Impact of extraction method on the quality of bull epididymal sperm

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Independent Project • 30 credits
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Faculty of Veterinary Medicine and Animal Science
Veterinary Medicine Programme

Uppsala 2024

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Extraktionsmetodens påverkan på spermiekvaliteten hos bitestikelspermier från tjur

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Credits:	30 credits
Level:	Second cycle, A2E
Course title:	Independent Project in Veterinary Medicine
Course code:	EX1003
Programme/education:	Veterinary Medicine Programme
Course coordinating dept.:	Department of Clinical Sciences
Place of publication:	Uppsala
Year of publication:	2024
Cover picture:	Terese Compier

Keywords:	sperm quality, extraction method, bovine, epididymis, cryopreservation, viability, motility, membrane integrity, chromatin structure, reactive oxygen species, post-mortem

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Abstract

Epididymal sperm are a potential resource enabling the preservation of valuable genes in populations where collection of ejaculated semen is not possible. There are many suggested methods on how to extract epididymal sperm, although comparison of some of these and establishment of a gold standard method have not been thoroughly studied – especially not in bovines. In this study the aim was to evaluate and compare two of the most used extraction methods in bulls, evaluating sperm quality characteristics to establish which method is to be preferred. The material used, consisting of pairs of bull testes, was provided by a local abattoir, and the extraction, as well as the initial analysis before cryopreservation, was executed the same day. The sperm collected using the cutting method (A), where incisions are made into the caudae epididymidis and the exuding sperm suspension is collected, had a significantly lower total and progressive motility than the vas deferens retrograde flushing method (B) prior to cryopreservation. This difference also applied to the kinematic parameters curvilinear velocity, straight line velocity, average path velocity and amplitude of lateral head displacement, as assessed by computer assisted sperm analysis (CASA). Another important aspect favoring Method B is that it exhibited a trend towards significance regarding a higher number of spermatozoa obtained, a desirable property when aiming to extract epididymal sperm from genetically valuable individuals.

Regarding the flow cytometry analyses plasma membrane integrity, reactive oxygen species and sperm chromatin structure, no differences were detected between the methods. Following the first analyses the samples were cryopreserved and subsequently analysed again after thawing. There were multiple differences between the samples prior to cryopreservation and post-thawing, showing a decrease in characteristics post-thawing for all quality analyses except for the chromatin structure and the reactive oxygen parameter Live SO^{+}. No significant differences or correlations were found in sperm characteristics between the left and right testes, or between scrotal and testicular circumference. A weak correlation with age was, however, discovered implying that older bulls have better sperm motility and viability; the presence of testicular adhesions was associated with impaired sperm quality. All in all, this implies that the retrograde flushing method is to be preferred when extracting epididymal sperm, to acquire a higher total number of motile sperm in the fresh sperm sample. Regarding cryopreservation, a different protocol should be evaluated to achieve better post-thaw sperm quality. One should also bear in mind that sperm quality in younger bulls might not be as good as in slightly older ones.

Keywords: sperm quality, extraction method, bovine, epididymis, cryopreservation, viability, motility, membrane integrity, chromatin structure, reactive oxygen species, post mortem

Sammanfattning

Bitestikelspermier är en potentiell resurs som kan möjliggöra bevarandet av värdefullt genetiskt material i populationer där uppsamling av ejakulat inte är möjligt. Det finns flera föreslagna metoder för hur extraktionen av bitestikelspermier bör gå till, men jämförelse av metoderna har inte utförts i någon omfattande utsträckning hos oxdjur. Etablering av en gold standardmetod har således uteblivit. Denna studies syfte är att utröna och jämföra hur två av de vanligast förekommande metoderna påverkar spermiekvaliteten hos tjurar för att därefter kunna fastställa vilken metod som är att föredra. Materialet bestående av tjurtestiklar tillhandahölls av ett lokalt slakteri och spermie-extraktion likväl som initial analys av proverna innan nedfrysning utfördes samma dag. Metoden innefattade anläggande av snitt i cauda epididymidis (A) följt av aspiration av framläckande spermiesuspension innebar signifikant lägre totalt och progressiv motilitet hos spermierna före nedfrysning jämfört med metoden där spermier retrogradt spolas genom vas deferens (B). Denna skillnad gällde även parametrarna ”curvilinear velocity”, ”straight line velocity”, ”average path velocity” och ”amplitude of lateral head displacement” som beräknats med hjälp av datorassisterad spermieanalys (CASA). En annan viktig aspekt som talar för att Metod B är att föredra är att den uppvisade en trend mot signifikans gällande större antal extraherade spermier vilket är en mycket önskvärd egenskap vid extraktion av bitestikelspermier från genetiskt viktiga individer.

För analyserna utförda med flödescytometri inkluderande membranintegritet, reaktiva syreföreningar och kromatinstruktur kunde ingen signifikant skillnad identifieras metoderna emellan. Efter den första analysen kryopreserverades proverna och upprepande analys utfördes efter upptining. Det fanns flera skillnader mellan proverna innan frysning och efter upptining, som tillsammans tydde på sämre spermiekvalitet efter upptining. Detta omfattade samtliga kvalitets-analyser förutom kromatinstruktur och parametern Live SO^{+} inom analysen för reaktiva syreföreningar. Spermiekvaliteten hos proverna jämfördes även avseende vänster och höger testikel samt för skrotal och testikulär omkrets utan att någon signifikant skillnad eller korrelation kunde identifieras. En svag korrelation hittades dock med antydan att äldre tjurar har bättre spermie-motilitet och viabilitet, samt att en signifikant skillnad kunde fastställas där förekomst av tesikulära adherenser innebar en sämre spermiekvalitet. Sammantaget tyder detta på att extraktionsmetoden där spermier utvinns genom retrograd genomspolning är att föredra vid extraktion av bitestikel-spermier för att förvärva en högre motilitet i färska spermieprov. Angående nedfrysningen bör ett annat protokoll utvärderas för att uppnå en bättre spermiekvalitet efter upptining. Man bör också ha i åtanke att yngre tjurar kanske inte bidrar med lika god spermiekvalitet som de något äldre tjurarna.

Nyckelord: spermiekvalitet, extraktionsmetod, nötkreatur, epididymis, kryopreservation, viabilitet, motilitet, membranintegritet, kromatinstruktur, reaktiva syreföreningar, post mortem

Table of contents

List of tables

List of figures

Abbreviations

ALH	Amplitude of Lateral Head Displacement
ATP	Adenosine Triphosphate
BCF	Beat Cross Frequency
cAMP	Cyclic Adenosine Monophosphate
CASA	Computer Assisted Sperm Analysis
DCFDA	Dichlorodihydrofluorescein Diacetate
DFI	DNA Fragmentation Index
DNA	Deoxyribonucleic Acid
FSH	Follicle-stimulating Hormone
GnRH	Gonadotropin-releasing Hormone
HDS	High DNA Stainable Sperm
HE	Hydroethidine
HO	Hoechst 33258
LH	Luteinizing Hormone
LIN	Linearity
MI	Plasma Membrane Integrity
PI	Propidium Iodide
PKA	Protein Kinase A
PM	Progressive Motility
ROS	Reactive Oxygen Species
SACY	Soluble Adenylyl Cyclase
SCSA	Sperm Chromatin Structure Assay
SD	Standard Deviation
STR	Straightness
TM	Total motility
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VSL	Straight Line Velocity
WOB	Wobble

1. Introduction

The extraction of spermatozoa from the epididymis in bulls post mortem is of interest to enable the preservation of genetic material from genetically valuable individuals. This might be useful in case of sudden trauma, unexpected death or for the preservation of wild bovine species where the individuals cannot be handled and thereby present a huge difficulty in collecting semen. (Kaabi et al. 2003; Martins et al. 2007) The viability of the recovered sperm samples from these individuals depends on multiple factors such as the interval between the animal's death and sperm recovery (Kaabi et al. 2003; Martins et al. 2009), the storage temperature before recovery (Bertol et al. 2013) and if the sperm sample is to be used fresh or cryopreserved. Cryopreservation enables the material to be used at any time after the death of the animal, although it requires knowledgeable technicians, the right equipment and transportation in a cold environment to a laboratory, all of which are crucial to sperm viability (Martins et al. 2009).

The extraction of the spermatozoa is usually performed by making incisions in the cauda epididymidis and pressing against the region while collecting the spermatozoa that are released (Kaabi et al. 2003; Martinez-Pastor et al. 2005, 2006; Goovaerts et al. 2006; Martins et al. 2007). This method is called the cutting method and differs from the retrograde collecting method, in which semen extender is used to flush through the vas deferens and caudal epididymis in a retrograde manner. The sperm suspension is then collected while dripping out through a cut in the distal part of the cauda epididymidis (Bruemmer 2006; Martinez-Pastor et al. 2006). These two methods have been compared with a third method called the float-up method, where incisions are made in the cauda epididymidis and it is submerged in semen extender (Cary et al. 2004; Bruemmer 2006; Turri et al. 2012). The retrograde flushing method is known to provide a higher total amount of collected sperm than the float-up in stallions (Bruemmer 2006), although the comparison of the cutting and retrograde flushing methods has not been thoroughly studied. The purpose of this study was to compare the two extraction methods of cutting and retrograde flushing of the bovine cauda epididymidis in order to establish a preferred method of use. This was done by evaluating sperm quality characteristics before and after cryopreservation, including motility, plasma membrane integrity, reactive oxygen species and sperm chromatin structure.

2. Literature review

2.1 Anatomy of the male reproductive organs

The reproductive system of the bull has major functions that revolve around spermatozoa, including the production, transport and transfer of these male germ cells (Hopper 2014). The genital organs of the male bovine include the left and right testis within the scrotum, the bilateral epididymides, the vas deferens, the spermatic cord, the vesicular gland, the ampulla of ductus deferens, the prostate, the bulbo-urethral gland, and the penis (Dyce et al. 2010).

2.1.1 Scrotum and testes

The scrotum is essentially a skin and smooth muscle pouch and is dual-chambered, enveloping the two testes (Sjaastad et al. 2016). Rudimentary teats are often found on the cranial face of the scrotum, and the skin is covered with fine hair since a thicker coat could impair temperature regulation (Dyce et al. 2010). Since the male gametes require a lower temperature during development than can be found within the abdomen, the scrotum has an important job of regulating heat and this is executed partly by the tunica dartos. Tunica dartos is a fibromuscular layer adherent to the scrotal skin that can contract and relax and thereby push the testes closer to the body or allow them to move away from the body. The cremaster muscle in the spermatic cord elevates or lowers the scrotum and its contents, according to the environmental conditions (Dyce et al. 2010; Sjaastad et al. 2016). Another important way of regulating the heat is through countercurrent heat exchange between the warm arterial blood (37-38 °C) and the colder venous blood (approx. 33 °C) as the coiling spermatic artery is in contact with the veins of the pampiniform plexus returning from the testes (Sjaastad et al. 2016). In case of fat build up around the pampiniform plexus, the heat exchange is inadequate and results in abnormalities in spermatogenesis (Fayrer-Hosken 1997). These vessels are part of the spermatic cord, which also includes the vas deferens, nerves and lymphatic drainage (Hopper 2014).

The two testes make up the male gonads and are positioned side by side within the scrotum, suspended with their long axis vertically from the spermatic cord. They are oval and elongated dorsoventrally, and the epididymal border is found on the caudomedial aspect of the testis (Dyce et al. 2010; Budras et al. 2011). In all ruminants, especially the small ones, the testes are considered large in relation to body size. The size of the testes is measured by scrotal circumference and is very important since a greater circumference is associated with a higher capacity for sperm production (Fayrer-Hosken 1997; Senger 2012). The size depends on the age of the individual, as well as breed and nutrition (Fayrer-Hosken 1997), but can also be affected by various pathologies. This includes epididymitis which cause swelling with up to a 10-fold increase in size of the cauda epididymidis, or testicular hypoplasia causing a reduction in testicular size (Zachary 2017).

The testis as well as the spermatic cord is covered by two layers of peritoneum, the one closest to the parenchyma being the thicker capsule tunica albuginea and the outer layer being the tunica vaginalis (Dyce et al. 2010). Tunica vaginalis is an evagination of the peritoneum through the vaginal ring and consists of a parietal and a visceral layer forming a fold. The vaginal cavity between them contains a small amount of serous fluid. The testicular parenchyma is mostly (90% in bulls) consisting of seminiferous tubules supported by lobules. These lobules are created when the tunica albuginea radiates septae that terminate in mediastinum testis (Fayrer-Hosken 1997; Dyce et al. 2010). The seminiferous tubules open up into rete testis within the mediastinum, which is connected to the caput epididymidis and enables transport of spermatozoa (Fayrer-Hosken 1997).

The seminiferous tubules, where spermatogenesis takes place, are lined by Sertoli cells supporting the developing spermatocytes by producing hormones and growth factors, but also provides protection. The inside of the seminiferous tubules is shielded by the blood-testis barrier to avoid attack by the immune system on haploid cells. This is possible due to the basal lamina of the myoid cell layer, Sertoli cells and the tight junctions between the Sertoli cells keeping up a strong barrier (Fayrer-Hosken 1997). Leydig cells, which have an important endocrine role, are positioned in islands between the seminiferous tubules (Dyce et al. 2010).

2.1.2 Epididymis

The epididymis is an organ on the caudomedial aspect of the testis consisting of a single convoluted duct compacted by connective tissue (Dyce et al. 2010). It can be divided into three distinct parts: the head (caput epididymidis), the body (corpus epididymidis) and the tail (cauda epididymidis). These macroscopically separated parts do not have quite such a clear division from a functional perspective. The head

is connected to the rete testis by multiple efferent ductules that pierce the tunica albuginea and merge into the epididymal duct. This duct, which in cattle is a total of about 60 meters long, continues through the body of the epididymis, which is attached to the testis forming the testicular bursa. (Dyce et al. 2010; Sjaastad et al. 2016) The tail of the epididymis is firmly attached to the testicle by the ligamentum proprium and the parietal layer of tunica vaginalis forming the inferior ligament of the epididymis. The tail of the epididymis eventually tapers and joins the vas deferens with its narrow lumen and thick muscular wall enabling semen transportation (Fayrer-Hosken 1997; Dyce et al. 2010).

The epididymal functions are many. It includes sperm transport from rete testis to vas deferens by rhythmical contractions of the muscular wall of the duct, sperm concentration by absorption of redundant fluid, sperm protection from the external environment, from reactive oxygen species by excretion of antioxidant enzymes and from the immune system through the blood-epididymis barrier (James et al. 2020). The epididymis also functions as a location for storage of spermatozoa prior to ejaculation and is essential for sperm maturation during the epididymal transit where spermatozoa acquire motility and fertility capabilities.

2.1.3 Accessory sex glands

The accessory sex glands contribute to the composition of the seminal plasma and in bulls they include the vesicular gland, the ampulla of the ductus deferens, the prostate and the bulbourethral glands (Hopper 2014). The vesicular glands produce the majority of the seminal fluid volume, are lobulated and of substantial size; in the adult bull they are between 10x3 cm and 15x5 cm (Dyce et al. 2010). The size and function depends on the levels of testosterone in the individual, supported by the fact that the glands undergo involution in castrated males (Sjaastad et al. 2016). The vesicular gland lies dorsal to the bladder and lateral to the ureter and ampulla (Budras et al. 2011). The ampulla is a cylindrical enlarged part of ductus deferens; the prostate consists of two parts, with the disseminate part running along the urethra for 12-14 cm, and a compact part called the body of the prostate found dorsally in the first part of the urethra. The bulbourethral glands are bilateral and are the most caudal glands, they produce a watery secretion that is used to flush the urethra prior to the main ejaculate (Dyce et al. 2010).

The seminal plasma is mainly produced by the accessory sex glands but also contains secretions from the testes and epididymis (Poiani 2006). Spermatozoa and seminal plasma together make up the ejaculate (Poiani 2006), which in cattle has a total volume of about 4-8 ml (Sjaastad et al. 2016). The seminal plasma has an important role of delivering the spermatozoa to the female but also provides a protective environment and nutrients, e.g. containing the energy source fructose

which supplies an energy source for sperm motility (Poiani 2006; Sjaastad et al. 2016).

2.2 Endocrinology of the male reproductive system

The testicular functions are strictly regulated by hormonal mechanisms depending on gonadotropins, including luteinizing hormone (LH), follicle-stimulating hormone (FSH) and prolactin (PRL), as well as steroids, including androgens and estrogens (Sjaastad et al. 2016). The primary hypothalamic hormone regulating the levels of LH and FSH from the pituitary gland is gonadotropin-releasing hormone (GnRH) (Hopper 2014). The level of GnRH is determined by spontaneous rhythms, sensory impulses and the negative feedback from testosterone produced by the testes, briefly explained in Figure 1. The testosterone can also act directly on the pituitary gland reducing its response to GnRH and thereby the release of LH and FSH. The release of GnRH is occurring in a pulsatile fashion multiple times throughout the day, resulting in fluctuating levels of especially LH but also FSH and the hormones that these two gonadotropins regulate.

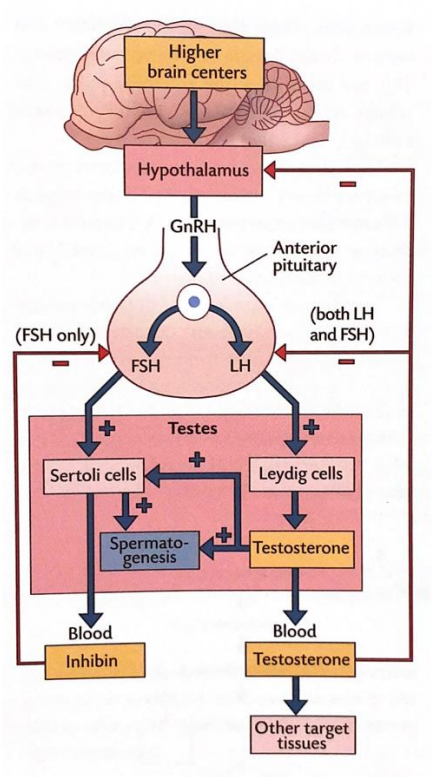

Figure 1: The hormonal regulation of the secretion of testosterone and the effect of testosterone on spermatogenesis (Sjaastad et al. 2016). Used with permission from author K. Hove.

The production of testosterone by the Leydig cells in the testicular parenchyma is impacted by LH and testosterone then binds to the cells by plasma membrane receptors (Hopper 2014). This binding will stimulate the production of cyclic

adenosine monophosphate (cAMP) and thereby increase the amount of cholesterol converted to pregnenolone, which is a precursor to most steroid hormones (Hopper 2014), including testosterone secreted by the Leydig cells (Sjaastad et al. 2016). Testosterone then stimulates spermatogenesis by indirectly stimulating the Sertoli cells. In some tissues testosterone is converted into dihydrotestosterone which has a higher affinity for androgen receptors.

Follicle-stimulating hormone acts upon the Sertoli cells, stimulating them to synthesize several factors that are important in supporting spermatogenesis (Sjaastad et al. 2016). It also affects the Sertoli cell secretion of the hormone inhibin which is used as a negative feedback regulator. When the rate of spermatogenesis is too low, inhibin levels are decreased and FSH will thereby increase. Conversely, when the production of sperm is rapid, FSH secretion will be reduced as inhibin levels rise. This network of negative feedback loops makes FSH less dependent on the release of GnRH than LH.

2.2.1 Puberty

Puberty is a process which the domestic bull usually enters at approximately 8-12 months of age (Sjaastad et al. 2016). It is defined as when mature sperm appear in the ejaculate and the animal becomes able to reproduce. At the onset of puberty, pulsatile GnRH secretion is increased, stimulating the release of gonadotropins from the pituitary gland. Until this point in life, the reproductive organs have been growing at the same pace as the rest of the body (isometric growth pattern), but at puberty the hormones released from the gonads cause the genitals organs to grow more rapidly (allometric growth pattern). Testosterone and dihydrotestosterone play an important role in this process. When production increases in the Leydig cells, testosterone diffuses into the seminiferous tubules and stimulates spermatogenesis, but it also diffuses into the systemic circulation. The free active fraction of testosterone not only stimulates growth of the external and internal genitalia, but it also effects secondary sex characteristics including sex drive behaviour, increased muscle mass and secretion of pheromones. Dihydrotestosterone is the most important androgen during puberty in some tissues, including the penis and prostate.

2.3 Spermatogenesis

Spermatogenesis is the combined process of spermatocytogenesis and spermiogenesis, including all steps from spermatogonia to spermatozoa and briefly explained in Figure 2 (Fayrer-Hosken 1997; Sjaastad et al. 2016). In the adult bull, the entire process takes place in approximately 60 days and during this period each

gram of testicular tissue can produce a total of 11 to $12x10^6$ sperm cells (Fayrer-Hosken 1997). The process is located in the seminiferous tubules, where the Sertoli cells play a very important role of supplying nutrients and regulating the maturation of germ cells (Sjaastad et al. 2016). The Sertoli cells are connected to the basal lamina and to each other with tight junctions, extending to the lumen of the seminiferous tubules. These tight junctions divide the tubules into two compartments, the basal compartment and the adluminal compartment. As the germ cells develop, they move through the tight junctions towards the tubular lumen until they lose contact with the Sertoli cells and are transported by the tubular fluid to rete testis.

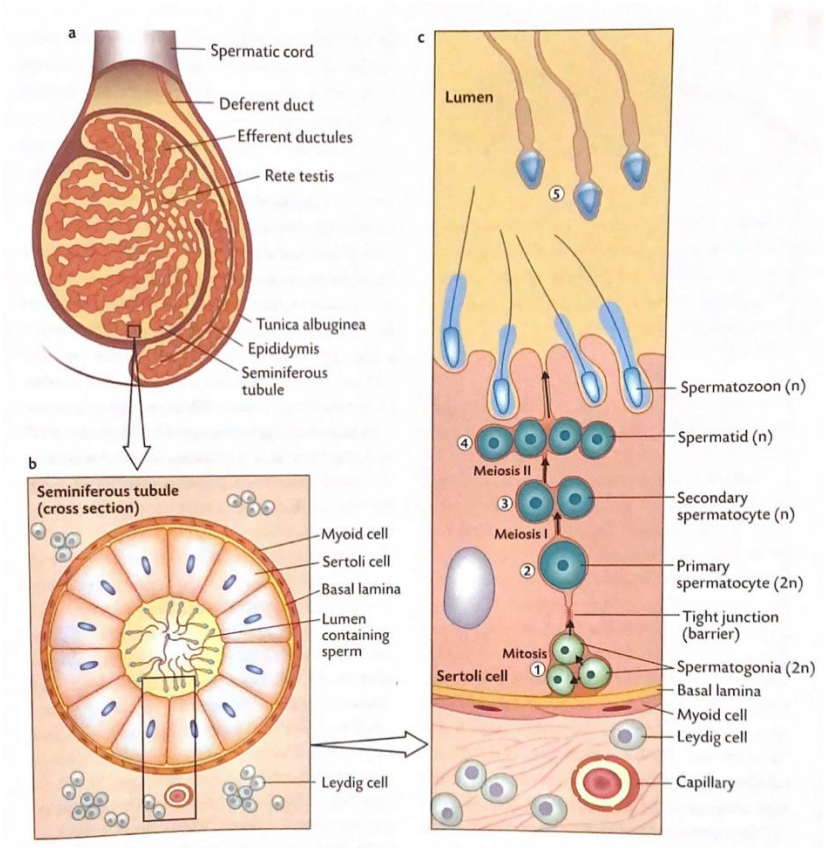

Figure 2: Spermatogenesis. a. Longitudinal section of the testes and epididymis. b. Cross-sectional enlargement of a seminiferous tubule. c. Relationship of the Sertoli cells to developing sperm (Sjaastad et al. 2016). Used with permission from author K. Hove.

2.3.1 Spermatocytogenesis

Spermatocytogenesis is the first part of the spermatogenesis where the spermatogonia form primary and secondary spermatocytes, and the spermatocytes then develop into spermatids (Fayrer-Hosken 1997). The spermatogonia are undifferentiated diploid stem cells located in the seminiferous tubules at the level of the epithelial basal lamina (Sjaastad et al. 2016). They divide continuously by mitosis

to produce new spermatogonia, and when the individual has gone through the process of puberty, some of the spermatogonia enter the luminal compartments. Within the luminal compartment the spermatogonia produce primary spermatocytes by ongoing mitotic division (Fayrer-Hosken 1997). The primary spermatocytes enter meiosis involving two cell divisions, Meiosis I and Meiosis II (Sjaastad et al. 2016). Before the process of miosis begins, the DNA is duplicated, and during Meiosis I when each primary spermatocyte divides into two secondary spermatocytes, they each receive one set of chromosomes creating two haploid cells. During meiosis, the process of crossing over and random distribution of each pair of chromosomes enables an enormous increase of variation in the genetic material. The secondary spermatocytes then undergo Meiosis II as an ordinary cell division. This starts with two haploid double stranded cells and ends up with four haploid single-stranded cells, producing round spermatids.

2.3.2 Spermiogenesis

Spermiogenesis is the second part of spermatogenesis where the spermatids produced during spermatocytogenesis are transformed into spermatozoa (Fayrer-Hosken 1997). It includes four stages of cellular metamorphosis, during which the spermatid with its large round nucleus is differentiated to a spermatozoon with a head, midpiece and tail shown in Figure 3. The process takes place within the adluminal compartment between adjacent Sertoli cells. The first of the four phases is the Golgi phase, with fusion of the small Golgi vesicle within the cytoplasm of the spermatid into an acrosomic vesicle including several hydrolytic enzymes (Senger 2012; Hopper 2014), which enables the spermatozoon eventually to penetrate into the oocyte (Sjaastad et al. 2016). During this phase of spermiogenesis, the centrioles migrate to opposite pole of the cell to the vesicle. Subsequently, the proximal centriole is converted into the attachment point of the tail. (Senger 2012; Hopper 2014; Li et al. 2021).

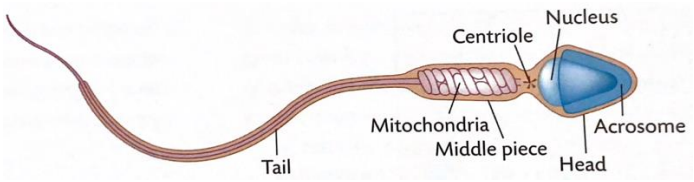

Figure 3: Spermatozoon from a bull (Sjaastad et al. 2016). Used with permission from author K. Hove.

During the second phase of spermiogenesis, the cap phase, the acrosomic vesicle migrates to form a cap over the nucleus (Senger 2012; Hopper 2014). The distal centriole forms the axoneme which is composed of nine microtubules around two central filaments. Within the third phase, the acrosomal phase, the nucleus and

cytoplasm undergo elongation and end up in the head region of the spermatid while the acrosome continues to enfold the nucleus. (Hopper 2014). During this elongation the fourth phase, i.e. the maturation phase, begins. By migration, the mitochondria gather around the flagellum, creating the midpiece. The mitochondria produce ATP from fructose in seminal plasma and is used to propel the tail, making the cell motile (Sjaastad et al. 2016). During spermiogenesis, the histones within the nucleosomes are replaced by protamine, leading to the genetic material being highly condensed and allowing the nucleus to shrink in size (Meistrich et al. 2003; Li et al. 2021). During maturation, the cytoplasm and unwanted organelles of the spermatozoa are reduced to a minimum, and the cytoplasmic residue gradually falls off (Sjaastad et al. 2016; Li et al. 2021).

2.4 Sperm quality parameters

2.4.1 Motility

Motility is described as the ability of spermatozoa to swim and is the most common way to assess sperm viability (Senger 2012). Spermatozoa acquire the ability to be motile during maturation in the epididymis, though the actual movement is initiated only after activation by seminal plasma (De Jonge & Barratt 2006). This is partly due to its content of the ATP sources glucose and fructose, with glycolysis being the most important energy-generating pathway inducing sperm motility (Mukai & Okuno 2004). Seminal plasma also contains bicarbonate (HCO_3^-), another essential molecule enabling sperm motility (De Jonge & Barratt 2006). Bicarbonate both activates the sperm atypical adenylyl cyclase (SACY) and opens Ca^{2+} channels by raising the pH, thereby increasing the levels of cAMP and Ca^{2+} (Wennemuth et al. 2003). Another important factor initiating motility is Protein kinase A (PKA), the activity of which is stimulated by the increase in cAMP.

Sperm motility is measured objectively using computer assisted sperm analysis (CASA) (De Jonge & Barratt 2006). This method requires videomicrography to measure the proportion of moving cells as well as other motility related parameters shown in Table 1. The evaluation is to be done at a temperature of 38 °C since lower temperatures can unfavourably affect the motility (Senger 2012). This can be done by incubating the samples before analysis and by using a heated stage during microscopy.

Table 1: Parameters analyzed using CASA, as well as their respective explanations.

Parameter	**Explanation**
Total motility (TM) [%]	The proportion of spermatozoa that is motile.
Progressive motility (PM) [%]	The proportion of spermatozoa that are moving forwards.
Curvilinear Velocity (VCL) [μm/s]	The measure of the travel rate of the sperm head over a given time.
Straight Line Velocity (VSL) [μm/s]	The average velocity of the sperm head along a straight line between its first and last position during a given time.
Average Path Velocity (VAP) [μm/s]	The velocity of the sperm along the average path, taking VCL and VSL in account.
Amplitude of Lateral Head Displacement (ALH) [μm]	The lateral deviation of the sperm head from its spatial average trajectory.
Beat Cross Frequency (BCF) [Hz]	The average rate at which the curvilinear sperm track crosses with its average path trajectory (how often the head is crossing the average path trajectory).
Wobble (WOB)	Departure of actual sperm track from average path, WOB = VAP/VCL
Linearity (LIN)	The linearity of the curvilinear trajectory, VSL/VCL x 100.
Straightness (STR)	The STR of the sperm average path, VSL/VAP x 100.

2.4.2 Plasma membrane integrity

The plasma membrane of the mammalian sperm is a structure with multiple functions, overall protecting the cell from extracellular damage and responding to physiological challenges (Tapia et al. 2012). Both the head and tail of the spermatozoon are covered by the sperm plasma membrane, also referred to as plasmalemma (De Jonge & Barratt 2006). It is mainly composed of lipids as in somatic cells, but the composition of the included lipids is different in the sperm cell. The most common ones are glycerophospholipids (GPLs), creating a two-layered structure with the hydrophobic tails of the lipids turned inwards towards each other. Since the sperm plasma membrane contains a high amount of polyunsaturated fatty acids it has great fluidity, but this increases the risk of lipid peroxidation by reactive oxygen species (Sikka 1996; Wathes et al. 2007).

The contents of the plasma membrane contribute to multiple properties affecting sperm fertilization capacity including motility, capacitation, the acrosome reaction and the spermatozoon binding to the oocyte (Gautier & Aurich 2022). The aim when using assisted reproductive technologies is to preserve plasma membrane integrity, although it is affected by actors such as nutrition, reproductive stage, age and season that are not associated with the handling of the semen after ejaculation.

Cryopreservation is one important factor negatively affecting the integrity of the sperm membrane, also called cryodamage (Tapia et al. 2012). It was formerly commonly believed that this was due to the formation of intracellular ice crystals in cases of too rapid cooling and exposure to concentrated solutions when water progressively turned to ice if the cooling process was too slow. However, more recently, cryodamage has been shown to be almost certainly the result of osmotic stress occurring especially during the thawing process (Peña et al. 2011; Tapia et al. 2012). During freezing, extracellular ice crystal formation takes place, creating a hyperosmotic environment that causes the spermatozoa to lose intracellular water. The thawing process then has a reverse effect, with a hypotonic environment causing the sperm intracellular water levels to rise again. The osmotic stress caused by both freezing and thawing leads to plasma membrane damage due to the shrinkage and swelling of the sperm cell.

Staining of spermatozoa using SYBR14 and propidium iodide (PI) (Live-Dead Sperm Viability Kit L-7011; Invitrogen, Eugene, OR, USA) is preformed to analyse plasma membrane integrity (Garner et al. 1994; Morrell et al. 2010, 2017) These stains enable a fluorescence-based analysis that distinguishes live and dead cells, resulting in the three parameters shown in Table 2. Propodium iodide, which fluoresces red, can only enter the spermatozoa if the plasma membrane is damaged, whereas SYBR14, which fluorescence green, can penetrate the intact plasma membrane. The analysis is preformed using a flow cytometer, and the live bovine sperm cells with intact cell membranes will fluoresce bright green whilst the damaged cell membranes fluoresce red.

Table 2: Parameters measured when analysing plasma membrane integrity (Morrell et al. 2017).

Parameter	**Explanation**
Live Spermatozoa	SYBR14 positive, PI negative. (+/–) Intact membrane, live spermatozoa.
Dying Spermatozoa	SYBR14 negative, PI positive. (–/+) Affected membrane, moribund spermatozoa.
Dead Spermatozoa	SYBR14 positive, PI positive. (+/+) Damaged membrane, dead spermatozoa.

2.4.3 Reactive oxygen species

Reactive oxygen species (ROS) occur as by-products of the normal metabolism in all cellular systems and have at least one unpaired electron within the outer shell, making them very reactive (Sidney J. Stohs 1995). In semen it can also arise from abnormal or dead spermatozoa, leukocytes and immature sperm cells and will increase in the event of an inflammatory response (Shannon & Curson 1972;

Agarwal et al. 2006). The production of ROS is driven by enzymes, or takes place within the mitochondria through electron transfer to oxygen (Agarwal et al. 2014; Bollwein & Bittner 2018). Sperm-specific ROS can mainly be traced to the mitochondrial electron transport system, and the levels may increase further in case of abnormal oxygen supply or mitochondrial dysfunction due to cryopreservation (Koppers et al. 2008). Since spermatozoa are naturally rich in mitochondria, any abnormality or dysfunction will increase the production of ROS and thereby affect both sperm function and motility (Agarwal et al. 2014). When the production of ROS overwhelms the antioxidative capacity of the fluids bathing the sperm, the condition of oxidative stress arises (Sies 1993).

Spermatozoa are sensitive to oxidative stress due to their plasma membrane and mitochondrial membrane being rich in polyunsaturated fatty acids (Sikka 1996). These acids can be damaged in the presence of ROS by lipid peroxidation, resulting in decreased sperm motility, sperm apoptosis and poor reproductive outcome. Lipid peroxidation is considered to be the key mechanism of ROS-induced sperm damage.

However, at a controlled level, ROS play an important role in many physiological processes (Bansal & Bilaspuri 2010). This includes the capacitation of the spermatozoa, when they mature to be able to fertilize the oocyte, but also enables hyperactivation when spermatozoa become highly motile, which is essential for locating the oocyte for fertilization. Even the acrosome reaction and the fusion between sperm and oocyte demands the presence of ROS, indicating the importance of oxidation. This desirable amount of oxidative stress is sometimes referred to as “oxidative eustress” (Upadhyay et al. 2022).

Flow cytometry can be used to analyse ROS following multiple steps of staining (Cojkic et al. 2023). Hoechst 33258 (HO) is the first stain to be added, enabling the differentiation between live and dead spermatozoa. The following stains, Hydroethidine (HE) (HE; Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) and Dichlorodihydrofluorescein diacetate (DCFDA) (DCFDA; Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) are used to detect the molecules superoxide (O^{2-}) and hydrogen peroxide (H_2O_2). The analysis thus provides the following parameters: ROS Live $H_2O_2^-$, ROS Live $H_2O_2^+$, ROS Dead $H_2O_2^-$, ROS Dead $H_2O_2^+$, ROS Live SO^-, ROS Live SO^+, ROS Dead SO^+.

2.4.4 Sperm chromatin structure

Chromatin is the complex of DNA and proteins that together form chromosomes within the cell nucleus (Meistrich et al. 2003). Sperm chromatin structure is a parameter of sperm quality giving information about the stability of the sperm

genome and DNA damage. During spermatogenesis, many complex changes take place affecting sperm chromatin. This includes the replacement of histones by protamines, enabling condensation of the sperm DNA which protects the genetic information during transfer through the male and female reproductive tracts and the process of fertilization. Despite condensation, the DNA is still susceptible to damage (De Jonge & Barratt 2006). The damage may not lead to deviating sperm morphology or motility, but might reduce fertilization potential and will inhibit early embryonic development. The damage can be caused by numerous factors, but ROS is a significant cause leading to DNA-strand breakage (Evenson 2022).

Sperm Chromatin Structure Assay (SCSA) is a well-established method used to examine sperm DNA integrity and chromatin structure, developed by Donald Evenson (Evenson et al. 1980). The sperm samples are treated with a strong acid to denaturate DNA with single- or double-stranded breaks, leaving the nuclei condensed enough to be stained for evaluation with flow cytometry (De Jonge & Barratt 2006). After the acid treatment, the sample is stained with a metachromatic dye, acridine orange, which stains double-stranded DNA green and single-stranded DNA red. The ratio of the red staining compared to the total staining is called the DNA fragmentation index (%DFI), measuring the extent of single- and double-strand breaks (Evenson 2022). The excess nuclear histones can also be measured, analysed as high DNA stainable sperm (%HDS) (Evenson 2016).

3. Materials and methods

3.1 Experimental design

The experimental design is illustrated in Figure 4, showing the samples way from collection of testes at the local abattoir, to the extraction using Method A or B, pre-freezing analysis, cryopreservation, thawing and finally the post-thawing analysis.

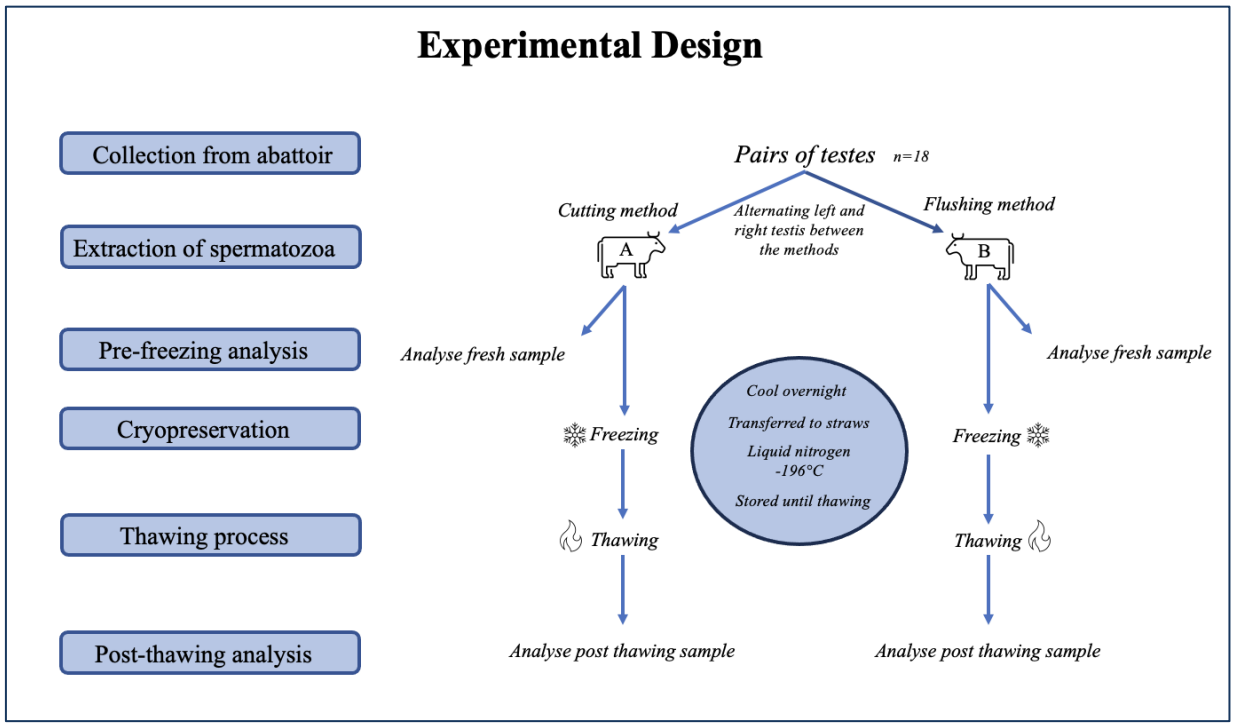

Figure 4: Experimental design diagram of the study.

3.2 Testes collection and transportation

Collection of testes for this project was made possible by a local abattoir and the material was picked up on the morning of the first day of sample analysis. After slaughter, the testes were put aside at room temperature and labeled by the abattoir personnel, keeping track of breed and age. After collection, the testes were

transported at room temperature by car for approximately 20 minutes to the Centre for Veterinary Medicine and Animal Science at the Swedish University of Agricultural Science.

3.3 Dissection and extraction

The testes were kept in room temperature (22 °C ± 2 °C) after arrival. The scrotal circumference was measured using a ReliaBull measuring tape, firmly pushing the testes into the lower part of the remaining scrotum and measuring the circumference at the widest point. The circumference of each testis once removed from the scrotum with the tunica vaginalis still intact was also measured to compare the size of the two testes from the same bull. Information about each bull's breed and age as well as the presence of any abnormality of the testes was noted. This included four of the testes showing slight adhesions and one testis a haemorrhage in the surrounding tissues. Tunica vaginalis was cut open using a pair of scissors, and the epididymis located. Using blunt dissection technique, the corpus epididymis was dissected away from the testis followed by the cauda epididymis. The cauda epididymis and the vas deferens was cut off proximally to maintain enough tissue used during the extraction. The epididymides were labelled to keep track of which bull and testes they originated from. The testes were discarded after dissection and the epididymides transported to the main lab to be prepared for the extraction, shown in Figure 5. The extraction was performed at a minimum of 3 hours post slaughter.

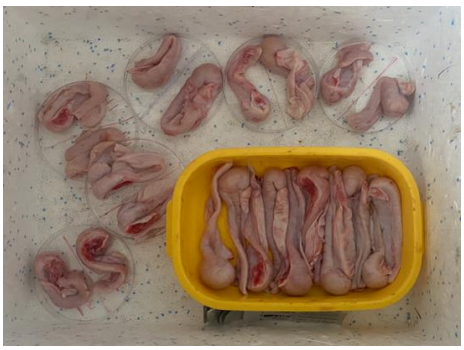

Figure 5: Picture of the epididymides ready to be transported to the main lab. Image by author.

After the dissection the epididymides from the same bull were used for one of two extraction methods as illustrated in Table 3, alternating between the left and right epididymis.

Table 3: Illustrating the alternating extraction method between each bull's left and right epididymis.

Bull	Left Epididymis	Right Epididymis
1	Method A	Method B
2	Method B	Method A

Method A: The cutting method. The epididymis was placed in a petri dish and a 5-10 mm longitudinal incision into the cauda epididymis was made with a scalpel. Gentle pressure was applied on the tissue and the semen that leaked out through this incision was aspirated with a disposable pipette. Areas with blood contamination were avoided. The contents were placed in an Eppendorf tube with the correct marking and mixed with 1-1.5 mL semen extender at room temperature (22 °C ± 2 °C) depending on the batch. The first batch was mixed with 1 mL semen extender, but due to very high concentration obtained, the second batch was mixed with 1.5 mL.

Method B: The retrograde flushing method. The distal part of the cauda epididymis was cut off using a pair of scissors. The epididymis was held by one hand over a petri dish and the cut off end of vas deferens was fixed between thumb and index finger. A blunt 19 g needle connected to a rubber-free 5 mL syringe containing 5 mL semen extender at room temperature was inserted into the vas deferens. The semen extender was then flushed in a retrograde manner and exited together with the content of sperm through the incision in the cauda epididymis. The fluid collected in the Petri dish was mixed and pipetted into two Eppendorf tubes containing 1.5 mL each.

3.4 Labelling of tubes and straws

Eppendorf tubes (1.5 mL) used for the extracted semen were labelled according to the predetermined plan; an example of one pair of testes is shown in Table 4.

Table 4: Labeling system of the Eppendorf tubes prior to extraction.

Bull, nr	Left or Right Epididymis	Extraction method	Pre-freeze Sample	Post thawing Sample
1	Left (L)	A	1.L.A.C	1.L.A.F
1	Right (R)	B	1.R.B.C	1.R.B.F

Ahead of the staining protocol for the analysis made with the flow cytometry, the tubes were labelled to distinguish between the intended analysis and applied stains. An example of one pre-freezing sample and one post-thawing sample is respectively illustrated in Table 5 and Table 6.

Table 5: Labeling system for the pre-freezing samples before staining and Flow cytometry analysis.

Pre-freezing	CASA	MI (SYBR)	ROS	SCSA
1.L.A.C	1.L.A.C	1.L.A.C.S	1.L.A.C.R	1.L.R.C

Table 6: Labeling system for the post-thawing samples before staining and Flow cytometry analysis.

Post-thawing	CASA	MI (SYBR)	ROS	SCSA
1.L.A.F	1.L.A.F	1.L.A.F.S	1.L.A.F.R	1.L.A.F

In preparation for the freezing process, straws (0.25 mL from Cryo-Vet, Z.A. de Rolin, Quebriac, France) were labelled manually to distinguish each sample from the others. Different coloured straws were used and labelled with varying number of lines and different marking pen colours. The number of straws produced was also noted as well as the storage unit post freezing. A picture and an example of this system is shown below in Figure 6 and Table 7.

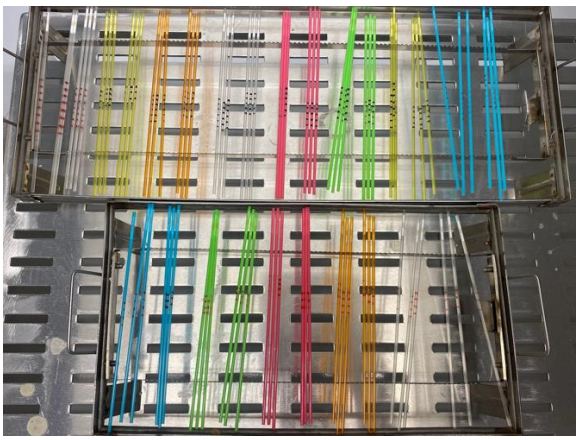

Figure 6: Picture of the straws used in during cryopreservation of the second batch of samples. Image by author.

Table 7: Labeling system of the sperm straws before freezing.

	Colour of straw	Lines	Writing colour	Number of straws
1.L.A.C	White	I	Blue	3 straws
1.R.B.C	White	II	Blue	3 straws
2.L.B.C	Blue	I	Blue	3 straws
2.R.A.C	Blue	II	Blue	3 straws
3.L.A.C	Green	I	Blue	2 straws
3.R.B.C	Green	II	Blue	3 straws
4.L.B.C	Pink	I	Blue	2.5 straws
4.R.A.C	Pink	II	Blue	2.5 straws

3.5 Sperm preparation and evaluation

The sperm concentration of each extracted sample was measured prior to any analysis using the Nucleocounter SP-100 (Chemometec, Allerød, Denmark) for sample 1.L.A to 11.R.B. Due to the sudden availability of a large number of samples, the supply of cassettes was exceeded and the concentration of the remaining 14 samples including 12.L.B to 18.R.A had to be evaluated manually in a Bürker chamber. For the latter, 1000 µL of physiological saline solution (3% NaCl) was mixed with 100 µL of the well-mixed sperm sample. 10 µL of this mixture was then pipetted into the prepared Bürker chamber into which it was drawn by capillary action. The chamber was placed in a moist chamber a few minutes to allow the sample to settle. Using a microscope, the sample was then viewed using the x40 magnification. The sperm cells within a total of 25 squares (24 on the diagonals as well as one random square) within the Bürker chamber were counted, only counting the heads within the square and the sperm overlapping the top or right border of the square. These numbers were added together, resulting in the concentration of the sample. Both methods are pictured below in Figure 7.

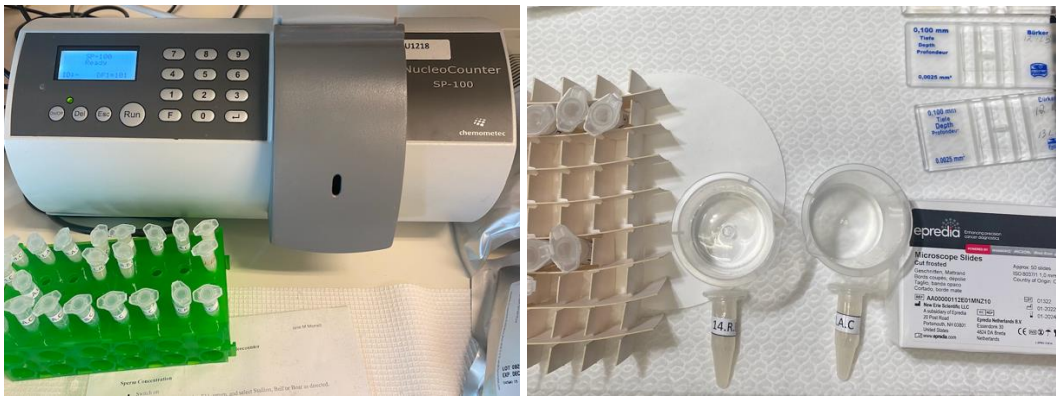

Figure 7: Pictures of the two methods used to evaluate the concentration of the samples, the Nucleocounter SP-100 to the left and the Bürker chamber preparation to the right. Images by author.

After establishing the concentration of each sample, they were diluted with semen extender at room temperature (22 °C ± 2 °C) into new Eppendorf tubes, creating the desired concentration for the upcoming freezing of 69 x 10^6 per mL. Any samples (4 in total) with a concentration lower than this were left unadjusted.

3.5.1 Computer assisted sperm analysis – CASA

The sperm samples were analysed using CASA after adjusting the concentration. The samples were thoroughly mixed by turning the tube over multiple times and a 5 µL drop was then pipetted onto a glass slide. Any air bubbles were removed and a coverslip placed on top. The slide was left to incubate on a 38 °C Minitüb HT300 heated stage connected to a Zeiss AxioStar Plus binocular microscope for a few

minutes to reach the desired temperature. The CASA was preformed using the AndroVision CASA software (Minitüb, Tiefenbach, Germany) and the following parameters were examined and documented for each sample: total motility (TM), progressive motility (PM), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), amplitude of lateral head deviation (ALH), beat cross frequency (BCF), wobble (WOB), linearity (LIN) and straightness (STR). The motility of each sample was evaluated in 5 or 8 different fields of view depending on the observer, as shown in Figure 8. This resulted in the analysis of >1000 spermatozoa except for the samples with a lower concentration than 69 x 10^6 per mL where <1000 spermatozoa were observed.

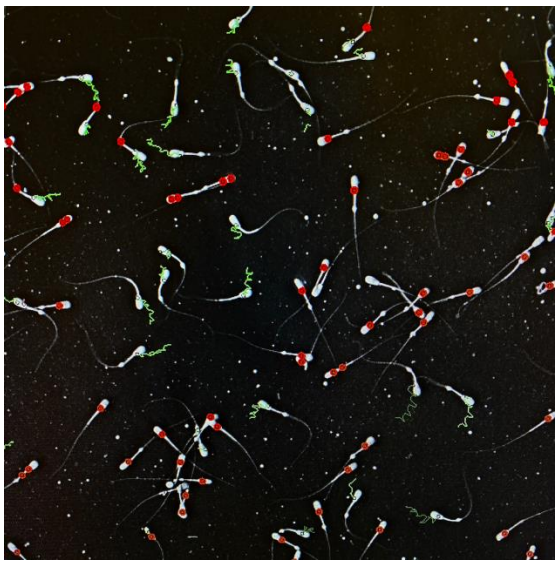

Figure 8: The green and red markings of the CASA distinguishing between mobile and non-mobile spermatozoa. Image by author.

3.5.2 Flow cytometry

Prior to the flow cytometry analysis the samples were stained with various fluorescent stains; one to study plasma membrane integrity, one to look at reactive oxygen species and another analysing chromatin structure. All of these stained aliquots were individually prepared from the same pre-freezing sample. Knowing the concentration of each sample ahead of the staining protocols enabled the calculation of the volume needed from the stock sample for each of the analyses. The flow cytometer used for the measurements was a BD FACSVerse Flow Cytometer (Becton Dickinson and Company, San José, CA, USA).

Sperm plasma membrane integrity

The stain SYBR14 was prepared by adding 0.5 µL SYBR14 to 25.4 µL Buffer B (Patent pending; J.M. Morrell and H. Rodriguez-Martinez). An aliquot of the

sample containing 2 x 10^6 spz/mL was calculated by dividing the desired concentration by the concentration of the stock sample. The calculated volume was aspirated into a new tube marked according to the intended analysis together with Buffer B to create a total volume of 300 µL. 0.6 µL of the prepared SYBR14 stain was then pipetted into the same tube, followed by 3 µL Propidium iodide (PI). The sample was incubated at 37.5 °C for 10 minutes before analysis using flow cytometry and the spermatozoa were characterised as live, dying and dead depending on their membrane integrity, as seen in Figure 9.

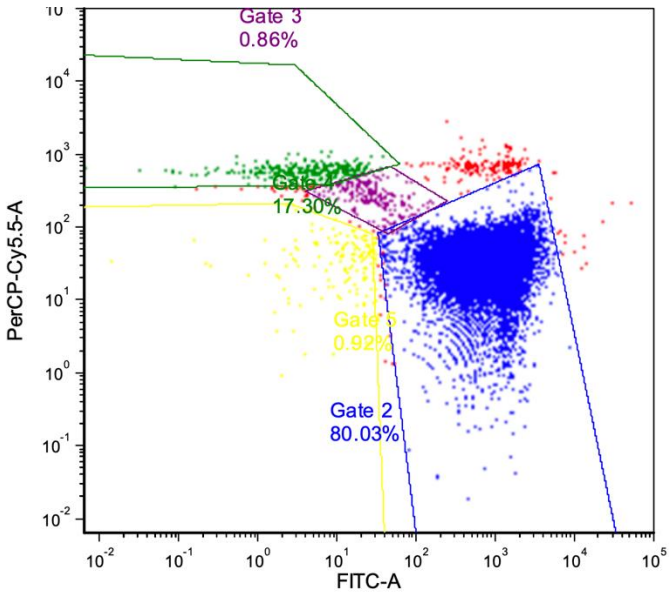

Figure 9: An example of a graph generated by evaluating the samples for sperm plasma membrane integrity using the flow cytometer. Gate 2 (blue) shows the proportion of live spermatozoa, gate 3 (purple) the proportion of dying spermatozoa and gate 4 (green) the proportion of dead spermatozoa.

Reactive oxygen species

As for the sperm plasma membrane integrity, an aliquot of the sample containing 2 x 10^6 spz/mL was calculated and aspirated into a new tube marked according to the intended analysis together with Buffer B, creating a total volume of 300 µL. The semen was stained using 3 µL HO, 3 µL HE and 3 µL DCFDA and was then mixed. The sample was incubated at 37.5 °C for 30 minutes before analysis using flow cytometry. The following parameters and proportions were analysed with Figure 10 as an example: Live hydrogen peroxide negative, Live hydrogen peroxide positive, Dead hydrogen peroxide negative and dead hydrogen peroxide positive, Live sulfoxide negative, Live sulfoxide positive and Dead sulfoxide positive.

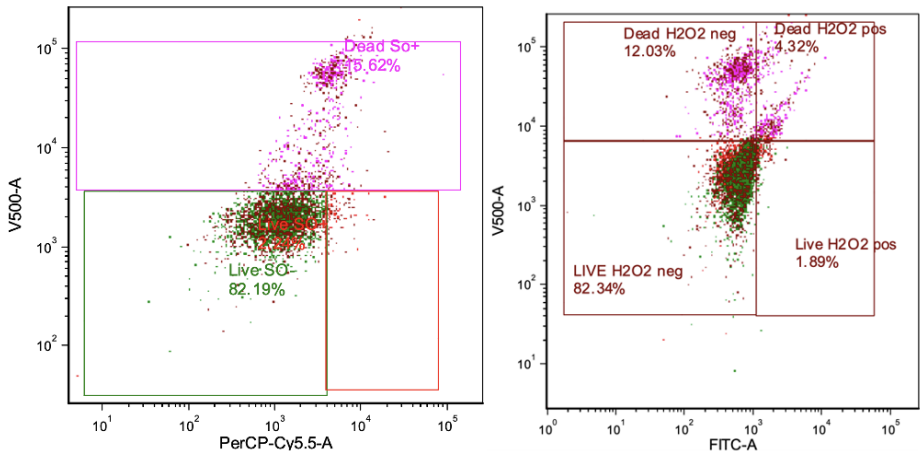

Figure 10: An example of the graphs generated by evaluating reacting oxygen species using the flow cytometer.

Sperm Chromatin Structure Analysis

50 µL of the stock sample was diluted with 50 µL of TNE buffer containing Tris-HCl, NaCl and EDTA, and immediately frozen with liquid nitrogen at -196 °C. The samples were stored in -80 °C until evaluation by flow cytometry. Post-thawing on ice the sample was diluted with more TNE, using 10 µL of the sample and 90 µL of TNE. This solution was then mixed with 200 µL of an acid detergent solution that was left to act for exactly 30 seconds, and 600 µL of the staining solution acridine orange (AO) was then added. Within 3-5 minutes post-staining the samples were analysed using flow cytometry.

3.5.3 Cryopreservation and post-thawing evaluation

The stock samples were stored overnight at 5 °C after the analyses, and the following morning they were prepared for freezing. Working within a cold bench at 5 °C, the samples were thoroughly mixed and then manually aspirated (0.25 mL) into pre-labelled sperm straws using a 1 ml syringe and a rubber tube connected to the closed-off end of the straw. The straws were heat-sealed and placed on a floating rack 4 cm above liquid nitrogen for 20 minutes, and were then plunged into the liquid nitrogen and stored until the following analysis.

The thawing was done by putting the frozen straws into water bath at 38 °C for 12 seconds and the semen was then transferred into pre-labelled tubes. The samples that were previously labelled as e.g. ‘1.L.A.C’ were now labelled as ‘1.L.A.F’ indicating that the sample had been frozen (F). The CASA and FC analyses were carried out in the same manners as for the pre-freeze samples.

3.5.4 Statistical analysis

The results of the analyses performed during this study were compiled in an Excel file (Microsoft, 2023). The statistical analysis was then preformed using the MATLAB (Version 23.2.0 (R2023b), The MathWorks Inc. Natick, Massachusetts) software programme. The descriptive statistics such as mean, median, range and standard deviation were all calculated using the varfun function. Data of the sperm quality parameters were analysed using the paired t-test when comparing the same sample prior to cryopreservation and post thawing, and the two-sample t-test was used when comparing the two different methods, analysing the effect of adhesions and a large number of samples at once. The Pearson r correlation test was used to investigate the correlation between the evaluated parameters with age, scrotal and testicular circumference. The alpha value was set at 5%, and differences at $p < 0.05$ were considered statistically significant.

4. Results

4.1 Number of extracted sperm and concentration

Both extraction methods gave a wide range of concentration, illustrated in Table 8. There was no significant difference between the extraction methods considering concentration, but there was a trend towards significance for a higher number from Method B than from Method A (p=0.0543). The presence of adhesions or other macroscopic abnormalities did not affect the number of sperm recovered.

Table 8: The number of extracted sperm obtained by both extraction methods described as mean, standard deviation (SD), median and range as well as the associated p-value. The unit is x10^6 spermatozoa/mL.

Sperm Count	Mean ± SD	Median	Range
Method A	436.59 ± 208.25	401.35	988.5–147.2 = 841.3
Method B	743.36 ± 762.25	432.15	2967.6–30.9 = 2936.7
p-value: 0.0543			

4.2 Macroscopic abnormalities of the testes

Compared to the results of the testes with no remark of abnormalities, the testis with nearby haemorrhage showed no significant difference. The four testes with adhesions showed significant difference throughout multiple of the parameters, both pre-freezing and post-thawing, concluded in Table 9, 10 and 11. This difference showed that the adhesions most definitely had a negative effect on the sperm quality.

Table 9: The CASA parameters (Mean ± SD) of the pre-freeze samples showing significant difference comparing the testes with adhesions to the ones with no macroscopic abnormality.

CASA	TM[%]	PM[%]	VCL[µm/s]	VSL[µm/s]	VAP[µm/s]	ALH[µm]	BCF[Hz]
Adhesion *Mean ± SD*	37.58 ± 8.79	36.56 ± 8.34	92.12 ± 6.33	34.89 ± 3.16	43.75 ± 3.17	1.01 ± 0.07	6.29 ± 1.46
No abnormality *Mean ± SD*	66.35 ± 16.35	65.24 ± 16.66	169.09 ± 53.04	65.52 ± 23.13	80.63 ± 27.37	1.76 ± 0.50	8.87 ± 2.24
p-value	0,001	0,001	0.004	0.007	0.006	0.003	0.016

Table 10: The Reactive oxygen species (ROS) and Sperm Plasma Membrane Integrity (SPMI) parameters (Mean ± SD), including the proportion of live/dead spermatozoa and H2O2 (hydrogen peroxide) or SO (sulfoxide) positive (+) or negative (-) spermatozoa, of the pre-freeze samples showing significant difference comparing the testes with adhesions to the ones with no macroscopic abnormality.

ROS & SPMI	Live spermatozoa	Dead spermatozoa	Live H2O2-	Dead H2O2-	Live SO-	Dead SO+
Adhesion *Mean ± SD*	49.19 ± 5.03	42.18 ± 3.30	62.83 ± 6.93	34.43 ± 6.75	60.69 ± 9.0	35.64 ± 7.39
No abnormality *Mean ± SD*	65.34 ± 16.97	24.09 ± 14.30	79.19 ± 13.43	18.20 ± 13.44	75.47 ± 13.83	20.32 ± 13.28
p-value	0,035	0,009	0.012	0.012	0.023	0.016

Table 11: The parameters (Mean ± SD) analysed, straight line velocity (VSL), average path velocity (VAP) and the proportion of live/dead spermatozoa and H2O2 (hydrogen peroxide) or SO (sulfoxide) positive (+) or negative (-) spermatozoa, of the post-thawing samples showing significant difference comparing the testes with adhesions to the ones with no macroscopic abnormality.

Post-thawing	VSL[µm/s]	VAP[µm/s]	Live H2O2-	Live SO-	Dead SO+
Adhesion *Mean ± SD*	6.38 ± 3.49	9.60 ± 4.95	11.41 ± 6.60	8.53 ± 5.70	86.81 ± 8.57
No abnormality *Mean ± SD*	12.21 ± 6.47	16.74 ± 7.92	27.20 ± 14.31	24.71 ± 13.90	69.56 ± 16.19
p-value	0,044	0,045	0.019	0.015	0.023

4.3 The effect of age, scrotal and testicular circumference

There were no significant differences between the left or right testis considering any of the parameters, including both CASA and the Flow Cytometry analyses. Despite the varying scrotal circumference within the group of bulls, between 29.0-40.0 cm, there were no significant correlations between this measurement and any of the parameters analysed. Some of the bulls with a larger circumference scored a low motility rate, while some of the bulls with a smaller scrotal circumference scored a relatively high motility rate. This pattern continues when comparing the parameters with the testicular circumference ranging between 18.0-24.0 cm with no significant correlation between the factors. A weak positive significant correlation ($r = 0.33–0.36$, $p < 0.05$) was found between the bull age in months ranging between 16-23 months, and the pre-freezing CASA parameters VSL, VAP, LIN and STR, an example shown in Figure 13. This implies that the older the bull the better the sperm motility. A weak age correlation was also discovered regarding the viability parameter Live spermatozoa, with a weak positive correlation ($r = 0.22$, $p = 0.031$) compared in Figure 14. This is interpreted as older bulls having a better sperm viability as well.

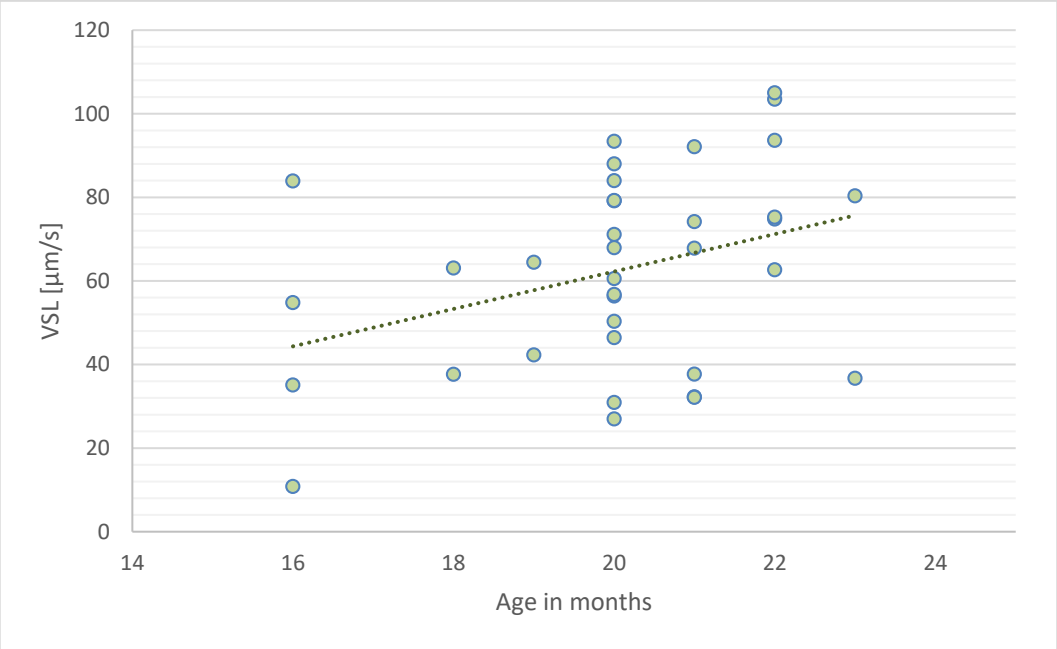

Figure 11: The significant positive correlation between Straight Line Velocity (VSL) and the bulls age in months illustrated in a plot with a regression line. r = 0.352, p = 0.035.

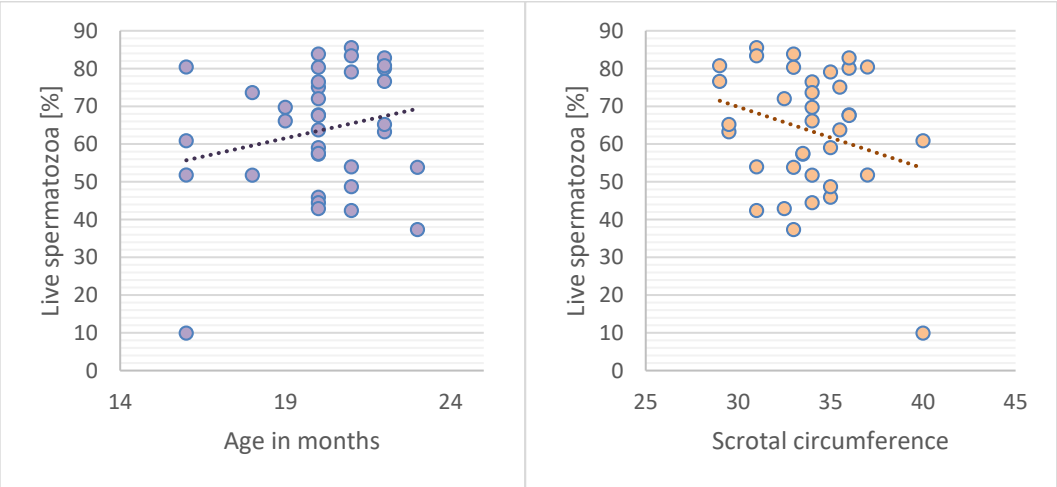

Figure 12: ***Left****; The significant positive correlation between the viability parameter Live spermatozoa and age in months illustrated in a plot with a regression line. r = 0.22, p = 0.031.* ***Right****; The non-significant negative correlation between the viability parameter Live spermatozoa and scrotal circumference illustrated in a plot with a regression line. r = -0.26, p = 0.124.*

4.4 Extracting a large number of samples at once

During one occasion, a sudden availability of a large number of samples (n = 26, from 13 bulls) meant that the time of extraction relative to the time post slaughter varied moderately between the first and last extractions. The exact passed time was not noted, however preforming the extractions all together this day took about 1.5-2 hours. Comparing the samples from the first 4 bulls (n = 8) with the samples from the last 4 bulls (n = 8), a significant difference was discovered between the early and late extractions. This applies to both CASA and ROS parameters pre-freezing as shown in Table 12, and was maintained for ROS parameters post-thawing shown in Table 13.

Table 12: The pre-freezing parameters showing significant difference between the early and late extractions. Including computer assisted sperm analysis parameters ALH (Amplitude of Lateral Head Displacement) and STR (Straightness) as well as reactive oxygen species parameters, evaluating the proportion of live/dead spermatozoa, H2O2 (hydrogen peroxide) or SO (sulfoxide) positive (+) or negative (-).

Pre-freezing	ALH [µm]	STR	Live H2O2-	Dead H2O2-	Live SO-	Dead SO+
Early extraction *Mean ± SD*	2.17 ± 0.42	0.79 ± 0.03	87.14 ± 8.01	10.81 ± 7.95	82.67 ± 9.24	14.00 ± 9.45
Late extraction *Mean ± SD*	1.57 ± 0.48	0.82 ± 0.02	76.03 ± 11.47	22.23 ± 11.33	72.84 ± 11.72	23.95 ± 11.04
p-value	0.009	0.028	0.021	0.018	0.042	0.037

Table 13: The post-thawing reactive oxygen species parameters showing significant difference between the early and late extractions, evaluating the proportion of live/dead spermatozoa, H2O2 (hydrogen peroxide) or SO (sulfoxide) positive (+) or negative (-).

Post-thawing	Live H2O2-	Dead H2O2-	Live SO-	Live SO+	Dead SO+
Early extraction *Mean ± SD*	30.09 ± 14.40	66.38 ± 14.47	29.64 ± 13.59	1.71 ± 0.89	69.05 ± 13.96
Late extraction *Mean ± SD*	9.43 ± 3.79	85.75 ± 3.42	7.23 ± 3.92	3.23 ± 1.63	91.07 ± 3.34
p-value	0.001	0.001	0.001	0.018	0.001

4.5 CASA

4.5.1 Comparing method A and B

When comparing the two methods A and B, a difference was found for both total and progressive motility ($p < 0.05$) as illustrated in Figure 15. Significant difference was also found for the CASA parameters VCL, VSL, VAP and ALH, shown in Figure 16. These parameters were all in favour of Method B. The remaining parameters including STR, BCF, WOB and LIN did not show a statistically significant difference between the two methods, compiled in Table 14.

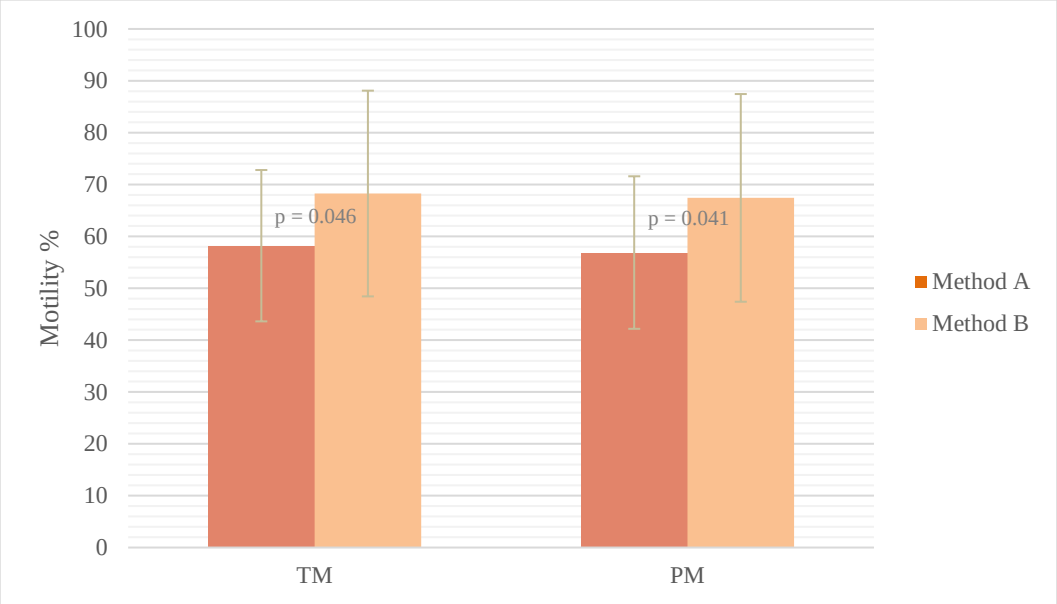

Figure 13: The motility parameters (Mean ± SD) total motility (TM) and progressive motility (PM) showing significant difference between the pre-freezing samples of Method A and B.

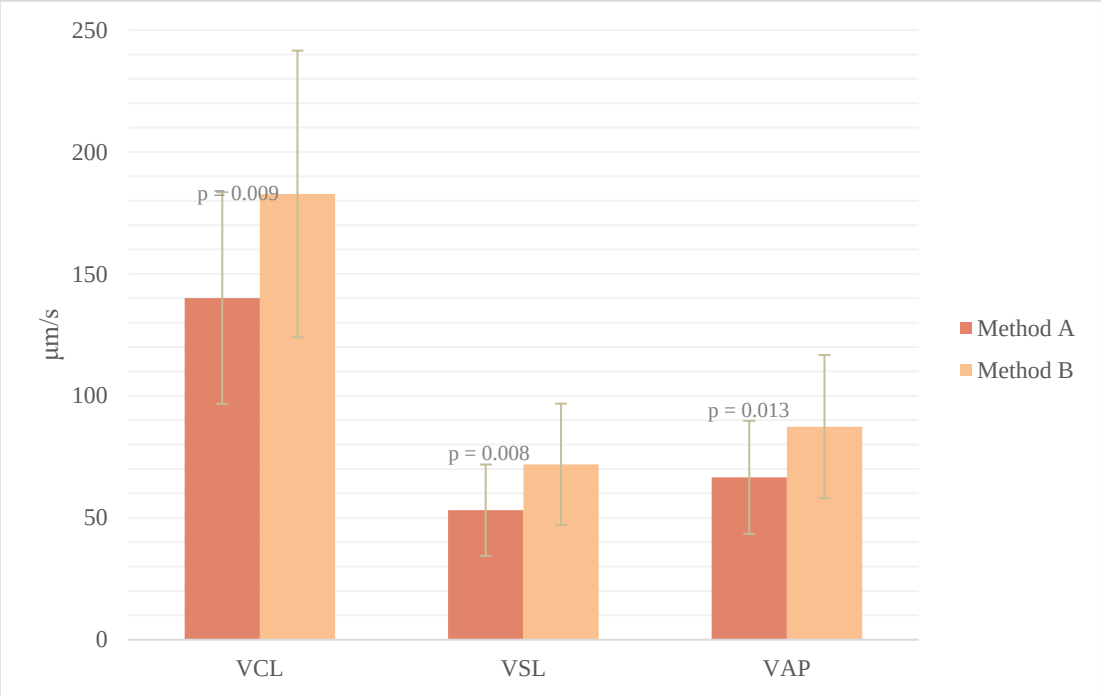

Figure 14: The CASA parameters (Mean ± SD) curvilinear velocity (VCL), straight line velocity (VSL) and average path velocity (VAP) showing significant difference between the pre-freezing samples of Method A and B.

Table 14: The CASA parameters of the pre-freeze samples, expressed as the parameter mean and standard deviation (SD) together with the associated p-value.

CASA	TM[%]	PM[%]	VCL[µm/s]	VSL[µm/s]	VAP[µm/s]	ALH[µm]	BCF[Hz]	WOB	LIN	STR
Method A *Mean ± SD*	58.20 ± 14.59	56.88 ± 14.71	140.09 ± 43.39	53.10 ± 18.74	66.60 ± 23.20	1.50 ± 0.41	8.48 ± 1.66	0.47 ± 0.03	0.37 ± 0.02	0.80 ± 0.03
Method B *Mean ± SD*	68.27 ± 19.84	67.42 ± 20.03	182.78 ± 58.79	71.90 ± 24.90	87.34 ± 29.40	1.87 ± 0.59	8.69 ± 2.80	0.47 ± 0.03	0.39 ± 0.04	0.82 ± 0.04
p-value	0.046	0.041	0.009	0.008	0.013	0.016	0.392	0.453	0.156	0.063

Post thawing there were no significant differences between the two extraction methods for the CASA parameters, except for BCF where Method B had a significantly higher value. This is concluded in Table 15.

Table 15: The CASA parameters (Mean ± SD) of the post-thawing sample with the associated p-value.

CASA	TM[%]	PM[%]	VCL[µm/s]	VSL[µm/s]	VAP[µm/s]	ALH[µm]	BCF[Hz]	WOB	LIN	STR
Method A *Mean ± SD*	16.31 ± 7.10	14.77 ± 6.86	34.53 ± 12.65	10.11 ± 5.33	14.16 ± 6.51	0.45 ± 0.15	3.64 ± 1.04	0.40 ± 0.06	0.28 ± 0.07	0.69 ± 0.08
Method B *Mean ± SD*	20.46 ± 11.63	19.07 ± 11.14	43.29 ± 18.68	12.72 ± 7.23	17.38 ± 8.91	0.56 ± 0.22	4.87 ± 1.78	0.39 ± 0.08	0.27 ± 0.08	0.68 ± 0.14
p-value	0.103	0.087	0.055	0.114	0.112	0.052	0.009	0.221	0.328	0.430

4.5.2 The impact of freezing the samples

The pre-freeze samples showed distinct difference ($p \leq 0.0000107$) compared to the post thawing samples for all the CASA parameters, confirming a decreased motility after freezing. This is illustrated in Figure 17, 18 and 19.

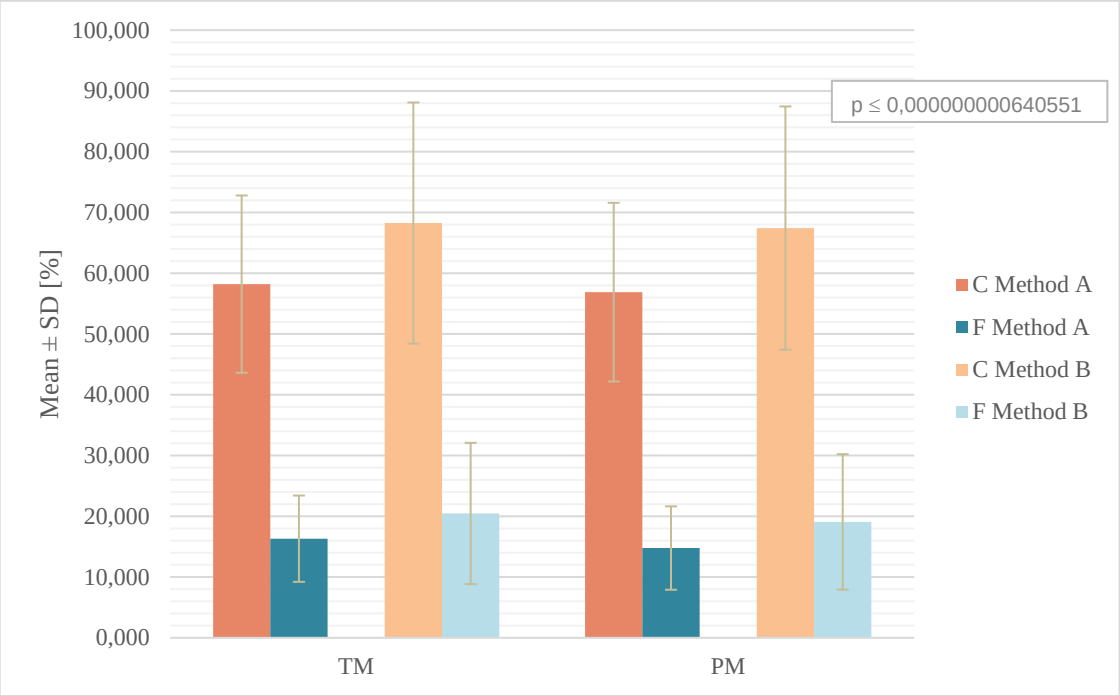

Figure 15: The comparison of motility parameters (Mean ± SD) total motility (TM) and progressive motility (PM) between the pre-freeze (C) and the post thawing (F) samples.

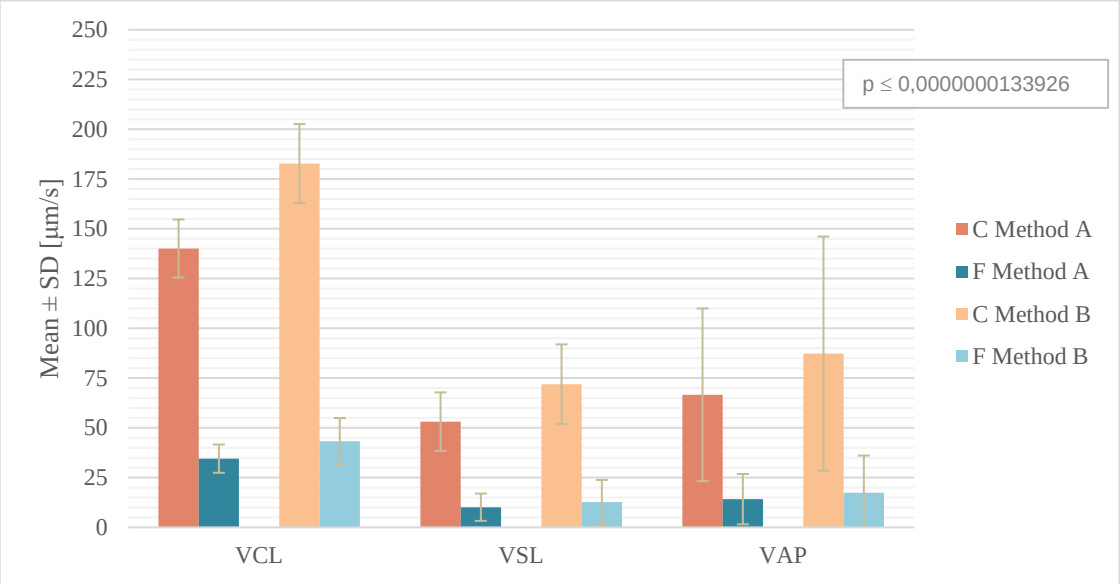

Figure 16: The comparison of CASA parameters (Mean ± SD) curvilinear velocity (VCL), straight line velocity (VSL) and average path velocity (VAP) between the pre-freeze (C) and the post thawing (F) samples.

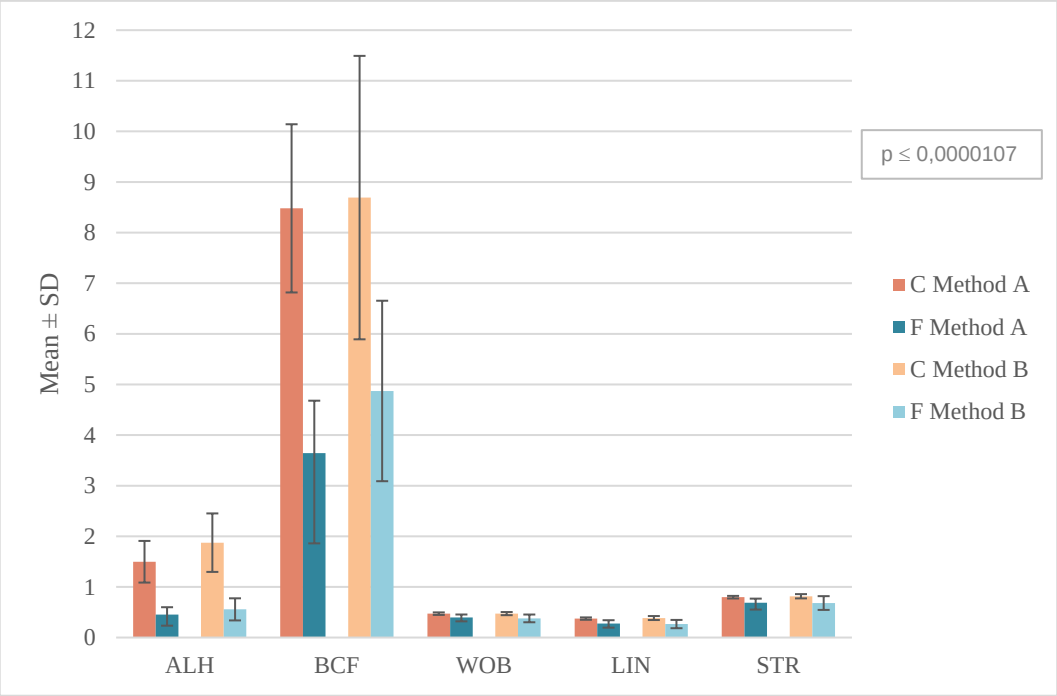

Figure 17: The comparison of CASA parameters (Mean ± SD) including amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), wobble (WOB), linearity (LIN) and straightness (STR) between the pre-freeze (C) and the post thawing (F) samples. The units of each parameter are listed in Table 1.

4.6 Flow cytometry

4.6.1 Sperm plasma membrane integrity

The analysis of sperm plasma membrane integrity included the three parameters of live spermatozoa, dying spermatozoa and dead spermatozoa [%], all illustrated in Figure 20. There was no significant difference between Method A and B in either the pre-freezing or post thawing samples. The pre-freezing and post thawing samples showed a clear difference with p-values ≤ 0. 00334 for all parameters.

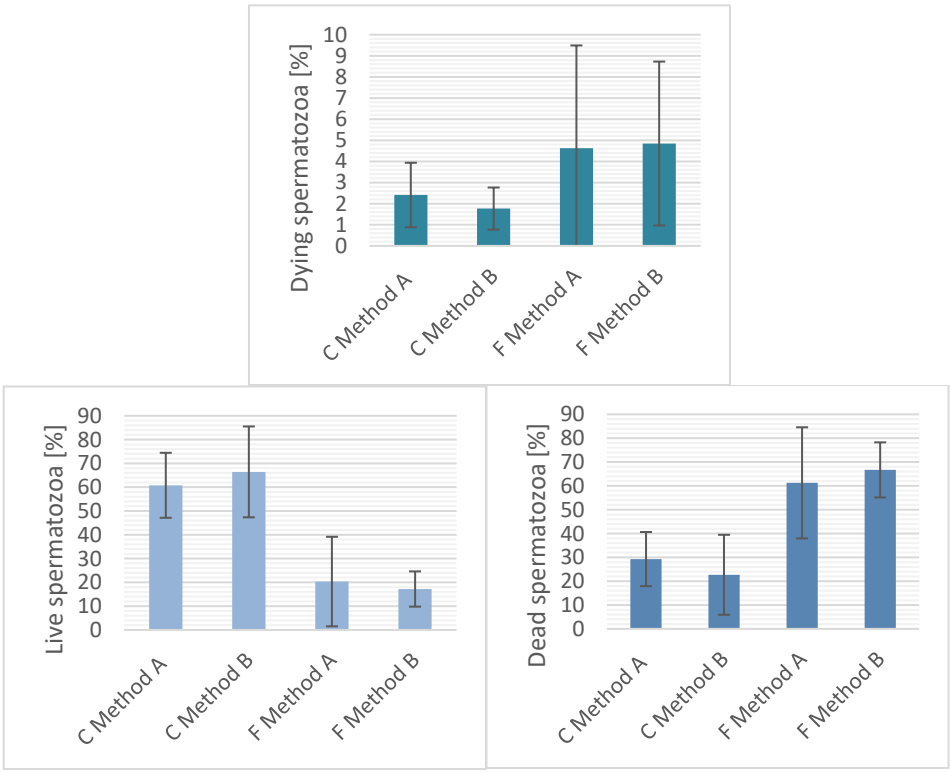

Figure 18: Sperm Plasma Membrane Integrity illustrated with the mean and standard deviation [%] of live, dying and dead spermatozoa in the pre-freeze (C) and post thawing (F) samples, comparing Method A and B.

4.6.2 Reactive oxygen species

There were no significant differences between Method A and B for the analysed reactive oxygen species as shown in Table 16. Comparing the pre-freeze and post thawing groups, there were clear differences for all parameters except for Live SO Positive for both methods, illustrated in Figure 21.

Table 16: Reactive oxygen species parameters, evaluating the proportion of live/dead spermatozoa, H2O2 (hydrogen peroxide) or SO (sulfoxide) positive (+) or negative (-), comparing Method A and B (Mean ± SD) pre-freezing and post thawing.

ROS Pre-freezing	Live H2O2 -	Live H2O2 +	Dead H2O2 -	Dead H2O2 +	Live SO -	Live SO +	Dead SO +
Method A *Mean ± SD*	75.13 ± 10.68	0.72 ± 0.47	22.51 ± 10.93	1.88 ± 1.00	70.63 ± 11.52	4.40 ± 2.78	24.91 ± 10.45
Method B *Mean ± SD*	79.74 ± 16.07	1.43 ± 2.34	17.41 ± 15.79	1.52 ± 0.70	76.91 ± 15.62	3.66 ± 1.76	19.33 ± 15.62
p-value	0.159	0.112	0.134	0.116	0.090	0.172	0.109

ROS Post Thawing	Live H2O2 -	Live H2O2 +	Dead H2O2 -	Dead H2O2 +	Live SO -	Live SO +	Dead SO +
Method A *Mean ± SD*	24.10 ± 12.62	2.93 ± 3.49	66.58 ± 15.92	7.14 ± 8.05	20.75 ± 11.15	7.64 ± 7.86	72.61 ± 14.02
Method B *Mean ± SD*	25.70 ± 16.69	3.43 ± 4.56	62.80 ± 21.42	8.70 ± 11.65	24.10 ± 16.88	5.07 ± 4.65	71.62 ± 10.12
p-value	0.374	0.357	0.276	0.322	0.244	0.122	0.431

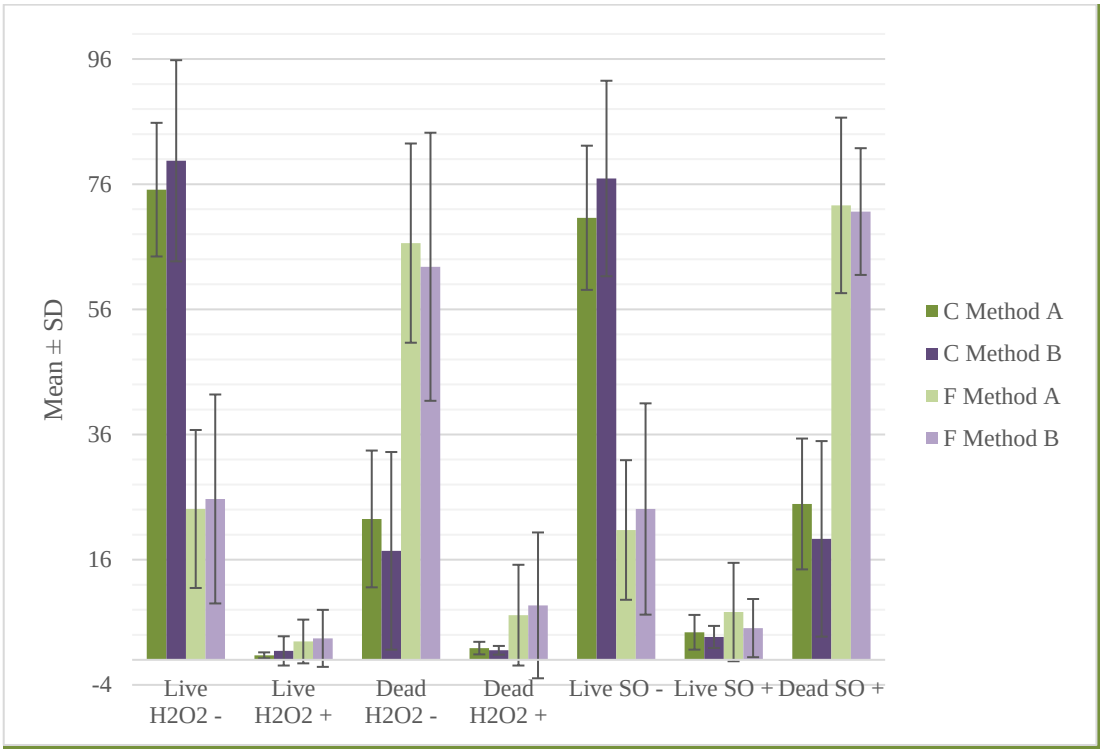

Figure 19: Reactive oxygen species parameters, illustrating the proportion of live/dead spermatozoa, H2O2 (hydrogen peroxide) or SO (sulfoxide) positive (+) or negative (-), showing the results of Method A and B (Mean ± SD) both pre-freezing (C) and post thawing (F).

4.6.3 Sperm chromatin structure

There were no differences in %DFI between Method A and B, or between pre-freeze and post thawing samples. There was a wide range of values as illustrated in Table 17.

Table 17: Sperm chromatin structure analysis result in %DFI comparing Method A and B (Mean ± SD, median and range) pre-freezing (C) and post thawing (F). The p-values ranged from 0,242-0,496 for the comparison between methods and groups.

SCSA	Mean ± SD	Median	Range
C Method A	3.89 ± 4.43	2.83	19.23
C Method B	4.79 ± 6.58	2.48	22.81
F Method A	4.19 ± 3.92	3.12	17.02
F Method B	4.17 ± 4.93	2.94	21.64

5. Discussion

The aim of this study was to compare the two extraction methods of cutting (A) and retrograde flushing (B) on the quality of bull epididymal sperm, evaluating motility, viability, reactive oxygen species and chromatin structure. The study was originally intended to be carried out on bison bulls, since collecting epididymal sperm postmortem would enable the preservation of genetic material from genetically valuable individuals. However, the bison bulls in question were exported to another country to become breeding bulls instead. Therefore, the decision was made to evaluate and compare the methods on epididymal sperm from slaughtered beef bulls as an experimental model, providing better material access. However, during the collection process numerous unpredictable events took place. This included many of the bulls being too young to include in the study and many pre-planned collection days when no adult bulls were slaughtered. Other issues disturbing the time schedule included mechanical trouble at the abattoir causing the slaughter to be cancelled as well as problems with the flow cytometry machine in the laboratory. Despite these unfortunate circumstances enough material was eventually collected and analysed to enable the two methods to be compared.

5.1 Evaluation and comparison of the two methods

When analysing the concentration of the extracted samples, it was confirmed that both Method A and B provided a wide range of sperm concentration without any significant difference between them. Method B however provided 4 individual samples with a concentration of <69 $x10^6$ spermatozoa/mL meaning that the concentration when freezing these could not be standardised. The reason for this was most likely a dilution effect from the amount of semen extender used to flush through the vas deferens. An excessive dilution of sperm to 20 $x10^6$ spermatozoa/mL is known to significantly reduce the proportion of motile spermatozoa post thawing, including the same CASA parameters as in this study: TM, PM, VAP, VSL, VCL, ALH, BCF, STR and LIN (Lone et al. 2022). The reason behind this reduction might be due to the decreasing levels of motility stimulating factors in diluted semen (Karan et al. 2018; Chakraborty & Saha 2022), or increased levels of ROS (Armstrong et al. 1999) resulting in the spermatozoa being immobilised (Peris et al. 2007). In this study the lowest concentration of a

sample pre-analysis was 10.3 $x10^6$ spermatozoa/mL, followed by 23.0, 24.0 and 56.1 $x10^6$ spermatozoa/mL. In hindsight it might have been better to use a smaller amount of extender to flush through the epididymides using Method B, or to have excluded these samples from the study when the low concentration was registered. However, an important aspect is that Method B, despite providing 4 low concentration samples, also provided the sample with the highest concentration. Method B also generated a larger sample volume (3 mL), more than twice the size of Method A (1-1.5 mL). Comparing the sperm count of the two methods, there is a trend towards significance ($p = 0.0543$) implying that Method B obtains a higher total number of spermatozoa. This property is both beneficial and desirable when the aim is to extract epididymal sperm from a genetically valuable individual but has to be furthered examined to determine the difference with certainty. In future studies, this might be done by expanding the sample group.

A significant difference was found when comparing Method A and B regarding the parameters total motility and progressive motility, with Method B excelling. This significance also applied to the CASA parameters curvilinear velocity, straight line velocity, average path velocity and amplitude of lateral head displacement. A possible explanation behind this difference was the fact that Method B involved less contact between the spermatozoa and the instruments and materials used during the extraction. The retrograde flushing meant that the spermatozoa was diluted with semen extender early on in the process, partly protecting it from direct contact with the pipette and Eppendorf tubes. Using Method A the spermatozoa was in direct contact with both the scalpel, pipette and Eppendorf tube prior to the addition of semen extender, which might explain the less favorable results. In addition, the incision used in Method A resulted in more contamination with blood. Though these areas were avoided while aspirating sperm, blood contamination likely took place to some extent. Using Method B, despite cutting through a greater portion of the cauda epididymidis, the bleeding was limited and no visual blood contamination was noticed during the extraction. Blood contamination in bulls is known to increase the presence of head-to-head agglutination, however a decreased motility was not significant (Verberckmoes et al. 2004). In fact, head-to-head agglutination in vitro was demonstrated to maintain sperm motility and viability instead of reducing it (Umezu et al. 2020). Blood had a negative effect post thaw on canine spermatozoa, exerted by blood on both motility parameters, membrane integrity and acrosomal status. (Rijsselaere et al. 2004). This is partly explained by rising levels of hemoglobin from red blood cell hemolysis after the process of freezing and thawing, as well as the presence of white blood cells producing ROS (Rijsselaere et al. 2004; Verberckmoes et al. 2004). Although this does not explain the difference between methods, it might have induced the generally sharply declining parameters for the post-thawing samples in comparison to the results prior to cryopreservation. This clearly significant difference is additionally most likely

due to cryodamage (Peña et al. 2011; Tapia et al. 2012), but it might also have been affected by the fact that the coverslips had not been pre-heated on the 38 °C heated stage during the CASA analysis of the post thawing samples including both methods. Cooling of the sperm cells too rapidly between 30 °C and 0 °C can cause the detrimental phenomenon ‘cold shock’, where lethal stress is induced (Watson 2000). This was an unfortunate mishap that could have had great impact on the motility results, and in worst case it might have obscured any difference between Method A and B post thawing.

5.2 Factors possibly affecting the outcome

Another factor affecting the concentration of the sperm samples is how it was calculated. Due to the sudden availability of a large number of samples, the supply of cassettes needed to analyse the concentration with the Nucleocounter SP-100 was exceeded. For the last 14 samples the concentration was thereby evaluated manually in a Bürker chamber. Unfortunately, no samples underwent both methods, and it is thereby not known how accurate the concentrations counted with the Bürker chamber were. However, several previous studies have presented that the two methods are comparable with correlated concentrations measured (Morrell et al. 2010; Egeberg et al. 2013; Gloria et al. 2023). Even if it is not desirable using different methods to evaluate concentration, there was no other possible solution in the circumstances. Since the same method was used to determine the concentration within each pair of samples in the present study, the potential impact of using different methods was considered to be negligible.

The same sudden material supply ended up affecting the time needed to perform the extractions on one occasion, causing the time of extraction relative to the time post slaughter to vary. There were significant differences between the samples extracted early and late that day, showing that the earlier extracted samples had a higher ALH and lower STR pre-freezing than the later extracted samples. The samples of the early extractions also has a larger proportion of spermatozoa that were Live H_2O_2 negative and Live SO negative than the late extractions. In turn, the samples of the late extractions had a higher proportion Dead H_2O_2 negative, Live SO positive and Dead SO positive spermatozoa. These significant results implies that the extra time taken to do a large number of extractions (n = 26) at one occasion affected the outcome. This finding leads us to suggest that the number of samples done in one day should be limited. Cooling the sperm samples as soon as they have been extracted instead of keeping them in room temperature prior to analysis might also be beneficial and should be considered in future studies.

No significant correlation was found regarding scrotal and testicular circumference in relation to any of the parameters analysed; however it is widely accepted that a larger scrotal circumference correlates with increased fertility (Fayrer-Hosken 1997; Senger 2012). The result might depend on the relatively small sample group of 18 bulls in total, but the presence of adhesions indicating earlier occurring inflammation on some of the testes (4 in total) also affected the outcome. The testes with adhesions provided spermatozoa with a significant lower sperm quality throughout many parameters, most likely because inflammation impairs normal scrotal and testicular thermoregulation, and normal sperm production and maturation (Wolfe 2018). Adhesions can form due to infection, exposure to cold inducing scrotal frostbite, trauma, or epididymitis and orchitis. Adhesions between the testis, epididymidis and parietal vaginal tunic occur at a higher incidence in older animals (Foster 2016). Whilst finer and thread-like fibrous adhesions are normally found near the cauda epididymidis of bulls, more extensive presence is a sign of periorchitis. Theses adhesions usually start off as fibrinous during the acute phase of inflammation, but later on turn fibrous. It is not known what caused the adhesions seen in the bulls in this study but one possibility is that the animals cause trauma to each other, leading to mild inflammation and limited adhesion formation.

The CASA analysis was performed by two observers due to increasing workload when a large number of samples had to be analysed on the same day. This ended up being a source for inter-observer variation since one of the observers analysed 5 fields of view, and the other one 8 fields of view. However, >1000 spermatozoa were analysed in all samples with the corrected concentration of 69 $x10^6$ spermatozoa/ml. In the samples with a lower concentration this directly affected the number of spermatozoa analysed, the lowest count being 292. This is still within margins as a minimum of 200 spermatozoa should be counted when assessing sperm motility, but in low concentration samples it might be enough to evaluate as few as 100 spermatozoa (Centola 2018).

In this study the testes and epididymides from the bull were used as each other's controls, and the two methods that are compared are evaluated using sperm from different testes. This is current practice and occurred in many previous studies, but according to Goovaerts et al. 2006 the equality of sperm quality between the two epididymides is not fully comparable. A deviation of around 20% was established for most semen parameters comparing paired caudae epididymides. This implies that the difference between methods in this study might instead be due to a variation between the testes of the bull. However, there are limited options of experimental designs to avoid this problem. The one established as the best option in this case ended up being alternating the method between the left and right epididymides. Testing the sperm motility and viability of each epididymides prior to the experiment would also not have been possible. However, since there were no

significant differences in results between the left and right testis and the selection group was large enough, the approach of dividing the epididymides into extraction method groups and alternating side seems to have worked well.

6. Conclusions

The results of this study showed a significant difference prior to cryopreservation between the two epididymal sperm extraction methods, with the retrograde flushing method providing multiple desirable properties regarding motility and sperm count. The post thawing samples showed significantly less favorable sperm quality throughout all quality analyses except for the chromatin structure and reactive oxygen parameter Live SO^{+}. A weak correlation was seen indicating that the older bulls in the study had better sperm motility and viability, and significant difference showed that the presence of testicular adhesions impair the sperm quality. The results implies that the retrograde flushing method is to be preferred when extracting epididymal sperm in future studies, but regarding the cryopreservation a different protocol should be evaluated to hopefully better preserve sperm quality post thawing.

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Popular science summary

Traditionally, the collection of bovine spermatozoa is limited to ejaculated semen, thus the option of collecting semen in case of sudden life-threatening injury or death of a valuable bull is not available. Due to their wild nature, non-domesticated species such as wisents, bisons, wild yaks and buffalos cannot be handled to a degree that allows this type of collection method without it posing a risk of injury. Some of these species' existence is somewhat threatened, with the wild yaks being classified as vulnerable by the International Union for Conservation of Nature. Another example is the wisent which might not be considered endangered anymore, but the species still face challenges regarding limited genetic diversity and habitat loss. Improving the accessible genetic variety within the species and ensuring a continued preservation, the collection and storage of sperm samples could support their long-term survival. Establishing methods to extract sperm samples from genetically valuable individuals after death does ensure genetic material within the species that were previously lost due to slaughter or trauma. Although the method of extracting spermatozoa from the epididymis enables the development of genetic diversity, the preferred extraction method has not yet been established.

The extraction of spermatozoa from the epididymis can be done in multiple different ways, but the three most common ones include:

- *Cutting method:* Incisions are made into the tissue and sperm protruding can be collected.
- *Floating method:* Incisions are made into the tissue and the epididymis is placed within fluid semen medium, diluting the sperm collected into the fluid.
- *Flushing method*: Semen extender fluid is used to flush through the tissue and simultaneously flushes out the sperm cells.

Once collected, the sperm samples can be kept fresh at a temperature of 4 °C or frozen and stored in liquid nitrogen at -196 °C. The choice of storage method can vary depending on when the sample is planned to be used. Frozen spermatozoa can be used at any time after the death of the animal, but it does require knowledgeable technicians, advanced equipment and refrigerated transportation to a laboratory in order to remain viable. At this stage the sample can also be analysed regarding sperm quality parameters, for instance by investigating the motility of the

spermatozoa. One commonly used method in order to do this is computer assisted sperm analysis (CASA), where the proportion of moving sperm cells and other motility parameters are evaluated using a computer connected microscope. It is important that the sperm sample and the equipment it is in contact with retains a temperature of 38 °C during the analysis since the sperm otherwise might experience a temperature shock negatively effecting the motility. Other analyses preformed using a flow cytometer include plasma membrane integrity in order to establish the sperm viability, reactive oxygen species to investigate the presence of oxidative stress and sperm chromatin structure to examine the presence of DNA damage.

Two of the extraction methods, the cutting and the flushing method, are evaluated and compared in this study. The aim was to establish the preferred extraction method between the two. It was originally planned to study wisent bulls, but due to material shortage the decision was made to compare the methods using epididymal sperm from slaughtered beef bulls instead. The bull testes were collected at a local abattoir and the sperm samples extracted using one of the two methods. The sperm quality of the samples was then analysed as previously described, and then frozen within liquid nitrogen at -196 °C. On a later occasion, the samples were thawed and the analysis repeated.

Results showed that the flushing method provided a significantly higher motility rate than the cutting method across many of the CASA motility parameters. In addition, the flushing method also provided a larger sample volume and a higher total number of spermatozoa, although this needs to be studied further to determine with certainty. All of the samples collected during the study showed a generally higher motility and viability rate before freezing than after thawing. A weak correlation was also discovered showing that older bulls produced spermatozoa with better quality, and that the presence of adhesions on the testes was consistent with lower sperm quality. Altogether, this implies that the flushing method would be the preferred extraction method used to collect sperm from a genetically valuable individual. However, the quality of sperm is best assured in fresh samples since the freezing and thawing processes clearly had a negative effect on the sperm. If the intention is to freeze the sperm gathered, a different freezing protocol and approach should be evaluated and then hopefully considered in order to better preserve the sperm quality.

Acknowledgements

I cannot begin to express my appreciation for the outstanding support of my supervisor, Jane Morrell, who has assisted me throughout the project and put forward inventive solutions when unexpected challenges have arisen on a short notice. I also want to extend my sincere thanks to Ziyad Al-Kass who has been a great support and provided guidance during the preparation and handling of the material, as well as the analysis of the samples. I also want to express my gratitude towards Mohua Dasgupta who on a short notice was able to facilitate the workload when we went off schedule. Thanks to the local abattoir and their involved personnel for their selfless cooperation and contribution of material to this project. Finally, a special thank you to my mom Angelica, for always supporting and encouraging me throughout all my years of study and through the process of writing this project.

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