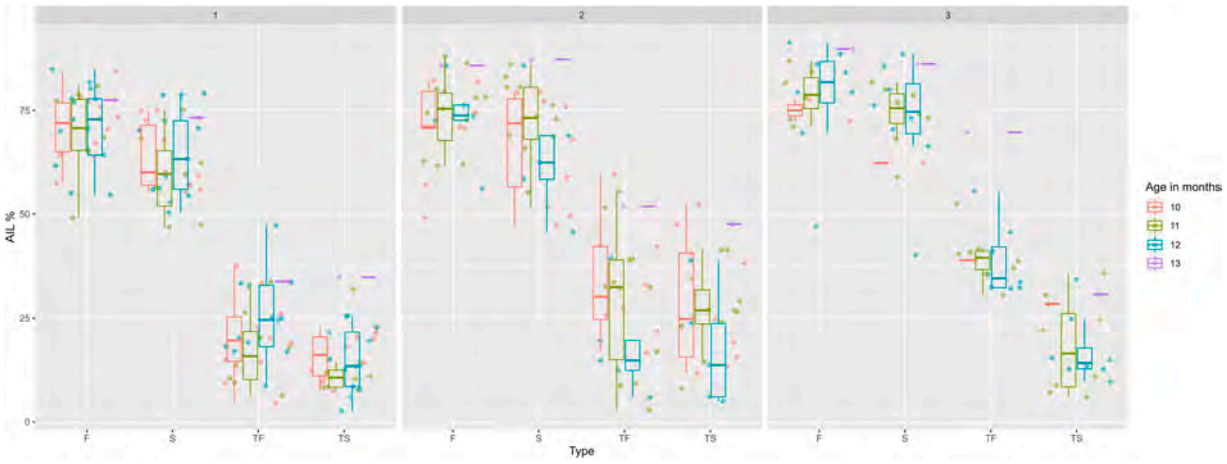

Fig. 2. Proportion of live spermatozoa with intact acrosome (AIL) presented as individual data points and population mean ± SEM for Types (Fresh - F, Stressed - S, Thaw Fresh - TF and Thaw Stressed – TS) for four age groups presented for three clusters.

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 μm^2 and 43.8 μm^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

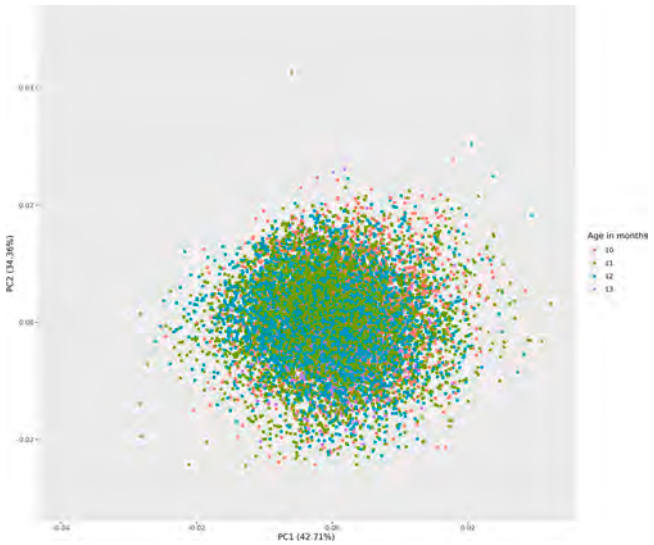

Fig. 3. Principal component analysis (PCA) in 2D. The first two Principal Components: Principal Component 1 and Principal Component 2 (PC1 and PC2) with the proportion of explained variance are presented. Head morphometry of the individual spermatozoa. The colour indicates the bull's age in months.

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

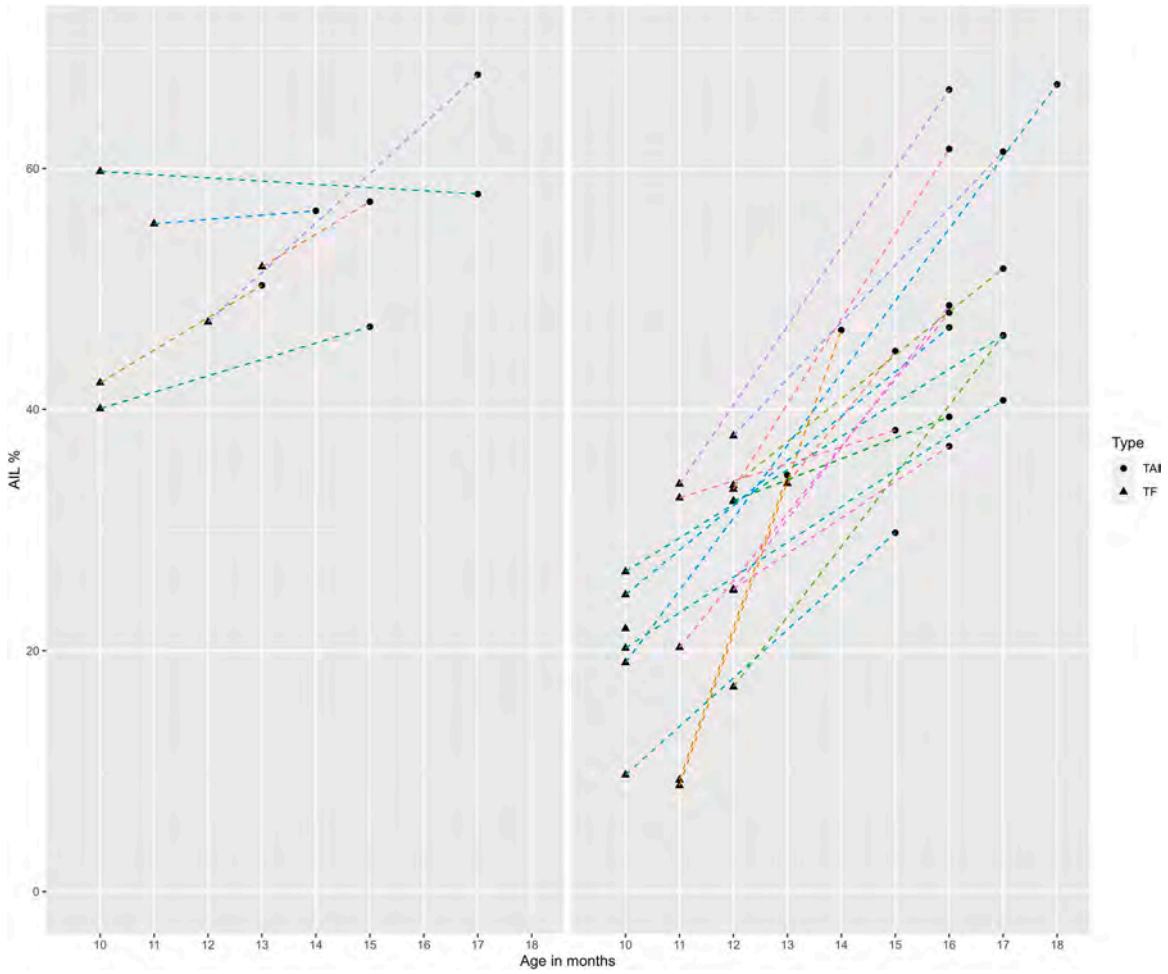

Fig. 4. Change in proportion of live spermatozoa with intact acrosome (AIL) for post-thaw fresh from the performance testing station (TF) and post-thaw from AI station (TAI) from 25 bulls with NR56 values. Data are presented as individual data points for each condition connected by a line to emphasise 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.

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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

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Associations between insulin-like factor 3, scrotal circumference and semen characteristics in young Norwegian Red bulls

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ABSTRACT

With the integration of genomic selection in the cattle artificial insemination **(AI)** industry, bulls are selected for their semen production capacity and fertility at a younger age than previously. Norwegian Red bull calves selected as candidates to become future AI bulls based on their genomic breeding value are kept in a performance testing station from around the age of 3–12 months, allowing for sample collection and analysis of different parameters during their pre- and peripubertal period. Insulin-like factor 3 **(INSL3)** is a small peptide hormone specifically secreted by the mature Leydig cells of the testes. In the foetus, it induces the first phase of testicular descent and is considered to reflect Leydig cell development during puberty; it could therefore be an interesting early indicator of future semen production capacity. The main objective of our study was to evaluate the relationship between INSL3, scrotal circumference **(SC)**, and semen characteristics. This is the first time INSL3 was measured in the Norwegian Red population. We collected blood samples for analysis of INSL3 from 142 Norwegian Red bulls at the performance testing station and measured their SC on the same day. Altogether, measurements were made at four time points: upon arrival at the performance testing station (quarantine **(Q)**: 2–5 months) and later at approximately 6, 9 and 12 months of age. Information on season and place of birth were made available from the database of the breeding company Geno, together with data on semen characteristics from the test station and the AI station. The median SCs for age groups Q, 6, 9, and 12 were 15, 21.5, 29, and 34 cm, respectively. INSL3 was shown to be positively correlated with SC (R = 0.4) but not with any of the semen characteristics. Similarly, we found no correlation between SC and sperm characteristics from data on ejaculates analysed at the performance testing station and AI station. The mean sperm volume for the 31 selected bulls with at least 10 ejaculates produced in the AI station increased from 2.3 ml at the performance testing station to 6.4 ml at the AI station. The corresponding increase in mean sperm concentration was from 497 million/ml to 1 049 million/ml. We conclude that INSL3 exhibits high inter-individual variability in the Norwegian Red bull population, which cannot be explained by the parameters measured in this study. At present, INSL3 cannot be used as a biomarker of sperm production in this breed.

Implications

Cattle breeders require an early indicator of future semen production capacity and potential fertility in bull calves for effective selection of future breeding sires. Insulin-like factor 3, produced by Leydig cells in the testes, could serve as a biomarker of sperm production onset. Although we found a moderate correlation between insulin-like factor 3 levels and scrotal circumference in young Norwegian Red bull calves, there was high individual variation, rendering insulin-like factor 3 problematic as a biomarker of sperm production onset, and thus spermatogenesis, in this breed at present. More work is needed to understand factors influencing the inter-individual variability in insulin-like factor 3 in young bulls of this breed.

Introduction

In the past decade, the introduction of genomic selection in the dairy breeding industry enabled a large number of candidate

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breeding bulls to be evaluated, increased the accuracy of predicted breeding values in young animals and shortened the generation intervals significantly (Taylor et al., 2018). As a consequence, bulls are introduced into semen production at a young age, and the breeding industry faces a challenge in predicting fertility and semen production capacity as early as possible. (Fair and Lonergan, 2018; Taylor et al., 2018; Brito et al., 2021). The shift caused interest in possible indicators that could be used to identify bulls which can produce semen samples at a young age. The reproductive potential of young bulls is limited by the amount of semen they can produce as well as its quality. Identification of breed-specific indicators that can predict a bull's future performance are needed (Byrne et al., 2018; Taylor et al., 2018; Brito et al., 2021).

Bull breeding soundness evaluation **(BBSE)** aims to identify sub-fertile bulls in herds under field conditions (Barth, 2018). There are several existing systems of BBSE around the world, and not all countries are bound to fulfil the requirements listed in these systems (Garcia-Paloma, 2015; Barth, 2018). This BBSE cannot predict fertility but is a simple and repeatable means of assessing breeding potential (Fordyce et al., 2006; Irons et al., 2007). Scrotal circumference **(SC),** sperm morphology and motility assessment are required measurements across all BBSE systems (Barth, 2018; Waite et al., 2019). Scrotal circumference is an important reproductive trait that is easily measureable, with moderate to high heritability (Ferreira et al., 2021). Bulls with larger SC were shown to reach puberty earlier than bulls with a smaller SC, and SC was positively correlated with the proportion of normal sperm, increased sperm output and fertility outcomes. The crucial time for rapid testicular growth in bulls happens during the peripubertal phase. It was shown that age, breed, nutrition and BW can influence SC (Bollwein et al., 2016; Penitente-Filho et al., 2018; Waite et al., 2019).

Under the influence of LH**,** the Leydig cells of the mammalian testis are responsible for the production of androgens, which are essential for the development of the male reproductive system and sperm production. Mature mammalian Leydig cells also specifically secrete a small peptide hormone, insulin-like factor 3 **(INSL3)** (Ivell et al., 2013). INSL3 can also cross the blood-testis barrier, thus being detectable in luminal fluid from seminiferous tubules, rete testis, epididymis and blood (Ivell et al., 2013). Previous findings showed that INSL3 mRNA is exclusively expressed in the testis, and INSL3 production depends on the number and developmental stage of the Leydig cells in the testis. LH influences the differentiation of Leydig cells, consequently influencing INSL3 production (Anand-Ivell et al., 2019). In female mammals, concentrations of INSL3 in peripheral blood are very low or undetectable; the exception is a female carrying a male foetus (Kibushi et al., 2016). Large amounts of INSL3 secreted by the foetal testes under the control of LH induce the first phase of testicular descent (Ivell and Anand-ivell, 2009). For several mammalian species, including bovine, INSL3 was shown to be an accurate measure of Leydig cell development during puberty (Johansen et al., 2014; Anand-Ivell et al., 2019). INSL3 was used as a candidate biomarker for the assessment of puberty in dairy bulls showing the effect of a high plane of nutrition in the first six months on spermatogenesis (Anand-Ivell et al., 2019). The same authors showed that INSL3 is negatively correlated with the timing of puberty and positively correlated with total testis weight at 18 months.

Our hypothesis was that INSL3 could be a potential biomarker of sperm production onset in Norwegian Red **(NR)** bulls. Our main aim was to investigate the relationship between INSL3, scrotal circumference and semen characteristics. We also explored potential factors influencing individual variability in INSL3 concentration in NR bulls during their peripubertal age, such as season and location of birth.

Material and methods

Animals

The breeding organisation, Geno, buys approximately 150 NR bull calves annually for their performance testing programme selected on their genomic breeding value. Six groups of bull calves, aged 2–5 months (median age 16 weeks), arrive at the testing station per year and are quarantined for two weeks upon arrival. During the testing period, bulls are housed in the same group of 10 individuals. Bulls are fed concentrate according to age and grass silage ad libitum. All bulls at the station are tested for temperament, conformation, and sperm quality. At around 12 months of age, they are approved or rejected for the artificial insemination **(AI)** station. Annually, 50–60 bulls are accepted to become AI bulls. This study was performed during a period from September 2020 until December 2021 and included 142 bulls enrolled in the performance testing programme during this period.

Scrotal circumference measurements and blood collection for measurement of insulin-like factor 3

The measurement of SC of NR bulls was done at four time points: upon arrival at the performance testing station (quarantine **(Q)**: age 2–5 months) and later at approximately 6, 9 and 12 months of age. During the procedure, qualified veterinarians used scrotal tape for SC measurement for all bulls while restrained in stocks. Blood samples from the jugular vein were collected into whole blood tubes by a qualified veterinarian on the same day as SC measurements were made. Blood samples were transported to the university laboratory and stored at 4 °C to clot for serum. The next day blood serum was separated and transferred into clean test tubes and aliquoted. The aliquots of serum – 2 × 250 µl per bull were stored in the freezer at −20 °C until analysis. One of these aliquots was sent to the University of Nottingham on dry ice and used for the analysis of INSL3. The number of animals sampled was 96 in quarantine, 111 at 6 months, 137 at 9 months and 123 at 12 months. Due to technical issues, the schedule of the project, and removal of animals from the unit, a few observations in each age group were lost. Overall, samples were collected from 142 NR bulls, with 82 bulls being sampled at all four time points; 34 were accepted to the AI station.

Insulin-like factor 3 assay

INSL3 peptide was measured using a bovine-specific time-resolved fluorescent immunoassay **(TRFIA)**, following the detailed procedure described in Anand-Ivell et al. (2019). Serum samples were diluted threefold in assay buffer, and the dilution factor was used in calculating the actual INSL3 serum concentration. The bovine INSL3 TRFIA assay range was 0.02–16 ng/ml. Each assay plate was run with the same standard range, as well as positive and negative controls. The intra- and inter-plate coefficients of variation were intra: <1% at all concentrations, and inter: 8.0, 3.3 and 4.4% at the lower, middle, and upper range limits, respectively. This assay recognises no other structurally related peptides (Anand-Ivell et al., 2011; 2019). The final INSL3 concentrations calculated for undiluted serum were used for all further data analysis.

Semen characteristics data

Geno made available data from the 142 young NR bulls included in this study. The semen characteristics data included the ejaculate volume (ml) and sperm concentration (million/ml) measured by a photometer (Bovine Photometer n°932, IMV technologies) of the

last ejaculate in sperm quality testing. Semen production data from the AI station included volume and concentration for the 10 first ejaculates from the 34 bulls that became AI bulls. The acceptance criteria for semen samples used for commercial use at the AI station are sperm concentration above 390 million/ml, prefreeze motility >70% and postthaw motility >50%.

Statistical analysis

All statistical analyses were performed with the use of R Studio version 1.4.0 (https://www.r-project.org/). Linear mixed-effects models were fitted and analysed with function lmer from R package lme4 (Bates et al., 2015). We collected information on the postcode of the farm where the bull calves were born, and the date of birth. Norway is divided into nine counties represented by the first number in the postcode. We extracted the county numbers from the postcode and created a 9-level factor variable representing the geographical birthplace of the calves. The number of bulls per county mirrors the distribution of the cattle industry in Norway, with a minimum of one bull calf from county 9 and a maximum of 46 bull calves from county 7. Date of birth was used to create the birth season in four classes as follows: season 1: December 2020 to March 2021 (n = 39); season 2: April to June 2021 (n = 21); season 3: July to September 2021 (n = 55); season 4: October to December 2021 (n = 27). To examine which fixed effects had a significant effect on the response variables, INSL3 and SC, we used the following mixed linear repeatability model with fixed effects of birth county (9 classes), birth season (four classes) and age at measurement (four groups: Q, 6, 9, and 12 months) and random effect of animal:

$$Y_{ijkl} = \text{county}_i + \text{season}_j + \text{age}_k + \text{bull}_l + \text{eijk}_l$$

where Y_{ijkl} is the observation of INSL3 or SC for bull *l* at the age *k* from county *i* born in season *j*.

Fixed effects of county and season were not significant for any of the variables and were therefore excluded from further analyses. To assess possible associations between the response variables and the semen characteristics, ejaculate volume and sperm concentration of last ejaculate were added to the model as a linear regression term (one at the time).

$$Y_{kl} = \text{age}_k + b * \text{semen Characteristics} + \text{bull}_l + e_{kl}$$

Here, b is the regression coefficient for the linear regression of volume or concentration on the response variable. For the 34 bulls accepted to the AI station, additional analyses were performed to examine possible associations to semen production at the AI station. Information on the ejaculate volume and sperm concentration from the first 10 ejaculates at the AI station were used, and the mean value was included as a linear regression term in a model similar to the one above.

Results

Distribution and variability of insulin-like factor 3 and scrotal circumference

The insulin-like factor 3 concentrations (ng/ml) showed large variation between bulls at all time points and exhibited an increasing trend over time (Fig. 1), but mean values for the age groups were not significantly different. The highest variation of INSL3 concentrations (SD = 2.37 ng/ml) was observed at 8–10 months of age. When comparing INSL3 from AI-accepted bulls vs all individuals, we observed larger variation among AI-accepted bulls in all age groups. The greatest difference between the variation of INSL3 of all individuals and AI-accepted bulls was in the 4–5 months group (SD = 2.34–3.13 ng/ml, respectively). The visual distribution of SC for all individuals is presented in Fig. 2. We observed a clear increasing trend in time which is also evident in the summary of the descriptive statistics in Table 1. Mean and median SC values in each age group were very close, a characteristic of symmetric distribution. The standard deviation around the mean was below 3 cm. We found no statistically significant difference in mean SC between bulls accepted to the AI station and rejected ones for any of the age classes. Table 2 provides an overview of sperm characteristic volume (ml) and sperm concentration (million/ml) of individual bulls from the performance testing station and the continuation of their production at the AI station. It also exhibits the proportion of accepted doses as well as prefreeze and postthaw motility of the ejaculates from the AI station. The mean volume for the population of selected 31 bulls with at least 10 ejaculates produced in AI station increased from 2.3 ml at the performance testing station to 6.4 ml at AI station. The corresponding increase in mean concentration was from 497.75 million/ml to 1 049.68 million/ml.

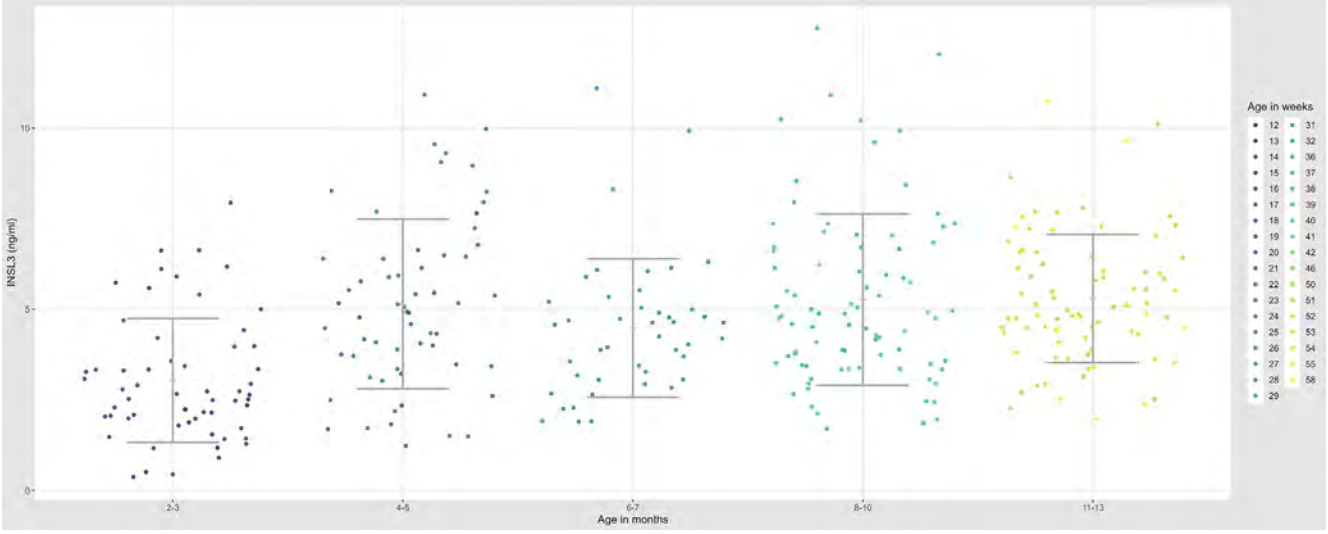

Fig. 1. Distribution of insulin-like factor 3 (INSL3) (ng/ml) concentration in peripheral serum among bulls during the pre- and peripubertal periods according to age in weeks. Data are presented as individual data points in ng/ml and means ± SEM. To clearly show the change over time, age in months for the quarantine group is separated into 2–3 and 4–5 months since it has the highest age range.

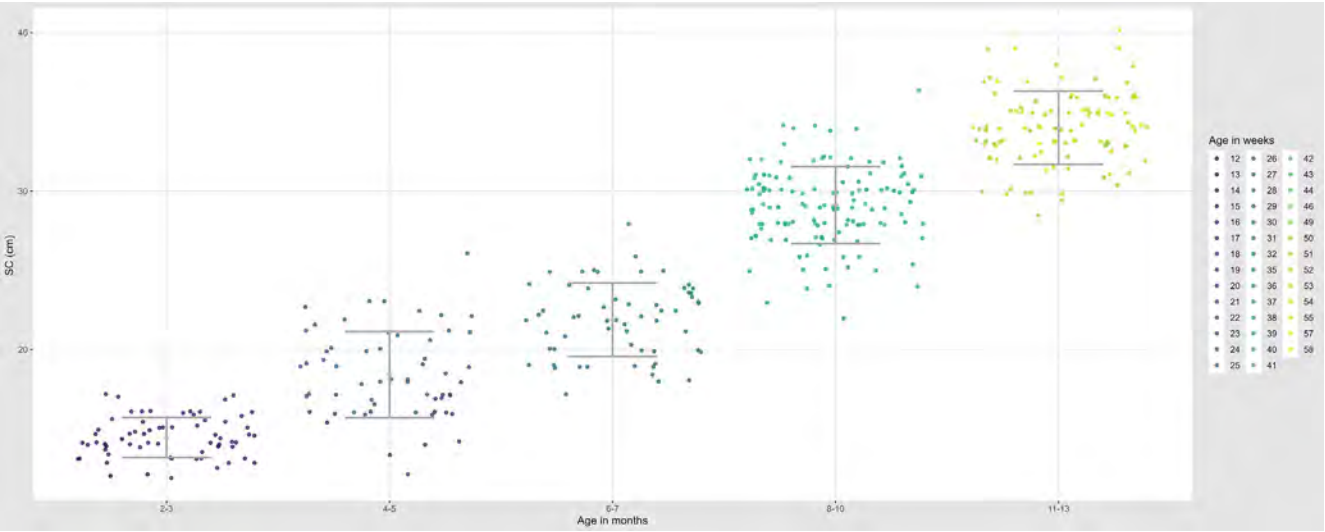

Fig. 2. Distribution of scrotal circumference (SC) (cm) in pre- and peripubertal bulls corresponding to age in weeks. Data are presented as individual data points in cm and means ± SEM. To clearly show the change over time, age in months for the quarantine group is separated into 2–3 and 4–5 months since it has the highest age range.

Table 1
Descriptive statistics for scrotal circumference (cm) of pre- and peripubertal Norwegian Red bulls for all age groups.

Age group	n	Mean ± SD	Median	Min	Max
Q[1]	94	15.0 ± 1.60	15	12	19
6	113	21.4 ± 2.51	21.5	16	28
9	137	29.1 ± 2.45	29	22	36.5
12	122	34.0 ± 2.24	34	28.5	40

Abbreviations: Q = Quarantine, n = Number of analysed bulls.
Samples were collected from 142 Norwegian Red bulls, with 82 bulls being sampled at all four time points; 34 were accepted to the artificial insemination station. Some bulls were only collected 1–3 times.
[1] Quarantine - age 2–5 months.

Relationship between scrotal circumference and insulin-like factor 3

Fig. 3 shows insulin-like factor 3 levels plotted against scrotal circumference measured on the same day. Pearson correlation analyses indicated a moderate positive correlation $R = 0.4$ between INSL3 (ng/ml) levels and SC (cm) with a 95 percent confidence interval of 0.31–0.47.

Mixed model – can we explain part of the variability in insulin-like factor 3 by chosen parameters?

The fixed effect of age at measurement had a significant effect ($P < 0.001$) for both INSL3 and SC. Estimated coefficients for the linear regression of sperm volume and concentration of the last ejaculate from the performance testing station on the response variables INSL3 and SC were not significantly different from 0 (P-value from 0.15 to 0.88). The same was true for mean sperm volume and concentration of the first 10 ejaculates from the AI station (P-value from 0.90 to 0.99).

Discussion

The objective of this study was to investigate the level and variation of insulin-like factor 3 concentration in peripheral serum during the pre- and peripubertal periods of young NR bulls. Our results demonstrated that the concentration of INSL3 (ng/ml) in NR bulls is characterised by high individual variability at all time points. Although these results differ from published studies on young bull calves and Japanese Black beef bull calves, where the population exhibits low variation between individuals (Sakase et al., 2018; Anand-Ivell et al., 2019), the trend of INLS3 levels over time shows a tendency to the same pattern. This resemblance suggests that factors influencing the INSL3 levels might be breed- and population-specific. Interestingly, a similar high inter-individual variability in INSL3 was reported in young men; Anand-Ivell et al. (2021) studied a Swedish cohort of 18-year-old men and showed an approximately 10-fold range of INSL3 values (0.74–8.2 ng/ml). In our NR young bulls at 9 months of age, we observed a similar range of values 1.70–17.49 ng/ml.

By using available information about the bull's birth month and geographical area of birth, we aimed to find if those parameters can explain the high inter-individual variability of INSL3 in our NR population. Aravindakshan et al. (2000) studied differences in the timing of reproductive maturation and developmental pattern of gonadotrophin secretion of Hereford × Charolais bull calves born in spring or autumn. Bull calves born in autumn had lower LH concentrations up to 18 weeks of age which might cause higher variation in the onset of puberty (Aravindakshan et al., 2000). A similar study conducted on Bos indicus Brahman bull calves concluded that bull calves born in spring reached sexual maturity earlier than those born in autumn (Tatman et al., 2004). Since INSL3 is LH-dependent, we hypothesised that we would observe the differences in INSL3 concentration for different birth seasons. Kenny and Byrne (2018) stressed the importance of early life management since the plane of nutrition before 6 months of age determines the age at puberty. The season of birth and geographical area could indirectly affect the plane of nutrition before the introduction to the performance testing station and consequently affect the onset of puberty. Contrary to expectations, our study

Table 2
Descriptive statistics of semen characteristics of the last ejaculate from the performance testing station, the first 10 ejaculates from the artificial insemination (AI) station, and the % of accepted doses from the 31 bulls selected to be AI bulls with at least 10 ejaculates produced.

	Performance testing station – last ejaculate		Artificial insemination station – 10 first ejaculates				
Bull	Volume of last ejaculate (ml)	Concentration of last ejaculate (million/ml)	Volume (ml) mean ± SD	Concentration (million/ml) mean ± SD	% of accepted doses	Prefreeze motility mean ± SD (%)	Postthaw motility (%) mean ± SD
1	2	140	7.73 ± 3.06	578.66 ± 161.37	50	76.66 ± 5.77	28.33 ± 2.89
2	1	920	4.84 ± 1.38	796.6 ± 272.59	50	79 ± 4.18	57 ± 2.74
3	2.5	930	4.35 ± 1.81	1 398.75 ± 481.16	60	78.75 ± 2.5	57.5 ± 5
4	3	850	4.96 ± 0.75	940.83 ± 289.23	60	77.5 ± 4.18	55 ± 7.07
5	3.5	210	5.38 ± 1.25	1 298.6 ± 471.21	60	80 ± 5	51 ± 12.45
6	1	390	4.66 ± 2.16	720.6 ± 172.44	70	79 ± 2.23	50 ± 10.61
7	3.5	770	8.2 ± 1.13	1 078.5 ± 75.66	70	80 ± 0	57.5 ± 3.54
8	3.5	460	4.62 ± 2.29	748 ± 318.25	70	78.57 ± 2.43	56.42 ± 9.88
9	3.5	780	4.75 ± 1.72	1 067.14 ± 414.61	80	75 ± 4.08	51.42 ± 8.02
10	1.5	1 150	4.9 ± 1.96	1 388.6 ± 438.70	80	77 ± 2.73	41 ± 8.94
11	2	400	5.81 ± 2.43	894.87 ± 298.78	80	76.25 ± 4.43	43.75 ± 14.82
12	3	450	11.82 ± 1.22	736 ± 135.88	80	79.28 ± 1.89	44.28 ± 9.32
13	2.5	380	6.27 ± 1.73	1 196.28 ± 348.11	90	78.57 ± 2.43	57.14 ± 5.67
14	3	100	7.4 ± 2.01	1 005 ± 386.46	90	78.75 ± 4.43	58.75 ± 5.18
15	2	640	8.57 ± 2.13	1 025.88 ± 304.55	90	80 ± 4.33	52.77 ± 6.67
16	1.5	500	8.23 ± 2.72	1 423.11 ± 365.40	90	80 ± 3.54	50 ± 5.59
17	4.5	410	5.95 ± 1.30	816.33 ± 423.18	100	79.16 ± 2.04	49.16 ± 12.81
18	2	450	6.77 ± 1.53	1 147.5 ± 360.07	100	78.75 ± 6.29	60 ± 7.07
19	2	360	5.6 ± 1.94	828.44 ± 321.29	100	77.77 ± 2.63	51.11 ± 5.46
20	2	720	4.87 ± 1.90	1 291.37 ± 365.92	100	78.75 ± 3.53	56.25 ± 7.91
21	3.5	510	5 ± 1.66	1 079.22 ± 195.01	100	78.88 ± 2.20	57.77 ± 9.72
22	2.5	400	6.55 ± 1.14	875.8 ± 281.54	100	77 ± 4.21	55.5 ± 11.17
23	1.5	680	4.36 ± 1.30	1 382.3 ± 367.01	100	79 ± 3.94	54 ± 9.07
24	1	800	5.41 ± 1.42	1 077.8 ± 199.05	100	79.5 ± 3.69	60.5 ± 7.25
25	2	680	8.9 ± 2.15	1 389 ± 408.44	100	74 ± 2.24	49 ± 7.42
26	3	770	6.18 ± 2.09	1 332.75 ± 236.38	100	76.25 ± 3.54	40.62 ± 10.50
27	5	440	7.55 ± 1.43	1 137.8 ± 294.38	100	77.5 ± 4.25	55 ± 6.67
28	3.5	960	9.74 ± 2.65	862.4 ± 183.86	100	79.5 ± 4.38	59 ± 9.66
29	0.5	500	7.51 ± 1.76	1 321.4 ± 335.10	100	77 ± 4.83	54.5 ± 7.62
30	3.5	170	8.8 ± 1.10	1 232.44 ± 263.93	100	80 ± 2.5	54.44 ± 5.83
31	4	700	10.28 ± 2.63	1 372.5 ± 458.31	100	78 ± 2.58	49.5 ± 10.39

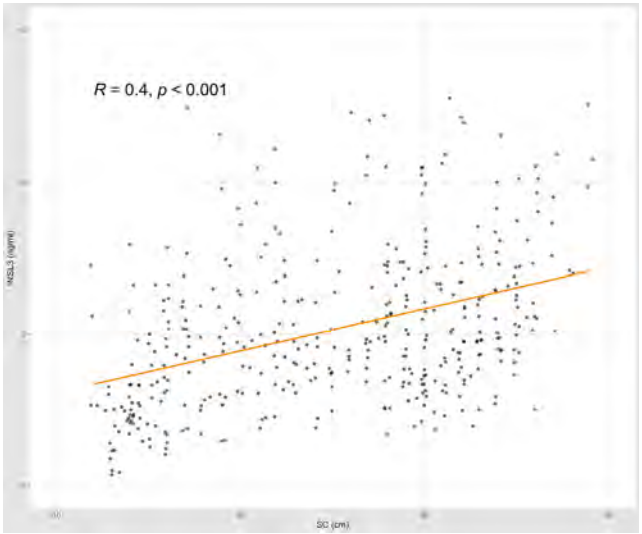

Fig. 3. Relationship between the concentration of insulin-like factor 3 (INSL3) (ng/ml) and scrotal circumference (SC) (cm) measured on the same day in pre- and peripubertal bulls. Regression line shows a moderate positive relationship between INSL3 (ng/ml) and SC (cm) R = 0.4.

did not find any significant effect of birth season or geographical area on INSL3 levels. The other research question was to measure the association between INSL3 and SC for our NR population. Previous studies have demonstrated that the SC of young HF and Japanese Black beef bull calves positively correlated with INSL3 (ng/ml) (Sakase et al., 2018; Anand-Ivell et al., 2019). In a study on young HF bull calves, the authors showed a weak positive correlation R = 0.31 between INSL3 levels at 8 months and SC at 12 months (Anand-Ivell et al., 2019). For Japanese Black beef bull calves, 1–7 months old, a correlation of R = 0.64 was found between SC and INLS3 levels measured on the same days. Our study found a moderate positive correlation R = 0.4 between INSL3 (ng/ml) levels and SC (cm) from NR bull calves from 2 to 12 months of age measured at 4-time points. However, these comparisons should be viewed with caution because of the differences in age and frequency of measurement and the predictive vs descriptive approach. This study aimed to evaluate the association between selected semen characteristics from performance testing and AI station and INSL3 or SC. We found no significant association between these parameters. It is established that young bulls have lower sperm quality and higher variability in volume and concentration between ejaculates (Murphy et al., 2018). This was the reason we also included 10 first ejaculates from the AI station in the analysis. Weerakoon et al. (2018) showed that prepubertal Japanese Black beef bulls with lower sperm motility and a high proportion of morphologically abnormal spermatozoa had decreased INSL3 concentration compared to bulls with normal semen parameters. However, they set up the specific cut-off point for the semen quality parameters and compared INSL3 between two groups, normal and abnormal, which excludes direct comparison. As Anand-Ivell et al. (2011) described, INSL3 is a foetal gender-specific hormone, with concentrations significantly increasing during mid-gestation in cows carrying a male foetus. Anand-Ivell et al. (2011) showed no breed-specific differences in maternal peripheral INSL3 levels for Angus and Brahman cows. However, foetal INSL3 levels ranged between 1 and 5 ng/ml and differed significantly between pure breed and crossbreed foetuses. The impli-

cation is that paternal genetics plays a role in determining INSL3 levels (Anand-Ivell et al., 2011). Moreover, this might be important for further research to explain the high inter-individual variability in the NR population. Research questions that could be studied include the measurement of bovine foetal INSL3 from mid-gestation until the peripubertal period ending at around 12 months to explore the source of variation. Our research showed that the NR breed exhibits a heterogeneous distribution of INSL3 in all age groups. Since the 1970s, Geno introduced broader breeding goals focused on health and fertility ('Norwegian Red breeding program', 2020). In contrast, Holstein Friesian cattle were bred predominantly for milk yield for several decades. Effective population size (a measure of the number of unrelated individuals in a population) for the NR population in the last 5 years was: 239 in 2017, 242 in 2018, 246 in 2019, 250 in 2020 and 253 in 2021. In comparison, the effective population size of Holstein cattle calculated based on the pedigree record from the Canadian Dairy Network was equal to 58 (Makanjuola et al., 2020). These differences between breeds might affect the variability in certain traits, such as INSL3. Several questions remain unanswered, and we propose including genetic parameters in further studies to confirm the paternal effect suggested by Anand-Ivell et al. (2011).

Conclusion

This research extends our knowledge about INSL3 levels in pre- and peripubertal bulls. The results of this investigation show that INSL3 in the Norwegian Red bull population exhibits high inter-individual variability, which cannot be explained by the season and location of birth and might be connected to the large effective population size of the NR cattle population. It was also shown that INSL3 is positively correlated with scrotal circumference. We conclude that high inter-individual variability in INSL3 prevents us from using INSL3 as a biomarker of sperm production onset in this breed at present. Further experimental investigations are needed to identify the source of variation.

Ethical approval

Ethical approval was not required in this study. We worked at the performance testing station under the supervision of a qualified veterinarian employed at the breeding company Geno. The performance testing station fulfils EU requirements for the housing of bulls.

Data and model availability statement

The data/models were not deposited in an official repository. The data/models that support the study findings are available from the authors upon request.

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Declaration of interest

The authors declare no conflict of interest.

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Deep learning–based automated measurements of the scrotal circumference of Norwegian Red bulls from 3D images

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ABSTRACT

The main aim of this study was to create an automated method for the measurement of the scrotal circumference (SC) of Norwegian Red bulls using 3D images of the scrotum based on convolutional neural networks. The study population was bull calves recruited for performance testing before the selection of bulls for semen production in the breeding program. Bulls were measured at four different time points: upon arrival in quarantine (Q) and thereafter at approximately 6, 9 and 12 months of age. Both 3D images and manual SC measurements were performed at all time points. In our approach, SC could be calculated without direct contact with the bull, using only 3D images and a simple, user–friendly application into which mentioned images are uploaded. The results show that SC measurements obtained using semantic segmentation are comparable with manual measurements. The mean prediction error was significantly different between age groups Q, 6, 9 and 12, and it was -3.07 cm, -3.02 cm, -1.79 cm and -1.11 cm, respectively. The results show a significant difference in the measurement error of the SC based on the quality of the images. Images were categorised into three quality groups. For good prediction accuracy, we recommend capturing 3D images of quality 2 – full circle from individuals older than 6 months.

1. Introduction

In dairy farms, automated herd control systems are used for multiple physiological and behavioural traits measurements such as estrus detection, calving time, lameness or pH of the rumen [1,2]. Scrotal circumference (SC) is an essential part of the breeding soundness evaluation of bulls due to its high repeatability and moderate to high heritability (from 0.36 to 0.69) [3]. Automation of SC measurement and implementation into feeding stations would be a valuable tool for performance testing stations and bovine semen collection centre. SC of bulls follows the sigmoidal growth pattern starting with an increase before six months of age and rapid growth during the peripubertal phase. It is broadly agreed that larger SC is associated with early puberty onset, increased sperm output and better fertility outcomes [3–7]. SC is influenced by age, body weight, nutrition (especially before 6 months of age) and breed with individual differences [5–7]. Traditional measurements of scrotum circumference are based on manual handling, using a measuring scrotal tape. This activity is associated with HSE (Health, Safety and Environment) issues for the technician and a stressful situation for the animal. Using 3D cameras combined with automatized image analysis might possibly constitute a good alternative.

Artificial Neural Networks (ANNs) are a type of mathematical model inspired by biological neural networks designed to mimic learning processes in the human brain. An ANN architecture is based on neurons (also called perceptrons) grouped in layers connected with each other using weights. The information is transferred from the input layer onto the output layer. ANN weights are fitted in a backpropagation process using a stochastic gradient descent algorithm to minimise a loss function, which corresponds to "learning" how to perform a specific task (regression, classification) by an ANN. Convolutional Neural Networks (CNNs) are a subclass of ANNs designed to perform complicated tasks on visual imagery (e.g. images, videos, spectrograms, holograms). CNNs are widely used for image classification, image segmentation, object detection, optical character recognition, etc. In contrast to classical ANNs, CNNs learn local filters that can be applied to the image data to extract interesting features. In CNNs those filters are calculated automatically in the backpropagation process, similar to those in ANNs [8–10].

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3D camera scanning combined with Convolutional Neural Networks (CNNs) or other machine learning algorithms was already studied with success in the field of animal production for body condition scoring of dairy and beef cattle [11–13], pigs [14] and horses [15]. Recently the 3D imaging combined with CNNs was used for supernumerary teat classification of Norwegian Red cattle udder [16]. Yang *et al.* [17] created a portable non–contact 3D measurement system for dairy cow body using smartphones. The point cloud hole completion method they used worked regardless of the posture of the animal, with relative errors for different traits from 2 to 6%. They showed that a low–cost method can be introduced for accurate non–contact measurement of livestock.

Deep learning methods such as CNNs were used in the field of reproduction to classify the human spermatozoa into WHO [18] shape–based morphology categories [19]. Butola *et al.* [20] combined a partially spatially coherent digital holographic microscope (PSC–DHM) for quantitative phase imaging (QPI) with deep neural networks (DNN) to differentiate with high accuracy normal human spermatozoa from abnormal. Another research used U–net image segmentation to automate the identification of the different stages of spermatogenesis in rats applied on stained testis tissue [21]. As seen in the presented examples, computer vision methods based on deep learning are increasingly used in fields of reproduction and other branches of biology, genetics, medicine and agriculture.

Our research's main aim was to explore the potential of using convolutional neural networks for automated SC measurements. Which would be of great interest to breeding companies and increase the accuracy of the SC measurements during breeding soundness evaluation. Our second objective was to investigate differences in prediction accuracy between age groups and image quality. Further, our practical goal was to create a framework of what is a good quality image to be directly uploaded into our user–friendly app and provide a quick and easy way of SC measurement in bulls without direct contact with the animals.

2. Materials and methods

2.1. Animals

Geno (*Geno*), the breeding organisation for Norwegian Red (NR), each year buys approximately 150 NR bull calves for their performance testing program [22]. Individuals aged 3-5 months arrive in 5-6 groups per year at the testing station and are quarantined for two weeks upon arrival. After isolation, they are housed in groups of 10 and consequently kept in the same group for the whole duration of the performance testing period. Bulls are subject to temperament, conformation, and andrology testing at the station. Around 12 months of age, they are approved or rejected to the bovine semen collection centre. Bulls are fed concentrate according to the age and grass silage ad libitum. This study was performed during a period for 1.5 years and included bulls enrolled in the performance testing program during this period.

2.2. Manual Scrotal Circumference measurements

The SC of NR bulls were measured manually at four–time points: upon arrival at the performance testing station (3-5 months) and later at approximately 6, 9 and 12 months of age. The bulls were restrained during the procedure. Three qualified veterinarians under the supervision of centre veterinarian measured the SC manually by scrotal tape for all bulls and time points.

2.3. 3D Scrotal Circumference measurements

After manual SC measurements, each bull's scrotum was photographed using a handheld device consisting of an Intel Real Sense d415 camera connected with a tablet by a stick [23]. The camera was carefully placed on the floor between the bull's legs. Qualified personnel assisted the procedure by keeping the tail of the restricted animal. One image per bull was captured at each time point. In total, four images per bull were taken. For five individuals, multiple images on the same day were captured and used for quality control of the method. Depth images were saved in the OneDrive cloud and used for further analysis. The number of animals per age group per measurement is shown in Table 1. Due to technical issues, the schedule of the project, and losses in animals, a few observations in each age group were lost. The proportion of the training, validation and test data were 60%, 20%, and 20%, respectively.

Table 1
Number of animals per age group (Q[1],6,9,12 months) per method of measurement used in our study.

Age group	n SC [2]	n 3D [3]
Q	96	76
6	111	99
9	137	131
12	123	116

[1] Quarantine - age 3-5 months
[2] Number of bulls from which scrotum circumference (SC) was manually measured
[3] Number of bulls from which 3D pictures of scrotum were collected

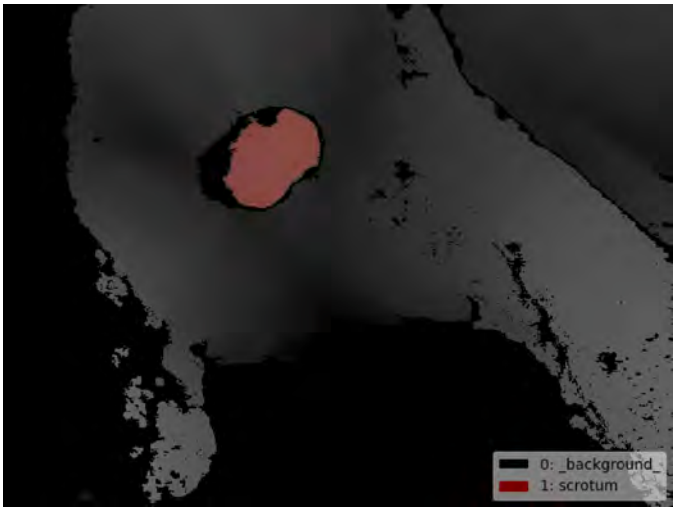

Fig. 1. Image segmentation visualisation. An example of a true scrotum segmentation mask (1) overlapped with the original 3D image of bull.

2.4. Artificial Neural Network (ANN)

In our approach, we decided to use CNNs, specifically the U–Net architecture; it is already successfully used in biomedicine biology and genetics for image semantic segmentation [24–26]. In an image classification task, we are interested in predicting the correct label for the whole image (e.g. cat or dog). In the semantic segmentation task, this approach was extended for each pixel of the image. This created a ground truth segmentation mask, showing the localisation of pixels (objects) belonging to the same class. This predicted segmentation mask was compared with the ground truth segmentation mask to check if a prediction was correct or not (Fig. 1). This task's most common compatibility measures are the Dice coefficient, the IoU coefficient and the Tversky coefficient which represents coverage between ground truth and predicted segmentation masks. In our approach, we created ground truth segmentation masks of the bull scrotum using the 'labelme' tool [27]. For each image the scrotum boundary was outlined using 30 to 40 unique points. Pixels inside the boundary were marked as 'scrotum' and outside the boundary as 'background'. Those segmentation masks were used to train, validate and test the U–Net model.

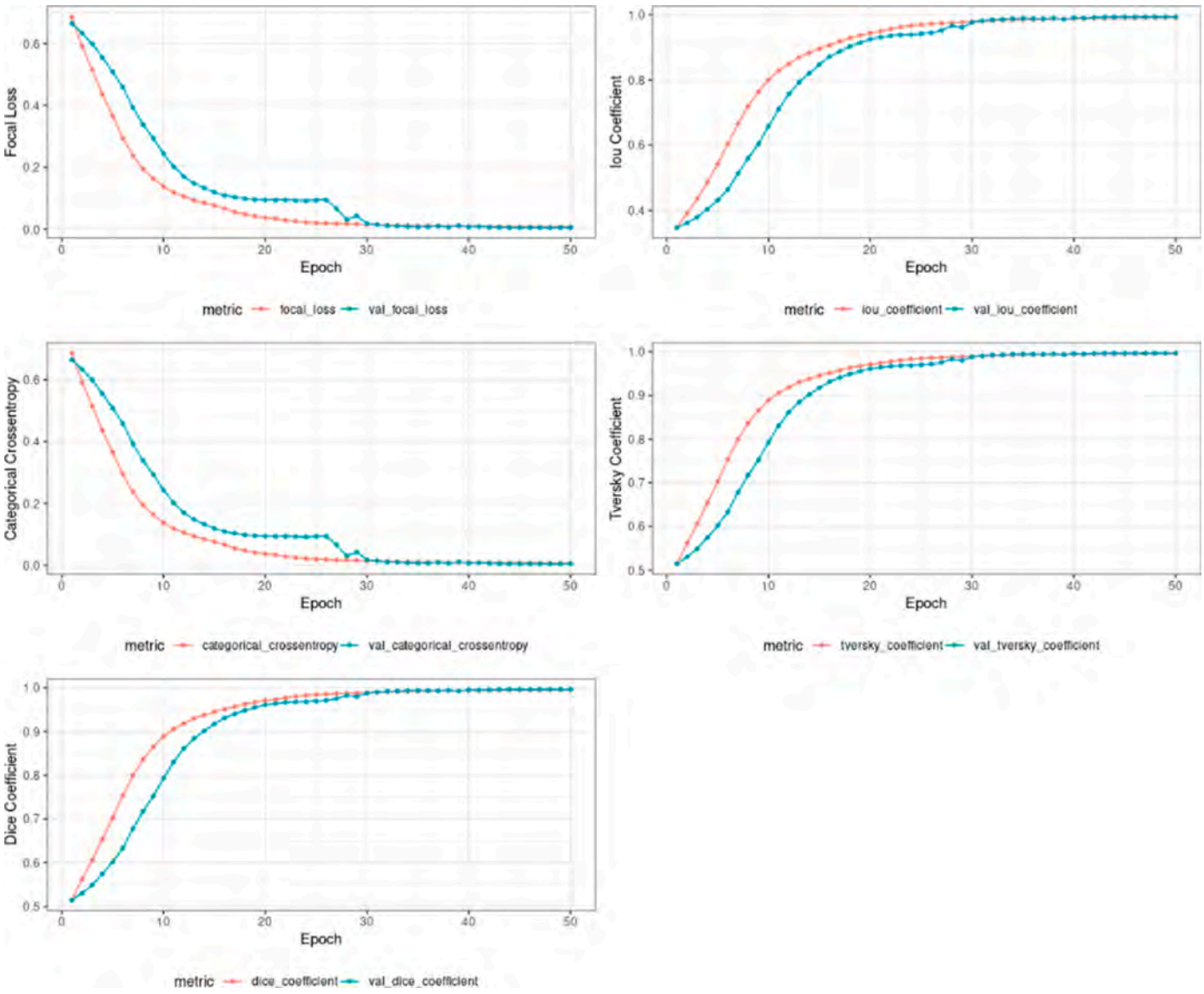

Fig. 2. Learning curves for Focal loss, IoU coefficient, Categorical Crossentropy, Tversky coefficient and Dice coefficient, respectively for training (red) and validation (blue) sets.

2.5. Model architecture

In the U–Net model used in our study for scrotum segmentation, the input image was a tensor of shape 256 × 512 × 1, rescaled from the original image 640 × 480 × 1. Our model was composed of 5 double–convolutional downscaling blocks – each block included two iterations of 2D convolution with the same padding (the activation map had the same shape as input) and batch normalisation layers with ReLU activation followed by 2D max–pooling and dropout layers – and 5 deconvolutional upscaling blocks – each block included 2D deconvolution and concatenation layers followed by two iterations of 2D convolution with same padding (the activation map had the same shape as input) and batch normalisation layers with ReLU activation. Downscaling and upscaling blocks were connected with a bridge composed of two iterations of 2D convolution with same padding (the activation map had the same shape as input) and batch normalisation layers with ReLU activation. The model was fitted using Adam optimizer with a Focal loss function (Fig. 2). The model was build, trained and validated using 'pyplatypus' software (https://github.com/maju116/pyplatypus).

Fig. 3. Multiple objects as a result of segmentation. Example of predicted scrotum segmentation mask containing 2 objects (scrotum and incorrect artefact).

2.6. Connected–component labeling (CCL) algorithm

For most of the images, the predicted segmentation mask contained one solid object, which was expected and desirable. Some artefacts that created a second smaller object for the remaining images were found in the predicted segmentation mask (Fig. 3). To solve this problem, we used a connected–component labeling (CCL) algorithm (also known as blob extraction or region labeling) to count the number of solid objects in a predicted segmentation mask [28]. In the case of finding more than

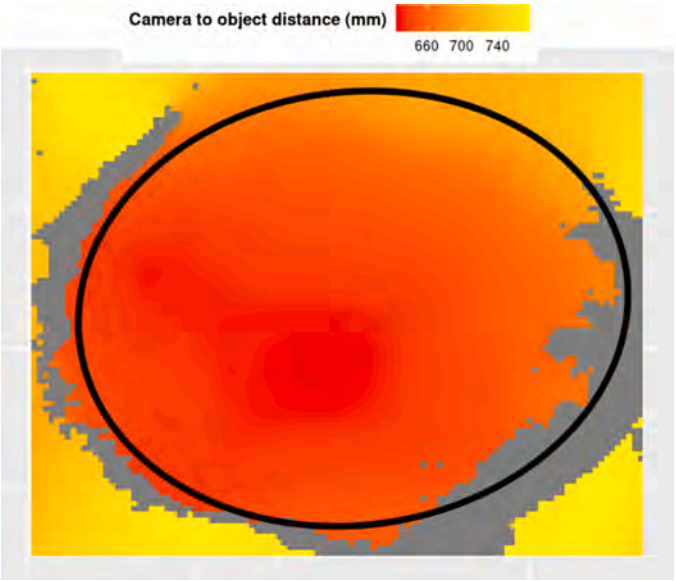

Fig. 4. Direct Linear Least Squares fitting of an ellipse. An example of an ellipse fitted onto the predicted scrotum segmentation mask using the Direct Linear Least Squares method is plotted onto the original depth image. The colour scale represents the camera to object distance in mm. The grey represents NA.

one object, only the object with the highest area would stay on the segmentation mask.

2.7. Direct Linear Least Squares fitting of an ellipse

After segmentation and cleaning faze, the Direct Linear Least Squares algorithm was used to fit an ellipse onto the boundary of a segmented mask (Fig. 4) [29,30].

From this fit, semi–major and semi–minor axes of the ellipse were calculated using angles per pixel from the median camera to object distance. The angles used in the angles per pixel calculation 66.5° x 40.5° were selected from the metafile. The ellipse perimeter was calculated using the Pade approximation [31].

2.8. Validation of capturing the 3D SC images and statistical analysis

To validate the method, multiple images from the same individuals were analysed and subsequently compared with their predicted 3D SC. The results were repeatable with a difference of +/-1cm. If the difference was higher, the quality of the image was low. Metafiles from the 3D camera contained angle range DPFOV: 65°±2° x 40°±1°, identical for every image. The angles are required to calculate the angle per pixel, which were further used for the 3D SC calculations. To choose one pair of angles, we predicted the 3D SC for a combination of angles and compared the results, which showed a difference of +/-1cm. We chose the following pair of angles based on this validation: 66.5° x 40.5°.

The predicted 3D SC values and their corresponding manual measurements were used for the calculation of mean prediction error (MPE), mean squared prediction error (MSPE) and mean percentage prediction error (MPPE) adjusted by the mean of the group for different age groups (Q,6,9,12 months) and different image quality categories (0,1,2). The data were tested for normality, and the nonparametric Scheirer–Ray–Hare test was performed to evaluate significant differences in MPE $p < 0.01$ between age groups and the image quality categories in each age group.

3. Results

The values of Dice, IoU and Tversky coefficients for the training, validation and test sets are represented in the Table 2. Mean prediction errors for each age group Q, 6, 9, 12 were -3.07 cm, -3.02 cm, -1.79 cm and -1.11 cm respectively. Fig. 7 shows the distribution of prediction error for each age group. Mean and percentage prediction errors were significantly different ($p < 0.05$) between the age groups (Table 3). Age group 12 showed significantly lowest MPE and MPPE. While capturing 3D images, we observed that natural or artificial light influenced the quality of the images significantly. Fig. 5 shows the 3D images and their matching RBG images of one individual in two different light conditions.

To improve future prediction accuracy, we conducted a test of image quality control. Each 3D image was rated based on the quality of the image. The scale was the following: 2 – full circle, 1– partial circle / "hanging testicle", 0 – not enough information/scrotum not well pronounced (Fig. 6).

The results show that the quality of image category 2 shows

Table 2
Values of Dice, IoU and Tversky coefficients for the training, validation and test sets.

Metric	Training set	Validation Set	Test Set
Dice	99,8%	99,5%	99,3%
IoU	99,3%	99,4%	99,2%
Tversky	99,4%	99,3%	99,2%

Table 3
Mean prediction error (MPE), mean squared prediction error (MSPE) and mean percentage prediction error (MPPE) adjusted by mean of the group for different age groups (Q[1],6,9,12 months). Based on the nonparametric Scheirer–Ray–Hare test, significant differences in MPE $p < 0.05$ between the age groups are marked by letters [a-d].

Age group	n images [2]	MPE [3]	MSPE[4]	MPPE [5]	mean SC (cm) [6]
Q [a]	57	-3.07 cm [cd]	16.6	-19.9 %	15 cm
6 [b]	88	-3.02 cm [cd]	13.9	-14.0 %	22 cm
9 [c]	124	-1.79 cm [abd]	7.89	-6.16 %	29 cm
12 [d]	101	-1.11 cm [abc]	7.46	-3.28 %	34 cm

[1] Quarantine – age 3-5 months
[2] Number of analysed images
[3] MPE – Mean of (SC-prediction)
[4] MSPE – Mean of (SC-prediction)^2
[5] MPPE – Mean of [(SC-prediction)/group_mean * 100%]
[6] Mean manually measured SC in cm

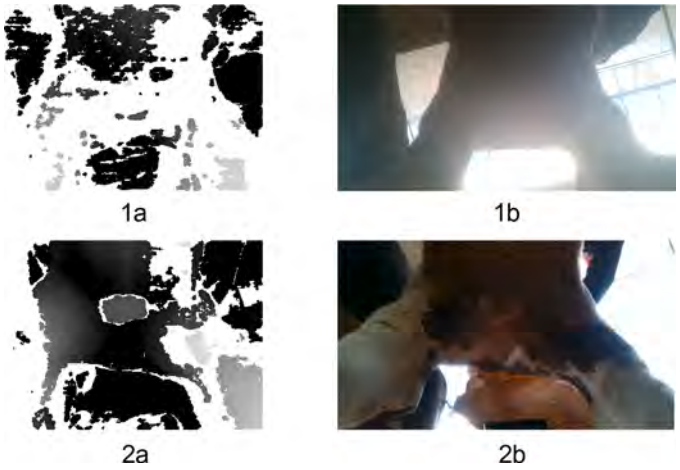

Fig. 5. Influence of natural light on 3D image quality for the same individual. 1a: 3D image with high exposure to natural light. 1b: RGB image matching 3D image, scrotum not visible. 2a: 3D image with the covered back of the bull to block natural light in the area of interest. 2b: RGB image matching 3D image, scrotum visible.

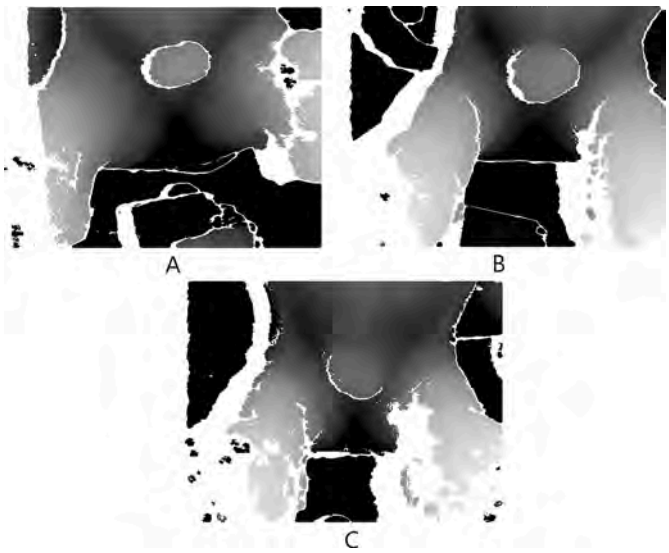

Fig. 6. Image quality categories. Examples of the 3D images from the category 2 – full circle (A), 1 – partial circle/" hanging testicle" (B), 0 – not enough information/scrotum not well pronounced (C).

significantly lower mean prediction error and mean percentage prediction error in age groups 6, 9 and 12 compared to image quality 0 and 1 as well as the mix of all images (Tables 3 and 4). For group Q, we can observe the lowest MPE and MPPE for image quality 0.

4. Discussion

Our results show that the quality of the image and the age of the bull are important variables affecting the accuracy of the prediction of SC based on 3D images. Three–month–old bull calves have small testicles that are not very well pronounced in the picture compared to older bulls. Furthermore, the intensity and quantity of natural and artificial light seem to influence the images' quality, as shown in Fig. 5. The Intel RealSense d415 depth sensor is mainly based on stereoscopy. Therefore, it is differently dependent on light conditions than sensors using structured light (like e.g. Kinect v1) or time of flight (like e.g. Kinect v2). In our case, strong backlight (Figs. 5, 1a and 1b) gave poorer image quality because it generates too large light contrasts in the scene, with a very light background compared with the relative dark scrotum area. On the other hand, outdoor light should improve the quality of depth images taken by the Intel RealSense d4XX cameras [32], but this probably requires that the object of interest is properly illuminated. Similarly, based on other types of depth camera technology, previous research has observed that strong illumination affects the quality of the depth images [33–35]. Azzari, Goulden and Rusu [33] showed that the quality of images increased with decreasing light exposure. Azzari, Goulden and Rusu, [33] observed that sunlight and infrared radiation influenced the contrast of the camera's laser pattern, which caused the lower quality of the images.

Dice, IoU and Tversky coefficients used in our study as a performance measure for semantic segmentation were chosen already by others [36–41]. A high Dice, IoU and Tversky coefficients of our model can be explained by the fact that a scrotum is a big object easily detectable by the human eye, which could imply that it should be just as easy for an advanced computer vision algorithm as a convolutional neural network.

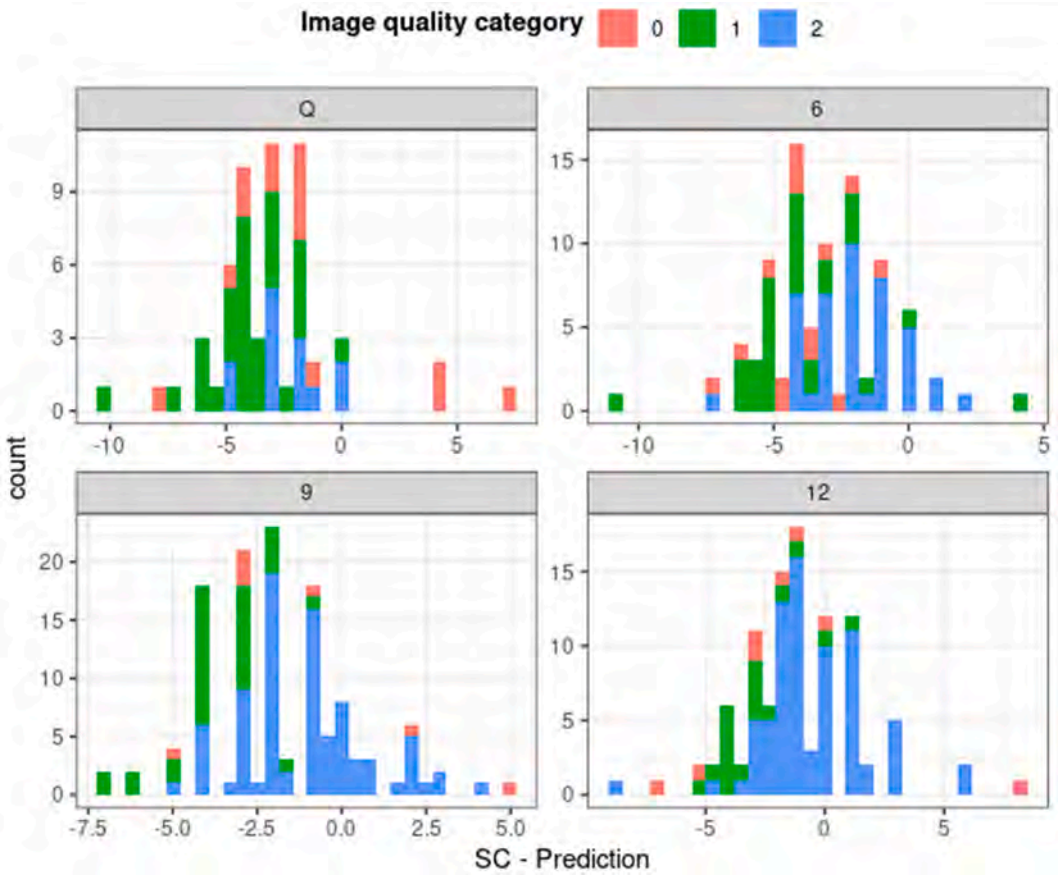

Fig. 7. Distribution of prediction errors. Histograms of prediction errors (SC – prediction) for different bull age groups (Q, 6, 9, 12) with the distinction of image quality (0, 1, 2).

Table 4
Mean prediction error (MPE)), mean squared prediction error (MSPE) and mean percentage prediction error (MPPE) adjusted by mean of the group for different age groups (Q[1],6,9,12 months) and different image quality categories (0[2],1[3],2[4]). Based on the nonparametric Scheirer–Ray–Hare test, significant differences in MPE $p < 0.01$ between the image quality categories in each age group are marked by letters [a-c].

Age group	Image quality category	n images [5]	MPE [6]	MSPE[7]	MPPE [8]	mean SC (cm) [9]
Q	0 [a]	14	-1.5 cm [bc]	16.9	-10 %	15 cm
Q	1 [b]	30	-4.1 cm [ac]	20.1	-26.3 %	15 cm
Q	2 [c]	13	-2.5 cm [ab]	8.31	-15.6 %	15 cm
6	0 [a]	14	-3.9 cm [bc]	17.4	-18.9 %	22 cm
6	1 [b]	31	-4 cm [ac]	22.2	-19.3 %	22 cm
6	2 [c]	43	-2 cm [ab]	6.80	-8.9 %	22 cm
9	0 [a]	7	-1.1 cm [bc]	11.7	-3.8 %	29 cm
9	1 [b]	33	-3.7 cm [ac]	15.5	-12.8 %	29 cm
9	2 [c]	84	-1.1 cm [ab]	4.58	-3.8 %	29 cm
12	0 [a]	8	-1.63 cm [bc]	20.1	-4.8 %	34 cm
12	1 [b]	18	-2.97 cm [ac]	11.1	-9.1 %	34 cm
12	2 [c]	75	-0.6 cm [ab]	5.21	-1.8 %	34 cm

[1] Quarantine – age 3-5 months
[2] Full circle
[3] Partial circle/"hanging testicle"
[4] Not enough information/scrotum not well pronounced
[5] Number of analysed images
[6] MPE – Mean of (SC-prediction)
[7] MSPE – Mean of (SC-prediction)^2
[8] MPPE – Mean of [(SC-prediction)/group_mean * 100%]
[9] Mean manually measured SC in cm

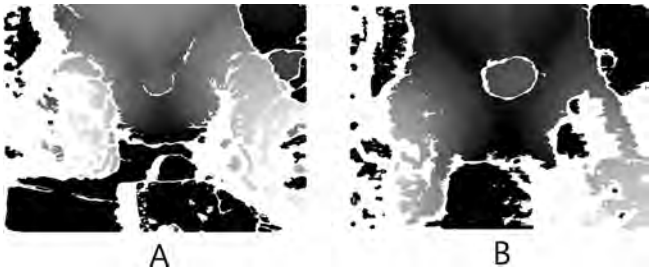

Fig. 8. Age differences and 3D image quality. Examples of 3D pictures of a three–month–old NR bull calf (A) and twelve–month–old NR bull (B).

Images used in the study are standardised since the scrotum, in most cases, can be found in the centre of the image. Similar methods to the ones used in our study were applied to the field of horticulture for the growth rate, size and yield measurement of the fruits and vegetables [33, 42–46]. The successful easy–to–use prediction of the in–field mango fruit size was performed with high accuracy by Wang, Walsh and Verma [46]. As a scrotum, the mango has an approximately elliptical shape. The authors used this to predict the mango's length and width, concluding that measurement error is caused by the not entirely elliptical shape of the fruits, which agrees with our findings. In our study, during manual measurement, bulls were moving from side to side, and as a reaction to the stressful situation, some bulls pulled the scrotum closer to the body using cremaster muscle, which might have influenced the measurements. Above mentioned variables assess our prediction error complex since we do not know the "real true" value. We believe that the best way of reducing prediction error is to standardise image quality. As our results demonstrate, the lowest MPE and MPPA were achieved for the image quality 2 in age groups 6, 9 and 12, making it the best candidate for the reference image quality for future analysis. The quality category 0 exhibits the lowest error measurements in age groups Q and 9, which we consider false due to the significant lack of information on the images of quality 0 (Fig. 8). This result could speak for the excellent performance of our segmentation model (dice coefficient – 99, 8%, 99,3%), which succeeded in classifying the scrotum of the incomplete image correctly. It is of importance to point out a very low number of images analysed in image quality category 0 in all age groups, which can explain this result. Future research should be devoted to the validation of the results described in this paper by capturing 3D SC images of only image quality 2 and performing manual SC measurements of the same individuals. Our results indicate that the mean prediction error and mean percentage prediction error in all age groups will not increase if the quality of the image, including light conditions, is taken into consideration. On top of a collection of new samples, data augmentation methods could be beneficial to model performance. Another interesting development opportunity would be machine learning model ensembling. We noticed that applying even a simple linear model (Fig. 9) on top of the previously described modelling methodology could potentially decrease prediction even more. This hypothesis, however, has to be confirmed.

5. Conclusion

To our knowledge, this is the first time SC measurements of bulls were automated with the use of convolutional neural networks and 3D images. This innovative approach, combined with a user–friendly application, allows a fast integration into breeding soundness evaluation of Norwegian Red bulls at the performance testing and bovine semen collection centres. To keep a high prediction accuracy, we recommend analysing individuals older than 6 months, paying attention to light conditions and capturing 3D images of quality 2 only.

Ethical statement

Ethical approval was not required in this study. We worked at the performance testing station under the supervision of a qualified veterinarian employed at the breeding company Geno. The performance testing station fulfils Norwegian legislation for the housing of bulls.

Role of the funding source

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CRediT authorship contribution statement

Joanna Bremer: Conceptualization, Methodology, Software, Validation, Investigation, Writing – original draft, Visualization, Project administration. **Michał Maj:** Conceptualization, Methodology, Software, Validation, Investigation, Formal analysis, Writing – original draft, Visualization. **Øyvind Nordbø:** Conceptualization, Methodology, Resources, Writing – original draft. **Elisabeth Kommisrud:** Conceptualization, Methodology, Writing – original draft, Project administration, Funding acquisition.

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.

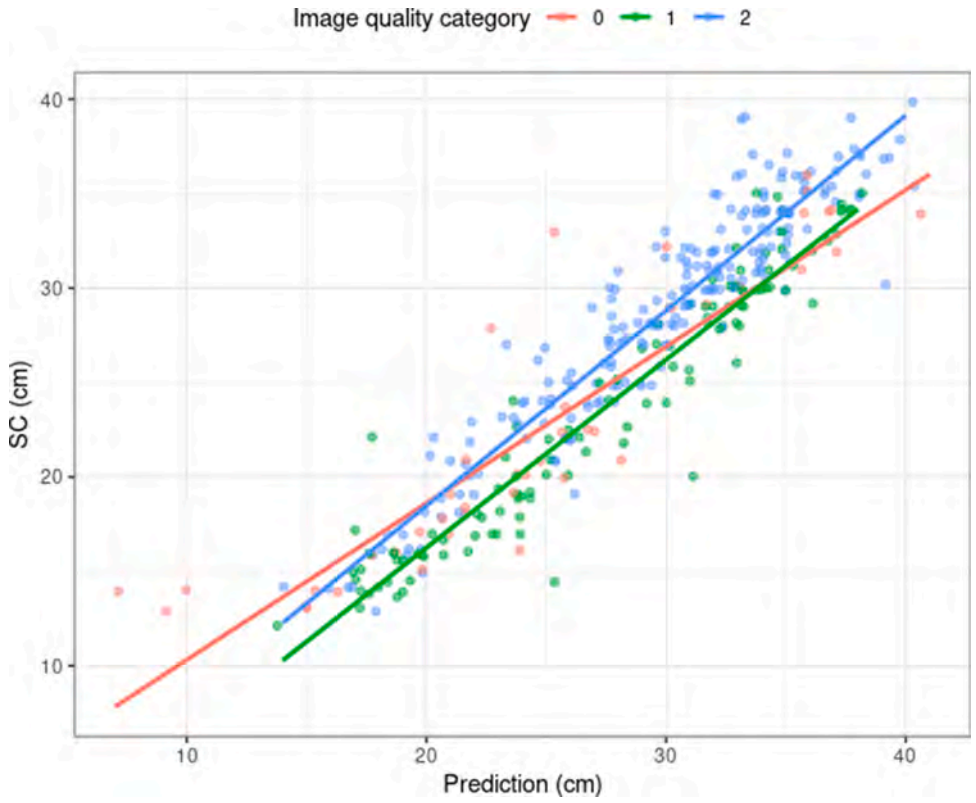

Fig. 9. Fitted linear regression models for the scrotum circumference (cm) based on the prediction (cm) value obtained from the fitted ellipse for different image quality (0,1, 2) groups.

Data availability

The authors do not have permission to share data.

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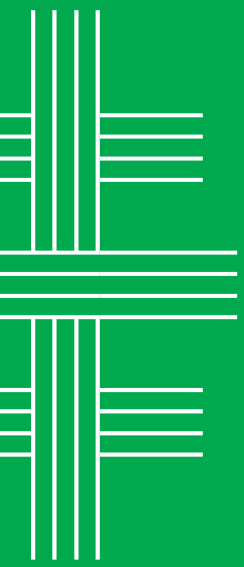

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With the application of genomic selection in dairy cattle breeding, the choice of elite sires is based on their estimated genomic breeding values instead of progeny testing. Consequently, bulls are introduced into semen production at a younger age. The main aim of this thesis was to identify novel early indicators of sperm production onset and maturity status of young Norwegian Red bulls during their performance test period, to provide insight into their potential future semen production, acceptance for the AI station, and field fertility.

The results of Paper 1 showed that by incorporating sperm stress tests, cryopreservation, and early morphology analysis, valuable insights into the maturity of bulls for sperm production could be gained. This approach would allow for the integration of younger bulls into semen collection, facilitating reduced generation interval and increased genetic gain.

The focus in Paper 2 was on investigating the potential of insulin-like factor 3 as a biomarker for predicting the onset of sperm production in young Norwegian Red bulls. Due to the substantial inter-individual variability in the Norwegian Red bull population, insulin-like factor 3 is currently not a reliable biomarker for predicting the onset of sperm production in this breed.

Paper 3 presents an automated method for measuring scrotal circumference of Norwegian Red bulls using 3D images and convolutional neural networks. The results showed that the automated scrotal circumference measurements were similar to manual measurements. This novel measurement method has the potential to be implemented at performance test stations and semen collection centers, providing a fast and efficient approach for assessing scrotal circumference.

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