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Reproductive potential and semen attributes in Norwegian Red bulls

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Birgitte Narud

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List of abbreviations

AI	Artificial insemination	IVP	*In vitro* production
Al	Aluminium	K	Potassium
ALH	Amplitude of lateral head displacement	LC	Liquid chromatography
AO	Acridine orange	LH	Luteinizing hormone
As	Arsenic	LIN	Linearity
ATP	Adenosine triphosphate	LINE-1	Long interspersed nuclear elements-1
Ba	Barium	LUMA	Luminometric-based assay
BCF	Beat cross frequency	mBBr	Monobromobimane
BSP	Bovine seminal plasma	Mg	Magnesium
Ca	Calcium	MOET	Multiple ovulation and embryo transfer
cAMP	Cyclic adenosine monophosphate	MS	Mass spechtrometry
CASA	Computer assisted sperm analysis	ncRNAs	Noncoding RNAs
CGI	CpG island	NDHRS	Norwegian dairy herd recording system
CpG	Cytosine residue followed guanine	NGS	Next-generation sequencing
CMA3	Chromomycin A3	Ni	Nickel
COCs	Cumulus–oocyte complexes	NMR	Nuclear magnetic resonance
Cu	Copper	NR	Non-return
DFI	DNA fragmentation index	NR56	56-day non-return
DMR	Differentially methylated regions	Pb	Lead
DNA	Deoxyribonucleic acid	PI	Propidium iodide
DNMTs	DNA methyltransferases	PKA	Protein kinase A
dsDNA	Double stranded DNA	PMT	Photo multiplier tube
dUTP	Deoxyuridine triphosphate	PNA	Arachis hypogea (peanut) agglutinin
ELISA	Enzyme-linked immunosorbent assay	PSA	Pisum sativum agglutinin
EV	Electronic volume	PTMs	Post-translational modifications
Fe	Iron	PTK	Protein tyrosine kinase
FL	Fluorescence detector	Ptyr-Ptase	Protein tyrosine phosphatase
FSC	Forward scatter	RESP18	Regulated endocrine specific protein 18
FSH	Follicle stimulating hormone	RLU	Relative luminescence unit
FITC	Fluorescein isothiocyanate	RNA	Ribonucleic acid
FSH	Follicle stimulating hormone	ROS	Reactive oxygen species
GC	Gas chromatography	RRBS	Reduced representation bisulfite sequencing
GLM	Generalized linear model	sAC	Soluble adenylyl cyclase
GnRH	Gonadotropin releasing hormone	SAS	Statistical analysis system
GS	Genomic selection	SCD	Sperm chromatin dispersion
HCO_3^-	Bicarbonate	SCSA	Sperm chromatin structure assay
HDS	High DNA stainability	Se	Selenium
HPLC-UV	High-performance liquid chromatography-ultraviolet	SNP	Single nucleotide polymorphism
ICP	Inductively coupled plasma	SSC	Side scatter
IVC	*In vitro* cultivation	ssDNA	Single stranded DNA
IVF	*In vitro* fertilization	STR	Straightness
IVM	*In vitro* maturation	TSSs	Transcriptional start sites

TUNEL	TdT-mediated dUTP nick end labelling
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight line velocity
WGBS	Whole-Genome Bisulfite Sequencing
WOB	Wobble
Zn	Zink
ZP	Zona pellucida
ZP3	ZP glycoprotein-3

List of papers

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II Khezri, A., B. Narud, E.B. Stenseth, T.T. Zeremichael, F.D. Myromslien, R.C. Wilson, R. Ahmad, and E. Kommisrud. 2020. Sperm DNA Hypomethylation Proximal to Reproduction Pathway Genes in Maturing Elite Norwegian Red Bulls. *Front Genet*. 11.

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IV Narud, B., A. Khezri, T.T. Zeremichael, E.B. Stenseth, B. Heringstad, A. Johannisson, J.M. Morrell, P. Collas, F.D. Myromslien, and E. Kommisrud. 2020. Sperm chromatin integrity and DNA methylation in Norwegian Red bulls of contrasting fertility. *Manuscript submitted to Molecular reproduction and development.*

Summary

Norwegian cattle breeding enjoys exceptional advantages due to a historically strong focus on long-term breeding programs with emphasis on factors such as health and fertility. With the recent introduction of genomic selection (GS) in cattle breeding, younger bulls have been recruited for semen production. Thus, the speed of cattle breeding has changed from a careful selection over generations with donor animals of known fertility, to rapid selection of young animals without any fertility records. Bull fertility has a major impact on the overall bovine reproductive efficiency, and good semen quality is essential for successful fertilization and subsequent embryo development. Therefore, it is of interest to the cattle breeding industry to have laboratory tests or seminal biomarkers that accurately and efficiently can predict the fertilization potential of a semen sample. The present thesis aimed to study sperm quality traits as well as underlying factors affecting reproductive potential of Norwegian Red bulls. The study populations were young bulls of both 14 and 17 months of age and a group of bulls with contrasting field fertility. The goal was to detect potential biomarkers related to bull maturation and fertility, for implementation at the breeding station.

Our results indicated that young bulls are mature enough for their semen to fulfil successful fertilization. However, several sperm attributes, including sperm hyperactivity and kinematic motility parameters, amino acid and trace element contents, differed between young bulls of 14 and 17 months of age. Sperm concentration and volume of the ejaculate increased significantly with increasing age, while the percentage of discarded batches decreased. Thus, reduced semen production efficiency in young bulls is a challenge, and it would be beneficial for the AI industry to identify biomarkers in semen that can predict bull maturity and reproductive performance. Further, we showed that reduced representation bisulfite sequencing (RRBS) in parallel with routine sperm quality analyses might provide additional information regarding reproductive capacity of young bulls. Sperm from 17 months old bulls were hypermethylated compared to sperm from 14 months old bulls, and differentially methylated regions identified can be linked with important sperm functions and hormonal pathways.

Sperm DNA fragmentation index (DFI) was negatively correlated with field fertility in bulls of contrasting 56-day non-return rate (NR56). Even though sperm attributes such as sperm motility were found to be associated with bull fertility, DFI was the only sperm quality parameter with a significant contribution to the model predicting bull fertility. Together with the sperm intracellular concentrations of aspartic acid, Fe and Zn, this prediction model

explained 59% of the variation in NR56. Further, multiple correlations were found between sperm metabolite contents, sperm quality parameters and NR56, indicating that metabolomics may be a useful tool in the identification of biomarkers for male fertility.

The *in vitro* fertilization (IVF) experiments showed that DFI and high DNA stainability (HDS) were negatively correlated with cleavage rate, but not with blastocyst rate, indicating that impaired chromatin integrity may affect the sperm cells ability to fertilize oocytes *in vitro*. An important factor that may contribute to the observed results was the relatively low levels of DNA fragmentation measured in the semen samples used for this study. Sperm protamination measured as protamine deficiency was associated with sperm DFI and HDS, but not with NR56 or with IVF outcomes. Furthermore, no associations were found between sperm thiols/disulfide bonds and DNA integrity or NR56. Sperm cells from bulls of low fertility were hypermethylated compared to sperm from high fertility bulls. The number of genes associated with biological processes related to different aspects of fertility and embryo development were higher in the hypermethylation group compared to the hypomethylation group, indicating that sperm hypermethylation is an aspect of male fertility problems. Genes annotated with differentially methylated cytosines were identified as participants in biological pathways important for bull fertility.

The results from the present thesis work show the importance of assessing multiple sperm characteristics when evaluating bull fertility. We further demonstrate that the conventional sperm quality parameters only partly explain the observed variation in bull fertility, and that underlying factors affecting fertility might be explained by the assessment of metabolites in semen and sperm DNA methylation. Further investigations are recommended to find specific biomarkers in semen that can help predict bull maturation and reproductive performance.

Sammendrag

Norsk storfeavl nyter eksepsjonelle fordeler på grunn av et historisk sterkt fokus på langsiktige avlsprogrammer med vektlegging av faktorer som helse og fruktbarhet. Nylig ble genomisk seleksjon (GS) introdusert i storfeavlen, noe som har resultert i at yngre okser blir rekruttert for sædproduksjon. Dette har ført til at hastigheten på norsk storfeavl har endret seg fra å være et tålmodig utvalg over generasjoner basert på dyr med kjent fruktbarhet, til rask seleksjon av unge dyr uten fertilitetsdata. Oksens fruktbarhet har stor innvirkning på den totale reproduksjonseffektiviteten hos storfe, og god sædkvalitet er avgjørende for vellykket befruktning og embryoutvikling. Derfor er det av interesse for avlsorganisasjonene å ha laboratorietester eller biomarkører som nøyaktig og effektivt kan forutsi befruktningspotensialet på hvert ejakulat. I denne avhandlingen ble spermiekvalitet samt underliggende faktorer som kan påvirke reproduksjonspotensialet hos Norsk Rødt Fe okser studert. Studiepopulasjonene bestod av unge okser med en alder på 14 og 17 måneder, samt en gruppe okser med ulik fruktbarhet i felt. Målet var å detektere potensielle biomarkører knyttet til oksens modenhet og fruktbarhet, for mulig implementering på avlsstasjonen.

Resultatene våre indikerte at unge okser er modne nok til at sæden deres er befruktningsdyktig. Videre ble det vist at flere sædparametere, inkludert hyperaktivitet og kinematiske motilitetsparametere, aminosyre og sporelementer i spermier, var forskjellig mellom unge okser på henholdsvis 14 og 17 måneder. Ejakulatets spermiekonsentrasjon og volum økte betydelig med økende alder, mens prosentandelen av kasserte sæddoser ble redusert. Dette viser at redusert sædproduksjonseffektivitet er en utfordring hos unge okser, og det vil være gunstig for avlsindustrien å identifisere biomarkører i sæd som kan forutsi oksers modenhet og reproduksjonsevne. Videre viste vi at redusert representasjons bisulfite sekvensering (RRBS) sammen med rutinemessige sædkvalitetsanalyser kan gi ytterligere informasjon om reproduksjonskapasiteten til unge okser. Spermier fra 17 måneder gamle okser var hypermetylert sammenlignet med spermier fra 14 måneder gamle okser, og differensielt metylerte regioner som kan knyttes til viktige sædfunksjoner og hormonelle synteseveier ble identifisert.

Spermienes DNA fragmenteringsindeks (DFI) var negativt korrelert med feltfertilitet hos okser med ulik 56-dagers ikke-omløpsprosent (NR56). Selv om parametere som spermie-motilitet var assosiert med oksenes feltfertilitet, var DFI den eneste sædkvalitetsparameteren med et betydelig bidrag til prediksjonsmodellen for oksefruktbarhet. Sammen med spermienes intracellulære innhold av asparaginsyre, Fe og Zn, forklarte denne prediksjonsmodellen 59%

av variasjonen NR56. Videre ble det funnet flere korrelasjoner mellom spermiers metabolittinnhold, sædkvalitetsparametere og NR56, noe som indikerer at metabolomics kan være et nyttig verktøy i identifiseringen av biomarkører for hanndyrfruktbarhet.

Forsøk med *in vitro* fertilisering (IVF) viste at DFI og høy DNA-fargeintensitet (HDS) var negativt korrelert med befruktningsraten, men ikke med blastocystraten, noe som indikerer at nedsatt kromatinintegritet kan påvirke sædcellenes evne til å befrukte egg *in vitro*. En viktig faktor som kan ha påvirket resultatene var de relativt lave nivåene av DNA-fragmentering i sædprøvene i denne studien. Spermienes protaminmangel var assosiert med DFI og HDS, men ikke med NR56 eller IVF-resultatene. Videre ble det ikke funnet noen assosiasjoner mellom spermienes tioler/disulfidbindinger og DNA-integritet eller NR56. Spermier fra okser med lav fruktbarhet var hypermetylert sammenlignet med spermier fra okser med høy fruktbarhet. Antall gener assosiert med biologiske prosesser knyttet til forskjellige aspekter av fruktbarhet og embryoutvikling var høyere i hypermetyleringsgruppen sammenlignet med hypometyleringsgruppen. Dette indikerer at hypermetylering av spermier kan være et aspekt ved fertilitetsproblemer hos hanndyr. Gener annotert med differensielt metylerte cytosiner ble identifisert som kjente deltakere til biologiske synteseveier som kan være viktige for hanndyrfruktbarhet.

Resultatene fra denne avhandlingen viser viktigheten av å undersøke flere egenskaper i sæd ved evaluering av fruktbarhet hos okse. Videre viste vi at de konvensjonelle sædkvalitets-parameterne kun delvis forklarer variasjonen i oksefertilitet, og at underliggende faktorer av betydning for fruktbarheten muligens kan forklares ved hjelp av analyser av metabolitter og DNA-metylering i spermier. Videre studier anbefales for å identifisere spesifikke biomarkører i sæd som kan bidra til å forutsi modenhet og reprodukskapasitet hos okser.

1. Introduction

1.1 General background

Fertility is a necessity for evolution, development and, above all, food production for an increasing world population expected to reach 8.6 billion people in 2030 (UN, 2017). In food-producing species such as cattle, reproductive success is critical for production efficiency. Current food production relies intricately on breeding programs, selecting individuals with preferable phenotypes harbouring advantageous alleles of genes for extensive propagation in the population (Dekkers, 2012). Artificial insemination (AI) is the most common used tool for introducing such genes into the population. It involves the introduction of sperm cells to the reproductive tract of recipient females to achieve fertilization without natural mating. Already in 1784, the Italian scientist Lazzaro Spallanzani successfully performed AI in a dog, resulting in three live born puppies. In the early 1900s, the Russian Professor Ilya Ivanovich Ivanov developed AI for several species including cattle (Ombelet and Van Robays, 2015). However, until the discovery of semen preservation there was no widespread use of AI. Polge et al. (1949) discovered that glycerol had remarkable properties in protecting sperm cells both at low temperatures and during freezing. Followed by the advancement of new semen extenders, packaging methods and freezing procedures through the 1950s, the methods for semen storage in liquid nitrogen were developed. AI with cryopreserved semen has several advantages such as accelerated spread of genetic diversity, world-wide distribution of genetically superior animals, reduced risk of disease transmission and cost-effectiveness (Ugur et al., 2019b; Valergakis et al., 2007). However, the process of freezing and thawing spermatozoa may strongly impair sperm quality (Yeste, 2016), mainly because of alterations to the sperm plasma membrane.

The rapid expansion of new tools and technologies in the livestock industry have had a remarkable impact on dairy cattle production during the last decades (Urrego et al., 2014). Along with the use of AI, new technologies such as semen sexing, *in vitro* fertilization (IVF), multiple ovulation and embryo transfer (MOET) combined with genomic selection (GS) contribute to maximize the genetic improvement of livestock (Schaten and Constantinescu, 2017). However, new inventions also cause new impediments for the industry. For instance, GS bulls are introduced into semen production at a young age, which may conflict with the onset of puberty. Furthermore, *in vitro* reproductive technologies may involve steps that exert environmental stress on sperm cells, oocytes and early embryos, which potentially can cause epigenetic changes and alterations in gene expression (Urrego et al., 2014).

Developments in assisted reproductive technology and cattle breeding have created a need for improved knowledge. Moreover, new technologies and research methods have led to a continuing search for novel seminal biomarkers, important for the livestock industry. In the present thesis, *in vitro* semen quality as well as sperm epigenetics and metabolomics were assessed in semen samples collected from Norwegian Red bulls. Sperm characteristics were investigated with focus on differences in the age of young bulls and field fertility.

1.2 Artificial insemination and cattle breeding in Norway

Cattle breeding in Norway enjoys exceptional advantages due to a historically strong focus on long-term breeding programs. Traditionally, quantitative breeding methods with progeny testing of large offspring groups have been applied for selection of animals possessing economically important traits. This breeding strategy has slowly created robust, healthy animals, with feed-efficient production (Ranberg et al., 2003; Refsdal, 2007). Several countries have experienced a decline in the reproductive performance in dairy cattle, due to decades of effective selection for high milk yield (Lucy, 2007; Nebel and McGilliard, 1993). This is not the case in Norway, where low heritability traits such as health and fertility were included as important traits at an early stage in the breeding program. High fertility currently represents a major competitive advantage for the Norwegian breeding industry on international markets, and the Norwegian Red is considered one of the most fertile cattle breeds worldwide (Ferris et al., 2014; Refsdal, 2007). It is of great importance that the Norwegian cattle breeding program continues to generate both healthy, fertile and production-efficient animals, thereby contributing to good animal welfare while strengthening the competitive advantages internationally.

Geno SA is the national breeding company for dairy cattle in Norway and is cooperatively owned by 8 400 Norwegian farmers. The main tasks of Geno are breeding and development of the Norwegian Red population. Geno has expanded internationally, and approximately half of the semen produced is exported to other European countries, North America and China (Geno, 2019). All inseminations performed in Norway, are recorded in The Norwegian Dairy Herd Recording System (NDHRS). This is a databank run by the dairy farmers cooperative, TINE BA. In addition to information regarding AIs, the database contains recordings from health cards, slaughterhouses and laboratory milk analyses. The AI technicians are employed by Geno and paid according to reported AIs (Ranberg et al., 2003), thus the field fertility data in Norway, calculated as non-return rate after 56 days (NR56), are considered highly reliable.

The introduction of GS, which is based on next generation sequencing using panels of increasing numbers of single nucleotide polymorphisms (SNPs) distributed over the whole genome, has caused a world-wide paradigm shift in animal breeding (Meuwissen et al., 2016). With GS, it is possible to estimate the animal breeding value without testing the performance of the animal itself or the performance of a larger number of offspring. This enables the selection of younger bulls for AI, thus reducing the generational interval and increasing the genetic gain (Murphy et al., 2018). In 2016, GS was implemented in the breeding system of Geno (Buskap, 2016), reducing the generation interval in bulls from 7 years to less than 2.5 years. Thus, the speed of cattle breeding has changed from patient and careful selection over generations with donor animals of known fertility, to selection of young GS-tested animals without any fertility records.

1.3 Spermatogenesis in bulls

Spermatogenesis, the process of producing spermatozoa, occurs in the seminiferous tubules of the testes (Figure 1). The production of sperm cells is under endocrine control by secretion of hormones from the hypothalamus, pituitary gland and the Leydig and Sertoli cells of the testes. The gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone are secreted in pulses with different duration and intervals, leading to the final development of spermatozoa (Senger, 2012). Spermatogenesis can be divided into three phases: the proliferation phase, the meiotic phase and the differentiation phase. In the proliferation phase, primordial germ cells multiply by a set of mitotic divisions, generating new spermatogonial stem cells and primary spermatocytes. The process of proliferation takes place at the basal compartment of the seminiferous epithelium (Pineda, 2003; Senger, 2012). During the meiotic phase, primary spermatocytes become secondary spermatocytes, which after a second meiotic division become haploid spermatids (Senger, 2012). The final stage of the spermatogenesis is the differentiation phase, also called spermiogenesis. Here, the round spermatids differentiate into spermatids at various stages of elongation, and finally into fully differentiated and highly specialized spermatozoa. During the differentiation process the sperm acrosome and flagellum develops, and the processes of elongation, nucleus condensation and removal of remaining cytoplasm are fulfilled. Finally, spermatozoa are released into the lumen of the seminiferous tubules, in a process recognized as spermiation. Spermiation is followed by passive transportation of the sperm cells into the epididymis where they continue to mature and acquire fertilizing competence (Senger, 2012;

Staub and Johnson, 2018). The complete process of spermatogenesis and epididymal maturation requires about 65 days in bulls (Fuerst-Waltl et al., 2006).

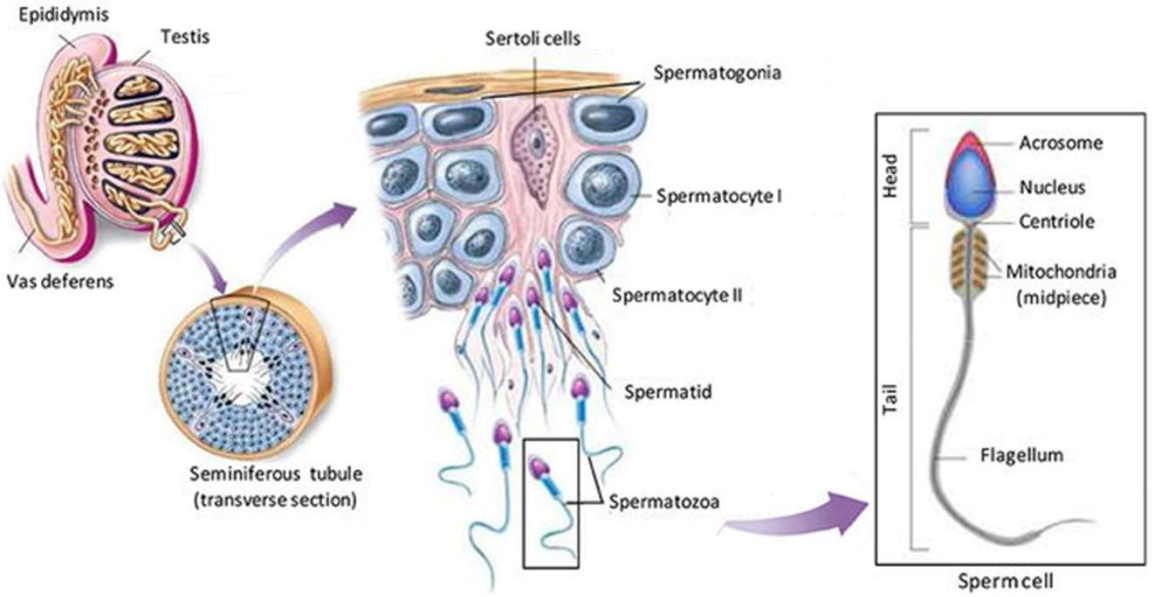

Figure 1. *The production of sperm cells (spermatogenesis) takes place within the seminiferous tubules of the testes. The process starts near the basal lamina of the seminiferous tubules, where diploid primordial germ cells (spermatogonia) undergo mitosis to produce diploid primary spermatocytes (spermatocyte I). Nearly half the primary spermatocytes produced remain near the basal lamina to continue to divide mitotically, while the other primary spermatocytes migrate toward the lumen of the seminiferous tubules and begin to undergo meiosis I, resulting in haploid secondary spermatocytes (Spermatocyte II). Further, the secondary spermatocytes divide through meiosis II and produce haploid spermatids. Through the final stage of spermatogenesis (spermiogenesis), the spermatozoa develop. Spermatozoa are transferred to the epididymis where they continue to mature and acquire fertilizing competence. The mature sperm cell consists of a head and tail region, where the tail can be divided into the mid-piece and the flagellum. The sperm head contains a compact nucleus, partly surrounded by the acrosome. Figure taken from Allais-Bonnet and Pailhoux (2014), with minor modifications.*

The accessory sex glands (vesicular glands/seminal vesicles, prostate, bulbourethral glands and ampulla) produce fluids, known as seminal plasma. Mixed with spermatozoa, the seminal plasma causes dilution and biochemical changes of the spermatozoa, which promotes sperm function and fertilization (Bromfield, 2016; Poiani, 2006; Senger, 2012). Upon ejaculation, the seminal plasma serves as a medium transporting the spermatozoa into the female genital tract (Bromfield, 2016). The volume of an ejaculate, sperm concentration and composition of seminal plasma vary between bulls, and may be affected by factors such as bull age, breed and season (Fuerst-Waltl et al., 2006; Snoj et al., 2013; Stålhammar et al., 1989).

The testicular size in bulls is positively associated with sperm production (Amann and DeJarnette, 2012; Schenk, 2018). Bull testes grow relatively slowly until approximately 25 weeks of age and then a rapid phase of growth occurs until puberty, at 37–50 weeks (9-12

months) of age, depending on breed and individual differences (Casas et al., 2007; Rawlings et al., 2008). Post-puberty, the testis continue to grow and spermatogenesis becomes more expanded and efficient, thereby increasing the sperm output (Schenk, 2018). Furthermore, semen quality improves as bulls mature, and spermatozoa normally fulfill quality criteria for motility and morphology tests around 16 months of age (Lambert et al., 2018). These improvements are likely associated with the normalization of spermatogenesis and epididymal function (Schenk, 2018).

1.4 Structure of the sperm cell

The mature spermatozoon is composed of a distinctive head, mid-piece and tail region (Figure 1), enclosed by a single plasma membrane. This compartmentalization, together with its streamlined shape, is important for the sperm cell to reach and fertilize the oocyte (Bonet et al., 2013; Flesch and Gadella, 2000). The sperm head is composed of a haploid nucleus with tightly packed DNA. During spermatogenesis, the majority of the core histones are replaced, first by their testis-specific histone variants, then by transition proteins, which are terminally replaced by protamines, resulting in chromatin hyper-compaction of the sperm nucleus (Figure 2). These steps minimize the volume of the sperm nucleus for efficient sperm transport and limit the possibility for DNA damage and mutagenesis (Bao and Bedford, 2016; Dogan et al., 2015). Furthermore, sperm protamination is important for the epigenetic regulation of gene expression (Carrell, 2012). A large secretory vesicle, called the acrosome, is located apical to the sperm nucleus. The acrosome contains hydrolytic enzymes, antigens and non-enzymatic acrosomal proteins that are necessary for sperm penetration through the zona pellucida during fertilization (Alberts et al., 2008; Berruti, 2016; Evans and Florman, 2002; Flesch and Gadella, 2000). Both the mid-piece and sperm tail are parts of a long flagellum that provides the spermatozoon with its motile force. Several mitochondria located in the mid-piece efficiently generate energy necessary for sperm movement. This is based upon the structure of the flagellar axoneme, which consists of two central singlet microtubules surrounded by nine evenly spaced microtubule doublets that further are surrounded by nine outer dense fibres. Dynein motor proteins that slide the microtubules by using energy from ATP, cause the flagellar movement. This enables the sperm cell to propel fast through aqueous medium and pass through the egg coat (Alberts et al., 2008; Jonge and Barratt, 2006).

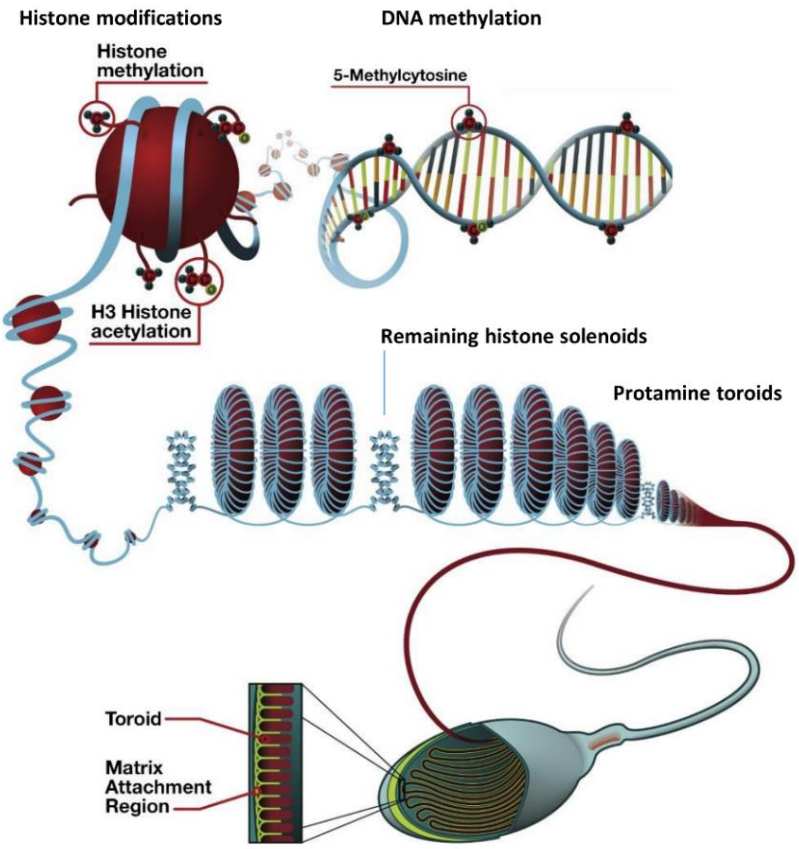

Figure 2. *During spermatogenesis, most histones are replaced by protamines, resulting in a higher order of DNA packaging in the sperm nucleus. However, some histones are usually retained. The protamines are coiled into toroids and attached to matrix attachment regions, while the histones are coiled into solenoids. The degree of sperm protamination may affect the epigenetic status of the sperm cell. Figure modified from Carrell (2012).*

1.5 Fertilization: the sperm cell's journey to and interaction with the oocyte

Following deposition of semen into the female reproductive tract, spermatozoa will spend most of their lifetime within the oviduct. The oviduct has shown to provide a suitable environment for sperm transport, storage, oocyte pick-up, fertilization and early embryo development. It consists of three parts: the uterotubal junction, the isthmus, and the ampulla. The uterotubal junction provides a barrier between the oviduct and the uterus to avoid entry of infectious microbes, but also regulates sperm cell entrance. The isthmus functions as an organ for sperm storage while the ampulla provides a favourable environment for fertilization and early embryonic development (Suarez, 2008). Sperm cells are differentiated cells with no active transcription or reparative mechanisms. Adequate environment within the female genital tract is therefore necessary for sperm survival and high reproductive performance (Rodriguez-Martinez, 2007; Suarez and Pacey, 2006). Sperm transport from the site of deposition to the site of fertilization involves dynamic interactions between the sperm cells and the female genital tract (Scott, 2000).

1.5.1 The formation of a sperm reservoir

The site of semen deposition is dependent on whether natural mating or AI is performed. Bypassing the vagina and cervix has shown to be advantageous for AI, thus the AI technician will transfer 5-20 million spermatozoa directly into the uterus (Suarez, 2007). After semen deposition, the spermatozoa will encounter rigorous filtration, causing the sperm number to decrease drastically. For sperm cells to travel through the uterotubal junction, specific molecules on the sperm surface are required (Cho et al., 1998; Suarez, 2016), in addition to normal morphology and motility. The rigorous filtration and selection of competent spermatozoa cause only a few thousand sperm cells to reach the isthmus (Suarez and Pacey, 2006). By binding to ciliated and non-ciliated epithelial cells, the sperm cells are trapped in the initial segment of the oviductal isthmus (Hunter, 2005; Hunter and Wilmut, 1984). This process seems to be facilitated by lectin-like proteins on the sperm head and specific carbohydrate moieties present in the oviductal epithelial cells (Suarez, 2002). The bovine seminal plasma (BSP) protein PDC-109 (BSP-A1/A2) has been identified as a fucose-binding protein that coat the acrosomal region of the sperm cell. Furthermore, two other proteins of the BSP family have been shown to enhance sperm-oviduct binding, namely the BSP-30-kDa protein and the BSP-A3 protein (Suarez, 2007). The BSPs binds to ligands containing fucose on the surface of cilia (Figure 3). The binding of sperm cells to the epithelium creates a functional sperm reservoir, where the fertilizing capacity of the spermatozoa can be maintained until ovulation occurs (Chian and Sirard, 1995; Pollard et al., 1991).

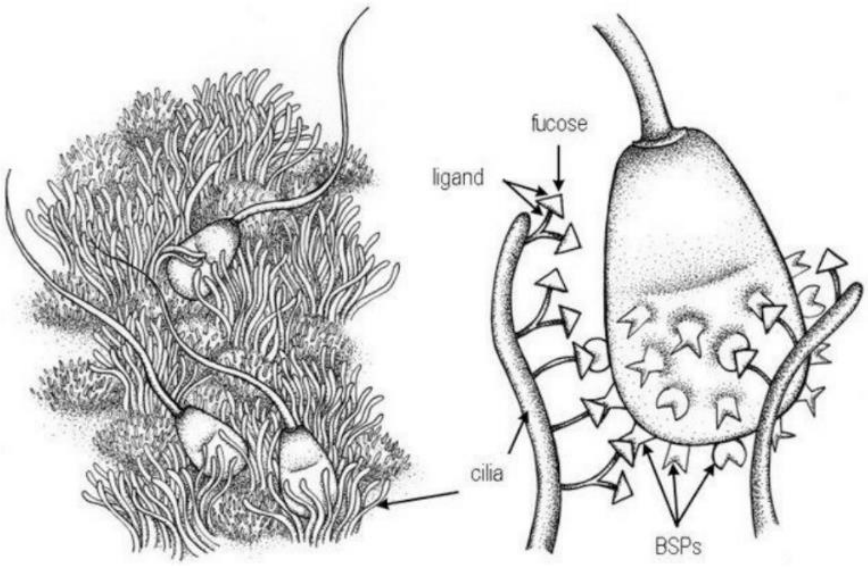

Figure 3. *Illustration of the interaction between spermatozoa and the oviductal epithelium, resulting in the creation of a sperm reservoir. The BSP proteins (PDC-109, BSP-A3 and BSP-30-kDa) on the sperm plasma membrane bind to ligands containing fucose on the surface of cilia. Figure taken from Suarez (2007).*

1.5.2 Sperm capacitation and hyperactivation

Sperm capacitation takes place *in vivo* during the transport through the female genital tract and is completed within the oviduct (Rodriguez-Martinez, 2007). Capacitation is the final step of sperm maturation, regulated by the microenvironment of the sperm reservoir to ensure that the sperm cells are in the proper state when ovulation occurs (Talevi and Gualtieri, 2010). Loss of cholesterol from the sperm membrane causes the membrane fluidity to change and allows influx of Ca^{2+} and HCO_3^- (Figure 4). This starts a cascade of intracellular signalling events leading to activation of soluble adenylyl cyclase enzymes in the cytosol, which produce cyclic adenosine monophosphate, stimulation of protein kinase A, and protein tyrosine phosphorylation, which is linked to the hyperactive swimming pattern of spermatozoa (De Jonge, 2017).

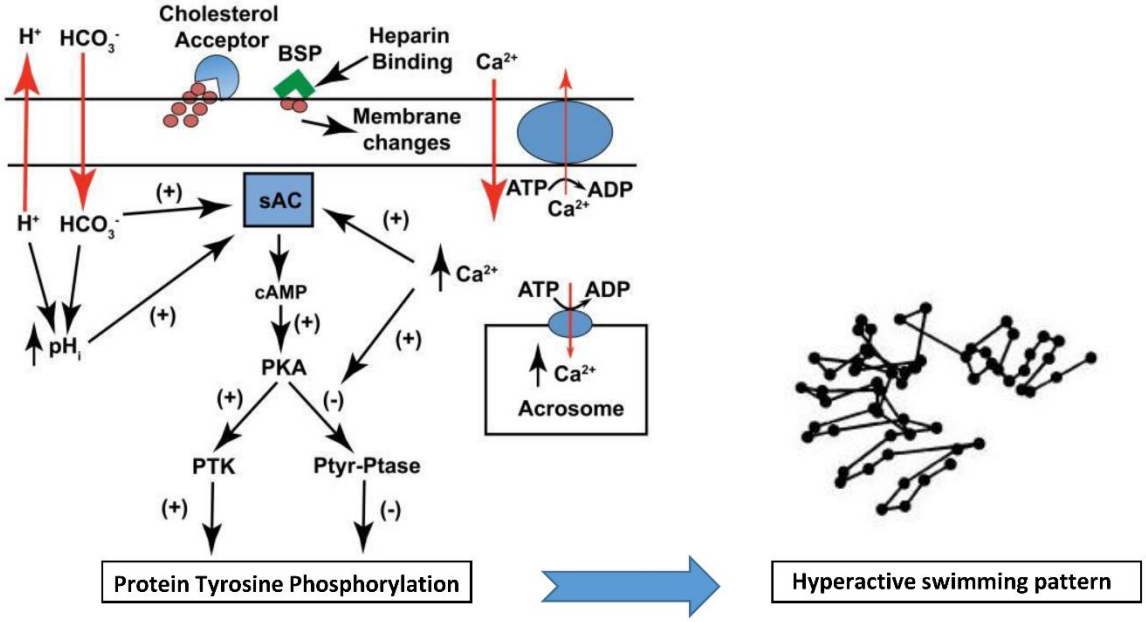

Figure 4. *Illustration of the main events of sperm capacitation. Loss of cholesterol from the sperm membrane causes the membrane fluidity to change and allows influx of Ca^{2+} and HCO_3^-. This is followed by a cascade of intracellular signalling events including activation of soluble adenylyl cyclase enzymes (sAC) in the cytosol, which produce cyclic adenosine monophosphate (cAMP), stimulation of protein kinase A (PKA), and protein tyrosine phosphorylation. Protein tyrosine phosphorylation is linked to sperm hyperactivity. Figure modified from Parrish (2014) and Cancel et al. (2000).*

Hyperactivity is a swimming pattern characterized by high amplitude and asymmetrical flagellar beating (Figure 4). Together with modifications of proteins on the sperm surface, hyperactivity could provide the force necessary to pull away from the epithelial cells (Suarez, 2008). There are specific signals that induce sperm release from the epithelium, and the spermatozoa may detach and reattach several times before leaving the storage region (Chang and Suarez, 2012). However, the signals that may pass between sperm and epithelium during

these interactions are still not completely understood (Suarez, 2016). The capacitated spermatozoon can later be activated to acrosome react and fertilize the oocyte. Hyperactive beating has shown to be critical for successful fertilization, as it in addition to allow sperm release from epithelial cells, enables spermatozoa to swim through mucus and penetrate the cumulus to reach the zona pellucida (ZP) (Suarez, 2016; Vadnais et al., 2007).

1.5.3 Sperm-oocyte interaction

Released spermatozoa continue their journey towards the ampullary-isthmic-junction, where fertilization takes place (Suarez and Pacey, 2006). Reaching the oocyte, the capacitated and hyperactive spermatozoa penetrate the layer of follicle cells and bind to the ZP (Figure 5). Specific zona-binding proteins on the sperm plasma membrane bind to ZP molecules on the surface of the ovulated egg. One of these ZP molecules, ZP3, initiates the acrosome reaction in sperm cells (Senger, 2012). A variety of hydrolytic enzymes are then released and will help the sperm cell tunnel through the ZP, reaching the perivitelline space. The plasma membrane of the spermatozoon and oocyte further fuse together and the cortical reaction takes place to prevent polyspermy. The final step of the fertilization process is the entrance of the sperm nucleus to the cytoplasm of the egg. Upon fertilization, the paternal chromatin is remodelled by the replacement of protamines with maternally derived histones. The decondensed paternal DNA expands, resulting in the formation of the paternal pronucleus. Fusion of the paternal and maternal pronucleus results in the diploid zygote, which ultimately will form a new individual organism (Champroux et al., 2018; Gadella and Luna, 2014).

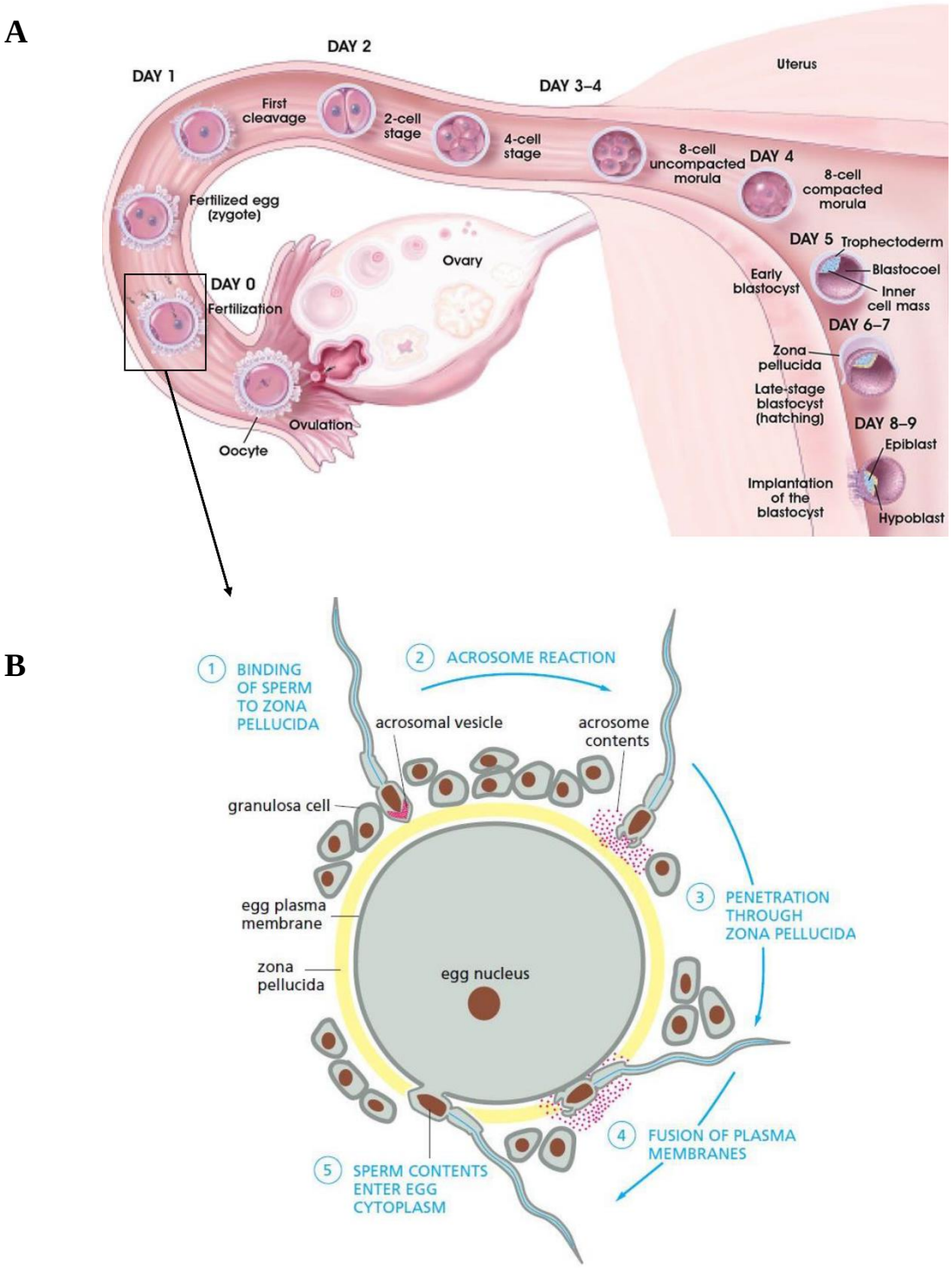

Figure 5. *Illustration of the main events during mammalian fertilization and early embryo development. A) Upon AI or natural mating, spermatozoa travel through the female reproductive tract towards the ampulla region of the oviduct, the site of fertilization. B) Capacitated spermatozoa can bind to the zona pellucida (ZP) of the egg (1), which permits the acrosome reaction to happen (2). The sperm cell releases its acrosomal content by exocytosis, which allows for penetration of the ZP (3) and fusion of the plasma membranes (4). The sperm nucleus enters the egg cytoplasm (5), where fusion of the paternal and maternal pronucleus results in a diploid zygote. The diploid zygote undergoes multiple mitotic divisions forming the morula, which further develops into the blastocyst. The blastocyst hatches before implantation and fetus development in the uterus (A). Modified from Alberts et al. (2008) and Hill (2020).*

1.6 Evaluation of bull fertility and quality of fertility data

In animal reproduction, fertility can be defined as the ability of the female to conceive and maintain pregnancy when served at the appropriate time in relation to ovulation. Several methods are available for measuring the reproductive performance of bulls, such as fertilization rate, conception rate, non-return (NR) rate and calving rate. The NR rate is commonly used in modern cattle reproduction management with large scale data (Pryce et al., 2004; Senger, 2012; Utt, 2016), and is calculated as the percentage of inseminated females that do not return to estrus within a stipulated time after AI (e.g. NR56 for 56 days) (Amann et al., 2018). Usually a difference near 20% between the NR rate and the actual prevalence of pregnancy is observed. This can be caused by several reasons including improper estrus detection, early embryonic death with irregular return to estrus interval, and management decisions not to rebreed (Amann et al., 2018; Utt, 2016). Due to the binominal nature of NR data (presented as either yes or no) the fertility estimates are largely affected by the number of AIs, and pooling of data is usually required to generate reliable average fertility calculations (Foote, 2003). It is important that non-bull factors that may influence the AI outcome, such as management and herd factors, AI personnel, season of AI and parity are carefully controlled (Amann and Hammerstedt, 2002; Coleman et al., 1985; Foote, 2003; Sellem et al., 2015). Furthermore, the credibility of field fertility data also depends on the number of AIs per bull and how the AI and its outcome is reported (Amann and Hammerstedt, 2002). As described previously, the practitioners performing AI in Norway are financially credited by Geno only when the AIs are registered in the database. Furthermore, recorded data must pass several quality controls before they are entered into the NDHRS. These procedures ensure the reliability of the recording system in Norway (Espetvedt et al., 2013).

1.7 Evaluation of semen quality

Fertility is a complex trait, depending on cellular and physiological processes in both the male and female. For years, researchers have attempted to develop tests that accurately predict the fertility potential of a semen sample. Still, there is no such test available. For the bovine AI industry, accurate timing of AI and appropriate herd management is also necessary to succeed (Sellem et al., 2015). A fertilization competent spermatozoon needs several attributes to be sufficiently expressed at the correct time and in the right environment. Some of the attributes essential for fertility are: intact plasma- and acrosomal membranes, adequate metabolism for energy production, progressive motility, capacity for hyperactive motility and a functional genome (Amann and Hammerstedt, 1993).

Bull fertility has traditionally been evaluated by test inseminations in the field (Hallap et al., 2004). Even though large insemination trials are considered reliable, these are both time consuming and expensive. It will be beneficial to the cattle breeding industry to have efficient laboratory tests that can predict the fertilization potential of semen samples by assessing *in vitro* sperm quality. Breeding companies will save both time and money, as the males with sub-optimal fertility can be eliminated from semen production and AI (Al Naib et al., 2011; Rodriguez-Martinez, 2006). Classical *in vitro* evaluation of semen quality at the AI stations, such as the assessment of sperm concentration, motility and morphology, is valuable for assessing the overall semen quality and for eliminating semen samples of poor quality. However, these methods are of limited value in assessing field fertility, i.e. select bulls with superior fertility from the more average fertility bulls (Hossain et al., 2011; Rodriguez-Martinez, 2006). Due to the complexity of the fertilization process and the fact that spermatozoa are highly differentiated cells with several specialized functions, combining sperm attributes that explains their ability to reach the oocyte, activate the oocyte, and sustain embryonic development has shown to be promising for prediction of male fertility potential (Kumaresan et al., 2017a). Flow cytometry and Computer Assisted Semen Analysis (CASA) are ideal techniques for high throughput and objective analyses of sperm functionality (Boe-Hansen and Satake, 2019).

1.7.1 Flow cytometry

Flow cytometry has become a common and widely used technique in veterinary and human andrology since its introduction in the 1970s (Pena et al., 2018). Initially this technique was focused on the analysis of sperm DNA (Meistrich et al., 1978; Van Dilla et al., 1977), and the first analysis of the chromatin structure of bull spermatozoa by flow cytometry was reported in 1980 (Evenson et al., 1980). Since then, multiple fluorescent probes have been introduced to study various sperm attributes, like the integrity of the plasma membrane, acrosome and mitochondrial function, DNA damage, capacitation status and level of reactive oxygen species (ROS) (Boe-Hansen and Satake, 2019; Pena et al., 2018). There is a continuous development of new fluorescent stains and techniques for flow cytometric evaluation of sperm cells.

By flow cytometry, a high number of cells can be analysed automatically at high speed in a short period of time (Gillan et al., 2005; McKinnon, 2018). In addition, multi-parametric flow cytometry assays may be conducted, where more than one sperm attribute can be assessed simultaneously. Thereby, the possibility to correlate sperm functionality to for example fertilization capacity increases. Furthermore, data from different subpopulations within a

sample can be obtained, which is important due to the heterogeneity of the semen sample (Boe-Hansen and Satake, 2019). Thus, analysis by flow cytometry is an objective and efficient method, with a high level of repeatability and sensitivity compared to classical microscopic analysis (Hossain et al., 2011).

The flow cytometer consists of one or more lasers emitting light at specific wavelengths, a flow cell where the cells are forced to pass through in a stream of single cells by the principle of hydrodynamic focusing, and several filters separating the emission signals. The sperm cells can be labelled with one or more fluorochromes of choice and used in either viable or fixed state (Gillan et al., 2005). As the labelled spermatozoa pass through the flow cell, the laser excites the fluorochromes. Photomultiplier tubes (PMT) or fluorescence detectors (FL) gather scattered and emitted light from particles and cells. The detected electrical signals are then transformed to a digital signal, which is transferred to a computer. Each PMT detects fluorescent light at a specific emission wavelength range, depending on the fluorescent filters and optical arrangement of dichroic mirrors. The final part of a flow cytometry experiment is the data analysis. Histograms are created for each single parameter, with the relative fluorescence from each FL plotted against the number of events. For multiple parameter approaches, two-dimensional diagrams plotting one parameter against the other in an X versus Y axis cytogram (dotplot) are used (McKinnon, 2018). Commonly, forward scatter (FSC) and side scatter (SSC) are used to identify and gate cells for size and granularity, respectively. By including unstained samples in the analysis, FSC/SSC can be used to identify the sperm population and gate out debris particles (Boe-Hansen and Satake, 2019). Computerized gating is also useful for selecting cell populations of interest and makes it possible to perform analysis on sub-populations within the semen sample. Some flow cytometers are based on the principle of electronic volume (EV) instead of FSC. Flow cytometers with EV for assessment of cell size are based on the Coulter principle, where the cell displaces an equivalent amount of liquid electrolyte as it passes through the flow cell. The displaced volume is measured as an electric pulse with a magnitude proportional to the cell volume. Identification of spermatozoa seems to be more accurate when using EV/SSC rather than FSC/SSC (Standerholen et al., 2014).

Plasma membrane integrity

One of the key elements for sperm quality and a necessity for successful fertilization is the intactness of the sperm plasma membrane, which often is referred to as sperm viability. The plasma membrane surrounds the entire spermatozoon, holding together its organelles and intracellular compartments. Most viability assays evaluate whether the plasma membrane is

intact or not. Non-viable cells can be identified by using membrane-impermeable nucleic acid stains like propidium iodide (PI), ethidium homodimer-1, Yo-Pro-1 or Hoechst 33258, that penetrate and bind to the DNA of cells with damaged membranes (Gillan et al., 2005). Viable cells can be identified using cell permeable fluorogenic esterase substrates like fluorescein diacetate (Matyus et al., 1984), carboxyfluorescein diacetate (Garner et al., 1986) and calcein asetomethyl ester (Donoghue et al., 1995). These dyes are non-fluorescent, but converted to fluorescent derivatives by intracellular esterase enzymes, and trapped by the intact plasma membrane, resulting in a fluorescing cell (Hossain et al., 2011). Today, membrane-permeant DNA fluorochromes like SYBR-14 (Garner and Johnson, 1995; Grundler et al., 2004; Zhou et al., 2010) and newly developed DNA stains, such as the SYTO® dyes (Boe-Hansen and Satake, 2019; Tárnok, 2008) are more commonly used. Fluorochromes for assessment of sperm viability by either one of the approaches are often used in combination. The most common combination is SYBR-14/PI, where a simultaneous estimation of the proportion of live (SYBR-14 positive) and dead (PI positive) cells is obtained. In addition, this combination gives information about the proportion of dying or damaged cells, represented as a moribund cell subpopulation (both SYBR-14 and PI positive cells). The SYBR-14/PI assay is especially useful for frozen-thawed semen with egg-yolk particles in the extender. Egg-yolk particles have scatter properties similar to those of sperm cells. Thus, elimination of non-sperm events by gating may be difficult, leading to a possible overestimation of viable cells (Nagy et al., 2003).

Acrosome integrity

Acrosome integrity is a prerequisite for fertilization, with prematurely acrosome-reacted spermatozoa being unable to penetrate the ZP. The most common method for acrosome integrity assessment is the use of fluorochrome-conjugated plant lectins that specifically bind to carbohydrate moieties of acrosomal glycoproteins. Labelling with plant lectins occurs only when the plasma membrane and outer acrosomal membrane is damaged (acrosome reacted sperm cells). *Pisum sativum* agglutinin (PSA) and *Arahis hypogaea* (peanut) agglutinin (PNA) conjugated with Fluorescein isothiocyanate (FITC) have been extensively used for assessing sperm acrosomal integrity (Boe-Hansen and Satake, 2019; Silva and Gadella, 2006). When evaluating spermatozoa diluted in egg-yolk, PNA is the lectin of choice, as PSA has shown a non-specific binding affinity to egg-yolk particles (Thomas et al., 1997). The lectin PNA comes from the peanut plant and binds to β-galactose moieties associated with the outer acrosomal membrane (Graham, 2001). During cell death, vesiculation of the plasma

membrane and acrosome occurs and results in what is termed a false acrosome reaction. The true acrosome reaction will only occur in viable and membrane intact spermatozoa (Didion and Graves, 1989). Thus, in order to identify the different sperm subpopulations within a semen sample it is important to simultaneously assess both sperm viability and the integrity of the acrosome (Kutchy et al., 2019; Nagy et al., 2003; Schulze et al., 2013; Standerholen et al., 2014; Thomas et al., 1997).

Chromatin integrity

The mammalian sperm chromatin has a unique compact structure, due to the replacement of histones by protamines during spermatogenesis. As sperm pass through the epididymis, their DNA is further stabilized by disulfide bonds. This complex packaging is fundamental for sperm DNA integrity and for protection of the paternal genome during transport through the male and female genital tracts (D'Occhio et al., 2007). However, sperm chromatin and DNA damage may occur and is recognized as one of the main causes of male infertility. Common causes of DNA fragmentation are defaults in the protamination process, apoptosis and insufficient protection against ROS (Hamilton and Assumpcao, 2020). Fertilization by a spermatozoon with DNA damage may affect embryonic development and survival, as the oocyte only is capable of repairing a certain degree of DNA damage (Johnson et al., 2011). Sperm DNA damage has been shown negatively correlated with fertility in several mammalian species including bulls (Gliozzi et al., 2017; Waterhouse et al., 2006), boars (Boe-Hansen et al., 2008; Myromslien et al., 2019), stallions (Morrell et al., 2008), mice (Li and Lloyd, 2020) and men (Evenson et al., 1999; Zeqiraj et al., 2018).

There is a variety of methods available to evaluate sperm chromatin integrity and the most used is the Sperm Chromatin Structure Assay (SCSA®). Alternative assays include the Terminal transferase dUTP nick end labelling (TUNEL) assay, the comet assay, the sperm chromatin dispersion (SCD) test and toluidine blue staining (Hekmatdoost et al., 2009; Martínez-Pastor et al., 2010). The pioneering work leading to the establishment of the SCSA® by Evenson et al. (1980) has led to hundreds of publications in animal and human reproduction, showing its usefulness for determining male fertility (Evenson, 2016). The susceptibility of DNA denaturation after incubation with an acid detergent is estimated by the assay (Evenson and Jost, 2001). The dye acridine orange (AO) is used for staining (Figure 6). The AO dye has metachromatic properties, which means that it expresses green fluorescence when attached to double stranded DNA (dsDNA, intact DNA) and red when attached to single stranded DNA (ssDNA, fragmented DNA). Upon denaturation, ssDNA is formed at sites

where the DNA is already broken. Thus, each sperm head yields a mixture of green and red fluorescence, depending on the degree of DNA fragmentation and the susceptibility of chromatin to denature. The damaged DNA is reported as DNA fragmentation index (DFI), calculated by the ratio of red fluorescence/total fluorescence (red + green). Furthermore, it is possible to estimate the amount of High DNA Stainable (HDS) sperm. The HDS sperm population in a semen sample has an unusually high amount of DNA staining due to an increased amount of retained histones and thus less chromatin condensation. These sperm cells are characterized as immature (Evenson, 2016; Evenson et al., 2002).

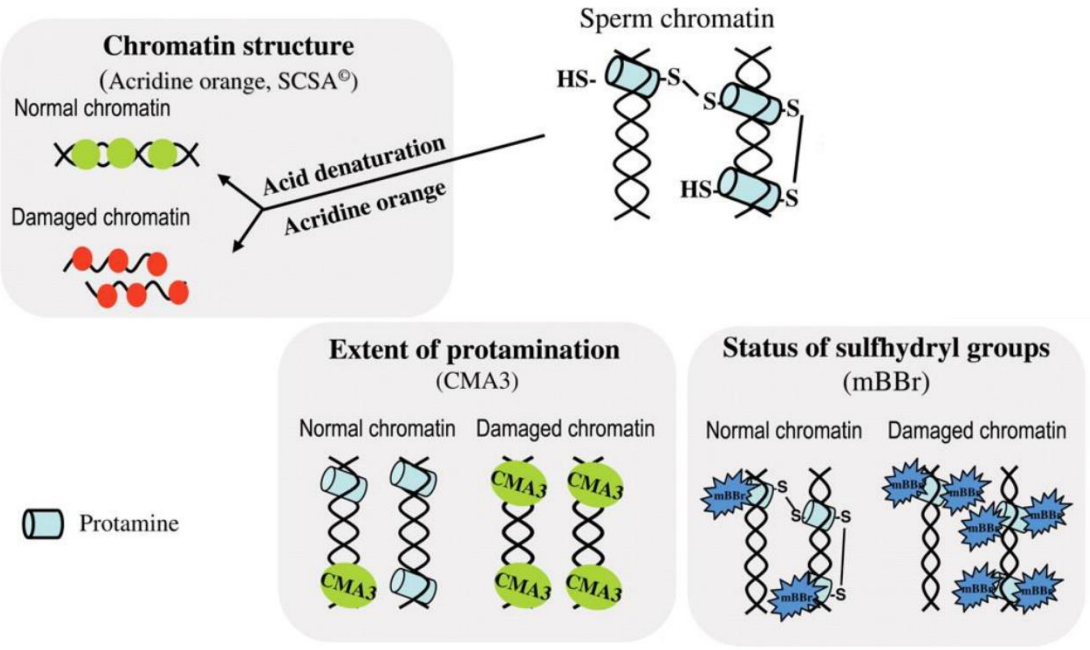

Figure 6. *Assays for sperm chromatin quality measurements. Sperm DNA is bound to protamines that contain disulfide bonds. The sperm chromatin structure assay, SCSA, measures the susceptibility of sperm chromatin to denaturation treatment. The dye fluoresces green when it binds to double-stranded DNA and red when it binds to single-stranded DNA. The amount of sperm protamines and chromatin compaction can be measured by the CMA3 and mBBr assays, respectively. Figure modified from Delbès et al. (2009).*

Chromatin integrity can also be assessed by the sperm protamine deficiency assay using the probe chromomycin A3 (CMA3) (Martínez-Pastor et al., 2010). As previously mentioned, sperm cells have a unique and highly compacted chromatin structure due to the histone to protamine transition. With the protamine deficiency assay, it is possible to assess the presence of protamines on sperm DNA. Protamines and CMA3 compete for the same DNA binding sites (Figure 6). Thus, a high level of CMA3 fluorescence indicates low protamination state of spermatozoa (Kumaresan et al., 2020). Sperm protamine deficiency has shown to be correlated with DNA damage in both bulls (Fortes et al., 2014) and men (Nasr-Esfahani et al., 2005). Furthermore, human studies suggest that protamine deficiency is associated with male

fertility (Ni et al., 2016; Zhang et al., 2006). The compactness of sperm chromatin is dependent on the number of disulfide bonds within and between protamines. The probe monobromobimane (mBBr) can be used in a thiol and disulfide bond assay to measure chromatin compaction (Figure 6). Combining the CMA3 and mBBr probes can give useful information regarding sperm protamination and the compaction of sperm chromatin (Martínez-Pastor et al., 2010). Recently, it was discovered that DNA fragmentation had a significant but weak positive correlation with free thiols and disulfide bonds of boar spermatozoa (Khezri et al., 2019). However, the relationship between sperm DNA integrity, protamine deficiency, the level of free thiol groups, semen quality parameters and infertility has not been fully established (Kumaresan et al., 2017b).

1.7.2 Sperm motility characteristics by CASA

Since the introduction of AI in cattle breeding, sperm motility has been one of the most widely used indicators of sperm quality and evaluation of male fertility. Traditionally, sperm motility is assessed by phase contrast microscopy, a method still widely used by cattle breeding centres. However, this is a subjective technique shown to be a poor predictor of fertility (Fitzpatrick et al., 2002; Liu et al., 1988). To minimize the subjectivity of the manual assessment of motility, Dott and Foster (1979) developed the first CASA system, that allowed for image capturing of spermatozoa and the analysis of their individual movement (Dott and Foster, 1979). Today, CASA provides an objective analysis with independent and detailed measurements on sperm motility in a sample, and most major andrology laboratories and AI stations have a CASA system (Boe-Hansen and Satake, 2019). The most commonly reported CASA parameters are curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN) and wobble (WOB) (Figure 7) (Mortimer, 2000). In addition, these parameter settings can be used to calculate total motility, progressive motility and hyperactivity.

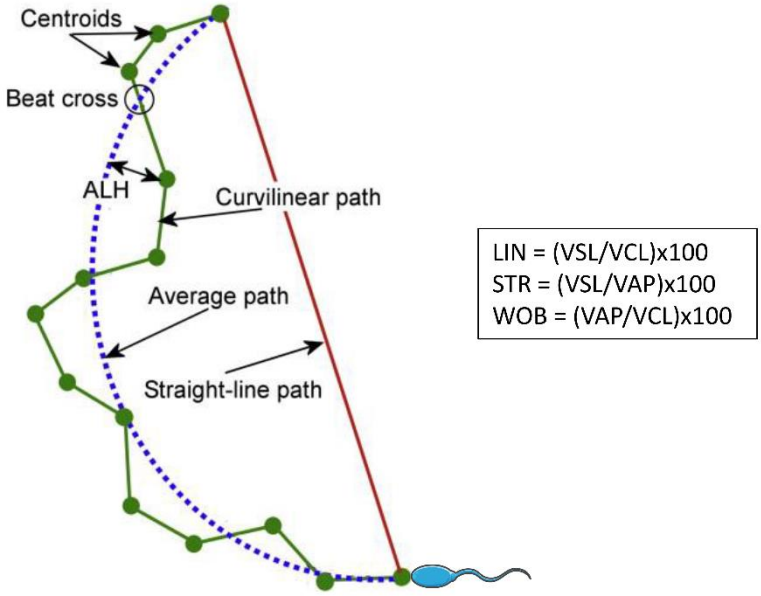

Figure 7. *Illustration of the motility characteristics important for sperm motility measurements by computer assisted sperm analysis (CASA). The centroids for each sperm head are recorded, and by using a path-finding algorithm, the lines and paths of the sperm across the field of view can be tracked. This results in a trajectory that provides the basic calculation of sperm motility characters and kinematics. The most commonly reported CASA parameters include curvilinear velocity (VCL;µm/s), average path velocity (VAP;µm/s), straight line velocity (VSL;µm/s), amplitude of lateral head displacement (ALH;µm/s), beat cross frequency (BCF; Hz), linearity (LIN), straightness (STR) and wobble (WOB). Figure modified from Amann and Waberski (2014).*

The CASA instrumentation commonly consists of a video camera connected to a microscope, a video capture card and a computer. The basic principle behind CASA is that the sperm cells are detected and visualized as white sperm heads on a dark background by using dark field/ negative phase contrast microscopy. Brightness of the sperm head is utilized to establish centroid positions in successive fields, which are identified and recorded by the system. The video camera records multiple microscopy frames of each sperm cell's track based on number of pixels covered by the sperm head and the number of frames. By using a path-finding algorithm connecting the centroids for individual sperm heads, the lines and paths of the sperm across the field of view can be tracked, and the sperm motility characters and kinematics can be calculated (Kathiravan et al., 2011; Mortimer, 2000). It is important to evaluate and standardize the set-up of the CASA system prior to analysis, and the technician should adjust the settings according to species-specific estimates. In order to successfully track sperm cells, the operator can select the number of frames analysed per second and the total number of analysed frames. Other important set-ups are intensity and size values, which are essential to discriminate sperm heads from debris particles resembling spermatozoa in pixel size. This is a potential source of error and is common when using semen extended with egg-yolk.

Furthermore, dilution medium, specimen chamber, imaging hardware and software, sperm concentration and technician, are factors that may affect accuracy and precision of the output values (Amann and Waberski, 2014; Kathiravan et al., 2011). If the density of sperm cells in the semen sample is too high, the spermatozoa may collide, change their trajectories and give incorrect kinematic computations. Thus, it is recommended to apply a diluted semen sample with sperm concentration below 40 x 10^6 cells/mL for CASA analysis (Mortimer et al., 1995).

Motility analysis by CASA allows for assessing the motility of individual spermatozoa, which generate large datasets. These datasets can be analysed using complex statistical methods such as cluster analysis, which group the spermatozoa into biologically relevant subpopulations (Amann and Waberski, 2014). Once identified, each subpopulation is characterized according to its average kinematic variables. For instance, a subpopulation with high velocity variables and high linearity variables can be defined as "fast, linear". Then, the frequencies of the subpopulations are used, rather than the mean of the kinematic variables themselves (Martínez-Pastor et al., 2011). Evaluation of sperm motility alone cannot provide an accurate prediction of the fertilizing potential of sperm in a semen sample. However, CASA can provide information important for sperm quality assessment and for research aimed at unravelling the diversity of sperm responses to changes in their microenvironment (Amann and Waberski, 2014).

1.7.3 Assessment of ATP levels in semen

Sperm flagellar movement is an adenosine triphosphate (ATP) dependent process driven by dynein proteins that causes sliding of microtubules. It is estimated that the majority of ATP (75%) produced by bovine spermatozoa is used to support motility (Bohnensack and Halangk, 1986). ATP can be formed by the two metabolic pathways of oxidative phosphorylation and glycolysis, both shown to occur in bovine sperm cells (Krzyzosiak et al., 1999). While oxidative phosphorylation takes place in mitochondria in the mid-piece of the sperm tail, glycolysis takes place in the head and principle piece of the flagellum. Oxidative respiration results in more ATP per mole glucose than glycolysis (Garrett et al., 2008). However, there is a disagreement as to which method of ATP production that is primarily used by the spermatozoa during fertilization (du Plessis et al., 2015). In addition to motility, mammalian spermatozoa use ATP to maintain the intracellular milieu and for other cellular processes such as capacitation, hyperactivation and the acrosome reaction (du Plessis et al., 2015). Although studies on the importance of ATP levels for sperm fertilization potential have shown contradictive results, sperm ATP production in bovine semen, based on both oxygen

consumption and lactate production, has been shown associated with NR rates (Garrett et al., 2008). A common way to determine the ATP content in semen samples is by bioluminescent assays or kits (Berg et al., 2018; Boulais et al., 2017; Li et al., 2016; Tremoen et al., 2018). An example of such an assay is the CellTiter-Glo® Luminescent cell-viability assay from Promega, which is based on the measurement of ATP in an ATP dependent luciferase reaction. Luciferin is added to the lysed cell sample and is converted to oxyluciferin by the ATP-dependent Ultra-Glo™ Luciferase. The reaction creates a light signal proportional to the number of living cells that can be measured by a luminometer, permitting direct quantitation of ATP (McElroy and DeLuca, 1983). The data recorded (measured in relative luminescence unit (RLU)) is converted to the corresponding ATP value in nM using a standard curve.

1.8 *In vitro* production of bovine embryos

The first human IVF baby was born in 1978, and since then the technology has been applied in several other species, including cattle (Sirard, 2018). In the late 1980s, the first calves obtained exclusively by *in vitro* embryo production (IVP) were reported (Goto et al., 1988). The process of IVP involves *in vitro* maturation (IVM) of oocytes to the matured metaphase II stage, IVF and *in vitro* culture (IVC) of embryos to the desired stage of development, usually the blastocyst stage (Ferre et al., 2019; Wrenzycki, 2018). Over the past decades, bovine IVP has been improved by an increased understanding of gamete and embryo requirements, metabolism, and pre-implantation embryonic development (Ferre et al., 2019). Today, IVP of bovine embryos is widely implemented in cattle breeding and production, particularly in South America, North America and Europe. The implementation of ultrasound-guided ovum pickup, semen sexing and GS has opened new possibilities for the selection of oocyte donors and embryos (Ferre et al., 2019; Stroebech et al., 2015). This has increased the application of commercial IVP (Sirard, 2018), and in 2016 the number of transferable IVP embryos exceeded those derived *in vivo* (Ferre et al., 2019). By using SNP chips, it is possible to screen thousands of genetic markers from small biological samples, such as ear skin biopsies or embryo biopsies. In Norway today, genomic selected heifers are used for embryo production and recipient heifers or cows become mothers to potentially elite bulls. This is part of Geno's breeding goal for increased breeding progress (Geno, 2020). By introducing GS even earlier, at the pre-implantation embryo stage, further genetic gain and progress can be achieved (Hall et al., 2013; Kasinathan et al., 2015). However, the procedures of IVP, embryo biopsies, and the subsequent cryopreservation of embryos all have to be successful in order to succeed with this in a commercial perspective. As many of these processes are highly complex, it is still a

challenge for the breeding companies to implement and commercialise the technology (Sirard, 2018).

Oocytes for IVP can be obtained by ultrasound-guided follicular aspiration in live animals or post-mortem by follicular aspiration of ovaries collected from the slaughterhouse. Oocytes are usually aspirated from antral follicles of 2 to 8 mm in size (Ferre et al., 2019). Completion of both nuclear and cytoplasmic changes during oocyte maturation is a prerequisite for successful fertilization and pre-implantation development (Eppig, 1996). In cattle, maturation occurs in the pre-ovulatory follicle within the last 24 h before ovulation (Hall et al., 2013). In addition to oocyte maturation, successful IVF requires appropriate sperm selection, capacitation and IVF media (Wrenzycki, 2018). Sperm separation procedures such as Percoll and BoviPure® centrifugation are important for the IVF outcome. Motile spermatozoa are separated from non-motile, and seminal plasma, debris, cryoprotective and infectious agents are removed (Centola et al., 1998; Samardzija et al., 2006). The sperm cells must also be treated with capacitating factors such as heparin, in order to penetrate the ZP (Parrish et al., 1986). Upon fertilization, major activation of the embryonic genome results in the first lineage segregation into trophoblast and inner cell mass. This occurs usually at the 8-cell stage in cattle, and is accompanied by changes in the chromatin structure (Memili and First, 1999). Blastulation occurs at day 7-8, followed by hatching from the ZP around day 8-9 (Hall et al., 2013). *In vitro* produced embryos both have different morphology and behave differently from *in vivo* derived embryos, and they have reduced capacity to survive cryopreservation due to altered cellular and molecular properties. Improvements of culture media have therefore been important to increase embryo development and survival after cryopreservation (Block et al., 2010; Sirard, 2018). One important finding has been that serum supplementation is one of the main causes of large offspring syndrome (van Wagtendonk-de Leeuw et al., 2000). Although the procedures for IVP have been improved over the years, an average of only 30-40% *in vitro* matured oocytes reach the blastocyst stage (Ferre et al., 2019; Meirelles et al., 2004). This may be caused by several factors including the follicular status from which the oocyte is obtained, incomplete cytoplasmic oocyte maturation and poor culture conditions, leading to impaired embryonic genome activation (Sirard et al., 2006).

The procedure of IVP has been investigated as a possible tool to predict male fertility, but there are inconsistent results whether the resulting cleavage and/or blastocyst rates correlate with bovine field fertility or not. In a study by Zhang et al. (1997), field fertility measured as NR56 was found to be positively correlated with both cleavage and blastocyst rate. However,

they also observed large individual variations between the bulls. Al Naib et al. (2011) observed that sperm from high fertility bulls tended to have higher cleavage rates compared to bulls of lower field fertility. However, the further development into blastocysts was not significantly affected by the bulls fertility status (Al Naib et al., 2011). In contrast to these studies, Kropp et al. (2017) did not find any significant correlation between field fertility and cleavage rate nor blastocyst rate. The conflicting results between studies are likely caused by variations in IVP procedures across the different research groups. Moreover, the number of ejaculates used per bull and how the field fertility data are collected and presented may contribute to the inconsistent results (Larsson and Rodríguez-Martínez, 2000). Whether *in vitro* embryo production can be correlated to field fertility or not, it is still a useful tool for assessing the effect of different sperm treatments on embryo quality. For instance, IVP has been used to study the effect of sperm oxidative stress and DNA quality (Fatehi et al., 2006; Simoes et al., 2013; Wyck et al., 2018), and different cryopreservation techniques (Anzar et al., 2019; Seshoka et al., 2016).

1.9 Finding new fertility markers in bull semen

For years, the paternal contribution to fertilization and the developing embryo was underestimated, and most of the research focused on the female. It is now well understood that the sperm cell not only delivers DNA to the oocyte, but rather a package including transcription factors, epigenetic modifications, RNAs and cell signalling molecules (Krawetz, 2005; Kropp et al., 2017). With the introduction of the 'omics' era, the search for novel male fertility and infertility biomarkers increased drastically. Technologies such as transcriptomics, proteomics, metabolomics, genomics and epigenomics have made it possible to identify fertility associated biomarkers, with the potential to improve the reproductive efficiency of livestock. Each of these fields offers the possibility to understand the molecular events linked to sperm physiology, which are important as they serve as the fundament for identification of factors associated with male reproductive performance (Carrell et al., 2016; Long, 2020).

1.9.1 Metabolomics

Metabolomics refers to the systematic identification and quantification of small, low molecular weight metabolites (<1500 Da), such as organic acids, amino acids, amines, lipids, nucleosides, vitamins and minerals (Xiao et al., 2012; Zhao et al., 2018). Metabolomics investigations can be categorized as either targeted or untargeted. The targeted approach focuses on the analysis of specific metabolites known to be related to certain metabolic pathways, while the untargeted approach is a method for global detection and quantification

of metabolic changes (Xiao et al., 2012). Metabolites are present both in seminal plasma and spermatozoa and may affect downstream changes in gene and protein expressions, thus contributing to the regulation of bull fertility (Velho et al., 2018). Metabolomics has been utilized to identify potential fertility and infertility biomarkers in both seminal plasma and sperm cells from different mammalian species, including bulls (Kumar et al., 2015; Menezes et al., 2019; Velho et al., 2018) and men (Qiao et al., 2017; Zhao et al., 2018). Velho et al. (2018) identified 63 metabolites in bull seminal plasma and suggested that 2-oxoglutaric acid and fructose could be potential biomarkers of bull fertility. Later the same group reported 22 distinct metabolites in sperm cells, where the five metabolites gamma-aminobutyric acid, carbamate, benzoic acid, lactic acid, and palmitic acid were statistically different between groups of high and low fertility bulls (Menezes et al., 2019; Velho et al., 2018).

The main biochemical compounds found in bovine sperm cells and seminal plasma are amino acids and peptides. Amino acids of seminal plasma and sperm have a variety of functions, including reducing free radicals and protect against oxidative stress, inhibit lipid peroxidation, and modulate osmotic mechanisms. In addition, amino acids play an important role in metabolic processes involved in sperm maturation, energy metabolism, motility, and viability (Cheah and Yang, 2011; Ugur et al., 2019a). In bull seminal plasma, the amino acids citrate, tryptamine, isoleucine, and leucine have been suggested as potential fertility markers (Kumar et al., 2015). Sperm cells and seminal plasma are also rich in trace elements, which may influence semen quality and the subsequent male fertility. Trace elements are necessary for the activities of many enzymes in metabolic pathways, and play a crucial role in biological processes like testicular development, spermatogenesis, sperm motility, and fertilization (Kasperczyk et al., 2015; Wong et al., 2001). While some elements such as calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), selenium (Se), and zinc (Zn) have been shown to be essential for reproduction and can protect sperm against oxidative stress, other elements such as aluminium (Al), lead (Pb), nickel (Ni), and arsenic (As) may have an adverse effect on reproduction (Aguiar et al., 2012; Kasperczyk et al., 2015; Klein et al., 2014). In addition, alterations in concentrations of trace elements, outside an optimal range, is likely to have a negative effect on semen quality. For instance, excess levels of Cu and Fe have a toxic effect on spermatozoa due to pro-oxidative properties (Aydemir et al., 2006).

Due to the complexity of the metabolome and the variation among metabolites in molecular weight, polarity, and solubility, it is impossible to utilize a single method for analysing all metabolites simultaneously. Therefore, it is necessary to combine data from multiple

instruments (Putri et al., 2013). Multiple analytical platforms are available for the study of metabolites. Among them, mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the most applied. The growth in metabolomics during the last years has been driven by the development of MS (Alonso et al., 2015). Mass spectrometry offers a quantitative analysis of metabolites with high sensitivity and selectivity. Different chromatography techniques can be coupled with MS, such as gas chromatography (GC) and liquid chromatography (LC). By combining LC to MS (LC-MS) a wide range of metabolites can be identified and quantified. Due to the high throughput, soft ionization, and good coverage of metabolites, there has been an increased popularity for LC-MS as the platform for metabolomic studies (Putri et al., 2013; Zhou et al., 2012). Inductively coupled plasma mass spectrometry (ICP-MS) is one of the most sensitive, precise and accurate methods for determination of trace elements in biological samples such as semen (Aguiar et al., 2012). Surprisingly, the number of studies using ICP-MS for detection of trace elements in livestock biological samples are scarce, even though trace elements as micronutrients are highly important for animal health and productivity.

1.9.2 Epigenetics

Epigenetics is the study of heritable changes in gene expression caused by modifications, without altering the underlying DNA sequence (Champroux et al., 2018; Feil, 2006). Epigenetic modifications include DNA methylation, post-translational modifications (PTMs) of histones, noncoding RNAs (ncRNAs) and chromatin remodelling (Figure 8). These processes alter gene expression by either activating or inactivating genes, which can be either beneficial or detrimental to the organism (McSwiggin and O'Doherty, 2018; Triantaphyllopoulos et al., 2016). The epigenome is dynamic throughout life and is affected by different environmental exposures that may lead to altered phenotypes (Bernstein et al., 2007; Triantaphyllopoulos et al., 2016). Epigenetic studies have the potential to elucidate factors that might improve the breeding of cattle, as it is possible to retrieve information regarding the heritability of diseases and complex traits, such as male infertility. The transmission of epigenetic information from one generation to the next, leading to phenotypic variation without direct environmental influences, is termed transgenerational epigenetic inheritance (Feeney et al., 2014).

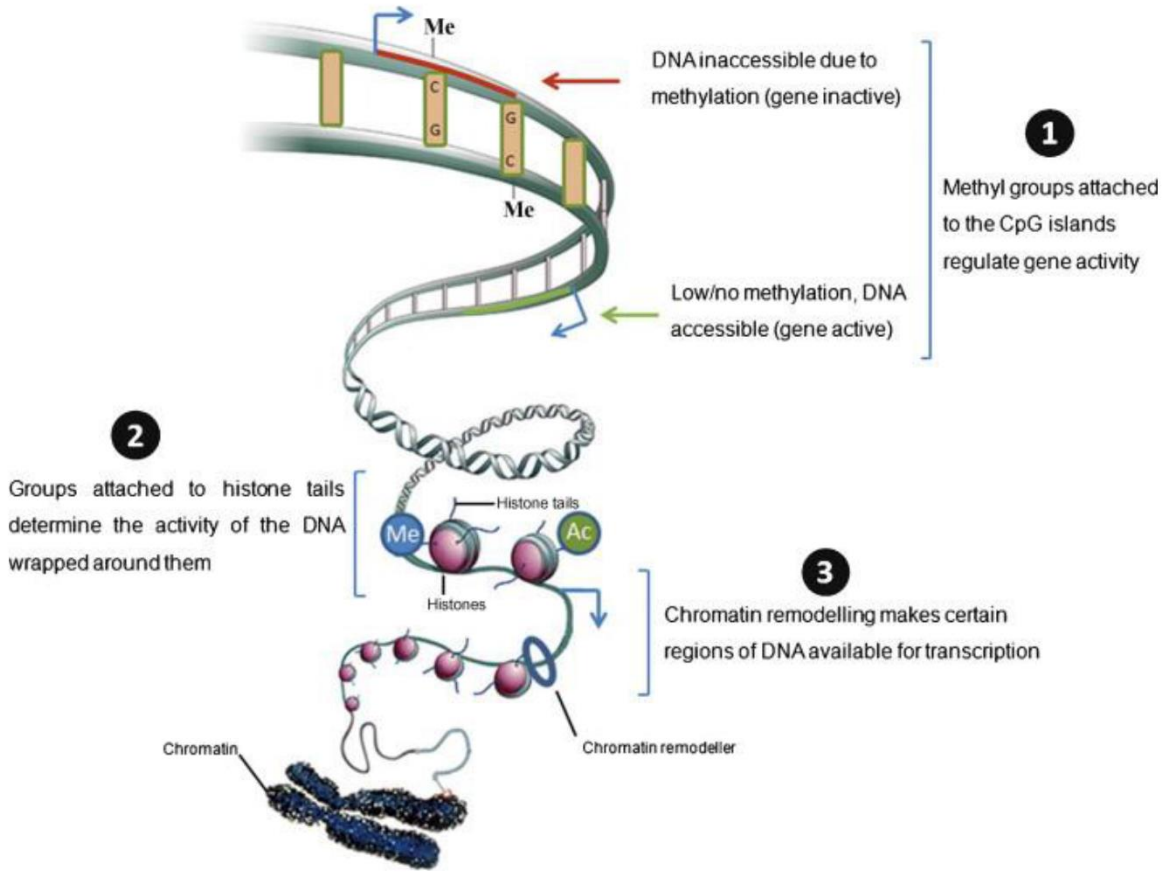

Figure 8. *Illustration of the major epigenetic mechanisms affecting gene activity, including DNA methylation (1), histone modifications (2) and chromatin remodelling (3). These epigenetic modifications alter gene expression by either activating or inactivating genes. Figure taken from Rajender et al. (2011).*

DNA methylation is essential to mammalian development and it is the epigenetic modification that is most thoroughly studied (Smith and Meissner, 2013). During DNA methylation a methyl group is added to the 5th carbon of cytosine, resulting in the formation of 5-methylcytosine. Cytosine methylation in mammals occurs generally at cytosine residues that are followed by a guanine residue (CpG dinucleotides). Regions that possess high CpG density are termed CpG islands (CGI) with generally low levels of methylation (hypomethylation). Other CpGs (in a non-CGI context) are usually hypermethylated (Messerschmidt et al., 2014; Zhou et al., 2016). Hypermethylation of DNA in CGIs is associated with shutdown of gene expression or transcriptional silencing, while hypomethylation in these regions is associated with gene expression (Figure 8) (Rajender et al., 2011). DNA methylation is catalysed by a family of DNA methyltransferases (DNMTs), where DNMT3A, DNMT3B, and DNMT3L are responsible for *de novo* methylation, while DNMT1 is involved in methylation maintenance (Champroux et al., 2018). The degree of DNA methylation is different in sperm cells and oocytes, and in bovine gametes, sperm cells are found to be highly methylated compared to

oocytes (Duan et al., 2019). Two major reprogramming events occur during mammalian development, both involving genome-wide erasure (de-methylation) and re-establishment of DNA methylation patterns (*de novo* methylation). The first occurs in primordial germ cells and the second during embryo development (Prokopuk et al., 2015). When primordial germ cells enter the genital ridge, a genome-wide demethylation occurs, which is followed by *de novo* methylation to establish the new sex-specific imprints during gametogenesis. In the male, the reestablishment of DNA methylation takes place in mitotically arrested pro-spermatogonia and is completed before birth (McSwiggin and O'Doherty, 2018; Moore and Oakey, 2011). Following fertilization and during embryo development, both the paternal and maternal methylation marks are erased and replaced by embryonic marks important for appropriate development. *De novo* methylation, occurring at the 8- to 16-cell stage, establishes the basic somatic methylation pattern (McSwiggin and O'Doherty, 2018; Triantaphyllopoulos et al., 2016).

DNA methylation has emerged as a promising indicator of male infertility (Kumaresan et al., 2020). Studies of sperm DNA methylation in bulls (Kropp et al., 2017), buffalos (Verma et al., 2014) and men (Alkhaled et al., 2018) have shown that DNA methylation signatures differ between individuals of contrasting fertility status. This is promising for the search for biomarkers in bovine semen that can be used to evaluate sperm quality and predict bull fertility. There are several techniques available for analysis of DNA methylation. One of the most applied techniques involves PCR amplification and sequencing of bisulfite-converted genomic DNA (Meissner et al., 2005). Bisulfite treatment converts unmethylated cytosines into uracil while the methylated cytosines are unchanged (Frommer et al., 1992). Bisulfite-based strategies coupled with DNA sequencing allow quantitative, site-specific methylation analysis, and can both be done using the whole genome or a reduced, specific subset of the genome. Whole-Genome Bisulfite Sequencing (WGBS) requires sufficient read depths to reliably determine methylation status, and the sequencing costs are high for organisms with large genomes such as mammalian species (Doherty and Couldrey, 2014). With reduced representation bisulfite sequencing (RRBS) it is possible to do a genome wide methylation analysis with reduced sequencing requirements, thus lowering the costs. RRBS allows for preferential selection and sequencing of CpG-rich regions (CGIs) by adding restriction enzymes (typically *Msp*I) to digest the DNA during the fragmentation step. *Msp*I cuts at 5'-CCGG-3' sites, and since the genome generally has low levels of CpGs except for promoters/CpG islands, the RRBS methodology is largely capturing only these regions for

further analysis (Doherty and Couldrey, 2014; Meissner et al., 2005). Analysis of DNA methylation by RRBS has been conducted for bovine somatic tissues and sperm cells (Khezri et al., 2020; Perrier et al., 2018; Zhou et al., 2016).

2. Aims of the thesis

The main aim of this PhD project was to elucidate underlying factors influencing fertility in modern, efficient livestock production. Furthermore, it was desirable to find suitable sperm quality parameters/biomarkers related to bull fertility and/or maturation that might be implemented in breeding systems to select young superior bulls for semen production and AI. The study population for this project was semen samples from Norwegian Red bulls in regular semen production, and fertility data obtained from the national AI recording database and the NDHRS. The following studies were designed to reach the aims of the thesis:

- Evaluate sperm quality parameters, semen metabolite levels and fertility in young Norwegian Red bulls of 14 and 17 months of age (Paper I).
- Examine the DNA methylation patterns of sperm cells obtained from bulls at 14 and 17 months of age and investigate if the DNA methylome of young bulls provide additional information to age related sperm quality differences (Paper II).
- Develop a method for investigating intracellular metabolites in the viable sperm population of frozen-thawed semen samples (Paper III).
- Investigate the relationship between sperm quality parameters, sperm intracellular metabolites and field fertility, expressed as NR56, using frozen-thawed semen samples from Norwegian Red bulls of contrasting fertility (Paper III).
- Reveal a suitable prediction model that can help explain the variation in NR56 observed between AI sires (Paper III).
- Investigate the relationship between chromatin integrity and bull fertility by studying differences in chromatin integrity parameters, *in vitro* embryo development, and sperm DNA methylation signatures between Norwegian Red bulls of contrasting NR56 (Paper IV).

3. Results: summary of individual papers

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

In this study, we aimed to evaluate sperm quality parameters, seminal metabolites, semen production capacity and fertility in young Norwegian Red bulls. Ejaculates were collected from 25 bulls, both at 14 and 17 months of age, while semen production and field fertility data were gathered for all bulls in production from December 2017 throughout 2019. The results showed that the proportion of hyperactive spermatozoa and the kinematic parameters VAP, VCL and ALH significantly increased with age in both fresh and frozen-thawed semen samples. Furthermore, 14 amino acids in seminal plasma and 10 amino acids in sperm cells were significantly different between the two age groups. This included arginine, glutamine, cysteine and proline, reported involved in protection against lipid peroxidation and oxidative stress, sperm motility and glycolysis. The levels of the trace elements K and Ba in seminal plasma and sperm cells were significantly higher in samples collected at 17 months compared to at 14 months. Sperm concentration and volume of the ejaculate increased significantly with increasing age, while the percentage of discarded batches decreased. The bulls' field fertility (NR56) were to a limited extent influenced by age (0.75 vs. 0.74). However, 1% unit in difference is unlikely to be of biological importance. These results showed that several sperm attributes, including amino acids and trace elements are potential parameters to differentiate between young bulls, even though the age difference was only three months. However, the results further confirm that young peri-pubertal bulls are mature enough for their semen to fulfil successful fertilization. Reduced semen production efficiency in young bulls is a challenge, and it would be beneficial for the AI industry to identify biomarkers in semen that can predict bull maturity and subsequent reproductive performance.

Paper II: Sperm DNA Hypomethylation Proximal to Reproduction Pathway Genes in Maturing Elite Norwegian Red Bulls

The objective of this study was to assess sperm chromatin integrity, viability, motility and ATP content in fresh and frozen-thawed semen samples from nine Norwegian Red bulls at both 14 and 17 months of age. In addition, reduced representation bisulfite libraries constructed according to two protocols, the Ovation® RRBS Methyl-Seq System and a previously optimized gel-free method, were sequenced to study sperm DNA methylation in frozen-thawed semen samples from seven of these bulls. The main aim was to determine if sperm DNA methylation could provide additional information to age related sperm quality differences. The results showed that fresh semen samples collected from the bulls at 17 months of age had significantly higher sperm concentration, total motility and progressive motility compared to the samples collected at 14 months. In both fresh and frozen-thawed semen, the percentage of DNA fragmented sperm cells significantly decreased with increasing age. Libraries from the Ovation method exhibited a greater percentage of read loss and shorter read size following trimming. Therefore, it was decided to conduct downstream analyses using RRBS libraries constructed based on the gel-free optimized method. Similar global sperm DNA methylation, but differentially methylated regions (DMRs) were revealed between the two age groups. Pathway analysis showed that genes annotated with DMRs having low methylation differences (less than 10%) and DMRs having between 10 and 25% methylation differences, could be associated with important hormonal signalling and sperm function relevant pathways, respectively. Thus, although the global sperm DNA methylation levels were similar in samples collected from bulls at 14 and 17 months of age, this study shows that regions with DNA methylation differences can be identified and linked with pathways and genes important for sperm quality and maturation.

Paper III: Differences in sperm functionality and intracellular metabolites in Norwegian Red bulls of contrasting fertility

The relationship between sperm functionality parameters, sperm intracellular metabolites and field fertility of 37 Norwegian Red bulls was investigated. In order to assess intracellular metabolites in viable sperm cells from frozen-thawed semen samples, a novel method was developed prior to this study. Based on retrospective field fertility data (NR56), 18 bulls with inferior fertility and 19 bulls with superior fertility were selected for the study. The associations between sperm parameters/metabolites and fertility were assessed using unpaired t-test and Pearson correlation analysis. Forward stepwise multivariate regression analysis was conducted to determine which combination of parameters that best could explain the bulls NR56. The chromatin integrity parameters, DFI and HDS, correlated negatively with NR56, whereas several sperm motility parameters correlated positively with NR56. The intracellular sperm concentrations of cysteine and glutamic acid correlated negatively with NR56, while the concentrations of aspartic acid, leucine and serine showed a positive NR56-correlation. The concentration of the trace elements Fe, Al and Zn, correlated negatively with NR56. Significant correlations were also found between several metabolites and sperm functional attributes. The regression analysis revealed that the best predictor of NR56 was a model containing DFI, aspartic acid, Fe and Zn. This model explained 59% of the variability in NR56. In conclusion, bulls of high and low NR56 differed in several aspects of sperm functionality and metabolome characteristics. The multiple correlations between metabolites and sperm parameters, as well as the correlation with NR56 indicate that a convergence of these different technologies can increase the knowledge of factors influencing and predicting bull fertility.

Paper IV: Sperm chromatin integrity and DNA methylome in Norwegian Red bulls of contrasting fertility

In this study, the relationship between different sperm chromatin integrity parameters, *in vitro* embryo development, sperm DNA methylation signatures and bull fertility was investigated. Frozen-thawed semen samples from 37 sires with contrasting fertility levels, expressed as NR56, were used for the analyses of sperm chromatin integrity. Protamine deficiency, thiols and disulfide bonds were assessed and compared to previously published data for DFI and HDS (Paper III). *In vitro* embryo development and sperm DNA methylation profiles were assessed using semen samples from 16 of the 37 bulls. The percentages of DFI and HDS correlated negatively with bull fertility and positively with sperm protamine deficiency ($p < 0.05$). Significant differences in cleavage and blastocyst rates were observed between the fertility groups ($p < 0.05$). However, once fertilization occurred, further development into blastocysts did not show any association to the bulls' NR56. While the global CpG methylation levels were similar between the groups of high and low fertility bulls, the differential methylation analysis showed that sperm samples from bulls of low NR56 were hypermethylated compared to sperm from bulls of high NR56. Pathway analysis showed that genes annotated with differently methylated cytosines could participate in different biological pathways having important biological functions related to bull fertility. In conclusion, this study shows that sperm DNA integrity is significantly associated with field fertility and *in vitro* fertilization capacity of Norwegian Red bulls. Spermatozoa of low-fertile bulls are hypermethylated compared to those of high-fertile bulls. Genes annotated with differentially methylated cytosines were identified as participants in different biological pathways important for bull fertility.

4. Discussion

4.1 Methodological considerations

4.1.1 Flow cytometry

Flow cytometry is an objective method for the assessment of multiple sperm attributes, and provides several advantages such as high sensitivity, repeatability and speed (Graham, 2001). However, the instrument set up, technician and proper sample preparation are factors important to obtain reliable results. For each flow cytometry experiment (Paper I-IV), optical alignment using uniformly fluorescent beads (Flow-Check™ Fluorospheres, Beckman Coulter Ltd) was conducted. Furthermore, the same technician conducted all flow cytometry analyses within the same procedure. Proper experimental controls are also important, and unstained sperm samples were included as negative controls for each experiment. The flow cytometry procedures used in this thesis were well-established protocols, and further optimization of fluorochrome concentrations, incubation time and inclusion of single stained samples for PMT adjustments were therefore not necessary.

The semen samples analysed during this thesis work were diluted with a semen extender containing egg-yolk. Egg-yolk particles are of comparable size to spermatozoa and may therefore be incorrectly included in the flow cytometry analysis, which may cause an overestimation of the proportion of unstained cells (Petrunkina and Harrison, 2010). For the assessment of sperm viability/plasma membrane integrity, it is argued that including a membrane impermeable dye like PI alone is not sufficient, as PI only detects dead cells and may cause mis-estimation of the cell populations. It is recommended to include a sperm selection marker, such as SYBR14, to identify non-sperm particles by their lack of SYB14/PI staining. In study I, PI was included alone for the analysis of sperm viability, due to the original design of the study where PI was combined with an oxidative stress marker (CellROX green). Because of large variations in the results for the oxidative stress probe, it was decided that the results could not be published. This is a weakness of study I, however, we believe that the published viability results can be considered as reliable. The Cell Lab Quanta^{TM} is based on EV instead of FSC, and it has previously been shown that the inclusion of a sperm identification marker is unnecessary for the Quanta flow cytometer as EV is more sensitive in identification of spermatozoa (Standerholen et al., 2014). Furthermore, the reliability of our PI data has been verified from both previous studies (unpublished data), and by positive and negative control samples (not shown).

The Cell Lab Quanta™ flow cytometer is equipped with only one laser (488 nm argon laser) as light source for excitation, and three detection channels. This caused some constrains regarding fluorochromes and number of combined markers that could be used for the studies. For analysis of DNA fragmentation, the well-established and patented SCSA® protocol was followed as described in detail by Evenson and Jost (2001). Other methods available for analysis of DNA fragmentation like the TUNEL, SCD and comet assays, all have variations in their protocols. Thus, the SCSA is claimed to be the only reliable method for data comparison between studies (Evenson, 2016). Through hundreds of publications in different mammalian species, the SCSA has been validated as a highly useful test for determining male reproductive performance. The SCSA has mainly been used to determine the percent of sperm cells with fragmented DNA (% DFI). However, the method is unique compared to other assays as it also allows for the evaluation of HDS (Evenson, 2016).

For the analyses of protamine deficiency, thiols and disulfide bonds (paper IV), cryopreserved semen samples were analysed by the Swedish University of Agricultural Sciences (SLU). To avoid ejaculate differences, the straws shipped to SLU were from the same batches as the straws analysed in our lab. The levels of thiols and disulfide bonds were analysed using mBBr. However, the protocol for mBBr staining was initially modified since these analyses were performed with a FACSVerse flow cytometer (FACSVerse, BD Biosciences, Franklin Lakes, NJ), using FSC/SSC for selection of the sperm cell population. To avoid the interference of egg-yolk particles in the analysis, PI was used as a sperm cell marker. This was possible as the sperm cells were fixated and dead, hence they could all be stained by PI. However, it was observed that PI quenched the mBBr-fluorescence. Therefore, each sample was measured twice, both with and without PI, and the PI data was used only as a further control to discriminate debris from spermatozoa and was not used for quantitative purposes.

4.1.2 Computer-assisted sperm analysis

Sperm cell motility is considered an essential parameter for the evaluation of semen quality and is routinely used in the control program at breeding stations. Today, subjective motility evaluations are largely replaced by CASA, which is an objective method for the analysis of sperm motion (Gliozzi et al., 2017). Even though CASA is reported as an objective method, there are several factors affecting the repeatability of the results, such as chamber depth, instrument settings, semen diluents, and sperm cell concentration (Bompart et al., 2018; Harstine et al., 2018). Disposable chambers with a depth of 10 or 20 µm (depending on the species) are commonly used for sperm motion analyses. If the depth of the chamber is to

narrow, the spermatozoa will not swim in their normal manner (Amann and Waberski, 2014). Further, applying samples with too high concentrations of sperm cells may cause the spermatozoa to collide, changing their natural trajectories and kinematic computations (Rijsselaere et al., 2002). To prevent inaccurate motility results in the current thesis work, the same technician, trained on how to prepare the samples and operate the instrument, conducted all motility analyses by CASA. Slides with fixed chamber depths of 20 µm were filled with 26 x 10^6 cells/mL, which follows the recommendations stated by Mortimer et al. (1995). Particles resembling sperm cells in size is another source of error, and is especially a problem when analysing semen diluted with egg-yolk (Kathiravan et al., 2011). In the current work, all semen samples were extended with egg-yolk, and debris particles were observed for all samples analysed by CASA. However, the set-ups for intensity and size values largely prevented false detection, and the few particles incorrectly recognized as sperm cells were manually removed prior to further analyses.

Analysing sperm motion by CASA gives the opportunity to assess all the kinematic parameters defining motility, both in a single cell manner and by calculating the mean for each sample. The single cell data can be analysed using statistical methods such as cluster analysis, to group the sperm cells into subpopulations based on their swimming patterns (Amann and Waberski, 2014). Such analyses may add useful information to semen quality that can be difficult to detect when considering only the average motility parameters of a semen sample (Ibănescu et al., 2018). Analysis of subpopulations was not conducted in this project. However, it would be interesting to perform such analysis in the future, applying the motility data we already have gathered during this thesis work.

4.1.3 *In vitro* production of embryos

Successful IVP procedures require completion of both adequate nuclear and cytoplasmic changes during oocyte maturation (Eppig, 1996), as well as appropriate sperm selection techniques, capacitation and fertilization/cultivation media (Wrenzycki, 2018). During the last decades, significant progress has been made in the development of culture media to achieve a higher total embryo production with improved embryo quality (Hansen, 2006) and reduction of the occurrence of the large offspring syndrome (Farin et al., 2001). In 2013, IVF bioscience developed a serum-free ready-to-use media suited for maturation, fertilization and culture (Hyttel et al., 2019). In the current project, we used the commercially produced media from IVF biosciences (Falmouth, UK), which is optimized for bovine IVP. Commercially produced media offer improved batch-to-batch consistency and less contamination compared to media

produced in house (Chronopoulou and Harper, 2014). It has been demonstrated that using the entire media system from IVF bioscience provide superior results due to the synergy of the media when they are used together (Nielsen et al., 2014).

In general, we followed the procedure and recommendations of IVF bioscience. The oocyte material collected for the IVP study were abattoir-derived. We did not have any information regarding the oocyte donors; hence, we did not know their age. The age of the female has shown to affect the oocytes developmental competence (Steeves et al., 1999). However, since oocytes from multiple females were pooled and 180 randomly selected oocytes were used per bull, it is unlikely that the age of the donors affected our results. The cumulus–oocyte complexes (COCs) were aspirated from follicles sized 3 to 15 mm in diameter. Only good quality COCs (grade I and II according to Blondin and Sirard (1995)) were selected for maturation. Oocytes harvested from follicles less than 3 mm in diameter may not have completed the growth phase and may lack the required mRNA and proteins to sustain development (Stroebech et al., 2015). Thus, these follicles were avoided.

In bovine IVF, the minimum number of spermatozoa required per oocyte may vary due to variation between bulls and breeds. However, a concentration of 1 to 2 million sperm cells/mL is commonly used (Ferre et al., 2019; Ward et al., 2002). The sperm concentration can significantly influence the results of cleavage and blastocyst rates, and a compromise must be found between having sufficient number of sperm cells to ensure acceptable fertilization rates while ensuring minimal incidences of polyspermy (Ward et al., 2002). We decided to adjust the sperm concentration such that each sperm sample added to the oocytes contained 1×10^6 progressive motile spermatozoa/mL. Hence, the observed difference between a low and high fertile bull was not affected by the sperm motility percentage.

The IVF experiment conducted in study IV included a limited number of bulls, and only one ejaculate per bull. Thus, the results should be interpreted with caution and the experiments should be repeated with more bulls and multiple ejaculates. Even though our bulls had a wide range concerning NR56, the bulls were all approved for AI and therefore had acceptable semen quality. Nevertheless, we were able to detect significant differences in cleavage rate and total blastocyst rate between the bulls of low and high NR56. It is recognised that individual bulls differ in their ability to fertilize by IVF (Marquant-Le Guienne et al., 1990; Ward et al., 2001; Zhang et al., 1997). This was also observed in study IV (results not presented), and could possibly be related to corresponding differences in the ability of their sperm cells to undergo

capacitation *in vitro* (Zhang et al., 1997). Screening of bulls of unknown NR56 for selection of superior fertility bulls might therefore be difficult, as some bulls with high NR56 do not perform satisfying in IVF and vice versa. However, IVF can be a useful tool for assessing the effect of different treatments on sperm cells, such as testing different methods for semen preservation (Larsson and Rodríguez-Martínez, 2000). In study IV, we were particularly interested in investigating the relationship between *in vitro* fertilization/embryo development and sperm chromatin integrity parameters.

4.1.4 Metabolomics

In livestock research, there are few studies focusing on metabolites in relation to reproductive performance (Goldansaz et al., 2017). Thus, there are several knowledge gaps that can be filled, and study I and III of this thesis were designed for this purpose. A good metabolomics study should use more than one analytical platform (Goldansaz et al., 2017), and in the present thesis both LC-MS and ICP-MS were applied. Other techniques could have been used such as NMR and gas chromatography coupled to mass spectrometry (GC-MS). As LC-MS and GC-MS are more sensitive methods, they are often preferred over NMR (Emwas et al., 2019). The use of ICP-MS for measuring the level of seminal trace elements brings new knowledge to the field, as the number of such studies in bulls are scarce.

The reliability of livestock metabolomic analyses depends on factors such as animal diet and sampling time (Goldansaz et al., 2017). The bulls included in the present project were uniformly raised and fed. However, it is possible that the bulls' diet consisting of roughage and straw was affected by the natural changes in climate and soil compositions during the season. The number of samples analysed therefore becomes highly important to equalize the possible differences caused by seasonal variations in diet. In study I, semen samples were collected from the same 25 bulls at 14 and 17 months of age. The 25 bulls were divided in three groups, corresponding to the time they reached the age of interest and semen was collected. Thus, semen from bulls of the different groups were collected at different seasons, and this group/season effect was included in the statistical model. In study III, semen samples from 37 bulls were included and the season for semen collection was evenly distributed between the bulls of both fertility groups and unlikely to affect the results.

For the analysis of metabolites in neat semen (Paper I), centrifugation was used to separate seminal plasma and sperm cells. As centrifugation may alter the sperm surface, it was hypothesized that it could affect the true metabolome of the sperm cells. Therefore, a

centrifugation force of only 110 x G was used. It can be discussed if 110 x G is enough to settle the live sperm cells in the pellet. However, a clear separation of the pellet and supernatant was observed and the supernatant was immediately aspirated after centrifugation to avoid the spermatozoa to swim up. Still, it is possible that a few sperm cells were included in the seminal plasma samples, but it is unlikely to cause much of an impact on the overall results.

Cryopreservation is known to have a detrimental effect on sperm viability (Ugur et al., 2019b). In study III, we hypothesized that dead spermatozoa might leak some of the intracellular metabolites due to damaged sperm membranes. Since the different bulls would have different levels of viable spermatozoa, this could potentially affect the results. Thus, a method for evaluating intracellular metabolites in the viable sperm population was developed. To our knowledge, this is the first study where only viable sperm cells of frozen-thawed semen have been analysed for intracellular metabolites. In both study I and III, the data was scaled prior to statistical analyses to compare samples of equal sperm numbers. The metabolomics approach initiated in this thesis work could be further complemented with other omics techniques (e.g. proteomics, transcriptomics etc.).

4.1.5 Epigenetics

Global DNA methylation is the most studied epigenetic modification and can be assessed by a variety of methods. The introduction of next-generation sequencing (NGS) platforms allowed for substantial analysis of the methylation status of CpG sites and construction of DNA methylation's genomic maps at a single base resolution (Barros-Silva et al., 2018). For profiling of whole genome methylation, methods such as high performance liquid chromatography-ultraviolet (HPLC-UV), liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), luminometric-based assay (LUMA), long interspersed nuclear elements-1 (LINE-1)/pyrosequencing, and enzyme-linked immunosorbent assay (ELISA)-based methods are available. For the identification of differentially-methylated regions, methods including WGBS, RRBS, different kits followed by NGS, and array or bead hybridization can be used (Kurdyukov and Bullock, 2016).

Bisulfite sequencing is considered as the "gold standard" for DNA methylation analysis. Bisulfite treatment of DNA mediates the conversion of unmethylated cytosine to uracil while methylated cytosine residues, resistant to this conversion, remain unchanged (Frommer et al., 1992; Kurdyukov and Bullock, 2016). After bisulfite conversion, genomic DNA is amplified by PCR and sequenced at high depth, yielding quantitative measurements of individual

cytosine methylation (Lee et al., 2014). With the advent of NGS, bisulfite sequencing can be extended to DNA methylation analysis across the entire genome. The method of WGBS is the most comprehensive, but also the most expensive method especially for organisms with large genomes such as the mammalian species (Doherty and Couldrey, 2014). Of note, bisulfite conversion may be followed by PCR amplification (instead of high-throughput sequencing) using specific primer pairs providing amplicons covering regions of interests, cloning of the PCR products into *E. coli* and sequencing a number of clones (typically ≥ 10). Analysis of the sequences provides a quantitative single-base resolution of cytosine methylation (Clark et al., 1994)

In this thesis work, RRBS was conducted for the analysis of sperm DNA methylation (Paper II and IV). The method of RRBS was designed by Meissner et al. (2005) and Gu et al. (2011), and allows for preferential selection and sequencing of CpG-rich regions. This results in the sequencing of a subset of DNA fragments from the genome, which is likely to contain the majority of regions relevant for DNA methylation analysis, thus reducing the cost (Doherty and Couldrey, 2014). In RRBS, restriction enzymes (usually *Msp*I, which has a recognition sequence of 5′-C^CGG-3′) are added to digest the DNA during the fragmentation step, thus every fragment produced contain at least one CpG dinucleotide. Since the genome generally has low levels of CpGs except for in genes, promoters and CpG islands, RRBS is largely capturing only these regions for further analysis (Doherty and Couldrey, 2014; Meissner et al., 2005). By combining different restriction enzymes, whole-genome CpG coverage can be altered to exclude or include certain regions of interest such as CpG island shores, which are known to play an important role in various biological processes (Wang et al., 2013). For the RRBS analysis in study II and IV, a combination of the enzymes *Msp*I and *Taq* α1 was used. In a study in humans, the combination of these two restriction enzymes was reported to improve coverage across all classes of sequences containing CpGs (Lee et al., 2014).

Comparison of RRBS with other NGS-based technologies has shown that most methods produce similar results (Bock et al., 2010; Harris et al., 2010). However, the optimal sequencing strategy may depend on the amount of DNA, as well as the desired genome coverage and sequencing depth (Gu et al., 2011). The coverage is recognised as the average number of times a particular nucleotide is represented in a collection of random raw sequences (Sims et al., 2014). In addition to the choice of enzymes, the coverage can be varied to some extent by size-selection, and it is possible to perform *in silico* analysis to determine whether regions of interest would be sufficiently covered. The appropriate coverage for an experiment

is determined on a case-by-case basis, and varies depending on the NGS and sample type. The general recommended coverage for identification of DMRs by bisulfite sequencing is 5-15x (ABM, 2020; Ziller et al., 2015). In study II, the average read coverage was 15.9x, and downstream analyses were performed based on all covered CpGs. However, in study IV, the read coverage was 30.5x of the *in silico* created RRBS genome, and downstream analyses were performed based on CpGs with equal or more than 5x coverage. A 10x coverage was also tested for study IV, however, this resulted in a lower number of differently methylated cytosines. The selection of *q*-value, percentage of methylation difference and number of samples included for the analysis of differential methylation may affect the outcome. In our studies, these parameters were selected based on experience and relevant publications.

As explained above, bisulfite treatment of DNA cause unmethylated cytosine to be converted to uracil. The subsequent mapping of the short reads to a reference genome allows interpretation of methylated versus unmethylated cytosines. Thus, the evaluation of DNA methylation is highly dependent on mapping of the reads to a reference genome (Tran et al., 2014). A great challenge with DNA methylation analysis is to be able to map millions of reads within reasonable time and with high mapping efficiency (the percentage of reads that are mapped to a reference genome). Several tools have been developed to map bisulfite converted sequence reads and determine cytosine methylation states, including Bismark (Krueger and Andrews, 2011), which was used in this thesis (Paper II and IV). Most bisulfite sequencing mappers first conduct some sequence conversions either on the reads or the reference genomes or both, and then use regular aligners such as Bowtie, Bowtie2 (Paper II and IV), SOAP and BWA to map short reads to a reference genome. In a comparison of five different programs, Bismark was found to perform best, concerning mapping efficiency (Tran et al., 2014). Thus, the results of methylation analyses may be affected by the different choice of tools for indexing and mapping. Furthermore, changing parameters in the programs may affect the mapping results. The parameters used in study II and IV were set to give high mapping efficiency. However, the parameters were carefully adjusted as allowing an increase in the number of mismatches not only increases the mapping efficiency, but also cause an increase in false positives (Tran et al., 2014). The unique mapping efficiencies with the reference genome of 33.1% (Paper II) and 34.8% (Paper IV) were higher than previously results reported for RRBS libraries in bull sperm cells (Jiang et al., 2018).

In study II, a comparison of a gel-free multiplexed technique and the Ovation® RRBS Methyl-Seq System was conducted, to find the best-suited protocol for sperm DNA methylation

analysis. Our results showed that libraries from the Ovation method exhibited a greater percentage of read loss and shorter read size following trimming. This is important, as longer reads tend to align better with reference genome in Bismark (Tran et al., 2014). Based on these observed differences, it was decided to conduct downstream analyses using RRBS libraries constructed based on the gel-free multiplexed method.

A limitation of our work regarding the study of epigenetics is that we only examined sperm DNA methylation and none of the other epigenetic modifications in spermatozoa, such as histone PTMs. Moreover, in study IV, it would have been interesting to investigate the DNA methylation pattern of the IVP embryos and correlate the findings to the observed DNA methylation of sperm samples. However, there was not sufficient time to perform these analyses in the course of this PhD project.

4.1.6 Experimental design for the study of age effects (study I and II)

The aim of study I and II was to improve the knowledge on how age of young peri-pubertal bulls might affect different aspects of semen quality, semen production traits and fertility. Furthermore, it was of interest to evaluate if semen metabolites and sperm DNA methylation signatures could provide additional information to the possible age-related sperm quality differences. Semen samples were collected from the bulls at the age of both 14 and 17 months. The reason behind these age groups is that the Norwegian Red bulls arrive at the AI semen production centre at an age between 13 and 14 months. Due to adaption for the bulls, only the second ejaculate was collected for analyses. Therefore, 14 months was the first possible age that could be included for the studies. Furthermore, it has been observed that some of the bulls are kept for production only for a few months, due to reduced genetic value or due to other and younger bulls being superior and preferred. Thus, the second sampling was performed at 17 months to include sampling from all bulls once more. It can be debated that the age difference of only 3 months may cause problems when interpreting the results, as it can be difficult to address if the differences are due to age or season, or both. However, in our statistical models, it was corrected for the possible effect of season/time of semen collection. Our results showed that this short period in the bulls' life influenced several of the parameters examined. Even though these three months only represents 1.5 spermatogenesis periods, it is possible that the different stages of the cycle of the seminiferous epithelium may be influenced, thus giving at least 18 different stages being possibly influenced by age and testicular development.

4.1.7 Experimental design for the study of high and low fertility bulls (study III and IV)

Field fertility data must be recorded with high precision in order to establish reliable relationships between *in vitro* tests and fertility (Rodriguez-Martinez, 2003). The field fertility data in Norway, measured as NR56, are considered highly reliable. This is because AI technicians are employed by the breeding company and paid according to reported AIs (Ranberg et al., 2003). Furthermore, the NDHRS provides easy access to data on factors that may influence the fertility estimates and that should be included in the statistical analyses. These factors include herd ID, cow ID, parity, bull ID, date of semen collection, days since last collection, date of AI and AI personnel. In study III and IV, NR56 was calculated for 507 Norwegian Red bulls used in AI from 2013-2018. The AI doses from each bull were distributed randomly all over Norway. This minimizes bias from herd-management and the AI personnel performing the inseminations, thus these factors were not included in the statistical model. Another important factor for reliable fertility results is the number of AIs per bull. In these studies, the number of first AIs ranged from 496 to 8542 in the high-fertile group and from 204 to 976 in the low-fertile group. Although the fertility records of most bulls were based on a high number of AIs, three bulls with low NR56 had less than 500 AIs recorded. It is possible that their NR56 data would change if the number of AIs had increased. The total number of first AIs were 12 636 for the low-fertile group and 29 240 for the high-fertile group. This is probably influenced by the fact that several of the bulls with low fertility were eliminated from semen production when the first NR56 data was available.

An advantage with the Norwegian Red breed is the high field fertility level. This is, however, a challenge in research, as access to low-fertile bulls is limited. This was also a drawback for study III and IV, and resulted in a broader range of NR56 in the low-fertile group compared to the high-fertile group. Ideally, the low-fertile group should have been more homogenous and with even lower NR56. It is likely that a larger difference in NR56 between the bulls of each groups would have affected the results in paper III and IV. However, given the data material available for this research, the experimental design was good and there was a significant difference in NR56 between the two groups. In study IV, a selection of 16 bulls were conducted prior to the analysis of sperm DNA methylation and IVP of embryos. It would have been preferable to perform both RRBS analyses and IVP experiments for all 37 bulls. However, due to the costs of sequencing and that IVP experiments are highly time-consuming, the number of bulls had to be reduced.

One of the aims of study III was to find a prediction model that could explain the observed variation in bulls NR56. To provide a good relationship to fertility, several *in vitro* methods measuring one or more sperm attributes were assessed. Correlation analyses were conducted, both in-between parameters and between the parameters and NR56. Parameters that were significantly correlated with each other were not combined together in the regression model, as this may cause collinearity problems (Karoui et al., 2012). Forward variable selection was applied and the F statistic was calculated to determine the contribution of each independent variable to the model. The variable with the smallest p-value below the cut-off value 0.1, was kept in the model. Both the choice of selection method and the p-value set as selection criteria may affect the results. However, in this study, data analysis using both stepwise, forward and backward selections provided similar results. A weakness of paper III is the lack of validation of the suggested prediction model. However, as this was the first study of metabolites in frozen-thawed semen from Norwegian Red bulls, there were no data available to perform the validation.

4.2 Sperm quality in relation to field fertility

Before being introduced to the breeding program, bulls are normally subjected to breeding soundness evaluation, which includes measurements of ejaculate volume, sperm concentration, motility and morphology (Correa et al., 1997). Even though this evaluation excludes bulls of poor semen quality from the breeding program, it is insufficient in discriminating bulls of superior fertility from the bulls of lower performance (Larson and Miller, 2000). With the introduction of GS, the search for new and more rapid methods for accurate fertility prediction has become even more crucial. The young selected sires are typically used only for a short period, after which they are replaced by the next generation of genetically superior bulls (Holden et al., 2017). This high-turnover rate of AI bulls leaves insufficient time to assess the reproductive performance of a bull before its semen is widely distributed in the field.

4.2.1 The number of sperm cells in the AI dose

The true fertility potential of a semen sample is closely associated with sperm quality. However, the outcome of a perfectly timed AI will also be affected by the number of sperm cells in the AI dose (Amann and DeJarnette, 2012; Amann and Hammerstedt, 1993; Amann et al., 2018). This can be explained by the principle of compensable vs. un-compensable sperm quality parameters and their relationship to threshold sperm numbers per dose (DeJarnette et al., 2004; Saacke et al., 2000). The threshold in sperm numbers is the minimum number of

spermatozoa required to reach maximum fertility. The threshold limit depends on the ratio of compensable and un-compensable sperm traits, hence it can vary widely between individual bulls or ejaculates (Kastelic, 2013). Concerning compensable sperm quality traits, the inseminated females will respond to increasing numbers of sperm per AI dose with increased conception rates. In contrast, un-compensable sperm quality traits are those for which the field fertility outcome does not change as the number of sperm per AI dose increases (Amann and DeJarnette, 2012). Compensable traits are considered important for sperm transport and function in the female reproductive tract, while un-compensable traits are associated with the ability to sustain fertilization and embryonic development (Kastelic, 2013).

The standard number of sperm cells per AI dose used for Norwegian Red bulls at the time of the study was 12×10^6. Previously, it has been shown that 12×10^6 sperm cells/dose results in the same fertility outcome as AI doses with 18×10^6 sperm cells (Kommisrud et al., 1996). Thus, the sperm concentration of 12×10^6 cells/dose can be regarded as above the threshold of maximum fertility. It is a general desire within the livestock industry to reduce the number of sperm per dose without affecting the fertility. This will allow more doses to be produced from each ejaculate, thus being of economic importance for the breeding companies. Reduction of sperm numbers per AI dose has become especially essential after the shift towards the intensive use of young GS bulls, which tend to produce fewer sperm cells per ejaculate than the more mature bulls (Fair and Lonergan, 2018). The results of study I showed that 14 months old bulls had reduced ejaculate volumes and sperm concentrations compared to 17 months old bulls. Furthermore, the number of discarded semen doses decreased significantly with increasing age. The high number of discarded doses and low sperm concentration in the ejaculates from the youngest bulls must have been costly for the breeding company. With the new era of GS, some AI companies start semen collection even earlier when the sires are at the age of 10-12 months old, and this will probably be the future in Norway as well. With this in mind, it would be interesting to investigate semen quality and production efficiency of even younger bulls, and test if further reduction of the sperm number per AI dose affects the fertility results of young bulls.

4.2.2 Sperm quality traits essential for sperm transport and oocyte interaction

Associations between single sperm attributes and fertility have been widely demonstrated (Saacke et al., 2000). However, due to the complexity of events involved in fertilization, it is generally recognised that a single sperm quality parameter alone is unable to reflect the real fertility potential of a semen sample (Gliozzi et al., 2017; Kumaresan et al., 2017a; Oliveira et

al., 2012; Sellem et al., 2015). Fertility estimations should involve assessments of parameters related to the spermatozoa's ability to reach and fertilize the oocyte, such as progressive motility (Farrell et al., 1998; Gliozzi et al., 2017; Puglisi et al., 2012), plasma membrane and acrosome intactness (Christensen et al., 2011; Kumaresan et al., 2017), metabolism for energy production (Garrett et al., 2008), and hyperactive motility (Suarez et al., 1991; Talevi and Gualtieri, 2010). All these parameters are claimed to be compensable sperm traits, and they are important to AI as they govern the minimal numbers of sperm required for an inseminate to reach maximum fertility (Saacke et al., 2000).

Although there are conflicting results regarding the correlation between sperm motility and field fertility (Bailey et al., 1994; Farrell et al., 1998; Gillan et al., 2008; Januskauskas et al., 2003; Stålhammar et al., 1994), sperm motility is considered as an important indicator for fertilization potential (Kathiravan et al., 2011; Mortimer, 1997; Rodriguez-Martinez, 2006). In study III, total motility, progressive motility, hyperactivity, and the kinematic parameters VAP, VCL, VSL, STR, and ALH correlated positively with NR56. However, the linear regression model indicated that none of the motility parameters significantly contributed to the variation in bull fertility (Paper III). Bulls of high NR56 had sperm cells with higher levels of hyperactive motility compared to bulls of low NR56 (Paper III). Furthermore, there was a significant difference in hyperactivity between semen samples collected at 14 and 17 months, with the proportion of hyperactive spermatozoa increasing with age (Paper I). A previous study by Shojaei et al. (2012) indicated that sperm cells from high fertility bulls are in transition to a hyperactivated motility pattern. This transitional phase, often termed a progressive hyperactive phase, is likely important for proper sperm transport (Suarez, 1988). Therefore, it may be hypothesised that the increase in hyperactivity observed for bulls of high NR56 (Paper III), reflects that their sperm cells are in a progressive hyperactivated motility phase with enhanced ability to reach and fertilize the oocyte. Although superior levels of sperm hyperactivity were observed in semen samples from 17 months old bulls compared to 14 months old bulls (Paper I), this did not reflect the NR56 data of the two age groups. This may be explained by the number of sperm cells per AI dose being above the threshold for maximum fertility, and that hyperactivity alone is unlikely to significantly affect the reproductive potential of the bulls.

During the spermatozoa's journey to the site of fertilization, they undergo a sequence of activities requiring energy, including sperm motility, capacitation, hyperactivation, acrosome reaction and fertilization (du Plessis et al., 2015). Thus, it is hypothesised that sperm ATP

content is associated with reproductive performance. Reports concerning the impact of sperm ATP content on reproductive performance is limited. However, a study in bovine demonstrated an association between sperm ATP production and field fertility (Garrett et al., 2008). Previous research has further shown associations between sperm ATP content and motility of mammalian spermatozoa (Januskauskas and Rodriguez-Martinez, 1995). In study III, no correlation between sperm ATP content and NR56 was observed, which is in agreement with the findings of Berg et al. (2018). The sperm ATP content was generally higher in fresh semen samples compared to frozen-thawed samples (Paper I). It is well-known that the processes of freezing and thawing can have detrimental effects on sperm cells due to changes in temperature, induction of osmotic stress, and formation of ice crystals (Gürler et al., 2016). Cryopreservation caused reduction in sperm motility and viability, which explains the decreased levels of sperm ATP in frozen-thawed samples (Paper I).

Sperm plasma membrane integrity is essential for sperm survival inside the female reproductive tract and for successful fertilization (Flesch and Gadella, 2000; Oliveira et al., 2012). Furthermore, acrosome integrity is essential for the acrosome reaction to happen, which is critical for penetration of the zona pellucida and subsequent fertilization (Birck et al., 2010). Most researchers agree on that plasma membrane and acrosome integrity are important parameters when evaluating the fertilization potential of a semen sample (Januskauskas et al., 2003; Oliveira et al., 2014; Silva and Gadella, 2006; Tartaglione and Ritta, 2004). However, there are also studies reporting no significant relationship between these integrity parameters and bull fertility (Berg et al., 2018; Cumming, 1995; Waterhouse et al., 2006). Although higher proportions of acrosome intact live spermatozoa and lower proportions of acrosome reacted dead spermatozoa were observed in semen from high fertility bulls compared to the low fertility bulls in study III, no significant correlations were found between these parameters and NR56.

As all previously mentioned sperm quality parameters are considered compensable sperm traits, and the sperm number per AI dose is above the threshold for maximum fertility, it is unlikely to find consistent associations between these parameters and fertility. Due to the relatively high number of sperm cells per AI dose, possible defects in compensable sperm traits may have been masked. Furthermore, the bulls included in this study are pre-selected for AI based on their semen quality, such as post-thaw sperm motility > 50%. When these compensable sperm attributes were included in the linear regression model, none of them contributed to the explanation of observed variation in NR56 (Paper III). Using lower sperm

numbers per AI dose could provide important basic knowledge on these compensable sperm traits and their association with NR56. Additionally, expanding the study to include more bulls, or a greater range of fertility scores, may change the relative importance of the various variables. However, finding bulls of low NR56 is challenging, due to the exceptionally high field fertility of Norwegian Red bulls.

4.2.3 The importance of sperm DNA integrity in relation to fertility

Mammalian fertilization and subsequent embryo development depend, in part, on sperm DNA integrity (Kumaresan et al., 2020). Interestingly, sperm cells with damaged DNA can be viable, motile, and even able to fulfil fertilization (Yamauchi et al., 2012). The effect of sperm DNA damage on the developing embryo depends on the balance between the type and amount of DNA damage and the capacity of the fertilized oocyte to repair the sperm DNA damage (García-Rodríguez et al., 2018). Any sperm DNA damage that the oocyte is unable to repair may negatively affect embryo development (Ménézo et al., 2010). In study III, sperm DNA fragmentation assessed by the SCSA was negatively correlated with field fertility. Furthermore, DFI was the only sperm quality parameter assessed that significantly contributed to the fertility prediction model. These results corroborates a previous study in Norwegian Red bulls, where the relationship between multiple sperm traits and field fertility was evaluated, and only variables related to sperm DNA damage showed significant contribution to the model (Waterhouse et al., 2006). Sperm DNA fragmentation is considered an un-compensable sperm parameter, i.e. a reduction in fertility outcome is expected regardless of the number of inseminated sperm cells. Previous studies in bulls have confirmed the negative association between sperm DNA integrity and field fertility (Boe-Hansen et al., 2018; Kumaresan et al., 2017a).

There are several explanations to the origin of sperm DNA damage, but three common hypotheses have been suggested: 1) defects in DNA compaction or ineffective repair of DNA stand breaks during the reconfiguration of DNA, associated with the replacement of histones by protamines, 2) abortive apoptosis, and 3) insufficient protection against ROS (Aitken and Baker, 2004; Boe-Hansen et al., 2018; Ni et al., 2016). Reactive oxygen species are thought to represent the main cause of sperm DNA damage, possibly in combination with incomplete chromatin condensation (Aitken and Baker, 2004; Ni et al., 2016). However, there are also extrinsic factors that may have an effect on sperm DNA quality, such as age, testicular temperature, infections, semen handling, sex sorting, season and methods of semen collection (Kumaresan et al., 2020). The relationship between age of young bulls and DNA integrity was

assessed in study I and II. While the results of study I showed no correlation between sperm DNA damage and age of young bulls, the results of study II showed that the percentage of DNA fragmented sperm cells significantly decreased with increasing age in both fresh and frozen-thawed semen samples. However, the results of study I are more representative due to the higher number of bulls included in the study (25 bulls vs. 9 bulls in study II). In agreement with the results of study I, the integrity of sperm chromatin assessed in young bulls with the mean ages of 13, 18 and 24 months showed no association with age (Fortes et al., 2012). Interestingly, Carreira et al. (2017) reported that young bulls (1.8 to 2 years) have higher levels of DNA fragmentation and deficient protamination, indicating a state of immaturity, compared to adult bulls (3.5 to 7 years).

The percentages of spermatozoa with DNA damage ranged from 1.0 to 6.5% in study III, with a mean of 3.5% in low-fertile bulls and 1.8% in high-fertile bulls. Thus, the level of DNA damage in bull semen was generally low compared to in humans, where DFI levels of 20-40% have been reported and associated with failure to maintain pregnancy (Evenson et al., 2002; Kumaresan et al., 2020). For bovine semen samples, DFI values of 7 to 10% have been reported as indicators of low AI success (Karoui et al., 2012). Even though the mean DFI values for the groups of low and high NR56 bulls were below the threshold observed by Karoui et al. (2012), our results showed that applying the SCSA might be important for the identification of bulls with reduced reproductive potential. DNA fragmentation has been reported a “tip of the iceberg” effect, meaning that although the reported percentage DFI in a semen sample is low, the same DNA abnormalities likely exists to a lesser extent throughout the sperm population, and in a sufficient amount to cause subfertility (Evenson and Jost, 2001). As mentioned previously, all bulls included in the studies of this thesis were pre-selected based on e.g. sperm post-thaw motility > 50%. Hence, it is likely that the inclusion of true sub-fertile or even infertile bulls in study III would have resulted in higher observed DNA fragmentation in the low-fertile group.

The major use of the SCSA test has been to determine the percentage of sperm cells with fragmented DNA. However, by including the determination of the percentage of HDS sperm, it is also possible to characterize sperm chromatin defects (Evenson, 2016). The HDS sperm population in a semen sample has an abnormally high level of (green) DNA staining caused by lack of full protamination. Thus, more nuclear histones are retained, leading to looser compaction of the chromatin structure. The HDS sperm population is characterized as immature spermatozoa (Evenson, 2016; Evenson et al., 2002). In study III, the percentage

HDS correlated negatively with NR56, indicating that the bulls of low fertility had a higher proportion of retained nuclear histones. This corroborates findings in humans, where high HDS has been reported to have a negative effect on pregnancy outcome (Evenson et al., 1999), and to be predictive of pregnancy failure (Evenson, 2013). However, it is not fully clear from published articles whether HDS is associated with reproductive performance (Speyer et al., 2015). In a study by Boe-Hansen et al. (2006), higher HDS appeared to be favourable to IVF and pregnancy outcome. Interestingly, the results of study I and II indicated that sperm cells from 17 months old bulls were more immature (higher HDS) compared to at 14 months. This might have been influenced by more frequent semen collections in the oldest bulls. However, this should be further investigated in a larger study.

The results of study IV showed that both DFI and HDS were negatively correlated with cleavage, but not to blastocyst rate. Furthermore, high fertility bulls had significantly higher cleavage rates compared to low fertility bulls, but the subsequent development into blastocysts was unaffected by bull fertility. Based on these findings it can be speculated that sperm DNA damage mostly affects the spermatozoa's ability to fertilize the oocyte. This is in agreement with a previous study in bulls, where sperm DNA damage caused by increased oxidative stress was found to affect cleavage rate (Simoes et al., 2013). In contrast, others have reported that DNA fragmentation in bull sperm does not impair *in vitro* fertilization but rather the further embryonic development when the blastocyst stage is reached (Fatehi et al., 2006). It can be hypothesised that the level of fragmented sperm DNA in the present study where within the range of damage that the (successfully fertilized) oocytes managed to repair. Oocyte repair of DNA damage is, however, commonly followed by early embryonic death, implantation failure, chromosomal abnormalities and higher abortion rate (Tesarik et al., 2004). Thus, this might explain the strong negative association observed between sperm DNA fragmentation and NR56.

During spermatogenesis, histones of round spermatids are replaced by transition proteins and ultimately by protamines in elongated spermatids (Kumaresan et al., 2020). This process results in sperm chromatin compaction and renders the spermatozoa transcriptionally and translationally silent besides protecting the DNA from damage (Simon et al., 2011). However, not all histones are replaced by protamines in mature sperm DNA. Researchers have demonstrated that mammalian spermatozoa generally retain between 1 and 15% histones, depending on the species (Samans et al., 2014). Furthermore, it has been reported that abnormal protamination has a negative effect on semen quality and fertility in bulls (Carreira

et al., 2017; Dogan et al., 2015) and humans (Aoki et al., 2006; Aoki et al., 2005). Protamine deficiency is likely one of the contributing factors to sperm DNA damage (Boe-Hansen et al., 2018). Fortes et al. (2014) demonstrated a clear association between sperm protamine content and sperm DNA damage in bulls. The results of study IV corroborate these findings as sperm DNA damage (both DFI and HDS) correlated positively with the degree of protamine deficiency. However, there were no associations between sperm protamine deficiency and field fertility (NR56) or IVF outcomes. A recent study reported that sperm protamine deficiency might not have a strong biological impact on bull *in vitro* fertility (Castro et al., 2018). This may be due to the generally low levels of protamine deficiency reported in bovine spermatozoa (Castro et al., 2018; Kipper et al., 2017). A generally low level of protamine deficiency in bovine sperm cells, and the lack of significant differences between the two fertility groups, might also explain the absence of correlations between NR56 and disulfide bonds/thiols. Study IV further showed that DNA integrity was not associated with either disulfide bonds or thiol status. In contrast, studies in humans have shown that semen samples from oligozoospermic or infertile men contain fewer disulfide bonds compared with normozoospermic samples, and that DNA integrity is associated with the level of thiol groups (Rufas et al., 1991; Zini et al., 2001). Again, our results might have been different if true sub-fertile bulls or bulls with higher levels of DNA damage had been included in the study.

Overall, the findings of study III and IV indicate that the SCSA could be a promising tool in selecting bulls of superior fertility since sperm DFI and HDS significantly correlated with NR56 (Paper III), and that DFI accounted for significant variations in NR56. Breeding companies should consider assessing sperm DNA damage in addition to the conventional semen analyses, as this could provide additional useful information in identifying subfertile bulls. Assessment of sperm DNA integrity becomes especially important when working with assisted reproductive technologies, such as IVP of embryos, where the natural selection of good quality sperm cells is excluded.

4.3 The search for new biomarkers in bovine semen

In the present thesis, sperm quality parameters such as motility and DNA fragmentation correlated with field fertility. However, our results as well as the results of previously reported papers have demonstrated that *in vitro* sperm quality tests by methods such as CASA and flow cytometry only explains a part of the variation in bull fertility (Sellem et al., 2015). It is apparent that additional tests should be applied, thus, the present thesis work also included analyses of seminal metabolites and sperm DNA methylation. The aim was to advance our

current understanding of the multifactorial processes related to male maturation and fertility and for the potential detection of new seminal biomarkers in Norwegian Red bulls.

4.3.1 Metabolomic markers

Metabolomics has become important for the discovery of seminal biomarkers as low molecular weight compounds may provide a clear picture of the regulatory pathways within sperm cells (Menezes et al., 2019). To our knowledge, metabolomics of bovine seminal plasma and sperm cells has previously only been conducted using fresh semen samples (Kumar et al., 2015; Menezes et al., 2019; Velho et al., 2018). Recently, 22 distinct metabolites were identified in spermatozoa from Holstein bulls (Menezes et al., 2019). In a study by Velho et al. (2018), the metabolites 2-oxoglutaric acid and fructose of seminal plasma were suggested as potential biomarkers of bull fertility. Unlike these studies, we have applied targeted metabolomics, with a special focus on amino acids and trace elements. Our results (Paper I and III) show that amino acids and trace elements found in seminal plasma and sperm cells may contribute to biological processes related to sperm function, fertility and age-related differences between bulls. For instance, arginine and glutamine were positively correlated with the degree of acrosome intact live spermatozoa and several of the sperm motility parameters, including sperm progressivity (Paper III). Progressive motility has previously been associated with arginine and glutamine levels of human spermatozoa (Engel et al., 2019). Glutamic acid has been identified as the most abundant amino acid of bull seminal plasma (Ugur et al., 2019a), and was the most abundant amino acid in both spermatozoa and seminal plasma of our studies (Paper I and III). Glutamic acid was further positively associated with the percentage of DFI and HDS (Paper III). The trace elements Al and Fe, which were significantly different between high vs. low fertility bulls, were also positively correlated with DFI (Paper III). This is in agreement with previous studies, reporting that Fe may cause an increase in sperm DNA damage (Perera et al., 2002) and that Al may impair sperm quality and increase oxidative stress in sperm (Martinez et al., 2017).

Several amino acids and trace elements associated with bull age (Paper I) and/or fertility (Paper III) are reported involved in protecting sperm cells against lipid peroxidation and oxidative stress, including arginine, glutamine, glutamic acid, cysteine, proline and Zn (Fallah et al., 2018; Krishnan et al., 2008; Rudolph et al., 1986; Rushworth and Megson, 2014; Srivastava et al., 2000; Srivastava et al., 2006; Zhu et al., 2017). As we did not measure the level of ROS in the semen samples, it is unclear how the metabolites were involved in protection against oxidative stress in our studies. However, the results of study I may indicate

that differences in the level of metabolites in both seminal plasma and spermatozoa collected from bulls at 14 and 17 months of age, affect important processes such as the ability to withstand negative consequences of ROS. Furthermore, oxidative stress is linked to DNA damage and male infertility (Agarwal et al., 2014). Thus, the lower observed percentage DFI in sperm cells from high-fertility bulls may indicate that sperm from these bulls were better protected against ROS compared to low-fertility bulls (Paper III).

In study III, sperm DFI together with the intracellular sperm concentration of aspartic acid, Fe and Zn were found to explain 59% of the total variation in NR56. Aspartic acid of bull seminal plasma has previously been shown to correlate with fertility (al-Hakim et al., 1970). As mentioned above, the level of Fe is positively associated with sperm DNA damage, while Zn works as an antioxidant protecting sperm cells from ROS. It is possible that applying the suggested prediction model may provide enough information for breeding companies to select bulls of superior sperm quality, and that the suggested metabolites may function as sperm fertility biomarkers. However, before recommending this to AI companies, the model needs to be validated using a larger number of bulls with known fertility. Nevertheless, the multiple correlations found between metabolites and *in vitro* sperm parameters, as well as with NR56 indicate that metabolomics may be a useful tool in the identification of male fertility markers.

With the increased physiological pressure on young GS bulls, finding biomarkers that can predict the maturity and subsequent reproductive performance of individual bulls would be highly valuable for the AI industry. The overall results of this thesis work indicate that several sperm attributes, including amino acids and trace elements have the potential to differentiate between young bulls based on the quality of their ejaculates, even though the age difference was only three months. However, further investigations including a larger number of bulls are necessary to identify if any of these parameters can be used as maturity biomarkers in the future.

4.3.2 Epignetic markers

Epigenetics play a crucial role in regulation of gene expression (Rajender et al., 2011). The epigenetic modification investigated in the current thesis was sperm DNA methylation, which has emerged as one of the most promising indicators of male infertility (Urdinguio et al., 2015). The differential methylation analysis in study II showed that sperm from 14 months old bulls were hypomethylated compared to sperm from 17 months old bulls. Even though most of the identified DMRs displayed less than 10% methylation difference, our results indicate

that there is a relationship between DMRs and sexual maturation in young bulls. This corroborates a study in Holstein bulls where hypermethylated regions were found in sperm cells from 16 months old bulls compared to 10 months old bulls (Lambert et al., 2018). In study IV, sperm cells from bulls of low fertility were hypermethylated compared to sperm from bulls of high fertility. This is in agreement with previous findings in human (Camprubí et al., 2016; Luján et al., 2019) and buffalo sperm (Verma et al., 2014). In contrast, a study in Holstein bulls indicated that spermatozoa from high fertility bulls had more hypermethylated regions compared to low fertility bulls (Kropp et al., 2017). The contradictive results may be explained by the different techniques used for the study of sperm DNA methylation and/or by the method and reliability of the fertility measurements.

For the pathway analyses during this thesis work, we were particularly interested in genes associated with biological processes and molecular functions related to sexual maturity (Paper II) and fertility (Paper IV). The results of study II showed that the number of genes related to androgen signalling, steroid hormone biosynthesis, steroid hormone receptor signalling, spermatogenesis and developmental growth were higher in the hypomethylation group compared to the hypermethylation group of 14 months old bulls. Furthermore, genes related to steroid hormone biosynthesis were identified exclusively in the hypomethylation group (Paper II). Given their annotated molecular functions, such genes may contribute to age dependent differences in young bulls. However, this should be further investigated. The number of genes associated with biological processes related to different aspects of fertility and embryo development were higher in the hypermethylation group compared to the hypomethylation group of low-fertile bulls (Paper IV). Genes such as transition protein 2 and T-box transcription factor T, involved in penetration of zona pellucida, were exclusively identified in the hypermethylation group of bulls with low fertility. This may indicate that sperm hypermethylation is an aspect of male fertility problems, and further analyses should be conducted to elucidate the molecular basis of how male infertility develops. One of the genes identified with a single and specific biological process related to fertilization was the regulated endocrine specific protein 18 (RESP18) gene in the hypermethylated group of low fertility bulls. Interestingly, the RESP18 gene was previously identified as hypermethylated in low-fertile buffalo bulls (Verma et al., 2014). It would be interesting to perform a larger study, including more bulls and *in vitro* produced embryos, to further study the RESP18 gene and elucidate if this may be an epigenetic marker for predicting bull fertility potential.

In study II, the disulfide bond pathway was exclusively identified in the hypermethylation group of 14 months old bulls. Disulfide bonds are essential for protamine function and DNA packaging in bull sperm chromatin (Hutchison et al., 2017). The results of both study I and II indicated that sperm cells from 14 months old bulls had higher degrees of chromatin compaction (lower HDS) compared to 17 months old bulls. These results suggest a possible link between sperm DNA hypermethylation and DNA packaging via protamine function in young bulls. Even though we could not find a significant association between global DNA methylation and protamine deficiency in the high vs. low fertility bulls (Paper IV), it would be interesting to further investigate the relationship between protamine deficiency and DNA methylation in young bulls. The pathway analysis in study II showed that genes annotated with DMRs could be associated with hormonal pathways such as GnRH, estrogen and oxytocin signalling. Previously, Lambert et al. (2018) identified that DMRs in sperm cells from bulls at 10 and 16 months of age were associated with pathways related to sperm function, including androgen hormone signalling. These findings highlight the importance of hormonal signalling in bull development and sexual maturation.

The pathway analysis in study IV showed that genes annotated with differentially methylated cytosines could be associated with pathways related to fertilization and embryonic development, which might explain the lower fertility performance of bulls with low NR56. For instance, pathways such as oxytocin signalling, calcium signalling, ion channel/transport, voltage-gated channel, developmental proteins and vascular endothelial growth factor (VEGF) signalling were discovered exclusively in the hypermethylation group of bulls with low NR56. Interestingly, metal binding and metabolic pathways were found exclusively in the hypermethylation group of low-fertile bulls (Paper IV), corroborating our findings that several sperm amino acids and trace elements are associated with field fertility and that metabolomics may be a useful tool in the identification of biomarkers for male fertility (Paper III).

The present DNA methylation studies identified genes and pathways related to bull sperm maturation (Paper II) and fertility (Paper IV), however, the results should be interpreted with caution. In both studies, some genes that annotated with differently methylated cytosines were found to overlap between the hypo- and hypermethylation groups. It is not clear how transcriptional regulation can be exerted via transcriptional start sites (TSSs) proximal to both hypo- and hypermethylated regions. Furthermore, due to high degree of transcriptome inactivity of sperm cells, unknown reproductive capability of the females included in the studies and the fact that spermatozoa are a heterogeneous population, it is premature to draw

solid conclusions. Nevertheless, our results raise the possibility that the identified genes of both studies might contribute to the observed phenotypes, and further studies should be conducted to identify if any of these genes can be used as maturity/fertility biomarkers in the future.

5. Conclusions

In the current thesis work, we demonstrated that the combination of different flow cytometry methods and CASA enabled assessments of multiple sperm characteristics and were valuable tools for the assessment of sperm quality in relation to age differences and bull fertility potential. We further demonstrated that the conventional sperm quality parameters only explain part of the variation observed in bull fertility, and that underlying factors affecting fertility might be explained by the assessment of metabolites in semen and sperm DNA methylation. Further investigations are required to find specific biomarkers that can contribute to predict the reproductive performance of a bull based on its ejaculate quality.

Specific conclusions:

- Even small age differences in young bulls may significantly affect sperm quality, semen metabolite contents and production efficiency. However, 14-15 months old Norwegian Red bulls are mature enough for their semen to obtain successful fertilization.
- Sperm DNA methylation assessed by RRBS identified differences in methylated regions between bulls being 14 and 17 months of age. The majority of identified DMRs were hypomethylated in 14 months old bulls. The identification of DMRs and related pathways/genes offers useful insight into the reproductive capacity of young bulls.
- A method for the investigation of intracellular metabolites in the viable sperm population of frozen-thawed semen samples was successfully developed.
- Bulls of contrasting fertility differed in several aspects of sperm functionality and intracellular metabolite contents. Multiple correlations between metabolites, sperm quality parameters and NR56 indicate that metabolomics may be a useful tool in the identification of biomarkers for male fertility.
- A potential prediction model including the parameters DFI, aspartic acid, Fe and Zn explained 59% of the variability in bull field fertility.
- Sperm DNA integrity is significantly associated with field fertility and *in vitro* fertilization capacity of Norwegian Red bulls. Furthermore, sperm cells from bulls of inferior fertility have less compact chromatin structure and are hypermethylated compared to bulls of superior fertility.
- Differentially methylated cytosines identified as participants in biological pathways important for bull fertility were discovered in low- vs. high-fertility bulls.

6. Future perspectives

Based on the observed differences in sperm quality between bulls in relation to their age (Paper I and II) and fertility (Paper III and IV) it is desirable to focus future research on the understanding of such variations, and especially continue the search for novel biomarkers in semen. Study I and III revealed that several metabolites in semen are correlated with different aspects of sperm quality and fertility, indicating that metabolomics may be a useful tool in the identification of age and fertility related seminal markers. However, this should be further investigated using a higher number of bulls. Furthermore, it would be interesting to couple metabolomic data to other "omics" such as genomic and transcriptomic data, which may improve the future screening and selection of superior AI bulls. Several of the discovered metabolites in semen were reported to be involved in protecting sperm cells against damage from ROS. Oxidative stress in spermatozoa is linked to DNA damage, which was the sperm quality parameter strongest associated with NR56. Thus, a screening of ROS in semen is a possible future approach.

With the discovered differences in sperm quality, metabolite content, semen production efficiency and DNA methylation signatures between semen samples collected at 14 and 17 months of age, it would be interesting to do a further investigation of even younger bulls. To evaluate the effects of compensable sperm traits, a decrease in the sperm number per AI dose should be tested. With the breeding company in mind, it would be desirable to design a study with focus on IVP of embryos, embryo biopsies for GS and cryopreservation of the embryos. A successful procedure for GS at the pre-implantation stage and commercialisation of such a technology would benefit the whole AI industry.

The study of sperm DNA methylation patterns revealed several genes and pathways that offer useful insight into bull maturation and reproductive capacity. However, further studies are required to elucidate if the discovered genes may be used as epigenetic markers in the future. The study of other epigenetic modifications such as histone PTMs and non-coding RNAs may provide increased knowledge of the epigenetic landscape of bovine spermatozoa. To better elucidate how sperm DNA methylation affects fertility, the methylation pattern of IVP embryos should be assessed. Furthermore, to answer how transcriptional regulation can be exerted via TSSs proximal to both hypo- and hypermethylated regions, further research using transcriptome analysis of IVP embryos is recommended.

An epidemiologic study recently demonstrated a 50% decline in human sperm numbers and quality over the past 50 years (Levine et al., 2017). Research on the causes of this continuing decline is urgently needed, and animal models such as bovine are suitable for this purpose. The development of a male infertility diagnostic will be highly useful and remove a significant economic and social burden from our society. Thus, future studies should open for collaboration between the agricultural/animal science and human medicine. Such synergy between the professions may allow for new knowledge and competences.

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Sperm DNA Hypomethylation Proximal to Reproduction Pathway Genes in Maturing Elite Norwegian Red Bulls

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Genomic selection in modern farming demands sufficient semen production in young bulls. Factors affecting semen quality and production capacity in young bulls are not well understood; DNA methylation, a complicated phenomenon in sperm cells, is one such factors. In this study, fresh and frozen-thawed semen samples from the same Norwegian Red (NR) bulls at both 14 and 17 months of age were examined for sperm chromatin integrity parameters, ATP content, viability, and motility. Furthermore, reduced representation bisulfite libraries constructed according to two protocols, the Ovation® RRBS Methyl-Seq System (Ovation method) and a previously optimized gel-free method and were sequenced to study the sperm DNA methylome in frozen-thawed semen samples. Sperm quality analyses indicated that sperm concentration, total motility and progressivity in fresh semen from 17 months old NR bulls were significantly higher compared to individuals at 14 months of age. The percentage of DNA fragmented sperm cells significantly decreased in both fresh and frozen-thawed semen samples in bulls with increasing age. Libraries from the Ovation method exhibited a greater percentage of read loss and shorter read size following trimming. Downstream analyses for reads obtained from the gel-free method revealed similar global sperm DNA methylation but differentially methylated regions (DMRs) between 14- and 17 months old NR bulls. The majority of identified DMRs were hypomethylated in 14 months old bulls. Most of the identified DMRs (69%) exhibited a less than 10% methylation difference while only 1.5% of DMRs exceeded a 25% methylation difference. Pathway analysis showed that genes annotated with DMRs having low methylation differences (less than 10%) and DMRs having between 10 and 25% methylation differences, could be associated with important hormonal signaling and sperm function relevant pathways, respectively. The current research shows that RRBS in parallel with routine sperm quality analyses could be informative in reproductive capacity of young NR bulls. Although global sperm DNA methylation levels in 14 and 17 months old NR bulls were similar, regions with low and varying levels of DNA methylation differences can be identified and linked with important sperm function and hormonal pathways.

Keywords: Norwegian Red, bulls, puberty, sperm, DNA methylation, RRBS

INTRODUCTION

Epigenetics is a phenomenon where gene expression is regulated without any changes in DNA sequence, rather being modulated via changes in DNA methylation, histone post-translational modification, and interaction of transcriptional factors with small RNAs (Donkin and Barres, 2018). Epigenetic changes in sperm cells are even more complex compared to somatic cells for two main reasons. First, during primary phase of spermatogenesis, where germ cells develop to spermatids, DNA methylation is initially erased, becoming re-established later. Moreover, during spermiogenesis, where spermatids further differentiate to spermatozoa, the majority of histones are gradually replaced by protamines (O'Doherty and McGettigan, 2015; McSwiggin and O'Doherty, 2018). In recent years, different methods have been developed to study DNA methylation. Reduced representation bisulfite sequencing (RRBS) is an efficient and high-throughput method, allowing the study of DNA methylation profiles at single-base resolution, while experiment costs are kept low (Meissner et al., 2005). Previous studies have used RRBS to investigate DNA methylation profile in different bovine somatic tissues as well as bull sperm cells (Zhou et al., 2016; Perrier et al., 2018).

Sexual maturation in bulls is a hormone-regulated process lasting up to 50 weeks of age (Rawlings et al., 2008). Previous research has demonstrated that semen quality is closely correlated with different environmental factors and animal age. For instance, it has been shown that sperm morphology, concentration and motility positively correlated with age in young tropical composite bulls (Fortes et al., 2012) and Austrian Simmental bulls (Fuerst-Waltl et al., 2006). Although several studies reported low methylation levels in different genomic features of bull sperm cells (Perrier et al., 2018; Zhou et al., 2018; Kiefer and Perrier, 2019), the methylation level is dynamic and recent evidence suggests that the bull sperm methylome correlates with age (Lambert et al., 2018).

Norwegian Red (NR) is a highly fertile breed with e.g., low incidence of calving difficulties and mastitis (Refsdal, 2007; Ferris et al., 2014). Historically the NR breeding program was based on progeny testing of sires. However, starting from 2016, genomic selection was implemented in the NR breeding program and top NR elite bulls are today selected based on genomic breeding values. Despite the fact that NR is widely employed for artificial insemination (AI) in Norway and a very good record of genetic information has been build up during the last 40 years, little is known about the NR sperm methylome. In addition, because of short generation intervals due to genomic selection, there is more demand hence more physiological pressure for semen production from young NR bulls. The objective of this research is to determine if sperm DNA methylome could provide additional information to age related sperm quality differences. For this purpose, sperm samples from the same NR bulls at 14 and 17 months of age were used for analyzing chromatin integrity, viability, ATP content, and motility parameters. Furthermore in order to assess the sperm DNA methylome, two different RRBS protocols including Ovation RRBS Methyl-Seq System (as a, simple, fast, and scalable solution) and a gel-free based RRBS protocol which previously was optimized to study boar sperm DNA methylome (Khezri et al., 2019), were implemented.

MATERIALS AND METHODS

In the present study, the sperm quality traits including chromatin integrity parameters, ATP content, viability and motility parameters were analyzed in semen from NR elite bulls, at both 14 and 17 months of age. Furthermore, following comparison of two different protocols for constructing RRBS libraries, sperm DNA methylation in both age groups was analyzed.

Semen Collection and Sample Preparation

Bulls in this study, were raised, cared for and fed standardized diet at Geno SA (Geno Breeding and AI Association, Hamar, Norway), AI station. Ejaculates were collected from nine young genomic selected NR bulls with unknown fertility at 14 and 17 months of age and processed by the breeding company Geno SA. Prior to dilution, sperm cell concentration in each ejaculate was calculated using an Accucell® spectrophotometer (IMV Technologies, L'Aigle, France). The semen was diluted in a two-step procedure using Biladyl extender (13500/0004-0006; Minitübe, GmbH, Tiefenbach, Germany). After first dilution, samples were taken for fresh semen analyses and simultaneously used for subjective quality control analysis. Ejaculates with motility above 70% and morphological abnormalities below 20% were further diluted with the glycerol-containing fraction (1:2) to a final concentration of 12×10^6 sperm cells per insemination dose, filled into 0.25 ml standard French mini straws (IMV, L'Aigle, France), and cryopreserved as previously described (Standerholen et al., 2014). Cryopreserved doses were later prepared for sperm quality analyses and DNA extraction by thawing the semen doses for 1 min in a water bath at 37°C. To minimize the influence of possible variation between straws, semen from two straws/ejaculate were pooled and mixed before analyses.

Sperm Quality Analyses

Sperm Chromatin Integrity Assessment

Sperm chromatin integrity assessment was performed using sperm chromatin structure assay (SCSA) (Evenson and Jost, 2000; Boe-Hansen et al., 2005). Using this assay, two different chromatin integrity parameters, including DNA fragmentation index (DFI) and high DNA stainability index (HDS), were measured.

In brief, both fresh and frozen-thawed semen samples were diluted to 2×10^6 cells/ml using TNE buffer (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4). Diluted samples were denatured for 30 s by adding an acid detergent solution (0.38 M NaCl, 80 mM HCl, 0.1% Triton-X 100, pH 1.2). Denatured samples were stained with acridine orange (AO) staining buffer (37 mM citric acid, 0.126 M Na_2PO_4, 1.1 mM EDTA, 0.15 M NaCl and 0.6 μg/ml of AO, pH 6.0) and were incubated for 3 min at room temperature. Two technical replicates were considered for each sample and 5000 sperm cells per replicate

were analyzed using a pre-AO saturated flow cytometer equipped with a blue laser (488 nm) (Cell Lab QuantaTM SC MPL flow cytometer, Beckman Coulter, Fullerton, CA, United States). Laser stability was controlled (at the beginning of the experiment and after every fifth sample) using a bull reference sample with pre-identified DFI and by re-setting the mean green and red fluorescence signals to 425 ± 5 and 125 ± 5, respectively. Following AO staining, double- and single-stranded DNA emitted green (collected using a 525 nm band pass filter) and red fluorescence (collected using a 670 nm long pass filter), respectively. The percentage of red and green fluorescence was determined using the FCS Express 6 flow cytometry data analyzer software (*Denovo* Software, Los Angeles, CA, United States). Based on the ratio of red/(red + green), the DFI percentage was calculated. Furthermore, HDS sperm cells, which are considered as sperm cells with an incomplete chromatin condensation, were identified according to a high incorporation of AO into double-stranded DNA.

Assessment of Sperm Viability

Sperm viability and data analysis were performed using the flow cytometry system described above and Kaluza® software, Version 2.1 (Beckman Coulter Ltd.). Frozen-thawed semen samples were diluted in SP-Talp media (105 mM NaCl, 3.1 mM KCl, 0.4 mM $MgCl_2$, 2.0 mM $CaCl_2{\cdot}2H_2O$, 0.3 mM $NaH_2PO_4{\cdot}H_2O$, 1 mM sodium pyruvate, 21.6 mM sodium lactate, 20 mM Hepes, 20 mM Hepes salt, 5 mM glucose, 50 μg/ml gentamycin) to a concentration of 1×10^6 sperm cells/ml. Two technical replicates were considered per sample. Sperm suspensions were stained with 0.48 μM propidium iodide (PI, Sigma-Aldrich) and incubated for 10 min prior to flow cytometric analysis. PI fluorescence was detected using a 670 nm long pass filter (FL3), and gating was performed to reveal sperm cells population (based on electronic volume) and percentages of living spermatozoa as previous described (Standerholen et al., 2014).

Sperm ATP Content

The ATP content was measured in frozen-thawed semen samples using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega; Madison, Wisconsin). A total volume of 60 μl of semen (3×10^5 sperm cells) was added to a white 96-well microtiter plate (NUNC®, Denmark) and mixed with 60 μl CellTiter-Glo® reagent. To induce cell lysis, the mixture was gently shaken for 2 min in a rotary shaker (IKA® MS 3 digital, United States), followed by 15 min incubation at room temperature to stabilize the luminescence. The bioluminescence signal was measured in relative luminescence units (RLU) using a FLUOstar OPTIMA multiwell plate reader (BMG LABTECH GmbH, Offenburg, Germany), equipped with MARS data analyzer software (Version 1.10, BMG LABTECH, Germany). Obtained RLU signals were converted to a corresponding ATP value in nM according to a prepared standard curve. ATP values obtained from the average of three technical replicates per sample. Then the value further corrected for the percentage of motile sperm cells before statistical analyses.

Sperm Motility Analysis

Sperm motility analysis were performed using the SCA evolution CASA system (Microptic SL, Spain), equipped with a phase contrast Eclipse Ci-S/Ci-L microscope (Nikon, Japan), a BASLER Ace acA780-75 gc digital camera (Basler Vision Technologies, Ahrensburg, Germany) and Sperm class analyzer software (v 6.1.0.0). Fresh and thawed semen samples were incubated for 15 min at 37°C, and diluted (1:2) with pre-warmed PBS buffer (37°C) before analysis. A volume of 3 μl of diluted samples was loaded into the chamber of a pre-warmed (37°C) 20 μm depth Leja-4 slide (Leja products, Nieuw-Vennep, the Netherlands). Analyses were performed using two technical replicates per sample, under a 10x objective and on the pre-heated thermal stage (37°C) of the phase contrast microscope. Eight or more microscope fields with at least 800 cells per sample were analyzed. Bull sperm cells were detected based on head area (20–80 μm^2) and camera setting of 45 frames per sec. The motility parameters measured were total motility, progressive motility, and hyperactive motility. In addition, other information regarding to sperm motion kinetics including curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), straightness of the average path [STR (%) = VSL/VAP], linearity of the curvilinear path [LIN (%) = VSL/VCL], Wobble [WOB (%) = VAP/VCL], lateral displacement of sperm head (ALH, μm) and beat cross frequency (BCF, Hz) were obtained. Sperm cells were defined as static and progressive motile if VAP < 10 μm/s and STR > 70μm/s, respectively. Sperm cells with VCL > 80μm/s, ALH > 6.5μm and LIN < 65% were defined as sperm cells with hyperactive motility.

RRBS Library Preparation

Prior to RRBS library construction, DNA from frozen-thawed sperm samples of seven bulls at, both 14 and 17 months of age, was extracted using a Maxwell 16 Benchtop instrument (Promega Corporation, United States) at Biobank AS, Hamar. Isolated DNA was quantified using Qubit dsDNA BR assay kit (Thermo Fisher Scientific, United States) and further diluted to 20 ng/μl in low TE media [10 mM Tris, pH 8.0 (Calbiochem, United States), 0.1 mM EDTA, pH 8.0 (Calbiochem, United States)]. Libraries for sperm DNA methylation analysis were constructed using the RRBS approach and according to two different RRBS protocols.

RRBS Library Preparation Using Ovation® RRBS Methyl-Seq System (Ovation Method)

In this method Ovation® RRBS Methyl-Seq System (NuGEN Technologies, San Carlos, CA, United States) was employed and RRBS libraries were constructed using 100 ng genomic DNA, according to the manufacturer's protocol with slight modifications.

Briefly, genomic DNA was digested overnight at 37°C using *Msp*I and Taq α1 enzymes (New England Biolabs, United States). After digestion, AMPure XP beads (Beckman Coulter, United States) were added (2x) and samples were kept at room temperature for 30 min. Then by putting the samples on a side magnet, supernatant was removed and beads were washed twice with 100% EtOH. Dried beads were re-suspended

in 10 μl elution buffer (Qiagen, Germany) and fragmented DNA was ligated with adapters by incubation at 25°C for 30 min followed by 70°C for 10 min. Adapter ligated fragments were final repaired at 60 and 70°C each for 10 min. The fragments were further size selected by adding 1.5x of 20% PEG 8000/2.5 M NaCl (Amresco Inc., United States) followed by incubation for 30 min at room temperature. The supernatant was removed as previously described and after washing the beads twice with 70% EtOH and drying, the beads were re-suspended in 25 μl elution buffer (Qiagen, Germany). Eluted products were subjected to bisulfite conversion using EpiTect kit (QIAGEN, Germany) following the manufacturer's protocol designated for DNA extracted from FFPE tissues. Bisulfite converted DNA, was amplified using 10 cycles of PCR. Amplified libraries were purified by adding 1x SPRI AMPure XP beads followed by incubation for 30 min at room temperature. Supernatant was removed, beads were washed with 70% EtOH and re-suspended in elution buffer. Eluted beads were further placed on a side magnet and purified libraries were transferred to a clean tube.

RRBS Library Preparation Using a Gel-Free Multiplexed Method (Gel-Free Method)

In this method RRBS libraries were constructed using a gel-free multiplexed technique (Boyle et al., 2012), which we previously optimized it to study boar sperm DNA methylome (Khezri et al., 2019). The protocol was consisted of the following steps.

First, genomic DNA (100 ng) was digested as described in section "RRBS Library Preparation Using Ovation® RRBS Methyl-Seq System (Ovation Method)." Gap filling and A-tailing steps were carried out by adding 1 μl of Klenow fragment (New England Biolabs, United States) along with 1 μl of dNTP mixture containing 10 mM dATP, 1 mM dCTP, and 1 mM dGTP (New England Biolabs, United States) to fragmented DNA. The processed DNA was incubated for 20 min at 30°C followed by 20 min at 37°C. After incubation, fragmented DNA was size selected (300–500 bp) by adding a 2x AMPure XP beads (Beckman Coulter, United States). After incubation in room temperature for 30 min the supernatant was removed as previously described in section "RRBS Library Preparation Using Ovation® RRBS Methyl-Seq System (Ovation Method)" and beads were washed and re-suspended in 20 μl elution buffer (Qiagen, Germany). Adapter ligation was performed by adding 2 μl of NEXTflex^{TM} Bisulfite-Seq barcodes (Bio Scientific Corporation, United States) and ligase mixture to eluted DNA followed by overnight incubation at 16°C. Adapter-ligated DNA again was size selected by adding 1.5x of 20% PEG 8000/2.5 M NaCl (Amresco Inc., United States) followed by incubation at room temperature for 30 min. The product was placed on a side magnet; supernatant was removed, beads were washed two times in 70% EtOH and were re-suspended in 25 μl elution buffer (Qiagen, Germany). Prior to fragment amplification, different PCR amplification cycles (10, 13, 16, and 19 cycles) were tested. PCR products were run on a gradient 4–20% Criterion precast polyacrylamide TBE gel (Thermo Fisher Scientific, United States). Gradient gel further stained with SybrGold (Thermo Fisher Scientific, United States) and the efficiency of protocol were evaluated based on the appearing DNA bands. Later, size selected DNA fragments were bisulfite converted and product was cleaned up according to recommended protocol in the QIAGEN EpiTect kit (Gu et al., 2011). At the last step, converted DNA, PCR amplified using 13 amplification steps (PCR Primer 1: 5′- AATGATACGGCGACCGAGATCTACAC-3′, PCR Primer 2: 5′-CAAGCAGAAGACGGCATACGAGAT-3′) and PCR product (libraries) were further cleaned up using 1x SPRI beads as described earlier for the Ovation method.

Illumina Sequencing

Eluted RRBS libraries from both protocols were quantified using the PicoGreen dsDNA absorbance method and were sent to Norwegian Sequencing Center. Sequencing was performed using Illumina HiSeq 2500 in the paired-end (2 × 150 bp) mode.

Bioinformatics Analyses

Illumina Reads Quality Assessment and Trimming

The quality of paired-end Illumina reads first was assessed using fastQC software (v 0.11.8 for Linux). For reads obtained via the gel-free protocol, Illumina adapters and low-quality sequences (below 20 bp and Phred score of 30) were trimmed using Trim-galore software (v 0.4.4 for Linux) (Martin, 2011). For reads obtained via the Ovation protocol, manufacturer's recommendations were followed for quality control and adapter trimming[1]. Then, additional nucleotides from the 5′ ends of adapter-removed reads, were further trimmed using a NuGEN-developed 'trimRRBSdiversityAdaptCustomers.py' script in Python (2.7.5 for Linux).

Mapping the Clean Reads With Reference Genome

Bull reference genome (*bosTau 9*) was downloaded from the UCSC database (UCSC, 2018) and was indexed using bismark_genome_preparation option in Bismark (v 0.19.0 for Linux) (Krueger and Andrews, 2011). After initial assessment of libraries (**Table 2**), only reads from the gel-free protocol were mapped to the reference genome. The mapping was carried out using Bismark and bowtie2 aligner (v 2.3.2 for Linux) (Krueger and Andrews, 2011) with following parameters [-n 0 −l 20 and –score-min (L, 0, −0.6)]. All covered Cs were used for calculation of global CpG methylation level in Bismark using following formula:% of global methylation = 100 * number of methylated Cs/number of methylated Cs + number of unmethylated Cs.

Differential Methylation Analysis

In this study differentially methylated regions were identified between control (17 months old bulls) and test (14 months old bulls) groups. In brief, SAM-sorted alignment files from Bismark were analyzed using the methylKit package (v 1.6.1) (Akalin et al., 2012) in Rstudio (v 1.1.453 for Linux). First, reads containing CpGs with more than 99.9th percentile coverage were filtered out. Every single C was considered to calculate differentially methylated regions (DMRs). For this purpose, the genome was divided in 1000 bp tiles with sliding step 1000 bp, containing at least three mutually covered Cs in the CpG context. Average DNA methylation of each tile was calculated and in order to determine

[1]https://github.com/nugentechnologies/NuMetRRBS

DMRs with *q-value* < 0.05 (filtered DMRs onward), logistic regression with a sliding linear model to correct for multiple comparisons was applied. In this study, hypermethylation and hypomethylation are defined as any positive and negative value for DMRs in the test group compared to the control group, respectively. Later, DMRs were categorized as those with < 10% differential methylation ($DMRs_{<10\%}$), those showing 10–25% ($DMRs_{10-25\%}$) and those exhibiting > 25% methylation differences ($DMRs_{>25\%}$) and were used for downstream analyses.

Annotation of Differentially Methylated Regions

BED files containing gene and CpG annotation for the *bosTau9* assembly were downloaded from the UCSC table browser (UCSC, 2018). All DMRs with any level of hypo/hyper methylation were annotated with nearest (no specific cut off) transcriptional start site (TSS), genes elements (exons, introns, promoter, intergenic regions) and CpG features (CpG islands, CpG shore, other) using Genomation package (v 1.14.0) in Rstudio. Promoters and CpG shore were defined as ± 1000 bp and ± 2000 bp of the TSS and CpG islands, respectively.

Pathway Analysis

Corresponding GenBank accession IDs to annotated TSSs, were submitted to DAVID Bioinformatics resources for functional annotation (Huang da et al., 2009) for Gene Ontology (GO) analysis. Gene enrichment for each identified pathway was calculated using Fisher's exact test and *p-value* was Benjamini adjusted for multiple testing and set to 0.05.

Statistical Analyses

Statistical analyses were performed in Rstudio (v 1.1.383 for windows). In order to compare sperm quality parameters in fresh and frozen-thawed samples from 14 and 17 months old bulls, linear mixed models within the lme4 package were established using quality parameters of sperm cells and bulls age as response and independent variables, respectively. In addition, animals, semen concentration at the time of semen collection and pedigree information were included as random effects. The level of significance (*p-value*) was set to 0.05 except for DFI and HDS where, in order to minimize type I error, *p-values* were Bonferroni adjusted to 0.025. Results were plotted using GraphPad Prism (v 6.01 for Windows, GraphPad Software, San Diego, CA, United States). Venn diagrams were constructed using Venny online platform (Oliveros, 2015). Pathway analysis results were plotted using ggplot2 package (v 3.1.0) in Rstudio (Wickham, 2016).

RESULTS

Sperm Quality Analyses in Young Norwegian Red Bulls

Sperm quality analyses results showed that some of the parameters were significantly different between the 14 and 17-months old bulls (**Table 1**). For instance, sperm concentration in 17 months old bulls was significantly higher compared to those 14 months old. Furthermore, both fresh and frozen-thawed samples from 14 months old bulls showed higher DFI compared to the 17 months old group. In addition, fresh sperm cells from 17 months old bulls showed significantly higher HDS (less condensed DNA) compared to those 14 months old. However, no significant changes in HDS between 14 and 17 months. of age were observed in frozen-thawed semen samples. The results further indicated that total sperm motility and progressivity in fresh semen from 17 months old bulls were significantly higher compared to 14 months old bulls. However, in frozen-thawed semen none of the sperm motility parameters was significantly different in bulls 14 months, compared to 17 months of age. Other sperm motion kinetic parameters showed no significance differences between 14 and 17 months old bulls (**Supplementary Table 1**).

Bioinformatics Analyses of RRBS Data

Comparison of RRBS Data Obtained Based on Ovation and Gel-Free Protocols

Table 2 compares the summary statistics for RRBS data obtained from two protocols. Surprisingly, no Illumina adapter contamination was detected for reverse reads in libraries constructed using Ovation method while both forward and reverse reads from RRBS libraries constructed based on gel-free

TABLE 1 | An overview of results (mean ± SEM) for different sperm quality parameters analyses for both fresh and frozen-thawed semen samples in 14 months (*n* = 9) and 17 months (*n* = 8) old Norwegian red bulls.

	Fresh semen		Frozen-thawed semen	
	14 months	**17 months**	**14 months**	**17 months**
Sperm concentration (10^6 cells/ml)	974 ± 83.80	1330 ± 133.40*	NA	NA
DFI	2.78 ± 0.30	1.83 ± 0.20*	2.02 ± 0.13	1.64 ± 0.20*
HDS	0.67 ± 0.07	0.80 ± 0.10*	0.64 ± 0.05	0.88 ± 0.15
ATP (nM/10^6 cells)	NA	NA	1.22 ± 0.13	1.16 ± 0.06
Viability (%)	NA	NA	62.15 ± 5.32	65.64 ± 2.85
Motility (%)	87.09 ± 1.51	98.10 ± 0.30*	58.45 ± 5.10	63.47 ± 2.95
Progressivity (%)	72.90 ± 2.00	87.23 ± 1.00*	52.16 ± 5.52	59.26 ± 3.16
Hyperactivity (%)	24.34 ± 1.83	24.21 ± 3.51	10.44 ± 2.24	12.50 ± 1.47

*Asterisks indicate a significant difference between age in fresh or frozen-thawed samples based on linear mixed model. *p < 0.05 for all parameters except for DFI and HDS, where *p < 0.025 (Bonferroni corrected). NA, data not available; DFI, DNA fragmentation index; HDS, high DNA stainability; nM, nanomolar.*

TABLE 2 | An overview of summary statistic for RRBS libraries constructed based on Ovation RRBS Methyl-Seq and our previously optimized method (gel-free method).

RRBS protocol	Adapter contamination	Read loss after trimming	Reads length (bp) distribution after trimming	
Ovation method	Detected only in forward reads	51%	< 50	34%
			50–99	40%
			100–150	26%
Gel-free method	Detected in both reads	8%	< 50	4%
			50–99	29%
			100–150	67%

Paired-end reads from both methods were quality checked using fastQC software and trimming for Ovation libraries was performed according to manufacturer's instructions. Read loss percentage was calculated as number of reads after trimming/number of reads before trimming. Bp, base pair.

method, showed Illumina adapter contamination. After quality control and trimming, 51% of reads were discarded in Ovation libraries, whereas quality control and trimming resulted in only 8% read loss in gel-free method. After trimming, reads with length < 50 bp and 100–150 bp were corresponding to 4 and 67% of all reads in libraries made according to gel-free protocol, respectively. Whereas reads with similar size in Ovation libraries were about 34 and 26% of total reads, respectively. This is particularly important, as longer reads tend to align better with reference genome in Bismark (Tran et al., 2014). Therefore, based on observed differences, we decided to conduct downstream analyses using RRBS libraries constructed based on the gel-free optimized method.

Basic Statistics of RRBS Libraries Constructed Based on Gel-Free Method

Using an in-house bioinformatics pipeline and after trimming the Illumina reads, 91% of reads were retrieved in libraries constructed based on the gel-free protocol. As shown in **Table 3**, this was equal to an average of 7.7 million reads per sample, 33.1% unique mapping efficiency, 15.9x read coverage and 99.1% conversion rate. Overall, minimum variation was observed between samples from different individuals and age regarding to retrieved clean reads, mapping efficiency, global CpG methylation, and bisulfite conversion rate (**Table 3**). Furthermore, CpG statistic revealed that generated libraries in average covered 4.4 million CpG, with methylation average of 40%. Mixed models indicated that none of mapping efficiency, global CpG methylation level and conversion rate parameters were significantly different ($p < 0.05$) in 14 months compared to 17 months old bulls. Cluster analysis based on methylation value of CpG_{W1000} (i.e., CpGs that have fallen into a 1000 bp tiles across the genome) in each sample, showed that samples are distributed in two main clusters. However, within the main clusters, samples from the same individuals but different age always sub-clustered together (**Figure 1**). Furthermore, Pearson's correlation coefficient based on methylation value of CpG_{W1000} indicated a very high positive correlation between samples in term of global methylation profile (Pearson's correlation coefficient ≥ 0.95) (**Supplementary Table 2**).

Differential Methylation Analysis

Differential methylation analysis were performed using a tile-based approach. This resulted in identification of 131,073 DMRs between test (14 months old) and control (17 months old) bulls. However, after setting the level of significance to *q-value* < 0.05, a total number of 6426 DMRs (filtered DMRs) were detected with varying levels of methylation ranging from 0 to 38%. Majority of filtered DMRs (60%) were found to be hypomethylated in the 14 months old group relative to the control group (**Figure 2A**). Distribution of DMRs exhibiting varying degrees of methylation differences in hypomethylation and hypermethylation groups were similar; ∼70% of DMRs showed less than a 10% difference in methylation, and ca. 1.5% of DMRs had over a 25% difference in methylation levels (**Figures 2B,C**).

Annotation of Differentially Methylated Regions With Gene and CpG Features

In this study, all filtered DMRs with any level of methylation differences ($DMRs_{<10\%}$, $DMRs_{10-25\%}$ and $DMRs_{>25\%}$), were considered for downstream analyses. The filtered DMRs were annotated with gene and CpG features. The analyses showed that, on average, 65% of the filtered DMRs were present in the intergenic regions. Annotation of DMRs in both hypomethylation and hypermethylation groups within promoters and introns showed similar trend. For instance, $DMRs_{<10\%}$ and $DMRs_{>25\%}$ were the major groups that annotated within promoter and intron regions, respectively (**Figure 3A**). For CpG features, on average, over 85% of filtered DMRs in both hypomethylation and hypermethylation groups were annotated within regions outside of CpG islands (CGI)/CpG shores. A majority of annotated DMRs within these external regions exhibited methylation differences exceeding 25%. Only around 15% of filtered DMRs were annotated within CGI/CpG shores and most showed less than a 10% methylation difference (**Figure 3B**).

Next, the nearest transcription start sites (TSSs) to filtered DMRs were extracted (**Figures 4A,B**). This resulted in a greater number of TSSs in the hypomethylation group (2982 TSSs) compared to the hypermethylation group (2129 TSSs). However, in both hypomethylation and hypermethylation groups, a majority of TSSs were associated with $DMRs_{<10\%}$, followed by $DMRs_{10-25\%}$ and $DMRs_{>25\%}$. Furthermore, 474 TSSs associated to $DMRs_{<10\%}$ were annotated with both hypo- and hypermethylated regions. This number for the $DMRs_{10-25\%}$ was 156 TSSs and no commonly annotated TSS was found between hypo- and hypermethylated regions in the $DMRs_{>25\%}$ group (**Supplementary Figure S1** and **Table 3**).

Pathway Analysis

Genes whose TSSs were annotated with DMRs were first identified and subsequently subjected to pathway analysis. We were particularly interested in genes associated with biological processes and molecular functions related to sexual maturity such as androgen signaling, steroid hormone biosynthesis,

TABLE 3 | An overview of basic statistic for RRBS libraries constructed based on the gel-free protocol.

Sample ID	Total reads	Clean reads after trimming	Read coverage (X)	Unique mapping efficiency (%)	Global CpG methylation (%)	Number of covered CpGs	Bisulfite conversion rate (%)
14 A	8,092,814	7,424,375	15.3	32.3	40.4	4,287,779	99.2
17 A	8,769,052	8,037,908	16.2	29.2	38.4	4,161,727	99.2
14 B	8,187,841	7,514,351	15.4	30.5	38.0	3,867,873	99.2
17 B	7,916,740	7,242,150	15.0	30.2	37.6	3,941,237	99.2
14 C	9,235,837	8,452,463	17.4	33.3	40.3	4,792,957	99.2
17 C	8,208,151	7,515,657	15.3	33.0	39.9	4,267,681	99.2
14 D	8,608,751	7,932,141	15.6	33.3	39.6	4,529,706	99.3
17 D	6,947,434	6,395,163	12.4	32.6	39.6	3,918,954	99.2
14 E	7,745,029	7,098,249	14.6	33.9	40.2	4,230,165	99.2
17 E	8,179,041	7,538,949	15.7	34.4	40.7	4,384,431	99.2
14 F	9,561,827	8,631,458	18.2	33.9	41.2	4,855,729	99.0
17 F	11,204,211	10,288,338	21.6	34.8	41.4	5,383,672	98.9
14 G	6,739,955	6,197,336	13.1	35.2	40.7	3,947,850	98.8
17 G	8,406,205	7,643,950	16.4	37.0	42.7	4,819,724	98.7

Libraries were constructed from sperm DNA collected from the same young Norwegian red bulls (n = 7) at two different ages. Letters A to G prefixed by 14 or 17, indicating different bulls of age 14 and 17 months, respectively. Clean reads were obtained after adapter and low-quality trimming of Illumina sequencing reads (total reads). Read coverage was calculated by number of bp in the clean reads/number of bp at in silico MspI-digested bosTau9 genome. Mapping efficiency shows the percentage of uniquely mapped clean reads with the reference genome. CpG methylation shows the percentage of global methylation in clean reads. Downstream analyses were performed based on all covered CpGs. Bisulfite conversion rate shows the proportion of Cs, which converted to uracil during bisulfite conversion process.

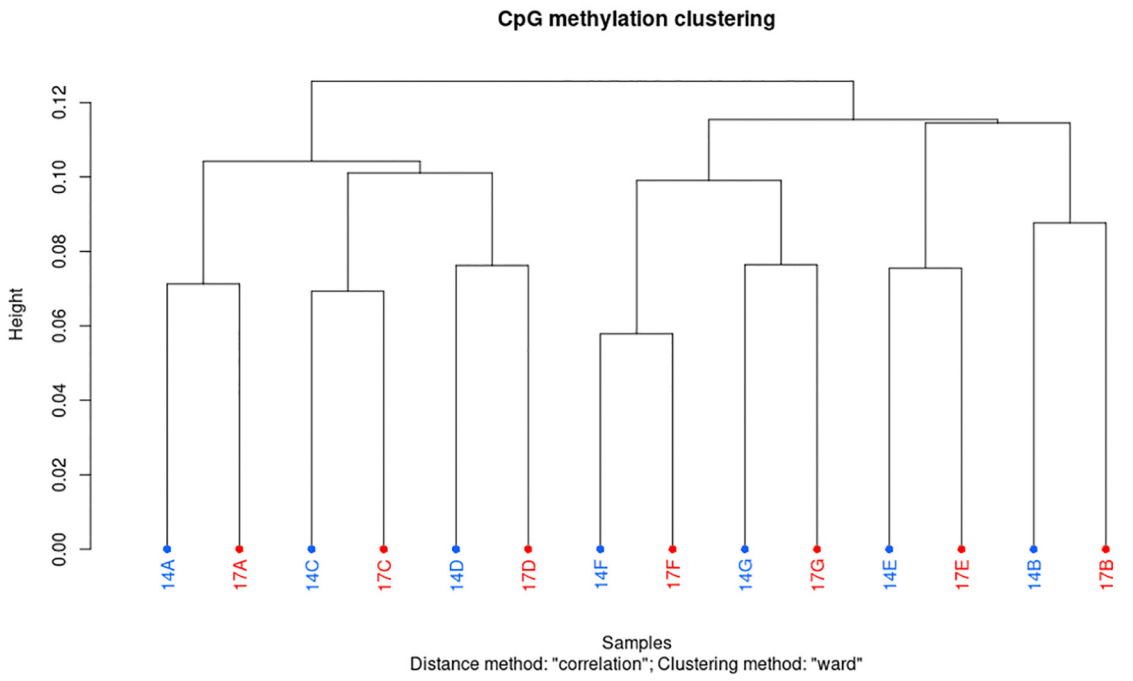

FIGURE 1 | Hierarchical clustering of samples based on their CpG_{W1000} methylation levels in sperm DNA from Norwegian red bulls. Letters A to G prefixed by 14 or 17, indicating different bulls of age 14 and 17 months, respectively.

steroid hormone receptor signaling, spermatogenesis and developmental growth. **Figures 5A,B** show that the numbers of such genes were higher in the hypomethylation group compared to the hypermethylation group. Steroid hormone biosynthesis, identified exclusively in hypermethylation group. Moreover, genes whose TSSs were annotated with the $DMRs_{<10\%}$ and $DMRs_{>25\%}$ groups represented those functions to the greatest and least extent, respectively. In both hypo- and hypermethylation groups, functions including spermatogenesis, followed by steroid hormone receptor and energy homeostasis, were represented by the highest numbers of genes (**Supplementary Table 4**).

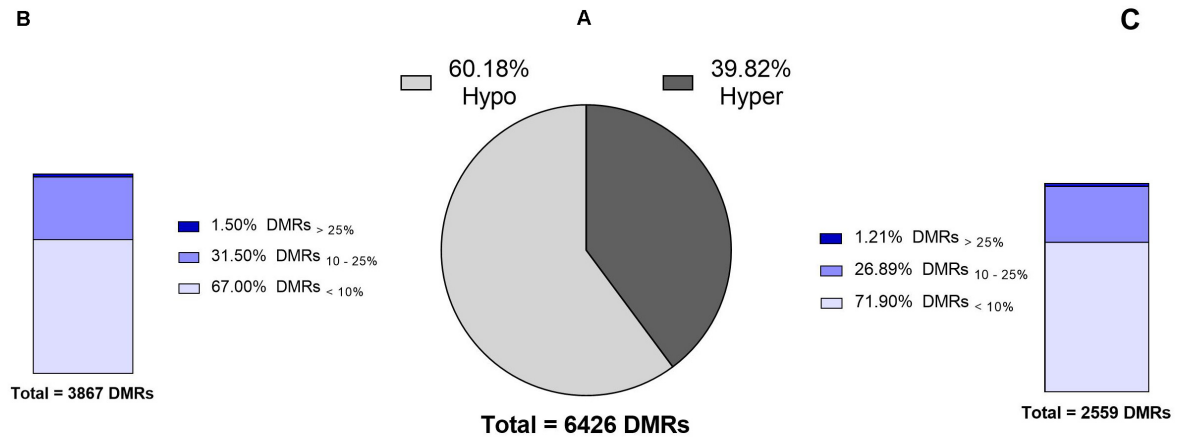

FIGURE 2 | Numbers and levels of significant DMRs identified in the sperm DNA methylome between identical 14 months old bulls (test) and 17 months old bulls (control). **(A)** Total number of significant DMRs indicating that 60% are hypomethylated in the test group compared to the control group. **(B,C)** Different levels of hypo- and hypermethylated regions in the test group compared to the control group. Explanation of percentage ranges: $DMRs_{<10\%}$ indicating regions with less than 10% difference in DNA methylation, $DMRs_{10\text{-}25\%}$ indicating regions having between 10 and 25% difference in DNA methylation, $DMRs_{>25\%}$ indicating regions having over 25% difference in DNA methylation.

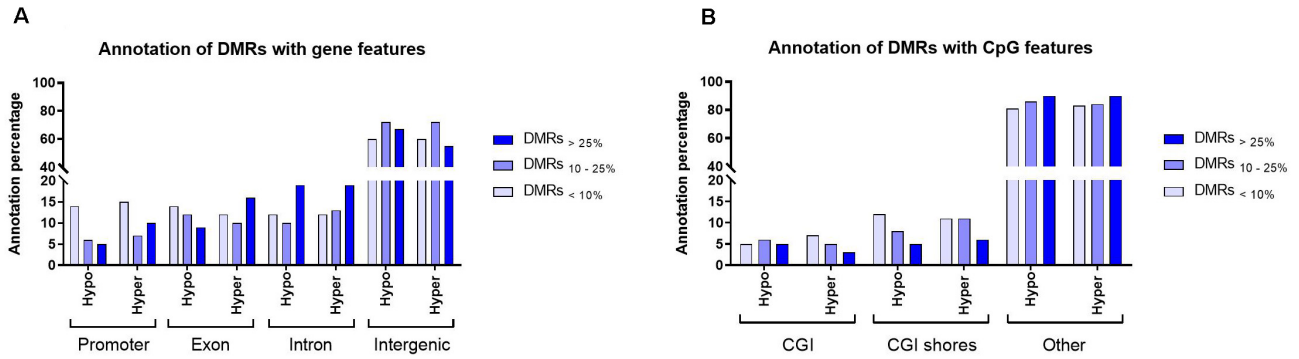

FIGURE 3 | An overview of the distribution of filtered DMRs in the *bosTau9* genome. **(A)** Annotation of the hypo- and hypermethylated regions with gene features. **(B)** Annotation of the hypo- and hypermethylated regions with CpG features. Hypo, hypomethylated regions; Hyper, hypermethylated regions; CGI, CpG island. Explanation of percentage ranges: $DMRs_{<10\%}$ indicating regions with less than 10% difference in DNA methylation, $DMRs_{10\text{-}25\%}$ indicating regions having between 10 and 25% difference in DNA methylation, $DMRs_{>25\%}$ indicating regions having over 25% difference in DNA methylation.

As shown in **Figure 6**, similar to biological processes and molecular functions, the majority of identified pathways (49 pathways) were in association with genes annotated with $DMRs_{<10\%}$. Only eight pathways were linked to genes annotated with $DMRs_{10-25\%}$. None of the identified pathways exhibited significant association with $DMRs_{>25\%}$. Some of the hormonal pathways (gonadotropin-releasing hormone, estrogen and oxytocin signaling) and sperm function related pathways (disulfide bond and glycoprotein) were exclusively identified in the hypermethylation group of test samples (14 months old bulls). In other words, genes associated with those pathways were annotated with hypomethylated regions in more mature, 17 months old, bulls. Although the number of annotated TSSs to DMRs (with any level) was higher in hypomethylation groups compared to the hypermethylation groups (**Figure 4**), the number of pathways represented by genes harboring those TSSs showed an opposite trend (**Figure 6**). However, the majority of identified pathways in the $DMRs_{<10\%}$ hypomethylation group exhibited stronger *p-values* (**Figure 6A**).

DISCUSSION

In this study, sperm quality was assessed in 14 and 17 months old NR bulls. Furthermore, DNA methylation patterns were elucidated in sperm cells from the same individuals using RRBS data generated from comparative library construction where two protocols were tested.

Our results showed that the number of sperm cells in ejaculates from NR bulls significantly increased with aging. These results support previous findings where total sperm count was higher in post-pubertal Holstein bulls compared to 4 months younger bulls (Devkota et al., 2008; Wu et al., 2020). Sperm DFI results (**Table 1**) indicate that DNA integrity

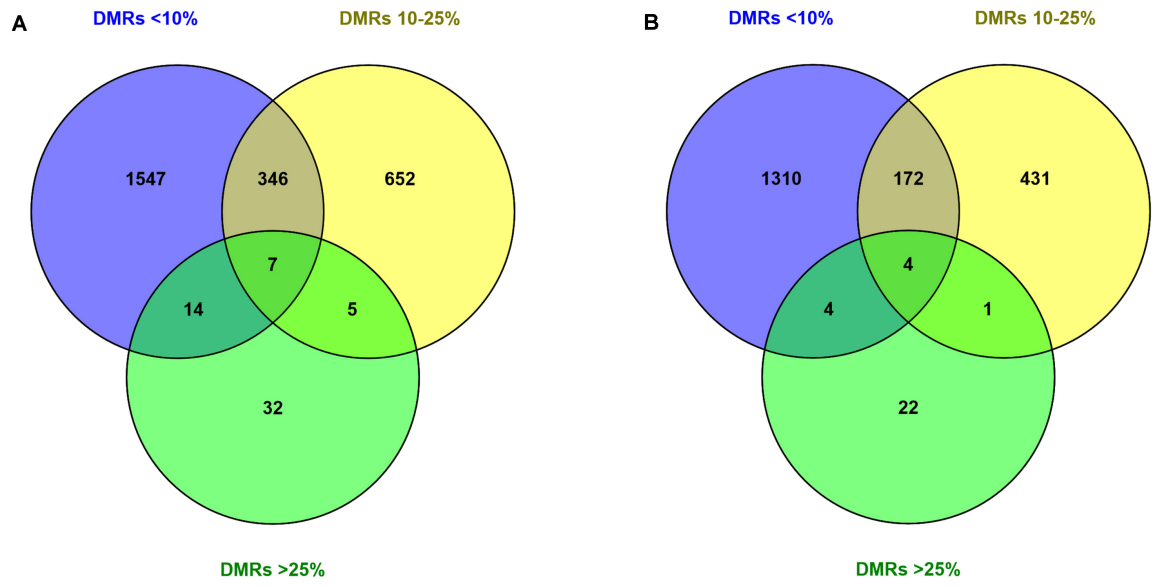

FIGURE 4 | Annotation of the filtered DMRs with varying degrees of methylation difference to the nearest TSSs. Numbers inside the circles, showing the number of closest unique TSSs (duplicated TSSs removed) to DMRs in **(A)** hypomethylation and **(B)** hypermethylation groups. Explanation of percentage ranges: $DMRs_{<10\%}$ indicating regions with less than 10% difference in DNA methylation, $DMRs_{10\text{-}25\%}$ indicating regions having between 10 and 25% difference in DNA methylation, $DMRs_{>25\%}$ indicating regions having over 25% difference in DNA methylation.

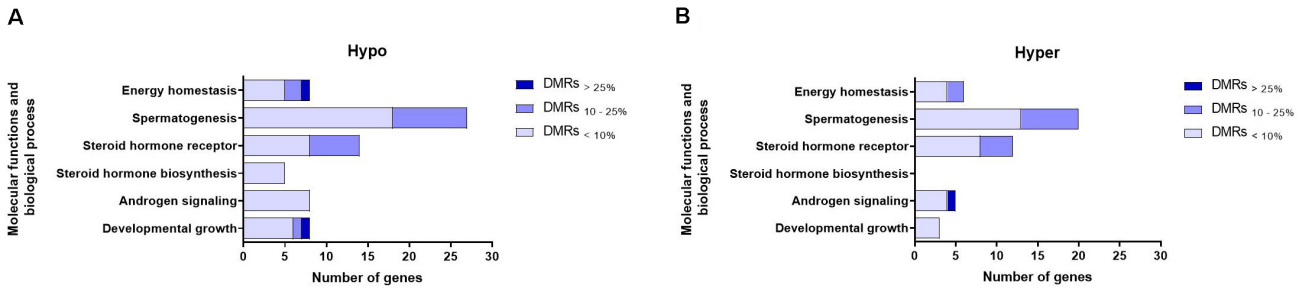

FIGURE 5 | Numbers of genes representing different molecular functions and biological processes related to sexual maturation in **(A)** hypomethylated (Hypo) and **(B)** hypermethylated (Hyper) groups and displayed according to their association with DMR-groups exhibiting varying degrees of methylation differences. Explanation of percentage ranges: $DMRs_{<10\%}$ indicating regions with less than 10% difference in DNA methylation, $DMRs_{10\text{-}25\%}$ indicating regions having between 10 and 25% difference in DNA methylation, $DMRs_{>25\%}$ indicating regions having over 25% difference in DNA methylation.

also improves with aging. This can be explained by ongoing sperm nucleus development from 14 to 17 months of age as it has been reported that sperm DNA is more compact in older bulls rendering it less prone to fragmentation (Andrabi, 2007). In addition, sperm DFI was reduced in frozen-thawed semen samples compared to fresh samples in both age groups. It seems possible that sperm cells with higher DFI in fresh semen may not tolerate the cryopreservation process and became degraded during cryopreservation and thawing, hence not falling into the gate defined as sperm cells based on electronic volume in the flowcytometry analysis. However, the observed differences are small and less likely to be of biological importance. The HDS results presented here indicate that the degree of sperm chromatin compactness was reduced in fresh NR ejaculates at 17 compared to 14 months of age. This must, however, be further investigated using a larger number of samples. Furthermore, both total sperm motility and sperm progressivity were significantly increased in fresh semen from bulls of age 17 compared to 14 months. This trend was also observed for frozen-thawed samples, although the changes were not significant. These results are in agreement with observations from previous research where sperm cell motility in fresh samples were positively correlated with increasing age in Holstein bulls (Devkota et al., 2008; Murphy et al., 2018) and Nelore bulls (Reis et al., 2016). Overall, the results from sperm quality analyses show that sperm cells from NR bulls at 17 months of age displayed higher sperm quality compared to 14 months of age. Although sexual maturation lasting up to 50 weeks of age (Rawlings et al., 2008), previous evidences have documented that bull sperm quality increased even after puberty (Brito et al., 2004). Therefore,

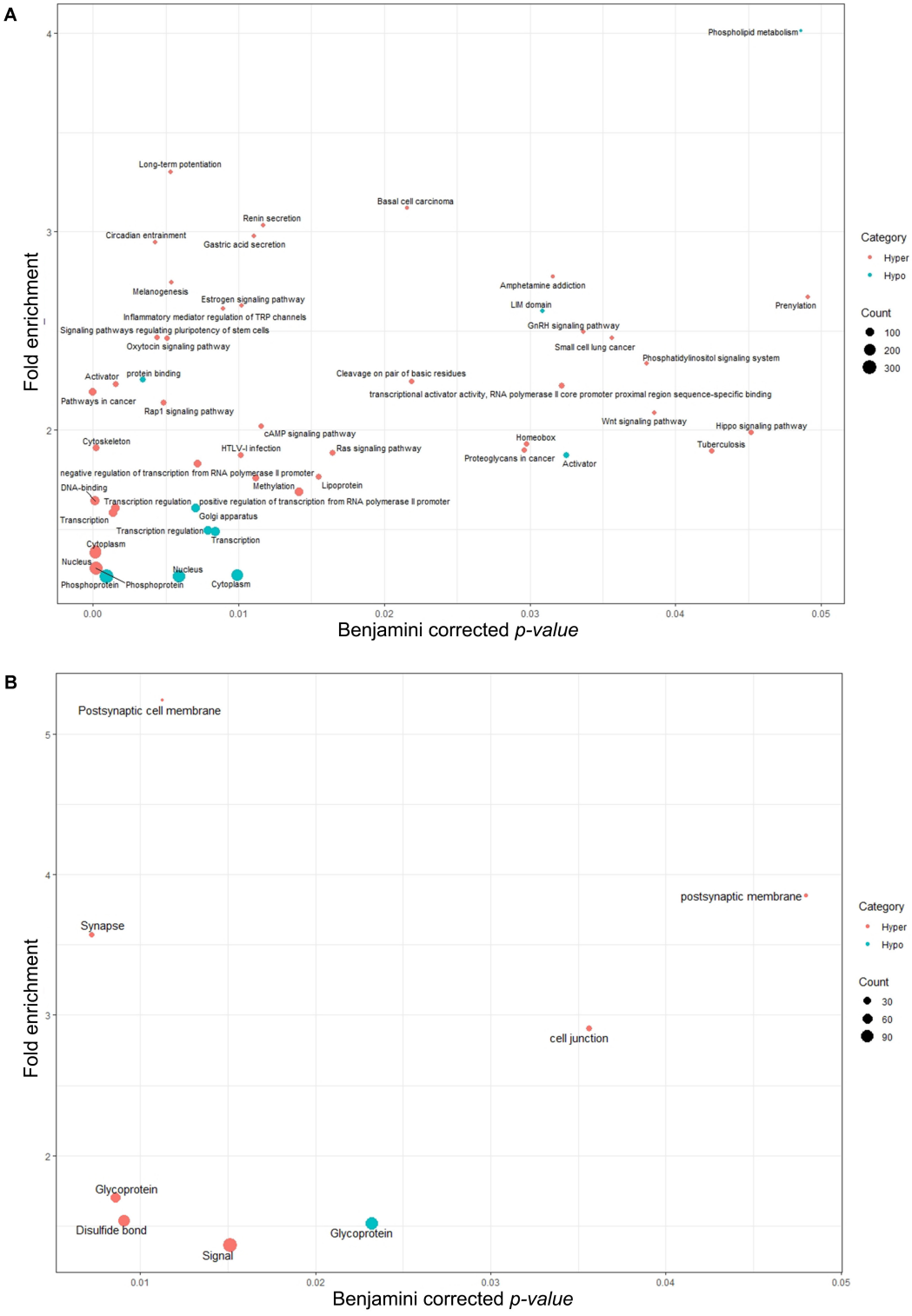

FIGURE 6 | Pathway analysis for annotated genes associated with **(A)** filtered DMRs with less than a 10% methylation difference and **(B)** filtered DMRs with a 10–25% methylation difference. No significant pathways were identified for annotated genes associated with DMRs over 25% methylation difference. GO terms are plotted in function of their Benjamini corrected *p*-value (*x*-axis) and fold enrichment (*y*-axis). Gene count size key shows the number of genes involved in that particular pathway. Hypo, hypomethylated regions (referring to TSSs annotated with hypomethylated regions in test group); Hyper, hypermethylated regions (referring to TSSs annotated to hypermethylated regions in test group).

these results are likely to be related to the well-known sexual maturation process.

Read quality control is an important initial step in next generation sequencing data processing. The Trim-galore software has been recommended for trimming the low-quality reads in RRBS libraries (Wreczycka et al., 2017). Although RRBS libraries from the Ovation method were constructed, evaluated and trimmed according to criteria recommended by the manufacturer, surprisingly we did not detect any Illumina adapter sequences in the reverse reads of libraries. This observation might partially be explained by the sequencing technique and source of DNA. Although according to manufacture recommendation, Ovation method is compatible with paired-end sequencing, previous results employing this method, sequenced the libraries in a single-end mode, and not paired-end, mode (Pilsner et al., 2018; Chen et al., 2019; Paul et al., 2019; Rashid et al., 2020). The same studies applied this method to study DNA methylome in rat, mice and humans brain/sperm cells while, according to manufacturer's recommendation, the Ovation method is designed to generate RRBS libraries from human genomic DNA (NuGEN, 2020). Length distribution after trimming also revealed that libraries constructed using the Ovation method had several peaks reflecting different fragment sizes specifically of short length, whereas reads from the gel-free method revealed only one major peak of fragments greater than 130 base pairs long. This is an important factor as it has been shown that longer reads align better and more specifically to a reference genome (Tran et al., 2014). To our knowledge, this was the first time the Ovation® RRBS Methyl-Seq system has been applied to study DNA methylation in bull sperm. However, further work is required to successfully adopt Ovation system for studying the bull sperm DNA methylome.

Basic statistics of sequencing results from the RRBS libraries constructed using the gel-free protocol indicated very consistent and reproducible bisulfite conversion between samples. The average conversion rate of 99.1% is higher and equal to previously published whole genome bisulfite sequencing (WGBS) (Duan et al., 2019) and RRBS results (Jiang et al., 2018) for bull sperm cells, respectively. Although in this study, the *bosTau9* genome was used as the reference genome and some relaxed alignment parameters were applied, the average mapping efficiency of 33.1% was much higher than previously reported results for RRBS libraries in bull sperm cells (Jiang et al., 2018).

Results further indicated a 40% global DNA methylation level in NR bull sperm cells. Previous studies showed that, in general, global DNA methylation level is low in bull sperm cells. For instance, using a luminometric methylation assay an average of 45% (Perrier et al., 2018) and using RRBS, an average of 35% (Jiang et al., 2018) global CpG methylation in bull sperm cells has been reported. Similarly, low global CpG methylation was also reported for ten different cattle tissues using RRBS (Zhou et al., 2016). Previously, we reported an average of 33% of global methylation in boar sperm cells using a gel-free RRBS technique (Khezri et al., 2019). However, it has been shown that global sperm DNA methylation in bulls can reach 75% as documented using WGBS (Zhou et al., 2018). One should keep in mind that the applied RRBS method in this study focuses on a small subset (CpG island) of the compact sperm genome where methylation levels are generally low (Suzuki and Bird, 2008). In addition, differences in global bull sperm DNA methylation described in the literature might be explained by different laboratory techniques, instrumental platforms, bioinformatics workflows, reference genome versions utilized for read alignment and interspecies differences in sperm DNA methylation patterns.

Here, no significant associations between sperm global CpG methylation and age were found. These findings are further supported by Pearson correlation and cluster analyses, where a high positive correlation between samples from both age groups was observed. In addition, samples from both age groups of the same individuals always clustered together, which suggests that, in this study, individual effects on global sperm DNA methylation are probably more pronounced than age effects. Considering uniform condition and environment for raising and feeding the bulls, it is least likely that individual differences in DNA methylation here is driven by environmental factors. However, in addition to environmental factors, it has been shown that individual differences in sperm DNA methylation may be explained by epigenetic polymorphism phenomenon and interindividual genetic diversity (Kiefer and Perrier, 2019). In agreement with global sperm DNA methylome results presented here, previous research reported that DNA methylation levels in bull sperm is dynamic during puberty, becoming stable after the age of 1 year (Lambert et al., 2018). In parallel with global methylation analysis, differential methylation analysis, showed an increasing trend of DNA methylation in the control group (sperm DNA from 17 months old bulls) compared to test group (sperm DNA from 14 months old bulls). Although, 70% of identified differentially methylated regions, displayed less than 10% methylation difference, we believe that this further highlights the possibility of an existing relationship between differentially methylated regions and sexual maturation in NR bulls. This hypothesis is supported by previous studies in Holstein bulls where more methylated regions were found in sperm cells from 16 months bulls compared to 10 months bulls (Lambert et al., 2018). Similar findings were reported in one Japanese black bull (at 14, 19, 28, 54, and 162 months of age), where authors identified eight CpGs that exhibited an age-dependent increase in their methylation levels (Takeda et al., 2019).

The distribution of DMRs demonstrated here showed that the majority lay within intergenic regions and regions outside CpG Islands/CpG shores. Similar trends have been reported in boar (Hwang et al., 2017; Khezri et al., 2019) and bull (Jiang et al., 2018; Perrier et al., 2018) sperm DNA. Previous research has shown that CpG Islands and CpG shores, in parallel with promoters, play an important role in regulation of transcription (Deaton and Bird, 2011; Long et al., 2017). Although only a small percentage of DMRs were annotated with CpG Island/CpG shores and promoters here, the majority of annotated DMRs exhibited less than a 10% methylation difference. This further suggests similar DNA methylation profiles in these regions in sperm samples from NR bulls at age 14 and 17 months.

GO analysis results for the $DMR_{>25\%}$ group further showed that molecular functions/biological processes such as energy

homeostasis, developmental growth and androgen signaling could be driven by Cytochrome B5 Reductase 4 (*CYB5R4*), Phospholipase C Beta 1 (*PLCB1*) and NK3 homeobox 1 (*NKX3-1*) genes, respectively. However, these genes are not specific for reproduction or sexual maturation. For instance, previous research demonstrated that the *CYB5R4* gene could be consider as one of the candidate gene for quantitative trait locus studies for the oleic acid percentage in Japanese Black cattle (Kawaguchi et al., 2019). In other research, the *PLCB1* gene was identified in oxidative stress response and heat tolerance in Dehong humped cattle (Li et al., 2020). Furthermore, the transcription factor *NKX3-1* was proposed as a possible regulator of gene expression in the endometrium of cattle who received n-3 polyunsaturated fatty acid as a feed supplement (Waters et al., 2014). In addition, steroid hormone biosynthesis was the only biological process that was exclusively identified in the $DMR_{<10\%}$ hypomethylation group. Several genes also identified, such as cytochrome P450 superfamily members (*CYP1B1, CYP11A1, and CYP2E1*), steroid 5 alpha-reductase 2 (*SRD5A2*) and steroid sulfatase (*STS*) are annotated to be involve in steroid hormone biosynthesis. Given their annotated molecular functions/associated biological processes, genes identified in this study may contribute to age-dependent reproductive capacity in NR bulls.

Our analyses did not show any significant pathways connected to genes annotated with $DMR_{>25\%}$. These findings are in line with previous research from Holstein bulls, where no significant DMR-associated pathways were found in sperm samples collected at 12 and 16 months of age (Lambert et al., 2018). For the $DMR_{10-25\%}$ group, a total number of eight significant pathways including sperm-relevant pathways such as "disulfide bond" and "glycoprotein" in 14 months old NR bulls were identified. "Disulfide bond" was exclusively identified in the hypermethylation group. It has been shown that disulfide bonds are essential for protamine function and DNA packaging in bull sperm chromatin (Hutchison et al., 2017). Although the number of bulls was limited here, fresh semen samples from 14 months old NR bulls exhibited higher degree of chromatin compaction compared to 17 months old bulls (**Table 1**). These results suggest a possible link between sperm DNA hypermethylation and DNA packaging via protamine function. Similar possible contribution of DNA methylation to nucleosome rigidity via histone function, has previously been suggested in human somatic cells (Choy et al., 2010; Lee and Lee, 2012). Furthermore, the pathway "glycoprotein" was identified in both hypo and hyper $DMR_{10-25\%}$ with a stronger *p-values* in the hypermethylation group. "Glycoproteins" have been identified in the sperm plasma membrane and play an important role in mammalian fertilization (Tecle and Gagneux, 2015). Further research is required to shed light on compositions of sperm glycoproteins during bull sexual maturation. The highest numbers of identified pathways with significant *p-values* were found to be related to genes annotated with $DMR_{<10\%}$. In the study conducted by Lambert et al. (2018), identified DMRs in sperm cells from bulls at 10 and 16 months of age were associated with pathways related to sperm function, including androgen hormone signaling. Here, we identified other hormonal pathways such as GnRH, estrogen and oxytocin signaling pathways, which were exclusively related to $DMR_{<10\%}$. This further emphasizes the importance of hormonal signaling in development and sexual maturation. However, pathway analysis results need to be interpreted with caution for two main reasons. First, it has been recommended to avoid using differential DNA methylation level cut off percentages less than 5% in DMR-analysis due to the minimal effects on gene expression they exercise (Wreczycka et al., 2017). Second, a moderate number of genes annotated with DMRs overlapped between hypo- and hypermethylation groups. How transcriptional regulation can be exerted via TSSs proximal to both hypo- and hypermethylated regions is not clear, especially in sperm cells that are relatively transcriptionally silent. Therefore, further research using transcriptome analysis of *in vitro* produced embryos, fertilized with sperm cells from wider age groups of young NR bulls is recommended.

CONCLUSION

The purpose of the present research was to study the sperm DNA methylome, in parallel with sperm quality assessment, in similar NR bulls both at 14 and 17 months of age. Although the number of tested bulls were limited, the present study found that with increasing age of young bulls, sperm quality increased. Furthermore, a gel-free, multiplexed method to construct RRBS libraries from frozen-thawed bull sperm cells was found to be reproducible. The current results showed that sperm DNA methylation in 14- and 17-months-old NR bulls was similar globally, while marginally different regionally. Taken all together, identified DMRs even with low levels of methylation differences, in parallel with sperm quality results, offers some useful insight into the reproductive capacity of genomic selected young NR bulls.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB37763.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because sperm cells that we used in this research routinely collected from bulls owned by breeding company Geno in Norway. However, the bulls were housed and cared for according to international guidelines and regulations for keeping bulls in Norway, at Geno artificial insemination (AI) station, in Hamar, Norway.

AUTHOR CONTRIBUTIONS

AK performed the sperm motility assay, bioinformatics and biostatistics analyses with inputs from RA as well as RW and

wrote the manuscript. BN, E-BS, and TZ performed and drafted the viability assay, sperm chromatin integrity analysis and ATP content assay, respectively. E-BS prepared RRBS libraries with inputs from RW. FM and EK did conceptualization and original project draft. All authors were involved in the planning of the experiments and provided useful inputs, interpreted the data, read, edited, and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00922/full#supplementary-material

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Differences in sperm functionality and intracellular metabolites in Norwegian Red bulls of contrasting fertility

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ABSTRACT

In the dairy breeding industry, prediction of bull fertility in artificial insemination (AI) is important for efficient and economically sustainable production. However, it is challenging to identify bulls with superior fertility applying conventional *in vitro* sperm assays. In the present study, sperm functionality was investigated to identify a multivariate model that could predict fertility. Two groups of young Norwegian Red bulls were selected, one with inferior fertility (18 bulls) and one with superior fertility (19 bulls) based on non-return rate after 56 days (NR56). Frozen-thawed semen doses were analysed for sperm chromatin integrity, viability, acrosome integrity, motility, and ATP content. A targeted approach was used to study intracellular concentrations of amino acids and trace elements in viable sperm cells. Significant differences between the two groups of bulls were observed, both for sperm functional attributes and intracellular concentrations of metabolites. Pearson correlation analyses indicated a negative relationship between NR56 and chromatin integrity parameters, DNA fragmentation index (DFI) and high DNA stainability (HDS). Several motility parameters correlated positively with NR56. The concentrations of cysteine and glutamic acid in sperm cells correlated negatively with NR56, while the concentrations of aspartic acid, leucine and serine showed a positive NR56-correlation. The sperm intracellular concentrations of the trace elements Fe, Al and Zn, correlated negatively with NR56. Correlations were observed between several sperm parameters and metabolites. Stepwise multiple regression analysis indicated that the best predictor of NR56 was a model containing %DFI, together with the intracellular sperm concentration of aspartic acid, Fe and Zn. This model explained 59% of the variability in NR56.

1. Introduction

Artificial insemination (AI) revolutionized cattle breeding in the 1950s. With the introduction of genomic selection (GS), breeding of dairy cattle has undergone a new paradigm shift allowing faster genetic progress [1]. This has increased the demand for using young bulls for semen production and AI. The bulls are typically in production only for a short and intensive period of time before they are replaced by younger, genetically superior bulls [2]. This shortens the available time to assess the fertility status of a bull before its semen is widely distributed. A common measure of the reproductive performance of AI bulls is non-return (NR) rate: the percentage of inseminated females that do not return to estrus within a specific time after the first AI [3]. The reliability of NR data

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depends on the number of AIs per bull and how the AI and its outcome is reported, being considered highly reliable in Norway [4].

Before Norwegian Red bulls are selected as AI bulls, they are subjected to a breeding soundness evaluation, including conventional semen analysis to determine sperm concentration, morphology, and motility. Infertile bulls are normally identified and excluded by this method. However, it is challenging to predict and distinguish bulls with superior fertility from those of lower performance [5,6]. It would benefit the AI industry to have a reliable *in vitro* method, which, at an early stage, could distinguish superior bulls from the rest. It has been demonstrated that parameters such as sperm motility [7–9], viability [10,11] acrosome integrity [12] and DNA integrity [7,13] correlate with field fertility. However, it is unlikely that the fertility of a bull can be predicted by a single sperm attribute alone. The fertilisation process is complex and it is expected that a multifactorial approach is needed [11,14,15].

Recently, metabolomics has been introduced to identify potential fertility and infertility biomarkers in seminal plasma and sperm from both men [16,17] and bulls [18–20]. Metabolomics is the study of small, low molecular weight metabolites. Metabolites are defined as final products of metabolic pathways and play significant roles in sperm physiology such as energy metabolism, motility and regulation of metabolic activities [16]. In a recent study, 63 metabolites were identified in bull seminal plasma [19]. Later, the same group reported 22 distinct metabolites in spermatozoa from bulls with either high or low fertility [20]. A common approach for studying metabolites in semen has been to separate seminal plasma from the sperm pellet by centrifugation, allowing for analysis of metabolites in both seminal plasma and spermatozoa. This does not, however, take into account differences between viable and non-viable cells, the viable cells having capacity to fertilize the egg. To the best of our knowledge, there is no study yet focusing on intracellular metabolites of frozen-thawed, viable sperm cells.

The aim of this study was to identify parameters that, in combination, could be used to discriminate bulls with superior fertility from bulls of lower performance. This was conducted by assessing several sperm attributes associated to fertility, including sperm metabolites, in frozen-thawed semen from bulls with contrasting NR rates after 56 days (NR56).

2. Material and methods

2.1. Animals and semen processing

Cryopreserved semen samples from 37 Norwegian Red bulls of known field fertility were provided by the breeding company Geno (Geno Breeding and AI Association, Hamar, Norway). NR56 was used as a measure of bull fertility. In Norway, the insemination technicians are employed by the breeding company and paid according to reported AIs, thus the NR data are considered highly reliable [4]. Breedings were reported to the Norwegian Dairy Herd Recording System (NDHRS), where data on AI were made available. Least square mean (LSmean) for NR56 was calculated for 507 Norwegian Red bulls used in AI from 2013 to 2018. The General Linear Model (PROC GLM in SAS®) included effects of bull, AI month and year, parity of the female and repeated AI within 1–4 days. Based on these results, a group of 19 bulls with high NR56 (hereafter referred to as HF) and a group of 18 bulls with low NR56 (hereafter referred to as LF) were selected for the study. LSmean for NR56 ranged from 0.76 to 0.78 for HF and from 0.46 to 0.65 for LF. The total number of first AIs were 29 240 for HF (range: 496 to 8542) and 12 636 for LF (range: 204 to 976). All bulls were in regular semen production, with a mean (±SD) age corresponding to the collection date of ejaculates analysed *in vitro* being 517 (±162) days for HF and 459 (±35) days for LF. For details on numbers of AIs and bull age, see Table 1. Post collection, the semen samples were diluted to a final concentration of 12×10^6 spermatozoa per AI dose in French mini straws (IMV, L'Aigle, France). This was performed in a two-step dilution procedure in Biladyl® extender containing glycerol (Minitube, Verona, WI, USA, 13 500/0004–0006) and fresh egg yolk. Only ejaculates with sperm concentration >390 million/mL, subjective total motility >70% pre-freeze, > 50% post-thaw and normal morphology >85% were used for AI. Cryopreservation was performed according to standard procedures [21] and the semen straws were stored in liquid nitrogen (−196 °C) until used. For *in vitro* analyses, the semen doses were thawed for 1 min in a water bath at 37 °C. For sperm functionality analyses, two semen doses from each bull were thawed and mixed together. For analysis of metabolites, seven semen straws from each bull were mixed.

2.2. Motility measurements by CASA

Analysis of sperm motility characteristics was performed using the SCA evolution CASA system (Sperm Class Analyzer® version 6.1, Microptic SL, Barcelona, Spain), equipped with a phase contrast Eclipse Ci-S/Ci-L microscope (Nikon, Japan), and a Basler digital camera (Basler Vision Technologies, Ahrensburg, Germany). The semen samples were incubated at 37 °C for 15 min and diluted (1:2) with pre-warmed PBS to a final concentration of 26×10^6 cells/mL. Then, 3 μL of the diluted sample was loaded into a pre-warmed 20 μm depth Leja-4 chamber slide (Leja products, Nieuw-Vennep, the Netherlands). A minimum of 8 microscope fields and at least 800 cells were analysed per sample and all samples were analysed in duplicate. The instrument settings were 45 Hz frame rate and 30 frames captured per sample, with sperm cell detection based on head area of 20 μm^2 to 80 μm^2. The kinematic parameters measured were: average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), straightness (STR) of the average path defined as VSL/VAP (%), linearity (LIN) of the curvilinear path defined as VSL/VCL (%), Wobble (WOB) defined as VAP/VCL (%), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz). Total motility (MOT) was defined as sperm cells with VCL > 15 μm/s, progressive motility (PROG) was defined as sperm cells with STR > 70 μm/s. Sperm cells with VCL >80 μm/s, ALH >6.5 μm and LIN <65% were defined as hyperactive (HYP).

2.3. Flow cytometry

All sperm functionality analyses by flow cytometry were performed with a Cell Lab Quanta TM SC MPL flow cytometer (Beckman Coulter, Fullerton, CA, USA). For each day of analysis, the flow cytometer was checked for optical alignment by running Flowcheck™ beads (6605359, Beckman Coulter), and a negative control (unstained semen sample) was included for each run. A 488 nm argon laser was used as light source for excitation.

2.3.1. Analysis of sperm plasma membrane and acrosome integrity

For the analysis of sperm plasma membrane integrity, the semen samples were stained with propidium iodide (PI, LIVE/DEAD® kit, L7011, Invitrogen) which discriminates between live and dead spermatozoa by binding only to DNA of sperm cells with damaged membranes. Lectin peanut agglutinin (PNA) from *Arachis hypogaea* (peanut) conjugated with Alexa Fluor 488 (PNA– Alexa 488, L21409; Invitrogen, Paisley, UK) was used to identify acrosome-reacted spermatozoa. A SP-Talp (105 mM NaCl, 3.1 mM KCl, 0.4 mM $MgCl_2$, 2.0 mM $CaCl_2 \cdot 2H_2O$, 0.3 mM $NaH_2PO_4 \cdot H_2O$,

Table 1
Details of fertility, age at semen collection for *in vitro* analyses and numbers of inseminations for the bulls used in the study. The age is expressed as days and NR56 is expressed as LSmean.

Low NR56				High NR56			
Bull	Age	NR56	No. AIs	Bull	Age	NR56	No. AIs
LF1	481	0.46	689	HF1	447	0.76	831
LF2	439	0.53	803	HF2	478	0.76	821
LF3	542	0.55	805	HF3	474	0.76	842
LF4	443	0.58	695	HF4	449	0.76	651
LF5	500	0.61	713	HF5	1083	0.76	8542
LF6	413	0.62	840	HF6	428	0.76	686
LF7	432	0.63	837	HF7	458	0.76	922
LF8	460	0.63	976	HF8	449	0.76	986
LF9	510	0.63	791	HF9	467	0.76	857
LF10	454	0.64	922	HF10	787	0.76	4156
LF11	489	0.64	318	HF11	422	0.76	611
LF12	421	0.64	652	HF12	422	0.76	825
LF13	453	0.64	333	HF13	423	0.77	764
LF14	487	0.65	204	HF14	553	0.77	710
LF15	414	0.65	751	HF15	571	0.77	1069
LF16	434	0.65	708	HF16	419	0.77	2149
LF17	440	0.65	700	HF17	453	0.78	767
LF18	457	0.65	899	HF18	485	0.78	496
				HF19	556	0.78	2555

1 mM sodium pyruvate, 21.6 mM sodium lactate, 20 mM Hepes, 20 mM Hepes salt, 5 mM glucose, 50 μg/mL gentamycin) staining solution of fluorochromes PNA-Alexa 488 and PI was prepared with stock concentrations of 0.05 μg/mL and 0.48 μM, respectively. Sperm samples, at a concentration of 1×10^6 sperm cells/mL, were stained and incubated for 10 min at room temperature (RT) prior to flow cytometric analysis. All samples were analysed in triplicate. Identification of spermatozoa and exclusion of debris particles was performed by Electronic Volume- (EV) and Side Scatter- (SS) signals, as previously described by Standerholen et al. [21]. PI fluorescence was detected using a 670 nm long pass filter, while PNA-Alexa 488 fluorescence was detected using a 510-to-540 nm band pass filter. Gating was performed to reveal four sub-populations: acrosome-intact and live spermatozoa (AIL), acrosome-intact and dead spermatozoa (AID), acrosome-reacted and live spermatozoa (ARL) and acrosome-reacted and dead spermatozoa (ARD). The data generated was further analysed by Kaluza® Analysis software, Version 2.1 (Beckman Coulter Ltd).

2.3.2. Analysis of sperm chromatin integrity

Sperm chromatin integrity was analysed using the Sperm Chromatin Structure Assay (SCSA), as previously described by Waterhouse et al. and Evenson and Jost [13,22]. The semen samples were diluted in TNE buffer (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4) to a final concentration of 2×10^6 sperm cells/mL in a volume of 200 μL. Next, 400 μL acid detergent solution (0.38 M NaCl, 80 mM HCl, 0.1% (w/v) Triton X-100, pH 1.2) was added, followed by exactly 30 s of incubation at RT. Further, 1.2 mL acridine orange (AO) staining solution (6 μg/mL AO (A3568, Life Technologies, OR, USA)) in a buffer containing 37 mM citric acid, 0.126 M Na_2HPO_4, 1.1 μM EDTA, and 0.15 M NaCl (pH 6) was added. After 3 min setup mode, data acquisition started, in which 5000 events were captured for each sample at a rate of ~200 events/sec. Signals were separated by a 550 nm dichroic long pass mirror, a 525 nm band pass filter detected the green fluorescence while a 670 nm long pass filter detected the red fluorescence. The flow cytometer was AO-saturated by running AO equilibration solution (1.2 mL AO staining solution and 400 μL acid detergent solution) through the system for 5 min prior to sample analysis. To control the stability of the laser, the mean green and red fluorescence signals were set to 425 ± 5 and 125 ± 5, respectively, first at the start of analysis and later after every fifth sample was analysed. This was performed using semen samples from a bull of known DNA fragmentation index (DFI) in a bivariate cytogram. The FL1 (green) was presented on the x-axis and FL3 (red) on the y-axis of cytogram, both on a linear scale. Analysis in FCS Express 6 Flow cytometry Software (Denovo Software, Los Angeles, CA, USA) revealed the percentage of red (ssDNA) and green (dsDNA) fluorescence. Based on a histogram of the fluorescence ratio red/(red + green), the spermatozoa with fragmented DNA (%DFI), average extent of DNA fragmentation (mean DFI), and variation in extent of DNA fragmentation (SD DFI) were calculated. The bivariate cytogram was used to determine the percentage of spermatozoa with high DNA stainability (%HDS).

2.4. ATP content by luminescence assay

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) was used for measuring ATP levels in bull semen. The semen samples (60 μL, 3×10^5 sperm cells) were added to a white 96-well microtiter plate (NUNC™, Denmark) before the addition of 60 μL CellTiter-Glo® reagent. The mixture was gently shaken for 2 min on a rotary shaker (IKA® MS 3 digital, USA) prior to 15 min incubation at RT. A FLUOstar OPTIMA multiwell plate reader (BMG LABTECH GmbH, Offenburg, Germany) with MARS data analysis software (Version 1.10, BMG LABTECH, Germany) was used for measuring the bioluminescence. Samples were analysed in triplicate, and the data recorded was measured in relative luminescence units (RLU). Using a prepared standard curve, RLU values were converted to corresponding ATP values in nM. Results are presented as μM ATP per million cells, and corrected for the percentage of viable spermatozoa, as measured by flow cytometry.

2.5. Analysis of intracellular sperm metabolites

2.5.1. Chemicals and reagents

All solvents, acids and salts used for LC-MS mobile phases and preparation of extracts were purchased from Sigma-Aldrich (Darmstadt, Germany) and were of LC-MS or pro analysis quality. Water was of MilliQ quality. All standards were obtained from Sigma Aldrich and internal standards were obtained from either Sigma Aldrich or TRC (North York, Canada). Standards for ICP-MS analysis were obtained from Inorganic Ventures (Christiansburg, USA).

2.5.2. Isolation of viable sperm cells by single layer colloid centrifugation

Single layer centrifugation (SLC) was conducted with a species-specific colloid, Bovicoll (patent application submitted, J. M. Morrell), according to Nongbua et al. [23] with modifications. The frozen-thawed semen (190 μL) was carefully layered on top of 1 mL Bovicoll prewarmed to room temperature in a 15 mL Falcon tube. The samples were centrifuged at 300×*g* for 20 min using a swing out rotor before the supernatant (seminal plasma, semen extender and most of the colloid) was removed. The pellets containing viable sperm cells were aspirated to sterile 2 mL tubes. To wash and remove remaining colloid material, the sperm pellets were carefully re-suspended in 200 μL PBS and centrifuged at 300×*g* for 1 min in two repeating steps. After washing, the sperm pellets were pipetted into 15 mL 50% MeOH and snap frozen in liquid nitrogen to lyse the sperm cells before metabolomic analysis. The samples were thawed and frozen in three cycles before they were freeze-dried. Before the last cycle of snap freezing, 15 mL of Milli-Q water was added to prevent thawing of the sample during the freeze-dry procedure. The samples were re-suspended in water and divided into aliquots for quantitative analysis of amino acids and elemental analysis. Due to a problem during the sample preparation for one of the semen samples, the following analyses were conducted for 36 bulls (19 samples from HF and 17 samples from LF bulls).

2.5.3. Quantitative analysis of amino acids

Samples were derivatised by propyl chloroformate (PCF) prior to analysis. The freeze-dried sample aliquot was re-suspended in methanol and the dissolved material was collected and dried using a speed-vac. A mix of deuterated amino acids (internal standard-mix) was added followed by 1 M NaOH (390 μL), 1-propanol (335 μL) and pyridine (65 μL), followed by addition of the derivatisation reagent PCF (80 μL). For extraction of the derivatised amino acids, a 400 μL volume of chloroform was added followed by 50 mM $NaHCO_3$ (400 μL). Vortex mixing was performed after each addition of solvent and reagent. A 200 μL aliquot of the chloroform phase was thereafter transferred to a clean vial and air-dried. The derivatised amino acids were dissolved in methanol prior to analysis.

Analysis was performed on an Agilent 1290 Infinity II LC system (Agilent, Santa Clara, USA) coupled to an Agilent 6495 QqQ mass spectrometer. The QqQ-MS was equipped with a jet-stream ESI source operated in positive mode. The QqQ-MS was operated in dynamic MRM mode (delta Rt = 1 min) with unit mass resolution for both mass filters. The MRM transitions for standards and internal standards and the employed collision energies, gas temperatures and flows, are given in Table 2. Amino acids (Alanine, Arginine, Asparagine, Aspartic acid, Cysteine, ɣ-Aminobutyric acid (GABA), Glutamic acid, Glutamine, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Ornithine, Phenylalanine, Proline, Serine, Threonine, Tryptophan, Tyrosine, and Valine) were analysed by LC-MS/MS following PCF derivatisation. The chromatographic separation was performed in reversed phase mode employing an Ascentis Express C8 (2.1 × 150 mm, 2.7 μM) column (Sigma-Aldrich), and gradient elution using 25 mM Formic acid as eluent A, and acetonitrile as eluent B, at a flow rate of 0.3 mL/min. The gradient used started at 35% B and was increased stepwise to 90% B at 14 min. Complete washout was performed by increasing to 100% B before the column was reconditioned with the starting conditions. The column thermostat was maintained at 35 °C and the autosampler at 6 °C. The injection volume was 2 μL. Mixed standards at 0-0.1-1-10-50-100-500-1000-4000 μM were used for calibration and quantitation. Internal standards were used in the analysis.

2.5.4. Analysis of trace elements by ICP-MS

Sample preparation prior to ICP-MS analysis involved digestion of the sperm samples in HNO_3 in a microwave digestion unit. The freeze-dried sample aliquot was mixed with 2.5 mL of 50% HNO_3 and digestion was performed in a Milestone Ultraclave (Sorisole, Italy) using a preset 8-step digestion program at 160 bar and increasing temperature stepwise from 50 °C to 245 °C. ICP-MS analysis of Mg, Al, P, S, K, Ca, Cr, Mn, Fe, Zn, As, and Ba was performed on an Agilent 8800 Triple Quadrupole ICP-MS (G3663A) (Agilent, Santa Clara, USA) mass spectrometer connected to a SPS4 autosampler. A Peltier-cooled (2 °C) spray chamber with a Micro-Mist nebulizer was used as the introduction system. The RF Power was set to 1550 W and the RF Matching to 1.80 V, and the nebulizer gas was set at 1.05 L/min. The cell gases used were He and O_2 at 4.3 and 1 mL/min, respectively. Analysis was performed in MS/MS mode. Extract voltage 1 and 2 were set at 0 and -195V, respectively.

2.5.5. Scaling of data

The number of viable sperm cells in a semen sample varied between individual bulls (49.57 ± 12.83 (LF) and 58.87 ± 8.41 (HF)). After Bovicoll centrifugation, there are mostly only viable spermatozoa in the pellet, and therefore differences in sperm number between samples may be observed. Thus, it was necessary to scale the data in order to adjust for differences in cell number between the samples. A scaling factor was determined based on the quantified amounts of a selection of amino acids in the samples after Bovicoll isolation of viable sperm cells. Eight amino acids (Aspartic acid, Glycine, Leucine, Lysine, Methionine, Proline, Threonine and Tyrosine) were included in the scaling factor, chosen based on their co-variance in the sample series, and their similar profiles in all samples (supplementary material, Fig. S1). The average concentrations for these eight amino acids were calculated and the ratio of observed concentration in each bull sample to this average was determined, giving each bull sample its own scaling factor. This scaling factor was used to compensate for different sperm numbers in sample material by dividing the observed concentrations by the values of corresponding scaling factors for the different bull samples. The scaling was performed for both the amino acid and trace element concentrations.

2.6. Statistics

The statistical analyses were performed using SAS Version 9.4 for Microsoft Windows (SAS Institute, Cary, NC, USA). The data was tested for normal distribution by the Shapiro-Wilk test. The parameters that did not show a normal distribution were log-transformed prior to further statistical analysis. Differences in sperm parameters and metabolites between HF and LF bulls was assessed by unpaired *t*-test. In cases where normal distribution was not achieved after log transformation, the non-parametric Wilcoxon test was applied. Correlation coefficients (Pearson) were calculated and considered statistically significant when $p < 0.05$. The correlation between sperm parameters/metabolites and NR56 was assessed, followed by the correlations between sperm functionality parameters and metabolites. To determine which combination of parameters that best could explain the bulls NR56, a forward stepwise multivariate regression analysis was conducted (PROC REG). The NR56 LSmean was the dependent variable and the sperm functional parameters and metabolites shown to correlate with NR56 were the predictive independent variables. Forward variable selection was applied where the selection started with only the intercept term in the model. For each of the independent variables the F statistic was calculated to determine each variable's

Table 2
Method parameter (multiple-reaction monitoring and retention time (RT)) and instrument settings for LS-MS determination of amino acids.

	Precursor ion	Product ion	CV (V)	RT (min)	Internal standard
Alanine	218	130	8	4.3	D3-Alanine
Arginine	303.2	70.1	44	1.1	13C6-Arginine
Asparagine	243.1	157	5	3.9	D3-Asparagine
Aspartic acid	304.4	216	10	6.6	
Cysteine	336	248	8	9.2	D2-Cysteine
GABA	232	85.9	16	4.3	D6-GABA
Glutamic acid	318	84	25	6.9	D5-Glutamic acid
Glutamine	275	84.2	28	2.2	13C5-Glutamine
Glycine	204	75.9	12	3.6	13C2Glycine
Histidine	370	109.9	40	5.6	13C6-Histidine
Isoleucine	260	172	8	8.3	
Leucine	260	172.1	8	8.3	D10Leucine
Lysine	361	301.1	4	5.9	13C6Lysine
Methionine	278	189.9	4	5.8	13CD3Methionine
Ornithine	347.2	287.2	5	5.2	D6Ornithine
Phenylalanine	294.3	206.1	8	7.9	D5Phenylalanine
Proline	244.2	156.2	10	5.1	D3Proline
Serine	234.2	60.1	24	2.7	D3Serine
Threonine	248	74.1	15	3.2	
Tryptophan	333	245.1	20	6.8	D5Trypthophan
Tyrosine	396	222.2	20	10.3	13C9-Tyrosine
Valine	246	158.2	5	6.5	D8Valine

Instrument settings; Capillary voltage (CV) = 4 kV, Nebulizer pressure = 40 psi, drying gas flow = 20 L/min, gas temperature = 210 °C, Fragmentor voltage = 380 V, Sheath gas temperature = 400 °C, sheath gas flow = 11 L/min, iFunnel positive high/low pressure RF = 150/60, and negative high/low pressure RF = 90/60.

contribution to the model. The variable with the smallest p-value below the cut-off value 0.1, indicating statistical significance, was kept in the model.

3. Results

3.1. Sperm functionality

Differences in sperm attributes between HF and LF bulls are presented in Table 3. For the chromatin integrity parameters, lower %DFI ($p < 0.001$) and HDS ($p < 0.01$) were observed in sperm cells from HF bulls compared to LF bulls. ATP levels, as well as the sperm motility parameters MOT, PROG, HYP, VAP, VCL, VSL, STR and ALH, were higher in semen from HF vs. LF bulls ($p < 0.01$). For sperm viability and acrosome integrity, higher proportions of live and AIL spermatozoa and lower proportions of ARD spermatozoa were observed in the HF group compared to the LF group ($p < 0.05$).

Table 3
Mean and standard deviation for sperm functionality parameters of bulls with low (n = 18) and high (n = 19) NR56 rates. Significant differences between the groups are marked by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Sperm parameter	Low NR56	High NR56
DFI (%)	3.50 ± 1.44	1.84 ± 0.80***
HDS (%)	4.31 ± 1.65	2.93 ± 1.15**
Live (%)	49.57 ± 12.83	58.87 ± 8.41**
AIL (%)	46.15 ± 13.16	55.82 ± 8.92*
ARL (%)	3.40 ± 1.73	3.07 ± 1.60
AID (%)	17.37 ± 3.88	16.13 ± 4.01
ARD (%)	33.08 ± 12.41	24.93 ± 6.48*
ATP (nM)	1.12 ± 0.47	1.52 ± 0.36**
MOT (%)	40.31 ± 14.32	51.92 ± 11.17**
PROG (%)	34.45 ± 14.64	48.28 ± 10.87**
HYP (%)	8.95 ± 4.51	16.69 ± 5.98**
VAP (μM/s)	82.76 ± 14.29	94.03 ± 4.94**
VCL (μM/s)	161.34 ± 28.39	184.26 ± 11.91**
VSL (μM/s)	64.83 ± 12.13	75.77 ± 5.35**
STR (%)	75.01 ± 4.85	79.45 ± 2.87**
LIN (%)	41.65 ± 4.17	44.14 ± 3.89
WOB (%)	53.50 ± 2.77	54.03 ± 3.19
ALH (μM)	4.75 ± 0.78	5.36 ± 0.49**
BCF (Hz)	20.81 ± 1.69	21.29 ± 1.53

DFI = DNA fragmentation index, HDS = high DNA stainable, AIL = acrosome intact live, ARL = acrosome reacted live, AID = acrosome intact dead, ARD = acrosome reacted dead, MOT = motile, PROG = progressive, HYP = hyperactive, VAP = velocity average path, VCL = velocity curvilinear, VSL = velocity straight-line, STR = straightness, LIN = linearity, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat-cross frequency.

Sperm functionality parameters were further assessed by Pearson correlation analysis to identify possible relationships to NR56. The results (Table 4) indicate that %DFI had the strongest (negative) correlation to NR56. DNA stainability (%HDS) was also correlated negatively with NR56, whereas the sperm motility parameters MOT, PROG, HYP, VAP, VCL, VSL, STR, and ALH correlated positively with NR56. The remaining sperm parameters did not show any significant correlations with NR56 ($p > 0.05$).

3.2. Concentration of sperm intracellular metabolites

The intracellular sperm concentrations of cysteine and glutamic acid were lower in semen doses from HF than from LF bulls ($p < 0.01$), while the concentrations of threonine, serine, leucine ($p < 0.05$) and aspartic acid ($p < 0.01$) were higher in HF compared to LF bulls (Table 5). Pearson correlations between amino acid concentration and NR56 were significantly different from zero ($p < 0.05$) for cysteine ($r = -0.37$), glutamic acid ($r = -0.39$), aspartic acid ($r = 0.46$), leucine ($r = 0.34$), and serine ($r = 0.36$).

The sperm intracellular concentrations of Al, Fe and Zn were significantly lower in sperm cells from HF bulls compared to LF bulls (Table 5). Pearson correlation coefficient values between trace element concentrations and NR56 were significantly different from zero ($p < 0.05$) for Fe ($r = -0.61$), Al ($r = -0.38$) and Zn ($r = -0.39$).

3.3. Correlation of sperm parameters and intracellular metabolites

Pearson correlation analyses indicated that there were correlations ($p < 0.05$) between several of the sperm functionality parameters and intracellular amino acid concentrations (Table 6). Arginine, glutamine, cysteine and threonine were correlated to most sperm attributes, with correlations to parameters such as chromatin integrity, viability, acrosome integrity, ATP level and

Table 4
The Pearson correlation coefficient (corr) and p-values for the correlations between NR56 and sperm parameters assessed for the bulls (n = 37). Only parameters showing significant correlation ($p < 0.05$) with NR56 are listed in the table.

Sperm parameter	Corr	p-value
DFI (%)	−0.57	0.0003
HDS (%)	−0.37	0.026
HYP (%)	0.42	0.010
VAP (μM/s)	0.42	0.010
VCL (μM/s)	0.44	0.007
VSL (μM/s)	0.44	0.006
STR (%)	0.40	0.013
ALH (μM)	0.41	0.012

DFI = DNA fragmentation index, HDS = high DNA stainable, HYP = hyperactive, VAP = velocity average path, VCL = velocity curvilinear, VSL = velocity straight-line, STR = straightness, ALH = amplitude of lateral head displacement.

several different sperm motility characteristics. Arginine, threonine, aspartic acid, glutamic acid and serine correlated with chromatin integrity parameters. Furthermore, correlations were observed between several of the trace elements and sperm functionality parameters (Table 7). The trace elements, whose concentrations correlated with most sperm attributes, were S, Ca, Mn, Fe and Ba. They correlated with i.e. chromatin integrity, acrosome integrity, viability and motility parameters. The intracellular concentration of Mg correlated positively with several kinematic motility parameters. The sperm concentrations of Al, S, K, Ca, Fe and Ba correlated positively with %DFI.

3.4. Identification of sperm parameters and metabolites associated with NR56

Stepwise multiple regression analysis with a variable selection criteria of $p < 0.1$, indicated that the best predictor of bulls' NR56 was a model containing %DFI and the intracellular sperm concentration of aspartic acid, Fe and Zn:

NR56 = 0.051 + (0.004 x aspartic acid) - (0.076 x Fe) - (0.067 x Zn) - (0.052 x DFI).

This model explained 59% of the variability in NR56.

4. Discussion

In the present study, a retrospective approach was used to study sperm attributes in selected groups of bulls with high and low NR56. Significant relationships between sperm functional parameters, metabolites and NR56 rates were revealed. To our knowledge this is the first study focusing on metabolomics of the viable fraction of frozen-thawed semen samples. The multiple correlations between metabolites and sperm parameters, as well as the correlation to NR56 indicate that metabolomics can be a useful tool in the identification of biomarkers for male fertility.

Table 5
Mean and standard deviation for amino acid and trace element concentrations (μM) of frozen-thawed viable sperm cells from bulls (n = 36) of low and high NR56. Only metabolites with significant differences between the fertility groups are presented: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Metabolite	Low NR56	High NR56
Aspartic acid	74.65 ± 3.96	78.75 ± 3.82**
Cysteine	18.05 ± 3.83	16.01 ± 2.46**
Glutamic acid	252.39 ± 9.46	242.77 ± 8.21**
Leucine	134.29 ± 13.78	142.57 ± 7.24*
Serine	124.70 ± 9.12	131.08 ± 5.68*
Threonine	92.77 ± 8.42	87.78 ± 5.12*
Al	0.13 ± 0.03	0.10 ± 0.04**
Fe	0.105 ± 0.046	0.066 ± 0.018***
Zn	0.080 ± 0.020	0.066 ± 0.024*

Several sperm attributes are presumed important for fertilising potential [5,24]. In the present study, differences between two contrasting groups of bulls were found for sperm motility, viability, acrosome integrity and ATP level. However, only motility parameters were significantly correlated to NR56. Motility is commonly believed to be one of the most important sperm attributes associated with fertility [25]. However, there are conflicting results regarding correlation between sperm motility and field fertility [8,26–29]. Based on motility kinematics, sperm from HF and LF bulls appeared to be in different stages concerning the presence of a hyperactive motility pattern. The HF bulls had sperm cells with increased levels of VCL and ALH, which was reflected in the corresponding observed increase in hyperactive motility. These results corroborate those from a previous study [30], where it was suggested that sperm cells from HF bulls are in transition to a hyperactivated motility pattern. This transition phase, which is termed a progressive hyperactivated motility phase, has been described also by others [31,32]. It has been suggested that the transitional phase plays an important role in sperm transport [31]. Thus, it may be hypothesised that the increase in hyperactivity observed for HF bulls in the present study reflects their sperm being in a progressive hyperactivated motility phase with enhanced ability to reach and fertilize the oocyte.

For the SCSA-derived chromatin integrity parameters, significantly lower %DFI and %HDS were observed in sperm from HF bulls compared to LF bulls. While DFI represents the spermatozoa with DNA damage, HDS is thought to represent immature spermatozoa with incomplete chromatin condensation due to lack of full protamination [33,34]. Our results may indicate that LF bulls have spermatozoa with less compact chromatin and more DNA strand breaks compared to HF bulls. Sperm %DFI was the attribute correlating strongest with NR56 and was also the only sperm parameter with a significant contribution to the model predicting NR56. This corroborates results from previous studies in bulls, where %DFI was found to correlate significantly with field fertility [7,11,13,28,35]. In agreement with our findings, Waterhouse et al. [13] tested the relationship between multiple sperm parameters and field fertility, and reported that only variables related to sperm DNA damage contribute significantly to the model. The main causes of DNA fragmentation are failure in the replacement of histones by protamines during sperm maturation, apoptosis and insufficient protection against reactive oxygen species (ROS) [36,37].

While DNA fragmentation is considered an uncompensable sperm attribute, motility, viability, acrosome integrity and ATP level are considered compensable traits [38,39]. It is possible that the observed differences between HF and LF bulls for the compensable sperm traits are camouflaged by the relatively high number of spermatozoa used per AI dose in the present study. This may explain why the regression model demonstrated the uncompensable trait %DFI to be the best indicator of fertility among the sperm parameters analysed. Given that most sperm quality traits are correlated to some extent, the results of regression studies can be influenced by collinearity problems [40]. To avoid this problem, we tested the correlation among the sperm functional attributes and avoided combining highly correlated parameters in the model.

Metabolomics of bovine seminal plasma and sperm cells has to our knowledge only been conducted using fresh semen samples [18–20]. Recently, 22 distinct metabolites were identified in spermatozoa from Holstein bulls [20]. In the present study, we used frozen-thawed semen samples, which also are used for AI and thus correspond better to the NR56 data. Cryopreservation is known to have a detrimental effect on sperm viability [41]. We hypothesised that the dead spermatozoa may leak out some of the intracellular metabolites due to damaged sperm membranes. This may affect the

Table 6
Pearson correlation coefficient values between sperm functional parameters and amino acid concentrations in bovine sperm cells that were significantly ($p < 0.05$) different from zero. Only amino acids and sperm attributes showing significant correlation are listed.

	Arg	Asp	Cys	GABA	Glu	Gln	His	Ile	Lys	Ser	Thr
DFI	−0.34		0.53		0.40					−0.33	0.35
HDS		−0.39			0.35					−0.41	
AIL	0.46		−0.45			0.45					−0.48
ARL	−0.34		−0.22			−0.38	0.36				
ARD	−0.42		0.46			−0.34					0.52
ATP	0.52		−0.21			0.56					−0.40
MOT	0.55		−0.37			0.44					−0.52
PROG	0.63		−0.36			0.49					−0.58
HYP	0.58		−0.33					−0.34	0.34		−0.59
VAP	0.65					0.56					−0.47
VCL	0.67					0.45			0.34		−0.57
VSL	0.55					0.55					−0.36
STR				0.34		0.40					
LIN						0.33					
ALH	0.55								0.35		−0.54
BCF	0.43					0.46					

DFI = DNA fragmentation index, HDS = high DNA stainable, AIL = acrosome intact live, ARL = acrosome reacted live, ARD = acrosome reacted dead, MOT = motile, PROG = progressive, HYP = hyperactive, VAP = velocity average path, VCL = velocity curvilinear, VSL = velocity straight-line, STR = straightness, LIN = linearity, ALH = amplitude of lateral head displacement, BCF = beat-cross frequency, Arg = arginine, Asp = aspartic acid, Cys = cysteine, GABA = gamma aminobutyric acid, Glu = glutamic acid, Gln = glutamine, His = histidine, Ile = isoleucine, Lys = lysine, Ser = serine, Thr = threonine.

results, depending on the proportion of live/dead spermatozoa in the different samples. Thus, we emphasized to select the viable sperm population in the present study. We found the concentrations of several amino acids to correlate significantly with NR56 as well as with different sperm parameters. Arginine and glutamine in human sperm cells have previously been demonstrated to correlate with sperm progressivity [42], which is in agreement with our findings. In a study by Zhao et al. (2018) asthenozoospermic patients (men with poor sperm motility) had reduced levels of several amino acids including leucine, glutamic acid, cysteine and tryptophan [17]. Our results for bull spermatozoa also showed a significantly lower concentration of leucine in sperm cells from LF bulls. However, the group of LF bulls also showed higher concentrations of glutamic acid and cysteine compared to HF bulls. Glutamic acid is the key component of glutathione, which has been demonstrated to protect cells from damage caused by ROS and lipid peroxidation [43]. Furthermore, glutamic acid has been identified as the most abundant amino acid of bull seminal plasma [44], and was also the most abundant amino acid in sperm cells in our study. A derivative of L-cysteine, N-acetyl-cysteine, is an antioxidant and contributes to glutathione synthesis [45,46]. It can be speculated that the observed decrease in glutamic acid and cysteine concentrations in HF bull sperm cells is a consequence of their consumption of glutamic acid and cysteine for protection against oxidative stress. Oxidative stress is linked to DNA damage and male infertility [47], and may contribute to the explanation of lower observed %DFI in sperm cells from HF bulls. Both glutamic acid and cysteine correlated significantly with %DFI. Aspartic acid of bull seminal plasma has previously been shown to correlate with fertility [48]. We found a significant positive correlation between aspartic acid and bulls' NR56, with aspartate being the only amino acid exhibiting a significant contribution to the model predicting NR56. Furthermore, we found aspartic acid to correlate negatively with %HDS. This may indicate that aspartic acid is involved in the packaging of chromatin and maturation of spermatozoa. Interestingly, a study performed on human seminal plasma and sperm cells found D-aspartic acid (D-Asp) to be related to the quality of semen, with higher levels of D-Asp in men with normal semen quality compared to oligoasthenoteratospermic donors. Based on these results, they hypothesised that D-Asp could have a specific role in spermatogenesis and that it is involved in sperm maturation and fertility [49].

Both seminal plasma and sperm cells are known to contain a variety of trace elements; some are essential for proper sperm cell function and fertility, while others may have an adverse effect on reproduction [50,51]. In the present study, concentrations of Al, Fe and Zn in spermatozoa were found to be significantly higher in bulls of low vs. high NR56, and they were correlated significantly with NR56. A high level of Fe in human seminal plasma was shown to have a negative impact on sperm motility [52] and it has been reported that Fe can cause an increase in sperm DNA damage [53]. This corroborates our findings, where the level of Fe correlated positively with %DFI and exhibited negative correlation with several of the sperm motility parameters. Zinc is an essential element for male fertility and works as an antioxidant protecting sperm cells from ROS [54,55]. The lower levels of Zn in HF bulls may be explained by the same hypothesis as stated above, that sperm cells from HF bulls exhaust more Zn in their protection against oxidative damage. Metals such as Al have a toxic effect on sperm

Table 7
Pearson correlation coefficient values between sperm functional parameters and trace element concentrations in bovine sperm cells that were significant ($p < 0.05$) different from zero. Only elements and sperm attributes showing significant correlation are listed.

	Mg	Al	S	K	Ca	Mn	Fe	As	Ba
DFI		0.39	0.35	0.33	0.39		0.46		0.52
AIL			−0.39		−0.45	−0.38	−0.33		−0.47
ARL								−0.37	
ARD			0.48	0.39	0.50	0.42	0.34		0.49
ATP			−0.28		−0.35	−0.25	−0.34		−0.44
MOT			−0.38		−0.44	−0.35	−0.43		−0.44
PROG			−0.36		−0.43	−0.40	−0.45		−0.44
HYP			−0.42		−0.45	−0.39	−0.38		−0.45
VAP	0.34					−0.35	−0.35		−0.32
VCL						−0.40	−0.34		−0.35
VSL	0.39						−0.35		
STR	0.39							0.38	
LIN	0.40								
WOB	0.36								
ALH						−0.39			−0.35
BCF	0.39								

DFI = DNA fragmentation index, AIL = acrosome intact live, ARL = acrosome reacted live, ARD = acrosome reacted dead, MOT = motile, PROG = progressive, HYP = hyperactive, VAP = velocity average path, VCL = velocity curvilinear, VSL = velocity straight-line, STR = straightness, LIN = linearity, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat-cross frequency.

cells [56]. In a study in rats, exposure to Al was shown to impair spermatogenesis, sperm quality and to increase oxidative stress and inflammation [57]. Our results showed a significant correlation between Al levels and %DFI, with LF sperm cells displaying significantly higher levels of both Al and %DFI.

In the regression model based on a variable selection criteria of $p < 0.1$, the sperm functionality parameter, %DFI, together with the sperm intracellular concentrations of aspartic acid, Fe and Zn, were identified as predictors of bulls' NR56. Together, these parameters explained 59% of the variation in NR56. Although this is a notable regression, the model does not explain the total variation in fertility. This could be partially due to the fact that the bulls used in the study were preselected based on breeding soundness evaluation, as is customary at the AI station. If subfertile bulls had been included in the study, the prediction model might have been even stronger. The fertility data used in the present study were based on AIs with a sperm number per AI dose of ~12×10^6. A reduction in the sperm number per semen dose might have yielded other results regarding compensable sperm parameters. Before recommending this prediction model to AI companies, it needs to be validated using a larger number of bulls with known fertility. As this was the first time that metabolites were included as a method of analysis for Norwegian Red bulls, we did not have data available to perform such a validation. However, our results show strong correlations between fertility and %DFI, which corroborates findings of several others [7,11,13,28,35], and may indicate that conventional semen analysis at the bull station could benefit from augmentation with analysis of DNA integrity. In this study, we presented correlations identified between sperm functional parameters and metabolite concentrations. However, sperm functionality traits also correlate with each other and this is important for the implementation of the quality control program at AI stations. Due to the high degree of correlation among sperm traits, it is debatable whether a new analytical method gives novel information or simply provides an alternative technique to measure what is already known [58]. Nonetheless, the multiple correlations between metabolites/trace elements and sperm parameters found in the present study may suggest that a convergence of these different technologies can increase the knowledge of factors influencing and predicting bull fertility.

In this study, there was greater variation in LSmean NR56 for LF bulls (0.46–0.65) than for HF bulls (0.76–0.78). This may explain why larger SDs were observed for several of the sperm parameters and metabolite concentrations for LF bulls. It would be preferable to have a homogenous groups representing both HF and LF bulls. However, the numbers of bulls with inferior fertility are scarce and to obtain enough bulls in the LF group, a larger variation in NR56 had to be accepted. The number of AIs underlying the NR56 data affects the reliability of the data [58]. Three LF bulls had less than 500 inseminations recorded, and their NR56 data might have changed if the number of AIs had been higher.

In conclusion, bulls of high and low NR56 rates differed in several aspects of sperm functionality and metabolome characteristics. In particular, the combination of %DFI, and sperm concentrations of aspartic acid, Fe and Zn seem to predict bulls' NR56 rates.

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CRediT authorship contribution statement

Birgitte Narud: Data curation, Formal analysis, Visualization, Writing - original draft. **Geir Klinkenberg:** Conceptualization, Methodology, Writing - review & editing. **Abdolrahman Khezri:** Data curation, Formal analysis, Writing - review & editing. **Teklu Tewoldebrhan Zeremichael:** Data curation, Formal analysis, Writing - review & editing. **Else-Berit Stenseth:** Data curation, Formal analysis, Writing - review & editing. **Anna Nordborg:** Data curation, Formal analysis, Writing - original draft. **Tonje Husby Haukaas:** Data curation, Writing - original draft. **Jane M. Morrell:** Resources, Writing - review & editing. **Bjørg Heringstad:** Formal analysis, Writing - review & editing. **Frøydis Deinboll Myromslien:** Conceptualization, Writing - review & editing. **Elisabeth Kommisrud:** Project administration, Conceptualization, Data curation, Writing - original draft.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2020.07.005.

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IV

Sperm chromatin integrity and DNA methylation in Norwegian Red bulls of contrasting fertility

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Abstract

In this study, the complexity of chromatin integrity was investigated in frozen-thawed semen samples from 37 sires with contrasting fertility, expressed as 56-day non-return rates (NR56). Protamine deficiency, thiols and disulphide bonds were assessed and compared to previously published data for DNA fragmentation index (DFI) and high DNA stainability (HDS). In addition were *in vitro* embryo development and sperm DNA methylation assessed using semen samples from 16 of these bulls. The percentages of DFI and HDS were negatively associated with NR56 and cleavage rate, and positively associated with sperm protamine deficiency ($p < 0.05$). Significant differences in cleavage and blastocyst rates were observed between bulls of high and low NR56. However, once fertilization occurred, further development into blastocysts was not associated with NR56. The differential methylation analysis showed that spermatozoa from bulls of low NR56 were hypermethylated compared to bulls of high NR56. Pathway analysis showed that genes annotated to differentially methylated cytosines could participate in different biological pathways and have important biological roles related to bull fertility. In conclusion, sperm cells from Norwegian Red bulls of inferior fertility have less compact chromatin structure, higher levels of DNA damage and are hypermethylated compared to bulls of superior fertility.

Keywords: bull sperm, non-return rate, chromatin integrity, *in vitro* fertilization, sperm DNA methylation

1. Introduction

The main task of the sperm cell is to deliver the paternal genome to an oocyte during fertilization. Sperm DNA integrity is crucial for successful fertilization and subsequent embryo development (Kumaresan, Das Gupta, Datta, & Morrell, 2020). The sperm cell has a unique chromatin structure where most of the histones are replaced by smaller proteins, called

protamines, during spermatogenesis (Champroux, Cocquet, Henry-Berger, Drevet, & Kocer, 2018). This histone to protamine transition facilitates tight packaging of DNA in the sperm nucleus. However, sperm DNA is still susceptible to damage (McSwiggin & O'Doherty, 2018). A wide range of intrinsic and extrinsic factors may cause sperm DNA fragmentation, including oxidative stress, abortive apoptosis, deficiencies in recombination, age, semen handling, thermal stress, vaccinations and bacterial infections (González-Marín, Gosálvez, & Roy, 2012; Kumaresan et al., 2020). Studies have shown that the degree of sperm DNA fragmentation is negatively correlated with bovine field fertility (Gliozzi, Turri, Manes, Cassinelli, & Pizzi, 2017; Narud et al., 2020; Waterhouse et al., 2006) and the outcome of *in vitro* fertilization (IVF) (Fatehi et al., 2006; Simoes et al., 2013). Further, the histone to protamine ratio in sperm cells affects male fertility, where infertile men possess sperm cells with higher proportions of retained histones compared to fertile men (X. Zhang, Gabriel, & Zini, 2006). Protamine deficiency may be considered as a contributing factor to DNA instability and damage (Boe-Hansen, Fortes, & Satake, 2018). The highly compacted structure of sperm chromatin is dependent on the number of disulphide bonds within and between protamines (Oliva, 2006). Assessment of free thiols and disulphide bonds in combination with protamine deficiency analysis may provide useful information regarding sperm chromatin compaction (Martínez-Pastor et al., 2010). However, there are disagreements between studies whether or not the degree of protamination of bull spermatozoa are associated with sperm chromatin integrity (Castro et al., 2018; Fortes et al., 2014). Fertilization of an oocyte by a sperm cell with damaged DNA may affect embryo development negatively and contribute to diseases in future generations, as the oocyte only has the ability to correct a certain degree of DNA damage (Johnson et al., 2011; Ménézo, Dale, & Cohen, 2010).

Sperm cells deliver epigenetic components to the oocyte, essential to successful fertilization and embryonic development (Kropp et al., 2017). The term epigenetics refers to

the study of heritable changes in gene expression that occur without altering the DNA sequence (McSwiggin & O'Doherty, 2018). The most thoroughly studied epigenetic modification is DNA methylation, which has emerged as a promising indicator of male infertility (Kumaresan et al., 2020). The mechanism behind DNA methylation is an addition of a methyl group to the 5th carbon of a cytosine immediately followed by a guanine (CpG dinucleotide) (Alkhaled, Laqqan, Tierling, Lo Porto, & Hammadeh, 2018; Urrego, Rodriguez-Osorio, & Niemann, 2014). Sperm DNA methylation signatures have been associated with the fertility status of bulls (Kropp et al., 2017), buffalo (Verma et al., 2014) and men (Alkhaled et al., 2018). Studies on DNA methylation in bull spermatozoa are, however, still limited. Increased knowledge within the field would benefit livestock breeding by providing information regarding possible heritable traits and predisposition of diseases (Triantaphyllopoulos, Ikonomopoulos, & Bannister, 2016). Reduced representation bisulphite sequencing (RRBS) is a high-throughput and cost-efficient DNA methylation analysis method, allowing the study of DNA methylation at a single-base resolution (Doherty & Couldrey, 2014; Meissner et al., 2005). Analysis of DNA methylation using RRBS has previously been conducted for bovine somatic tissues and spermatozoa (Khezri et al., 2020; Perrier et al., 2018; Zhou et al., 2016).

Recently, we showed that the chromatin integrity parameters DNA fragmentation index (DFI) and high DNA stainability (HDS) in sperm cells from Norwegian Red bulls were significantly negatively associated with field fertility, expressed as 56-day non-return rate (NR56) (Narud et al., 2020). Here, we evaluated several parameters related to sperm chromatin integrity in Norwegian Red bulls of contrasting NR56. The analyses included evaluation of protamine deficiency, free thiols and disulphide bonds, in addition to *in vitro* embryo development and sperm DNA methylation signatures.

2. Material and methods

2.1 Animals and semen processing

Cryopreserved semen samples were provided by the breeding company Geno (Geno Breeding and Artificial Insemination (AI) Association, Hamar, Norway). Bulls were raised and fed uniformly and cared for according to the Norwegian Animal Welfare Act (LOV 2009-06-19 no. 97). The semen production procedures were in compliance with European Union Directive 88/407.

Sires were selected based on their field fertility performance, measured as NR56, as previously described by Narud et al. (2020). In brief, data on AIs were collected from the Norwegian Dairy Herd Recording System (NDHRS). The General Linear Model (PROC GLM in SAS®) was used to calculate least square mean (LSmean) NR56 for 507 Norwegian Red bulls used for AI in the period 2013-2018. The model included effects of bull, month and year of AI, parity of the female and repeated AI within 1-4 days. Based on these results, a group of 19 bulls with high NR56 (hereafter referred to as HF) and a group of 18 bulls with low NR56 (hereafter referred to as LF) were selected. LSmean NR56 ranged from 0.76 to 0.78 for HF and from 0.46 to 0.65 for LF. All bulls were in regular semen production, with a mean (± SD) age corresponding to the collection date of ejaculates analysed *in vitro* being 517 (± 162) days for HF and 459 (± 35) days for LF bulls. For the *in vitro* production (IVP) of embryos and the analysis of sperm DNA methylation by RRBS, eight bulls with highest and lowest NR56 were selected. Pedigree information was considered to avoid including closely related individuals.

Post collection, ejaculates with motility above 70% and morphological abnormalities below 15% were diluted to a final concentration of 12 x 10^6 spermatozoa per AI dose in French mini straws (IMV, L'Aigle, France). A two-step dilution procedure with Biladyl® extender containing glycerol (Minitube, Verona, WI, USA, 13500/0004-0006) and fresh egg yolk was applied. Cryopreservation was performed according to standard procedures (Standerholen,

Myromslien, Kommisrud, Ropstad, & Waterhouse, 2014) and the semen doses were stored in liquid nitrogen (-196 °C) until use.

2.2 Preparation of samples for protamine deficiency and thiol assays

Two semen straws per bull were thawed at 37°C for 12 sec and mixed. For four of the bulls, only one straw was available. The concentration of spermatozoa was determined with a Nucleocounter SP-100 (Chemometec, Allerød, Denmark). Further, each sample was split in two, one part of the sample was diluted in 400 µL TNE-buffer (0.01 mol/L of Tris-HCl, 0.15 mol/L of NaCl, and 1 mmol/L of EDTA, pH 7.4) and used for analysis of protamine deficiency, while the other was diluted in 1100 µL TNE-buffer and used for analysis of thiols and disulphide bonds. In both sample preparations, the final concentration was 2 x 10^6 cells/mL. Quadruplicates of both sample types were then snap-frozen in liquid nitrogen.

2.3 Analysis of protamine deficiency

The level of sperm protamine deficiency was assessed using Chromomycin A_3 (CMA3; SigmaAldrich), as described by Zubkova, Wade, and Robaire (2005) with minor modifications. Briefly, snap-frozen samples were thawed on ice and washed with PBS by centrifugation (300 x g; 10 min). The resulting sperm pellet was resuspended in 80 µL McIlvaine's buffer (17 mL 0.1 mol/L citric acid mixed with 83 mL 0.2 mol/L Na_2HPO_4 and 10 mmol/L $MgCl_2$, pH 7.0) containing 0.25 mg/mL CMA3. Further, the samples were incubated in the dark for 20 min at 37°C and washed in 400 µL PBS by centrifugation (300 x g; 10 min). Pellets were resuspended in 400 µL PBS containing 3.2 µL propidium iodide (PI, 2.4 mM solution; Molecular Probes). A flow cytometer (FACSVerse, BD Biosciences, Franklin Lakes, NJ) equipped with a blue laser (488 nm) was utilized for analysis of the samples. Gating of the sperm cell population was performed using forward scatter (FSC) and side scatter (SSC) and sperm cells were further identified by propidium iodide (PI) positive signal collected via 586/42 bandpass filter. After

excitation with a violet laser (405 nm), the CMA3 fluorescence from gated cells was collected through a 528/45 bandpass filter.

2.4 Analysis of thiols and disulphide bonds

Free thiols, total thiols and disulphide bonds in bull spermatozoa were analysed using monobromobimane (mBBr; Molecular Probes), according to the previously described method by Zubkova et al. (2005) and Seligman, Kosower, Weissenberg, and Shalgi (1994) with some modifications. Briefly, the samples were thawed on ice and divided into two tubes, each containing 1×10^6 sperm cells. The first tube was incubated with 1 mmol/L of 1,4-dithiothreitol (DTT, Sigma-Aldrich) for 10 min at 37 °C, while no DTT was added to the second tube. After centrifugation of both tubes (300 x g, 10 min), the pellets were resuspended in 100 µL PBS containing 0.5 mmol of mBBr solution. Both tubes were incubated in the dark for 10 min at 37 °C, washed with 500 µL PBS twice by centrifugation (300 x g, 10 min). The pellets were resuspended in 500 µL PBS and analysed with a FACSVerse flow cytometer (BDBiosciences) after excitation of mBBr by a 405 nm violet laser. The mBBr fluorescence was collected by a 528/45 bandpass filter, and gating of the sperm cell population was done using FSC and SSC. In order to calculate disulphide concentrations, the fluorescence signals of free thiols (mBBr fluorescence from non-DTT-treated sample) were subtracted from fluorescence signals of total thiols (mBBr fluorescence from DTT-treated sample), thereafter that value was divided by two. After the initial run, 4 µL PI (2.4 mM solution, Molecular Probes) was added to each sample, and the samples were analysed again, this time also collecting the PI-fluorescence through a 586/42 bandpass filter. These data were used only as a further aid to discriminate debris from spermatozoa, and were not used for quantitative purposes, since the PI was found to quench the mBBr-fluorescence.

2.5 *In vitro* production of bovine embryos

In vitro production of embryos was conducted using media from IVF biosciences (Falmouth, UK). Eight bulls with low NR56 and eight bulls with high NR56 were used for IVF. For each experiment, oocytes were randomly split into groups of 30 oocytes and two groups were fertilized with frozen-thawed semen from the same bull. Two LF and two HF bulls were used for fertilization in each test week. In addition, a reference bull with known IVF performance was used as control throughout the study. Each IVF experiment was repeated three times and a total of 180 oocytes per bull were included.

2.5.1 Oocyte collection and *in vitro* maturation

Bovine ovaries were collected at a local slaughterhouse and transported in warm (30 to 33 °C) saline solution (0.9% NaCl, 2.5 mL 1% kanamycin) to the laboratory within 4 h. Cumulus–oocyte complexes (COCs) were aspirated from follicles sized 3 to 15 mm in diameter and collected in a 50 mL falcon tube containing 140 µL heparin (5.000 IU/mL). Groups of good quality COCs (grade I and II according to Blondin and Sirard [1995]) were selected, washed and transferred in groups of 30 to 4-well plates (NuncTM, 734-2693, Thermo Scientific, VWR Norway) containing 500 µL of BO-IVM media. The COCs were matured for 22 to 24 h at 38.8°C under an atmosphere of 6% CO_2 in air with maximum humidity.

2.5.2 *In vitro* fertilization and culture

After maturation, the COCs were washed and transferred to new 4-well plates containing 500 µL BO-IVF media, and kept in the incubator (38.8°C, 6% CO_2, maximum humidity) while the semen samples were prepared. Two semen straws per bull were thawed in a water bath at 37°C for 1 min and the semen was transferred to the bottom of a 15 mL falcon tube containing preheated (36°C) BO-SemenPrep. The semen samples were centrifuged (330 x g, 5 min), the supernatant was removed, and 4 mL of BO-SemenPrep was added to the pellet. The centrifugation step was repeated, followed by removal of the supernatant, and computer-

assisted sperm analysis (CASA) was used for assessing sperm motility and concentration. Sperm was added to each group of oocytes with a final concentration of 1 x 10^6 progressive motile spermatozoa/mL, followed by 18 h incubation (6% CO_2, 38.8°C, maximum humidity). The presumptive fertilized oocytes were denuded by vortexing, before they were washed and transferred to culture plates containing 500 µL BO-IVC media with oil overlay. Culture was performed in a humidified atmosphere of 7% O_2, 6% CO_2 and 87% N_2 at 38.8°C. At day 3 post-fertilization, the cleavage rate was recorded. Further, at day 8 the blastocyst rate was calculated based on the total number of oocytes. The number of cleaved cells that developed further into blastocysts was also recorded.

2.6 RRBS library preparation and illumina sequencing

Semen samples from eight bulls with low NR56 and eight bulls with high NR56 were utilized for the construction of RRBS libraries using a gel-free multiplexed technique (Boyle et al., 2012), previously optimized to study sperm DNA methylation in boar (Khezri et al., 2019) and bull (Khezri et al., 2020). The protocol consisted of the following steps.

An amount of 100 ng genomic DNA was digested overnight at 37°C using *MspI* and *Taqα1* enzymes (New England Biolabs, USA). Fragmented DNA was subjected to Gap filling and A-tailing by adding 1 µL of Klenow fragment (New England Biolabs, USA) and 1 µL of dNTP mixture containing 10 mM dATP, 1 mM dCTP and 1 mM dGTP (New England Biolabs, USA). The processed DNA was further incubated for 20 min at 30°C followed by 20 min at 37°C. Size selection (300-500 bp) was performed by adding a 2x SPRI AMPure XP beads for 30 min at RT (Beckman Coulter, USA). Then samples were placed on a side magnet, the supernatant was removed, and beads were washed and re-suspended in 20 µL elution buffer (Qiagen, Germany). Size selected DNA samples were further prepared for Illumina sequencing by ligating the standard Illumina adapters using 2 µL of NEXTflex™ Bisulfite-Seq barcodes (Bio Scientific Corporation, USA) and ligase mixture, followed by incubation overnight at 16

°C. Adapter ligated DNA samples were again subjected to size selection by adding 1.5x of 20% PEG 8000/2.5 M NaCl (Amresco Inc., USA) followed by 30 min incubation at RT. The supernatant was discarded as described previously and beads were washed twice using 70 % EtOH followed by re-suspension in 25 µL elution buffer (Qiagen, Germany). Size selected DNA fragments were bisulfite converted and the product was cleaned up according to recommendations in the QIAGEN EpiTect kit (Gu et al., 2011). Prior to amplification of bisulfite converted DNA fragments, various PCR amplification cycles (10, 13, 16 and 19 cycles) were tested. In order to evaluate the efficiency of the protocol by observing DNA bands and to determine the appropriate PCR amplification cycle, the PCR product were analysed on a gradient Criterion precast polyacrylamide TBE gel (4 – 20%) (Thermo Fisher Scientific, USA). The PCR product (after 13 amplification cycles), were further cleaned up by adding 1x SPRI AMPure XP beads followed by incubation for 30 min at RT. The supernatant was discarded and the beads were washed with 70% EtOH. After 30 sec incubation at RT, EtOH was removed, and beads were allowed to dry for 5 min before resuspension in 25 µL elution buffer. Eluted beads were placed on a side magnet for 5 min prior to transfer of the supernatant (purified libraries) to a clean tube. The DNA concentration of eluted RRBS libraries were measured using PicoGreen dsDNA absorbance method. The prepared libraries were sent to Norwegian Sequencing Centre where sequencing was performed by Illumina HiSeq 4000 in paired-end (2 × 150 bp) mode.

2.7 Bioinformatics analyses

2.7.1 Illumina reads quality assessment and trimming

Quality of paired-end Illumina reads was assessed using fastQC software (v 0.11.8 for Linux). Illumina adapters and low-quality sequences (below 20 bp and Phred score of 30) were trimmed using the Trim-galore software (v 0.4.4 for Linux) (Martin, 2011).

2.7.2 Mapping the clean reads with reference genome

Bull reference genome (*bosTau 9*) was downloaded from the UCSC database (UCSC, 2018) and indexed using bismark_genome_preparation in Bismark (v 0.19.0 for Linux) (Krueger & Andrews, 2011). Mapping was performed using the Bismark tool and bowtie2 aligner (v 2.3.2 for Linux) (Krueger & Andrews, 2011) with the following parameters [-n 0 -l 20 and --score-min (L, 0, -0.4)]. Global CpG methylation level in Bismark was calculated for all covered cytosines (Cs) using the following formula: % of global methylation = 100 * number of methylated Cs / number of methylated Cs + number of unmethylated Cs.

2.7.3 Differential methylation analysis

Differentially methylated cytosines (DMCs) were identified using reads with CpGs ≥ 5x coverage depth (CpG_{5x}) between control (eight HF bulls) and test (eight LF bulls) groups, using the methylKit package (v 1.6.1) (Akalin et al., 2012) in Rstudio (v 1.1.453 for Linux). Before the analysis, reads containing CpGs with more than 99.9th percentile coverage were excluded. After applying logistic regression analysis with a sliding linear model to correct for multiple comparisons only DMCs with ≥10% methylation difference and *q-value* < 0.05 (filtered DMCs onwards) were considered for downstream analysis. In this study, hypermethylated and hypomethylated Cs are defined as differential methylation over 10% or smaller than -10% in the test group compared to the control group, respectively.

2.7.4 Annotation of differentially methylated cytosines

BED files containing gene and CpG annotation for the *bosTau9* assembly were downloaded from UCSC table browser (UCSC, 2018). Genomation package (v 1.14.0) in Rstudio was employed to annotate filtered DMCs with nearest transcriptional start site (TSS), gene elements (exons, introns, promoters, intergenic regions) and CpG features (CpG islands, CpG shores, other). In this study, promoters and CpG shores were defined as ± 1000 bp and ± 2000 bp of the TSS and CpG islands, respectively.

2.7.5 Pathway analysis

Corresponding GenBank accession IDs to annotated TSSs were submitted to DAVID Bioinformatics resources for functional annotation (Huang da, Sherman, & Lempicki, 2009) for Gene Ontology (GO) analysis. Gene enrichment for each identified pathway was calculated using Fisher's exact test and the *p*-value was Benjamini corrected for multiple testing and set to 0.05.

2.8 Statistical analyses

The statistical analysis of the different *in vitro* sperm quality traits and IVP experiments were performed using SAS Version 9.4 for Microsoft Windows (SAS Institute, Cary, NC, USA). The data was tested for normal distribution by the Shapiro-Wilk test. Parameters that did not show a normal distribution (DFI and HDS) were log-transformed prior to further statistical analysis. Differences in sperm chromatin integrity parameters between HF and LF bulls were assessed by unpaired t-test, including the folded F test for equality of variances. Differences in cleavage and blastocyst rate (calculated as % development from fertilized oocytes and % development from cleaved cells) between LF and HF bulls were studied using mixed linear model (proc mixed). Fertility group [0 (LF) or 1 (HF)], replicate of bull (1, 2, 3) and test week (1, 2, 3, 4) were included in the model as fixed effects, and bull (1 to 16) was included as random effect. Correlation coefficients (Pearson) were calculated and considered statistically significant when $p < 0.05$.

The RRBS results were plotted using GraphPad Prism (v 6.01 for Windows, GraphPad Software, San Diego, CA, USA). The Venny online platform (Oliveros, 2015) and ggplot2 package (v 3.1.0) in Rstudio (Wickham, 2016) were employed to construct Venn diagrams and plot pathway analysis results, respectively.

3. Results

3.1 Sperm chromatin integrity analyses

Sperm quality parameters related to chromatin integrity for the two fertility groups are presented in Table 1. As previously reported in Narud et al. (2020), higher percentages of DFI ($p < 0.001$) and HDS ($p < 0.01$) were observed in sperm cells from LF bulls compared to HF bulls. The mean levels of free thiols, total thiols, disulphide bonds and protamine deficiency were not significantly different ($p > 0.05$) for LF bulls compared to HF bulls.

The chromatin integrity parameters were further subjected to Pearson correlation analysis to identify possible relationships with NR56 and among parameters. Table 2 shows that DFI and HDS correlated negatively with NR56. Furthermore, DFI and HDS correlated positively with each other and with protamine deficiency. The number of disulphide bonds was strongly (positively) correlated with total thiols.

3.2 IVF and embryo development

A significantly higher proportion of oocytes cleaved after fertilization with sperm from bulls of high (71.3% ± 10.6) compared to low (53.8% ± 13.7) NR56 (Figure 1). Further, there was a significant higher proportion of blastocysts at day 8 for the HF bulls (27.9% ± 6.1) than for the LF bulls (20.5% ± 6.3). The proportion of cleaved cells that developed further into day 8 blastocysts was not significantly different between the two fertility groups (HF: 39.1% ± 6.3, LF: 38.0% ± 4.8).

3.3 Correlations between NR56, chromatin integrity traits and *in vitro* embryo development

Pearson correlation analysis indicated a positive relationship between NR56 and *in vitro* embryo development (Table 3). The cleavage rate was negatively correlated with DFI and HDS ($p < 0.05$). There were no significant correlations between the proportion of cleaved cells that further developed into day 8 blastocysts and any of the chromatin integrity parameters.

3.4 Basic statistics of RRBS libraries

An overview of the basic statistics for the RRBS libraries is presented in Table 4. After removing low quality reads and Illumina adapters, 94% of reads were saved. The RRBS libraries had an average of 14.6 million reads per sample and 30.5x read coverage compared to *in silico* created RRBS genome. Trimmed reads had on average 99% conversion rate and mapping against reference genome resulted in 34.7% unique mapping efficiency. Furthermore, on average, 741,000 CpG_{5x}, with methylation average of 42.8% were covered in RRBS libraries. None of the parameters for mapping efficiency, global CpG methylation level and conversion rate were significantly different ($p < 0.05$) between LF bulls compared to HF bulls. The correlation between global CpG level and embryo development was also assessed; however, no significant correlations were detected.

Cluster analysis according to CpG_{5x} methylation value showed that the samples were not clustered based on fertility performance (Figure 2). Furthermore, Pearson's correlation coefficient based on the same criteria revealed a high positive correlation between samples regarding CpG_{5x} methylation profile (Pearson's correlation coefficient > 0.94) (Supplementary Table S1).

3.5 Differential methylation analysis

By applying a significance cut-off equal to q-value < 0.05, a total number of 16,542 DMCs were detected with varying degree of methylation, ranging from 0 to 75%. Majority of DMCs (56%) were found to be hypermethylated in the LF group compared to the HF group (Figure 3A). After applying a 10% methylation cut-off, 65% of DMCs had over 10% methylation difference (filtered DMCs) in both hypo- and hypermethylated groups, which were further used for downstream analyses (Figure 3B).

3.6 Annotation of differentially methylated cytosines with gene and CpG features.

Annotation of filtered DMCs in both hypomethylation and hypermethylation groups with gene features revealed similar trends. For instance, 70% of the filtered DMCs were present in the intergenic regions followed by introns, exons and promoters (Figure 4A). Furthermore, over 85% of filtered DMCs in both hypomethylation and hypermethylation groups were annotated within regions outside of CpG islands (CGI) / CGI shores. Only around 15% of filtered DMCs were annotated within CGI / CpG shores (Figure 4B).

3.7 Pathway analysis

Prior to pathway analysis, $DMCs_{\geq 10\%}$ were annotated with the nearest transcription start sites (TSSs) (Figure 5A). Annotation results showed a greater number of TSSs in the hypermethylation group (3274 TSSs) compared to the hypomethylation group (2727 TSSs). A total number of 1287 TSSs were harboured with both hypomethylated and hypermethylated Cs. Pathway analyses showed that TSSs in hyper- and hypomethylated groups, contributed to a total number of 50 and 23 significant pathways, respectively. Among those, 18 pathways were detected as mutual pathways associated with TSSs harboured with both hypo- and hypermethylated Cs (Figure 5B).

We were particularly interested in genes associated with biological processes related to fertility. Figure 6 shows that the number of such identified genes were higher in the hypermethylation group compared to the hypomethylation group. Genes related to the penetration of zona pellucida was identified exclusively in the hypermethylation group. In both hypo- and hypermethylation groups biological processes including *in utero* embryo development, followed by fertilization and embryo implantation, were represented by the highest numbers of genes (Supplementary Table S2).

Pathway analysis showed that the majority of identified mutual pathways between hypomethylated and hypermethylated groups exhibited similar *p*-values (Supplementary Figure S1). Moreover, the majority of exclusively identified pathways (32 pathways) were in association with genes close to hypermethylated cytosines. Only five pathways were linked to genes close to hypomethylated cytosines (Figure 7). Several pathways were exclusively identified in the hypermethylation group of test samples (LF bulls). This included hormonal pathways such as oxytocin signalling and ion related signalling pathways such as calcium signalling, ion channel/transport and voltage-gated channel, which all play a direct role in the fertilization process. Furthermore, pathways involved in embryonic development such as developmental proteins and vascular endothelial growth factor (VEGF) signalling pathway were exclusively identified in the hypermethylation group of LF bulls.

4. Discussion

In the current study, various chromatin integrity parameters in semen samples from 37 Norwegian Red bulls of low and high NR56 were analysed. Furthermore, 16 of these bulls were selected for IVP of embryos and investigation of sperm DNA methylation profiles by RRBS.

As we previously reported, significantly lower percentages of DFI and HDS were observed in sperm cells from bulls of high NR56 compared to bulls of low NR56 (Narud et al., 2020). In the present study, sperm DFI and HDS correlated positively with protamine deficiency. This corroborates the results of a previous study in bovine (Fortes et al., 2014), and may indicate that LF bulls have spermatozoa with less compact chromatin and more DNA strand breaks compared to HF bulls. Failure in the replacement of histones by protamines during spermatogenesis, abortive apoptosis and insufficient protection against reactive oxygen species (ROS) (Hamilton & Assumpcao, 2020) may have contributed to our results. The protamine structure in the sperm nucleus is strongly stabilized through the formation of disulphide bonds

between cysteine residues of adjacent protamine molecules (Oliva, 2006). Recently, our group discovered that DNA fragmentation had a significant but weak positive correlation with free thiols and disulphide bonds of boar spermatozoa (Khezri et al., 2019). In the present study however, no associations were found between sperm thiols / disulphide bonds and DNA integrity or field fertility (NR56). This was in contrast to findings in humans, where semen samples from oligozoospermic or infertile men were found to have fewer disulphide bonds compared with normozoospermic samples, and where DNA integrity correlated positively with the level of thiol groups (Rufas et al., 1991; Zini, Kamal, & Phang, 2001). However, the reproductive performance of the different species may explain the contradictory results between the studies, as breeding bulls and boars are more fertile compared to men with infertility problems.

Spermatozoa from HF bulls displayed an increased ability to fertilize oocytes *in vitro* and resulted in significantly higher cleavage rates and total blastocyst rates than LF bulls. However, by measuring the number of cleaved cells that developed further into blastocysts, no differences were detected between the fertility groups. Our results corroborates the findings of Al Naib, Hanrahan, Lonergan, and Fair (2011), which observed that once fertilization occurred the following embryo development into blastocysts was not influenced by the bulls' fertility status. Furthermore, Ward et al. (2001) observed that bull's field fertility measured as non-return rate after 150 days correlated with cleavage rate, while B. R. Zhang, Larsson, Lundeheim, and Rodriguez-Martinez (1997) found a positive correlation between NR56 and both cleavage and blastocyst rate. In contrast, Kropp et al. (2017) found no difference in cleavage or blastocyst rates between sires of contrasting fertility. The discrepancy between studies may be explained by factors such as the reliability of fertility data, number of ejaculates used per bull, how the experiments are conducted in lab and the range in fertility among the bulls included (Larsson & Rodríguez-Martínez, 2000).

The correlation analysis between NR56, chromatin integrity traits and *in vitro* embryo development showed that both DFI and HDS were negatively correlated to the cleavage rate, but not to the blastocyst rate, indicating that impaired chromatin integrity may affect the sperm cells ability to fertilize oocytes in IVF. This was further corroborated by our findings that HF bulls had higher cleavage rates, but that the subsequent development into blastocysts was unaffected by bulls' fertility group. Thus, based on these results we can assume that sperm DNA damage mostly affects the sperm cells' ability to fertilize the oocyte. This is in agreement with the findings of a previous study, where sperm DNA damage, caused by increased oxidative stress, was found to affect cleavage rate (Simoes et al., 2013). However, others have reported that DNA fragmentation in bull sperm does not impair *in vitro* fertilization but rather the further embryonic development when the blastocyst stage is reached (Fatehi et al., 2006). An important factor that may contribute to the observed results is the relatively low levels of DNA fragmentation in the Norwegian Red bulls of this study (ranging from 1.0 to 6.5%). It can be hypothesised that the level of fragmented DNA is within the range of damage that the oocytes manage to repair (García-Rodríguez, Gosálvez, Agarwal, Roy, & Johnston, 2018). However, such repair is usually followed by early embryonic death, implantation defect, chromosomal abnormalities and higher abortion rate (Tesarik, Greco, & Mendoza, 2004), which might explain the strong negative association observed between DNA damage and NR56. This highlights the importance of elucidating sperm DNA integrity, especially when working with assisted reproductive technologies, such as IVF, where the natural selection of good quality sperm cells is excluded.

The correlation analysis further indicated that there were no associations between sperm protamine deficiency and NR56 or with IVF outcomes. This is in agreement with a recent study by Castro et al. (2018), reporting that protamine deficiency in bovine spermatozoa might not have a strong biological impact in explaining difference of *in vitro* fertility between bulls due

to the generally low levels of protamine deficiency in bovine spermatozoa (Castro et al., 2018; Kipper et al., 2017).

In this study, the average global CpG methylation level was 42.8%. Recently, we reported similar results (global CpG methylation level of 40%) for bull sperm samples collected from young Norwegian Red bulls (Khezri et al., 2020). These results are in agreement with previous studies, where average global CpG methylation levels of 35% and 45% in bull sperm cells have been reported, using RRBS and a luminometric methylation assay, respectively (Jiang et al., 2018; Perrier et al., 2018). In the present study, the differential methylation analysis showed higher level of CpG methylation (hypermethylation) in samples from bulls of low NR56 compared to bulls of high NR56. Similar results have previously been reported for humans, where 74% of DMCs were hypermethylated in infertile patients (Camprubí et al., 2016). In addition, the present findings are supported by a study in buffalo, where a higher number of genes were hypermethylated in sub-fertile compared to high-fertile bulls (Verma et al., 2014). In contrast, Kropp et al. (2017) detected a higher level of methylated regions in sperm cells from high fertility Holstein bulls. This may be explained by the different techniques used for the study of sperm DNA methylation.

The regional analyses showed that on average 70% of the filtered DMCs were present in intergenic regions and over 85% of filtered DMCs were annotated within regions outside of CGI / CGI shores. These results are in agreement with previous studies on sperm DNA in bull (Jiang et al., 2018; Khezri et al., 2020; Perrier et al., 2018) and boar (Khezri et al., 2019). However, the results for intergenic regions are in contrast with data published in infertile human patients where only 33% of identified DMCs were annotated with intergenic regions (Camprubí et al., 2016).

In this study, functional analysis was used to identify some of the gene's biological processes related to fertility. The results indicated that a high number of genes represent

biological processes such as *in utero* embryo development, fertilization and embryo implantation, in both hypo- and hypermethylation groups, but with most genes represented in the hypermethylation group. For instance, genes such as transition protein 2 (TNP2) and T-box transcription factor T (TBXT), involved in penetration of zona pellucida, were exclusively identified in the hypermethylation group. Previous studies in mice have reported a relationship between premature translation of TNP2 mRNA and the number of immobile and deformed sperm cells (Tseden et al., 2007), and that deletion of the TNP2 gene may result in less condensed sperm chromatin (Zhao et al., 2001). Data from Chinese Holstein bulls showed that the relative mRNA expression of TNP1 gene was significantly associated with the degree of sperm cell deformities (S. Zhang et al., 2015). Currently, there are no data available on the role of TBXT gene in male fertility; therefore, this would be a fruitful area for further research.

One of the challenges in interpreting functional analysis results is that genes could be involved in several biological functions, simultaneously. Although several genes involved in fertilization were identified in this study, only three genes were identified with a single and specific biological process related to fertilization. These genes were zona pellucida binding protein (ZPBP) and regulated endocrine specific protein 18 (RESP18) in hypermethylated group and Glioma Pathogenesis-Related 1-like protein 1 (GLIPR1L1) in hypomethylated group. Previous studies clearly demonstrated the significant role of GLIPR1L1 in sperm-oocyte binding in mice and bovine (Caballero et al., 2012; Gaikwad et al., 2019; Gibbs et al., 2010). Furthermore, mice lacking ZPBP genes produced abnormal sperm cells with decreased ability to penetrate zona pellucida (Lin, Roy, Yan, Burns, & Matzuk, 2007). In this study, the RESP18 gene was connected to *in utero* embryo development. Previous studies reported that RESP18 may have an important role in the development of nervous, cardiovascular, endocrine, renal and reproduction systems (Atari, Perry, Jose, & Kumarasamy, 2019). Furthermore, in agreement

with our result, RESP18 was previously identified as hypermethylated in low fertile buffalo bulls (Verma et al., 2014).

We have identified pathways related to fertilization and embryonic development that might explain the lower fertility performance of bulls with low NR56. For instance, oxytocin signalling, calcium signalling, ion channel/transport, voltage-gated channel, developmental proteins and vascular endothelial growth factor (VEGF) signalling pathways were discovered exclusively in the hypermethylation group of the test group (bulls with low NR56). In addition, we have recently shown that several intracellular sperm amino acids and trace elements are associated with field fertility and suggested that metabolomics may be a useful tool in the identification of biomarkers for male fertility (Narud et al., 2020). In accordance with this hypothesis, metal binding and metabolic pathways were found exclusively in the hypermethylation group, which might also explain the lower fertility in LF bulls. However, due to the high degree of transcriptome inactivity of sperm cells and unknown reproductive competence of females in this study, conclusion of involved pathways / genes in fertility output of LF bulls must be done with caution.

In conclusion, we show here that sperm DNA integrity is significantly associated with field fertility and *in vitro* fertilization capacity of Norwegian Red bulls. Spermatozoa of low-fertility bulls are hypermethylated compared to those of high-fertility bulls. Genes annotated to differentially methylated Cs were identified to participate in different biological pathways important for bull fertility.

Conflict of interest

The authors have no conflicts of interest to declare.

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Availability of data

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Table 1. Sperm quality parameters related to chromatin integrity in semen samples from bulls of low (*n* = 18) and high (*n* = 19) 56-day non-return rates (NR56). Results are presented as range (min-max) and mean ± SD. For protamine deficiency, *n* = 15 and *n* =18 for bulls of low and high NR56, respectively.

		Low NR56	High NR56
DFI (%)[1]	min-max	1.67 - 6.5	1.0 - 4.0
	mean ± SD	3.5 ± 1.4	1.8 ± 0.8 **
HDS (%)[1]	min-max	1.5 – 7.2	1.6 – 5.1
	mean ± SD	4.3 ± 1.7	2.9 ± 1.2 *
Free thiols (mFI)	min-max	3609.0 - 11,303.0	6470.0 - 10,849.0
	mean ± SD	8184.0 ± 2144.0	8464.1 ± 1233.7
Total thiols (mFI)	min-max	32,115.0 - 47,524.0	30,121.0 - 47,372.0
	mean ± SD	40,593.3 ± 5023.8	41,659.4 ± 4087.2
Disulphide bonds (mFI)	min-max	11,721.0 - 20,190.0	11,482.0 - 19,722.0
	mean ± SD	16,204.7 ± 2378.6	16,597.6 ± 2018.4
Protamine deficiency (mFI)	min-max	2429.0 - 4305.0	2347.0 - 3475.0
	mean ± SD	3134.5 ± 455.1	2890.7 ± 347.3

Abbreviations: NR56, 56-day non-return rate; DFI, DNA fragmentation index; HDS, high DNA stainable; mFI, mean fluorescence intensity.

[1]Data from Narud et al. (2020).

Asterisks indicate significant differences between the groups; *($p < 0.01$), ** ($p < 0.0001$).

Table 2. Pearson correlation coefficients and *p*-values (in brackets) between sperm chromatin integrity traits and NR56 (*n* = 37, except for PD where *n* = 33).

	NR56	DFI	HDS	FT	TT	DB
DFI	-0.57					
	(<.0001)*					
HDS	-0.37	0.63				
	(0.03)*	(<.0001)*				
FT	-0.01	-0.09	0.07			
	(0.97)	(0.59)	(0.68)			
TT	0.13	-0.13	-0.19	0.29		
	(0.43)	(0.43)	(0.27)	(0.08)		
DB	0.14	-0.10	-0.22	-0.09	0.93	
	(0.40)	(0.55)	(0.19)	(0.59)	(<.0001)*	
PD	-0.23	0.40	0.45	0.21	0.07	-0.01
	(0.20)	(0.02)*	(0.01)*	(0.23)	(0.71)	(0.96)

Abbreviations: NR56, 56-day non-return rate; DFI, DNA fragmentation index; HDS, high DNA stainable; FT, free thiols; TT, total thiols; DB, disulphide bonds; PD, protamine deficiency.

Asterisks indicate significant correlations ($p < 0.05$).

Table 3. Pearson correlation coefficients (corr.) between NR56, the chromatin integrity parameters and cleavage rate and total blastocyst rate (n = 16).

	Cleavage rate		Blastocyst rate T	
	Corr.	*p*-value	Corr.	*p*-value
NR56	0.56	0.025*	0.50	0.051*
DFI (%)	-0.56	0.025*	-0.45	0.084
HDS (%)	-0.50	0.048*	-0.29	0.274
FT (mFI)	0.43	0.100	0.34	0.204
TT (mFI)	0.22	0.408	0.40	0.127
DB (mFI)	0.01	0.969	0.23	0.388
PD (mFI)	-0.08	0.789	0.17	0.587

Abbreviations: NR56, 56-day non-return rate; DFI, DNA fragmentation index; HDS, high DNA stainable; FT, free thiols; TT, total thiols; DB, disulphide bonds; PD, protamine deficiency; mFI, mean fluorescence intensity; Blastocyst rate T, % blastocysts developed from the total number of oocytes.

Asterisks indicate correlations significantly different from 0 ($p \leq 0.05$).

Table 4. Summary statistics for RRBS libraries. Libraries were created from sperm DNA collected from bulls with high (HF) and low (LF) NR56 (n = 8 in each group). Clean reads were obtained after adapter and low-quality trimming of Illumina sequencing reads (total reads). Read coverage was calculated by dividing the number of bp in the clean reads by the number of bp at *in silico* MspI-digested *bosTau9* genome. Mapping efficiency shows the percentage of clean reads that mapped with unique positions at *bosTau9* genome. Global CpG methylation indicate the percentage of methylated CpGs regardless of depth in clean reads. Downstream analyses were performed based on CpGs with equal or more than 5x depth coverage. Bisulphite conversion rate shows the percentage of Cs that converted to uracil during the bisulphite conversion process.

Bull ID	Total reads	Clean reads after trimming (percentage of retrieved reads)	Read coverage (X)	Unique mapping efficiency (%)	Global CpG methylation (%)	Number of 5x CpGs	Bisulphite conversion rate (%)
HF 1	13 657 477	12 754 713 (93.4)	26.5	36.9	43.3	660 513	99.1
HF 2	14 127 957	13 309 272 (94.2)	28.0	35.6	42.6	657 272	99.0
HF 3	15 231 439	14 336 622 (94.1)	30.0	33.9	41.9	700 034	99.1
HF 4	17 031 740	15 961 764 (93.7)	34.3	30.2	44.0	645 608	99.0
HF 5	20 397 755	19 240 081 (94.3)	39.9	34.3	42.2	1 361 145	99.0
HF 6	15 623 143	14 728 278 (94.3)	30.8	35.9	43.1	840 029	99.0
HF 7	12 725 025	11 911 015 (93.6)	25.7	36.2	43.2	537 377	99.0
HF 8	11 189 589	10 489 998 (93.7)	22.8	38.6	45.0	533 764	99.0
LF 1	12 484 969	11 708 651 (93.8)	24.7	36.0	42.9	462 950	98.9
LF 2	16 053 221	15 124 114 (94.2)	30.4	33.3	42.6	684 065	99.0
LF 3	15 345 745	14 462 408 (94.2)	29.2	32.4	42.2	573 138	98.8
LF 4	19 046 631	18 537 954 (97.3)	39.5	31.6	39.6	943 073	98.9
LF 5	18 701 716	17 621 093 (94.2)	36.2	30.4	41.1	740 494	99.0
LF 6	16 619 280	15 562 528 (93.6)	32.8	37.3	44.9	937 896	98.9
LF 7	13 472 993	12 679 214 (94.1)	25.8	34.7	42.9	513 328	99.0
LF 8	15 603 965	14 562 799 (93.3)	32.6	38.7	44.1	1 072 109	99.1

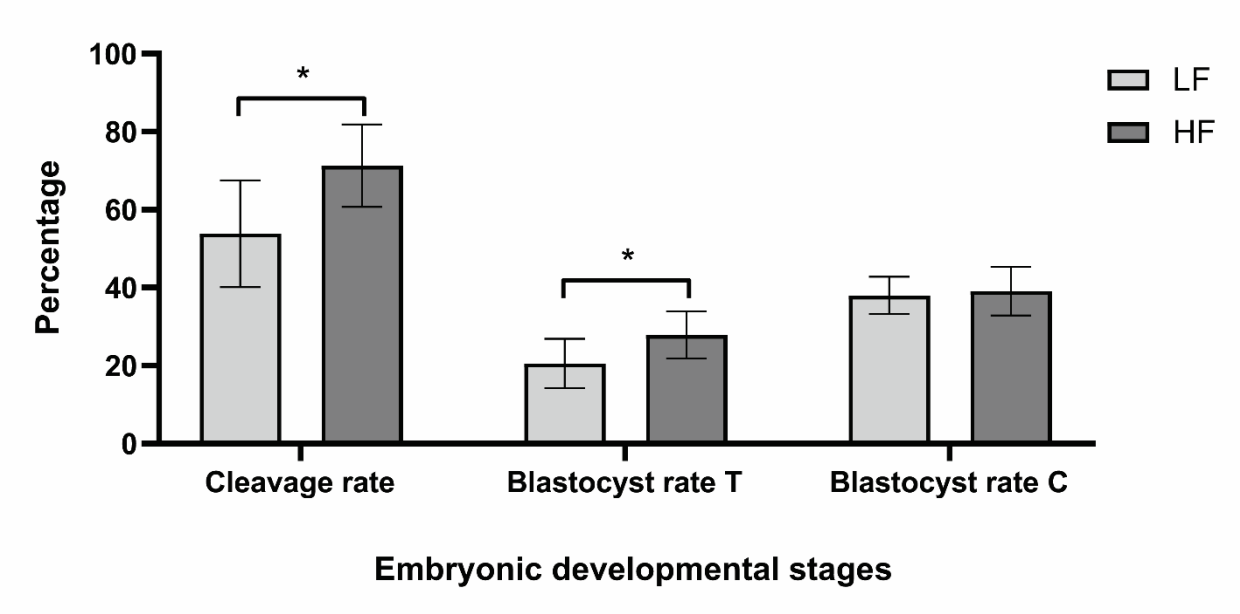

Figure 1. Mean cleavage and blastocyst rates (SD given as whiskers) following in vitro fertilization of oocytes with sperm from bulls with high (HF) and low (LF) 56 days non-return rate (n = 8 in each group). Asterisks indicate a significant difference between the two fertility groups ($p < 0.05$) based on a linear mixed model. The blastocyst rate was assessed on day 8 and is expressed as Blastocyst rate T: % blastocysts developed from total number of oocytes and Blastocyst rate C: % blastocysts developed from the cleaved oocytes.

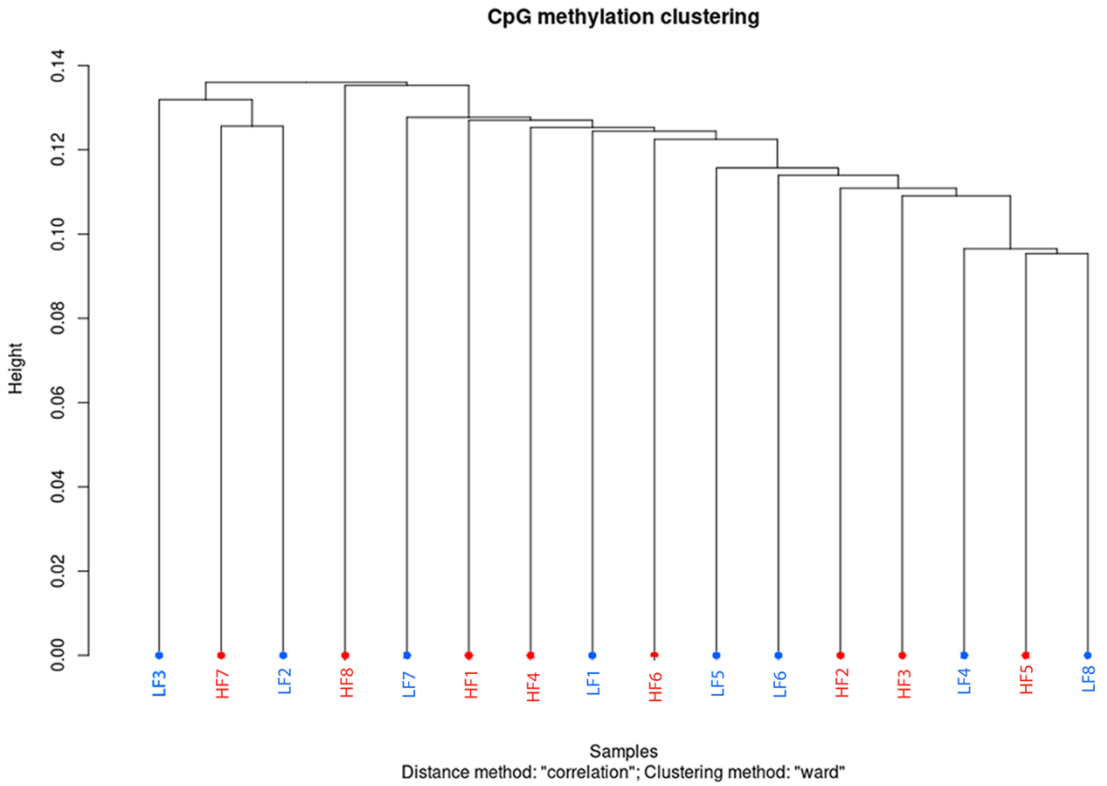

Figure 2. Hierarchical clustering of samples based on their CpG_{5x} methylation levels in sperm DNA (*n* = 16). Letters LF and HF prefixed by numbers indicates bulls with low and high NR56, respectively.

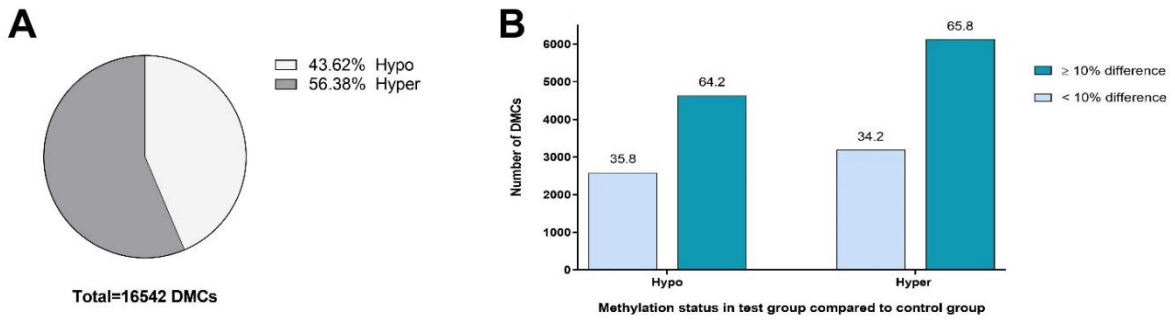

Figure 3. Numbers and degree of differentially methylated cytosines (DMCs) identified in the sperm DNA methylome between LF bulls (test) and HF bulls (control). A) Total number of significant DMCs indicating that 56% are hypermethylated in the test group compared to the control group. B) Levels of hypo- and hypermethylated cytosines in the test group compared to the control group. The numbers on the bars indicate the percentage in each respective bin. $DMCs_{<10\%}$ indicate cytosines with less than 10% difference in methylation level, while $DMRs_{\geq 10\%}$ indicate cytosines having equal and over 10% difference in methylation.

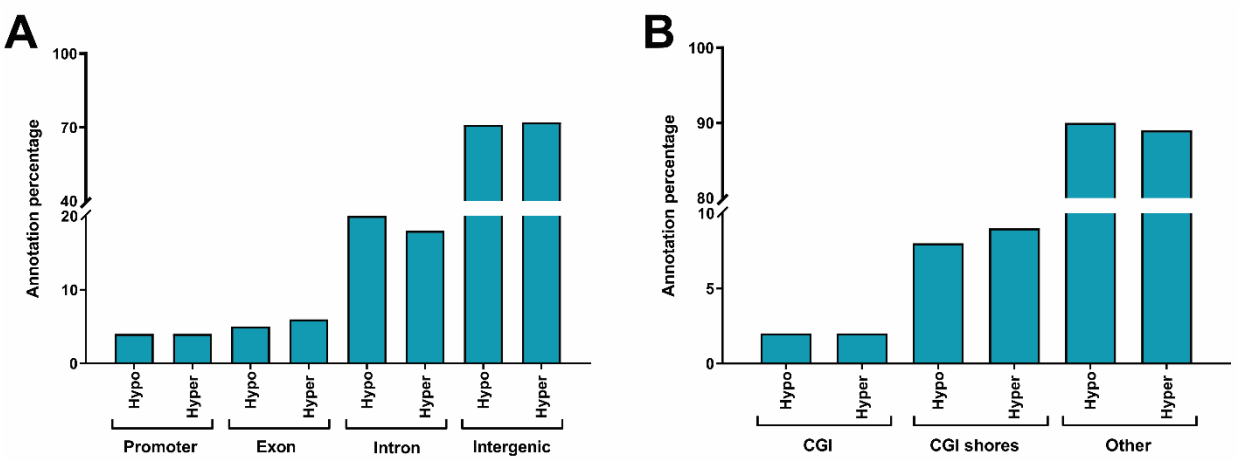

Figure 4. The distribution of filtered differentially methylated cytosines (DMCs) (methylation cut-off 10% and *q*-value < 0.05) in the *bosTau9* genome. A) Annotation of the hypo- and hypermethylated cytosines with gene features. B) Annotation of the hypo- and hypermethylated cytosines with CpG features. Hypo: hypomethylated cytosines; Hyper: hypermethylated cytosines; CGI: CpG island.

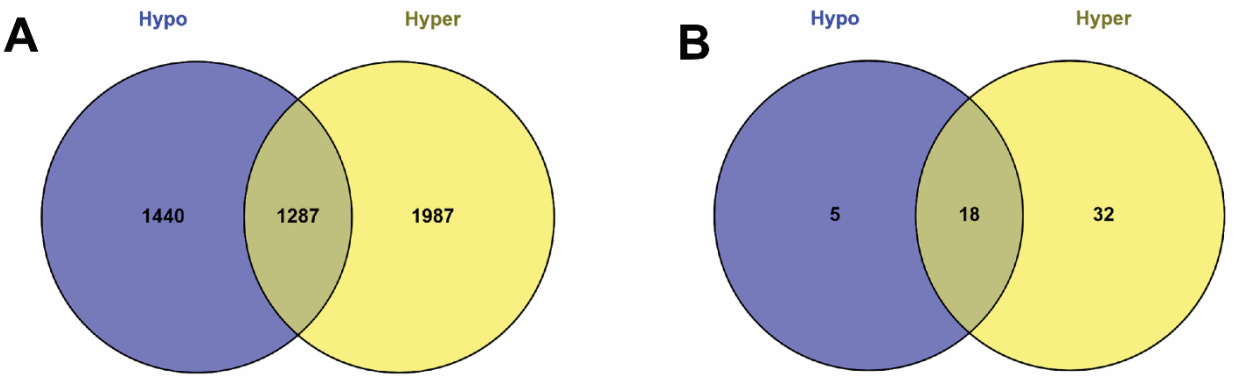

Figure 5. A) Annotation of the filtered $DMCs_{\geq 10\%}$ to the nearest TSSs. Numbers indicating the closest unique TSSs (duplicated TSSs removed) to $DMCs_{\geq 10\%}$ in hypomethylation (Hypo) and hypermethylation (Hyper) groups. B) number of significantly associated pathways to filtered $DMCs_{\geq 10\%}$ in hypomethylation (Hypo) and hypermethylation (Hyper) groups.

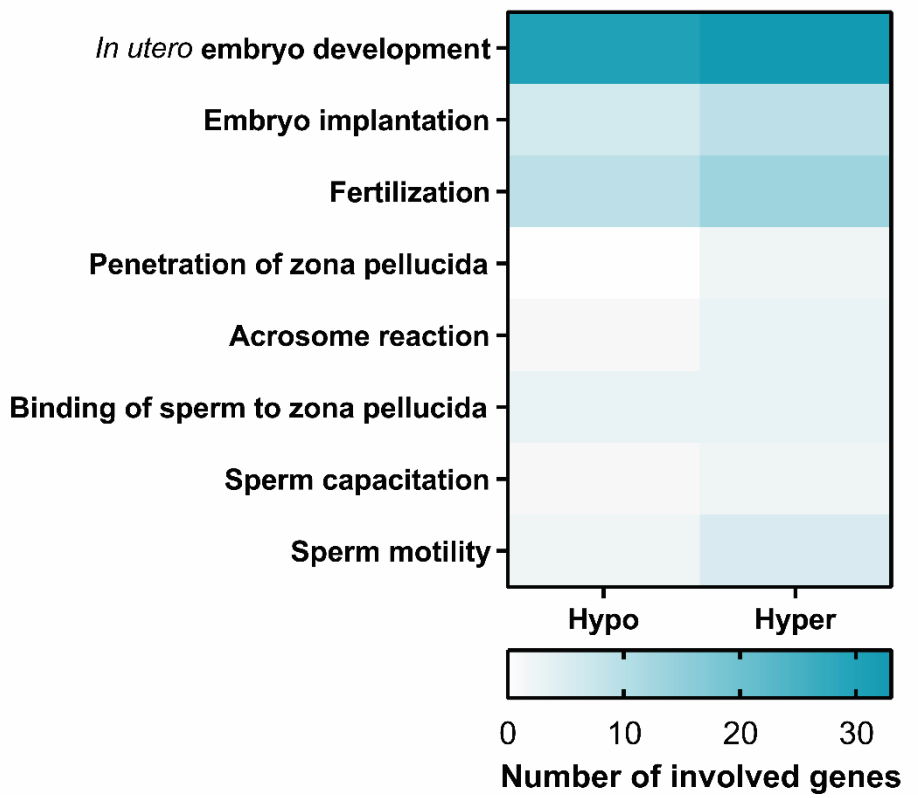

Figure 6. Number of genes representing different biological processes related to fertility, for which their TSSs were annotated with the $DMCs_{\geq 10\%}$ in hypomethylated (Hypo) and hypermethylated (Hyper) groups.

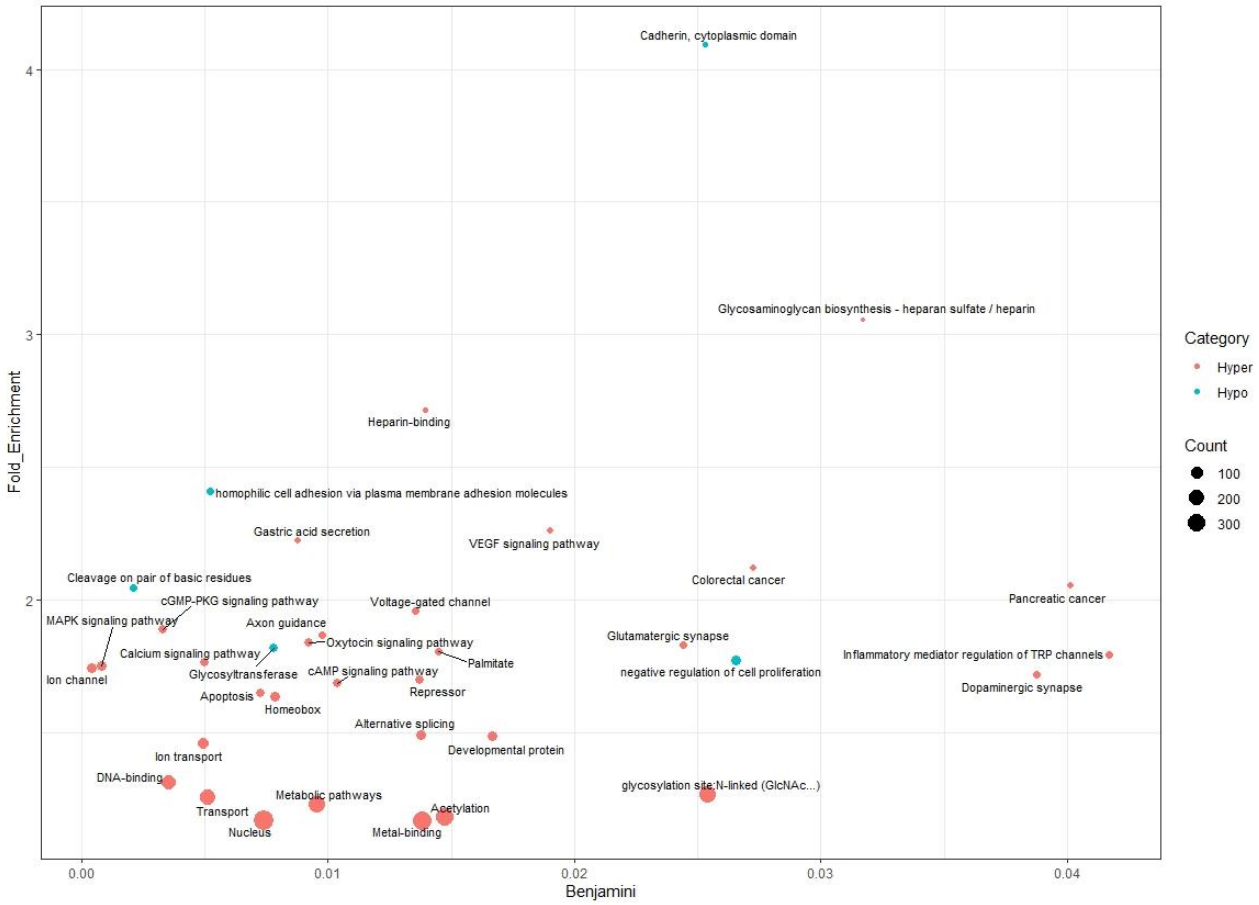

Figure 7. Exclusively identified pathways for annotated genes to DMCs≥10%. Pathways are plotted in function of their Benjamini corrected *p*-value (x-axis) and fold enrichment (y-axis). Gene count size key shows the number of genes involved in each pathway. Hypo; hypomethylated cytosines (referring to TSSs annotated with hypomethylated cytosines in test group), Hyper; hypermethylated cytosines (referring to TSSs annotated to hypermethylated cytosines in test group).

Supplementary material

Table S1. Correlation analysis based on methylation value of CpG 5x in each sample. Numbers in each cell represent the pairwise Pearson's correlation scores. LF: low fertile bulls, HF: high fertile bulls.

	HF1	**HF2**	**HF3**	**HF4**	**HF5**	**HF6**	**HF7**	**HF8**	**LF1**	**LF2**	**LF3**	**LF4**	**LF5**	**LF6**	**LF7**
HF1															
HF2	0.949														
HF3	0.949	0.953													
HF4	0.947	0.947	0.947												
HF5	0.953	0.954	0.957	0.952											
HF6	0.948	0.950	0.949	0.948	0.953										
HF7	0.945	0.952	0.948	0.944	0.951	0.946									
HF8	0.944	0.948	0.946	0.944	0.950	0.944	0.943								
LF1	0.946	0.949	0.950	0.946	0.953	0.947	0.945	0.944							
LF2	0.947	0.953	0.950	0.945	0.952	0.948	0.948	0.944	0.946						
LF3	0.946	0.949	0.947	0.944	0.950	0.947	0.945	0.942	0.943	0.946					
LF4	0.952	0.956	0.954	0.953	0.959	0.954	0.952	0.952	0.951	0.952	0.951				
LF5	0.949	0.953	0.951	0.947	0.955	0.951	0.948	0.946	0.947	0.949	0.949	0.955			
LF6	0.948	0.951	0.950	0.949	0.956	0.949	0.944	0.947	0.950	0.947	0.945	0.954	0.951		
LF7	0.946	0.952	0.950	0.944	0.952	0.945	0.946	0.945	0.946	0.947	0.946	0.952	0.950	0.949	
LF8	0.951	0.956	0.955	0.953	0.959	0.953	0.952	0.951	0.954	0.952	0.950	0.959	0.954	0.955	0.952

Table S2. List of genes (corresponding GenBank accession IDs to annotated TSSs) involved in different biological processes relevant to field fertility in bulls. Differentially methylated cytosines with over 10% methylation difference were annotated with TSSs.

Hypermethylation		**Hypomethylation**	
Gene accession no.	**Biological process**	**Gene accession no.**	**Biological process**
NM_001014389	Fertilization	NM_001007814	Fertilization
NM_001242571	Fertilization	NM_001076209	Fertilization
NM_001015572	Fertilization	NM_001077869	Fertilization
NM_001076209	Fertilization	NM_001191264	Fertilization
NM_001031750	Fertilization	NM_001099105	Fertilization
NM_001105017	Fertilization	NM_174402	Fertilization
NM_001105617	Fertilization	NM_174817	Fertilization
NM_001099105	Fertilization	NM_001192910	Fertilization
NM_174402	Fertilization	NM_001104960	Fertilization
NM_001077869	Fertilization		
NM_176646	Fertilization		
NM_001105007	Fertilization		
NM_001166572	Fertilization		
NM_001046235	Sperm motility	NM_001324544	Sperm motility
NM_001192253	Sperm motility	NM_001193225	Sperm motility
NM_001075900	Sperm motility		
NM_001037320	Sperm motility		
NM_174199	Sperm motility		
NM_174018	Sperm capacitation	NM_174018	Sperm capacitation
NM_001075900	Sperm capacitation		
NM_001038611	Binding of sperm to ZP	NM_001076357	Binding of sperm to ZP
NM_173975	Binding of sperm to ZP	NM_001034595	Binding of sperm to ZP
NM_001076491	Binding of sperm to ZP	NM_001038086	Binding of sperm to ZP
NM_001075310	In utero development	NM_001192401	In utero development

NM_001038072	In utero development	NM_001076804	In utero development
NM_001076804	In utero development	NM_001007814	In utero development
NM_001192432	In utero development	NM_001076209	In utero development
NM_001105493	In utero development	NM_001077869	In utero development
NM_001046218	In utero development	NM_001193211	In utero development
NM_001205805	In utero development	NM_001191126	In utero development
NM_001076209	In utero development	NM_177504	In utero development
NM_001102354	In utero development	NM_001192432	In utero development
NM_001077921	In utero development	NM_001105493	In utero development
NM_001034414	In utero development	NM_001205805	In utero development
NM_181010	In utero development	NM_001193247	In utero development
NM_174308	In utero development	NM_001191418	In utero development
NM_001038670	In utero development	NM_001102354	In utero development
NM_001206019	In utero development	NM_001076797	In utero development
NM_001110207	In utero development	NM_001178103	In utero development
NM_001205310	In utero development	NM_181010	In utero development
NM_001083689	In utero development	NM_001206019	In utero development
NM_174068	In utero development	NM_001205310	In utero development
NM_001206854	In utero development	NM_001083689	In utero development
NM_001205886	In utero development	NM_174068	In utero development
NM_174798	In utero development	NM_001105424	In utero development
NM_181037	In utero development	NM_174798	In utero development
NM_001077869	In utero development	NM_174834	In utero development
NM_001097991	In utero development	NM_181037	In utero development
NM_174425	In utero development	NM_001193255	In utero development
NM_001144106	In utero development	NM_001304343	In utero development
NM_001113261	In utero development	NM_174734	In utero development
NM_001077897	In utero development	NM_001205893	In utero development
NM_001103221	In utero development	NM_001191145	In utero development
NM_001304343	In utero development		

NM_001076394	In utero development		
NM_001159566	In utero development		
NM_001014389	Embryo implantation	NM_001007814	Embryo implantation
NM_001242571	Embryo implantation	NM_001077869	Embryo implantation
NM_001075324	Embryo implantation	NM_001304343	Embryo implantation
NM_173931	Embryo implantation	NM_001191264	Embryo implantation
NM_001077869	Embryo implantation	NM_173931	Embryo implantation
NM_174444	Embryo implantation	NM_174615	Embryo implantation
NM_174445	Embryo implantation		
NM_001304343	Embryo implantation		
NM_174615	Embryo implantation		
NM_001192985	Penetration of ZP		
NM_174200	Penetration of ZP		
NM_174321	Acrosome reaction	NM_174817	Acrosome reaction
NM_174200	Acrosome reaction		
NM_173975	Acrosome reaction		

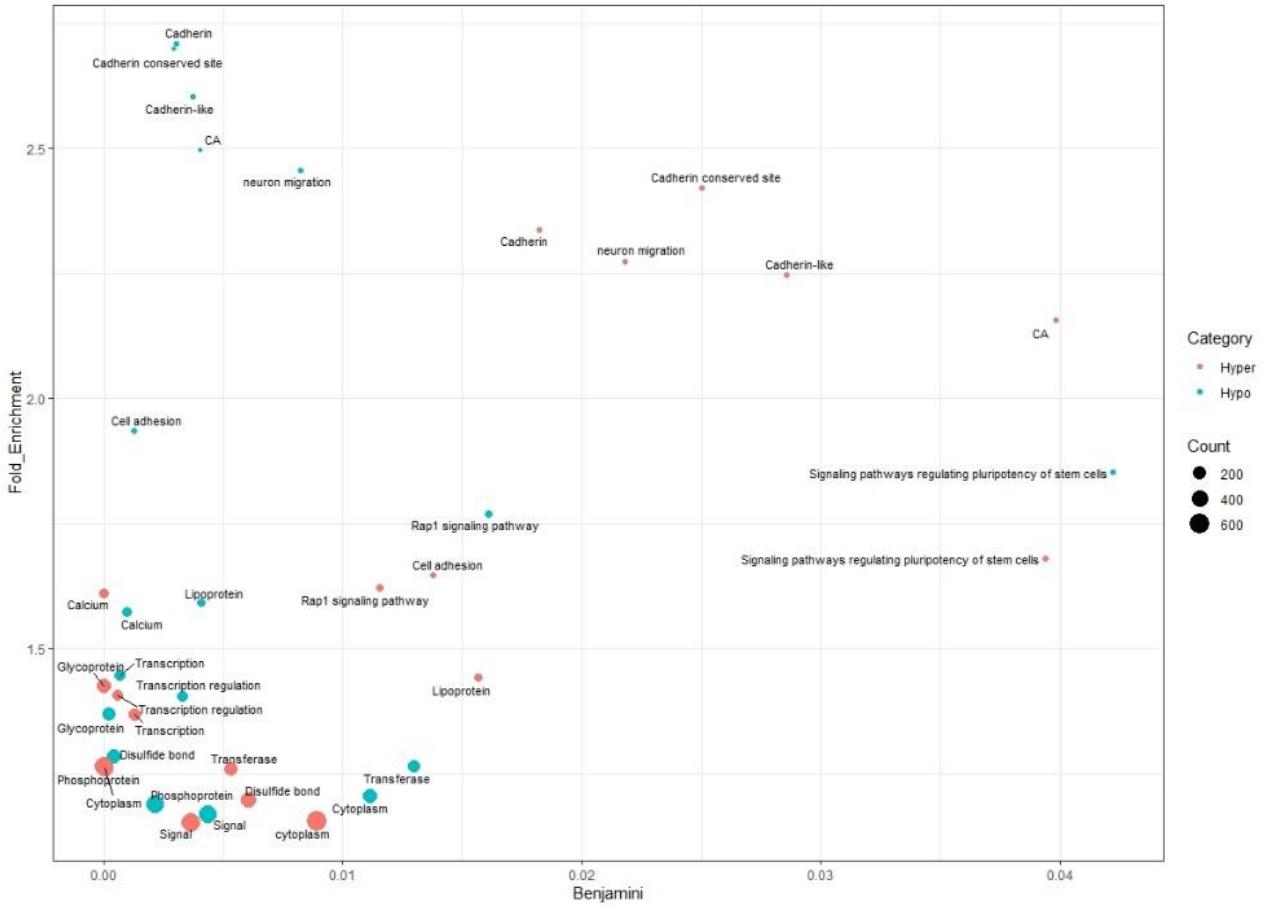

Figure S1. Mutually identified pathways associated with hypo- and hypermethylated cytosines. Pathways are plotted in function of their Benjamini corrected *p-value* (x-axis) and fold enrichment (y-axis). Gene count size key shows the number of genes involved in that particular pathway. Hypo; hypomethylated regions (referring to TSSs annotated with hypomethylated regions in test group), Hyper; hypermethylated regions (referring to TSSs annotated to hypermethylated regions in test group).