Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Production Animal Clinical Sciences

Reproductive potential and quality of SpermVital semen used for artificial insemination in cattle

Fruktbarhet og sædkvalitet ved bruk av SpermVital-sæd til kunstig inseminering i storfe

Halldor Felde Berg

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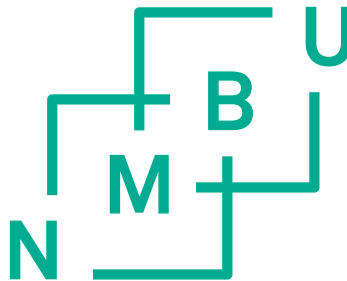
Philosophiae Doctor (PhD) Thesis

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Contents

Abbreviations

AI	Artificial insemination	IO56	Ikke-omløp etter 56 dager
AIL	Acrosome intact live	IVF	In vitro fertilization
AID	Acrosome intact dead	KS	Kunstig sædoverføring
ARD	Acrosome reacted dead	LIN	Linearity
ARL	Acrosome reacted live	LH	Luteinizing hormone
ALH	Amplitude of lateral head displacement	LN_2	Liquid nitrogen
AO	Acridine orange	Nm	Nanometer
ATP	Adenosine triphosphate	NDHRS	Norwegian Dairy Herd Recording System
ATPlive	ATP content of live sperm cells	NR56	56-day non-return
B	Biladyl (processed semen, standard)	NRF	Norsk Rødt Fe
BCF	Beat-cross frequency	OAM	Outer acrosomal membrane
BSP	Binder of sperm proteins	OXPHOS	Oxidative phosphorylation
Ca^{2+}	Calcium ion	P4	Progesterone
CASA	Computer-assisted sperm analysis	pCLE	Probe based confocal laser endomicroscopy
CL	Corpus luteum	$PGF_{2\alpha}$	prostaglandin F2α
CO_2	Carbon dioxide	PI	Propidium iodide
CPA	Cryoprotective agent	PMT	Photo multiplier tube
DFI	DNA fragmentation index	PNA	Arachis hypogea (peanut) agglutinin
DNA	Deoxyribonucleic acid	PSA	Pisum sativum agglutinin
dUTP	Deoxyuridine triphosphate	RLU	Relative luminescence unit
EMA	Ethidium monoazide	SAS	Statistical Analysis System
Em	Emission	SCSA	Sperm Chromatin Structure Assay
EY	Egg yolk	SSC	Side Scatter
Ex	Excitation	STR	Straightness
FL	Fluorescence detector	SV	SpermVital (processed semen, immobilized)
FSC	Forward Scatter	T	Triladyl (processed semen, standard)
FSH	Follicle stimulating hormone	TdT	Terminal deoxynucleotidyl transferase
FITC	Fluorescein isothiocyanate	T0	Post-thaw
GLM	Generalized Linear Model	T3	3 hours after thawing
GnRH	Gonadotropin releasing hormone	TUNEL	TdT-mediated dUTP nick end labelling
GS	Genomic selection	VAP	Average path velocity
GTE	Genital tract endoscopy	VCL	Curvilinear velocity
HCO^{3-}	Bicarbonate	VSL	Straight-line velocity
IAM	Inner acrosomal membrane	WOB	Wobble
IGF	Insulin-like growth factor	ZP	Zona pellucida

List of papers

Paper I. Berg, H. F., Kommisrud, E., Bai, G., Gaustad, E. R., Klinkenberg, G., Standerholen, F. B., Thorkildsen, L. T., Waterhouse, K. E., Ropstad, E., Heringstad, B., Alm-Kristiansen, A. H. (2018). Comparison of sperm adenosine triphosphate content, motility and fertility of immobilized and conventionally cryopreserved Norwegian Red bull semen. *Theriogenology*, 121, 181-187. (Study I)

Paper II. Berg, H. F., Kommisrud, E., Heringstad B., Gaustad, E. R., Bai, G., Stenseth, E. B., Witschi, U., Ropstad, E., Alm-Kristiansen, A. H. (2020). Post-thaw quality of spermatozoa immobilized in alginate gel before cryopreservation. Submitted to *Reproduction in Domestic Animals.* (Study II)

Paper III. Berg, H. F., Heringstad, B., Alm-Kristiansen, A. H., Kvale, V. G., Dragset, K. I., Waldmann, A., Ropstad, E., Kommisrud, E. (2019). Ovarian follicular response to estrous synchronization and induction of ovulation in Norwegian Red cattle. Submitted to *Acta Veterinaria Scandinavica*. (Study III)

Paper IV. Berg, H. F., Spang, H. C. L., Heringstad, B., Ropstad, E., Alm-Kristiansen, A. H., Kommisrud, E. (2020). Studies of gel with immobilized semen by intrauterine endoscopy post-artificial insemination. *Reproduction in Domestic Animals*, 00, 1–4. (Study IV)

Summary

The outcome of artificial insemination (AI) depends on multiple factors. These include the sperm cell quality, the method of semen processing, and the genital tract environment of the inseminated female. Successful AI in cattle also requires effective reproductive management, particularly concerning the detection of estrus and the correct timing of insemination relative to the occurrence of estrus and ovulation. Knowledge concerning the fields of male and female reproduction and reproductive management that can lead to an increase in reproductive success is of great interest to breeding organizations and cattle farmers throughout the world.

SpermVital AS is a subsidiary company of the Norwegian breeding and AI association, Geno, that has developed a semen processing technology in which sperm cells are immobilized in an alginate gel extender before cryopreservation. The SpermVital (SV) immobilization technology has been shown to have cryoprotective properties and results in an improvement in post-thaw sperm cell quality and lifespan compared with conventionally processed semen. The sperm cells are immobilized in the alginate gel to facilitate the gradual release of spermatozoa over a prolonged period *in utero* following AI. By extending the time in which viable sperm cells are present *in utero*, the SV technology could make the timing of AI more flexible relative to the time of ovulation and increase the probability of fertilization. As a result, the use of SV semen can contribute to greater room for adaptability in AI routines and increased cost efficiency through improved reproductive performance.

Similar fertility rates (non-return rate after 56 days, NR56) have been reported for Norwegian Red cattle following AI with SV semen compared with standard processed semen. However, recent improvements in SV technology have led to enhanced SV semen quality. Until now, SV semen has been produced with elevated sperm cell concentrations, but recent improvements in SV sperm cell quality could allow for reductions in the number of sperm cells per AI dose without compromising fertility.

In this thesis an AI trial with Norwegian Red cattle was conducted to examine the reproductive performance of SV semen doses containing fewer sperm cells. Competitive fertility rates (NR56) were shown after AIs with SV semen compared with those achieved with standard processed semen. We demonstrated that the sperm quality measured as motility, viability and ATP levels in SV processed semen was superior to that in standard processed semen, possibly enabling sperm cell numbers being reduced per AI dose without affecting reproductive performance.

The SV technology has been exported to several countries and used for AI in different cattle breeds. In this thesis, semen from different cattle breeds was characterized by *in vitro* sperm parameters and showed enhanced quality for SV semen compared with standard processed semen following incubation. These findings indicate that the SV technology is suitable for cryopreservation of semen from different cattle breeds.

In Norway and globally, there is a tendency towards an increase in herd size, making reproductive management more challenging. As a result, estrous synchronization has been a commonly implemented to improve reproductive performance. However, investigation of the response of Norwegian Red animals to such treatment has been limited. In this thesis, the use of a double-PGF$_{2\alpha}$/GnRH protocol in Norwegian Red heifers and cows provided no indications that animals of this breed respond differently to such synchronization than cattle of other breeds, despite previous findings indicating breed differences regarding estrous expression. Our findings provide some evidence that future research involving the synchronization of estrus and ovulation in combination with AI (timed AI) is applicable in Norwegian Red.

The assumed advantage of the SV immobilization technology is the increased flexibility regarding timing of AI relative to ovulation. Using intrauterine *in vivo* endoscopy, in this thesis we showed that SV gel can be retrieved *in vivo* 24 hours after insemination. Uterine incubation of the semen *ex vivo* demonstrated that high motility and viability were maintained overnight. The results indicate prolonged release of immobilized spermatozoa expressing high reproductive potential, possibly providing an extended window of time for successful AI in cattle.

The findings of the current thesis have a direct practical and economic impact concerning the production and use of SV semen. In particular, we have demonstrated that sperm cells processed by the SV technology are of high quality, and that AIs with SV semen containing fewer sperm cells result in competitive fertility.

Sammendrag

Utfallet av kunstig sædoverføring (KS) avhenger av flere faktorer, blant annet sædkvalitet, metoden for sædprosessering og miljøet i kjønnsorganet hos det inseminerte hunndyret. Vellykket KS hos storfe krever også riktig brunstobservasjon for å kunne anslå riktig tidspunkt for inseminering i forhold til brunst og eggløsning. Økt kunnskap angående fruktbarhet som kan føre til en effektivisering i styringen av reproduksjon hos storfe er av interesse for avlsorganisasjoner og storfebønder verden rundt.

SpermVital AS er et datterselskap av det norske avlssamvirke, Geno SA, som har utviklet en prosesseringsteknologi for sæd der sædceller er immobilisert i en alginatgel før kryokonservering. Det er vist at SpermVital (SV) teknologien bidrar til beskyttelse av sædcellene mot frostskader som kan oppstå ved nedfrysning, noe som er vist å medføre en bedring i sædcellekvalitet og lengre levetid etter tining sammenlignet med konvensjonelt prosessert sæd. Immobiliseringen av sædcellene i alginatgel muliggjør en gradvis frigivelse av sædceller i uterus over lengre tid etter KS. Ved å forlenge tiden der levedyktige sædceller er til stede i uterus, kan SV-teknologien gjøre tidspunktet for KS mer fleksibelt i forhold til eggløsningstiden og øke sannsynligheten for befruktning. Følgelig kan bruk av SV-sæd bidra til økt fleksibilitet i forbindelse med KS og økt kostnadseffektivitet gjennom forbedret fruktbarhet.

Lik fruktbarhet (ikke-omløp etter 56 dager, IO56) er vist etter KS av Norsk Rødt Fe (NRF) med SV-sæd sammenlignet med konvensjonelt prosessert sæd. I den senere tiden er det påvist forbedringer i sædkvalitet i SV-prosessert sæd. Fram til i dag har SV-sæden blitt produsert med en forhøyet sædcelle-konsentrasjon. Imidlertid vil den påviste forbedringen i sædkvalitet kunne muliggjøre en reduksjon i antall sædceller per KS-dose uten at det går ut over fruktbarheten.

Vi har gjennomført et insemineringsforsøk med NRF for å undersøke fruktbarhet ved KS med SV-sæd med redusert spermiedose. Insemineringsforsøket viste lik fruktbarhet (IO56) mellom SV-sæd og standard prosessert sæd. *In vitro* undersøkelser av sæden viste bedre sædkvalitet målt ved motilitet, viabilitet og ATP innhold i SV-sæd sammenlignet med standard sæd, noe som kan forklare hvorfor KS med SV-sæd med redusert antall sædceller kunne gi like god fruktbarhet som kontrollsæden.

SV-teknologien eksporteres til flere land og brukes til KS i forskjellige storfe raser. Våre undersøkelser av sæd fra forskjellige storferaser med henblikk på ulike *in vitro* parametere viste

høyere kvalitet i SV-sæd sammenlignet med standard sæd etter inkubering. Funnene tyder på at SV-teknologien er egnet til bruk sammen med kryokonservering av sæd fra forskjellige raser.

I Norge og globalt sees det en økning i besetningsstørrelse, noe som kan være utfordrende i forbindelse med styring av fruktbarhet. Som følge av denne økningen, har det blitt mer vanlig å synkronisere dyrenes brunst for å oppnå god fruktbarhet. Foreliggende litteratur angående responsen på slik behandling hos dyr av NRF-rasen er imidlertid begrenset. Vi synkroniserte en gruppe NRF kviger med en dobbelt-$PGF_{2\alpha}$/GnRH-protokoll uten å finne indikasjoner på at dyr av NRF-rasen reagerer annerledes på slik synkronisering enn storfe fra andre raser, til tross for funn som viser raseforskjeller angående brunstuttrykk. Resultatene gir informasjon om det optimale tidspunktet for KS etter behandling med GnRH. Denne informasjon er også av stor verdi for fremtidig forskning, f.eks. i utformingen av kliniske KS-studier som undersøker ulike varianter av prosessert sæd og variasjoner i antall spermier per dose.

SV-teknologien antas å gi økt fleksibilitet når det gjelder tidspunktet for KS i forhold til ovulering. Ved intrauterin endoskopi viste vi at SV-gel kan påvises *in vivo* 24 timer etter inseminering. God spermiemotilitet og viabilitet ble påvist i SV prosesserte spermier etter inkubering over natt av sæd i uterus *ex vivo*. Resultatene indikerer en langvarig frigjøring av immobiliserte sædceller av god kvalitet, noe som kan muliggjøre vellykket KS over et utvidet tidsvindu hos storfe.

Funnene som ble gjort i dette prosjektet har en direkte praktisk og økonomisk innvirkning på produksjon og bruk av SV-sæd. Vi har vist at spermier som er prosessert med SV-teknologien innehar høy kvalitet, og at inseminering med SV-sæd som inneholder reduserte spermiekonsentrasjoner resulterer i konkurransedyktig fruktbarhetsresultater.

1. Introduction

1.1. Background

Artificial insemination (AI) is the deliberate introduction of sperm cells into the female reproductive tract to achieve pregnancy through *in vivo* fertilization, without the act of sexual intercourse. Leeuwenhoek (1678) was the first to observe sperm cells and, one century later, the first successful insemination was performed in a dog by Spallanzani (Foote, 2002). AI facilitates accelerated genetic improvement by the dissemination of desired male genes over a large geographical area, producing multiple offspring from sires of superior genetic value. The use of AI contributes to increased safety during the handling of animals and improved biosecurity, potentially resulting in more cost-effective management. SpermVital AS is a subsidiary company of the Norwegian breeding and AI association, Geno, which has developed a semen processing technology in which sperm cells are immobilized in an alginate gel extender before cryopreservation (Kommisrud et al., 2008). The SpermVital® (SV) immobilization technology has been shown to have cryoprotective properties, resulting in an increase in both sperm cell quality post-thawing and an extended lifespan compared with conventionally processed semen (Alm-Kristiansen et al., 2018a). The sperm cells are immobilized in the alginate gel to facilitate the gradual release of spermatozoa over a prolonged period *in utero* following AI. By prolonging the period in which viable sperm cells are present *in utero*, the SV technology has the potential to enable the timing of AI to be more adaptable relative to the time of ovulation, and thereby increase the probability of fertilization. The use of SV semen can therefore contribute to greater flexibility in AI routines and can result in increased cost efficiency through improved reproductive performance.

Following decades of effective selection for high milk yield in Holstein cattle, the most common breed of dairy cattle, a decline in reproductive performance was reported (Pennington et al., 1986, Dobson et al., 2008). The opposite trend has been observed in Norwegian Red cattle, where the breeding program over the past 50 years has prioritized fertility, alongside selection for improved health and milk production (Refsdal, 2007a). The high genetic potential concerning health and fertility in Norwegian Red has led to an increasing global interest in using Norwegian Red for crossbreeding (Heins et al., 2006, Cartwright et al., 2011). In addition to documented differences in fecundity, there are also marked breed differences between Holstein and Norwegian Red cattle in the expression of estrus, the latter demonstrating longer estrus and more frequent signs of estrus (Sveberg et al., 2015). Fertility rates (NR56) for Norwegian Red cattle following AI with SV semen have been reported to be similar to those

obtained with standard processed semen (Standerholen et al., 2015). However, recent advances in the SV technology have resulted in improved SV semen quality (Alm-Kristiansen et al., 2018a, Alm-Kristiansen et al., 2018b). In addition, promising fertility results (pregnancy rate) following estrous synchronization and early AIs in heifers with SV semen have been reported (Alm-Kristiansen et al., 2017). Nevertheless, until now, SV semen for AI has contained elevated sperm cell concentrations; i.e. $>20 \times 10^6$ sperm cells per dose. The recent improvements in SV sperm cell quality could enable the number of sperm cells per AI dose being reduced without compromising fertility.

Developments in SV technology has created the need for further knowledge. In the current thesis, *in vitro* semen quality, as well as reproductive performance, was investigated in cattle inseminated with SV semen. Additionally, the reproductive characteristics of Norwegian Red cattle were studied following estrous synchronization.

1.2. Artificial insemination in dairy cattle

AI is the predominant method of breeding in dairy herds worldwide; in Norway, nearly 90 % of all breedings are by AI. Geno breeding and AI association is the main actor in cattle breeding in Norway, extending internationally through cooperation with leading breeding companies in North America and Europe. At present, more than half of the semen that is produced in Norway by Geno is exported (Geno, 2018). Every AI performed in Norway with semen from Geno is reported to the company′s AI recording database, where data are validated and then transferred to the Norwegian Dairy Herd Recording System (NDHRS). The NDHRS is a centralized nationwide system; it consists of milk recordings, an AI database, a register of health recordings for individual animals and herds, results of laboratory milk analyses, and slaughterhouse recordings, i.a. (Espetvedt et al., 2013). Approximately 90 % of Norwegian dairy cattle are registered in the NDHRS.

The Geno database also contains estimated breeding values for Norwegian Red sires. Recently, Geno converted from the traditional progeny testing system, based on phenotypic evaluation of progeny, to genomic selection (GS). In the GS system, young bulls are selected for further breeding based on genomic breeding values. The fertility data used in this thesis were acquired before conversion to the GS system and are therefore based on the progeny testing system, in which approximately 300 bull calves were brought to Geno′s performance test station per year to test specific parameters including growth rate and semen quality (Geno, 2016). Each year,

around 120 of these animals are selected as test sires to produce semen from about the age of 15 months. Each bull sires 200-300 daughters, and information regarding their performance is used to predict breeding values for a pool of bulls from which approximately 10 "elite sires" are selected annually. In the Norwegian Red total merit index, milk production, fertility, udder quality, and health are emphasized relative to other traits.

1.3. Spermatogenesis

Spermatogenesis is the production of sperm cells that takes place in the seminiferous tubules of the testis, a process that takes 54 – 61 days in the bull. Spermatogenesis is regulated through the hypothalamic-pituitary-testicular axis, which functions by interactions between Leydig cells, Sertoli cells, and germ cells in the testis (Senger, 2015). Spermatogenesis consists of spermacytogenesis, by the proliferation of diploid spermatogonia through mitotic divisions into new spermatogonia and primary spermatocytes (Figure 1). Following meiosis and differentiation (spermiogenesis), the haploid secondary spermatocytes are formed from the diploid spermatogonia, which, after a second meiotic division, develop into spermatids. Apart from halving the number of chromosomes, meiosis contributes to genetic diversity due to the possibility of recombination. Spermiogenesis is the last step of spermatogenesis and consists of the morphological and functional differentiation of spermatids into spermatozoa. Differentiation of spermatids results in the elongation of the sperm cells, development of the acrosome membrane, condensation of the nucleus, and shedding of the cytoplasmic residue (Senger, 2015). Finally, spermiation takes place as spermatozoa are discharged into the seminiferous tubules, followed by transport of the sperm cells to the epididymis, where they are stored in the cauda until ejaculation.
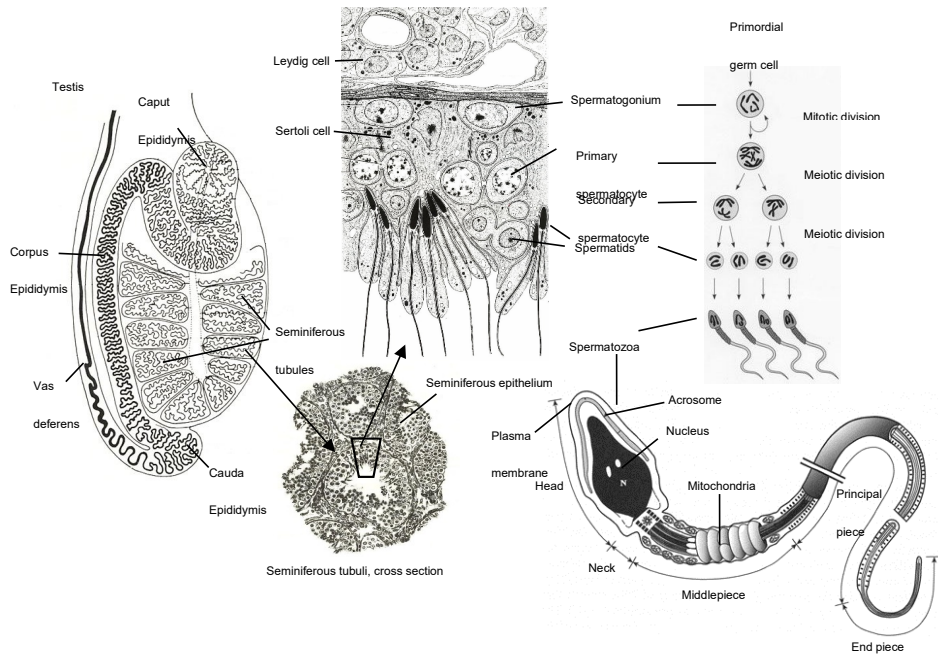
Figure 1. *The production of spermatozoa by spermatogenesis in the seminiferous tubules of the testis and sperm structure. Spermatogenesis is a synchronized process under endocrine regulation in the hypothalamic-pituitary-testis axis, which functions through interactions between Leydig cells, Sertoli, cells and germ cells. Haploid spermatozoa are formed from diploid spermatogonia by cell proliferation (spermacytogenesis), meiosis, and differentiation (spermiogenesis). After the release of spermatozoa from the seminiferous tubules (spermiation), they are transferred to the epididymis for further maturation while passing through the caput and corpus epididymis. The spermatozoa are stored in the cauda epididymis until ejaculation. The mature sperm cell consists of the head, neck, and tail, which is divided into three segments: the middle piece, principal piece, and end piece. The head contains the nucleus, which is partially surrounded by the acrosome and plasmalemma, whereas the tail contains the mitochondria. (Modified from Senger (1997), Hafez and hafez (2000), and Toshimori (2009).*

1.4. Preservation of bovine semen

Sperm cells are not adapted to long-term *in vitro* storage. *In vivo*, spermatozoa exist in a physiological environment regarding temperature, osmolality, and pH. Consequently, the preservation of semen for short-term and long-term *ex vivo* storage presents multiple challenges, as previously reported (Vishwanath and Shannon, 2000). The spermatozoa plasma membrane is the principal site of sublethal and functional damage following the preservation of semen (Holt, 2000a), as this is the cell's barrier against the surrounding environment. The nucleus and flagellum are other parts of the sperm cell that are prone to damage, and are therefore commonly used to evaluate fertilization potential. To prevent damage to the cell during processing and storage, semen extenders are used that have been designed to provide a

stable environment and eliminate bacterial growth, stabilize pH and osmotic pressure, and offer nutritional availability (Vishwanath and Shannon, 2000). The discovery that egg yolk (EY) could be used as a cryoprotective agent in semen processing by Phillips et al. (1939) was a breakthrough in the development of commercial semen processing technology (Phillips, 1939). EY functions as a source of lipoprotein to prevent cold shock during semen processing. Other substances of high molecular weight, such as milk, soybean lecithin, and coconut oil, have been demonstrated to possess similar properties that are suitable for the protection of sperm cells (Vishwanath and Shannon, 2000, Layek et al., 2016, Raheja et al., 2018). The use of semen extenders provides the opportunity to split ejaculates into multiple insemination doses for preservation; either short-term preservation in liquid form or long-term preservation by cryopreservation by submerging in liquid nitrogen (LN_2, -196 ºC). In the field, semen that has been processed for commercial purposes is usually stored in insulated aluminum containers for mobile use (10-40 L LN_2 capacity), divided into canisters and goblets that are submerged in LN_2. Each goblet holds semen doses preserved in straws, most commonly 0.25 mL Cassou mini-straws (IMV Technologies, L'Aigle, France) that are suitable for use with both frozen and liquid semen. The high cryotolerance of bovine semen in comparison with semen from other domestic species, such as porcine semen (Yeste et al., 2017), has resulted in the widespread use of cryopreservation in cattle breeding. The long shelf life and high biosecurity following freezing of semen have contributed to the widespread use of cryopreservation for bovine semen. However, in geographically restricted areas with a high density of dairy cattle, such as in New Zealand and Ireland, the use of liquid bull semen is common. The highly effective production of liquid semen is particularly relevant during seasonal breeding peaks and times of high demand for semen doses (Vishwanath and Shannon, 2000, Murphy et al., 2018a). The effective production of liquid semen is especially favorable for young, genomically selected bulls with high breeding values, as their ejaculates are of a lower volume than those of more mature bulls (Brito et al., 2002). The typical dose of liquid semen contains $5x10^6$ sperm cells compared with $15x10^6$ sperm cells for a typical frozen-thawed dose of semen (Vishwanath et al., 1996).

1.4.1. Cryopreservation of bovine semen and cryodamage

In the advent of modern reproduction science, vitrification was introduced as a method for semen preservation (Luyet and Hodapp, 1938). This method involves the transition of liquid semen to a non crystalline, glassy state by rapid freezing (Isachenko et al., 2004). The discovery of glycerol as a protectant of sperm cells during cryopreservation following slow freezing of

semen (Polge et al., 1949), combined with increased knowledge about cell responses to other cryoprotective agents (CPA), provided the entry into the widespread commercial use of AI (Lonergan, 2018). The high tolerance of bovine sperm in glycerol compared with that of sperm of other species, along with the low number of sperm cells required to achieve fertilization in cattle, are the main reasons for the great success of AI in the cattle industry (Holt, 2000b). Due to the arresting effect of subzero temperatures on the metabolic activity of the sperm cell and the discovery of glycerol as a CPA, solid CO_2 was initially used to preserve and store bovine semen at -79 ºC. In the 1950s it was shown that storage at -79 ºC did not completely arrest biological activity, and this was rectified by using LN_2 (-196 ºC). The development of containers with improved insulation (Foote, 2002) enabled the use of LN_2 in the field. In the process of equilibrium cooling (slow cooling) of cells for cryopreservation, a CPA is added to the cells in suspension before cooling to approximately 0 ºC. The semen is then brought to -80 ºC by slow cooling, at a rate of 5-10 ºC per minute, and thereafter rapidly frozen in LN_2 to -196 ºC for storage (Benson et al., 2012). The general effect of a CPA is to lower the freezing point of water and thereby create an osmotic gradient for the extraction of intracellular water to reduce the formation of intracellular ice crystals during freezing (Gunn, 2016). Other than EY, commonly used CPAs are glycerol and dimethyl sulfoxide (DMSO), both penetrating cryoprotectants that are able to cross the cell membrane, leading to the reduction of cell damage by preventing the concentration effects of extracellular media (Vishwanath and Shannon, 2000, Benson et al., 2012). Sucrose and dextran are non penetrating CPAs, which, in the event of freezing, result in extracellular ice-crystalline lattices forming that are believed to provide a shield for the protection of sperm cells (Nicolajsen and Hvidt, 1994).

Despite the cryoprotective effects of glycerol and other CPAs, a large proportion of sperm cells die or are impaired due to physical and chemical stress during cryopreservation and thawing (Watson, 1990), resulting in a decrease in fertility (Yeste, 2016). There are species differences in the amount of sperm cells required to achieve fertilization. Sperm physiology, female tract anatomy, sperm transport mechanisms, and the likelihood of identifying optimum timing and delivery of sperm cells are important variables that vary between species and that can potentially affect the fertility outcome (Holt, 2000b). Additionally, species differ in the susceptibility of their sperm to cryodamage; sperm from the bull and ram seem to withstand the harmful effects of freezing better than porcine and canine sperm. The high cryotolerance of bull and ram sperm cells is possibly due to the higher ratio of unsaturated/saturated membrane fatty acids and lower levels of cholesterol in bull and ram sperm membranes (White, 1993).

Knowledge about between species differences in sperm cell anatomy, physiology, and susceptibility to cryodamage with the use of semen cryopreservation is therefore crucial to achieving reproductive success. Furthermore, differences in the ability of sperm cells to endure freezing are seen between breeds and individuals (Holt, 2000b, Waterhouse et al., 2006b). This means that there is potential for improvement in cryopreservation by customizing semen processing media and the rates of cooling and thawing for semen from genetically valuable males.

1.4.2. Cryopreservation of sperm cells by immobilization – the SpermVital technology

In cattle breeding, semen cryopreservation, combined with the use of various semen extenders, has enabled long-term storage and transport of semen, thereby facilitating the dissemination of selected genes to a large geographical area. However, the advantages of cryopreservation combined with AI come with the cost of reduced fertility due to cryoinjury compared with natural mating (Holt, 2000b). As the primary goal of semen preservation is to minimize the gap in fertility, the development of new methods in semen processing has been important. Presently, most AI companies choose among a few prevailing proprietary brands of semen diluents for cryopreservation; e.g., Biladyl®, Triladyl® (both from Minitüb GmbH, Tiefenbach, Germany), Optidyl®, and Biociphos® (both from IMV, L'Aigle, France). In 2010, the SpermVital immobilization technology (SpermVital, Hamar, Norway) was introduced for commercial use, with the intention of improving cryoprotection during freezing and storage, and prolongation of the lifespan of sperm cells after AI.

Alginate capsules have been shown to be suitable as media for entrapment of live cells (Strand et al., 2000). The capsules are composed of gels containing divalent cations, typically Ca^{2+}, and block structures of guluronic acid in an alginate polymer chain. The relevant cells, e.g., sperm cells, are mixed with sodium alginate solution and dripped into another solution containing the cations, thereby creating gel spheres or capsules entrapping the cells (Strand et al., 2000). The alginate technology, in which sperm cells are encapsulated, has been previously described (Nebel et al., 1993, Vishwanath et al., 1997). The SV technology, however, uses a different approach than the encapsulation technique described previously (Kommisrud et al., 2008). The cells are embedded in a solid gel, in which calcium and alginate are the main components. The spermatozoa are embedded and immobilized in the alginate gel in order to increase spermatozoa survival during cryopreservation, to improve post-thaw viability, and to prolong the lifespan of

each cell (Standerholen et al., 2015). Additionally, the SV technology results in increased availability of spermatozoa; this is due to the gradual release of spermatozoa over a certain timespan in the uterus (Kommisrud et al., 2008). Until now, SV semen doses have contained a higher concentration of sperm cells than conventionally processed semen, due to the concept that as the gel dissolves gradually after insemination, more spermatozoa are needed to be certain of the availability of fertile spermatozoa. However, it has been reported that reduced sperm cell concentrations do not compromise fertility after AI with conventional semen (Schenk et al., 1987, Christensen et al., 2011). In Study I of the current project, the relationship between a reduction in sperm cell concentrations in SV semen and fertility was investigated.

1.5. Assessment of fertility and quality of fertility data

Fertilization is key to describing fertility, and is defined as the fusion of a spermatozoon with the ovum, followed by formation of a zygote (Amann et al., 2018). Due to the binomial nature of fertilization (yes or no), fertilization data often require pooling to generate a meaningful average fertility value for a male or female individual at any given time point or interval following natural mating or AI (Foote, 2003). In theory, fertilization rate could be expressed as the proportion of oocytes that are fertilized following sperm cell exposure, but as recording zygote formation is highly impractical from a clinical perspective, other methods of measuring fertility are necessary. Return to estrus after natural mating or AI is commonly used in modern cattle reproduction management to measure fertility. Fertilization will result in the absence of a new ovarian cycle, which is recorded as a lack of return to estrus (Senger, 2015). Non-return rates to estrus are then recorded as an indicator of pregnancy. The non-return rate is calculated as the proportion of inseminated females that are not subsequently re-inseminated within a specified interval after the first AI (Foote, 2003); e.g., NR56 for 56 days. For several reasons, including inadequate reproduction management and monitoring for estrus, a discrepancy of between approximately 10 and 20 % between the non-return rate and the actual prevalence of pregnancy is not uncommon (Garmo et al., 2008, Amann et al., 2018). Direct pregnancy diagnosis by transrectal palpation is a more precise method to assess fertility and has traditionally been used to determine the outcome of AI in cattle (Govind Purohit, 2010). Experienced bovine practitioners can perform precise pregnancy diagnostics as early as 35 days after AI. However, the use of transrectal ultrasonography has gradually replaced pregnancy diagnosis by transrectal palpation. This is likely due to ultrasonography being of greater accuracy than rectal palpation, particularly for less experienced practitioners. In addition,

ultrasonography provides the possibility of accurate detection of pregnancy as early as 28 days after AI, along with the opportunity to decide more precisely the ovarian status of cows that are not pregnant (Fricke et al., 2016).

The quality of the recorded reproduction data depends on the reliability of the personnel who report the AI data and the degree of validation of the recordings. In Norway, breeding personnel and bovine practitioners are reimbursed by Geno, thus motivating accurate reporting to the company's recording database. The data are then subject to several validation tests before they are entered into the NDHRS. These procedures ensure the credibility of the recording system (Espetvedt et al., 2013).

1.6. Acquisition of fertilizing potential and fertilization

The final stages of spermatozoa maturation take place in the epididymis before mixture with seminal fluid from the accessory sex glands, particularly the vesicular gland in the bull, and ejaculation. A variety of physiological functions have been suggested for seminal fluid, including provision of energy, regulation of sperm cell motility, and buffering (Noakes et al., 2009). In cattle, semen is ejaculated into the vagina from where spermatozoa must pass through the highly viscous mucus of the cervix. The cervix, with its labyrinthine folds and crypts, has been shown to act as a reservoir for sperm cells (Hawk, 1987b). To enable fertilization, the sperm cells must travel further through the female genital tract to the site of fertilization at the ampullar-isthmic junction of the oviduct, where they form a sperm reservoir (Rath et al., 2008). During this journey, physical and biochemical interactions between sperm cells and the genital tract environment result in the selection, storage, and preservation of the viability of sperm subpopulations, with specific characteristics concerning sperm quality (Suarez, 2016). Physical interactions include swimming responses to variations in the viscoelasticity of fluid and the anatomical microarchitecture of the genital tract lining. As sperm cells encounter walls, they tend to swim alongside them, just as they tend to orient their progressive movement into gentle fluid flows (Suarez, 2016). Biochemical interactions include the capacitation process, in which surface molecules of the spermatozoa bind with receptors on the epithelial lining of the uterotubal junction and oviduct to acquire fertilization competence. It has been shown that specific proteins on the surfaces of sperm cells interact with the uterotubal lining and are necessary for the opening of the uterotubal junction for the passage of sperm cells into the oviduct (Suarez, 2016).

As the time of ovulation approaches, sperm cells detach and reattach from the oviductal epithelium before moving out of the sperm cell depot area of the oviduct (Chang and Suarez, 2012). The interaction between sperm cells and the oviductal epithelium may contribute to maintaining fertilizing potential. In addition, the storage of spermatozoa in this reservoir has been shown to prevent polyspermic fertilization; only a few spermatozoa at a time pass through to the ampulla, where they can potentially reach oocytes for fertilization (Chian and Sirard, 1995). Carbohydrate moieties have been shown to contribute in the interactions between sperm cells and oviductal epithelium, acting as essential components or blockers of oviductal receptors for sperm cells (Lefebvre et al., 1997). However, these carbohydrates vary among species (Suarez, 2016). Essential components of the carbohydrate moieties have been found to be produced in the epididymis and accessory sex glands, and, in the bovine, this particularly applies to the fluid produced in the vesicular glands, which is key to the binding of sperm cells in the oviductal epithelium (Gwathmey et al., 2003). A group of these moieties, termed Binder of Sperm (BSP) proteins, is found in the bull and produced in the vesicular gland.

It has been reported that sperm cells attached to the oviductal epithelium remain alive longer than free sperm cells (Kawakami et al., 2001). Spermatozoa bound to this epithelium have been shown to have lower levels of Ca^{2+} than free sperm cells, whereas a rise in the levels of Ca^{2+} is associated with sperm capacitation. The protective effect from binding of sperm cells to the oviductal epithelium is possibly caused by inhibition of capacitation (Suarez, 2016). It has been shown that BSP proteins of bull semen contribute to extending the viability of spermatozoa by stabilizing their phospholipid membranes through reducing membrane fluidity and immobilizing cholesterol (Müller et al., 2002).

Detachment of sperm cells from the oviductal epithelium is an essential part of the capacitation process, and apparently renders the sperm cells competent for fertilization. Detachment of sperm cells from the oviductal lining is achieved by factors deriving from both the sperm cell and the oviductal epithelium. A reduction in the affinity of the binding of sperm cell receptors to the oviductal epithelium has been observed following modifications in sperm cell surface proteins, e.g., by adding a medium that induces the acrosome reaction (Lefebvre and Suarez, 1996). Furthermore, hyperactivation of sperm cell motility has been shown to provide the force required for the sperm cell to detach from the epithelium (Chang and Suarez, 2012). A reduction in binding sites in the epithelium, caused by the secretion of hormones known to play a role in reproductive physiology, could affect sperm cell release from the oviductal lining. There is evidence that preovulatory follicles secrete factors that induce capacitation and hyperactivation

of sperm cells, resulting in epithelial release of the sperm cells (Tollner et al., 2009). Similarly, capacitated sperm cells in bulls show a reduced ability to bind to the oviductal epithelium (Ignotz et al., 2001) following modification of BSP proteins after capacitation, and loss of the carbohydrate portions of molecules responsible for binding sperm cells to the epithelium (Demott et al., 1995).

Gamete interactions during fertilization in cattle (Figure 2), including binding of sperm cells to the ovum, result in an alteration of gamete specific components to zygotic arrangements (Sutovsky, 2018). In the bovine, the apical ridge of the sperm acrosome forms the point of contact with the zona pellucida (ZP) of the ovum. The acrosome consists of 3 layers, the outer and inner acrosomal membranes (OAM and IAM, respectively), which sandwich the acrosomal matrix and cover the subacrosomal perinuclear theca (Oko and Sutovsky, 2009). Modifications in the OAM that result from capacitation, expose sites for binding with the ZP, which, in the bovine, consist of glycosylated proteins that function as receptors for the initial sperm-oocyte contact. As the gametes link by the binding sites, acrosomal discharge of hydrolytic enzymes facilitates penetration of the ZP. The acrosome reaction results in loss of the OAM, with only the IAM left intact after penetration of the ZP (Gerton, 2002). Exposure of the IAM results in the display of oolemma binding proteins in preparation for sperm-oocyte binding, with protein receptors in the perivitelline space (Cuasnicú et al., 2016).

Gamete fusion is the process in which the fertilizing sperm cell adheres to the oolemma, resulting in fusion of the gametes and entry of the sperm pronucleus into the oocyte (Sutovsky, 2009). Multiple proteins are responsible for the interactions that occur between the sperm cell (particularly the sperm head equatorial segment) and oocyte during gamete fusion (Ito et al., 2010, Satouh et al., 2012). Gene knockout studies in mice have identified specific proteins and receptors as being potential mediators in the process of gamete adhesion and fusion; e.g., the sperm immunoglobulin cell adhesion protein IZUMO (Inoue et al., 2005) and its oocyte binding partner, the folate binding receptor JUNO (Bianchi et al., 2014). Both these proteins also exist in cattle gametes. Whereas the initial phases of gamete fusion are dependent on interactions between the equatorial segment of the sperm cell head, the post-acrosomal sheath becomes more involved in the subsequent steps (Sutovsky et al., 2003). It is assumed that the release of mediators from the post-acrosomal region activates the release of calcium from oocyte organelles, which, in turn, triggers a cascade that results in meiosis in the oocyte. These actions prevent polyspermy, pronuclear development, and, finally, cleavage of the embryo (Oko et al., 2017).
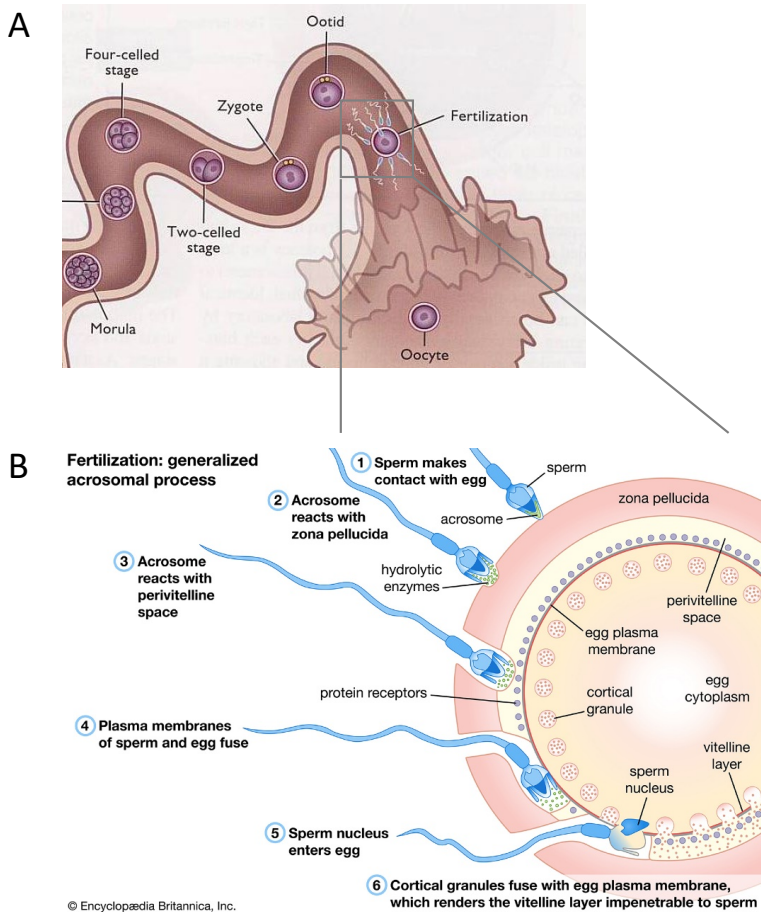
Figure 2. *The ovary, infundibulum, and anterior parts of the uterus, ovulation (A) and gamete fusion during fertilization (B). After AI or natural mating, sperm cells ascend the genital tract to the oviduct, i.e., the site of fertilization, where surface molecules of the spermatozoa bind with receptors on the epithelial lining of the oviductal epithelium to acquire fertilization competence. When a sperm cell has obtained fertilizing capacity (capacitation), it can bind to the zona pellucida of the oocyte by specific plasma proteins (1). The ensuing acrosome reaction (2) results in the discharge of acrosomal hydrolase to degrade the zona pellucida before binding to protein receptors attached to the vitelline layer (3). The fusion of the gamete plasma membranes then occurs (4), allowing the sperm nucleus into the ovum and its transformation into a pronucleus (5). Cortical granules subsequently fuse with the oocyte plasma membrane, which makes the vitelline layer impenetrable to spermatozoa, thereby acting to prevent polyspermy. The sperm and oocyte pronuclei finally form a diploid zygote, which then undergoes multiple mitotic divisions, resulting in the formation of a morula. The morula develops into a blastocyst before implantation and the growth of a fetus. Modified from Senger (2003) and Britannica (2019).*

1.7. The estrous cycle in cattle

Cattle of the *Bos taurus* species are polyestral, with a uniform distribution of estrous cycles throughout the year, except during pregnancy and the postpartum period (Noakes et al., 2009). The estrous cycle is defined as the interval from one estrus to the next, with a duration of about 21 days (18-24 days). The estrous cycle consists of the follicular phase (4-6 days), which is divided into proestrus and estrus, and which culminates in ovulation, and the luteal phase (14-18 days), which consists of metestrus and diestrus and is the period between ovulation and regression of the corpus luteum (CL) (Forde et al., 2011).

The estrous cycle of cattle is controlled by the hormones of the hypothalamus (gonadotrophin releasing hormone, GnRH), anterior pituitary (follicle stimulating hormone: FSH, and luteinizing hormone: LH), the ovaries (progesterone: P4, estradiol: E2, and inhibins), and the uterus (prostaglandin $F_{2\alpha}$: $PGF_{2\alpha}$). These control the estrous cycle by positive and negative feedback loops (Roche, 1996). The effect of GnRH on the estrous cycle is mediated by its control on the anterior pituitary, including the binding of GnRH to specific receptors on the surfaces of cells that produce gonadotrophs, resulting in a cascade of intracellular reactions and, finally, the release of LH and FSH (Weck et al., 1998).

Proestrus is the initial part of the follicular phase, characterized by $PGF_{2\alpha}$ induced luteolysis and a subsequent decline in P4 concentrations. The low P4 concentrations that are maintained throughout estrus lead to a surge of GnRH, resulting in an increase in blood levels of FSH and a subsequent rise in estradiol concentrations produced by the maturing dominant follicle (Figure 3). In addition, the GnRH surge induces pulsatile secretion of LH, with peaks in concentration every 40-70 minutes for 2-3 days, leading to ovulation of the dominant follicle (Roche, 1996). In animals ovulating spontaneously, ovulation occurs 10-14 hours after estrus. Following ovulation, the initial part of the luteal phase, known as metestrus, occurs over 3-4 days. A CL gradually forms, replacing the void of the erupted follicle by the growth of theca and granulosa cells that luteinize to produce progesterone. The elevated progesterone concentrations prepare for the establishment of pregnancy or for return to the estrous cycle (Niswender, 1981). The elevated levels of progesterone that are characteristic of the luteal phase of the estrous cycle partially obstruct the secretion of LH by negative feedback, only allowing secretion of high amplitude LH pulses at a low frequency, which is insufficient to trigger ovulation of the dominant follicle. The estrous cycle returns to estrus following luteolysis of the CL in response to $PGF_{2\alpha}$ secretion from the uterus (Hansel and Convey, 1983).
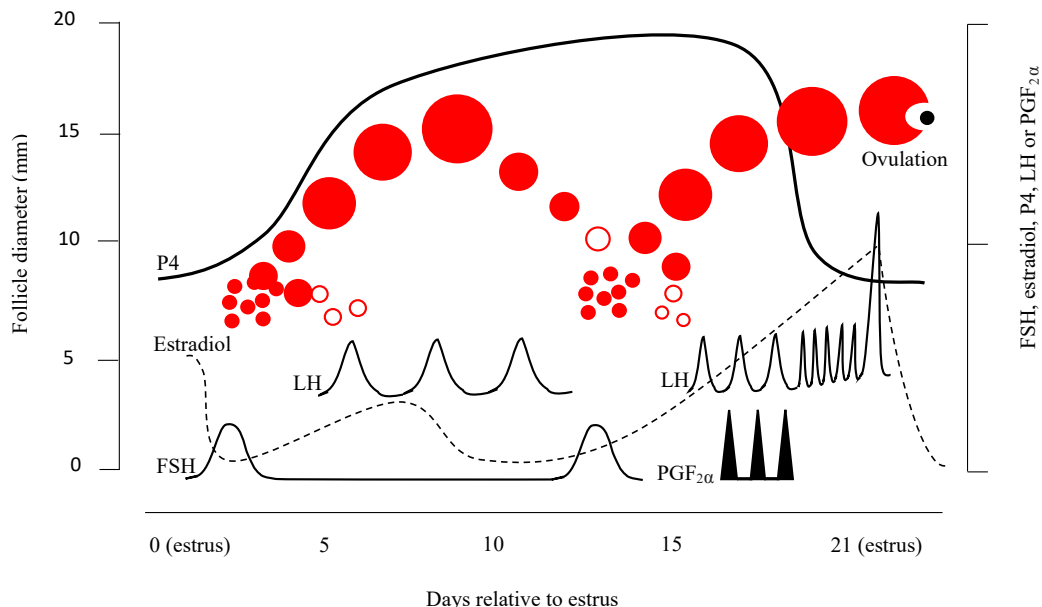
Figure 3. *The pattern of secretion of progesterone (P4), follicle stimulating hormone (FSH), estradiol, luteinizing hormone (LH), and prostaglandin (PGF); the growth of ovarian follicles during the estrous cycle is illustrated by circles (Forde et al., 2011, Stevenson, 2017). Follicular growth occurs in waves, which are led by a temporary rise in FSH concentrations. The onset of ovarian follicular maturity is detected as the pre-ovulatory follicle reaches about 10 mm in diameter (Sartori et al., 2001). Concurrent with follicular growth, circulating estradiol concentrations increase, followed by an LH surge and ovulation of the dominant follicle at about day 21 of the estrous cycle; atretic follicles (open circles). By Berg (2019).*

1.7.1. Growth of ovarian follicles and ovulation

The development of ovarian follicles begins at the embryonal stage, with the creation of a fixed number of primordial follicles that function as a reservoir for recruitment into the follicular waves of the estrous cycle following sexual maturity (Forde et al., 2011). Understanding the process that leads to the growth, selection, and ovulation of the dominant ovarian follicle is essential for the efficiency of reproductive management in cattle (Pursley et al., 1995). During the estrous cycle, there are commonly 2 waves of follicular growth in dairy cattle and 3 waves in heifers and beef cows (Savio et al., 1988). Each wave of folliclular growth consists of the emergence of a group of follicles, selection of a dominant follicle, and either ovulation or atresia of the dominant follicle. The arrangement of follicles in waves is established during the

gonadotrophin independent pre-pubertal period of development and the waves take place continuously throughout the estrous cycle, each lasting 7-10 days.

The initiation of gonadotrophin dependent follicle development is characterized by a transitory increase in FSH concentrations and the concurrent emergence of a cohort of 5-20 follicles with a diameter of at least 5 mm (Sunderland et al., 1994). The largest follicle of a follicular wave begins the process of deviation from other follicles in the same wave about 12 hours before a difference in size can be detected (Ginther, 2016). In contrast to its contemporaries, the dominant follicle expresses an increase in granulosa LH receptors and estradiol, making it susceptible to the increase in LH following the wave of FSH (Ginther, 2016). The increase in the size of the dominant follicle compared with other follicles in its cohort leads to an increase in its follicular estradiol and inhibin contents, which together inhibit the secretion of FSH from the pituitary gland by negative feedback (Ginther, 2000).

Multiple factors that are produced within the ovary influence the course of events in the estrous cycle. A group of peptide hormones, known as insulin-like growth factor (IGF), constitutes a superfamily, including specific ligands, receptors, and binding proteins, which directly contributes to the growth of the future dominant follicle and its ability to produce steroids (Canty et al., 2006). Indirectly, the secretion of IGF results in an increase in estradiol levels that causes negative feedback to the pituitary and hypothalamus. Similarly, cytokines of the transforming growth factor (TGF) superfamily, which have been identified in ovarian follicular fluid, have been shown to stimulate follicular production of estradiol (Knight and Glister, 2003).

The time for the onset of estrus following luteolysis is dependent on the maturation status of the dominant follicle (Austin et al., 1999). Spontaneous ovulation in cattle requires the presence of a mature dominant follicle with enough LH receptors and estradiol secretion to enable the follicle to be receptive to the preovulatory increase in LH (Ginther, 2016). Early research reported ovulation to occur approximately 25 hours after the LH peak (Schams et al., 1977). Ovulation of the dominant follicle can occur spontaneously after luteolysis or can result from hormonal treatment, with induction of a preovulatory LH surge achieved by using GnRH or estrogen. Artificial induction of ovulation can also be accomplished by using hormones such as porcine LH to simulate the preovulatory surge of LH. There is a potential downside to the induction of ovulation in cattle, as it may initiate the ovulation of immature follicles. Premature induction of ovulation results in less secretion of progesterone from the resulting CL than from a CL derived from an ovulatory follicle of a larger diameter, and this may lead to reduced fertility (Vasconcelos et al., 2001).

1.7.2. Corpus luteum – luteal activity

In the event of ovulation, theca and granulosa cells surrounding the ovulatory follicle gradually luteinize to form a CL, following luteinizing stimuli associated with LH, the main luteotropic hormone in the bovine (Alila and Hansel, 1984). After ovulation, a major role of the CL is to produce progesterone for a longer period to support pregnancy - should fertilization occur. In the mid-luteal phase of the estrous cycle, sustained high concentrations of circulating progesterone (P4) result in downregulation of the receptivity of luminal epithelial cells in the endometrium to P4. By day 16 of the estrous cycle, maternal recognition of the pregnancy signaling factor, interferon-τ, which is secreted by the bovine conceptus, is required to support pregnancy (Roberts, 2007). In the absence of interferon-τ, luteolysis of the CL by uterine binding of oxytocin and secretion of $PGF_{2\alpha}$ occurs, causing a return to ovarian cyclicity.

1.7.3. Timing of artificial insemination in cattle

Successful AI in cattle depends on the correct timing of insemination relative to the occurrence of estrus (Nebel et al., 1994) and ovulation (Walker et al., 1996, Roelofs et al., 2005). Pioneering research in the late 1940s established the optimal timing of AI to be within 13 – 18 hours before ovulation (Trimberger, 1948). However, optimal timing of AI may vary with parity (Roelofs et al., 2005), estrous length (Walker et al., 1996, Roelofs et al., 2005), expression of estrous signs, and breed (Burke et al., 1995, Sveberg et al., 2015). When AI is performed too early, sperm cells will likely be aged and incapable of fertilizing the oocyte (Hawk, 1987a). Late AI has also been shown to result in reduced fertility, probably due to aged oocytes (Hunter and Greve, 1997).

With the patented SV technology, sperm cells are immobilized in an alginate gel that enables their gradual release as the gel dissolves after AI (Kommisrud et al., 2008). Accordingly, the timing of insemination relative to estrous signs is assumed to be more flexible when using SV semen than when using standard processed semen (Figure 4). Pregnancy rates following single AIs with SV semen early in estrus have been compared with AIs using standard processed semen on two consecutive days (Alm-Kristiansen et al., 2017) and have shown equal fertility. Furthermore, it has been reported that SV sperm cells have prolonged viability compared with that of standard processed sperm cells (Alm-Kristiansen et al., 2018a). However, further investigations, including early, single inseminations with standard processed semen, are necessary in order that the potential of using SV semen for early AI can be fully realized.
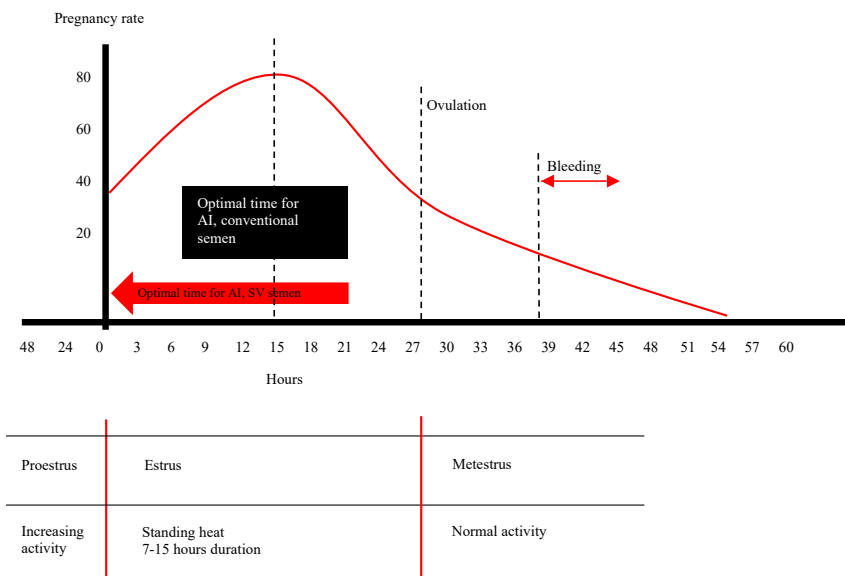
Figure 4. *Optimal time (hours) of insemination expressed as pregnancy rate using conventional semen or SpermVital (SV) semen. The follicular phase of the estrous cycle begins with proestrus (2 – 5 days duration), in which increased physical activity can be observed. The onset of estrus is observed by the onset of estrous behavior (sexual receptivity). The follicular phase and estrus terminate at ovulation, approximately 24 hours after the onset of estrus. The following stage of metestrus is characterized by initial bleeding and a cease in estrous behavior. Several effects contribute to the variety in optimal time of AI; e.g., breed, herd, and season, and, for conventional semen, AI is recommended within 6 – 18 hours following the onset of estrus. The sperm cell normally remains fertile in the female reproductive tract over an interval of approximately 24 hours following AI with conventional semen. Prolonged sperm cell survival has been shown in SV semen, which leads to an increase in the duration of the recommended interval for AI. This allows successful AI from the onset of estrus, and possibly earlier within the stage of proestrus. Modified from Refsdal et al. (2014).*

1.7.4. Estrous synchronization and ovulatory response

Appropriate timing of AI relative to estrus and ovulation is a prerequisite for reproductive success in cattle. During the past 40 years, a great diversity of timed AI protocols (protocols for synchronization of estrus and ovulation) has been applied worldwide to standardize AI management. However, the different protocols demonstrate variable reproductive efficiency (Meyer et al., 2007). In most synchronization protocols, $PGF_{2\alpha}$ and GnRH are given in a series of treatments in which the first administration of GnRH results in the regression or ovulation of the dominant follicle and initiates a new follicular wave in most of the treated animals (Thatcher et al., 1996). Ovsynch is one of the protocols that is most commonly used in

combination with timed AI, applying a luteolytic $PGF_{2\alpha}$ treatment, commonly 7 days after the first GnRH treatment, to regress the CL before a second treatment using GnRH to induce the LH surge that brings about ovulation (Thatcher et al., 2002). In Norway, another protocol for estrous synchronization is more commonly applied; the initial prostaglandin (PGF) analog treatment acts as a pre-synchronization step, synchronizing the animals into the first follicular wave of the estrous cycle (Meyer et al., 2007). The theory is that all animals will then be in the luteal phase of the estrous cycle 11 days later. The second PGF analog treatment mediates luteolysis of the mature CL and a drop in P4 concentrations, thus allowing low-amplitude LH pulses that stimulate the growth of the dominant follicle (Gong et al., 1995). As the follicle grows, estradiol concentrations increase, leading to the LH surge that precedes ovulation (Lucy et al., 1992). Research has revealed that administration of GnRH before the spontaneous LH surge increases the height of the LH surge compared with administration afterwards, or if no GnRH is given (Rosenberg et al., 1991). By simultaneously treating animals with a GnRH analog 48 hours after the final PGF analog treatment, the LH surge is triggered, bringing about the physiological steps that mediate ovulation (Thatcher et al., 2002). As a result, some standardization of the duration of folliculogenesis is achieved (Perry et al., 2007). Although there is evidence that the use of Ovsynch with timed AI results in higher pregnancy rates than with the latter synchronization protocol, the gap in fertility has been shown to decrease as days increase between the AI event and pregnancy check. This tendency can be explained by a relatively high proportion of embryonic loss subsequent to AI following Ovsynch (Meyer et al., 2007).

1.7.5. Detection of estrus

AI is the main breeding strategy in the modern reproductive management of dairy cattle herds. However, to succeed with bovine reproductive management, effective detection of estrus is essential (Andrews et al., 2008). A variety of methods and technologies have been developed for detection of the characteristic physiological changes that occur in estrous animals, including mount detectors, physical activity monitoring systems, measurement of vaginal conductivity or pH, body temperature, and hormone levels in blood and milk (Stevenson and Britt, 2017).

1.7.5.1.   Physical activity

Traditionally, cows have been monitored by a herdsman in the morning and afternoon using visual observation to detect standing estrus (animals standing to be mounted), resulting in AI based on the a.m./p.m. rule; i.e., the observation of standing heat in the morning results in an afternoon AI. Manual monitoring using visual observation excludes the observation of estrus over longer periods, and, for this reason, a wide variety of estrus-detection aids have been developed. Low-tech products, such as paint or chalk markers applied to the tailhead of cows, indicate standing estrus of the animal if the coloring is rubbed off. However, validation studies on the accuracy of such estrus-detection tools have demonstrated a high degree of variability in their precision (Roelofs et al., 2010, Stevenson, 2018) and laborious routines associated with the practical management of such tools. In around 2005, technologies enabling automated estrus-detection were made available to the dairy industry (Stevenson and Britt, 2017). These technologies include pedometry, activity monitoring, and milking systems with chemical sensors. One solution involves neck collars including a microprocessor and accelerometer for the measurement of animal movement. Evidence shows that the use of activity monitoring tags results in high accuracy in estrus detection (Roelofs et al., 2010), which, in combination with individual cow milk production data, is a powerful tool in reproductive herd management.

1.7.5.2.   Progesterone in milk

Between estrous cycles, a recurring pattern of fluctuations in P4 levels can be readily measured in milk. Milk P4 levels are elevated from 4 to 16 days after ovulation, before luteolysis occurs, resulting in a fall in P4 levels to a nadir (Forde et al., 2011). A P4 measurement assay is commonly used as a standard calibration tool to evaluate the accuracy of estrus-detection systems by measuring daily milk P4 levels until the drop in P4 that precedes ovulation by approximately 48 hours is detected (Mottram, 2015). Otherwise, the measurement of milk P4 can be used in daily reproductive herd management as an indicator of luteolysis and subsequent estrus (Nebel, 1988). An immuno-sensing test can indicate the presence of milk P4 by a reaction between the test reagent and the hormone, which is visualized as a change in sample color. Another approach to using P4 fluctuations in the estrous cycle to detect heat is by the application of biosensors, i.e., sensors that detect P4 by a change in the electrical signal, in combination with automated milk sampling in milk parlors or in automatic milking systems (Blom and Ridder, 2010). However, the decrease in milk P4 content following luteolysis is insufficient by itself for precise determination of the correct timing of AI because of large variations in time to

ovulation following luteolysis, which are probably due to individual variations in hepatic metabolism of steroid hormones (Wiltbank et al., 2006).

1.7.6. Transrectal ultrasonography

Transrectal ultrasonography is a commonly applied tool for the assessment of changes in ovarian features, such as luteal proliferation, follicular growth, and ovulation in cattle (Roelofs et al., 2004, Saumande and Humblot, 2005). Clinically controlled studies have examined whether frequent examinations with transrectal ultrasonography in cattle interfere with levels of reproductive parameters; however, no differences have been found between groups of animals subjected to ultrasonography and controls provided that the reproductive organs have been handled with care (Sirois and Fortune, 1988, Roelofs et al., 2004). Examination of ovulation and ovarian follicular development by transrectal ultrasonography has been shown to be an accurate and reliable method in cattle (Saumande and Humblot, 2005) compared with rectal palpation (Pieterse et al., 1990). However, there are challenges concerning the precise measurement of ovarian structures, which requires consistent positioning of the ultrasound transducer (Perry and Cushman, 2016).

1.8. Assessment of semen quality

A primary goal of the cattle breeding industry has been having the capability to predict bull fertility accurately. Following the shift from progeny-based selection to GS of bulls in cattle breeding, it has become increasingly important to be able to predict bull fertility. Application of the GS technology means that breeding values are available for bulls at an early age, resulting in a high turnover of young GS bulls at the premium level. Young bulls typically have a relatively low volume of semen and few sperm cells per ejaculate (Murphy et al., 2018b), which, in combination with a high throughput of selected bulls, emphasizes the importance of accurate prediction of bull fertility (Fair and Lonergan, 2018). Provided that the experimental design is adequate, and investigations conducted properly, then fertility results from AI trials are considered to give an accurate indication of bull fertility. However, such trials are expensive and time consuming. In addition, the widespread application of GS breeding presents a challenge to the execution of AI trials, due to the high turn-over of young bulls, potentially resulting in low numbers of AIs per bull. In order to support findings from AI trials, it has been common to implement complementary *in vitro* studies. Associations have been demonstrated

between the fertility of bull semen and *in vitro* parameters, such as motility (Sellem et al., 2015), intactness of DNA (Waterhouse et al., 2006a), and acrosome and plasma membrane integrity (Fatehi et al., 2006). However, results from experiments on bull semen that include such *in vitro* parameters are not always in concurrence, making assessment of *in vivo* fertility based on separate *in vitro* sperm characteristics incomplete. Nevertheless, studies on bovine semen have suggested that predicting fertility by combining several *in vitro* variables of semen is of increased reliability (Puglisi et al., 2012, Oliveira et al., 2013). Evidence shows an association between field fertility and a combination of *in vitro* variables by grouping kinetic parameters measured by computer-assisted sperm analysis (CASA), plasma and acrosome membrane integrity, and sperm DNA integrity measured by flow cytometry (Oliveira et al., 2013, Sellem et al., 2015).

1.8.1.   Computer-assisted sperm analysis

Since the introduction of AI in cattle breeding, sperm cell motility has played an important role in the evaluation of male fertility. Traditionally, sperm cell motility was analyzed subjectively, a practice still widely applied in cattle breeding centers. Over the past decades, however, CASA technology has followed the rapid progress of computer and imaging tools, enabling accurate characterization of sperm cell motility (Holt and Palomo, 1996) for evaluating semen quality (Amann and Waberski, 2014). A CASA system usually consists of a video camera attached to a microscope, a video frame grabber card, and a computer. During CASA, successive frames of a diluted semen sample contained in a shallow chamber slide are projected onto a detector array. Objects in the diluted semen with a specified concentration of pixels (centrioles) using darkfield and negative phase contrast or fluorescence microscopy (combined with fluorescence staining and laser excitation) are identified and recorded by proprietary software for processing sperm cell data (Kathiravan et al., 2011). The video camera records multiple microscopy frames of each sperm cell. The frames are then processed by the computer, resulting in a digital readout of the number of pixels that the sperm cell travels. Artifacts or debris in semen samples resembling spermatozoa in pixel size are potential sources of error. To reduce the chance of erroneous recording of particles, CASA technicians can adjust settings according to species specific estimates or, in some instruments, other settings can be used, such as the requirement of detecting a tail attached to the identified shape of a spermatozoa head (Wijchman et al., 1995).

Several different CASA systems are currently in use to assess sperm quality in a large proportion of cattle breeding centers and andrology laboratories (Noakes et al., 2009). Generally, images of semen samples taken by CASA are automatically captured at a frequency of 25 to 60 Hz (Morris et al., 1996), resulting in 25 or more frames per sample field and acquisition of data on multiple kinematic parameters for individual sperm cells and the sample population. Following the conversion of sperm cell motion from video to digital form, the data are processed in a multistep algorithm to identify the trajectory of the sperm cell (Katz and Davis, 1987). First, the positions of the sperm head centroid throughout a trajectory are determined by the computer as it searches for consecutive images within a zone of probability, which is set by the maximum distance a spermatozoon is expected to travel within the period. The two-dimensional coordinates of the sperm cell throughout its trajectory are then calculated, providing the basis for the calculation of a series of kinematic values (Figure 5). During the recording of sperm trajectories, values for all sperm cells in each frame are calculated simultaneously. For samples with high concentrations of sperm cells, the spermatozoa may collide, leading to changes in trajectories and faulty kinematic computations. It is therefore advised that sperm cell concentrations during CASA do not exceed 40×10^6/mL (Mortimer et al., 1995).
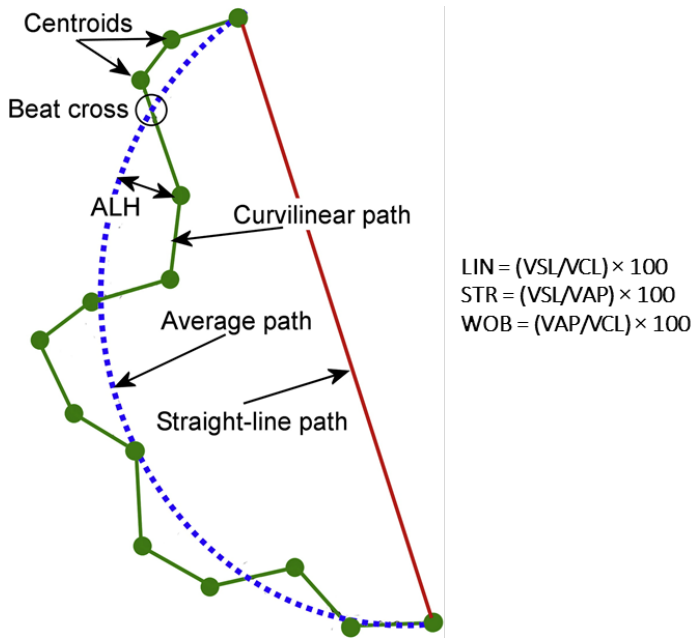
Figure 5 *Sperm cell trajectory measured by CASA. Centroids for each sperm head are recorded in the first field of a recording. Thereafter, the next field is analyzed, and the digitally recorded sperm head centroids are tracked by the computer within a zone of probability. Coordinates for the sperm cell's position throughout the period are then estimated, resulting in a trajectory that provides the basis for calculation of a series of kinematic values; the curvilinear velocity (VCL; μm/s) refers to the velocity of the sperm cell along its actual trajectory (curvilinear path), the average path velocity (VAP; μm/s) refers to the velocity along the average path, the straight-line velocity (VSL; μm/s) refers to the velocity along the path between the first and last coordinates recorded (straight-line path). Each centroid is characterized by the width of the lateral movement of the sperm head, referred to as amplitude of lateral head displacement (ALH; μm/s). The beat-cross frequency (BCF; Hz) is the number of times that the sperm head crosses the direction of movement per second. The form of the trajectory is shaped by the velocity values, which is why they are compared in ratios: linearity (LIN), straightness (STR), and wobble (WOB). Modified from Amann and Waberski (2014).*

The output of kinematic values from CASA for each sperm cell describes velocity, including the curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP), all given in units of μm/s. The VCL refers to the average velocity measured over the actual point-to-point track followed by the cell, always the fastest of the three velocity values. The VSL refers to the velocity between the first and the last coordinates of the trajectory. This is always the lowest of the three velocity values. The VAP refers to the velocity of the sperm cell as it travels the distance of the path following the average direction of movement over the observation period. In trajectories that are close to linear, the VAP is almost the same as the VSL, with the opposite being seen with very irregular trajectories with a high degree of lateral

deviation, when VAP is usually closer to VCL. The average path is calculated by smoothing the trajectory, i.e., averaging values of neighboring (x,y) coordinates along the sperm cell path (Mortimer, 1997). The width of the sperm head trajectory is described by the amplitude of lateral head displacement (ALH). The ALH refers to the lateral movement of the sperm cell head, and is measured as the deviation between the smoothed track relating to the calculation of VAP and the actual trajectory (Boyers et al., 1989). The amplitude in ALH includes the deviation of the sperm head to both sides of the averaged trajectory, rather than half the width as applied in mathematics. The frequency of the change in the direction of the sperm head is expressed as beat-cross frequency (BCF). The BCF is given in Hz and expresses the number of times the sperm head crosses the average path per second. The BCF is a useful parameter for monitoring flagellar beat patterns, but the recording of BCF values may be hampered by the CASA equipment if the BCF exceeds the frequency with which images are acquired (Mortimer and Swan, 1999).

Results show that different sperm cell subpopulations coexist in bovine ejaculates (Muiño et al., 2008). There are various potential reasons for heterogeneity of sperm cells and their grouping into subpopulations. During the final stages of spermatogenesis, sperm cells with differences in origin, and varying maturational status and age are mixed during storage in the epididymis before ejaculation. These variations are reflected by differences in morphology, motility, and viability, which are ultimately reflected in the fertilizing potential (Rodriguez-Martinez and Barth, 2007).

Knowledge about sperm cell biology is required to provide relevant *in vitro* evaluation of sperm cell quality by CASA through the study of subpopulations. This will enable researchers to decide on the settings for high and low values of kinematic traits and to set limits for a biologically meaningful range (Boyers et al., 1989). It has been reported that the distribution of cells into major subpopulations (clusters) based on motility criteria can be used to clarify cell responses to specific biological differences or stimuli, a method of investigation shown to be more informative than evaluation of total sample means (Satake et al., 2006, Henning et al., 2014). Variations in the abilities of semen from different bulls to withstand cryopreservation (freezability) have been shown (Parkinson and Whitfield, 1987), and this variation in freeze-thawed semen is possibly associated with the proportion of highly motile subpopulations in fresh semen (Martínez et al., 2006, Núñez-Martínez et al., 2006). These findings are supported by research in Holstein bulls, where ejaculates with the highest proportion of sperm cells in the rapid progressive subpopulation demonstrated superior resilience against cryopreservation

(Muiño et al., 2008). Subpopulations have also been observed to respond differently to specific stimulation from *in vitro* additives, like bicarbonate and caffeine, with some clusters not reacting to treatment at all (Verstegen et al., 2002). In addition, assessment of sperm cell subpopulations by CASA can be used to show differences in sperm cryotolerance between bulls of different breeds (Ntemka et al., 2016) and different age groups (Hallap et al., 2006).

Different sperm subpopulations can be identified through motility characteristics displayed by individual spermatozoa. Various procedures for multivariate clustering analysis are available for the processing of kinematic parameters generated by CASA. A cluster analysis includes data from individual spermatozoa, usually defined by the previously described CASA motility parameters, thus classifying each of the spermatozoa into a few subpopulations corresponding to their trajectories; each spermatozoon belongs to only a single cluster (Muiño et al., 2008). The clustering procedure applies Euclidean distances calculated for each of the kinematic variables included to determine the means of the observations assigned to each cluster. The number of applied clusters is based on previous assessment of hierarchical dendrograms (Holt, 1995). Cluster analyses of bovine spermatozoa have revealed 3 to 4 distinct subpopulations, one with highly active, non progressive spermatozoa, a second with relatively high velocity and highly progressive spermatozoa, and a third with low motility and non progressive and/or progressive spermatozoa (Muiño et al., 2009, Dorado et al., 2017, Kanno et al., 2017). Differences in the distribution of sperm cells into motility subpopulations can be assumed to reflect diversity in the sperm fertilization potential (Martínez-Pastor et al., 2011) and sperm cell resilience against cryodamage (Muiño et al., 2008).

Although CASA technology is not capable of precise prediction of fertility (Gadea, 2005), its relevance to sperm quality assessment has been demonstrated. Provided that there is adequate validation of the CASA system, it can firstly generate data for quality control of semen for commercial purposes, and, secondly, can aid in investigating sperm cell responses to specific stimuli during research in a controlled *in vitro* environment (Amann and Waberski, 2014).

1.8.2. Flow cytometry

Flow cytometry is a laser based, biophysical technology used for counting cells, sorting cells, and biomarker detection (Herzenberg et al., 2002). Cells are suspended in a stream of fluid and led through an electronic detection apparatus (Figure 6). Flow cytometry allows a high level of experimental repeatability, with simultaneous multiparametric analysis of the physical and

chemical characteristics of up to thousands of cells per sample (Gillan et al., 2005). Different organelles or cellular molecules marked with specific fluorochromes are excited by laser light of various wavelengths and forced through the flow cell in a single cell stream by hydrodynamic forces (Gillan et al., 2005). As the cells pass through the flow cell, light is spread as forward scatter or side scatter, bound fluorescent dyes are excited by a laser, and fluorescent light is emitted. Photomultiplier tubes (PMT) or fluorescence detectors (FL) gather and amplify forward scatter and side scatter (i.e., all light signals) (Brown and Wittwer, 2000), which is transferred to a computer that translates the analog signal to digital data. Each FL detects emitted light of a specific wavelength interval, depending on the dichroic mirrors and fluorescence filters used. Finally, the analog signals gathered by the optical system are translated into digital data for computer analysis by the electronic system (Brown and Wittwer, 2000). Storage of the data in standard flow cytometry files allows sperm cell analysis; typically the spermatozoa are assigned to only a few populations following manual classification. Single parameter histograms present the relative fluorescence from each FL plotted against the number of events, whereas cytograms with scatter plots can be used for simultaneous comparison of multiple parameters (Figure 7).

Classification of sperm cells into subpopulations is based on the correlation of different levels of intensity between the parameters of interest. Computer software sets gating parameters based on single cell data for the demarcation of subpopulations of potential interest. Multiple traits of the cell or biochemical attributes can be studied by flow cytometry, including viability, acrosomal status, sperm capacitation status, mitochondrial activity, oxidative stress, and chromatin status (Boe-Hansen and Satake, 2019). Gating is also used for the exclusion of cell populations without relevance to the current experiment or to eliminate cell debris.
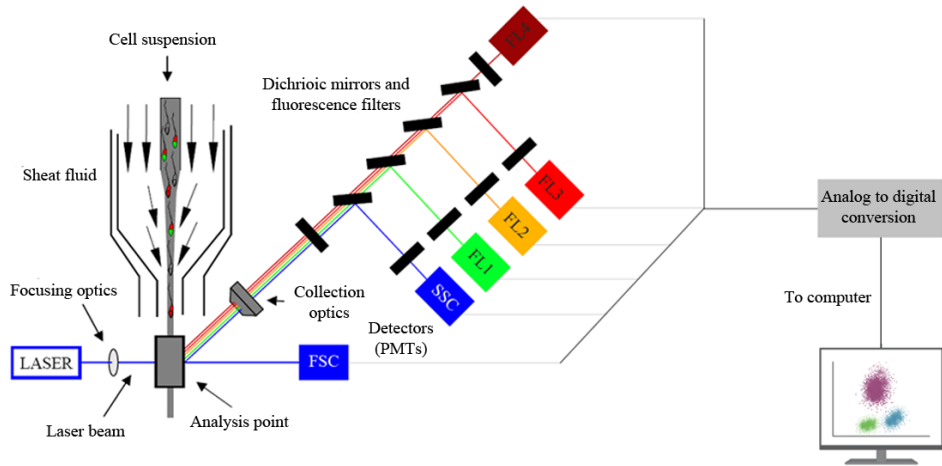
Figure 6. *The functional components of a flow cytometer showing the fluidic, optical, and electronic systems. The hydrodynamic forces of the sheath fluid create a stream of single cells in the cell suspension. The cells then pass through the analysis point (laser focal point) leading to scattering of light, and the excitation of bound fluorescent dyes and emission of fluorescent light (the fluidic system). As the fluorescent light reaches the dichroic mirrors and fluorescence filters, it is separated and led to specific detectors called photomultiplier tubes (PMTs; FL1, FL2, FL3, FL4) that enhance the signal. In addition to these tubes, detectors of forward scatter (FSC) and side scatter (SSC) comprise the optical system. Analog signals gathered by the optical system are then translated to digital data for computer analysis by the electronic system. Modified from Brown and Wittwer (2000b) and Waterhouse (2007). Adapted from Standerholen (2018).*

41
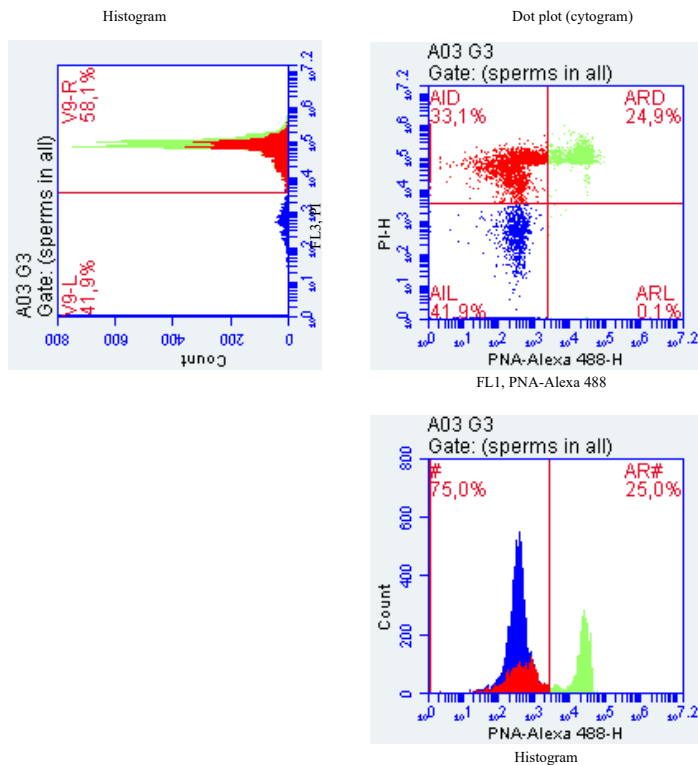
Figure 7. *Two-parameter (cytogram) and one-parameter (histogram) distributions of acrosome intact live (AIL), acrosome intact dead (AID), acrosome reacted dead (ARD), and acrosome reacted live (ARL) spermatozoa resulting from flow cytometry analysis of sperm cells stained with Arachis hypogea (peanut) agglutinin (PNA)-Alexa 488, and propidium iodide (PI). The cytogram shows a bivariate distribution of PNA-Alexa 488 fluorescence (detected in FL1) versus PI fluorescence (detected in FL3). By Bai, G. T. (personal communication, 2020) and modified by Berg, H. F. (2020).*

1.8.3. Flow cytometric assessment of sperm quality

Various biomarkers can be identified by probes or dyes for the flow cytometric analysis of sperm cell properties (Robles and Martínez-Pastor, 2013) such as viability, acrosomal status, and DNA integrity (Purdy and Graham, 2004, Sellem et al., 2015). Biomarkers can identify sperm cell attributes based on their ability to target and bind to proteins and lectin ligands that are specific to these attributes (Sutovsky et al., 2015). The optimal flow cytometric probe possesses a high degree of reactive sensitivity and specificity at low concentrations in detecting its complementary sperm cell molecule, is chemically well defined, easy to load into organelles, and non toxic (Wardman, 2007).

1.8.3.1.   Plasma membrane integrity

An intact plasma membrane is a prerequisite for viability as it keeps vital intracellular components in place and protected. In addition, the plasma membrane is essential for fusion of the gametes during fertilization. Flow cytometry has been proven to be a suitable tool for characterization of plasma membrane integrity, in combination with dyes such as propidium iodide (PI, ex/em 535/617 nm), Hoechst 33258 (ex/em 352/461 nm), and Yo-Pro-1 (ex/em 491/509 nm) that only bind to sperm cell DNA in sperm cells with damaged membranes (i.e., in moribund or dead cells). These are common viability stains that are membrane impermeable and can be excited by the 488-nm laser in most flow cytometers. As the dyes cross the damaged plasma membranes of moribund cells, they bind to nucleic acids from which light of specific wavelengths are emitted following laser induced excitation. These dyes are not suitable for staining in combination with fixation, as they cross the damaged membranes and are redistributed in the cell suspension following fixation. This does not apply to photoactivated ethidium monoazide (EMA, ex/em 462/625 nm), which binds covalently to DNA, and is also applicable in fixed cells with damaged membranes (Riedy et al., 1991).

Membrane integrity can also be studied using cell permeable acylated membrane dyes (Silva and Gadella, 2006). Amphipathic membrane probes of these dyes can pass through intact membranes of living cells due to their acetyl moieties that deacetylate by intracellular esterases on entering the intracellular space. In cells without intact membranes (dead cells), this reaction will not take place, which allows the dye to escape. In contrast, living cells are loaded with the dye SYBR–14 (ex/em 488/515 nm), which is a viability probe that stains both live and dead cells, but with different intensities (Garner and Johnson, 1995, Silva and Gadella, 2006). For frozen-thawed sperm cells, flow cytometric analysis is complicated by the presence of EY particles that possess scattering properties similar to spermatozoa. The elimination of non sperm events by gating is hampered greatly (Pena et al., 1999). The combination of SYBR–14 and PI is one of the most commonly used staining methods for viability analyses of frozen-thawed semen, as neither of these dyes binds to EY particles (Silva and Gadella, 2006). Alternatively, SYTO60 can be used for the identification of sperm cells in combination with PI (Alm-Kristiansen et al., 2017).

1.8.3.2.   Acrosome integrity

Acrosome integrity is a requirement for fertilization (Silva and Gadella, 2006), as the acrosome content is needed for penetration of the ZP of the oocyte, which precedes fusion of the gametes

(Rodriguez-Martinez, 2006). Acrosomal condition is assessed using fluorochrome conjugated lectins that bind to glucosidic residues on the inside of the acrosome membrane, thus marking sperm cells with compromised acrosome integrity (Martínez-Pastor et al., 2010). *Pisum sativum* (pea) agglutinin (PSA) (Tao et al., 1993) and *Arachis hypogaea* (peanut) agglutinin (PNA) (Tao et al., 1993, Nagy et al., 2003) are commonly used lectins that bind to α-mannose or α-galactose moieties and β-galactose moieties, respectively. These lectins are conjugated with fluorochromes such as fluorescein isothiocyanate (FITC, ex/em 490/525 nm), and R-phycoerythrin (RPE, ex/em 496, 546, 565/578 nm).

1.8.3.3. DNA integrity

Mammalian sperm chromatin is organized in a compact structure to transport DNA, successfully and without damage, throughout the male and female genital tracts. Evidence shows that sustained embryonal development and survival are associated with the degree of spermatozoa DNA integrity (Fatehi et al., 2006). Correlation between DNA integrity in bovine semen and fertility, expressed as non-return rates, has been demonstrated (Waterhouse et al., 2006a). Sperm cell DNA integrity is commonly analyzed by flow cytometry using the Sperm Chromatin Structure Assay (SCSA®) or the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) assay. The SCSA was performed as early as 1980 (Evenson et al., 1980), exploiting the increased susceptibility of damaged DNA to *in situ* acid induced denaturation. In a semen sample, intact, double-stranded DNA can be identified by green light that is emitted following staining with metachromatic acridine orange dye (AO, ex/em 500/525, 650 nm), whereas denatured single-stranded DNA is seen as red light. The resulting ratio of red:(red + green) fluorescence can then be used to estimate a DNA fragmentation index (DFI) (Evenson, 2016). The TUNEL assay applies an alternative method to study sperm cell DNA integrity by fluorescence, using direct conjugation of dUTPs, or indirect biotin conjugation, to the large amounts of exposed 3'-hydroxyl ends that are characteristic of areas containing DNA breaks (Sharma et al., 2013).

1.8.4. Sperm cell ATP

Uterine and oviductal tonus and abdominal contractions assist in the transport of sperm cells through the female genital tract to create a reservoir of sperm cells at the isthmic end of the oviduct (Suarez et al., 1995, Noakes et al., 2009). However, as the sperm cell is released from

the oviductal epithelium, it has been reported that motility are likely to be prerequisites for its ability to penetrate the cumulus matrix of the ovum (Myles and Primakoff, 1997). Sperm motility in mammals is supported by ATP metabolism in the mitochondria, in which protons are pumped across the inner mitochondrial membrane before they return, via ATP synthase, to form ATP from adenosine diphosphate and inorganic phosphate (Garrett et al., 2008). The ATP is then hydrolyzed, powering dynein proteins to slide microtubules in the tail of the sperm cell (Kamp et al., 1996, Minelli et al., 1999). Differences between various rodent species regarding spermatozoa ATP production and swimming performance have been reported, indicating that ATP content could be an interesting parameter concerning fertility (Garrett et al., 2008). In the bovine, it is estimated that a large proportion of ATP production in sperm cells (~75 %) is used to support motility (Bohnensack and Halangk, 1986). In mammalian sperm cells, ATP is synthesized either by oxidative phosphorylation (OXPHOS) or glycolysis, both of which occur in bovine sperm cells (Krzyzosiak et al., 1999). Bovine sperm ATP production, both measured as oxygen consumption and lactate production, has been associated with non-return rates following AI (Garrett et al., 2008). The OXPHOS of spermatozoa mainly occurs in the mitochondria of the midpiece of the tail, whereas ATP production resulting from glycolysis has been shown to take place in the distal parts of the spermatozoon flagellum (Turner, 2003). Findings differ between species regarding which of these metabolic pathways predominates for the synthesis of ATP; whereas both pathways contribute significantly to ATP synthesis in the bovine, in the horse and boar OXPHOS is the major pathway (Marshall et al., 1990, Tourmente et al., 2015). In addition, variation between individuals within a breed has also been reported (Garrett et al., 2008), indicating the complexity of energy metabolism in mammals.

A large proportion of semen doses used for AI in cattle are cryopreserved. During cryotreatment, there is the risk of inflicting damage to sperm cell organelles, including mitochondria, which can impede sperm cell ATP production and motility. Adequate levels of ATP in the sperm cell and its surroundings have the potential to affect semen quality (Luria et al., 2002, Thuwanut et al., 2015), possibly by facilitating sperm cell metabolism of Ca^{2+} as this has been shown to affect both spermatozoa motility and protein phosphorylation, one of the major signaling events during capacitation (Gadella and Luna, 2014).

Although findings on the importance of ATP levels for sperm cell fertilization potential are contrasting, several tests have been developed to measure energy content, most commonly by application of bioluminescence to measure total ATP content (Bennison et al., 2016, Li et al., 2016, Boulais et al., 2017). The CellTiter-Glo® Luminescent cell-viability assay from Promega

is one example of the various proprietary tests for ATP measurement. This assay measures ATP content using an ATP-dependent luciferase reaction, in which the enzyme catalyzes the conversion of luciferin. When ATP is the limiting factor in the luciferase reaction, the intensity of the emitted light is proportional to the concentration of ATP. Measurement of the light intensity using a luminometer permits direct quantitation of ATP (McElroy and DeLuca, 1983). The unit of measurement is a relative luminescence unit (RLU), allowing the calculation of ATP content according to a standard curve.

1.8.5. Use of endoscopy for *in vivo* reproductive research

Fertility results from breedings of a sufficient number of females are considered to be the optimal indicator of male reproductive performance, because such investigations include both female aspects and management factors that have the potential to influence the outcome. However, such investigations of reproductive performance are time consuming and expensive, which is why *in vitro* investigations that are designed to predict male reproductive potential have commonly been implemented (Martínez-Pastor et al., 2010, Puglisi et al., 2012, Sellem et al., 2015). However, such investigations are not capable of fully mimicking the physiological and biochemical interactions between sperm cells and the genital tract environment that result in the selection, storage, and maintenance of sperm viability of specific spermatozoa subpopulations (Suarez, 2016).

Cryopreservation causes significant damage to spermatozoa (Watson, 1990, Yeste, 2016), resulting in artificial remodeling of the sperm surface and internal components (Parks and Graham, 1992), possibly compromising their ability to survive. Such changes in sperm viability have been problematic to investigate due to the difficulty of replicating *in vitro* studies using models that mimic *in vivo* conditions, and the absence of techniques for the *in vivo* study of sperm following AI. In the past decade, however, researchers have made progress in the development of different methods for *in vivo* studies of sperm in the female genital tract (Druart et al., 2009, Rickard et al., 2014, Garcia-Pena et al., 2017, Radefeld et al., 2018). By using probe-based confocal laser endomicroscopy, combined with fluorescence staining of sperm cells, it has been possible to monitor sperm transit success through the reproductive tract (Rickard et al., 2014). The same study found indices suggesting that seminal plasma plays an important role in the survival and transport of sperm cells through the cervix of the ewe.

Furthermore, endoscopy in cattle, combined with AI, has previously been used successfully as a minimally invasive technique for research in bovine reproduction (Garcia-Pena et al., 2017, Radefeld et al., 2018). Significant reductions in the number of sperm cells have been observed during their travel to the site of fertilization following standard intrauterine AI in cattle (Rath et al., 2008). For this reason, recent research has examined the feasibility of performing endoscopy mediated intratubal insemination in the near vicinity of the site of fertilization (Radefeld et al., 2018). The researchers found that intratubal inseminations can be performed successfully with the guidance of transvaginal endoscopy, and that both sperm dose and time of insemination are factors that are potentially associated with reproductive success. Furthermore, it was shown that inseminations with sex sorted sperm cells could be successfully applied. A common trait among these imaging techniques is that they enable the study of semen in an *in vivo* environment that is biologically relevant.

2. Knowledge gaps and aims of the thesis

The long-term effective selection for increased milk production in Holstein cattle has been associated with a decline in reproductive performance. At the same time, selection for fertility has been prioritized in the breeding program of Norwegian Red cattle, leading to a global interest in Norwegian Red genetics and an increase in the use of Norwegian Red semen for crossbreeding. The increasing size of dairy herds, internationally and in Norway, has led to challenges in reproductive management, particularly concerning the detection of estrus and timing of AI. In response to these challenges, the SV immobilization technology has been developed to enable the prolonged release of sperm cells in the female, thereby increasing the flexibility of the timing of AI. However, continuous work towards the improvement of the reproductive performance of SV semen and sperm quality is necessary. Consequently, research that contributes to the elucidation of the reproductive characteristics of cattle and their response to the use of SV semen for AI is of considerable interest to dairy farmers.

The overall aim of this thesis was to acquire more knowledge about the SV alginate gel, particularly concerning dissolution, sperm quality (after immobilization and cryopreservation), and fertility following AI performed at normal timing relative to ovulation. In addition, it was desirable to learn more about the time to ovulation of Norwegian Red following estrous synchronization and induction of ovulation.

The first objective was therefore to compare sperm quality measured as ATP content, viability, acrosome intactness (Papers I and II), DNA integrity (Paper II), total motility, progressive motility, motility kinematic parameters (Papers I and II), and motility subpopulations (Paper II), for spermatozoa processed by the SV technology and the standard processing method, post-thaw and after thermal stress.

The second objective was to compare the fertility potential of semen processed by the SV technology using two different sperm cell concentrations and standard processed semen in a blinded field trail (Paper I).

The third objective was to assess the response of Norwegian Red heifers and cows to estrous synchronization; here the time elapsing between induction of ovulation by a GnRH treatment and ovulation was investigated by transrectal ultrasound following $PGF_{2\alpha}$ synchronization (Paper III).

The fourth objective was to use *in vivo* endoscopy to study the dissolution of the SV gel (Paper IV).

3. Results – summary of papers

Paper I: Comparison of adenosine triphosphate content, motility, and field fertility of immobilized and conventionally cryopreserved semen of Norwegian Red bulls

Fertility (non-return rate after 56 days, NR56) was examined for SpermVital (SV) processed semen and conventionally processed semen in Biladyl (B) extender. *In vitro* sperm quality post-thaw and after thermal stress, and potential correlations between *in vitro* sperm parameters and NR56, were assessed. Ejaculates from 16 Norwegian Red young bulls were split into three and processed as B semen (B15; 15×10^6 spermatozoa/dose) or by the SV technology (SV25; 25×10^6 spermatozoa/dose or SV15; 15×10^6 spermatozoa/dose) before cryopreservation. A total of 1400 semen doses were produced per bull and distributed throughout Norway for a blinded field trial. Fertility was recorded as NR56 after first AI (n = 7155). No differences in NR56 were detected in the field trial; least square means were 75.5% (B), 75.6% (SV25), and 74.8% (SV) (p > 0.05). Two ejaculates from each bull were randomly selected for *in vitro* experiments. B and SV semen samples were analyzed for motility by CASA, viability and acrosome integrity by flow cytometry, and ATP content by bioluminescence assay post-thaw and after thermal stress. Although there were no differences in total motility and progressive motility post-thaw (p > 0.05), after three hours incubation at 38 °C sperm motility and progressivity were higher for SV semen than for B semen (p < 0.05). The percentage of acrosome-intact live sperm cells was higher for SV semen than B semen at all time points analyzed (0 hours, 3 hours, 24 hours, p < 0.05). Whereas B semen showed higher ATP levels at 0 hours (p < 0.05), SV semen had higher ATP levels after 3 and 24 hours (p < 0.05). No association was detected between *in vitro* parameters and NR56 (p > 0.05). In conclusion, SV15, SV25, and B15 semen seemed to be associated with equal levels of fertility. However, there were differences in sperm quality, as measured *in vitro*, with sperm in SV semen having higher motility, viability, and ATP levels after thermal stress (p < 0.05).

Paper II: Post-thaw quality of spermatozoa immobilized in alginate gel before cryopreservation

The effects of cryopreservation and immobilization of semen in alginate gel were evaluated by assessment of sperm quality and the distribution of spermatozoa into different motility subpopulations. Semen was collected from 16 Norwegian Red bulls and six bulls from other breeds (Brown Swiss, Red Holstein, and Limousin). For the other breeds, two ejaculates per

bull were divided into two aliquots and processed in Triladyl (T) extender or immobilized in an alginate gel by the SpermVital (SV) technology before cryopreservation. Post-thaw (T0), sperm motility was studied by CASA, while viability, acrosome intactness, and DNA fragmentation were investigated by flow cytometry, and ATP levels were determined by a bioluminescence assay. Furthermore, viability and motility were evaluated after incubation in oviduct like conditions at T0 and after three hours (T3). Post-thaw percentage acrosome intact live (AIL), total motility, and progressive motility were higher for SV than T spermatozoa (p<0.05). However, neither ATP level nor percentage DFI differed significantly between SV and T spermatozoa. After thermal stress, ATP, AIL, total motility, and progressive motility were higher for SV than for T sperm cells (p<0.05). Division of sperm cells into motility subpopulations revealed that the proportion of rapid progressive spermatozoa was higher at T0 and maintained at higher levels over time for SV sperm cells. Likewise, Norwegian Red SV sperm cells sustained a higher amount of rapid progressive spermatozoa than conventionally processed semen. In summary, SV spermatozoa had enhanced viability, ATP content, total motility, and rapid progressive motility. The study indicates that the SV technology is suitable for cryopreservation of semen from different breeds.

Paper III: Ovarian follicular response to estrous synchronization and induction of ovulation in Norwegian Red cattle

In this study, ovarian follicular growth and ovulatory response to estrus and ovulation synchronization were examined in Norwegian Red heifers and cows. Estrous cycles in 34 heifers and 10 cows from 4 herds were synchronized with two $PGF_{2\alpha}$ analog treatments 11 days apart, followed by GnRH analog treatment for induction of ovulation. Thereafter, the ovaries were examined by ultrasonography at 3-hour intervals until ovulation. The luteolytic effect of the $PGF_{2\alpha}$ analog was verified in 9 of 10 cows by the progesterone content in milk. Maximum physical activity of the cows occurred on average 69 hours after $PGF_{2\alpha}$ analog treatment. An ovulatory response was recorded in 95.5% (42/44) of the animals. A significant difference in follicle size at ovulation was found between 2 of the herds. Animals with medium sized and large follicles and heifers aged >16 months ovulated earlier than other animals. The sequence of treatments that was applied in this study was shown to be effective for synchronizing and inducing ovulation within a relatively narrow time interval in Norwegian Red heifers and cows, and this is consistent with findings in other cattle breeds.

Paper IV: Studies of gel with immobilized semen by intrauterine endoscopy post artificial insemination

The *in vivo* dissolution of SpermVital (SV) alginate gel was examined over time by endoscopy and the quality of spermatozoa was assessed following *in utero ex vivo* incubation of the gel. *In vivo* endoscopy showed SV gel in the uterus 3, 6, 20, and 24 hours after AI, demonstrating the potential for release of spermatozoa to the uterus throughout this period. *In utero, ex vivo* incubation of the semen demonstrated that high motility and viability of sperm cells were sustained following overnight incubation. The results indicate a gradual release of immobilized sperm cells expressing high reproductive potential, which possibly results in an extended window of time for successful AI in cattle.

4. General discussion

4.1. Methodological considerations

The results presented in this thesis are based on 4 studies. Papers I, III, and IV were based on a combination of *in vivo* and *in vitro* research on Norwegian Red females and semen, whereas Paper II was based on *in vitro* research, including semen from Norwegian Red cattle and bulls of other breeds. Implications concerning the choice of materials and methods are presented in the following. A separate section of the thesis dedicated to Papers I-IV provides a detailed description of the materials and methods applied in each study.

4.1.1. Experimental design and statistics of the *in vivo* studies

4.1.1.1. The bull fertility trial

As reviewed by Amann et al. (2005), many of the studies that assess field fertility and its association with sperm quality are deficient regarding experimental design, thus resulting in vague conclusions. In the experimental design of the bull fertility trial of Paper I, NR56 was used as the fertility measure of semen from Norwegian Red young bulls. The reliability of NR56 data depends on: 1) the number of AIs per bull, ejaculate, or batch of production, and 2) the method of reporting inseminations, because the quality of AI data is dependent on the personnel who report the breedings and the extent of subsequent data validation. In Norway, the breeding personnel are reimbursed by Geno, and this motivates accurate reporting to the company's AI recording database. The data are then subjected to several tests for validation before they are entered into the NDHRS, a process that ensures the reliability of the data included (Espetvedt et al., 2013). Multiple parameters of importance for the estimation of fertility, such as herd and individual ID, parity, and recordings of disease of the bred female (Jansen and Lagerweij, 1987, Foote, 2003, Berry et al., 2011) are combined with bull ID, semen batch number, date of AI, and the ID of the AI technician in the NDHRS. The well-structured organization of recordings arranged by these parameters in the NDHRS facilitates correct assessment of their influence on the outcome of AI and, subsequently, an accurate estimate of bull fertility.

In the field fertility trial of Study I, no differences in NR56 between different semen extenders and sperm cell concentrations were found, nor was any association between NR56 and sperm cell quality parameters detected. In this case, the AIs were performed at standard timing relative to ovulation, which was based on observations of estrous behavior. These results concur with those from previous research comparing NR56 of SV semen (25×10^6 spermatozoa per AI dose)

to conventional semen (Standerholen et al., 2015). Nevertheless, the designs of AI trials that are described in the current literature (Standerholen et al., 2015, Alm-Kristiansen et al., 2017) have not been fully capable of revealing the advantages of SV semen compared with standard processed semen following early AI. For example, further investigations, including early, single inseminations with standard processed semen, are required to enable the potential of using SV semen for early AI to be fully revealed. However, if a study included early inseminations with SV semen and standard processed semen was used for control, reduced fertility would be expected for the control group, and this would probably require financial compensation of farmers.

Non-return rates for a male or a group of females have been considered to be a reasonably accurate indicator of reproductive performance. However, pregnancy rates are reported to be approximately 10 percentage points lower than non-return rates in previous research including Norwegian Red cattle (Garmo et al., 2008). In the fertility trial of Study I, inadequate reproduction management and monitoring for estrus are identified as potential reasons for imprecise estimation of non-return rates. However, the Geno distribution system for semen was applied in the fertility trial, resulting in a study population consisting of more than 3000 Norwegian Red dairy herds dispersed throughout Norway. It is therefore likely that any adverse effects associated with herd management or the AI technician were minimized, obviating inclusion of them in the statistical models. Association has been found between season of semen collection and semen quality (Graffer et al., 1988) and calving rate, but not NR56 (Haugan et al., 2005), in Norwegian Red bulls. In the present study, semen was collected during the autumn, which is why season of semen collection was not included in the statistical models. Parity and season (month) of AI have been reported to be associated with the outcome of AI (Haugan et al., 2005), which is why these effects were included in the statistical models of Study I. Furthermore, the bulls in the study were selected from a pool of individuals with sperm concentrations > 390 million/mL, motility > 70%, and normal morphology > 85%. The study could have been performed by including a group of bulls with high fertility and another group with low fertility, possibly adding contrast to the expression of the assessed *in vitro* parameters. However, due to the overall high fertility of the bulls and a lack of a noteworthy contrast in fertility, they were randomly selected. The study population of Study I consisted of 3298 Norwegian Red dairy herds, in which 7155 inseminations with semen from 16 bulls were performed in females of parity 1-9. In order to avoid biologically atypical females, only cows within the interval of 28 – 180 days in milk were included.

4.1.1.2. The estrus-synchronization study

Several studies led to the discovery and assessment of ovulation-synchronization programs used before timed AI in cattle (Giordano et al., 2012, Fricke et al., 2014, Stevenson and Britt, 2017). Study II describes the responses of Norwegian Red cattle after application of a two dose $PGF_{2\alpha}$ /GnRH-based protocol in 34 heifers and 10 cows in 4 herds during 3 seasons. This study provided results indicating that ovarian follicular responses in Norwegian Red are similar to those responses reported in Holsteins (Pursley et al., 1995). The included herds were typical of Norwegian dairy farms, being family owned and with 30–50 lactating cows. Sampling of animals was restricted by the number of accessible herds, their limited size, and some skewed distribution of calvings throughout the year. In consequence, the design of Study II was not optimal as potential confounders could not be sufficiently accounted for. Furthermore, monitoring of ovarian follicular development by transrectal ultrasound has previously been analyzed for its precision and its potential for inflicting adverse effects upon the animal being examined. Evidence shows that the technique is highly accurate, commonly with precision as in the present study, and, provided that handling is skilled, is without adverse clinical effects (Roelofs et al., 2004).

4.1.2. Technologies for assessment of sperm quality

4.1.2.1. Computer-assisted sperm analysis

Results from studies on the relationship between sperm cell motility data and fertility are inconsistent, with some reporting an association (Correa et al., 1997, Zhang et al., 1998a, Januskauskas et al., 2000), whereas others do not (Andersson et al., 1992) (Januskauskas et al., 1999). Nevertheless, sperm motility is largely considered to be an essential parameter for the assessment of semen quality, either by subjective evaluation or by automated semen analysis using an objective approach (Verstegen et al., 2002). CASA ensures a high degree of objectivity and is the preferred choice in large AI facilities. When the subjective method of motility assessment is used, it is difficult to separate variation caused by the operator from variation resulting from operator fatigue (Harstine et al., 2018). The CASA technology is used for semen analysis in multiple species, enabling precise and highly repeatable measurements of numerous specific sperm motion characteristics (Amann and Waberski, 2014). The use of CASA has contributed significantly to the removal of bias and variability caused by subjective assessment of semen quality by manual microscopic evaluation (Verstegen et al., 2002). Nevertheless, the outcome of semen analysis by CASA can be influenced by various factors, e.g., sperm cell

concentrations (Rijsselaere et al., 2002, Rijsselaere et al., 2005). Furthermore, the validity of CASA measurements depends on consistency in the preparation of semen samples. Use of fixed depth chamber slides has been recommended in order to standardize the inspection of samples in relation to the range volume and sperm cell concentration (Harstine et al., 2018). High concentrations of sperm cells during CASA will crowd the semen analysis chamber, leading to numerous collisions between sperm cells, and these will hamper the measurement of progressive motility (Rijsselaere et al., 2002, Hoflack et al., 2005). To prevent inaccurate measurements by CASA, semen samples used in the sperm velocity assessments of Papers I and II were maintained at concentrations within 20 to 30×10^6 spermatozoa/mL, which is within the range of general recommendations (Yeste et al., 2018).

Although incorporation of CASA routines into a semen quality program at commercial AI facilities provides objective assessment of sperm motility, cost-benefit considerations and examination of associations between CASA motility measurements and alternative methods of quality assessment, e.g., subjective motility evaluation or flow cytometric assays, should be evaluated before CASA replaces quality tests that have been previously validated. Although CASA is capable of generating large amounts of data for accurate determination of sperm motility characteristics, including various kinematic parameters, well-approved studies demonstrate somewhat diverging results concerning their significance in relation to the assessment of sperm quality (Harstine et al., 2018).

4.1.2.2.   Flow cytometric analysis

Flow cytometry enables the effective sorting and counting of many cells by highly specific biomarker detection, and allows a high level of experimental repeatability, with simultaneous multiparametric analysis of the physical and chemical characteristics of up to several thousand cells per second. To ensure reliable data output from the flow cytometer, appropriate calibration of the instrument is necessary before running cell samples. Optical alignments using fluorescent validation beads were run daily in advance of the flow cytometry analyses used in Papers I and II. Spermatozoa stained with the relevant fluorochromes used in a specific experiment, termed positive controls, were used in Study II for PMT adjustment and for spectral compensation of fluorescence emission overlap between detectors in multifluorochrome assays.

Data from cell samples that are generated by flow cytometry are usually collected using signal gating, by which particles with light scatter properties that differ from those of sperm cells are

eliminated from the analyses. Samples including particles with light scattering properties resembling sperm cells, such as cytoplasmic droplets, cell debris, or extender components (e.g., EY), may still be included as sperm events. Such recordings result in overestimation of the negative or positive fluorescence population, depending on the binding properties of the dye in question (Petrunkina and Harrison, 2011). A combination of fluorochromes is therefore required to avoid incorrect assessment of sperm cell events, as in Studies I and II by using SYTO® 60 Red Fluorescent Nucleic Acid Stain (SYTO60, S11342, Invitrogen), which stains the DNA of both live and dead sperm cells in conjunction with propidium iodide (PI, P4864, Sigma-Aldrich), which is membrane impermeable and therefore binds only the DNA of sperm cells with deteriorated membranes (dead or dying cells).

By using flow cytometry in Study I, significant differences in sperm cell quality were demonstrated between semen extender treatments; higher levels of spermatozoa viability (AIL) were demonstrated post-thaw and over time in SV semen than in conventional Biladyl® semen. However, these differences were not correlated to fertility as measured as NR56, possibly due to the relatively high number of sperm cells per dose and that viability is regarded as a compensable trait (Amann and DeJarnette, 2012). Furthermore, the bulls of Paper I displayed similar levels of fertility. Research including bulls of high and low fertility has demonstrated correlations between field fertility based on NR56 and intactness of the acrosomal membrane (Sellem et al., 2015, Kumaresan et al., 2017).

4.2. Relevance of the *in vivo* assessment of reproductive physiology and dissolution of SV gel in response to estrous synchronization in Norwegian red heifers and cows

For the past 50 years, Norwegian Red cattle has been the predominant dairy breed in Norway, a period during which the Norwegian Red breeding goals have largely focused on fertility and health, along with milk production. As a likely consequence of the long-term selection for improved fertility, recent research, including Study I, demonstrates highly satisfactory fertility of Norwegian Red cattle, with an average NR56 of 72.5 % (Standerholen et al., 2015, Geno, 2016, Berg et al., 2018). Following decades of effective selection for increased milk production in Holstein, adverse effects of associated responses concerning health and fertility have been observed (Pryce et al., 2014). In consequence, crossbreeding with Norwegian Red has become increasingly popular over the last 2 decades, due to its resolving effect on the decline in the health and fertility of Holsteins (Heins et al., 2006, Heins and Hansen, 2012). Thus, more than half of the 1.3 million doses of Norwegian Red semen that are produced annually are exported

to the international market (Geno, 2018). This represents a significant dissemination of Norwegian Red genetics, and this spurs the need for knowledge about the reproductive function and performance of the breed. Furthermore, the SV immobilization technology is applied in approximately 14% of the first AIs on the Norwegian market (personal communication, Steig, N.C., January 10, 2020), on top of which the technology is exported to countries such as Germany, Switzerland, Poland, Spain, Austria, Finland, and Belgium. Correct timing of AI in relation to estrus and ovulation is imperative for reproductive success. The use of SV semen for AI is assumed to enable the gradual release of sperm cells over a prolonged period *in utero* after AI (Kommisrud et al., 2008), thus making the timing of AI more flexible. It is therefore of great interest for dairy farmers from Norway, and also from countries that import the SV technology or Norwegian Red genetics (or both), to learn more about the reproductive characteristics of Norwegian Red heifers and cows, and their responses to the use of SV semen for AI.

Furthermore, reproduction management is of utmost importance to economic outcome, and there is a tendency towards an increase in the application of estrous synchronization worldwide. Up until now, hormone treatment has been restrictively used in Norway. Fertility traits have been included in the breeding programs for almost 50 years (Refsdal, 2007a), resulting in high and sustained fertility in Norwegian Red, despite increasing milk yield. This fact has reduced the need for estrous synchronization in Norway to some extent. However, there is a tendency for herd size to be increasing in Norway, making knowledge concerning the response of Norwegian Red cattle to estrous synchronization more relevant. Ovarian follicular development of cattle following spontaneous estrus (Mihm et al., 2002, Forde et al., 2011) and estrous synchronization are widely described, particularly for the Holstein breed (Pursley et al., 1995, Saumande and Humblot, 2005, Wiltbank and Pursley, 2014). However, differences exist in the duration and appearance of estrus between Holstein and Norwegian Red (Sveberg et al., 2015). A positive association between the size and maturity of the preovulatory follicle and fertility (pregnancy rate) in cattle has been reported (Waldmann et al., 2006, Perry et al., 2007). Although solid evidence of reproductive physiology concerning ovarian follicular development is established for Holstein cattle and for other breeds, characterization of the growth (size) of the ovulatory follicle over time in Norwegian Red has been limited. The heifers and cows in Study III were purebred Norwegian Red from herds that are representative, in terms of herd size and milk yield, of Norwegian dairy farms. The results of Study III showed that more than 95 % of the heifers and cows responded to estrous synchronization and induction of ovulation by ovulating within a relatively limited time interval, indicating that a standard ovarian

synchronization protocol could be successfully applied in Norwegian Red cattle. Considerations concerning the validity of the results presented in Study III have been previously discussed (Section 4.1.1.2.).

The relatively high fertility rate (NR56) that was demonstrated, regardless of semen processing method, in the Norwegian Red cattle of Study I has previously been reported for conventionally processed semen (Refsdal, 2007b), likely due to decades of systematic breeding for fertility traits in this breed (Garmo et al., 2008). In Study I, equally high fertility (NR56) was reported in Norwegian Red when using SV processed semen following AI at normal timing, which concurs with the results of studies in which SV semen was used for AI with standard timing (Standerholen et al., 2015) and also after early AI following estrous synchronization (Alm-Kristiansen et al., 2017). The latter more recent study showed promising fertility results (pregnancy rate) following early AI with SV semen.

However, additional information concerning the reproductive performance of SV is desirable. To date, bull sperm cell quality has been systematically characterized through a variety of *in vitro* parameters, commonly by the assessment of sperm viability (Martínez-Pastor et al., 2010) and motility (Amann and Waberski, 2014). Although Studies I and II demonstrated enhanced sperm motility, ATP content, and viability with SV semen, the NR56 of SV semen was similar to that of standard processed semen. The lack of association between sperm *in vitro* parameters and NR56 suggests a need for a different approach concerning the design of future studies. The inclusion of semen from groups of bulls with low and high fertility in an AI trial, combined with *in vitro* sperm quality examination, could result in the detection of differences in fertility that can be associated with sperm quality, as previously reported (Gliozzi et al., 2017). Furthermore, differences in fertility due to a contrast in sperm quality concerning compensable traits could be identified by lowering the sperm cell dose concentrations into the dose-response area of the fertility curve (Saacke, 2008).

The assumed advantage of the SV immobilizing technology is the increased flexibility regarding the timing of AI relative to ovulation, owing to the release of highly motile and viable sperm cells from the gradually dissolving SV alginate gel, as previously observed *in utero ex vivo* (Alm-Kristiansen et al., 2018a). However, evidence shows that fertility in cattle is affected by reproductive management factors, e.g., the ability to detect estrus and the correct timing of AI relative to estrus and ovulation (Amann et al., 2018), whereas after AI, sperm cells are influenced by numerous biological interactions in the female (Rath et al., 2008, Amann and DeJarnette, 2012).

The development of a technique that involves *in vivo* uterine incubation of semen could provide an improved method for evaluation of sperm quality. The initial solid consistency and gradual dissolution of the SV alginate gel following AI, enables the retrieval of SV semen by gentital tract endoscopy (GTE) at least 24 hours after insemination, as demonstrated in Study IV. In addition, attempts to detect the semen for longer periods of time indicated promising results. However, the presence of solid SV gel is a requirement for meaningful application of GTE in its current form, making the method irrelevant in combination with standard processed semen. During Study IV, attempts were made to extract SV semen from the uterus at certain time points after AI. However, due to practical limitations concerning the operation of accessory endoscopic tools, no findings were made with this approach. Further studies are necessary to determine the full potential of the GTE technique for evaluation of the SV gel. An improvement in SV gel staining to facilitate endoscopic retrieval and development of a method for gel extraction could enable *in vitro* analysis of the SV gel following AI. Nonetheless, procedures involving intrauterine endoscopy after AI in the ewe, including laparotomy and exteriorization of the anterior genital tract, have provided interesting findings by using probe-based confocal laser endomicroscopy for the assessment of *in vivo* sperm functionality (Rickard et al., 2014).

The abilities of sperm cells to endure cryopreservation vary between species (Parks and Graham, 1992), breeds, and individuals (Holt, 2000b, Waterhouse et al., 2006b). For example, semen from the bull and ram demonstrate a particularly high cryotolerance compared with semen from the boar (White, 1993), differences that indicate the potential for improvement in methods of semen processing. Cryopreservation of semen causes substantial damage to the spermatozoa of various species, inflicting a change in the degree of expression of sperm surface proteins between fresh and processed spermatozoa (Chen et al., 2014, Pini et al., 2016, Pini et al., 2018, Pérez-Patiño et al., 2019). The changes in surface molecules that are imposed on spermatozoa following cryodamage affect their interactive competence and viability within the genital tract of the female (Rickard et al., 2019). Experiences following the application of GTE in Study IV revealed a potential for further development of the method to perform intrauterine studies of SV processed semen, also presenting the opportunity to evaluate the influence of different semen processing methods on sperm cell quality.

4.3. Methods of semen processing using slow release technologies

In 1985, Nebel and associates proposed a controlled release of spermatozoa by their entrapment in capsules composed of gels containing alginate, divalent cations (typically Ca^{2+}), and poly-L-

lysine in an alginate polymer chain (Nebel et al., 1985). Although the technology of alginate encapsulation of bovine spermatozoa has evolved and shown promising results (Nebel et al., 1993, Conte et al., 2003, Weber et al., 2006), it has yet to be commercialized, likely due to technical challenges during processing.

In contrast with semen processing using encapsulation, methods of embedding sperm cells in a solid substance have been developed. It has been reported that rabbit sperm immobilized in an extender combining standard boar diluent and gelatin, display a higher degree of viability and acrosome intactness than conventional semen (Nagy et al., 2002). Improved sperm quality is possibly promoted by the solidifying effect of gelatin through its effect of reducing fluctuations in pH within the semen dose caused by the metabolic products of spermatozoa (Levis, 2000). The SV technology offers a similar embedding of sperm cells in a solid material, but this is composed of an alginate gel matrix (Kommisrud et al., 2008), rather than animal-based gelatin. Immobilization of sperm cells in the SV gel restricts their opportunity to move and likely supports the conservation of energy, as indicated in Study I, where motility and ATP levels of standard processed semen were compared with those of SV semen. Furthermore, the immobilization of spermatozoa in an alginate gel provides an environment that is potentially similar to that of the epididymal cauda regarding the degree of confinement and viscosity (Eksittikul and Chulavatnatol, 1986); an environment in which sperm cells are conserved until ejaculation. Previous studies evaluating the reproductive potential of SV semen in AI trials have discussed the importance of correct experimental design with respect to the timing of AI relative to the time of identification of estrus (Standerholen et al., 2015, Berg et al., 2018). Similar to the results obtained with semen processed by the encapsulation technology of Nebel et al. (1994), AIs performed in the latter parts of estrus would be potentially unfavorable to fertility when using the SV alginate gel and slow release of semen. As discussed in the evaluation of experimental design (Section 4.1.1.), a study comparing early AIs with SV and standard processed semen would be relevant for evaluation of the SV technology, but expensive.

4.4. Sperm quality in relation to fertility

In modern livestock breeding, bulls that have been proven suitable are kept at well-established AI stations to produce AI doses for the market from semen that has been pre-selected on the basis of a set of quality parameters. Breedings with inferior semen containing low sperm cell concentrations with poor motility or morphology and with genetically undesirable traits are therefore generally avoided. In the proven bulls of modern livestock breeding, the emphasis of

semen assessment is therefore to discover detectable variations in reproductive performance that can be associated with fertility.

In the bull fertility trial of Study I, differences in NR56 were not found when using different semen extenders and sperm cell concentrations, nor was any association detected between NR56 and the sperm cell quality parameters viability, motility, and ATP content. In this study, fertility after AIs with SV semen containing fewer sperm cells (15×10^6 sperm cells) compared with the commercially available SV semen (25×10^6 sperm cells) was investigated, and the same NR56 levels were achieved using the SV semen with 15×10^6 sperm cells. These levels of NR56 can possibly be explained by the fact that differences in fertility between bulls are often regarded as being compensable by the minimum number of sperm cells required to reach maximum fertility. This implies that SV insemination doses with 15×10^6 sperm cells per SV semen dose yield fertility levels that lie within the asymptotic area of the fertility curve. In future trials using SV semen, further examination of the effect from varying the numbers of cryopreserved sperm cells from ~1×10^6 to 20×10^6 per insemination on non-return rates, could provide an approximation of such a threshold value (Amann and DeJarnette, 2012). In the event of uncompensable sperm cell deficiencies, as occurs, for example, with sperm chromatin aberrations, a reduction in fertility would be anticipated, regardless of sperm cell dose concentration. In Study I, the overall NR56 (72.2 %) and NR56 after AI with SV semen were equal and relatively high; these values are similar to previous findings in Norwegian Red (Standerholen et al., 2015, Geno, 2016). It is therefore unlikely that the SV technology causes significant sperm cell DNA damage during preservation compared with standard semen processing. This assumption is supported by other recent DFI measurements in Norwegian Red semen (Alm-Kristiansen et al., 2018a) and also by the low levels of DNA fragmentation in SV processed semen from other breeds (Study II).

Assessment of sperm quality requires the choice of a reliable fertility outcome variable, such as pregnancy rate or days of non-return to estrus (Garmo et al., 2008) and selection of relevant independent variables for the prediction of fertility. Although associations between single sperm quality parameters and fertility in cattle have been widely demonstrated, evidence shows that by examining the relationship of a combination of parameters with fertility, stronger relationships may be identified (Puglisi et al., 2012, Oliveira et al., 2013, Sellem et al., 2015, Gliozzi et al., 2017, Kumaresan et al., 2017). Although superior levels of viability, ATP content, and motility were observed in SV semen compared with standard processed semen in Study I, an association between fertility (NR56) and sperm quality parameters was not found, either

individually or combined. Standerholen et al. (2015) demonstrated similar fertility between SV semen with 25×10^6 sperm cells per dose and standard processed semen with 12×10^6 sperm cells per dose. Similarly, there was no difference in non-return rates between SV and standard processed semen in the AI trial of Study I, using semen with 15×10^6 sperm cells per dose in both types of semen. However, the viability of the SV semen in the former study was inferior to that of standard processed semen (Standerholen et al., 2015), whereas the opposite relationship was found in Study I, indicating an improvement in sperm quality in SV processed semen. Reducing sperm cell concentrations in the SV semen of Study I to 15×10^6 sperm cells per dose without compromising fertility implies that this is a sperm cell concentration outside the dose-response area of the fertility curve (i.e., still within the asymptotic area of the curve). Improved SV sperm quality, expressed as enhanced viability, is another possible explanation for the sustained fertility despite reduced sperm cell concentrations. In Study IV, GTE following AI with SV semen demonstrated the gradual dissolution of SV gel, resulting in the prolonged release of sperm cells for at least 24 hours. However, due to the gradual release of spermatozoa and to ensure enough available sperm cells throughout this period, until now SV semen has been produced with elevated sperm cell concentrations, i.e., $>20 \times 10^6$ sperm cells per dose. The AIs of Study I were performed using standard timing. It has been reported that early AIs with SV semen containing 25×10^6 sperm cells per dose result in similar fertility (NR56) as double AIs with standard processed semen on two consecutive days (Alm-Kristiansen et al., 2017). Furthermore, the prolonged dissolution of SV gel shown by GTE in Study IV, along with the improved sperm quality of SV semen demonstrated in Study I and in earlier research (Alm-Kristiansen et al., 2018a), suggests that it would be worthwhile to conduct an early AI trial to investigate fertility after inseminations with lowered sperm cell concentrations per dose ($<15 \times 10^6$).

4.4.1. The relevance of sperm cell motility and ATP contents

During standard AI in cattle, semen is deposited in the caudal section of the uterine body. In order to enable fertilization, the sperm cells must traverse the female reproductive tract to the site of fertilization at the ampullaristhmic junction of the oviduct (Rath et al., 2008). Although abdominal contractions in the female play a major role in the transport of semen after deposition, a broad base of evidence shows that spermatozoa motility is positively associated with *in vivo* fertility (Barratt et al., 1993, Macleod and Irvine, 1995, Farrell et al., 1998, Puglisi et al., 2012, Sellem et al., 2015) and is an indicator of fertilization potential (Holt et al., 1985,

Chan et al., 1989, Sukcharoen et al., 1996, Mortimer, 1997, Rodriguez-Martinez and Barth, 2007, Muiño et al., 2008). In addition, physical interactions between sperm cells and the genital tract lining, which include progressive sperm cell movement in response to variations in the viscoelasticity of fluid and anatomical microarchitecture, have been shown (Suarez, 2016).

Despite broad evidence showing an association between motility and levels of reproductive success, no such relationships were found between NR56 and motility, progressive motility, or kinematic parameters of B15 or SV15 semen in Study I. This absence of association could be due to the relatively high number of sperm cells per dose and the compensable nature of sperm motility characteristics (Saacke, 2008, Amann and DeJarnette, 2012). The lack of association with NR56 can also be explained by the low range of variation in fertility among the Norwegian Red bulls used in the study (inclusion criteria for semen are described in Section 4.1.1.1). In contrast with the results of Study I, associations between fertility and sperm motility have been reported in studies that include groups of bulls with both high and low fertility (Sellem et al., 2015, Gliozzi et al., 2017). In addition, sperm numbers per AI dose in the latter study were just over the threshold amount of post-thaw, viable sperm per AI dose, possibly leading to further accentuation of differences in fertility that can be related to diversity in sperm motility between bulls.

However, the comparison of sperm motility between SV15 and B15 semen in Study I revealed a significantly higher proportion of motile and progressively motile SV15 spermatozoa following thermal stress for 3 hours post-thaw. These findings concur with previous evidence (Alm-Kristiansen et al., 2018b) and the results of Studies II and IV. GTE was used *in vivo* in Study IV to observe SV semen following AI. A gradual dissolution of the alginate gel was visualized in 4 recordings taken between 3 and 24 hours after AI. The gradual dissolution of the alginate gel was also observed *ex vivo* after uterine incubation in Study IV, indicating slow release of spermatozoa from the solid alginate gel.

In Study II, superior levels of motility and progressive motility, as measured by CASA following a 3 hour incubation at body temperature, were found in SV sperm cells. Most of the semen samples from the different Norwegian Red bulls included in Study I displayed superior levels, with little variation in motility in those from SV processing compared with standard processing. This pattern recurs in the findings of Study II, in which sperm motility from bulls of other breeds than Norwegian Red was examined, including categorization of sperm cells as described above (Section 1.8.1. Computer-assisted sperm analysis) into four motility subpopulations. The distribution of sperm into different motility subpopulations was compared

between standard processed semen (Biladyl or Triladyl) and SV semen, and also between semen from Norwegian Red bulls and bulls of other internationally prevalent breeds. The proportion of rapid progressive spermatozoa was higher for SV semen than for standard processed semen, post-thaw and following a 3 hour incubation. Other breeds showed a similar pattern in the distribution of sperm cells into the different subpopulations following a 3 hour incubation at body temperature, but the decline in RapidP sperm over time was lower in Norwegian Red semen than in semen from the other breeds of bull. Although the importance of grouping sperm cells into different motility subpopulations is not entirely understood, the proportion of rapid progressive cells seems to have some relevance to sperm cell quality. This is supported by findings that have shown that ejaculates with elevated subpopulations of rapid and progressive spermatozoa are less prone to cryodamage, as they demonstrate better post-thaw sperm viability than other groups of sperm cells (Muiño et al., 2008).

Male reproductive efficiency can be assessed by examining the ability of sperm cells to penetrate oocytes in an *in vitro* environment (Taberner et al., 2010). It has been reported that sperm cells from Holstein that swim with a high velocity and degree of straightness are more likely to bind to the ZP during *in vitro* fertilization (Ferraz et al., 2014). Interestingly, RapidP and RapidNP sperm cells in Study II were significantly more correlated with ATP and AIL than sperm cells from the Slow subpopulation. Differences in correlations between subpopulations are interesting, due to their potential association with reproductive performance. Furthermore, contrasts in sperm quality measured over time between standard processed semen and SV semen have been indicated in Studies I and II concerning sperm viability, levels of ATP, and motility. Taken together, these results concerning motility subpopulations found in different cattle breeds indicate an enhanced level of sperm quality following sperm immobilization in alginate gel, and this is possibly related to the cryoprotective ability of SV processing.

Following AI in cattle, spermatozoa travel from the uterine site of deposition to the oviduct. During this journey, the sperm cells undergo a sequence of activities that require energy, such as: capacitation, hyperactivation, acrosome reaction, and fertilization. Therefore, it could be hypothesized that the sperm ATP level is relevant in the characterization of sperm quality and reproductive potential. Interestingly, the ATP content of SV sperm cells has been reported to follow the trend of motility after incubation, showing sustained ATP levels compared with those of standard processed semen (Alm-Kristiansen et al., 2018b); this finding concurs with those of Studies I and II. As expected, the energy levels (ATP) measured in Studies I and II declined following incubation with thermal stress. Whereas post-thaw ATP levels were higher for

standard processed semen than for SV semen in Study I, and were equal between extenders in Study II, after incubation under thermal stress the levels were superior in SV semen in both studies.

Evidence concerning the impact of sperm ATP production on successful fertilization is limited. However, an association between ATP production in spermatozoa and the fertilizing ability of the bull has previously been reported (Garrett et al., 2008). The accessibility of ATP in spermatozoa is essential for the multiple cellular and biochemical processes necessary for successful fertilization, e.g., ion transport, protein phosphorylation (Miki, 2007, Garrett et al., 2008), and capacitation (Visconti et al., 2011). Sperm motility is a requirement for successful fertilization, a relationship that has been demonstrated in various studies (Zhang et al., 1998b, Kathiravan et al., 2008, Puglisi et al., 2012). Previous research has also shown that motility of mammalian sperm cells is associated with sperm ATP content (Januskauskas and Rodriguez-Martinez, 1995). This relationship is likely due to the ATP mediated interaction between dynein (ATPase) and microtubules in the flagellum (Bohnensack and Halangk, 1986). In Study I, an association was not found between ATP and fertility (NR56). The high quality of the included semen from the Norwegian Red bulls (motility $\geq$ 50 %) and high sperm cell concentrations per AI dose ($\geq$ 15 $\times$ 10^6), in combination with the compensable nature of sperm cell motility (Saacke, 2008, Amann and DeJarnette, 2012), are potential reasons for this lack of association. However, the prolonged conservation of energy stores that was demonstrated in SV semen after thermal stress, implies that semen processing by immobilization in alginate gel is beneficial to post-thaw bull sperm quality. It has also been recently reported that cryoinjury inflicted on spermatozoa by semen cryopreservation can result in alterations in sperm energy metabolism (Słowińska et al., 2018).

In Studies I and II, the ATP levels of SV spermatozoa were higher than in conventionally processed sperm cells after thermal stress, even after the transfer of semen from both processing methods to the same type of solution to eliminate the effect of extender. Viability measured as AIL, was also significantly higher for SV semen after incubation. These results are supported by findings from boar semen, in which a positive correlation was found between viability and ATP content (Nguyen et al., 2016). However, evidence of a positive relationship between sperm ATP content and viability can possibly be explained by positive correlations between the proportion of living sperm cells and sperm ATP content, as previously demonstrated during optimization of the CellTiter-Glo® Luminescent cell assay (Standerholen, 2018), which was also used in the current study. It is therefore appropriate to question whether sperm ATP content

is a direct determinant of sperm cell motility or whether it indirectly mediates motility through the general maintenance of multiple cell functions and viability.

Although findings concerning the relationship between spermatozoa ATP content and fertilization potential are diverging (Vigue et al., 1992, Garrett et al., 2008), a positive relationship between levels of ATP in hamster ejaculates and the number of zona free oocytes penetrated by sperm cells has been reported (Comhaire et al., 1983). A state of sperm cell hypermotility is assumed to be required for the attachment and release of spermatozoa to the epithelial lining of the oviduct, an interplay between the sperm cells and the oviduct that has been shown to contribute to fertilization competence (Chang and Suarez, 2012, Suarez, 2016). Activation of spermatozoa by multiple biochemical stimuli (capacitation) (Wassarman, 1999, Miller et al., 2016) from the female genital tract and the oocyte complex has been reported, likely leading to hypermotility following cellular influx of Ca^{2+} (Baldi et al., 1991). Vigorous beatings of the flagella then facilitate attachment of spermatozoa to the uterotubal lining, and penetration of the ZP of the oocyte in the event of fertilization. Interestingly, elevated and sustained levels of spermatozoa ATP content and progressive motility were observed in SV semen in Studies I and II, suggesting that the immobilization technology can contribute to improved fertilization potential of bovine semen. Results of Studies I and II revealed elevated levels of ATP in SV semen compared with standard processed semen after 3 and 24 hours of incubation, indicating an increase in the cryoprotection of spermatozoa ATP producing structures by the SV processing technology.

4.4.2. The significance of sperm cell membrane integrity

Sperm viability is essential for successful fertilization (Hossain et al., 2011). It is therefore useful to consider sperm traits concerning viability in the field of spermatology research (Studies I, II, and IV), as well as in the assessment of semen used for breeding. Sufficient viability of sperm is becoming increasingly relevant with the application of AI doses that contain low concentrations of sperm cells, as seen with GS young bulls (Fair and Lonergan, 2018) and in sexed semen (Seidel and Schenk, 2008).

As multiple interactions between sperm cells and the female genital tract take place after mating or AI, there is a fundamental need for an intact sperm cell membrane (Hung and Suarez, 2010). The process of fertilization consists of several steps that impose specific requirements on sperm functionality: establishment of an oviductal sperm reservoir, sperm capacitation within this

reservoir, gamete recognition, and adhesion of the spermatozoa to the oocyte, which is succeeded by incorporation of the sperm cell into the oocyte cytoplasm (Sutovsky, 2018).

The process of semen cryopreservation inevitably inflicts a certain degree of cryodamage on sperm cells. Normally, 40 to 50 % of sperm cells do not survive cryopreservation, even when standard cryoprotocols are followed (Holt, 2000a, Watson, 2000). However, differences in post-thaw viability of spermatozoa have been reported to be associated with the choice of semen processing method (Layek et al., 2016). Among other reasons, semen processing technologies applying the concept of slow release have been developed to improve post-thaw sperm quality and reproductive performance. Early (Nebel et al., 1985, Nebel et al., 1993) and more recent research studies on semen of cattle and water buffalos (Perteghella 2017) using alginate encapsulation of sperm have revealed similar levels of acrosome intactness and pregnancy rates to that of conventionally processed semen. Similarly, AIs with SV semen embedded in alginate gel resulted in equal NR56 to those of B15 semen in Study I. However, higher levels of post-thaw AIL were observed in SV semen than in standard processed semen following incubation for 24 hours in an environment mimicking oviductal conditions. Despite significant differences in AIL between processing methods, AIL was not associated with NR56. This lack of association can be explained by: 1) the relatively high number of sperm cells per dose and the compensable nature of acrosome integrity (Saacke, 2008), and 2) the limited range in fertility variation among the Norwegian Red bulls used in the study.

Nevertheless, the findings of Study I concerning acrosome integrity concur with previous findings (Alm-Kristiansen et al., 2018b) and the results of Study II. GTE was used *in vivo* in Study IV to observe SV gel following AI, and showed a gradual dissolution of the alginate gel up to 24 hours after AI. Uterine incubation of the SV gel *ex vivo* also showed a gradual dissolution of the gel and that high sperm cell viability was maintained overnight. This indicates a slow release of viable spermatozoa from the solid alginate gel. Moreover, Alm-Kristiansen et al. (2018) reported that post-thaw levels of viable acrosome intact spermatozoa were higher in SV semen than in the first commercialized production line of SV and standard processed Biladyl semen, findings that were supported by a decrease in acrosome reacted dead spermatozoa in SV semen. Similarly, enhanced levels of acrosome intactness, including enzymatic activity, were observed following encapsulation of boar semen compared with standard cryopreserved semen, up to 24 hours post-thaw (Faustini et al., 2004). Furthermore, a study using cryopreserved canine semen to compare processing with alginate gel microencapsulation with standard processing obtained comparable results, with elevated

viability and acrosome intactness in the former after incubation at body temperature (Shah et al., 2011).

It has been speculated whether structural differences in the lipid composition of the sperm cell membrane could play a role in the varying response to cryopreservation between bulls (Lemma, 2011, Grötter et al., 2019), manifested as "good" or "bad" freezers. Findings indicate that specific subpopulations within the same ejaculate are associated with variations in sperm functionality (Thurston et al., 1999), as explained by heterogeneity in spermatozoa head and tail structures (Thurston et al., 2001). In Studies I and II, enhanced viability and acrosome intactness were found in SV sperm cells. Additionally, both the rapid progressive and rapid non progressive motility subpopulations were significantly more correlated with AIL than the slow motility subpopulation when sperm cells were considered irrespective of semen extender (Study II). It has been argued that individual differences in cryotolerance, as observed by variations in the occurrence of subpopulations with high sperm quality, could reflect reproductive performance and provide a criterion for selection (Thurston et al., 1999).

5. General conclusions

In the current thesis, we demonstrated a competitive reproductive performance of SV semen compared with standard processed semen following AI in Norwegian Red cattle, despite using fewer SV processed sperm cells per AI dose than previously. We found that profiles of ovarian dynamics of Norwegian Red heifers and cows are compatible with successful synchronization of estrus and ovulation, thus indicating an interval for the optimal timing of AI. *In vivo, ex vivo in utero,* and *in vitro* studies indicated prolonged dissolution of the SV gel and gradual release of immobilized sperm cells expressing a high reproductive potential, possibly enabling an extended window of time for successful AI in cattle. The findings of the current thesis thus have a direct practical and economic impact concerning the production and use of SV semen.

Comparison of the *in vitro* sperm quality and fertility of immobilized and standard cryopreserved Norwegian Red semen led to the following specific conclusions:

- A reduction in sperm cell numbers in immobilized semen doses does not compromise fertility (NR56); sufficient sperm cells are released from the alginate gel after AI when performed at normal timing
- *In vitro* sperm quality measured as sperm motility, viability, and ATP contents is superior over time for sperm cells immobilized in alginate gel compared with those in standard cryopreserved semen

In vitro characteristics of semen from different cattle breeds following processing with the SV technology:

- *In vitro* sperm quality measured as sperm motility, viability, and ATP contents is superior over time in SV semen compared with standard processed semen following incubation
- The SV technology is suitable for the cryopreservation of semen from different breeds

Characteristics of the ovarian follicular response of Norwegian Red heifers and cows to a double-$PGF_{2\alpha}$/GnRH synchronizing protocol:

- The sequence of treatments was effective in synchronizing and inducing ovulation in the study animals and supports the use of the applied synchronization program as an effective preparation for timed AI
- The results are similar to those obtained from research with other cattle breeds

In vivo appearances of the SV gel with immobilized semen following a double-PGF$_{2\alpha}$/GnRH synchronizing protocol and AI:

- A gradual and prolonged *in vivo* dissolution of SV gel occurs up to 24 hours after AI
- Sustained levels of high sperm motility and viability are demonstrable following overnight *in utero ex vivo* incubation; this indicates prolonged release of immobilized spermatozoa expressing high reproductive potential, possibly resulting in an extended window of time for successful AI

6. Future perspectives

In the present thesis, we demonstrated the successful application of SV processed semen for AI in cattle and superior *in vitro* sperm quality. However, the design of the *in vivo* studies was not optimal for the assessment of SV performance following early AI relative to estrus and ovulation. Given the enhanced *in vitro* sperm quality of SV semen that was demonstrated and competitive fertility that was shown with SV15 (Study I), further reductions in sperm cell numbers per AI dose combined with early timing of AI would be interesting to investigate in the future. Differences in reproductive performance could be investigated in future trials by increasing the number of AIs and using pregnancy rates to increase the precision of fertility assessment.

In Study II, we applied the SV processing method to semen from bulls of different breeds, producing *in vitro* results that indicated superior sperm cell quality. However, these results are based on limited material, and more research is necessary for full evaluation of the breed aspect in relation to SV semen processing.

The application of a double-$PGF_{2\alpha}$/GnRH protocol in Norwegian Red heifers and cows (Study III) provided no indications that animals of this breed respond any differently to such synchronization than cattle of other breeds, despite findings that show breed differences concerning duration and expression of estrus. These findings offer assurance that future research involving synchronization of estrus and ovulation in combination with AI (timed AI) is applicable in Norwegian Red.

We followed the gradual *in vivo* dissolution of SV gel after AI by endoscopic monitoring (Study IV). It would be interesting to refine the GTE method to enable more exact assessment of how the SV processed semen responds to the *in vivo* environment of the female genital tract. Such assessment could include investigation of *in vitro* sperm quality characteristics following intrauterine extraction of SV semen after AI by using GTE.

The field of *in vitro* sperm quality assessment is constantly developing. We have demonstrated that sperm cells processed by the SV technology are of high quality. However, there is a continuing need for new *in vitro* methods to improve our understanding of the changes that are inflicted on sperm cells following SV immobilization and cryopreservation.

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Comparison of sperm adenosine triphosphate content, motility and fertility of immobilized and conventionally cryopreserved Norwegian Red bull semen

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ABSTRACT

Estrus detection and timing of AI remains a challenge in cattle breeding. Prolonging spermatozoa lifespan after AI, making sperm cells available over an extended period, could make timing of AI relative to ovulation less crucial and improve fertility. Immobilization of sperm cells by the patented SpermVital technology in an alginate gel will provide a gradual release of spermatozoa after AI. The first aim of this study was to examine fertility, measured as non-return rate after 56 days (NR56), of SpermVital (SV) processed semen with reduced sperm cell number per dose compared to earlier studies, and compare with conventionally processed semen in Biladyl, a proprietary version of the egg yolk Tris semen extender. The second aim was to examine in vitro sperm quality post-thaw and after thermal stress. The third aim was to examine potential correlations between in vitro sperm parameters and NR56. Ejaculates from 16 Norwegian Red young bulls were split in three, processed and cryopreserved as Biladyl semen (B15; 15 million spermatozoa/dose) or by SpermVital technology (SV25; 25 million spermatozoa/dose or SV15; 15 million spermatozoa/dose). 1400 semen doses were produced per bull and distributed throughout Norway for a blinded field trial. Fertility was recorded as NR56 after first AI (N = 7155). Two ejaculates from each bull were randomly selected for in vitro experiments. B15 and SV15 semen samples were analyzed for motility by computer-assisted sperm analysis, viability and acrosome integrity by flow cytometry and ATP content by bioluminescence assay, post-thaw and after thermal stress. The AI trial detected no differences in NR56; least square means being 75.5% (B15), 75.6% (SV25) and 74.8% (SV15) (p > 0.05). There were no differences in total motility and progressive motility post-thaw, however, after three hours incubation at 38 °C, SV sperm motility and progressivity were higher for SV15 than for B15 spermatozoa (p < 0.05). The percentage of acrosome intact live sperm cells was higher for SV15 than B15 spermatozoa at all timepoints analyzed (0 h, 3 h, 24 h, p < 0.05). B15 semen showed a higher ATP level than SV15 at 0 h (p < 0.05), while SV15 sperm cells had higher ATP levels after 3 and 24 h (p < 0.05). No association was detected between in vitro sperm parameters and NR56. In conclusion, SV15, SV25 and B15 semen yielded equal fertility after AI. However, there were differences in sperm quality, as SV15 spermatozoa displayed higher motility, viability and ATP levels after thermal stress than B15 spermatozoa (p < 0.05).

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1. Introduction

AI is the predominant method of breeding in dairy herds. To

succeed with insemination in cattle, AI must be timed relative to estrus and ovulation. Optimal timing of AI may vary with age [1], estrus length [1,2], estrous signs and breed [3]. The size of dairy herds has increased considerably in the past decades [4], making systematic estrus detection management more challenging. The diversity of estrous expression combined with challenges in management may influence the chance of correctly identifying estrus and optimal time of AI, which in turn will influence herd fertility.

Prolonged spermatozoa lifespan *in vivo* after AI could make timing of AI relative to time of ovulation less critical. By the patented SpermVital technology, sperm cells are frozen while immobilized in an alginate gel to facilitate release of sperm cells over a prolonged period *in utero* after AI [5]. Inseminations performed with SpermVital (SV) semen at normal timing relative to estrous signs have demonstrated comparable fertility to conventional semen [6]. Furthermore, AIs performed early in heat with a single SV semen dose resulted in comparable fertility rates to AIs using standard processed semen inseminated on two consecutive days [7]. In these studies, SV semen had more sperm cells than conventionally processed semen due to the theory that the gel dissolves gradually after insemination, i.e. more spermatozoa are needed to assure availability of fertile spermatozoa. Other studies have shown that reduced sperm cell concentrations have not compromised fertility after AI with conventional semen [8,9]. To ensure maximal fertility potential, the post-thaw surviving sperm cells must possess traits such as intact acrosomes, a high degree of intact DNA, active metabolism and maintenance of progressive motility; traits that can be measured by flow cytometry and computer-assisted sperm analysis (CASA) [10,11]. Association between AI fertility and sperm motility [12–14], levels of ATP [15] and viability [8] have been reported. Moreover, reliability of fertility prediction is reported to increase by combining several *in vitro* sperm quality parameters [13,16–19].

Recently, the post-thaw proportion of acrosome intact live (AIL) sperm cells of SV semen has been improved [20]. The present study was designed to examine the fertility potential of SV semen at normal timing with a reduced sperm cell number per dose compared to previous AI trials with SV semen containing 25 million sperm cells per dose [6]. Therefore, the first objective of this study was to compare the fertility potential of semen processed by the SpermVital technology with two different sperm cell concentrations to semen processed in Biladyl, a proprietary form of an egg yolk Tris semen extender, in a blinded field trial. The second objective was to compare sperm ATP content, motility and percentage AIL sperm cells after thawing of semen preserved by the two processing methods, as well as after post-thaw thermal stress. The third objective was to examine a potential association between AI fertility and sperm quality parameters.

2. Materials and methods

2.1. Ethical statement

In the present study, all procedures for semen processing were approved by the Norwegian Food Safety Authority and in accordance with EU Directive 88/407. The AI trial was performed in accordance with the Norwegian Animal Welfare Act (LOV 2009-06-19 no. 97).

2.2. Semen processing

Ejaculates were obtained from 16 Norwegian Red young bulls with a mean age of 460 days (range 438–502). Semen collection was performed routinely once a week using an artificial vagina. Three to five ejaculates were obtained from each bull to give a total

of approximately 1400 AI doses. Sperm concentrations were estimated by an Accucell® spectrophotometer (IMV Technologies, L'Aigle, France), while motility and morphology were evaluated by phase-contrast microscopy. Only ejaculates with sperm concentrations > 390 million/mL, motility > 70% and normal morphology > 85% were used for further processing.

Following collection from each bull, the semen samples were split in three aliquots before processing and freezing. One aliquot of semen was diluted in a two-step procedure using Biladyl (hereafter referred to as B15) extender (Minitube GmbH, Tiefenbach, Germany, 13500/0004–0006) to a final concentration of 15 million spermatozoa per straw (French mini straws, IMV, L'Aigle, France). The other two aliquots were processed by the patented SpermVital technology [5] to a final concentration of 15 and 25 million sperm cells per straw (hereafter referred to as SV15 and SV25, respectively). All processed semen samples were given unique batch numbers and cryopreserved as described earlier [7]. Only ejaculates with post-thaw motility ≥50% in all batches (B15, SV15, SV25) were used in the AI trial. Small ejaculates were split in two and processed as B15 and SV15, giving the following distribution of AIs by semen processing method: 38.0% (B15), 36.9% (SV15) and 25.1% (SV25).

2.3. AI trial

The trial was blinded, with processing method being unidentifiable to AI personnel. Equal numbers of straws from each batch within ejaculate were packed in ten-straw goblets and distributed to AI technicians and veterinarians throughout Norway. Batch numbers were later applied in the data collection from AIs. AIs were performed during a 7-month period with semen from 16 bulls and 186 batches. The study population consisted of 3298 Norwegian Red dairy herds dispersed throughout Norway. Breeding personnel were reimbursed by Geno, thus motivating accurate AI reporting to the company's AI recording database.

The outcome of the inseminations was measured as non-return after 56 days (NR56). Breedings and re-breedings were reported to the national AI recording database (Semindatabasen, Geno, Hamar, Norway) and the Norwegian dairy herd recording system (NDHRS). Additional data on females and herds such as parity, double insemination and month of AI were made available from the NDHRS. In 2016, the mean NR56 in Norway was 72.4% [21], whereas the overall mean in the present trial was 72.2%. Data from 7155 first AIs were obtained, of which 655 animals received double inseminations evenly distributed by extender and sperm cell concentration. Double insemination was defined as an additional insemination within 4 days of first insemination. Mean NR56, number of AIs and proportion of double AI by extender is presented in Table 1.

2.4. Experimental setup - in vitro sperm quality studies

Initial studies showed comparable post-thaw AIL, ATP content and subjective motility amongst the two groups of SpermVital

Table 1
Number of inseminations (*N*), mean non-return rate 56 days (NR56) and proportion of double AI for AI with split-sampled bull semen cryopreserved with Biladyl extender or by the patented SpermVital technology with two different sperm cell numbers per dose (B15; Biladyl 15 million, SV25; SpermVital 25 million, SV15; SpermVital 15 million).

	B15	SV25	SV15
N	2722	1795	2638
NR56 (%)	72.6	72.0	72.0
Double AI (%)	9.2	10.0	8.5

processed semen with different sperm cell numbers per dose. SV15 was used for further comparison with B15 semen. Two batches from each bull and semen processing method were randomly selected for *in vitro* studies of B15 and SV15. A total of 64 batches were analyzed as follows: two straws were thawed at 37 °C for 1 min, each straw transferred to 500 μl SpermVital dilution fluid, incubated for 15 min at 38 °C with gentle tilting and thereafter pooled to reduce the possible effect of straw variability. Biladyl and SpermVital semen have earlier shown to have different velocity and swimming patterns post-thaw [34]. To minimize the effect of extender, both B15 and SV15 were transferred to the same buffer: From each of the pooled samples, 900 μl were transferred to new Eppendorf tubes and centrifuged at 800 × g for 5 min. The pellet was then resolved carefully to the same sperm cell concentration in a total of 900 μl 38 °C modified Sp-TALP-H [22,23] (87 mM NaCl, 3.1 mM KCl, 0.4 mM MgCl₂, 0.3 mM NaH₂PO₄, 2.0 mM CaCl₂, 20 mM HEPES sodium salt, 20 mM HEPES acid, 10 mM NaHCO₃, 1 mM sodium pyruvate, 5 mM glucose, 21.6 mM sodium lactate, 6 mg/ml BSA, 50 μg/ml gentamicin, pH 7.4, osmolarity of 292 mOsm). From this solution, aliquots were taken out for simultaneous analysis of motility, ATP levels and flow cytometry as follows at time 0 (T0) and after three hours (T3, three hours after thawing) incubation at 38 °C. Flow cytometry and ATP level measurements were also performed after 24 h (T24) of incubation at 38 °C.

2.5. Computer-assisted sperm analysis (CASA)

Sperm motility parameters were assessed using a CASA system (Sperm Class Analyzer® version 6.1, Microptic SL, Barcelona, Spain) with a Basler digital camera (Basler Vision Technologies, Ahrensburg, Germany). The semen samples were incubated for 5 min at 38 °C, inverted 180° five times and 3 μl put on a Leja® 4 chamber slide (Nieuw-Vennep, The Netherlands). Each semen sample was analyzed twice (parallels), eight fields with an average of 800 cells per parallel. Instrument settings were 50 frames per second and 30 images per sample. Sperm cells were identified by sperm head area 30–70 μm². The following kinematic parameters were recorded: velocity average path (VAP, μm/s), velocity curved line (VCL, μm/s), velocity straight line (VSL, μm/s), straightness (STR) of the average path defined as the ratio VSL/VAP (%), linearity (LIN) of the curvilinear path defined as the ratio VSL/VCL (%), Wobble (WOB) was defined as the ratio 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.

2.6. ATP content

Energy levels were measured as ATP at T0, T3 and T24 by applying the CellTiter-Glo® Luminescent cell viability assay (Promega, Madison, WI, USA) and FLUOstar OPTIMA® luminometer (BMG LABTECH, San Diego, CA, USA) with MARS data analysis software (Version 1.10, BMG LABTECH, Germany). From each semen sample, two aliquots of 30 μl were diluted 1:6 in PBS, from which two parallels of 60 μl per dilution were mixed with 60 μl CellTiter-Glo® Reagent in a white flat-bottom 96 well microtiter plate (NUNC™, Denmark). First, the samples were incubated at room temperature for 2 min on an orbital shaker at 300 rpm to induce cell lysis. The samples were further incubated at room temperature for 15 min to stabilize the luminescent signal. Data were recorded as relative luminescence units (RLU) and ATP concentrations were determined by a standard curve. Results were presented as μM ATP per million cells.

2.7. Sperm plasma membrane and acrosome integrity by flow cytometry

From each semen sample, two parallels of 50 μl were analyzed for percentage of AIL sperm cells by flow cytometry at T0, T3 and T24. Sperm cells were stained at a concentration of 1–2 million cells/ml in 38 °C PBS with 0.2 nM SYTO® 60 Red Fluorescent Nucleic Acid Stain (SYTO60, S11342, Invitrogen) for all sperm cells, 7.5 μM propidium iodide (PI, P4864, Sigma-Aldrich) for dead sperm cells and 0.05 μg/ml Lectin PNA from *Arachis hypogaea* (peanut) conjugated with Alexa Fluor® 488 (PNA-Alexa 488, L21409, Invitrogen) for acrosome reacted sperm cells. The sperm samples were incubated 15 min at room temperature before flow cytometry analysis. The flow cytometer (BD Accuri™ C6 Cytometer, Franklin Lakes, New Jersey, USA) was equipped with a blue (488 nm) and red laser (633 nm), two light scatter detectors, and four fluorescence detectors with optical filters (FL1-4: 533/30, 585/40, 670LP, 675/25). Validation beads (Spherotech 8-peaks validation beads (FL1-FL3) and Spherotech 6-peaks validation beads (FL4), BD Biosciences, Franklin Lakes, New Jersey, USA) for all four fluorescence channels FL1-4 were run daily. Unstained sperm cells served as control, and single stained cell samples were used to set compensation. SYTO60 was excited by the 633 laser and detected in FL4. PNA-Alexa 488 and PI were excited by the 488 laser and detected in FL1 (533/30) and FL3 (670LP), respectively. SYTO60 stains the DNA of both live and dead sperm cells, while PI is membrane impermeable and therefore binds only DNA of sperm cells with deteriorated membranes. PNA is a lectin that binds glycoproteins in the acrosomal membrane and only stains positive for acrosome reacted spermatozoa. A cytogram of side scatter signal and SYTO60 identified sperm cells. To exclude debris further, a cytogram of SYTO60 and PI was used (both PI and SYTO60 negative). Finally, a cytogram of PNA and PI gated on sperm cells and excluding debris, was used to identify AIL sperm cells. For each parallel, about 10000 individual sperm cells were evaluated at flow rates of 300–500 sperm cells/s. Flow cytometry data was analyzed using BD Accuri™ C6 software.

2.8. Statistical analyses

Statistical analyses were performed using Statistical Analysis System (SAS® version 9.4) for Microsoft Windows (SAS Institute Inc., Cary, NC, USA). NR56 for first inseminations was analyzed using the Logistic Procedure. The effects of bull, semen processing method, double AI within 1–4 days, month of AI and parity on NR56 were estimated by the following model:

$$Y_{ijklmn} = \mu + B_i + S_j + DI_k + M_l + P_m + e_{ijklmn}$$

where:

Y_{ijklmn} = NR56 observation of heifer/cow n;
μ = overall mean NR56;
B_i = effect of bull,
i = 1 to 16;
S_j = effect of semen processing method,
j = 1 (B15), 2 (SV25) or 3 (SV15);
DI_k = effect of double AI within 1–4 days,
k = 1 (no) or 2 (yes);
M_l = effect of month of AI,
l = 1 to 7, signifying months December through June;
P_m = effect of parity,
m = 1 to 4, 1 applies to heifers, 2 to 1st parity, 3 to 2nd parity, and 4 to parity 3 and greater;
e_{ijklmn} = residual effect

Odds ratio (OR) estimates were used to assess the effect of exposure variables on the outcome variable NR56. Variables presenting 95% Wald confidence intervals not including the value 1 were considered to have a significant effect on NR56. In the present study, OR was calculated by dividing the odds for NR56 when using SV15 or SV25 semen by the odds for NR56 when using B15 semen. If the OR is 1, there is no difference. An OR > 1 gives an estimate of how much more likely it is not to return to heat when applying SV semen compared to if B15 semen is used.

The General Linear Models procedure in SAS was used to perform a least square analysis for the *in vitro* parameters. The effects of the exposure variables bull, semen processing and post-thaw time dependent stress test (T0, T3, T24) on the outcome variable; either being viability, motility or energy levels were accordingly estimated by the following model:

$$Y_{ijmn} = \mu + B_i + S_j \times T_m + e_{ijmn}$$

where:

Y_{ijmn} = observation of either AIL, motility (including progressive motility and kinematic parameters) or ATP level per semen sample;
μ = overall mean of AIL, motility or ATP level;
S_j = effect of semen processing method,
j = 1 (B15) or 3 (SV15);
T_m = effect of post-thaw time dependent stress test,
m = 0 (T0) or 3 (T3);
B_i and e_{ijmn} as previously stated.

Further, testing for the possible effect of proportion of spermatozoa AIL, motility (including total motility, progressive motility and kinematic parameters) and ATP, individually or combined, on outcome of AI measured as NR56, was performed using the following model:

$$Y_{ijkn} = \mu + B_i + S_j + DI_k + (b_1 \times AIL0) + (b_2 \times ATP0) + (b_3 \times motility0) + (b_4 \times AIL3) + (b_5 \times ATP3) + (b_6 \times motility3) + (b_7 \times AIL24) + (b_8 \times ATP24) + e_{ijkn}$$

where:

Y_{ijkn} = NR56 observation of heifer/cow n;
b_1 = regression of *AIL0*, AIL at T0 on NR56;
b_2 = regression of *ATP0*, ATP at T0 on NR56;
b_3 = regression of *motility0*, motility at T0 on NR56;
b_4 = regression of *AIL3*, AIL at T3 on NR56;
b_5 = regression of *ATP3*, ATP at T3 on NR56;
b_6 = regression of *motility3*, motility at T3 on NR56;
b_7 = regression of *AIL24*, AIL at T24 on NR56;
b_8 = regression of *ATP24*, ATP at T24 on NR56;

Table 3
Odds ratio (OR) for the effects of the listed predictor variables on the outcome variable non-return rate 56 days (NR56) after first AI with split-sampled bull semen cryopreserved with Biladyl extender or by the SpermVital technology in two different sperm cell numbers per dose (B15; Biladyl 15 million, SV25; SpermVital 25 million, SV15; SpermVital 15 million): semen processing and double AI (AI on two consecutive days = 1) with 95% Wald confidence limits.

Predictor variables	OR NR56	
Semen processing		
B15 vs SV25	1.03	(0.90–1.18)
B15 vs SV15	1.03	(0.91–1.16)
SV25 vs SV15	1.00	(0.88–1.15)
Double AI 0 vs 1	1.44[a]	(1.19–1.75)

[a] Significant difference (p < 0.05).

μ, B_i, S_j, DI_k, and e_{ijkn} as previously stated.

The data on sperm cell quality parameters are presented as mean (SD).

3. Results

3.1. Field fertility

Least square means for NR56 by semen processing method and parity are given in Table 2. Parity, bull and month of AI had an effect on NR56 (p < 0.05), whereas there was no significant difference in NR56 by semen extender or sperm cell number per dose (p > 0.05). Number of AIs per month was well balanced between B15, SV25 and SV15, whereas the distribution of AIs by month was skewed. January and February encompassed 61% of the total number of AIs, however, these AIs were well balanced between B15, SV25 and SV15, being 61.9%, 59.7% and 61.6%, respectively. AIs performed in May had a significantly higher NR56 than other months apart from June.

All parameters included in the model, except semen processing method had an effect on NR56 (p < 0.05). The OR for NR56 increased following double insemination (Table 3). In addition, OR was higher in heifers compared to later parities (p < 0.05), and in spring and summer months compared to fall and winter months (p < 0.05).

3.2. Sperm quality parameters

The results for AIL in B15 and SV15 spermatozoa post-thaw at T0, T3 and T24 are given in Table 4 The proportion of AIL sperm cells decreased over time (p < 0.05). In addition, AIL was higher for SV15 than B15 sperm cells at all timepoints (p < 0.05). On the other hand, ATP levels were higher for B15 than for SV spermatozoa at T0 (p < 0.05, Table 5.). However, at T3 and T24 ATP levels were higher for SV than for B spermatozoa (p < 0.05). Moreover, there was no

Table 2
Least square means for non-return rate 56 days after first insemination (NR56) for AIs with split-sampled bull semen cryopreserved with Biladyl extender or by the patented SpermVital technology in two different sperm cell numbers per dose (B15; Biladyl 15 million, SV25; SpermVital 25 million, SV15; SpermVital 15 million), parity (heifers: parity 0, cows: parity 1,2 and ≥ 3), and number of inseminations in each group (N).

	B15		SV25		SV15	
Parity	NR56	N	NR56	N	NR56	N
0	81.5[a]	989	77.9[ab]	689	80.6[a]	1027
1	71.6[b]	793	74.7[ab]	511	72.2[b]	738
2	73.5[ab]	463	79.9[ab]	266	71.3[b]	435
≥3	75.5[ab]	477	70.0[b]	329	75.0[ab]	438
Total	75.5	2722	75.6	1795	74.8	2638

*NR56 values with different superscripted letters differ significantly (p < 0.05).

Table 4
Percentage of acrosome intact live (AIL) sperm cells measured by flow cytometry post-thaw (T0) and after three (T3) and 24 h (T24) incubation for Biladyl (B15) and SpermVital (SV15) sperm cells. Results are shown as mean (SD).

Time	B15		SV15	
T0	51.2[a]	(8.9)	57.6[b]	(7.0)
T3	41.8[c]	(8.3)	52.9[a]	(6.1)
T24	30.7[d]	(6.2)	39.7[c]	(5.9)

[*]Values with different superscripted letters differ significantly (p < 0.05).

Table 5
ATP levels (µM ATP per 10^6 sperm cells) measured by bioluminescence assay post-thaw (T0) and after three (T3) and 24 h (T24) incubation for Biladyl (B15) and SpermVital (SV15) sperm cells. Results are shown as mean (SD).

Time	B15		SV15	
T0	2.57[a]	(0.55)	2.34[b]	(0.43)
T3	2.05[c]	(0.43)	2.30[b]	(0.46)
T24	1.25[d]	(0.39)	1.61[e]	(0.40)

[*]Values with different superscripted letters differ significantly (p < 0.05) based on pairwise t-tests.

decrease in ATP levels for SV15 from T0 to T3 (p > 0.05). For both AIL and ATP content, there were significant differences between bulls (p < 0.05).

The results for percentage total motile (MOT) and progressive motile (PROG) sperm cells for B15 and SV15 sperm cells at T0 and T3 are given in Table 6. There was no difference between B15 and SV15 at T0 (p > 0.05), however, after three hours of incubation at physiological temperature, SV15 spermatozoa maintained a higher proportion of MOT and PROG sperm cells than B15 sperm cells (p < 0.05). Further, there was difference in MOT and PROG by bull (p < 0.05), however, the tendency concerning how semen withstands the two preservation methods was similar between bulls.

Mean values for the kinematic parameters are shown in Table 7. The velocity parameters VCL and VAP decreased from T0 to T3 for B sperm cells (p < 0.05), as opposed to SV (p > 0.05). At T0 VCL was higher for B than SV (p < 0.05), nevertheless, VAP and VSL were higher for SV than B spermatozoa (p < 0.05). This is reflected in STR and LIN which were higher for SV sperm cells (p < 0.05). ALH was higher for B than SV spermatozoa at T0 (p < 0.05), otherwise there were no differences. BCF was higher for SV than for B sperm cells at both timepoints (p < 0.05).

No associations between sperm cell percentage AIL, motility, progressive motility or ATP levels, individually or combined, on the outcome of AI measured as NR56 (p > 0.05) were found.

4. Discussion

In the present study, conventionally cryopreserved semen (B15) and semen cryopreserved after immobilization in alginate gel by the SpermVital technology (SV15) were compared concerning field

Table 6
Percentage total motile (MOT) sperm cells and progressive motile (PROG) sperm cells measured by CASA post-thaw (T0) and after 3 h (T3) of incubation at 38 °C for Biladyl (B15) and SpermVital (SV15). Results are shown as mean (SD).

Parameter	Time	B15		SV15	
MOT (%)	T0	66.6[a]	(7.9)	67.0[a]	(6.8)
	T3	61.5[b]	(8.1)	66.2[a]	(6.3)
PROG (%)	T0	50.4[a]	(8.4)	52.9[a]	(7.6)
	T3	43.3[b]	(8.3)	51.5[a]	(7.1)

[*]Values with different superscripted letters within parameter differ significantly (p < 0.05).

Table 7
Kinematic parameters measured by CASA post-thaw (T0) and after 3 h (T3) incubation at 38 °C for Biladyl (B15) and SpermVital (SV15) sperm cells. Kinematic parameters: velocity average path (VAP, µm/s), velocity curved line (VCL, µm/s), velocity straight line (VSL, µm/s), straightness (STR) of the average path defined as the ratio VSL/VAP (%), linearity (LIN) of the curvilinear path defined as the ratio VSL/VCL (%), Wobble (WOB) defined as the ratio VAP/VCL (%), amplitude of lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz). Results are shown as mean (SD).

Parameter	Time	B15		SV15	
VAP	T0	72.8[a]	(7.0)	79.2[b]	(7.8)
	T3	67.4[c]	(8.2)	81.9[b]	(7.4)
VCL	T0	140.8[a]	(12.6)	126.4[b]	(10.1)
	T3	115.3[c]	(11.5)	130.1[b]	(10.2)
VSL	T0	61.4[a]	(7.1)	71.4[b]	(7.9)
	T3	57.2[a]	(8.2)	73.6[b]	(7.4)
STR	T0	69.8[a]	(2.5)	76.6[b]	(3.0)
	T3	67.5[c]	(3.0)	74.8[d]	(2.7)
LIN	T0	35.6[a]	(2.5)	47.1[b]	(3.9)
	T3	37.4[c]	(2.8)	46.1[b]	(3.2)
WOB	T0	46.8[a]	(1.9)	56.0[b]	(3.2)
	T3	50.0[c]	(2.5)	55.4[b]	(2.5)
ALH	T0	3.8[a]	(0.31)	2.9[b]	(0.18)
	T3	3.0[b]	(0.22)	3.0[b]	(0.21)
BCF	T0	18.8[a]	(1.3)	23.1[b]	(1.7)
	T3	18.7[a]	(1.5)	21.8[c]	(1.4)

[*]Values with different superscripted letters within parameter differ significantly (p < 0.05).

fertility, sperm AIL, ATP content and motility. No differences in NR56 were observed when using different sperm cell concentrations for SpermVital semen (SV15 and SV25) or B15 semen at normal timing of AI. Assessment of sperm quality post-thaw and over time revealed a higher percentage of AIL for SV15 than for B15 sperm cells at all timepoints. Interestingly, at T0 the ATP level was higher for B15 than SV15 spermatozoa, however, at T3 and T24 the ATP levels were higher for SV15 than for B15 sperm cells. In addition, it was observed that SV15 spermatozoa maintained higher motility at T3 than B15 spermatozoa.

In this field trial, there were significant effects of parity, bull and month of AI, which is in agreement with other studies [24,25]. The present study confirmed the results of Standerholen et al. [6], and additionally showed that SV15 AI doses release a sufficient number of cells to give comparable NR56 to B15 sperm cells at normal timing of AI. The number of AIs in this trial was large enough to detect a possible difference in NR56 above 4% (power of 0.9, significance level 0.05). A far greater sample size would have been required if differences in NR56 of a lesser magnitude were to be detected.

Accurate timing of AI relative to the occurrence of estrus is essential in modern dairy management. By prolonging spermatozoa viability, more living sperm cells will be present *in vivo* after AI over an extended time, potentially increasing the chance of fertilization. It has been reported that SpermVital semen has a prolonged lifespan *in vitro* in uterus mimicking conditions [21]. In the present study, spermatozoa were transferred to Sp-TALP-H post-thaw, mimicking conditions after release from the gel and transport to the oviduct, also resulting in higher survival levels for SV15 than B15 sperm cells.

After insemination, sperm cells travel from the uterus to the oviduct where capacitation, hyperactivation, acrosome reaction and fertilization take place in a coordinated fashion. All these events require energy. As expected, ATP levels in the present study decreased gradually over time during thermal stress. At T0, B15 spermatozoa yielded a higher ATP level than SV15 sperm cells. However, SV15 spermatozoa held elevated ATP levels over time. To our knowledge, little is reported on the association of ATP content to fertilization potential, but the production of ATP is reported to be linked to bovine sperm fertility [15]. Differences between various

rodent species concerning spermatozoa ATP production and swimming performance have been reported, indicating that ATP content could be an interesting parameter in relation to fertility [26,27].

Spermatozoa need to be motile to be able to fertilize the oocyte, and several researchers have reported correlation between motility and fertility [12,13,28–30]. In this study, no association between NR56 and sperm viability, ATP content, motility, progressive motility or kinematic parameters was found. However, this can be explained by the relatively high number of sperm cells per dose, and that viability and motility are considered to be compensable traits [31]. In addition, there was little variation in NR56 between bulls in the present experiment. However, research including groups of bulls with low and high fertility has shown correlation between field fertility and *in vitro* parameters [17–19]. Yet, comparison of B15 and SV15 semen revealed significant differences, as SV15 sperm motility and progressive motility were higher than for B15 sperm cells at T3, a tendency observed for most of the semen samples from the individual bulls. A better maintenance of motility for SV15 over time, combined with elevated ATP levels, could indicate enhanced conservation of energy store when bull semen is preserved by immobilization. Another explanation could be that the use of different extenders during the cryopreservation process influence sperm quality post-thaw.

Recently, Kumaresan et al. found correlation between NR56 and a combination of viability, acrosome intactness, reactive oxygen species and DNA integrity for bull semen [18]. However, no correlation among kinematic parameters and fertility was observed. On the other hand, Gliozzi and co-workers have recently shown a relationship between fertility in Holstein bulls and a combination of kinetic and flow cytometric parameters [19]. Viability, motility, progressive motility and linearity were all significantly higher in high-fertility bulls, and in their prediction model for fertility, kinematic parameters were included. Even though an association between swimming pattern and fertility was not found in the present study, it is interesting to note that SV15 sperm cells had a more linear swimming pattern than B15 semen. This confirms earlier results [34], and that the differences in swimming pattern is maintained when transferred to the same solution, eliminating the effect of extender and viscosity. A linear swimming pattern has also been associated with fertility in other species [32,33]. Hirai et al. reported a correlation between boars with high non-return rate and the amount of sperm cells with linear swimming pattern [32], while Vicente-Fiel et al. found that several kinematic parameters, including STR and LIN, were higher in high fertility rams [33].

In conclusion, a difference in fertility potential was not detected for SpermVital semen with sperm cell concentrations of 25 million or 15 million per AI dose when AI was performed at normal timing. In addition, it was found that AIL, ATP levels and motility were higher for SpermVital than Biladyl spermatozoa after thermal stress.

Acknowledgements

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II

Post-thaw quality of spermatozoa immobilised in alginate gel before cryopreservation

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1

Contents

Immobilisation of spermatozoa in alginate gel by the SpermVital technology has earlier been shown to be applicable for Norwegian Red bulls. The aim of this study was to evaluate the effect of immobilisation and cryopreservation of semen from other breeds on sperm cell quality, with emphasis on motility subpopulations. In addition, a secondary objective was to study the motility subpopulations of Norwegian Red spermatozoa immobilised in alginate. Semen was collected from six bulls (Brown Swiss, Red Holstein and Limousine), two ejaculates per bull, divided into two aliquots and processed in commercially available Triladyl (T) extender or immobilised in an alginate gel by the SpermVital (SV) technology before cryopreservation. For Norwegian Red semen, earlier obtained CASA data was further studied regarding subpopulations. Post-thaw percentage acrosome intact live (AIL), total motility and progressive motility were higher for SV than T spermatozoa ($p<0.05$), however, neither ATP level nor percentage DNA fragmentation index differed significantly. After thermal stress, ATP, AIL, total motility and progressive motility were higher for SV than for T sperm cells ($p<0.05$). CASA data was further used to divide the sperm cells into subpopulations according to swimming pattern. The proportion of rapid progressive spermatozoa was higher, and maintained higher over time for SV sperm cells, both for Norwegian Red and for semen from other breeds. In summary, SV spermatozoa had enhanced viability, ATP levels, total motility, and rapid progressive motility. The study shows that the SpermVital technology is suitable for the cryopreservation of semen from different breeds.

Keywords

1   INTRODUCTION

Cryopreservation of semen has been performed since the 1950s and is a method in continuous development for the improvement of fertility rates (Grötter, Cattaneo, Marini, Kjelland, & Ferré, 2019). One of these techniques is microencapsulation of sperm cells, which is intended to protect and maintain viability over time *in utero* to make the timing of insemination relative to ovulation less critical and thereby improve fertility (R. Nebel, Vishwanath, McMillan, & Pitt, 1996; R. L. Nebel, Vishwanath, McMillan, & Saacke, 1993). Sperm cells can also be immobilised by the SpermVital technology in a calcium alginate gel, allowing extended sperm survival at physiological temperatures and a gradual release of spermatozoa after insemination (Alm-Kristiansen et al., 2017; Standerholen et al., 2015). As opposed to conventional semen processing, the immobilisation of sperm cells in a solid alginate gel before cryopreservation enables the gradual release of viable spermatozoa with the potential for fertilization over a prolonged period. Furthermore, the immobilisation of sperm cells could decrease the metabolic rate and stabilize the plasma membrane during the freezing and thawing process (Alm-Kristiansen et al., 2018), possibly resulting in the conservation of ATP over time (Berg et al., 2018). Insemination trials in Norwegian Red based on 56 day non-return (NR56) following AI with conventional semen and SpermVital semen at normal timing relative to ovulation have demonstrated equal fertility (Berg et al., 2018; Standerholen et al., 2015). Furthermore, single dose inseminations with SpermVital performed at early timing relative to standard AI gave similar pregnancy rates to AIs with double inseminations (inseminations on two consecutive days with conventional semen) (Alm-Kristiansen et al., 2017).

Sperm quality parameters such as viability, acrosome intactness, and level of DNA integrity, which are crucial to sperm functionality, can be measured by flow cytometry (Gillan, Evans, & Maxwell, 2005). Further, to reach the site of fertilization, sperm cells must be motile (Correa,

Pace, & Zavos, 1997; Farrell, Presicce, Brockett, & Foote, 1998), and computer-assisted sperm analysis (CASA) is frequently used for objective assessments of motility (Amann & Waberski, 2014). Studies performed on spermatozoa from several species, including bovine (Muiño, Peña, Rodríguez, Tamargo, & Hidalgo, 2009; Muiño, Tamargo, Hidalgo, & Peña, 2008), have identified discrete sperm motility subpopulations showing differences in swimming patterns concerning velocity and progressivity (Martínez-Pastor, Tizado, Garde, Anel, & de Paz, 2011). Cluster analyses of motility of bull sperm cells have revealed three to four distinct subpopulations, one with highly active, non-progressive spermatozoa, a second with relatively high velocity and highly progressive spermatozoa and a third with slow motility and non-progressive and/or progressive spermatozoa (Dorado et al., 2017; Kanno et al., 2017; Muiño et al., 2009; Muiño et al., 2008). The observed subpopulations can be assumed to reflect diversity in sperm fertilization potential (Martínez-Pastor et al., 2011) and resilience against damage caused by cryopreservation (Muiño et al., 2009; Muiño et al., 2008).

Immobilisation of spermatozoa in alginate gel by the SpermVital technology is earlier shown to be applicable for Norwegian Red bulls (Berg et al., 2018; Standerholen et al., 2015). The aim of this study was to evaluate the effect of immobilisation and cryopreservation of semen from different breeds on sperm functionality with emphasis on motility subpopulations. The first objective was to compare the post-thaw quality of sperm cells immobilised by the SpermVital (SV) technology with those of the commercially available Triladyl (T) extender. The second objective was to study sperm cell motility, viability, acrosome integrity, and ATP level after thermal stress under oviduct-like conditions for three hours. The third objective was to divide the sperm cells into motility subpopulations, compare their distribution in T and SV semen and examine their correlations with viability and ATP. The fourth objective was to analyse the

distribution of motility subpopulations of Norwegian Red spermatozoa processed by the SpermVital technology.

2 MATERIALS AND METHODS

2.1 Ethical statement

All procedures for semen processing followed the EU Directive 88/407.

2.2 Chemicals

All chemicals were provided from Sigma Aldrich unless otherwise stated.

2.3 Semen processing

Semen from a total of six bulls (two from each of the breeds Brown Swiss, Red Holstein, and Limousine), from a commercial artificial insemination centre (Swissgenetics, Zollikofen, Switzerland), was used for the experiment. Two ejaculates of each bull fulfilling minimum standard, concentration > 390 million/mL, motility > 70% and normal morphology > 75%, were split in two equal parts and processed with either the commercially available Triladyl (Minitube GmbH, Tiefenbach, Germany) extender or by the SpermVital technology (hereafter referred to as T and SV, respectively) to standard concentrations of 15 and 25 million sperm cells per straw, for T and SV, respectively (French mini straws, IMV, L'Aigle, France). All semen samples were cryopreserved by standard procedure.

CASA data from semen of 16 Norwegian Red bulls were available from a previous study (Berg et al., 2018), and these data were further used to identify motility subpopulations in Norwegian Red SV semen as well, where the Biladyl (B) extender (Minitube GmbH, Tiefenbach, Germany) was used as control.

2.4   Experimental setup

Each straw was thawed at 37 °C for 1 minute and subsequently transferred to 500 µl pre-warmed SpermVital gel dissolution buffer and incubated for 15 min at physiological temperature with gentle tilting to dissolve the gel. From each ejaculate and cryopreservation method, samples from two straws were pooled before analysis. For the thermal stress test, 600 µl from each sample were centrifuged at 800 ×g for 5 min followed by careful resolving of the pellet to the same sperm cell concentration in preheated modified Sp-TALP-H (Parrish, 2014; Parrish, Susko-Parrish, Winer, & First, 1988) (87 mM NaCl, 3.1 mM KCl, 0.4 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 2.0 mM $CaCl_2$, 20 mM Hepes sodium salt, 20 mM Hepes acid, 10 mM $NaHCO_3$, 1 mM sodium pyruvate, 5 mM glucose, 21.6 mM sodium lactate, 6 mg/mL BSA, 50 µg/mL gentamicin, pH 7.4, osmolarity of 292 mOsm). Aliquots were analysed as below at time 0 (T0) and after three hours (T3, three hours after thawing) of incubation at 38 °C.

2.5   Computer-Assisted Sperm Analysis

Sperm motility parameters were assessed by CASA (Sperm Class Analyzer® version 5.4, Microptic SL, Barcelona, Spain). The semen samples were incubated for 5 min at 38 º C, rotated 180º five times before placing 3 µl on a Leja® 4 chamber slide (Nieuw-Vennep, The Netherlands). For each sample, two parallels were analysed in eight fields with a minimum total of 700 cells per parallel. The instrument was set to 25 frames per second, 25 images per sample and 11 VAP points to be used in cell path smoothing. Sperm cells were identified by a sperm head area of 30-70 μm^2. The following kinematic parameters were recorded: velocity average path (VAP, µm/s), velocity curved line (VCL, µm/s), velocity straight line (VSL, µm/s), straightness (STR) of the average path defined as the ratio VSL/VAP (%), linearity (LIN) of the curvilinear path defined as the ratio VSL/VCL (%), wobble (WOB) defined as the ratio VAP/VCL (%), amplitude of the lateral head displacement (ALH, µm) and beat cross frequency

(BCF, Hz). Motility (MOT) was defined as sperm cells with VCL > 15 µm/s, progressive motility (PROG) was defined as sperm cells with STR > 70. The use of limit settings in SCA 5.4 allows for classification of spermatozoa into motility subpopulations (Martínez-Pastor et al., 2011). Rapid progressive (RapidP) spermatozoa are characterized by a fast and linear swimming pattern, while rapid non-progressive (RapidNP) spermatozoa are characterized by a fast and non-linear movement. In this study, slow motile spermatozoa were not divided according to progressivity due to their low speed and therefore uncertain STR classification. The subgroups were defined as follows: RapidP; VCL > 50 µm/sec and STR > 70, RapidNP VCL > 50 µm/sec and STR < 70, slow motility; 15 µm/s < VCL < 50 µm/s and immotile; VCL < 15 µm/s.

2.6 Energy contents

The content of sperm cell ATP was measured by the CellTiter-Glo® Luminescent cell viability assay (Promega, Madison, WI, USA) and FLUOstar OPTIMA® luminometer (BMG LABTECH, San Diego, CA, USA) with MARS data analysis software (Version 1.10, BMG LABTECH, Germany) as in Berg et al. (2018). Briefly, two aliquots from each semen sample were further diluted and analysed in parallel by mixing with equal parts of CellTiter-Glo® Reagent in a white flat-bottom 96 well microtiter plate (NUNC™, Denmark). After incubation at room temperature for 2 min on an orbital shaker at 300 rpm and further for 15 min to stabilize the luminescent signal, data were recorded as relative luminescence unit (RLU) and ATP concentration was determined according to a standard curve. Results are shown as µM ATP per million cells.

2.7 Sperm plasma membrane and acrosome integrity

From each semen sample, two parallels were analysed for the percentage of acrosome intact live (AIL) sperm cells by flow cytometry. Sperm cells were stained at a concentration of 1-

2×10^6 cells/mL in preheated PBS with 0.2 nM SYTO® 60 Red Fluorescent Nucleic Acid Stain (SYTO60, S11342, Invitrogen) for all sperm cells, 7.5 µM propidium iodide (PI, P4864, Sigma-Aldrich) for dead sperm cells and 0.05 µg/mL Lectin PNA from Arachis hypogaea (peanut) conjugated with Alexa Fluor® 488 (PNA-Alexa 488, L21409, Invitrogen) for acrosome reacted sperm cells. The sperm samples were stained for 15 min at room temperature. The flow cytometer (BD AccuriTM C6 Cytometer, Franklin Lakes, New Jersey, USA) was equipped with a blue (488 nm) and red laser (633 nm), two light scatter detectors, and four fluorescence detectors with optical filters (FL1-4: 533/30, 585/40, 670LP, 675/25). Validation beads (Spherotech 8-peaks validation beads (FL1-FL3) and Spherotech 6-peaks validation beads (FL4), BD Biosciences, Franklin Lakes, New Jersey, USA) for all four fluorescence channels FL1-4 were run each day. For the identification of sperm cells, a cytogram of side scatter-signal and SYTO60 (excited by the 633 laser) in FL4 were used. PNA-Alexa 488 and PI were excited by the 488 laser and detected in FL1 (533/30) and FL3 (670LP), respectively. For each parallel, about 10,000 individual sperm cells were evaluated at flow rates of 300 to 500 sperm cells/s. Flow cytometry data were analysed using BD AccuriTM C6 software.

2.8   DNA integrity

The SCSA protocol was performed according to the procedure described by Evenson and Jost (2000) and Waterhouse et al. (2006). Semen samples were thawed and dissolved as above before dilution to 2×10^6 sperm cells/mL in TNE buffer (10 mM Tris-HCL, 0.1 M NaCl, 1 mM EDTA, pH 7.4) in a final volume of 200 µl. All solutions were kept on ice during the experiment. Thereafter, 400 µl acid detergent solution (0.38 M NaCl, 80 mM HCL, 0.1% (w/v) Triton X-100, pH 1.2) was added to the sample. After exactly 30 sec, 1.2 mL acridine orange (AO) staining solution (6 µg/mL AO [A3568; Life Technologies, OR] in a buffer containing 37 mM citric acid, 0.126 M Na2HPO4, 1.1 µM EDTA, 0.15 M NaCl, pH 6) was added to the

8

sample, which was placed in the flow cytometer and run in a set up mode for 3 min. Thereafter, 5,000 events were collected from each sample at a flow rate of ~200 events/sec. For sample analysis, a Cell Lab QuantaTM SC MPL flow cytometer equipped with an argon laser with excitation at 488 nm and 22 mW power (Beckman Coulter, Fullerton, CA) was used. The signals were separated by a 550-nm dichroic long pass mirror before collection through a 525-nm bandpass filter (green) and a 670-nm long-pass filter (red). Before sample analysis, the flow cytometry instrument was saturated with AO by running an AO equilibration solution (1.2 mL AO staining solution and 400 µl acid detergent solution) through the system for at least 5 min. Mean values for the green and red fluorescence signals were set at the beginning of each analysis and after analysing every fifth sample based on reference bull semen with a known DFI in a bivariate cytogram to control laser stability. The FL1 (green) was presented on the x-axis and FL3 (red) on the y-axis of the cytogram, both on a linear scale, with fluorescence signals set to 425 ± 5 and 125 ± 5, respectively. The percentage of red (ssDNA) and green (dsDNA) fluorescence was determined using FCS Express 6 Flow cytometry Software (Denovo Software, Los Angeles, CA, USA).

Based on a cytogram of the fluorescence ratio red/(red + green), percentage DFI (spermatozoa with fragmented DNA), mean DFI (degree of DNA fragmentation), SD DFI (heterogeneity of DNA fragmentation) and percentage HDS (percentage of spermatozoa with high green DNA stainability) were calculated.

2.9   Statistical analyses

Descriptive statistics for sperm viability, acrosome integrity, ATP content, motility, and DNA integrity were estimated using Statistical Analysis System (SAS® version 9.4) for Microsoft Windows (SAS Institute Inc., Cary, NC, USA). Descriptive statistics for motility sperm subpopulations were analysed by using the statistical program R, version 3.1.2 (http://www.r-

project.org). Mean and standard deviation (SD) are presented for each sperm quality parameter by semen processing method and time of incubation. An analysis of variance combined with a Tukey test was performed for multiple pairwise comparisons of the parameter means. Differences between parameter means were considered significant in the event of p-values < 0.05. A multivariate correlation analysis was performed using JMP software, version 15.0.0 for Microsoft Windows (SAS Institute, Inc, Cary, NC, USA) to examine the degree of correlation between the sperm quality parameters. Preliminary evaluation of semen showed that there were no differences in sperm quality between breeds (p>0.05) (Supporting information Table S1).

3   RESULTS

As shown in Table 1, the percentage of post-thaw MOT, PROG and AIL sperm cells was higher for SV than T (p<0.05), however, there was no significant difference in ATP (Table 1) or DNA integrity post-thaw between the two methods of semen processing (Table 2).

The mean values for AIL and ATP before and after thermal stress for three hours for T and SV sperm cells are given in Table 3. The percentage of AIL sperm cells was reduced over time for both T (p<0.05) and SV (p>0.05), however, the difference between T0 and T3 was smaller for SV than for T spermatozoa. Likewise, the ATP levels were higher for SV than T semen at T3 (p<0.05). However, the differences between extenders in MOT and PROG reductions over time were more pronounced (Table 4), as spermatozoa motility decreased more for T than for SV spermatozoa (p<0.05). The mean values for the kinematic parameters are shown in Supporting information Table S2. Velocity (VCL, VAP, VSL) decreased over time for T spermatozoa, but not for SV spermatozoa (p<0.05).

Motility was studied further by dividing the sperm cells into subpopulations according to velocity and swimming patterns (Figure 1). Regardless of the processing method, the motile

10

population was dominated by RapidP sperm cells, which also varied more than the other populations between treatments and over time ($p < 0.05$). The percentage of RapidP spermatozoa decreased more for T than for SV spermatozoa (Figure 1a, $p < 0.05$), while there was no significant difference in the slow population except for an increase at T3 for T spermatozoa ($p < 0.05$). In Norwegian Red semen, however, the subpopulation of RapidNP was higher for B spermatozoa than SV at both T0 and T3 (Figure 1b, $p < 0.05$), while the proportion of RapidP sperm cells was higher for SV than B spermatozoa at T3 ($p < 0.05$). Furthermore, there was a decrease in RapidP over time for B semen ($p < 0.05$), but not for SV semen ($p > 0.05$). A correlation analysis including AIL, ATP and motility subpopulations showed that the proportion of RapidP and RapidNP spermatozoa was highly correlated with AIL and ATP (Figure 2, Supporting information Table S3) and more so than the slow population ($p < 0.05$).

4   DISCUSSION

The SV spermatozoa in this study had higher AIL and motility over time than control semen, which is in accordance with previous results comparing SV semen to B semen following thermal stress (Alm-Kristiansen et al., 2018). This is possibly explained by the enhanced effect of SV processing on the survival of sperm cells, or a different response to Sp-TALP-H after processing in different extenders. The results indicate that processing by immobilisation using alginate gel can contribute to the conservation of energy over time and sustained motility.

 The higher ATP levels over time in SV than in T semen was in agreement with Berg et al (2018). It could be hypothesized that the different swimming patterns of spermatozoa lead to the consumption of different amounts of energy in the form of ATP. Correlation analysis of ATP, AIL, and the different motility subpopulations in the current study showed high correlations between rapid spermatozoa (RapidP and RapidNP) and AIL or ATP, while the slow

population showed less or non-significant correlation with AIL or ATP. These findings suggest that rapid cells consume more ATP or have the highest ability to produce ATP. As discussed in Berg et al (2018), the relationship between ATP and fertility is not well documented and needs further investigation. Nonetheless, an association is reported between the production of ATP and bovine fertility (Garrett, Revell, & Leese, 2008).

Contradictory results have been published concerning the relationship between kinematic parameters and fertility (Gliozzi, Turri, Manes, Cassinelli, & Pizzi, 2017; Kumaresan, Johannisson, Al-Essawe, & Morrell, 2017). However, Gliozzi et al (2017) reported an association between fertility (non-return rates) and a linear swimming pattern. Moreover, interesting findings are reported when organizing spermatozoa into different motility subpopulations according to sperm cell movement. Variability in subpopulations has been associated with ejaculate and the individual bull (Kanno et al., 2017; Muiño et al., 2008), sperm cryotolerance (Muiño et al., 2009; Muiño et al., 2008), fertility (Ahmed, Andrabi, & Jahan, 2016; Yániz, Palacín, Vicente-Fiel, Sánchez-Nadal, & Santolaria, 2015), and the ability to bind to the zona pellucida (Ferraz et al., 2014). Yaniz et al (2015) reported a relationship between high field fertility (lambing, yes or no) in rams and the proportion of sperm cells in the subpopulation with fast and linear movements. Likewise, subpopulations of rapid progressive sperm cells from buffalo have, combined with other sperm quality parameters (e.g. viability), been positively associated with fertility (pregnancy rate) (Ahmed et al., 2016).

The functionality of different motility subpopulations requires further investigation, however, it has been suggested that the proportion of rapid progressive cells after freezing-thawing is a good indicator of sample quality, while the slow population could be an indicator of reduced quality (Martínez-Pastor et al., 2011; Muiño et al., 2008). In the current study of motility subpopulations, the proportion of rapid progressive cells changed the most over time. In

addition, the proportion of RapidP spermatozoa decreased less over time in Norwegian Red semen than for other breeds. This could be explained by individual or breed differences, however, the processing of control semen was performed in different extenders, also potentially affecting the results. A pronounced decrease of RapidP spermatozoa compared to other subpopulations over time has also been reported previously (Muiño et al., 2009). Ferraz et al (2014) reported a significant positive correlation between sperm cells with high velocity and progressivity in frozen-thawed spermatozoa from Holstein bulls and the ability to bind zona pellucida and IVF-results, which indicates a favourable relationship between the amount of rapid progressive spermatozoa and fertilizing potential. Interestingly, in the current study, SV spermatozoa maintained a higher proportion of RapidP than control semen. However, further studies are needed to understand the functionality of these differences.

In summary, semen immobilised in alginate gel before cryopreservation had a higher percentage of AIL sperm cells, total motility, progressive motility, RapidP motility and ATP level than control semen. DNA integrity did not differ post-thaw. The results of the current study showed that the immobilisation of spermatozoa by the SpermVital technology effectively preserves the quality of semen from different breeds and maintains a high level of motility over time.

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CONFLICTS OF INTEREST

HFB, ERG, GB, EK, and AHAK are employees of SpermVital AS. Otherwise, the authors have no conflicts of interest to report.

DATA AVAILABILITY

Additional data can be acquired on request by contacting the corresponding author.

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Table 1 Mean (standard deviation) post-thaw percentage of acrosome intact live (AIL) sperm cells measured by flow cytometry, ATP levels (μM ATP per 10^6 sperm cells) measured by bioluminescence assay, percentage total motile (MOT) sperm cells and progressive motile (PROG) sperm cells measured by CASA for split-sampled cryopreserved semen using the Triladyl extender (T) or SpermVital immobilisation technology (SV).

| Parameter	T	SV
AIL	57.1[a] (6.4)	71.0[b] (5.5)
ATP	2.4[a] (0.3)	2.2[a] (0.3)
MOT	51.4[a] (11.8)	64.1[b] (9.0)
PROG	37.6[a] (10.2)	51.8[b] (8.2)

*Mean values within parameter with different superscripts differ significantly (p<0.05).

Table 2 Mean (standard deviation) percentage DNA fragmentation index (DFI), mean DFI, heterogeneity in the degree of denaturation (SD DFI) and high green DNA stainability (HDS) post-thaw of spermatozoa split-sampled and cryopreserved in Triladyl extender (T) or by the SpermVital (SV) technology.

Parameter	T	SV
DFI (%)	1.3[a] (0.4)	1.4[a] (0.3)
Mean DFI	216.2[a] (5.4)	220.5[a] (5.1)
SD DFI	15.1[a] (1.2)	14.5[a] (1.2)
HDS (%)	1.9[a] (0.7)	1.9[a] (0.5)

*Mean values within parameter with different superscripts differ significantly ($p < 0.05$).

Table 3 Mean (standard deviation) percentage of acrosome intact live (AIL) sperm cells measured by flow cytometry and ATP levels (μM ATP per 10^6 sperm cells) measured by bioluminescence assay for split-sampled semen cryopreserved using the Triladyl extender (T) or SpermVital immobilisation technology (SV) post-thaw (T0) in Sp-TALP-H and after three hours' incubation (T3).

Parameter	Time	T	SV
AIL	T0	53.7[ac] (6.8)	65.5[b] (6.9)
	T3	41.8[d] (5.6)	59.2[bc] (5.3)
ATP	T0	2.3[a] (0.3)	2.4[a] (0.3)
	T3	1.9[b] (0.3)	2.2[a] (0.2)

*Mean values within parameter with different superscripts differ significantly (p<0.05)

18

Table 4 Mean (standard deviation) percentage total motile (MOT) sperm cells and progressive motile (PROG) sperm cells measured by CASA for split-sampled semen cryopreserved using the Triladyl extender (T) or SpermVital immobilisation technology (SV) post-thaw (T0) in Sp-TALP-H and after three hours incubation (T3).

Parameter	Time	T	SV
MOT	T0	53.4^a (11.7)	70.5^b (10.3)
	T3	40.8^c (7.9)	63.6^{ab} (8.9)
PROG	T0	38.9^a (10.5)	55.5^b (8.9)
	T3	22.3^c (7.7)	47.9^d (9.2)

*Mean values within parameter with different superscripts differ significantly ($p < 0.05$)
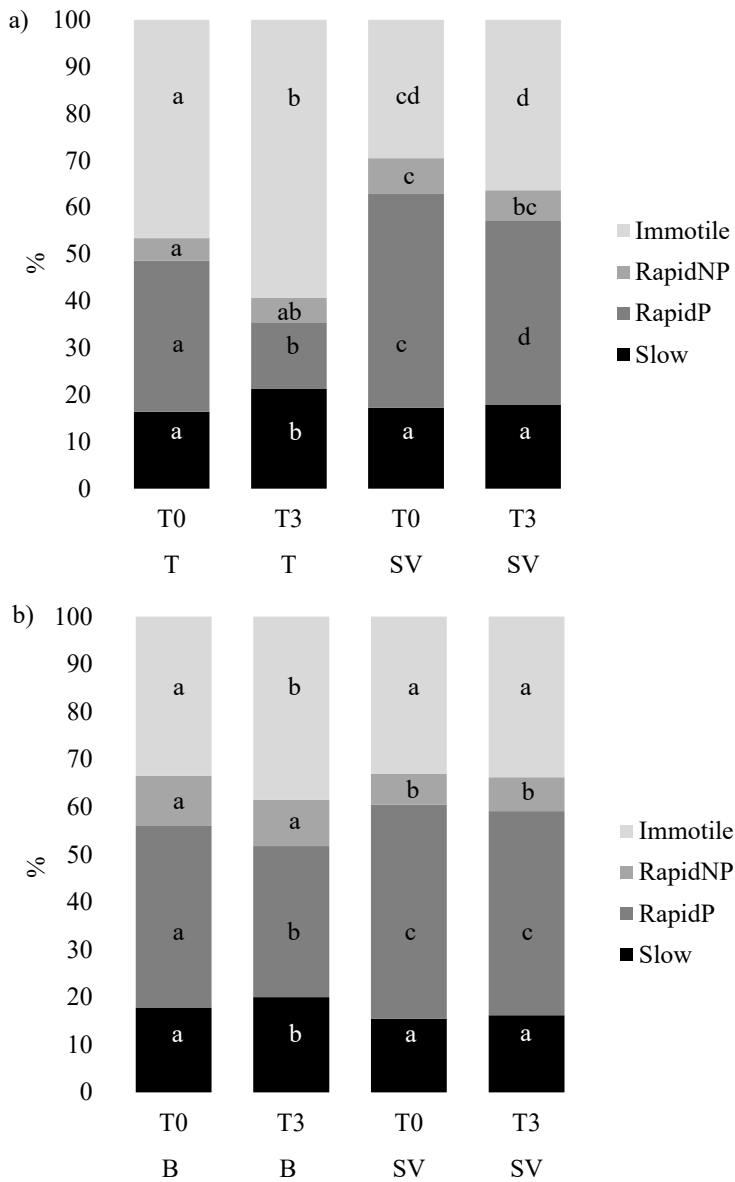
Figure 1 Distribution of sperm cells into motility subpopulations; Immotile, Rapid non-progressive (RapidNP), rapid progressive (RapidP) and slow motility, from bulls of a) different breeds (Brown Swiss, Red Holstein and Limousine) split-sampled and cryopreserved in Triladyl extender (T) or by the SpermVital (SV) technology, and b) Norwegian Red split-

sampled and cryopreserved in Biladyl extender (B) or by SV post-thaw (T0) in Sp-TALP-H and after three hours incubation (T3). Values within subpopulation with different letters differ significantly (p<0.05).
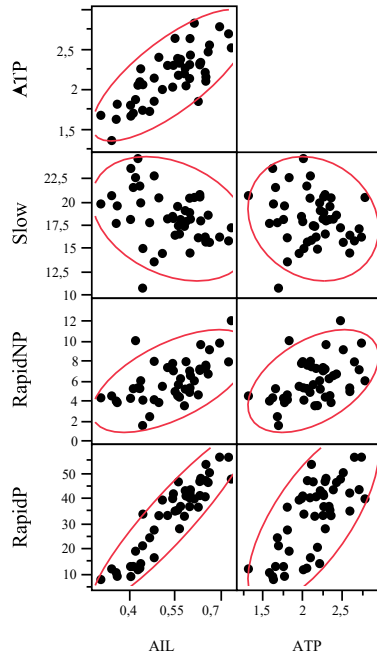
Figure 2 Scatterplot showing the proportion of acrosome intact live (AIL) sperm cells, ATP (μM ATP content per 10^6 sperm cells) and motility subpopulations classified as slow, rapid non-progressive (RapidNP) or rapid progressive (RapidP). Scatterplots are presented with 95% confidence interval ellipsoids.

Supporting information Table S1 Mean (standard deviation) sperm quality for different breeds (cryopreserved by SpermVital technology and Triladyl extender, analysed post-thaw and after three hours of incubation) of percentage acrosome intact live (AIL) sperm cells measured by flow cytometry, ATP levels (μM ATP per 10^6 sperm cells) measured by bioluminescence assay, percentage total motile (MOT) sperm cells and progressive motile (PROG) sperm cells measured by CASA divided by breed; there were no significant differences within parameter by breed (p>0.05).

Parameter	Brown Swiss	Limousine	Red Holstein
AIL	55.9 (10.9)	54.7 (12.3)	53.9 (9.3)
ATP	2.1 (0.3)	2.3 (0.3)	2.1 (0.4)
MOT	59.2 (13.5)	56.0 (16.0)	56.0 (15.2)
PROG	42.9 (14.5)	40.6 (17.1)	39.9 (15.1)

Supporting information Table S2 Mean (standard deviation) for kinematic parameters measured by CASA post-thaw (T0) in Sp-TALP-H and after 3 hours (T3) incubation at 38 °C for Triladyl (T) and SpermVital (SV) sperm cells. Kinematic parameters: velocity average path (VAP, μm/s), velocity curved line (VCL, μm/s), velocity straight line (VSL, μm/s), straightness (STR) of the average path defined as the ratio VSL/VAP (%), linearity (LIN) of the curvilinear path defined as the ratio VSL/VCL (%), Wobble (WOB) defined as the ratio VAP/VCL (%), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz).

Parameter	Time	T	SV
VAP	T0	77.2[a] (10.6)	82.7[a] (6.8)
	T3	45.9[b] (11.4)	82.4[a] (5.7)
VCL	T0	101.6[a] (12.8)	101.6[a] (8.6)
	T3	65.0[b] (12.2)	101.8[a] (6.9)
VSL	T0	68.0[a] (10.8)	72.5[a] (6.0)
	T3	35.6[b] (11.6)	72.6[a] (4.9)
STR	T0	87.8[a] (2.8)	87.6[a] (0.9)
	T3	76.7[b] (4.2)	88.2[a] (1.5)
LIN	T0	66.7[a] (5.0)	71.4[a] (2.3)
	T3	53.9[b] (5.8)	71.5[a] (3.9)
WOB	T0	75.9[a] (3.6)	81.5[b] (2.1)
	T3	70.1[c] (3.6)	81.0[b] (3.4)

ALH	T0	3.9^a (0.4)	3.2^b (0.4)
	T3	3.2^b (0.3)	3.3^b (0.4)
BCF	T0	10.1^a (1.0)	9.6^a (0.5)
	T3	7.3^b (0.9)	9.7^a (0.6)

[*]Values with different superscripted letters within parameter differ significantly ($p < 0.05$).

Supporting information Table S3

Pairwise correlations including percentage sperm cells classified as slow (Slow) motile, rapid non-progressive (RapidNP) motile or rapid progressive (RapidP) motile, percentage acrosome intact live (AIL) and μM ATP content per 10^6 sperm cells (ATP), as measured by CASA, flow cytometry and bioluminescence assay of semen pooled for time and processing method.

Parameters	r	L	U
ATP/AIL	**0,77**	0,62	0,87
Slow/AIL	**-0,34**	-0,58	-0,06
Slow/ATP	-0,17	-0,43	0,12
RapidNP/AIL	**0,59**	0,36	0,75
RapidNP/ATP	**0,48**	0,22	0,67
RapidP/AIL	**0,93**	0,87	0,96
RapidP/ATP	**0,71**	0,54	0,83

r = Pearson's correlation coefficient; 95 % confidence interval with L = lower and U = upper limit; values written in bold denote r ≠ 0, p<0.05; confidence intervals for r with no overlap differ significantly (p<0.05).

III

Ovarian follicular response to oestrous synchronisation and induction of ovulation in Norwegian Red cattle

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Abstract

Background: Oestrous synchronisation of cattle has been widely applied to accomplish simultaneous ovulation in animals and facilitate timed AI. The main aim of this study was to investigate the ovarian follicular growth and ovulatory response to oestrus and ovulation synchronisation in Norwegian Red heifers and cows. Oestrous cycles in 34 heifers and 10 cows from 4 herds were synchronised with two $PGF_{2\alpha}$ analogue treatments 11 days apart, followed by GnRH analogue treatment for induction of ovulation. Thereafter, the ovaries were examined by ultrasonography at 3 h intervals until ovulation.

Results: The luteolytic effect of the $PGF_{2\alpha}$ analogue was verified in 9 of 10 cows by progesterone contents in milk. Maximum physical activity of the cows occurred on average 69 h after $PGF_{2\alpha}$ analogue treatment. An ovulatory response was recorded in 95.5% (42/44) of the animals. A significant difference in follicle size at ovulation was found between 2 of the herds. Animals with medium sized and large follicles and heifers aged >16 months ovulated earlier than other animals.

Conclusions: The applied sequence of treatments in the study was shown to be effective in synchronizing and inducing ovulation within a relatively narrow time interval in the Norwegian Red heifers and cows, consistent with findings in other cattle breeds.

Key words: Oestrous synchronisation, ovulation, ultrasonography, Norwegian Red

Background

Synchronisation protocols are commonly applied in cattle to achieve simultaneous ovulation, allowing animals to be inseminated at a pre-set time, i.e. timed artificial insemination (AI). A variety of protocols have been developed for the reproduction management of cattle, especially in large herds, using different combinations of treatments with progesterone (P4), $PGF_{2\alpha}$ and GnRH to control the oestrous cycle and ovulation [1, 2]. Pre–synchronisation of follicular waves has been attempted using two–dose $PGF_{2\alpha}$ protocols and administering GnRH between $PGF_{2\alpha}$ treatments [3]. Wiltbank and Pursley (2014) achieved contrasting results using a selection of protocols and encouraged simplification. Competitive fertility results (high pregnancy rates and low embryonic loss) were demonstrated in a synchronisation procedure using only two $PGF_{2\alpha}$ analogue treatments, obviating the pre–synchronising steps used to align the follicular waves [4, 5].

Ovarian follicles in cattle are shown to develop in a wave-like pattern, during which the follicles pass through different stages of maturity (emergence, selection, deviation, dominance and atresia or ovulation) over a 7 to-10-day period [6]. Successful synchronisation of ovulation relies on the stage of follicular development at the time of GnRH treatment [7]. In spontaneously ovulating animals, the largest follicle of a follicular wave initiates the process of deviation from other follicles in the same wave approximately 12 h before a difference in their size can be detected [8]. This follicle differs from its contemporaries by having an increase in granulosa LH receptors and oestradiol, rendering it sensitive to the increase in LH following the wave of FSH [8]. In the case of synchronisation of ovulation by GnRH treatment, the diameter of the largest follicle at the time of administration may differ among animals. Interestingly, a positive correlation has been found at the time of GnRH treatment between the

size of the dominant follicle, circulating oestradiol and fertility [9]. Correspondingly, animals clearly displaying oestrus were reported to have larger ovulatory follicles [10].

In the past 50 years, the breeding goals concerning Norwegian Red have largely focused on fertility and health in addition milk production, resulting in a relatively high 56 d non-return rate in Norway of approximately 72.5% [11]. Moreover, Norwegian Red has been shown to have an increased oestrus length and more expression of oestrous signs compared to Holstein [12]. An association between the duration of oestrus and ovulatory response has been indicated [13]. Furthermore, cross-bred Holstein and Norwegian Red exhibit high reproductive performance; e.g. enhanced first service pregnancy rates, and considerably fewer days open during their first 5 lactations compared to pure Holstein [14]. Breed differences in oestrous expression and reproductive performance provide an incitement to study the ovulatory response to oestrous synchronisation in Norwegian Red.

The main aim of this study was to examine the ovarian follicular growth, ovulatory response and time to ovulation following synchronisation of oestrus and ovulation in Norwegian Red heifers and cows. Further, effects of herd, age and body condition score (BCS) were examined.

Methods

Animals

The experiment was performed in the period from May 2017 to March 2018 in south-eastern Norway. In total, 34 heifers in 4 dairy herds and 10 cows in one of these herds (identified as herd 4), all Norwegian Red cattle, held in free-stalls and fed a mixture of grass silage and grain concentrates supplemented with minerals, were included in this study. The heifers were 14-28 months old and cows were in parities 1-3. At the beginning of the study, the mean (range) for days in milk of the cows was 79.3 (60–111) and mean daily milk yield (range) was 32.8 kg

(23.4–40.8). Only animals with no record of reproductive, metabolic, mastitis or claw diseases within two months prior to the study were included. BCS was recorded and scored on a scale from 1–5 at the time of the initial treatment of the animals (Additional file 1) by using a visual scoring technique adjusted to Norwegian Red [15]. The study was performed in accordance with the Norwegian Animal Welfare Act (LOV 2009-06-19 no. 97).

Study design - Synchronisation and transrectal ultrasonography

Oestrus cycles of heifers and cows were synchronised with 500 µg cloprostenol (2 mL i.m. Estrumate vet., Intervet International B.V., Boxmeer, the Netherlands), a $PGF_{2\alpha}$ analogue. Estrumate was used for synchronisation according to manufacturer's instructions and the timing of treatments is illustrated in Figure 1. Ovarian structures were observed using a BCF Easi-Scan ultrasound scanner fitted with Easi-Scan Smart Display and a 7.5 MHz broadband straight linear rectal probe (BCF Technology Ltd, Scotland, UK). All examinations were conducted by the same veterinarian and assisting colleague. Transrectal ultrasound of the ovaries was used to confirm the presence of a mature corpus luteum (CL) on day 11 of the timeline, identifying animals potentially responsive to the luteolytic effect of the second cloprostenol treatment. One heifer was excluded from the trial at this stage due to lacking a mature CL.

Treatment with buserelin acetate (2.5 mL i.m. Receptal vet., Intervet International B.V., Boxmeer, the Netherlands), a synthetic GnRH analogue, was administered on day 13 at time defined as 0 h (T0). Transrectal ultrasonography of the ovaries was conducted at T0 and every 3rd hour thereafter until ovulation in the first herd (identified as herd 1, n=9) of the study. In this herd, no animals ovulated within 24 h post treatment with the GnRH analogue. For this reason, ultrasonography following the initial (T0) examination of the remaining herds was postponed 9 h, after which ultrasound examinations every third hour were applied until the animals had ovulated. Following rotation of the rectal probe about the dominant (largest observed) follicle, its position and size [16] was recorded using the electronic callipers of the

5

Easi-Scan Smart Display, until the maximum diameter was found. The persistence of dominant follicles was defined as time elapsed from T0 until their disappearance. Time of ovulation was assumed to be the midpoint between the first ultrasound measurement lacking the dominant follicle and the previous measurement 3 hours earlier, i.e. time of the first ultrasonography measurement after ovulation minus 1.5 h. Ovulation, i.e. disappearance of the dominant follicle, was confirmed by ultrasound examination and subsequent rectal palpation.

Progesterone in milk

Milk was sampled from the 10 cows for measurement of P4 [17] in the afternoon of day 11 (i.e. time of $PGF_{2\alpha}$ analogue administration) and day 12, and thereafter simultaneously with the ultrasonography examinations. Sampling continued once daily in the afternoon for two days following ovulation. All samplings were performed directly after hand strip milking, i.e. milk was drawn from the teat 5 times before sampling. Milk samples (10–20 mL), preserved with a bronopol tablet, were stored at −18 ºC until analysis. Concentrations of P4 in milk were measured by an enzyme immunoassay [17, 18]. The occurrence of complete luteolysis was detected by measurements showing P4 concentrations <2.5 ng/mL, as defined earlier [19]. However, one cow had P4 concentrations compatible with incomplete luteolysis, i.e. P4 concentrations remained high (≥2.5 ng/mL) within 72 h after $PGF_{2\alpha}$ analogue administration [19] and this sample was therefore removed from the P4 calculations.

Physical activity of the cows

Activity of the 10 cows was measured individually by an activity monitoring system (DeLaval DelPro, Tumba, Sweden) connected to a central computer in the automatic milking system (VMS, DeLaval). Activity levels were processed by DelPro 5.1 herd management software where activity measured in the past 6 h is compared to activity measured at the same time of day over the past 7 days and expressed as a relative activity ratio. For one of the cows, activity was not recorded due to technical malfunction of the monitoring equipment.

Data treatment and statistical analyses

Time to ovulation was defined as the number of hours from GnRH analogue treatment to the ultrasonography measurement where ovulation was detected minus 1.5 h. The size of the ovulating follicle was determined from the maximum diameter at the last measurement before ovulation. As ovulations occurred, the number of animals included in calculations for mean (standard deviation) diameter of ovulating follicles were progressively reduced. The data were analysed using Statistical Analysis System (SAS® version 9.4) for Microsoft Windows (SAS Institute Inc., Cary, NC, USA).

General Linear Model Analysis

The General Linear Models procedure (SAS® version 9.4) was used to perform a least square analysis for size of the ovulating follicle (outcome variable). The effect of herd, age, BCS and initial diameter of the dominant follicle at T0 on the outcome variable were estimated using the following model:

$Y_{ijkl} = \mu + H_i + A_j + B_k + I_l + e_{ijklm}$

where:

Y_{ijkl} = size of the ovulating follicle in animal m;

μ = overall mean size;

H_i = effect of herd, i = 1–4;

A_j = effect of age, j = 1–4, where 1 = heifer 14–15 months, 2 = heifer 16–18 months, 3 = heifer 19–28 months, and 4 = cow parity 1–3;

B_k = effect of BCS, k = 1–3, where 1 = (lean: 2.5–2.9), k = 2 (moderate: 3.0–3.4) or k = 3 (fat: 3.5–4.0);

I_l=effect of initial follicle size in 2 classes, where l=1 (diameter ≤10 mm) or l=2 (diameter >10 mm);

e_{ijklm}=residual effect.

A general linear model including the same effects, except that of initial follicle size, and additionally incorporating the size of the dominant follicle at the last measurement was used to analyse time to ovulation (outcome variable).

The study was conducted in herd 1 in May, herd 2 in November, herd 3 in February and herd 4 in February and March. Herd and season could therefore not be separated. Thus, the effect referred to as herd includes possible season effects.

Survival analyses

The Lifereg procedure (SAS® version 9.4) was used to perform a Cox regression, thus including time to ovulation for non-ovulating animals (n=2) as censored animals, rather than missing observations. The possible effects of herd, age, BCS, initial follicle size and size of the ovulating follicle classes as defined above, were tested on the outcome variable (time to ovulation) in the following model:

$H(t)=H_0(t)×exp(H_i+A_j+B_k+I_l+F_m)$

where

$H(t)$=the cumulative hazard of ovulating at time t;

$H_0(t)$=the cumulative baseline hazard of ovulating at time t;

F_m= effect of the size of the ovulatory follicle, m=1–3, where m=1 (small: 9 < 15 mm), m=2 (medium: 15 – 17.5 mm) or m=3 (large: 17.5 < 23 mm);

H_i, A_j, B_k, and I_l as previously stated.

The hazard ratio is the ratio of the hazard rates corresponding to two levels of one of the predictor variables. By exponentiation of the solution to the base of the natural logarithm (e^b), the hazard ratio is found.

Results

Of all animals, 95.5% showed an ovulatory response. The mean (SD) time to ovulation for these animals was 27.3 h (3.0), 27.0 (3.1) for heifers and 28.2 (2.6) for cows, with a range of 19.5–34.5 h (Additional file 2). Distribution of time to ovulation is presented in Figure 2. The earliest ovulations were observed 19.5 h after T0 (all heifers), whereas most ovulations (72.7% of all animals) were recorded between 25.5 and 28.5 h after T0. The majority of the cows (60%) ovulated 28.5 h after T0. At T0, the mean (SD) diameter of the dominant follicle measured 10.3 mm (2.9). From this point until ovulation, there was a gradual increase in follicle diameter (Figure 3). The mean (SD) diameter of the dominant follicles measured 1.5 h before the time defined as ovulation was 16 mm (3.0). The diameter at ovulation ranged from 9 to 23 mm (Figure 2).

The initial mean P4 concentration at −48 h, was 9.25 ng/ mL (range 3.5–20.4) (Figure 3). Subsequently, a rapid decline was observed over the following 24 h. Six of 10 cows reached a minimum P4 concentration 66–69 h after the last PGF$_{2\alpha}$ analogue treatment. From 96–120 h after the final PGF$_{2\alpha}$ analogue treatment, P4 concentrations remained low. One cow had particularly high initial P4 concentrations, remaining >4.0 ng/mL during the whole study (13 samplings from the last PGF$_{2\alpha}$ treatment until two days after ovulation), findings which are compatible with incomplete luteolysis [20]. Mean physical relative activity ratios are shown in Figure 3. The initial measurement at time −48 h showed a mean activity ratio of 98%, which was the lowest recorded value. Thereafter, the mean activity level increased to 110% 69 h after the final PGF$_{2\alpha}$ analogue treatment, when 5 of 9 cows had reached a maximum activity. A rapid decline in activity ratio was then observed up to 96 h, after which it stabilised.

Effects on the size of the ovulatory follicle are given as least square means (LSM) by herd, age, initial follicle size and BCS in Table 1. Ovulatory follicles were larger in herd 2 than in herd 1 ($P < 0.05$), and 16 to 18 months old heifers tended to have larger follicles than older heifers ($P=0.06$). Large follicles (>10 mm) at T0 tended to be larger at ovulation compared to other follicles ($P=0.07$). The correlation coefficient (95% CI) including the initial size of the dominant follicle and size at ovulation was 0.50 (0.21-0.71). No significant differences were found in LSM for time to ovulation between different levels of herd, age, size of the dominant follicle at the last measurement or BCS.

Cox regression analyses showed association between size of the ovulatory follicle and time to ovulation ($P < 0.05$) (Table 2). Medium-sized and large follicles ovulated earlier than small follicles ($P < 0.001$ and < 0.05, respectively). Further, heifers in the age groups 16–18 months and 19–28 months ovulated earlier than the younger heifers (14–15 months) ($P=0.05$ and $P < 0.05$, respectively). Animals with a moderate BCS tended to ovulate earlier than fat animals ($P < 0.08$). In two heifers, no ovulation was observed, although dominant follicles were observed in both animals during the study period. The final observations of these follicles showed that they were surrounded by an approximately 2 mm thin, speckled layer, indicating luteal tissue.

Discussion

In Norway, until recently, hormone treatment in cattle has been used restrictively, e.g. due to relatively small herds. However, increasing herd sizes makes knowledge on oestrous synchronisation more relevant. In the present study, a gradual increase in mean follicle diameter was recorded from the time of GnRH analogue treatment until ovulation. In total, 95.5% of the Norwegian Red heifers and cows responded to oestrous synchronisation (11 d double $PGF_{2\alpha}$ protocol) and induction of ovulation within 34.5 h of GnRH analogue treatment.

As explained by Meyer et al. (2007), two successive $PGF_{2\alpha}$ analogue treatments mediate luteolysis, align the animals into the oestrous cycle, resulting in a marked decline in P4 concentrations, which allows the LH surge that precedes ovulation [21]. In agreement with previous findings [19, 20], 9 of 10 cows in the current study showed a rapid decline in P4 concentrations to values <2,5 ng/ml within 24 h of $PGF_{2\alpha}$ analogue treatment, indicating complete luteolysis. One cow had milk P4 concentrations indicating incomplete luteolysis yet ovulated within the observed range. This could be due to a postponed but successful luteolysis or variation in P4 concentrations caused by heterogeneity in the time of sampling relative to milking [22]. Considerable individual variations in time to luteolysis after $PGF_{2\alpha}$ treatment have also been reported, likely due to diversity in the stage of oestrous cycles between animals at the time of treatment [23, 24]. Previous research shows an association between failure of luteolysis of seemingly mature CL and high milk yield and low milk protein content [19]. The cows in our study exhibited milk production consistent with earlier findings [19] and above the average in Norway.

Effective oestrous synchronisation relies on ovarian follicular maturity at the time of luteolytic $PGF_{2\alpha}$ treatment relative to the stage of follicular waves and the time of subsequent GnRH induction of ovulation [25]. Onset of ovarian follicular maturity is reported to be observed when the preovulatory follicle reaches about 10 mm in diameter [26]. In the current study, the size of preovulatory follicles at T0 were highly correlated to the size of follicles at ovulation, indicating that ovulatory response to GnRH treatment could be influenced by diversity in the maturity of preovulatory follicles. In the Cox regression, we found that ovulating follicles smaller than 15 mm ovulated significantly later than larger follicles. Our findings on preovulatory follicle size and time to ovulation are in agreement with previous observations [9] showing that small preovulatory follicles were associated with small ovulating follicles and a delayed time of ovulation, possibly caused by physiological immaturity during preovulatory

stages. Pre–synchronisation steps using GnRH were not included in the current study, potentially resulting in a greater variability in follicular maturity at the time of GnRH treatment (T0) and subsequent variation in follicular size at ovulation. However, the size of ovulating follicles in our study was comparable to other findings following follicular pre–synchronisation [27, 28], thus questioning the efficiency and necessity of pre–synchronisation steps in healthy animals.

Research in Holstein and Brown Swiss has shown that 80.0% of ovulating cows ovulated within the range of 26–30 h after GnRH analogue treatment [7]. Correspondingly, a recent study in Holsteins reported that 80.0% of the animals ovulated between 28–30 h following GnRH analogue treatment [29]. A comparable proportion of animals ovulated within a similar time interval in the current study. Sveberg et al. (2015) demonstrated more mounting and standing behaviour during oestrus in Norwegian Red compared to Holstein. Although a reduction in the expression of oestrous activity (e.g. less mounting and standing behaviour) has been observed over the past decades in Holstein compared to Norwegian Red [12], this does not seem to be related to an ovulatory response to synchronisation. Previous findings [27] and results of the present study indicate that the ovulatory response and time to ovulation following oestrous synchronisation is comparable between these dairy breeds. Our results showed a mean time to ovulation from GnRH analogue treatment of 27.3 h, with heifers ovulating slightly earlier than cows. Several factors contribute to the variety in optimal timing of AI, e.g. breed, herd, age and season. In general, AI is recommended within 12 – 18 h before ovulation [30]. Consequently, our advice is that AI should be performed 9 – 15 h after induction of ovulation.

Conclusions

The current study presents new information on the ovarian follicular dynamics and ovulatory response to oestrous synchronisation in Norwegian Red heifers and cows. The applied oestrous synchronisation induced ovulation on average 27.3 h after GnRH treatment in 42 of 44 animals, and 73% of the animals ovulated within a constricted time interval (25–28 h). In this study the temporal relationships between growth of the ovulatory follicle, time to ovulation, P4 concentrations and physical activity were investigated. Our findings indicate that the response to the applied synchronisation protocol follows a pattern which is in agreement with reports on other breeds. Norwegian Red heifers and cows expressed profiles of ovarian dynamics compatible with successful synchronisation of oestrus and ovulation when GnRH was administered 48 h after administering 2 doses of $PGF_{2\alpha}$ 11 days apart.

Authors' contributions

All authors participated in the planning of the study. The clinical work of the study was performed by HFB, VGK and KID. Statistical analyses were made by HFB and BH. The manuscript was drafted by HFB. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding

author on reasonable request.

Consent for publication

Not applicable.

Ethics approval

The study was performed in accordance with the Norwegian Animal Welfare Act (LOV 2009-06-19

no. 97) and in compliance with best practice of veterinary care.

Prior publication

Data have not been published previously.

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TABLES

Table 1 Least square means (LSM ± SE) of the size of dominant follicles at ovulation following oestrous synchronisation

Variable	Class	n	Size, LSM ± SE
Herd	1	9	[a]13.2 ± 1.5
	2	10	[b]19.6 ± 1.6
	3	6	14.8 ± 1.5
	4	19	15.8 ± 1.2
Age	Heifers 14–15 months	10	15.9 ± 1.0
	Heifers 16–18 months	13	17.1 ± 1.0
	Heifers 19–28 months	11	13.4 ± 1.5
	Cows parity 1–3	10	16.9 ± 1.6
Initial follicle size[†]	≤10.0 mm	21	15.4 ± 0.7
	>10.0 mm	23	16.3 ± 0.8
BCS	Lean (2.5–2.9)	6	15.5 ± 1.7
	Moderate (3.0–3.4)	20	15.2 ± 0.9
	Fat (3.5–4.0)	18	16.9 ± 0.8

Number of animals (n) and least square means (LSM) ± standard error (SE) for size of the dominant follicle (diameter, mm) at ovulation by herd, and classes of age, initial follicle size[1], and body condition score (BCS)

[a-b]LSM within variable and column with different superscripts differ; $P < 0.05$.

[†]Diameter of the dominant follicle at the time of GnRH analogue treatment (T0).

Table 2 Survival analysis of time to ovulation following oestrous synchronisation

Variable	Level	Estimate[†]	SE	Hazard ratio (e^b)
Herd	1	-	-	R
	2	0.14	0.12	1.15
	3	-0.05	0.06	0.95
	4	0.04	0.06	1.04
Age	Heifers 14–15 months	-	-	R
	Heifers 16–18 months	-0.10[*]	0.05	0.90
	Heifers 19–28 months	-0.20[*]	0.10	0.82
	Cows parity 1–3	-0.02	0.06	1.02
Initial follicle size[‡]	≤10.0 mm	0.05	0.04	1.05
	>10.0 mm	-	-	R
Diameter at ovulation[§, *]	9.0–15.0 mm	-	-	R
	15.1–17.4 mm	-0.15[**]	0.05	0.86
	17.5–23.0 mm	-0.12[*]	0.06	0.89
BCS	Lean (2.5–2.9)	0.07	0.07	1.07
	Moderate (3.0–3.4)	-	-	R
	Fat (3.5–4.0)	0.08	0.05	1.08

Hazard ratio for time to ovulation by herd, and classes of age, initial follicle size, follicle diameter at ovulation, and body condition score (BCS) in Norwegian Red cattle; time variable: hours from GnRH analogue treatment to ovulation; R, reference value and SE, standard error

[*]Overall association with time to ovulation or within variable difference relative to the reference value; $P \leq 0.05$.

[**]Within variable difference relative to the reference value; $P < 0.001$.

[†]Estimate for each parameter level.

[‡]Diameter (mm) of the dominant follicle at the time of GnRH analogue treatment (T0).

§Diameter (mm) of the dominant follicle at the time of ovulation.

FIGURES
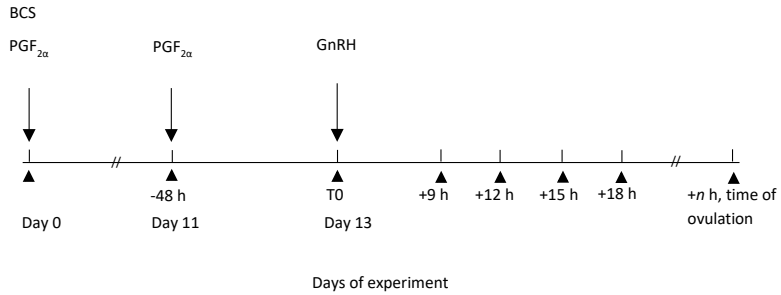
Figure 1 Timeline showing the synchronising protocol and sequence of animal treatments and recordings

$PGF_{2\alpha}$ analogue treatment ($PGF_{2\alpha}$) on day 0 and 11 and body condition scoring (BCS) on day 0; GnRH analogue treatment (GnRH) on day 13 at time 0 (T0); transrectal ovarian ultrasonography (▲) to identify animals with a mature corpus luteum on day 11 (-48 h) and from day 13 at T0 and every third hour from 9 h following T0 (+9 h) until the time of ovulation (+n h)
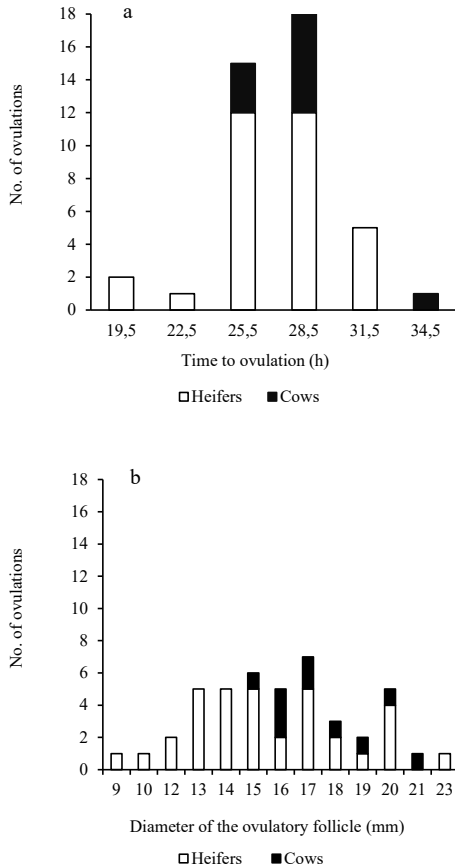
Figure 2 Ovarian follicular response to oestrous synchronisation

Response to PGF$_{2\alpha}$ analogue treatment (twice, 11 days apart) followed by GnRH analogue induction of ovulation 48 h later in heifers and cows. a) Distribution of time (h) to ovulation measured as time from GnRH analogue treatment to the final ultrasonography examination after ovulation minus 1.5 h and b) distribution of diameter (mm) of the ovulating follicle (at the last ultrasonography measurement); two heifers did not ovulate
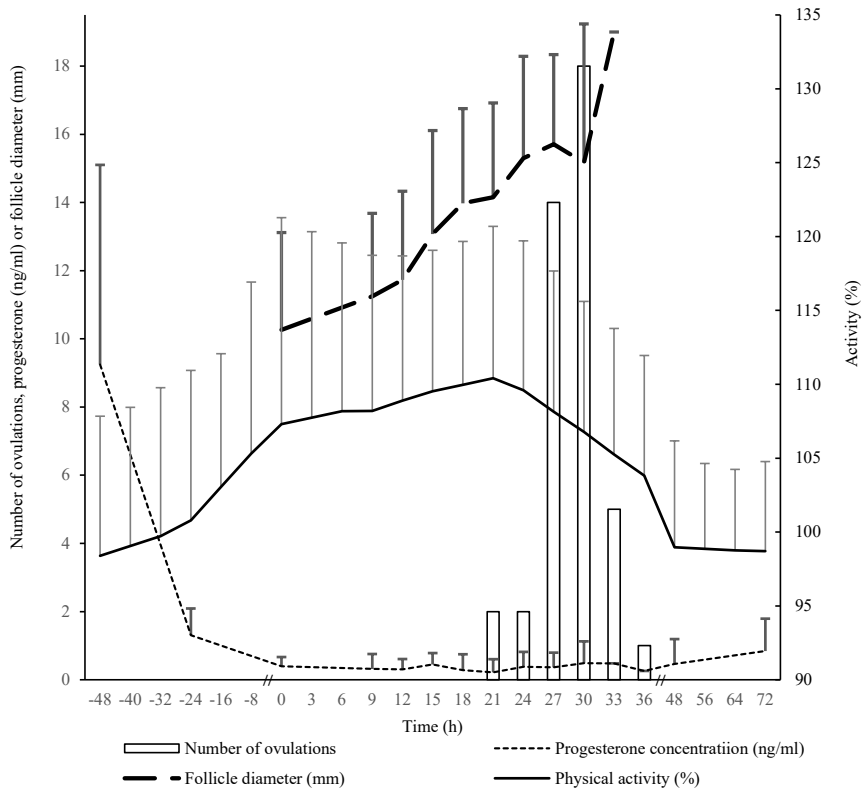
Figure 3 Physical activity, milk progesterone concentrations in cows, and follicular size and time to ovulation in heifers and cows; the results are presented as mean ± s.e.

Mean (SD) in cows (n=9) for physical activity (%) (activity measured in the past 6 h relative to activity measured at the same time of day in the past 7 days) measured by an automatic activity monitoring system and mean (SD) in progesterone (ng/ml) in milk given by time (h) from the last $PGF_{2\alpha}$ analogue treatment (time=-48 h); mean (SD) in heifers and cows (n=32 and 10, respectively) for the diameter (mm) of the dominant follicle measured until ovulation by ultrasonography following GnRH analogue treatment at time=0 h; number of ovulations at the point of final ultrasonography (1.5 h after ovulation) following GnRH analogue treatment at time=0 h; two heifers did not ovulate

ADDITIONAL FILES

Additional table 1 Body condition score of animals by herd and age

Category	Subcategory	n	BCS[†](SD)	Range
Herd	1	9	3.6 (0.3)	3.0–4.0
	2	10	2.8 (0.4)	2.5–3.5
	3	6	3.4 (0.2)	3.3–3.8
	4	19	3.3 (0.2)	3.0–3.8
Age	Heifers, all	34	3.2 (0.4)	2.5–4.0
	Heifers 14–15 months	10	3.2 (0.3)	2.5–3.5
	Heifers 16–18 months	13	3.4 (0.3)	3.0–4.0
	Heifers 19–28 months	11	3.0 (0.5)	2.5–4.0
	Cows parity 1–3	10	3.5 (0.1)	3.3–3.8
Total		44	3.3 (0.4)	2.5–4.0

Number of animals (n) mean (SD) and range for body condition score (BCS) grouped by subcategories within the categories herd and age

[†]BCS was recorded at the initial examination and treatment of the animals by using a visual scoring technique. BCS was measured on a scale from 1–5, where 1 is emaciated and 5 is severely over-conditioned animals.

Additional table 2 Time to ovulation and size of the ovulatory follicle after oestrous synchronisation

Level	N	Time (SD)	Range	Size (SD)	Range
Overall	42	27.3 (3.0)	19.5–34.5	16.1 (3.0)	9.0–21.0
Heifers	32	27.0 (3.1)	19.5–31.5	15.6 (3.2)	9.0–23.0
Cows	10	28.2 (2.6)	25.5–34.5	17.5 (2.0)	15.0–21.0

Mean (SD) and range for time to ovulation[†] and size of the ovulatory follicle[‡] overall, and in heifers and cows in response to oestrous synchronisation and induction of ovulation[§]; two heifers did not ovulate

[†]Hours between GnRH analogue treatment at time 0 on day 13 and the first ultrasonography measurement after ovulation minus 1.5 h (midpoint in time between the 3-hourly measurements).

[‡]Diameter (mm) of the dominant follicle at the time of ovulation; two heifers did not ovulate and were excluded from these calculations.

[§]$PGF_{2\alpha}$ analogue treatment on day 0 and 11; GnRH analogue treatment at time 0 on day 13.

IV

Reproduction in Domestic Animals **WILEY**

Studies of gel with immobilized semen by intrauterine endoscopy post-artificial insemination

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Abstract

An extended lifespan of spermatozoa following artificial insemination (AI) can make the timing of insemination less critical, as previously demonstrated with immobilized spermatozoa that are gradually released from an alginate gel. The purpose was to examine the in vivo dissolution of SpermVital (SV) alginate gel over time by endoscopy and secondly to assess spermatozoa quality after incubation of the gel. In vivo endoscopy showed SV gel in the uterus 3, 6, 20 and 24 hr after AI, demonstrating the potential release of spermatozoa to the uterus during this period. In utero ex vivo incubation of the semen demonstrated that high motility and viability of sperm cells was sustained following overnight incubation.

KEYWORDS

artificial insemination, cattle, endoscopy, immobilized spermatozoa

1 | INTRODUCTION

During standard AI in cattle, semen is deposited in the uterine body. To enable fertilization, the spermatozoa must travel through the female genital tract to the site of fertilization at the ampullaristhmic junction of the oviduct (Rath, Schuberth, Coy, & Taylor, 2008). Following AI, the selection and survival of spermatozoa, is affected by timing of AI, individual variation in the genital tract environment (Rath et al., 2008) and the choice of semen processing technology (Layek, Mohanty, Kumaresan, & Parks, 2016).

By the patented SpermVital (SV) technology, spermatozoa are immobilized in an alginate gel to enable their gradual release following insemination (Kommisrud, Hofmo, & Klinkenberg, 2008). Timing of AI relative to oestrous signs is assumed more flexible applying SV semen compared with standard semen. Alm-Kristiansen et al. (2017)

reported similar pregnancy rates between AIs using a single AI dose with immobilized SV semen early in heat and AIs using standard processed semen inseminated on two consecutive days. Further, in vitro evaluation of SV spermatozoa quality post-thaw and following incubation has demonstrated prolonged viability compared with standard processed semen (Alm-Kristiansen et al., 2018). Multiple physiological and biochemical interactions take place between spermatozoa and the female genital tract which result in the selection of specific sperm subpopulations after AI (Suarez, 2016). Therefore, it is interesting to follow the in vivo dissolution of SV gel by genital tract endoscopy (GTE).

The main objective of this case study was to examine the in vivo dissolution of SV gel by intrauterine endoscopy and to assess spermatozoa motility and viability over time by incubating SV semen in utero ex vivo.

Data material can be acquired on request by email: halldor.felde.berg@nmbu.no
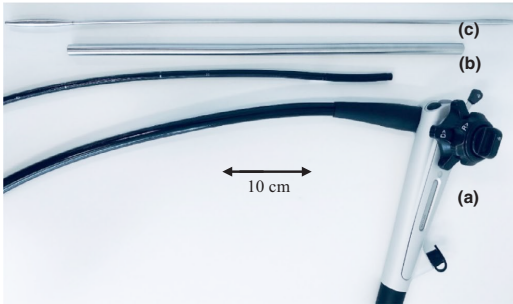
FIGURE 1 Endoscopy instruments: (a) endoscope with a controllable apex: external diameter = 7.9 mm, length = 140.0 cm, working channel = 2.8 mm (Karl Storz Silver Scope) (b) introducer, external diameter = 10.0 mm, internal diameter 9.0 mm, length = 42.0 cm (c) dilator, maximum diameter = 7.0 mm, length = 55.0 cm

2 | MATERIALS AND METHODS

2.1 | Ethical statement

The study was performed following criteria approved by the Animals in Science Regulation Unit of the Norwegian Food Safety Authority (FOTS No.: 20207).

2.2 | Semen processing

Two ejaculates were collected from one Norwegian Red bull (Geno Breeding and AI Association) within 15 min and pooled before split in two aliquots and processed using the SV technology in an alginate-based extender. One aliquot was added 0.23% Blue Dextran

(D4772, SigmaAldrich) to enable identification of SV gel by GTE, the other served as control. Semen samples were cryopreserved as described by Alm-Kristiansen et al. (2017). Blue Dextran staining did not compromise spermatozoa post-thaw quality.

2.3 | Animals

Three Norwegian Red cows (A, B and C: parity 3, 2 and 3, respectively) held in free-stalls, fed grass silage and grain concentrate with mineral supplement were included in the study. The animals had no record of disease within 2 months before the study.

2.4 | Oestrous synchronization

Oestrus was synchronized with cloprostenol (500 µg i.m. Estrumate vet., Intervet International B.V.). In accordance with the manufacturer's recommendation for cloprostenol used for synchronization, the animals were treated with one dose on day 0 and another on day 11. Transrectal ultrasonography (BCF Easi-Scan with 7.5 MHz broadband straight linear rectal probe, BCF Technology Ltd) of the ovaries was used to confirm presence of a mature corpus luteum on day 11, identifying animals potentially responsive to oestrous synchronization.
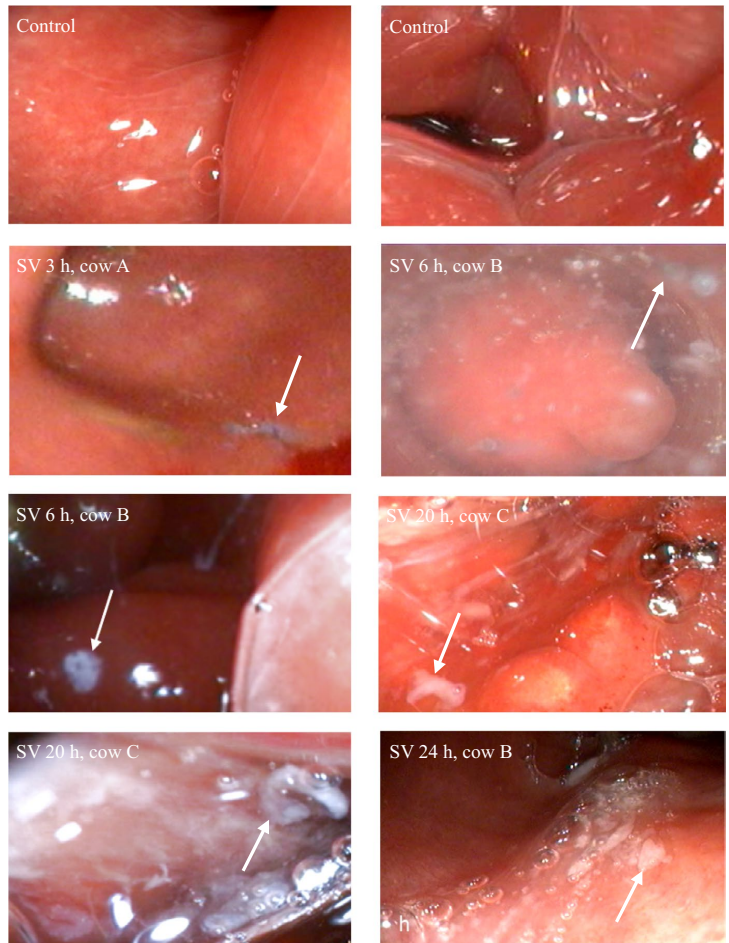
2.5 | Endoscopy

Directly after GnRH analogue (buserelin acetate, 10.5 µg i.m. Receptal vet., Intervet International B.V.) treatment on day 13 at the time defined as 0 hr the cows were inseminated with SV semen. To facilitate retrieval, 5 semen doses were consistently deposited at

Rectum

Endoscope
SpermVital
semen

Ovary
Uterus
Oviduct

Vagina Introducer Cervix
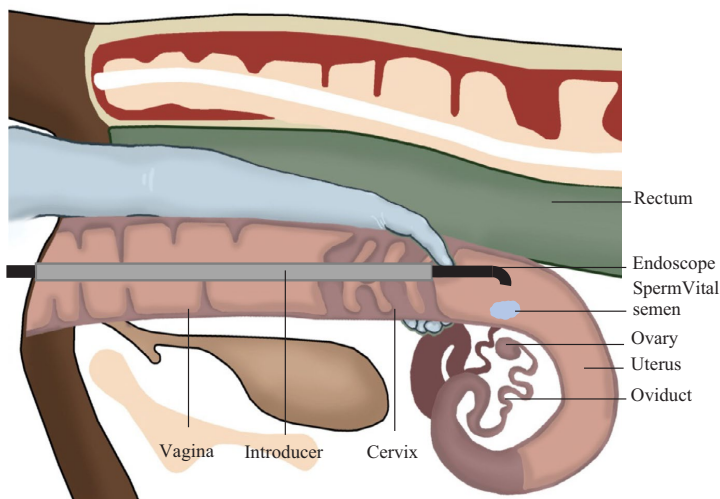
FIGURE 2 Genital tract endoscopy: introducer enabling passage of the endoscope through the cervix; SpermVital semen deposited by standard AI before endoscopy for in vivo monitoring

—**WILEY**

FIGURE 3 In vivo luminal view of the uterus; oestrous mucosa without stained SV fragments (transparent mucus with low viscosity, Control); SpermVital semen (arrows) stained with Blue Dextran 3 hr (SV 3 hr, cow A), 6 hr (SV 6 hr, cow B), 20 hr (SV 20 hr, cow C) and 24 hr (SV 24 hr, cow B) following AI

one location in one of the uterine horns in each cow. A dilator was then inserted through the cervix until reaching the posterior parts of the uterine body. To facilitate penetration of the flexible endoscope (Karl Storz Silver Scope) through the tortuous cervix, an introducer was thread onto the cervical dilator until reaching the anterior parts of the cervix (Figure 1).

By removing the dilator, passage of the endoscope through the cervix and access to the uterus was enabled (Figure 2). Dissolution of the SV gel was monitored by GTE 3, 6, 20 and 24 hr following AI. Images of the oestrous uterine mucosa without SV gel fragments were taken for control purposes.

2.6 | Spermatozoa quality in utero ex vivo

Bovine reproductive organs in pro-oestrus (n = 3) were inseminated with 2 SV doses in each uterine horn for incubation at room temperature. A section was made in the uterine horns 20 hr

post-insemination followed by sampling of the inseminated SV gel into a pre-warmed SV dilution buffer. The remaining gel from one AI dose was diluted in SV dilution buffer (500 µl, ~37°C) and incubated for 15 min at ~37°C with gentle tilting. Semen (5 µl) was placed on a pre-warmed microscope slide, and subjective motility was examined by phase-contrast microscopy at 37°C and 100× magnification. An aliquot of the SV semen sample was incubated for 10 min in room temperature with 37.5 µM PI for examination of viability by fluorescence microscopy as described (Alm-Kristiansen et al., 2018).

3 | RESULTS

Fragments of SV gel were found in vivo in the uterine horns at 3 (cow A), 6 (cow B), 20 (cow C) and 24 hr (cow B) after AI. In vivo, these fragments were discerned from the uterine tissue and the transparent oestrous mucus discharge by the Blue Dextran staining and their indented shape (Figure 3; video provided in Video S1). Since

fragments of gel were difficult to extract in vivo, sperm motility and viability was only examined after incubation in utero ex vivo. Initial measurements of post-thaw motility and viability means (*SD*) were 52% (3) and 53% (5), while for sperm from gel incubated overnight in utero ex vivo the corresponding values were 38.3% (12) and 52% (2), respectively. The SV gel was partly dissolved following overnight incubation.

4 | DISCUSSION

In this study, non-invasive GTE was used in vivo to monitor SV semen following AI. A gradual dissolution of the alginate gel was visualized in 4 recordings 3–24 hr after AI. The gradual dissolution of the alginate gel was also observed ex vivo after uterine incubation. This indicates a slow release of spermatozoa from the solid alginate gel as shown previously (Alm-Kristiansen et al., 2018), and as in the present study demonstrating viable and motile spermatozoa following overnight incubation. A study applying cryopreserved canine semen to compare processing with alginate gel microencapsulation to standard processing shows similar results, with elevated motility and viability in the former after incubation at body temperature (Shah et al., 2011).

Limitations of the GTE technique were encountered reaching the uterine lumen in animals with a highly tortuous cervix. Careful operating of the GTE equipment was required to avoid abrasions in the genital tract mucosa. The effective discrimination of SV semen from uterine tissue and exudate by GTE depended on the blue staining of the alginate gel. Decolourising of the gel was observed, particularly >6 hr' post-insemination and most prevalent in vivo, likely due to the extensive perfusion of the uterus in oestrus.

This case study showed that SV gel can be retrieved in vivo 24 hr after insemination. Uterine incubation of the semen ex vivo demonstrated that high motility and viability was maintained overnight. The results indicate a prolonged release of immobilized spermatozoa expressing high reproductive potential, possibly resulting in an extended window of time for successful AI in cattle.

CONFLICT OF INTEREST
None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS
HFB prepared trial design, performed animal clinical work, in utero ex vivo examinations, preparation of manuscript; HCLS performed clinical work, ex vivo examinations, semen in vitro analysis, preparation of manuscript; BH preparation of manuscript; ER preparation of manuscript; AHAK performed semen in vitro analysis, preparation of manuscript; EK prepared trial design, preparation of manuscript.

DATA AVAILABILITY
Additional data, for example endoscopy video material can be acquired on request by contacting the first author.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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