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Studies of Sex-sorted Bull Sperm Cells

Establishment of a New Potential Sperm Quality Test

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List of Abbreviations

AI - Artificial Insemination

NR56 - Non-return Rate 56 days

NCHRS - Norwegian Cattle Health Recording System

GnRH - Gonadotropin-Releasing Hormone

LH - Luteinizing Hormone

FSH - Follicle Stimulating Hormone

ATP - Adenosine Triphosphate

NADH - Nicotinamide Adenine Dinucleotide

OS - Oxidative stress

ROS - Reactive Oxygen species

MDA - Malondialdehyde

TBA - Thiobarbituric Acid

DAPI - 4′-6-Diamidino-2-Phenylindole

CASA - Computer Assisted Sperm Analysis

AIL - Acrosome Intact Live

MOT - Motility

PROG - Progressive

VCL - Curvilinear velocity

VAP - Velocity average path

VSL - Velocity straight line

STR - Straightness

WOB - Wobble

ALH - Amplitude of lateral head displacement

PBS - Phosphate buffer saline

PMT's - Photomultiplier tubes

SSC - Side Scatter

FSC - Forward Scatter

AIL- Acrosome Intact Live

PI - Propidium iodine

PNA - Peanut (*Arachis hypogaea*) agglutinin

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Abstract

Sex-sorting of sperm cells is a technology that is growing globally for the use in the breeding of cattle. Sorting of X- and Y-Chromosome bearing spermatozoa is done by flowcytometry before cryopreservation and it is important to be able to recognize possible damages caused by the methods, as well as having analytical tools to illustrate possible differences in fertility potential and bull to bull differences. The main objective for this thesis was to establish a motility stress test and a new analytical tool for measurement of lipid peroxidation as an indicator for oxidative stress, specifically designed for Norwegian Red sexed spermatozoa.

Various sperm quality parameters were analyzed for sex-sorted Norwegian Red spermatozoa, including motility by Computer Assisted Sperm Analyzer, ATP level and lipid peroxidation by plate reader and viability and acrosome integrity by flowcytometry after different time of incubation. The motility stress test showed a significant decline from pre and post incubation in total motility and progressive motility ($P<0.05$), therefore ATP level, MDA concentration, viability, and acrosome integrity were examined before and after incubation for 120 minutes at physiological temperature, in an effort to characterize possible causes for the decline in motility. The ATP measurement found a significant decline between pre and post incubation ($P<0.05$), likewise as the motility stress test, meaning that the loss of motility could be caused by low amounts of energy. Analysis of lipid peroxidation using the biomarker MDA showed that the loss of motility was not caused by lipid peroxidation. Thus, in further studies other types of oxidative stress biomarkers could be better suited for examining the relationship between motility and oxidative stress in sexed spermatozoa. However, the assessment of acrosome intact live spermatozoa post incubation showed that about half the sperm cells were still viable, meaning that some immotile spermatozoa may still have the ability to become motile again. This might prove to be a vital new parameter in terms of fertility prediction in sexed Norwegian Red spermatozoa for AI centers.

After the motility stress test and the new analytical tool for lipid peroxidation was established, sex-sorted spermatozoa from six Norwegian Red bulls with known field fertility were examined for total motility, progressive motility, velocity parameters (VCL and VAP) and lipid peroxidation (MDA). There were found some differences between high and low fertility groups, however further research must be conducted with an increase in the number of bulls to determine whether association between fertility and the quality parameters are significant.

1. Introduction

1.1 Fertilization in Bovines

Analogous to almost all mammals, bovines reproduce sexually by means of internal fertilization. In that way, a haploid sperm cell (spermatozoon) enters the female reproductive tract, encounters a haploid egg cell (oocyte), perforates the cumulus matrix, penetrates the zona pellucida, fuses the plasma membranes, and forms the diploid zygote, now carrying genetic information from both the male and female parent. To prevent the fusing with more than one spermatozoon (polyspermy), the oocyte is activated after a successful fertilization. From now on, the zygote reproduces itself via asexual mitosis, increasing the cell count and starting the formation of a new individual (Campbell et al., 2021, pp. 258–259; 1019–1022; Messinis et al., 2016). The fertilization in bovine is usually achieved through either natural mating or through artificial insemination (AI). The commercialization of AI was first introduced in the 1930s and enables sires with superior traits to pass on offspring. (DeJarnette et al., 2004) The use of AI has been the method of choice to spread favorable genes and improving the genetic traits of the herd (Vishwanath, 2003).

1.1.1 Breeding of Cattle

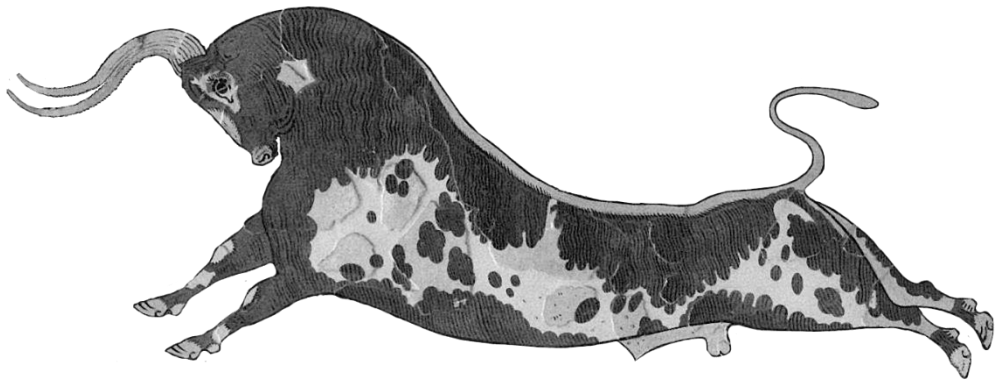

Figure 1: Diagram of the aurochs depicted in the bull-leaping fresco of the palace at Knossos, Crete. (Pinpin, 2007). CC BY-SA 3.0. (https://commons.wikimedia.org/wiki/File:Aurochs_animal-bw.png)

Only a few species on earth fulfill enough of the requirements necessary to be domesticated. Cattle ranks among those since they are herbivores, have short reproduction intervals, can be bred in captivity and handled under relative safety, serve as riding or draft animal, and provide not only meat but also milk and animal skin which can be turned into leather (Felius et al., 2014; Pitt et al., 2019).

Several successful domestication attempts in various regions could be proven with the earliest taking place more than 10,000 years ago. Over this time period the modern cattle (Bos taurus) descended from the aurochs (*Bos primigenius*) (Pitt et al., 2019). A representation of the aurochs (*Bos primigenius*) can be seen in Figure 1. The domesticated cattle differ from their wild counterpart in several traits, including body size, tail length, coat characteristics, horn features, fat distribution, and milk yield. Various regions produced distinct sets of characteristics, which progressed into modern cattle breeds we are familiar with today, such as the Norwegian Red, Holstein, and Friesian. Specializing on one or several productivity aspects or physical appearance (Gepts & Papa, 2003; Schafberg & Swalve, 2015; Vigne & Helmer, 2006).

Modifications in livestock were historically accomplished by artificial selective breeding, where only animals with desired traits are accepted into the breeding stock to increase the population faction carrying this characteristic phenotypically (Campbell et al., 2021, pp. 474–475). Nowadays, more precise methods can be applied, which not only improves the chances of breeding success, but also seeks to decrease the downfalls of naïve selection including the issue of inbreeding depression (Doekes et al., 2019; Gutiérrez-Reinoso et al., 2022; Weigel, 2001).

1.1.2 The Breeding Company Geno and The Creation of Norwegian Red

In Norway, the breeding company Geno emerged from a desire for cooperation between cattle farmers. To facilitate collaboration and improve on the unsatisfactory breeding results at that time, an orientation meeting was held in Hamar in 1935, during which the “Avlslaget for Hedmarksfe” was founded. The objective of the newly established farmers’ cooperative was geared towards increasing control and planning to optimize the local breeding practices, which – according to the farmer Ole Sandberg – were mostly left to chance beforehand (Geno, 2020b;

Ødegård, 2000). In this period, the focus in breeding lay on the cattle's ability to produce milk and meat while also being uncomplicated to feed and handle. To achieve progress, Helge Bækkedal insisted that a break with tradition was necessary, which prevented crossbreeding between the numerous local cattle breeds found in Scandinavia. Instead, breeding animals should be selected solely on their expression of desirable traits in the future (Geno, 2020b; Ødegård, 2000, p. 9).

In 1939, the term "Norsk Rødt Fe" (Norwegian Red Cattle) was coined, which was used both for the newly commenced cattle breed as well as the farmers' cooperative(Ødegård, 2000, p. 9). In the same year, several smaller Norwegian breeding associations joined, setting the foundation for the prevalent position which lasts to this day (Geno, 2020b; Ødegård, 2000, p. 9).

The success of the associations breeding strategies proved to be successful and lasting. At the beginning of the 1950s, the Norwegian Red breeding association placed increased emphasis on the technical and scientific development (Ødegård, 2000, pp. 9–11). Artificial insemination was introduced, which also resulted in the first cryopreservation (described in section 1.3) of bull sperm by Norwegian Red breeding association in 1953 (Geno, 2020b), a procedure where spermatozoa are frozen to be used at a later point without the necessity of a present bull. With the new technology at hand, the breeding goals could be broadened to now also include several metrics determining health and from the 1970s fertility of the animals (Ødegård, 2000).

The international market for Norwegian Red cattle grew over the years and in 1999, the name was changed to "Geno". Norwegian Red cattle ranks among the most sold cattle breeds worldwide and is very popular with European and North American farmers (Geno, 2020b, 2020c).

In the 2000s, Geno introduced genomic selection to further control for the development of the breed (Geno, 2020b), whereby genetic markers associated with desired breeding traits are analyzed to predict likely features of the offspring. To this day, research and technology about genetics, health, and reproduction play an important role in the operations of the company (Geno, 2020a).

In Norway the field bull fertility is determined by the non-return rate at 56 days since insemination ("NR56"), meaning the rate of inseminated heifers that were not inseminated again within the first 56 days after insemination, which is a metric used to approximate

successful inseminations per attempt (Costes et al., 2022). This data is documented in the Norwegian Cattle Health Recording System (NCHRS). In 2021, 98% of the dairy cow farmers in Norway were members and recorded their animals with the register. Therefore, it can be assumed that accurate fertility data can be derived from the database (Østerås et al., 2007; Vangen & Ferneborg, 2023).

The breed Norwegian Red was established in 1935, and originally started with cross breeding the Norwegian Hedmark cattle with the Scottish Ayrshire cattle. In the 1960s it was further cross bred with other Norwegian breeds to make the Norwegian Red cattle that we are familiar with today (Vangen, 2022).

In 1975 the Norwegian Cattle Health Recording System (NCHRS) was implemented, the system was created as a collaboration between the Norwegian veterinarian association, and the Norwegian Red Cattle cooperative which would later become Geno SA. The data from the national recording system shows that 94% of the Norwegian cattle are Norwegian Red and 4% are cross bred with Norwegian Red. The other breeds used in dairy production do not exceed more than 0.5% of the total population individually. Since 1975 the number of cows in the NCHRS has increased from 67% to 97% (Østerås et al., 2007).

The dairy breed Norwegian Red has been bred using a total merit index philosophy with a broad spectrum of traits for almost seven decades (Begley et al., 2009). During this time period traits for healthy cattle have been a high priority. The effects of crossbreeding Norwegian Red with Israeli Holstein cattle, resulted in a significantly lower risk for ketosis, metritis, and several calving issues (Rinell & Heringstad, 2018).

As a result of the focus on health instead of gross milk yield, Norwegian Red cattle fares better in comparison to the Holstein-Frisian breed regarding the incidence of infections like mastitis and the corresponding somatic cell count in milk. Additionally, Norwegian Red fared better when it comes to calving and stillbirths (Ferris et al., 2014).

1.2 Male Reproduction in Bovine

1.2.1 Anatomy and Physiology of the Male Reproductive Organs

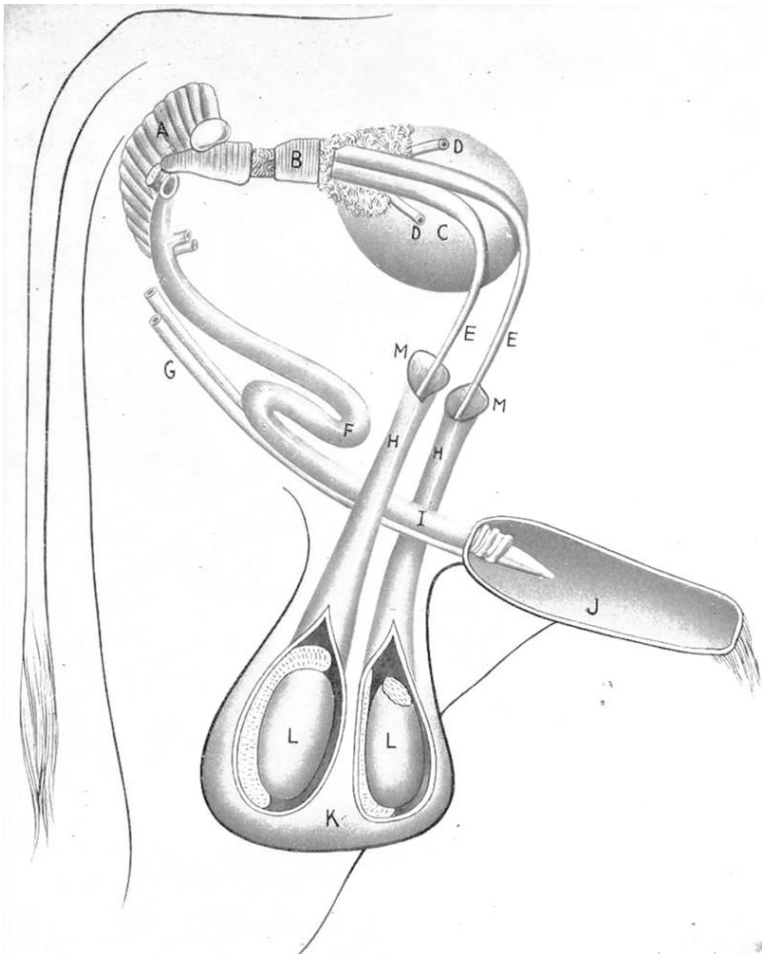

Figure 2: Schematic depiction of the male reproductive organs in a bull. C: bladder; D: urethrae; E: ductus deferentes with the ampulla widenings at the ends; L: testes with the epididymes; K: scrotum
(G. R. White, 1914). Public domain.
(https://picryl.com/media/animal-castration-a-book-for-the-use-of-students-and-practitioners-1914-14761514784-ba5b30)

The male reproductive system consists of the penis, the testes, and accessory sex glands, with the function of depositing viable sperm into the female reproductive system (Mawhinney & Mariotti, 2013).

The testes are located within a thin vascular structure called the scrotum as seen in Figure 2. The highly vascular tissue regulates the temperature during the sperm formation. To achieve normal sperm production, the temperature must be 4-5°C lower than the core body temperature (Fayrer-Hosken, 1997).

The epididymis, the ductus deferens, and the ampulla make up a total repository system of spermatozoa, referred to as the extragonadal reserves. The ampulla is an expanded segment of the ductus deferens, where the widening is caused by an increase of mucosa. In addition to their role in storing spermatozoa, the epididymis and ampulla also produce seminal plasma, which is also facilitated by the vesicular, prostate, and bulbourethral glands. In particular, the vesicular glands do not contain spermatozoa but instead function as a reservoir for a majority of the non-cellular fraction of the ejaculate, which is secreted directly into the pelvic urethra. The prostate gland is located next to the juncture of the bladder and the pelvic urethra, a section in where vast differences between species occur. Bulls have what is considered a urethral gland, due to the glandular tissue being elongated dorsally along the pelvic urethra, which is also referred to as a disseminate prostate (Senger, 2012, pp. 46; 70–74).

1.2.2 Spermatogenesis, Spermiogenesis, and Spermiation

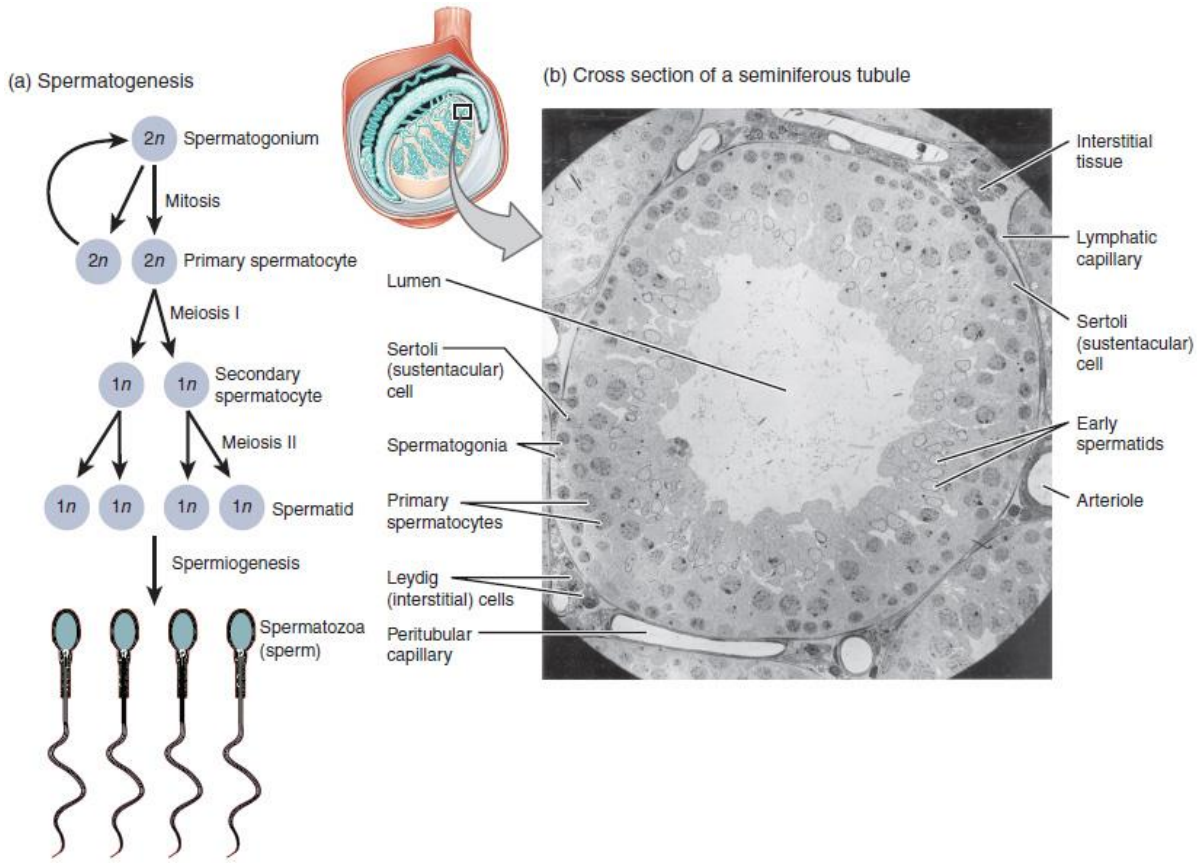

Figure 3: Diagram depicting spermatogenesis and spermiogenesis, and the lumen of the seminiferous tubules. (OpenStax College, 2013). CC BY 3.0. (https://commons.wikimedia.org/wiki/File:Figure_28_01_04.jpg)

Spermatogenesis is the process of developing spermatozoa within the seminiferous tubules and is divided into three phases: the proliferation phase of spermatogonia, followed by a meiotic phase and ultimately a third phase called differentiation as seen in Figure 3. In the proliferation phase, spermatogonia A go through mitosis and produce spermatogonia B. Due to the process of spermatogonia A renewal during the proliferation phase, spermatogonia B can regress to spermatogonia A which can develop into new spermatogonia B. During the meiotic phase, primary spermatocytes undergo meiosis I, enabling an increase in genetic diversity, due to cross over prior to the DNA replication. Thereafter, meiosis II follows where the secondary spermatocytes divide into haploid round spermatids. The third and final phase is often referred to as the spermiogenesis, during which the round spermatids differentiate into the exceptionally distinct spermatozoa (Senger, 2012, pp. 207–209).

In bulls, this process takes 61 days (Staub & Johnson, 2018). Neither this duration nor the amount of sperm cells produced is affected by the frequency of ejaculations (Senger, 2012, p. 203), but there is a significant correlation between the semen quality and amount, and the testicular circumference (Wahyudi et al., 2022).

During this phase of spermatogenesis, the round spermatids differentiate into spermatozoa, which are then capable of motility. To achieve this, the cells have to undergo several major changes: During the Golgi phase, an apical acrosome is formed while the axonemes develop on the opposing side, starting the developing of the flagella. In the following cap phase, the head membranes develop, which are an important defense mechanism for the spermatozoa. Afterwards, during the acrosomal phase, the laid-out head features are further enlarged, and the flagella receive a protective manchette made up by microtubules. The last phase of the differentiation is the maturation phase. The microtubular system within the manchette develops the post nuclear cap, and simultaneously the mitochondria relocate to the mid-piece of the tail. The mitochondria are positioned in a mitochondrial helix within the midpiece, which is the first part of the flagellum behind the distal centriole. The spermatozoon now has its correct shape, flagellum, and mitochondrial sheath seen in Figure 5 (Moreno et al., 2000; Senger, 2012, pp. 206–211).

The spermatogenesis is regulated through endocrine and paracrine pathways. Pulsative releases of hypothalamic gonadotropin-releasing hormone (GnRH) stimulate the pituitary gland to produce luteinizing hormone (LH) and follicle stimulating hormone (FSH). The high intratesticular concentrations of testosterone together with FSH are necessary to initiate and

continue the spermatogenesis. The transformation of testosterone into oestradiol and testosterone on its own function as negative feedback for GnRH released by the hypothalamus (Kraemer & Rogol, 2008).

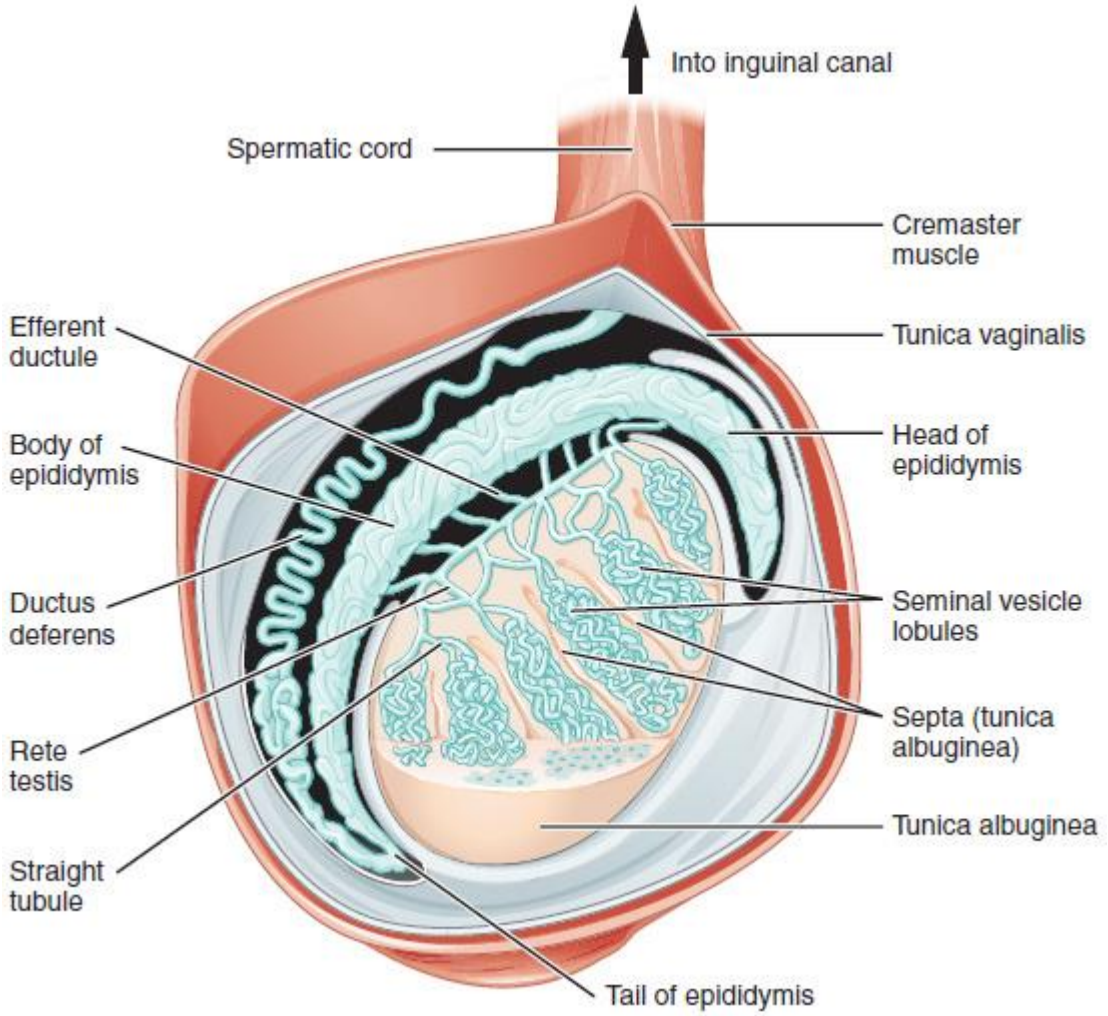

Figure 4: Diagram of a human testicle.
(OpenStax College, 2013). CC BY 3.0.
(https://commons.wikimedia.org/wiki/File:Figure_28_01_03.JPG)

The now mature spermatozoa are released into the lumen of the seminiferous tubules of the testes by spermiation, a complex process, where Sertoli cells support the reduction of cytoplasm and enabling transcriptional regulatory mechanisms to aid fertilization. In the following spermiation, the mature spermatozoa navigate through the testicle via the seminiferous tubules, the net-like rete testis, and the efferent ductules, to reach the epididymis as seen in Figure 4(O'Donnell et al., 2011).

1.2.3 Sperm Cell Physiology

The spermatozoon is a highly complex and differentiated cell and consists of two main sections: the sperm head and the sperm flagellum. The head of the spermatozoon comprises of the nucleus, the acrosome, and the post-nuclear cap, also known as the post-acrosomal sheath as seen in Figure 5. The flagellum or sperm tail enables motion and contains the capitulum, which connects the tail to the head, the middle piece, the principal piece, making up the majority of the tail, and the end piece (Senger, 2012, pp. 207–211; Sutovsky & Manandhar, 2006, pp. 3–7).

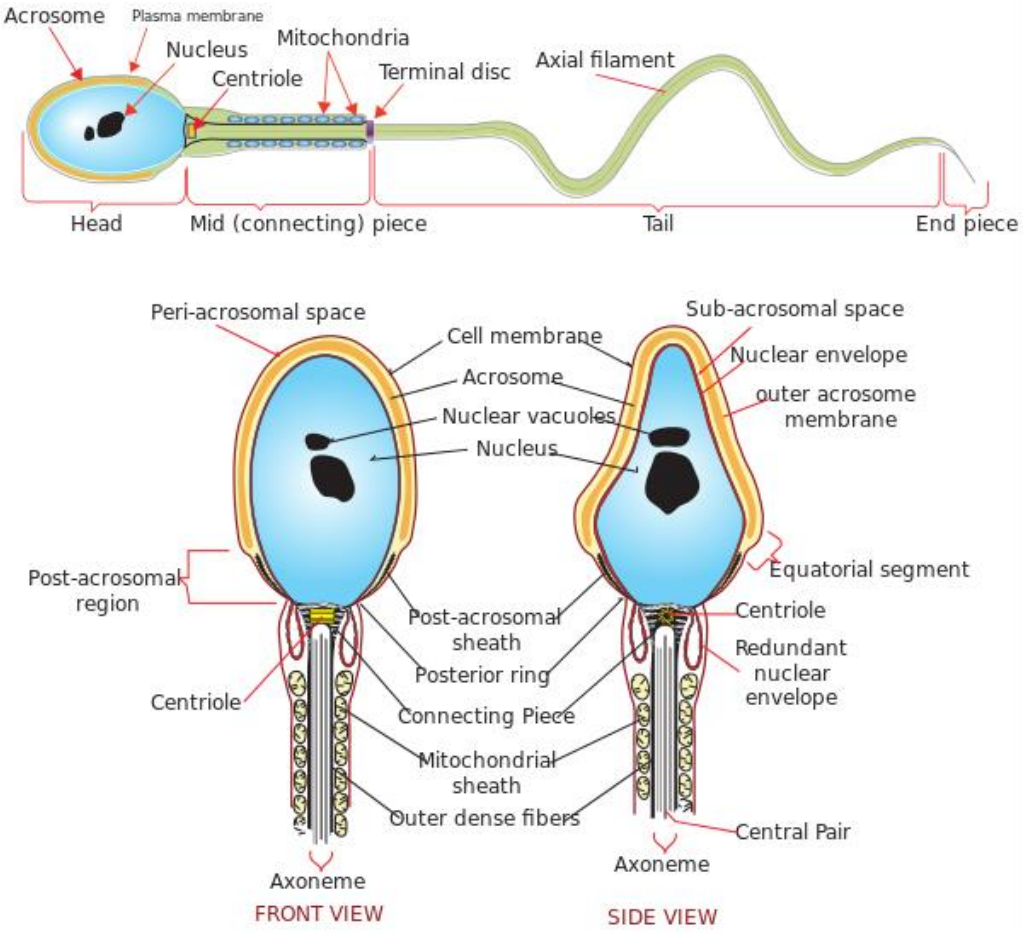

Figure 5: Diagram depicting a sperm cell from three different angles.

(M. R. Villarreal, 2006). Public domain.

(https://commons.wikimedia.org/wiki/File:Complete_diagram_of_a_human_spermatozoa_en.svg)

There is a significant variance between species to species both regarding the shape and size of the head as well as the tail (Gage, 1998). The nucleus in bovine sperm is responsible for a rounded head with an elliptical cross section (Senger, 2012, p. 211).

The nucleus contains the condensed DNA. In contrast to most somatic cells, the histones have been replaced by protamines, which enable a tighter packaging of the genetic material. This is achieved by disulfide bonds formed by the thiol groups of the protamine. This hypercondensation protects the genetic material against damage and allows for increased motility and effective penetration into the egg cell by providing a hydrodynamic shape. Additionally, the dense packing of the genetic material prevents transcription and therefore translation of the stored DNA (Senger, 2012, p. 211; Sutovsky & Manandhar, 2006, p. 3).

The apex of the sperm head is capped by the acrosome. Parallel to a lysosome organelle in somatic cells, it contains hydrolytic enzymes, which enable the spermatozoon to initiate the acrosome reaction, a process necessary for the spermatozoon to penetrate the membranes and the zona pellucida to ultimately fertilize the oocyte (Senger, 2012, pp. 211–212).

The function of the tail is to provide the cell with the ability to advance through the female reproductive tract and reach the oocyte to initiate fertilization. The mitochondrial helix sheath of the middle piece houses 75-100 mitochondria and provides the tail with the energy needed to power the propulsion of the spermatozoon. The axoneme of the principal and terminal piece contains 9 doublet microtubules that are positioned around 2 microtubules, that make up the moving component of the spermatozoon. On the outer most layer of the flagellum there are 9 coarse outer fibers, which is also named outer dense fibers. The axoneme and the outer coarse fibers provides the flagellum the structural integrity to enable propulsion (Senger, 2012, pp. 211–213).

1.2.4 Capacitation and Hyperactivation

To gain the ability to fertilize an egg cell, the mature spermatozoon has to go through a process called capacitation, which naturally occurs inside the female reproductive tract (Senger, 2012, p. 259). The change is facilitated by several chemical and physical factors, which include pH value, temperature, and various enzymatic substances (Purdy et al., 2022). Because of the great number of cofactors, capacitation is still not yet fully understood (Baro Graf et al., 2020).

Initially, reactive oxidative species (ROS; see also section 1.2.6) and multiple oxidants like $O_2^{\bullet-}$, H_2O_2, and $NO^{\bullet}$ are produced by the spermatozoa, reacting with their own plasma membrane. This activates adenylyl cyclase, the catalyzing enzyme for converting ATP into cAMP. (O′Flaherty, 2015).The presence of protein kinase A activated by cAMP can be used to determine capacitation in a laboratory environment (Mostek et al., 2021). To prevent the uncontrolled oxidation of the spermatozoa, they also produce antioxidant enzymes (O′Flaherty, 2015).

After successful capacitation, spermatozoa can shift from straight, linear motility to the faster paced, erratic hyperactive motility pattern (Senger, 2012, p. 260). This is caused by curling of the heads and faster movement of the flagella, assisting the journey through the mucous cervix into the uterus and oviduct to reach the oocyte (Swain et al., 2022). The capacitation itself, the acrosome reaction, and the motility require high amounts of energy, therefore energy management is an important factor for the effectiveness of spermatozoa (see also 1.2.5) (Amaral, 2022). The capacitation furthermore destabilizes the spermatozoon membrane, resulting in a faster cell death (Harrison, 1996).

During the oestrus phase of the sexual cycle in bovine, the grooved cervix produces two types of mucus: the less viscous, distally flowing sulfomucin in the center of the cervix and the highly viscous sialomucin inside the grooves. Only spermatozoa motile enough to navigate the thicker mucus between the folds are not flushed out by the sulfomucin. Therefore the bovine cervix has a selection bias towards higher motility (Senger, 2012, pp. 258–259).

Capacitated spermatozoa are able to induce the acrosome reaction, the ability to attach to the zona pellucida, a thick coating surrounding the oocyte, and further on release proteolytic enzymes to penetrate through to the membrane of the oocyte (Mostek et al., 2021; O′Flaherty, 2015). This ability is lost, if capacitation is reversed, occurring when spermatozoa are removed

from their specific capacitation environment and put into seminal plasma. Decapacitated spermatozoa do not lose the potential to capacitation (Senger, 2012, p. 260).

1.2.5 Adenosine Triphosphate Production and Energy Management to Maintain Sperm Function

The survival of spermatozoa are dependent on adequate energy production, as a lack of adenosine triphosphates (ATP) reduces sperm motility (Armstrong et al., 1999). In comparison to other cells, the ATP consumption is significantly higher, which is not only caused by the flagellar movement, but also by the energy required for the necessary protein modifications of the acrosome reaction.(Amaral, 2022; Miki, 2007).

To provide the required energy, two metabolic pathways are used by spermatozoa to gain ATP which are oxidative phosphorylation facilitated in the middle piece and glycolysis in the principal piece. Glycolysis uses glucose to produce pyruvate, the coenzyme nicotinamide adenine dinucleotide in its reduced form (NADH), and energy in the form of ATP molecules. Pyruvate can be further metabolized into acetyl CoA under aerobic conditions, or into lactate under anaerobic conditions, resulting in additional ATP molecules. Oxidative phosphorylation takes place in the mitochondria found in the middle piece, taking NADH, O_2 and succinic acid as substrates. The reactions ultimately end with the formation of H_2O and a large amount of ATP. Furthermore, pyruvate and lactate can also be metabolized by mitochondria utilizing the citric acid cycle (Amaral, 2022).

During the spermatogenesis, round spermatids favor lactate and pyruvate over glucose, the cells switch to using mainly glycolysis during the maturation in the epididymis. Nevertheless, the acrosome reaction requires either lactate or pyruvate to perform oxidative phosphorylation as well as glucose as a substrate for the pentose phosphate pathway to provide the nicotinamide adenine dinucleotide phosphate (NADPH) necessary for the fusion of the gametes (Miki, 2007).

While the motility of the spermatozoa does not require high levels of ATP, it is also linked to the tyrosine phosphorylation necessitated of hyperactivation, a state in which the spermatozoon shows very fast flagellar movement patterns, which helps with the penetration of the zona pellucida (Amaral, 2022; Miki, 2007).

Experiments with knockout mice showed, that mouse spermatozoa stay operational without the ability to perform oxidative phosphorylation, but fail to function without glycolysis (Miki, 2007).

1.2.6 Reactive Oxygen Species and Oxidative Stress

Oxidative stress is caused by an imbalance in production of Reactive Oxygen Species (ROS) and the antioxidant defense mechanisms to remove ROS. ROS is an inevitable consequence of oxygen dependent metabolism and is both a toxic metabolite and advantageous for regulation of crucial cell signaling. In intracellular cascade signaling the normal physiological levels of ROS provide vital signaling to support sperm maturation, hyperactivation, capacitation, acrosome reaction. When the amount of ROS surpasses the ability to be neutralized of the antioxidants or the quantity of antioxidants are weakened oxidative stress occurs. The excess ROS can cause damage to cellular components by lipid peroxidation, protein modifications, or DNA damage (Dutta et al., 2019).

When the oxidative stress caused by ROS affects lipids it severely impacts their function in the organism. This effect is especially detrimental in polyunsaturated fatty acids see Figure 6, which are a part of the phospholipid bilayer of cell membranes, causing them to fail by reducing the fluidity of the membrane which also results in a loss of membrane-bound protein function. Lipid peroxidation is most often caused by $HO^{\bullet}$ and $HO^{\bullet}_2$, which are the two ROS copiously generated under the influence of cell stress (Ayala et al., 2014).

The reaction of lipids with ROS results in various aldehydes, including malondialdehyde (MDA), which is believed to be the most mutagenic end product. Because of MDA's property of reacting with thiobarbituric acid (TBA) producing a brightly colored fluorescent compound, MDA is used as a biomarker for lipid peroxidation. TBA does not selectively react only with MDA, therefore the presence of certain substances can lead to false results (Ayala et al., 2014).

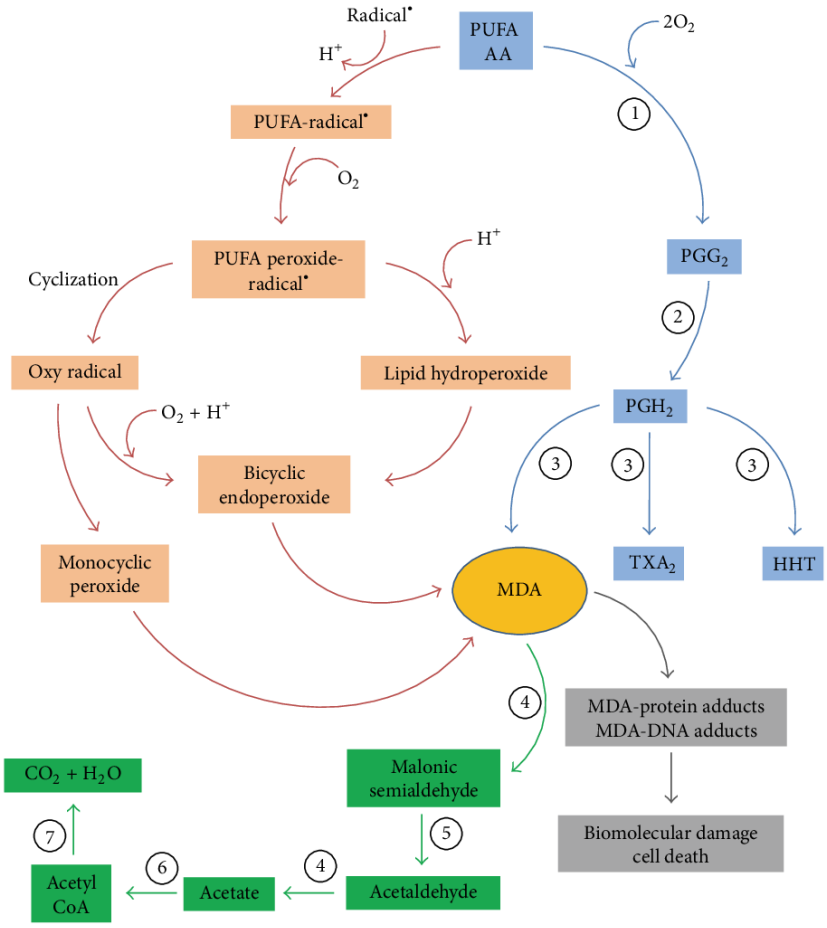

Figure 6: The lipid peroxidation of polyunsaturated fatty leading to the formation of the end product malondialdehyde (MDA) (Ayala et al., 2014).

High levels of oxidative stress have an overwhelmingly negative effect on sperm cell vitality, considering their relatively large volume made up by the vulnerable plasma membrane in comparison to the small cytoplasmic volume and its antioxidative capability. Both the disruption of antioxidative processes as well as an increase of ROS production can be a result of cryopreservation, therefore decreasing the sperm quality, by reducing the ability to fuse with an egg cell and motility through changes in membrane and flagellar proteins. Nevertheless, oxidative stress also regulates the capacitation and maturation in spermatozoa, which are crucial for successful fertilization (Bollwein & Bittner, 2018).

1.3 Cryopreservation of Semen

Artificial insemination presents a row of benefits to cattle farming by removing the necessity for a breeding bull on site with the heifer. Additionally, several reproductive advantages arise with AI, first and foremost the improvement of genetics. AI accounted for 85% of the Norwegian dairy herd in 2014 (Marstein & Sandnes, 2020, p. 7) In the US, 74% of dairy cattle are a result of AI (Dalton et al., 2021). In 2002, already 95% of the semen doses were deep-frozen, using various freezing techniques (Thibier & Wagner, 2002).

The method of cryopreservation began with the discovery of the cryoprotectant capabilities of glycerol (Polge et al., 1949), which was rapidly implemented by the Norwegian Red breeding association leading to the first cryopreserved Norwegian Red semen in 1953 (Geno, 2020b). It was understood that the spermatozoa need to be protected from cold shock damage during cooling, to uphold their ability to successfully fertilize oocytes. Freezing sperm cells without cryoprotectants results in the contained water to freeze into ice crystals, damaging the cells irrevocably, seen in Figure 7 (Ugur et al., 2019).

Possible cryoprotectants are hyperosmotic liquid media, that are not detrimental to the spermatozoa themselves. A often used solution for extending bull semen is tris egg yolk (Yánez-Ortiz et al., 2022), with a stable pH and a high concentration of low-density lipoprotein (LDL) glycerol which can diffuse into the phospholipid bilayer of cells to alleviate the diffusion of electrolytes, and several sources to support the sperm cells like glucose, antibiotics, and antioxidants (Yánez-Ortiz et al., 2022).

The lipid bilayer membrane of the spermatozoa is water permeable under physiologic circumstances. If water freezes to ice in the media surrounding the spermatozoa, it is no longer osmotically active, which is why the high concentration of dissolved particles will draw the liquid, intercellular water to the outside, reducing the likelihood of intercellular ice crystals, while also shrinking the cell (Yánez-Ortiz et al., 2022).

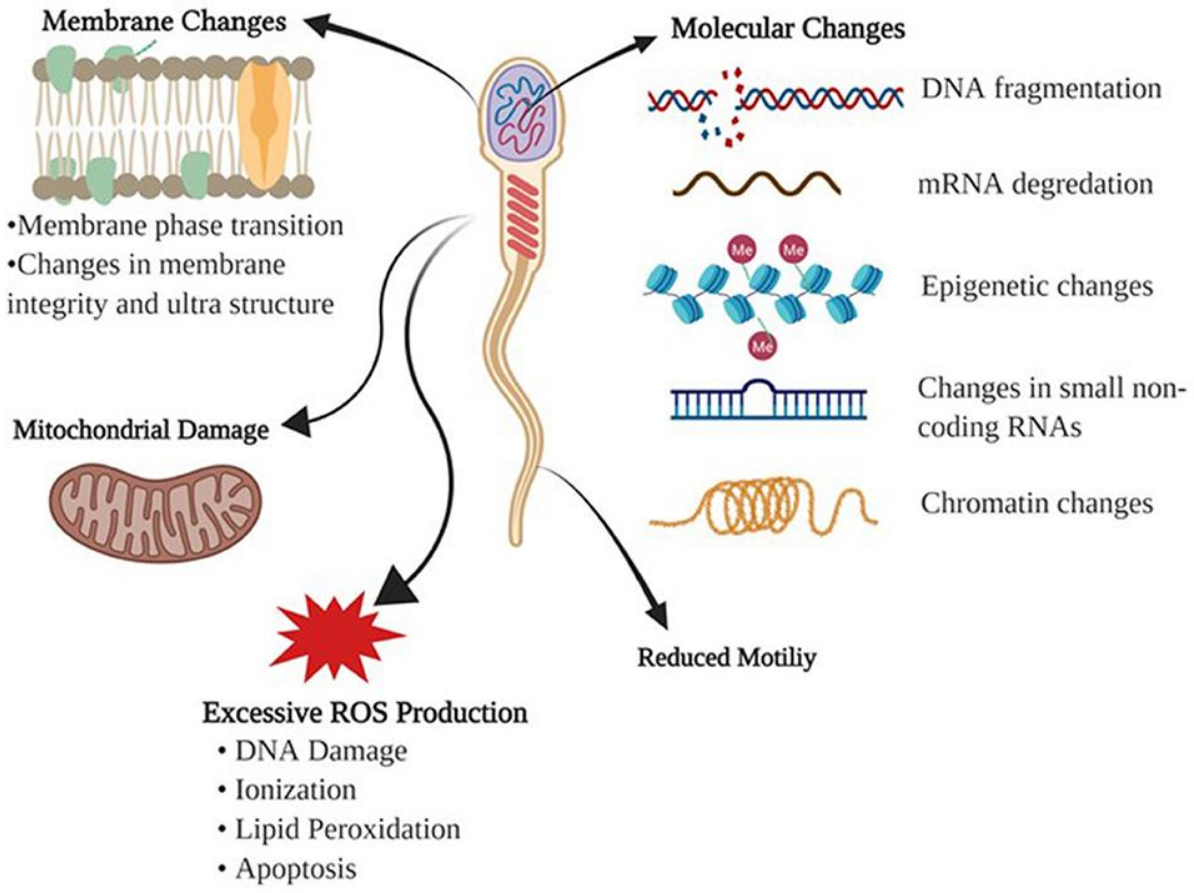

Figure 7: Visualization of physiological damages caused by the freezing and thawing process. Including mitochondrial damage, molecular changes affecting DNA, ROS production that can lead to reduced motility or ultimately apoptosis in spermatozoa. (Ugur et al., 2019)

During thawing, hypotonic media are used to revert the changes done by the cryoprotective, whereby water diffuses through the phospholipid bilayer into the spermatozoa. Although the membrane stays intact by these techniques, the quality of the spermatozoa will degrade in several aspects, including possible DNA and mRNA damages, acrosomal damage, and changes to the membrane and metabolic processes, which can cause a reduction of viable spermatozoa as well as negative changes in the embryonic development (Yánez-Ortiz et al., 2022).

While these effects are likely to occur in all viable sperm doses, there are differences in the extent of the damages found both in between bulls as well as in between straws of the same ejaculate. It is believed that a higher amount of seminal plasma proteins can increase the cryotolerance of spermatozoa. Since the seminal plasma is usually replaced by cryoprotectant media, readding it or replacing part of the egg yolk with bovine serum albumin during thawing can have a positive effect on the sperm quality (Yánez-Ortiz et al., 2022).

1.4 Sex-Sorting of Sperm Cells

The sexing of spermatozoa is now an important tool for farmers and are commercially sold worldwide, the benefit if predestination of the sex enables farmers to rapidly replace daughters for dairy production and then beef farmers could capitalize on the larger size of male offspring (Hohenboken, 1999). The predetermination and manipulation of the sex has been thought of and discussed since the time of the early Greeks when it was stipulated that the left testes were responsible for the male offspring and the right testes were responsible for the female offspring. Due to a lack of understanding in the principle of how sex is determined and finding reason in coincidences not related to the predetermination of sex, has caused several decades of experimenting in a journey to illuminate the matter (Garner & Seidel, 2008).

The first attempts in semen sexing were done in 1962, which proved mostly unviable. The first successful change in the sex ratios happened by using gradient methods including the Percoll gradient to increase the ratio of female offspring and discontinuous dextran density which show increased male offspring (Pinto-Pinho et al., 2023).

The approaches tried to exploit several differences found between X- and Y-chromosome bearing spermatozoa including size, motility velocity, pH differences, mass, electrophoresis, and macromolecules. Because of the minuscule of the differences, it was impossible to use these properties of an individual spermatozoon to accurately predict the sex of the possible offspring. Furthermore, since in vitro methods were only introduced in the 1980s, there existed no reliable way to determine and verify the sexing process in a cheap, time-effective way (Garner & Seidel, 2008).

With these new methods, the first sexing methods in live spermatozoa was published by de USDA Beltsville research group in 1983 and was done using the impermeable fluorochrome 4′-6-diamidino-2-phenylindole (DAPI) (Garner et al., 1983), which necessitated a compromised plasma membrane. Later this was performed using a membrane permeable DNA stain containing bisbenzimidazole known as Hoechst 33342. With the use of a permeable stain, it enables the sorting of live spermatozoa. The sorting of the two different populations was done by using flowcytometry shown in Figure 8. The DNA differences between x and y chromosome bearing spermatozoa are species dependent, and in bovine spermatozoa are there a difference of 3.9%. The differences between breeds can be seen in the height of the

datapoints of histogram, meaning that the height of the x peak and y peak are different depending on the breed of cattle (Garner et al., 1983).

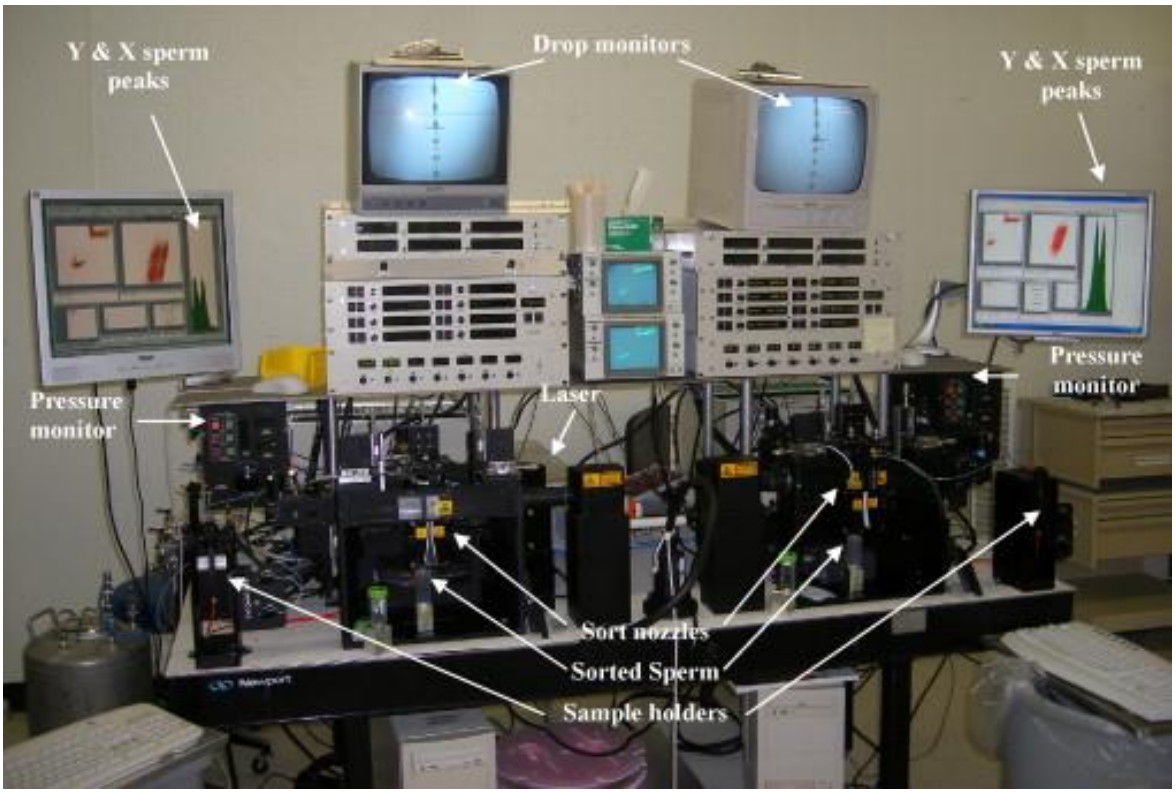

Figure 8: Representation of sex-sorting done with the patented nozzle developed by sexing technologies. To the far right are there two populations detected using Hoechst 33342 showing the x chromosome bearing and y chromosome bearing spermatozoa (Garner & Seidel, 2008).

The separation of x and y bearing spermatozoa are performed with the use of a vibrating crystal creating singular droplets containing a spermatozoon. Using an electrical field, the positive and negative fields was used to sort x for y chromosomes in different containers, while the droplets not containing a spermatozoon and the ones containing multiple spermatozoa passed through without applying any positive or negative field meaning they drop in the middle, see figure 9 (Garner & Seidel, 2008).

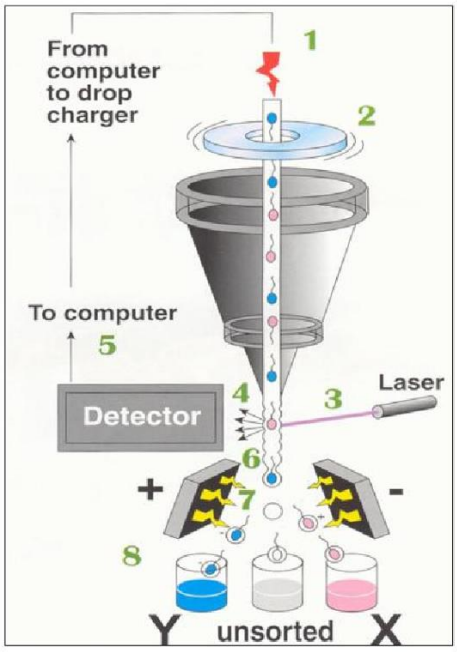

Figure 9: Schematic of the patented nozzle developed by sexing technologies; Vibrating crystal generating droplets containing singular spermatozoa, control of the preset criteria for sorting, excitation laser hit spermatozoa at the hydrodynamic focusing point, followed by conversion of the photo signal into electrical signal (Boro et al., 2016).

The detrimental forces caused by sorting semen are not yet fully known, the use of UV-light to detect the fluorescence of Hoechst 33342 has shown to negatively affect other cell types, meaning that the negative effect on spermatozoa needs further research. High dilution of the semen is also contributing to removing the natural defense found in the seminal plasma, meaning the spermatozoa are more prone to oxidative damage after dilution (Klinc & Rath, 2007). The use of DNA staining in differentiation between x chromosome and y chromosome bearing spermatozoa enables an elimination of spermatozoa containing compromised DNA (Blondin et al., 2009).

1.5 Sperm Quality Evaluation

There is a multitude of sperm parameters that can provide information regarding the sperm quality, some of the possible parameters range from motility, capacitation, oxidative stress, and to the levels of ATP (Lemma, 2012). Motility can be quantified with the use of kinematics parameters measuring the movement of the individual spermatozoa (Amann & Waberski, 2014). Measurement of the kinematics of spermatozoa is performed with computer assisted sperm analysis (CASA), which also provide data regarding the cell concentrations of the samples. Research has shown that there is usually a higher correlation with field fertility results when combining several parameters at once instead of single parameters(Sellem et al., 2015). The CASA allows measurements of numerous kinetic parameters for each spermatozoon, and due to the high number of cells analyzed it provides measurements high repeatability. Even though the CASA is proficient at measuring the motility *in vitro*, it does not necessarily represent the environment *in vivo*. The physiological alternations of the spermatozoa that occur in the female reproductive tract could be better measured using other methods (Waberski et al., 2022). The use of fluorescent probes and immune straining of specific structures within the spermatozoa might provide a more detailed measurement of sperm quality. The use of several methods of measuring sperm quality showed an increase in correlation with fields results (Sellem et al., 2015).

1.5.1 CASA

The idea of tracking spermatozoa originated in the early 1970's, the possibility of using manual tracking of spermatozoa in an attempt to quantify sperm motility, which was done using primitive techniques in microphotography and developed into microcinematography (S. Mortimer et al., 2015). The idea was further developed by David Katz and James Overstreet pioneering the method using rudimentary videomicrography. The work done by Katz group led to the basic principles we have today for characterizing kinetic movement and the use of 50 frames per second (Katz & Overstreet, 1981). While the first versions of CASA had its issues especially related to accurate measurements of human spermatozoa, the limitations were caused by primitive software and hardware and a lack of consensus in how measurements of motility should be conducted. The images had poor resolution and therefore problems with differentiating cellular debris from spermatozoa (S. Mortimer et al., 2015).

The WHO picked up this rapidly developing new method of quantifying sperm motility, adding it to their lab manual for sperm handling and standardizing the three-letter abbreviations and definitions we use today see Figure 10. (S. Mortimer et al., 2015).

The fist CASA were invented using a personal computer with the Cellsoft system in 1985. The use of personal computers enabled automatic individual spermatozoon tracking. The invention exploded the field and soon after gave rise to several to the known suppliers of CASA systems used today, IVOS, SpermVison, and SCA. During the last 30 years computer technology has had extraordinary improvements, enabling the modern-day CASA systems the possibility of accurate measurements of concentration, motility, viability, fragmentation, acrosome reacted, and morphology measurements (Van Der Horst, 2020).

CASA uses microscopic digital videos of sperm and processes the data by counting clusters of pixels to detect spermatozoa, a numeric value that is adjusted to the spermatozoa of the species at hand. After detecting the visible cells, an intricate path-finding algorithm is applied to track the movement from frame to frame (Kathiravan et al., 2011).

Several kinematic parameters can be measured using CASA, including total (MOT), progressive (PROG) motility, the curvilinear velocity (VCL), velocity of the average path (VAP), straight line velocity (VSL), and the amplitude of lateral head displacement (ALH) as seen in Figure 10.

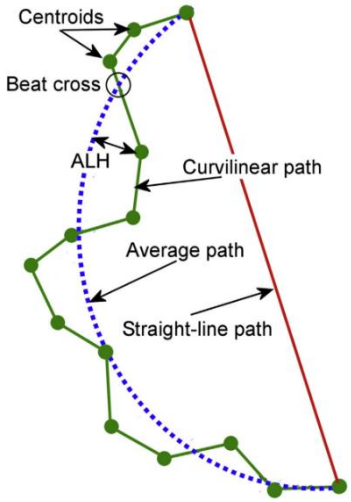

Figure 10: Kinematics measured by Computer Assisted Sperm Analysis. Centroids are the location of the head when each frame is taken, and the trajectory of the centroids make up the curvilinear line. The straight-line path is the linear line between the starting point and the end point, average path is the average of the curvilinear line, Amplitude of Lateral Head Displacement (ALH) the distance from the average path to the centroid, Beat Cross Frequency the frequency the head crosses the average path (Křížková et al., 2017).

1.5.2 Flowcytometry

The use of flowcytometry has become valuable method for measurement of sperm quality parameters such as viability, acrosomal status, evaluation of capitation, mitochondrial activity and changes associated with apoptosis (Martínez-Pastor et al., 2010).

The flowcytometry was invented by Dr Daniel Pinkel while working at the Lawrence Livermore National Laboratory. He was attempting to understand the effect of radiation on human DNA, they used spermatozoa from mice as a model, but due to the irregular shape of the mouse spermatozoon they could not measure the DNA damage accurately, the spermatozoon head shape of mice is represented in Figure 11. Until the creation of the flowcytometry where the instrument was able to reorientate the spermatozoa enabling accurate measurements of DNA (Garner & Seidel, 2008).

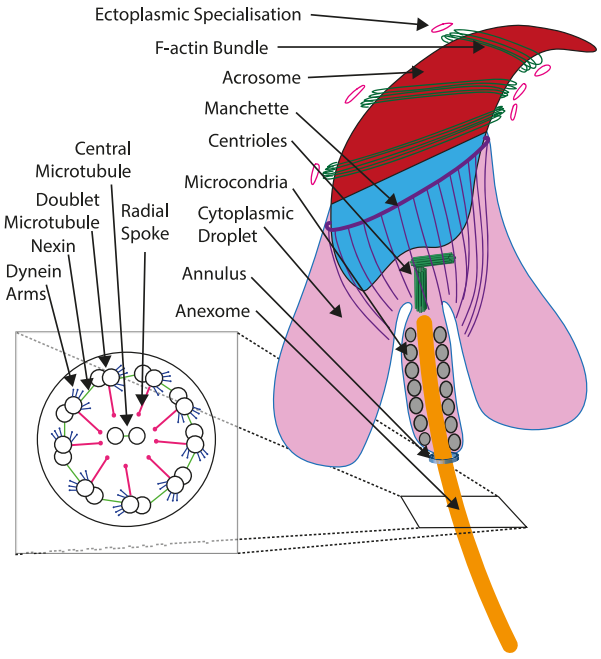

Figure 11: Representation of subcellular components and the shape the male spermatozoon of mice. (https://commons.wikimedia.org/wiki/File:Spermatozoa.svg)

The technology enables highly rapid and accurate measurements of single cells, where the cells pass through light sources and detectors translate the differences in light into electrical currents. The conversion of light into electrical signals are done by photodiodes or

photomultiplier tubes. The signals detected are either from scatter or fluorescent dyes (McKinnon, 2018).

Fluorescent dyes have a wide range of applications where they bind to for example protein, DNA, antibodies, membranes, or they function as an ion indicator. Florescent small molecules are often used to be conjugated with antibodies. Because of their variety and usefulness, they are used frequently in research. The flowcytometry method has been used in a multitude of biotechnological fields (McKinnon, 2018).

The principle of flowcytometry are fluidics, optics, and electronics. The fluidics system consists of the sheath fluid and the cells in a liquid (McKinnon, 2018). Another integral part of the fluids system is hydrodynamic focusing. Hydrodynamic focusing is accomplished when the microfluidics create laminar flow by differences in velocity for the sheath fluid and the sample fluid (Yang & Hsieh, 2007). This achieves a steady flow of singular cells passing the light source which is necessary for accurate and rapid detection.

The optics of a flow cytometer can be divided into two sections: one responsible for the excitation of fluorochromes and providing visible light, while the second part of the optics system revolves around directing the light into the detectors with the desired wavelength. The light is split with the use of dichroic lenses and mirrors, and the light gets focused by shaping lenses. To ensure the correct wavelength of fluorescent light, the light passes through bandpass filters which filter only the desired wavelength required for specific detectors (McKinnon, 2018).

The detectors are either photomultiplier tubes (PMT's) which are usually responsible for detecting the weaker signals like fluorescence and Side Scatter (SSC), or photodiodes which usually are responsible for detecting stronger light signals like Forward Scatter (FSC). The resulting analog signal from the detector is then transformed into electrical signals. The electrical signals generated form the electronic system is then ready to be analyzed by a computer (Adan et al., 2017).

Viability is most commonly detected with the membrane impermeable probe propidium iodine (PI) and ethidium homodimer (EH), in combination with a stain that is permeable to be able to detect a compromised plasma membrane. To detect acrosomal status can lectins bind to glucosidic residues within the acrosomal membrane such as *Arachis hypogaea* (peanut) agglutinin (PNA) conjugated with fluorochromes (Martínez-Pastor et al., 2010).

1.6 Aim of study

Sex-Sorting of semen is increasing as a technology used in breeding of cattle. However, the sorting process can be harmful for spermatozoa. To potentially increase profitability for both breeding companies and farmers, it is important to have tools to validate sperm quality in relation to fertility. In a production setting, it is limited for advanced analysis. This implies that a new test must be effective in terms of both time and its ability to verify sperm quality, while also overcoming the cost of new equipment and training of laboratory personnel.

The main objective of this master thesis is to get more knowledge of sperm quality for sex-sorted spermatozoa from Norwegian Red bulls through characterizing semen samples by establishing a stress test and a new analytical tool. This will be achieved through the following subgoals.

1. Establish a motility stress test for sex-sorted spermatozoa.
2. Study the motility over time for sex-sorted sperm with different fertility potential.
3. Establish a new analytical tool for studies of oxidative stress.
4. Examine viability, ATP content and level of oxidative stress in sex-sorted sperm cells.

2. Methods

2.1 Media and Chemicals

CellTiter-Glo® used for ATP assessment was provided by Promega (Promega, G7572, Madison, WI, USA), adenosine 5′-triphosphate disodium salt hydrate used for standard curves were provided by Sigma Aldrich (Sigma Aldric, A7699-1G). Lipid peroxidation (MDA) assay kit were provided by Sigma Aldrich (MAK085, Sigma Aldrich), the kit contained MDA Lysis Buffer (MAK085A), Phosphotungstic Acid Solution (MAK085B), BHT 100x (MAK085C), Thiobarbituric acid (TBA) (MAK085D), MDA Standard, 4.17 M (MAK085E). For measurement of acrosome integrity and viability by flowcytometry SYTO® 60 was provided by Invitrogen (Syto 60, S11342, Invitrogen), Propidium Iodine was provided by Sigma Aldrich (PI, P4864, Sigma-Aldrich), Peanut agglutin (PNA) conjugated with Alexa Flour® 488 was provided by Invitrogen (PNA-Alexa 488, L21409, Invitrogen).

2.2 Animal Material

AI doses were provided by Geno SA (Geno Breeding and AI Association, Hamar, Norway) production facility Store Ree Stange. Animals were kept according to the Animal Welfare Act (LOV 2009-06-19 nr 97: Lov om dyrevelferd).

For conventional AI doses, standard operating procedure consists of pooling two ejaculates collected within 15 minutes using an artificial vagina, passing ejaculates must have a measurement of motility above 70%, and morphology with less than 15% abnormal spermatozoa. The passing ejaculated was then extended using the two-step diluter Biladyl® (13500/0004- 0006; Minitube GmbH, Tiefenbach, Germany). Afterwards, it was cryopreserved and stored in liquid nitrogen at -196°C (Alm-Kristiansen et al., 2017).

Sexed AI doses are sold containing a minimum of 1.24 million progressive spermatozoa, with a 90% purity of x-bearing spermatozoa. Cryopreserved and stored in liquid nitrogen at -196°C.

Fertility data used for the High and Low fertility bulls were collected from the Norwegian Dairy Herd Recording System (NDHRS) and the national AI recording system database

(Semindatabasen) in June 2023. The fertility was determined using non-return rate 56 days, meaning that there was not register another insemination within 56 days, thus assuming pregnancy. The three high fertility bulls, bull nr 3, 4, and 6 have a NR56 of 70.5%, 66.9% and 70.7%, while low fertility bulls were bull nr 1, 2, and 5 with a NR56 of 59.5%, 59.8% and 59.8% respectively see table 1.

Table 1: Fertility field data represented as mean non-return rate 56 days (NR56), number of inseminations in the field, for high (3,4, and 7) and low fertility Norwegian Red bulls (1,2, and 5), the data was acquired from May 2023.

Bull Nr	NR56 (%)	Inseminations
1	59,5	424
2	59,8	380
3	70,5	1414
4	66,9	237
5	59,8	519
6	70,7	293

2.3 Experimental Design

The experimental design for the master thesis is shown in Figure 12. First, to elucidate the parallel variation, CASA experiments to validate parallel variation with ordinary preserved semen were performed. Thereafter, all experiment was done using sexed semen. The preliminary test of incubation intervals was preformed using two bulls, analyzed by CASA after incubation at 37 °C – for ten different timepoints where the number represent minutes T0, T5, T10, T20, T30, T45, T60, T90, T120, and T150.

From this experiment six different incubation intervals were selected T0, T10, T30, T60, T120, and T180. The motility stress test was performed with sexed semen from seven Norwegian Red bulls. The first group consists of seven bulls, whereby one of the bulls (denoted 7.1 and 7.2) provided two ejaculates.

Due to interesting motility results at 120 minutes we decided to extend the testing and measure additional sperm quality parameters, including ATP content by plate reader, the percentage of acrosome intact live (AIL) sperm cells by flowcytometry and measurement of the biomarker malondialdehyde (MDA) from a pathway initiated by oxidative stress with plate reader. For all experiments, two straws were thawed at 37 °C for 1 minute and pooled to reduce straw to straw variation.

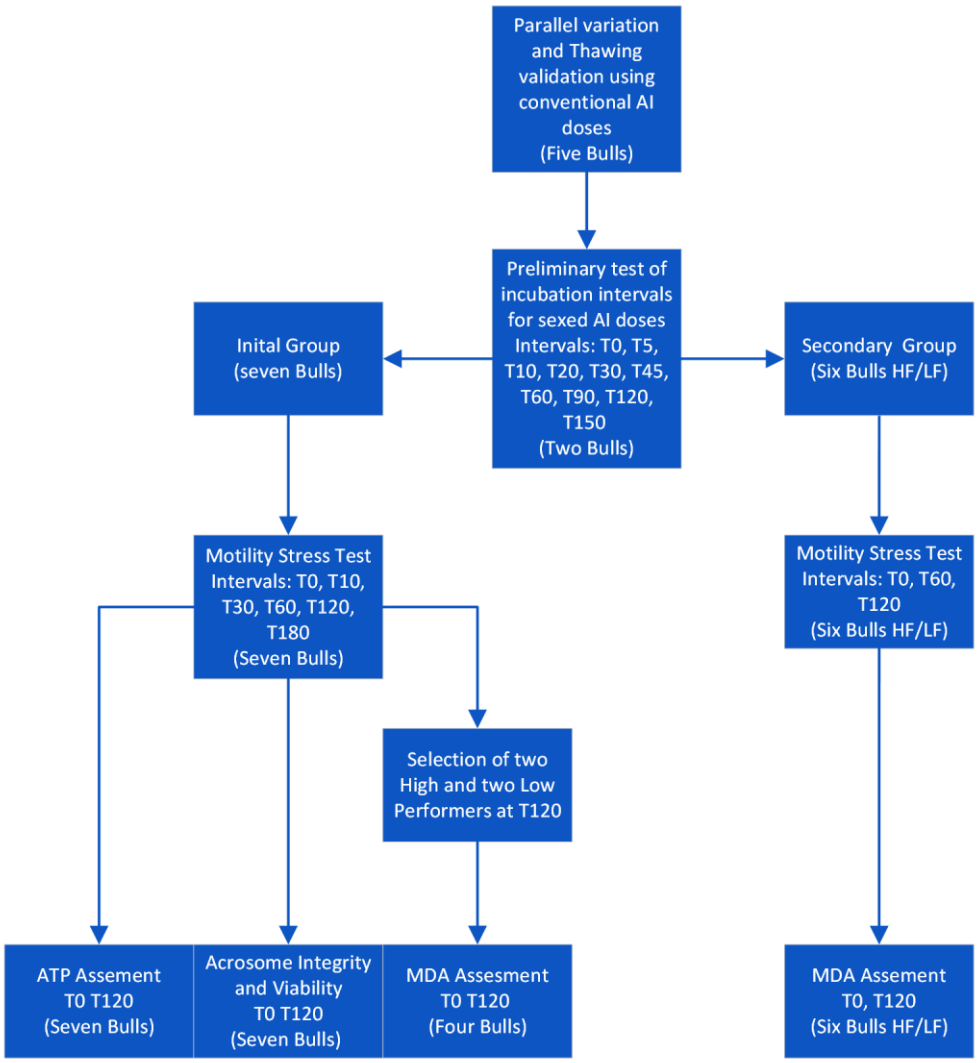

Figure 12: Flowchart visualizing the order of experiments conducted during this master's thesis, The initial group consisting of seven bulls were tested for Adenosine triphosphate (ATP), Acrosome intact live (AIL) and the two high and two low performers was tested for Malondialdehyde (MDA). The secondary group consists of six bulls with known fertility, three high fertility (HF) and low fertility (LF).

ATP and AIL were measured for seven bulls at T0 and T120. Then there was a selection of two high and two low performers at T120 from the motility stress test meaning that a total of four bulls from the initial seven bulls were tested for MDA.

The second group consisted of six bulls whereby three were considered low fertility and three bulls were considered high fertility. The motility stress test was performed for sex semen samples from six bulls with known field fertility data at three time points T0, T60, and T120 incubated at 37°C. Finally, MDA was assessed of the six bulls with known field fertility.

2.4 Measurment of motility by Computer Assisted Sperm Analyzer (CASA)

Motility and sperm kinetic parameters were measured using Computer Assisted Sperm Analyzer (CASA) instrumentation (Sperm Class Analyzer® version 6.6, Microptic SL, Barcelona, Spain). Images were captured using Basler acA780-75ge digital camera (Basler AG, Germany). Samples were incubated for 10 min at 38 °C before being diluted 1:3 in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4). Then 3 µl of the sample was loaded into Leja® 4 chamber slides (Nieuw-Vennep, The Netherlands), 20 microns chamber depth. The samples were left in the chamber for 1 min prior to assessment due to capillary forces and avoidance of drifting. Eight fields with a minimum of 500 cells total were captured per parallel. There was taken 30 frames for each field captured with a speed of 50 frames/s. Two replicates were performed for each sample and time point.

Spermatozoa were identified with area of head size set to 20-80 μm^2. Kinetic parameters measured: Velocity curved line (VCL, µm/s), velocity straight line (VSL, µm/s), velocity average path (VAP, µm/s), beat cross frequency (BCF, Hz) and amplitude of lateral head displacement (ALH, µm). Sperm cells were considered total motility (MOT) if VCL>15 µm/s, progressive motility was defined by STR>70. Linearity was defined by the ratio of (VSL/VAP) × 100 (LIN, %), Straightness was defined by the ratio of (VSL/VCL) × 100 (STR, %), Wobble was defined by the ratio of (VAP/VCL) × 100 (WOB, %).

2.5 Assessment of post-thaw ATP content

The amount of ATP was measured using CellTiter-Glo® luminescent cell viability assay (Promega, G7572, Madison, WI, USA). The conversion of luciferin into oxyluciferin was done by recombinant luciferase. The luciferase is unable to convert luciferin without the presence of ATP. The product oxyluciferin emit light which enable the measurement of luminance. Straws were thawed at 37 °C for 1 minute, then diluted with pre-heated PBS to 38 °C to achieve a concentration of 5×10^6 cells/ml. Equal parts of sample and CellTiter-Glo® reagent was added to each well. There was used a flat bottom opaque-walled 96 well microtiter plate (NUNC™, Roskilde, Denmark). Contents were mixed for 2 minutes using an orbital shaker set to 300 rpm (IKA® MS 3 digital, IKA®-Werke GmbH & Co, Staufen, Germany). Thereafter

incubated for 15 minutes in room temperature to stabilize luminescence signal. The luminescence signal was detected using FLUOstar® OPTIMA (BMG labtech GmbH, Offenburg, Germany). Raw data was analyzed using MARS data analysis software (Version 1.10, BMG labtech, Offenberg, Germany). Standard was made using adenosine 5′-triphosphate disodium salt hydrate (Sigma Aldric, A7699-1G) was used. ATP in each sample was calculated using the standard curve and the relative luminescence unit (RLU). The standard curve was accepted if r^2>0,99. ATP level per sample is given as µM per 10^6 sperm cells.

2.6 Assessment of oxidative stress by measurement of MDA level

The amount of MDA was measured using lipid peroxidation (MDA) assay kit (MAK085, Sigma Aldrich). The thiobarbituric acid and MDA forms a pink adduct which can be detected both fluorometric and colorimetric. The sperm cell samples were thawed for 1 minute in 37 °C. Two million spermatozoa per sample were washed twice with 1 ml PBS. The lysis buffer was made using 300 µl of MDA lysis buffer, 100 µl TCEP 100 mM and 4 µl 100xBHT. 300 µl the newly made lysis buffer was added per sample. The final concentration of TCEP 24,75 mM. Sonication was used during the lysis step to ensure proper lysis, and lysis was confirmed by visual inspection of the cells using a Nikon (Nikon, Japan) light microscope with 10x objective. After successful lysis the debris and insoluble particles were removed using centrifugation at 13 000 rpm. The standard curve was made measuring 20 nmole/well, 8 nmole/well, 4 nmole/well, 2 nmole/well, 0.8 nmole /well and 0.4 nmole/well. For each sample, 100 µl supernatant was mixed with 300 µl of Thiobarbituric acid (TBA) dissolved in glacial acetic acid and incubated at 95°C for 60 minutes. If turbidity occurred, the samples was filtered using 0.22-micron syringe filter. The samples were then measured colorimetrically with a clear 96 well plate (Nunc™, Roskilde, Denmark) with a plate reader (GloMax Discorvey®, Promega), at 490nm. The Promega software we used was version 3.1.0. The results are given as nmole MDA per sample.

2.7 Acrosome integrity and viability by flowcytometry

The Acrosome intact live (AIL) cells was determined using flowcytometry (Cytoflex, Beckman Coulter, California, USA) and raw data was analyzed using the Beckman Coulter CytExpert software. The sexed samples were diluted to a concentration of 1 million cells/ml. Differentiation between cells and debris was done using the red fluorescent nucleic acid stain SYTO® 60 (Syto 60, S11342, Invitrogen), which was excited by a 633nm laser, and fluorescence detected with a bandpass filter with a range of 675/25nm. The impermeable fluorochrome Propidium Iodine (PI, P4864, Sigma-Aldrich) was used to measure viability by detecting plasma membrane integrity. Peanut agglutin (PNA) conjugated with Alexa Flour® 488 (PNA-Alexa 488, L21409, Invitrogen) binds to glycoproteins present in cells where acrosome activation has occurred. Both PI and PNA-Alexa 488 was excited with the use of a laser at 488 nm, PI was detected using a 670/ nm band pass filter while PNA-Alexa 488 was detected using a band pass filter at 533/30 nm. The samples were measured at 0 minutes and 120 minutes. Validation beads were used daily. There were two replicates per sample and 10000 events were recorded.

2.8 Statistical Methods

When determining significance, the parametric paired T-test were used when analyzing dependent samples in this case where the same sample from the same bull has been run twice and the only difference was the incubation time. For independent samples meaning different bulls and different incubation times a two sampled t-test was used. And variance for each matrix was confirmed to be equal beforehand, if the variance was not confirmed a two-sample t test for unequal variance was used. If the data did not conform to the assumption of normality required for parametric statistical analysis, then the data underwent log transformation. If log transformation still could not provide a dataset with normal distribution the non-parametric Mann-Whitney test was used. Elucidation of parallel variation was confirmed using one way ANOVA testing. Statistics were done in Excel using the data analysis tool. Pearson correlation analysis was done using R-studio.

3. Results

3.1 Parallel Variation and Thawing Procedure Using Conventional AI Doses

First of all, we wanted to elucidate the variance between parallels and straws for bull semen samples. Sex-sorted semen doses are quite expensive, and for this experiment we therefore used conventionally cryopreserved semen in Biladyl extender See Figure 13.

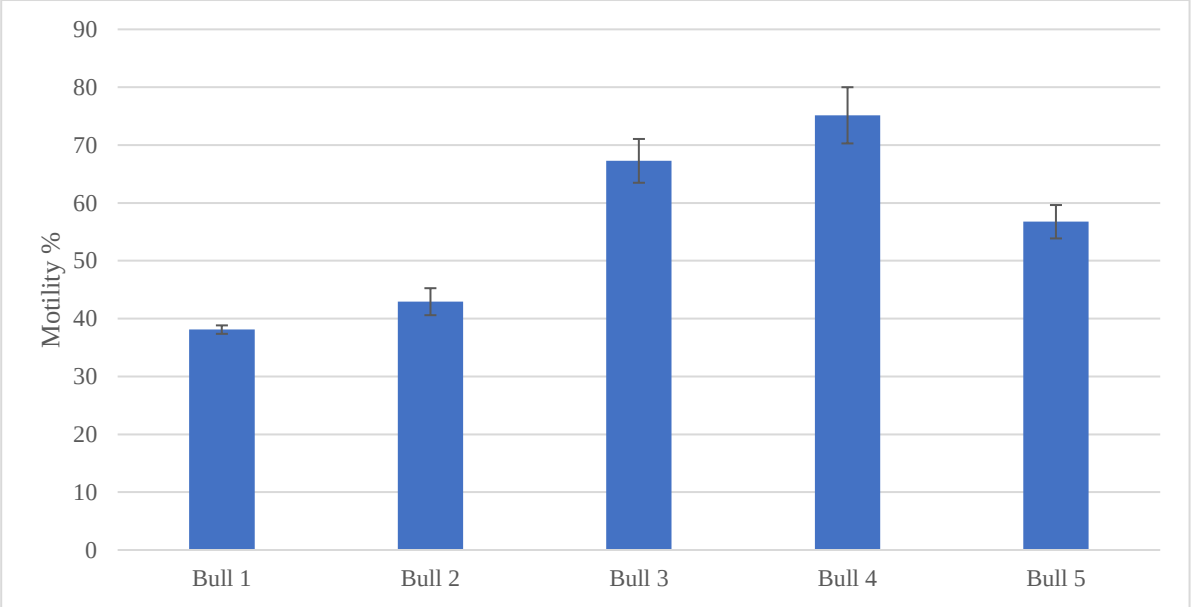

Figure 13: Post-thaw computer assisted sperm analysis measurements of total motility % (SD) in Norwegian Red sperm samples extended with Biladyl incubated at physiological temperatures. Two straws were thawed and then combined for each bull.

There was not found any significant difference between replicate 1,2 and 3 (P>0,05), therefore we could elucidate parallel variation and experiments from now on continued using Norwegian Red sexed samples. Furthermore, to reduce the straw-to-straw variation two straws were thawed and combined before each analysis.

3.2 Preliminary Motility Stress Test For Sex-Sorted Spermatozoa

The changes in the motility over-time are not very well known for sex-sorted spermatozoa, and therefore needed be determined with a preliminary study with semen from two bulls analyzed at several time points. As shown in Figure 14, there were relative minor changes in motility in the time period 0 minutes to 90 minutes, however, the motility declined significantly between T60 and T120 ($P<0,05$). The last time point still showed some progressive cells, so in the final experimental set up the last measurement was after 180 minutes (T180).

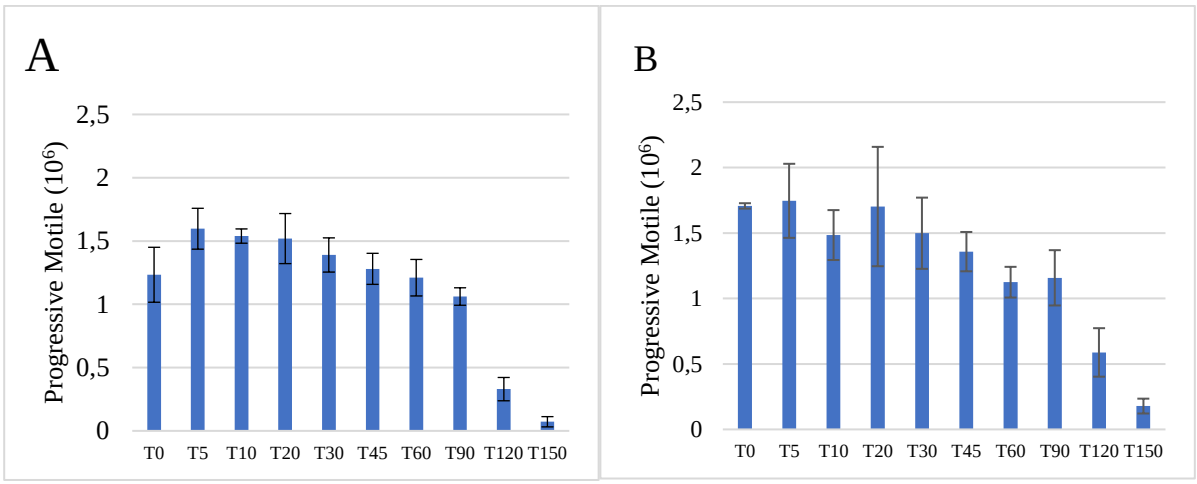

Figure 14: Preliminary post-thaw computer assisted sperm analysis, measurements of progressive motility in sexed bovine sperm samples after incubation at physiological temperature. Two straws were thawed and then combined for each bull before analysis. Two Norwegian Red bulls were tested represented by A and B. Results are shown as mean million progressive motile cells (SD).

3.3 Motility Stress Test of Sexed-Sorted Spermatozoa

Therefore, we next wanted to examine the motility of sex-sorted sperm cells from several bulls and performed a stress test as above for eight different ejaculates (from seven different bulls) at 0 min after thawing, 10 minutes, 30 minutes, 60 minutes, 120 minutes, and 180 minutes at 38°C. The results are shown in Figure 15-17. Total motility (MOT) decreased over time, and mostly between the 60- and 120-minute time points. The motility stress test showed that bull 3 had initially a higher number of motile sperm cells compared to the rest of the semen samples, yet at 180 minutes bull 2 showed to retain the motile sperm better. There was not a significant difference between T0 and T10, T30 and T60 ($P>0,05$), however, from T0 to T120 and T180 there was found a significant decrease in total motility ($P<0,05$) and from T60 to both T120 and T180 ($P<0,001$).

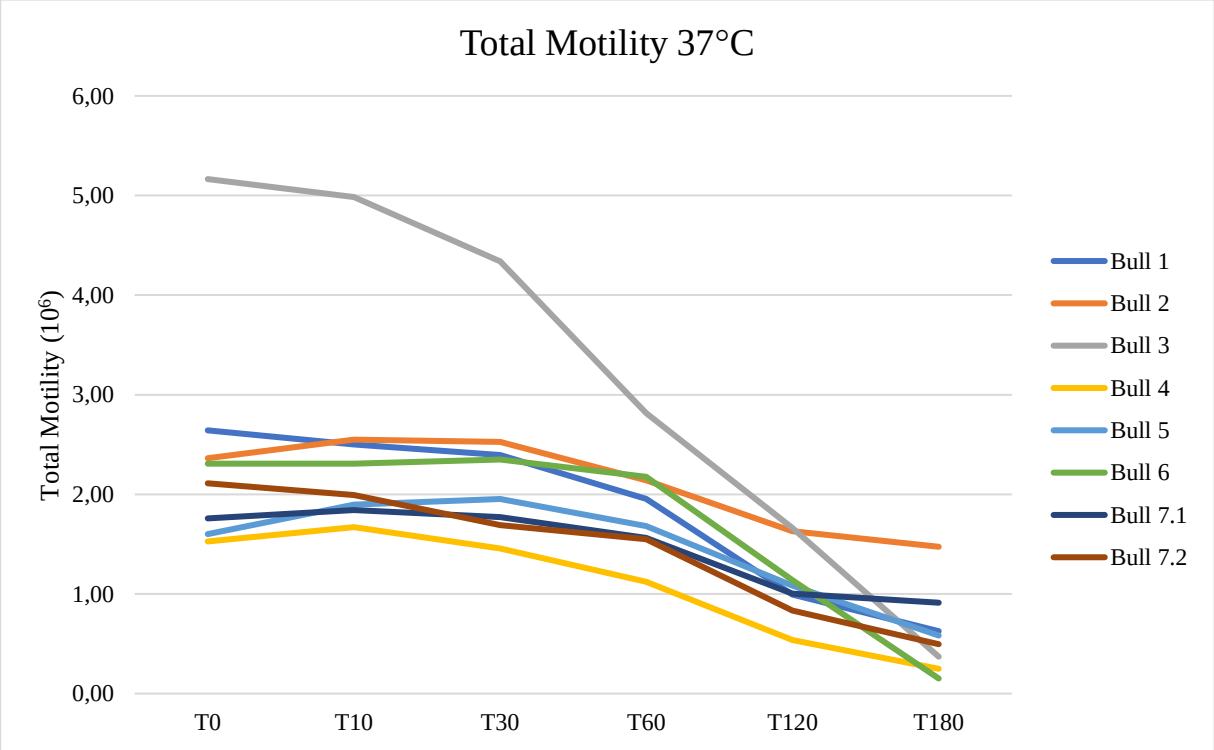

Figure 15: Computer assisted sperm analysis measurements of post-thaw total motility in sexed Norwegian Red sperm samples. Samples were incubated for T0 (0 minutes), T10 (10 minutes), T30 (30 minutes), T60 (60 minutes), T120 (120 minutes), and T180 (180 minutes) at physiological temperature. Two straws per bull were thawed and pooled before analysis.

Prominent differences for all bulls in progressive motility were observed from T60 to T120 (see Figure 16), with the exception of bull 3 where the greatest difference was from T10 to T60. As for total motility, there was not a significant difference in progressive motility between T0 and T10, T30 and T60 (P>0,05), but between T0 and T120/T180 and T60 and T120/T180 were there significant differences (P<0,001).

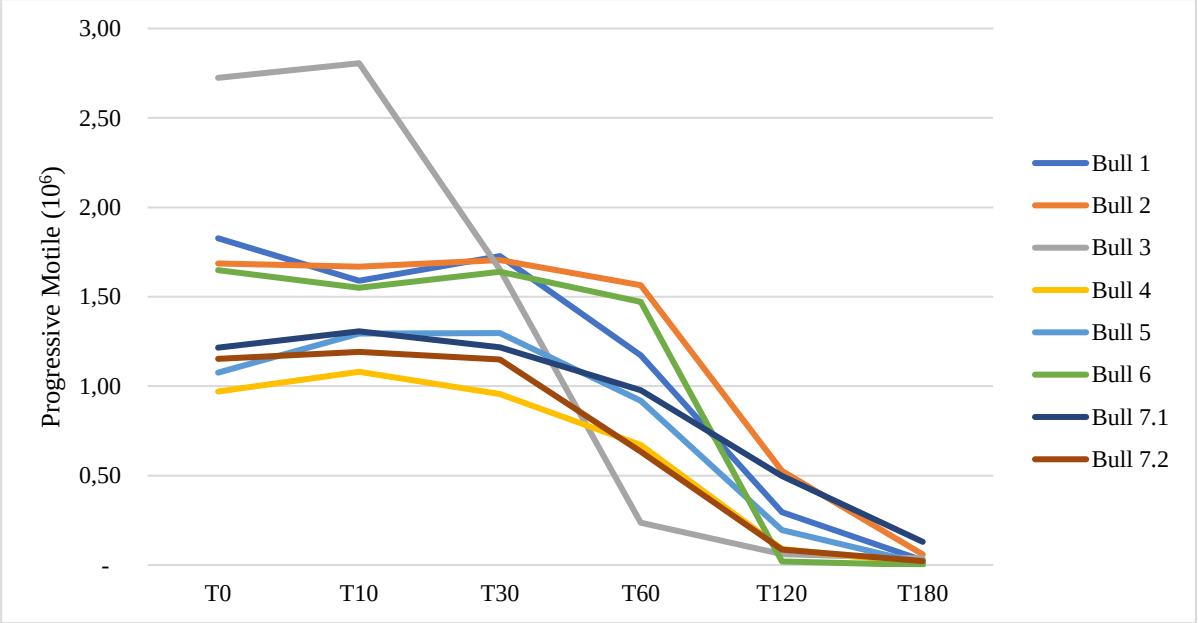

Figure 16: Computer assisted sperm analysis measurements of post-thaw progressive motility in sexed Norwegian Red sperm samples. Samples were incubated for T0 (0 minutes), T10 (10 minutes), T30 (30 minutes), T60 (60 minutes), T120 (120 minutes), and T180 (180 minutes) at physiological temperature. Two straws per bull were thawed and then combined.

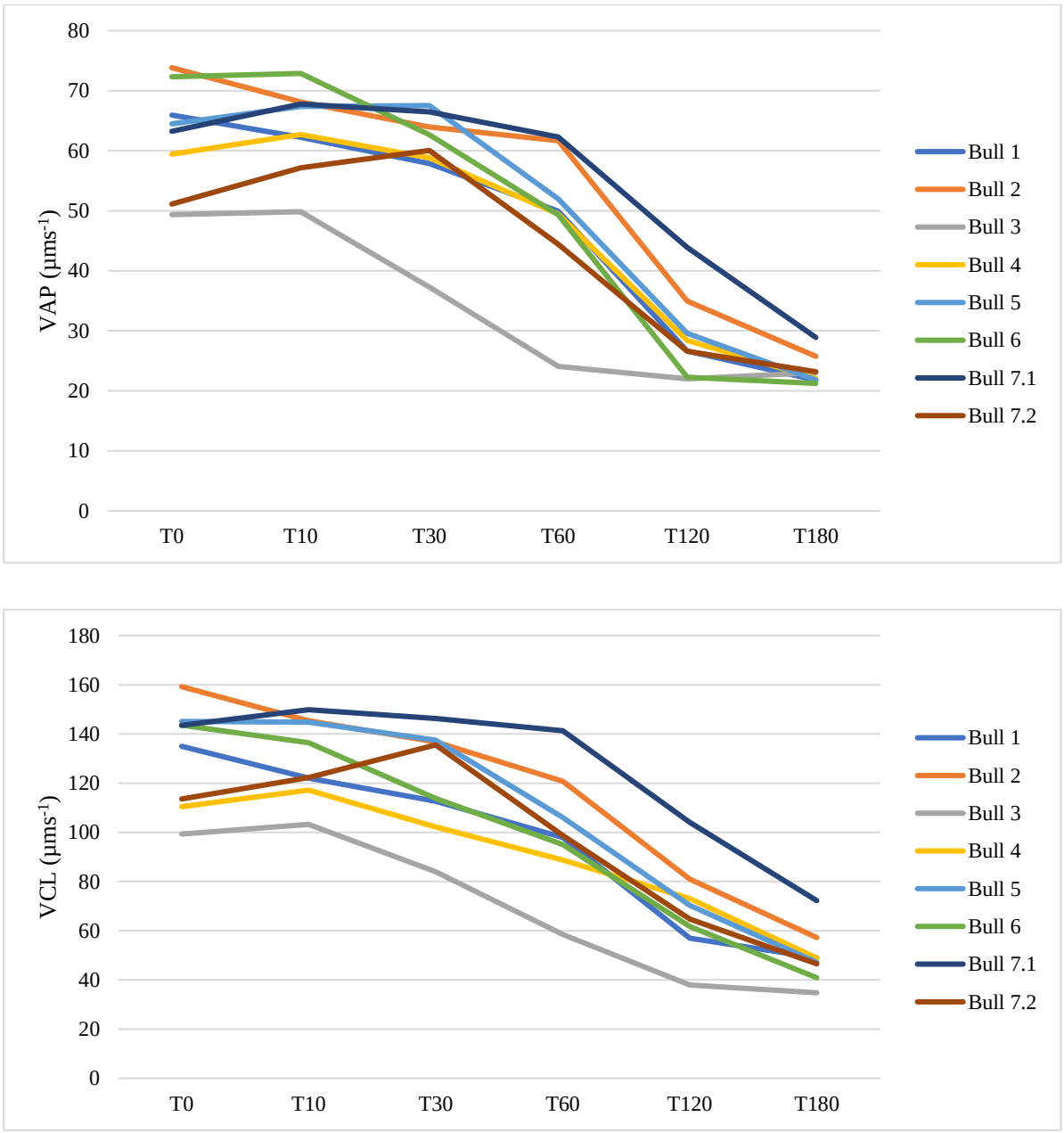

Figure 17: Computer assisted sperm analysis measurements of post-thaw velocity along the average path (VAP) and velocity along the curvilinear line (VCL), in sexed Norwegian Red sperm samples. Samples were incubated for T0 (0 minutes), T10 (10 minutes), T30 (30 minutes), T60 (60 minutes), T120 (120 minutes), and T180 (180 minutes) at physiological temperature. Two straws per bull were thawed and then combined.

3.4 Motility Stress Test of High and Low Fertility Bulls

The results from the thermal stress test on the high and low fertility groups consisting of three high fertility and three low fertility bulls, showed that the low fertility group had a higher total motility at T0 compared to the high fertility group, yet the high fertility group had a higher T120 then the low fertility group, as shown in Figure 18.

There was found a significant decrease in total motility within the low fertility group between T0 and T120 ($P<0,05$), however within the high fertility group and between T0 and T120 there was not found a statistically significant difference ($P>0,05$).

There was not found a significant difference in progressive motility between the high and low fertility groups at T0 and T120 ($P>0,05$). But within the high fertility group there was found a significant difference between T0 and T120 ($P<0.05$), likewise for the low fertility group ($P<0.01$).

The velocity parameters VAP and VCL for the high fertility groups was higher at T0 and T120 compared to the low fertility group. There was found a significant difference between T0 and T120 within high fertility bulls, likewise within the low fertility group ($P<0.05$). There was not found a significant difference between the high and low groups at T0, and at T120 ($P>0.05$).

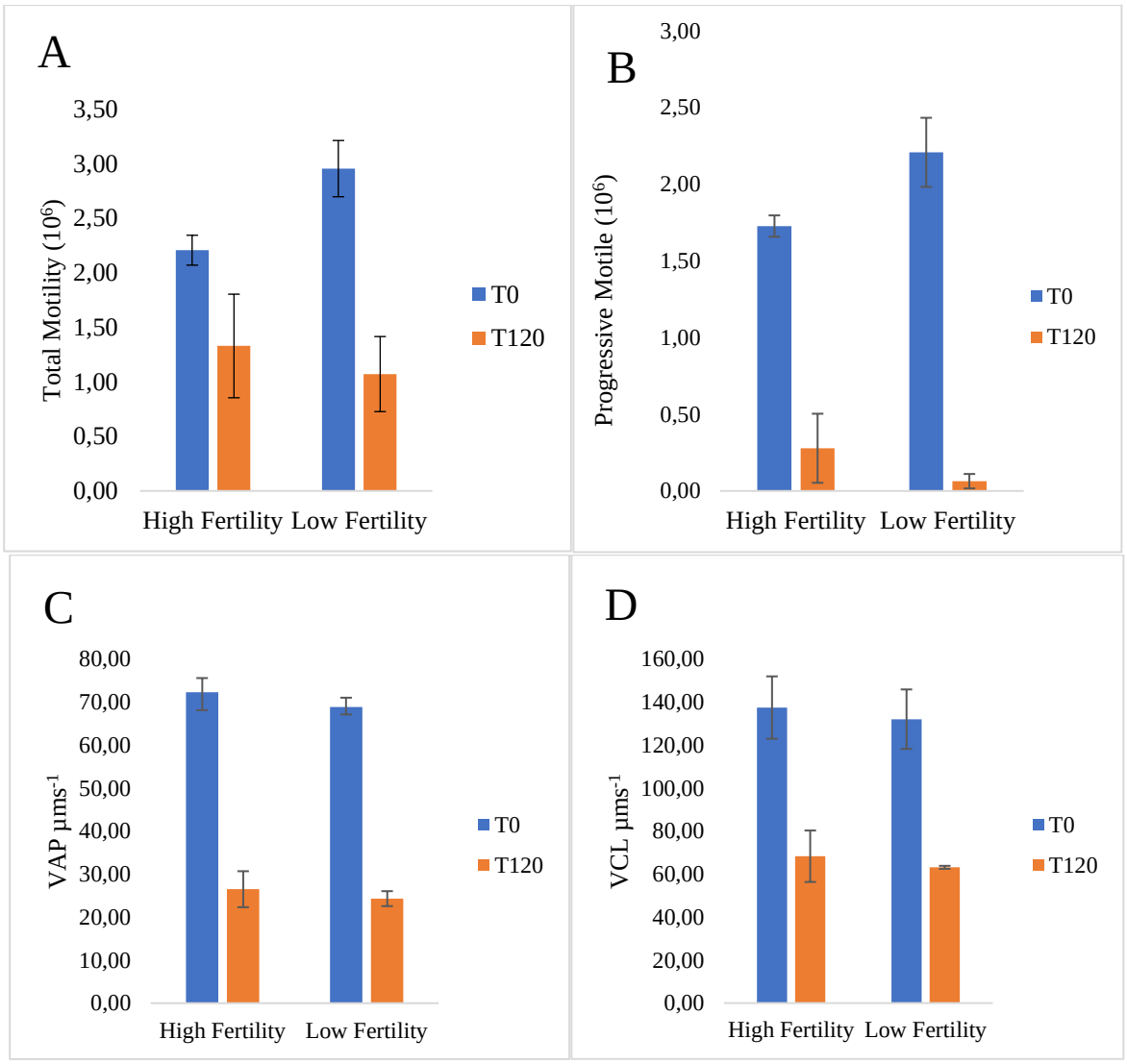

Figure 18: Post-thaw measurements of mean total motility (A), progressive motility (B), velocity along the average path (VAP) (C), and velocity along the curvilinear line (VCL) (D) measured with computer assisted sperm analysis. The experiment was performed with sex-sorted spermatozoa from three high fertility Norwegian Red bulls and three low fertility Norwegian Red bulls. Two straws for each bull were thawed and then pooled for each bull. Results are shown as mean total motility (SEM), progressive motility (SEM), Velocity average path (SEM), and velocity curvilinear line (SEM). Samples was measured after incubation at physiological temperature for T0 (0 minutes) and T120 (120 minutes)

When looking at bull to bull differences it showed that bull number 5 starts with the highest number of progressive cells at 10 minutes post-thaw. Notably bull nr 3 performed very well considering the retention of motile cells were 89% between T0 and T60 post-thaw. In comparison bull nr 1 had a retention of only 46% in progressive motile cells between the 10 and 60 minutes. This difference becomes even more apparent comparing the retention of

progressive cells between T60 and T120, whereby bull nr 3 had a retention of 48%, while bull nr 2 and bull nr 4 had no-measurable progressive cells retained as seen in Figure 19.

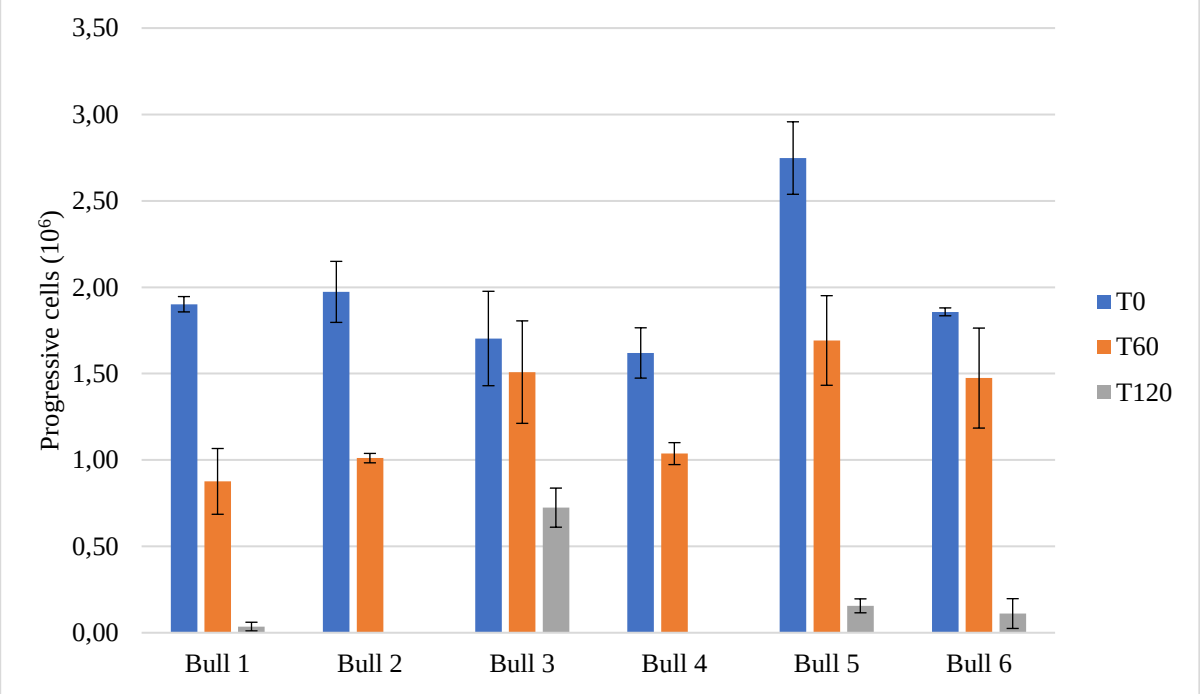

Figure 19: Computer assisted sperm analysis measurements of post-thaw progressive motility in sexed Norwegian Red sperm samples. Samples were incubated for T0 (0 minutes), T60 (60 minutes), and T120 (120 minutes) at physiological temperature. Two straws per bull were thawed and then combined. Results are shown as million progressive spermatozoa (SD).

3.5 ATP Assessment of Sex-Sorted Spermatozoa Over Time

Since the motility decreases over time, we wanted to examine the energy potential of the sperm cells by measuring the ATP level post-thaw and after incubation at physiological temperature for 120 minutes. As shown in Figure 20 there was an overall decrease in ATP concentration from T0 to T120.

($P<0,001$). The mean value of samples measured at T0 was 0.90 µM ATP per million with a variance of 0.010, while the mean value for samples measured at T120 was 0.16 µM ATP per million with a variance of 0.011.

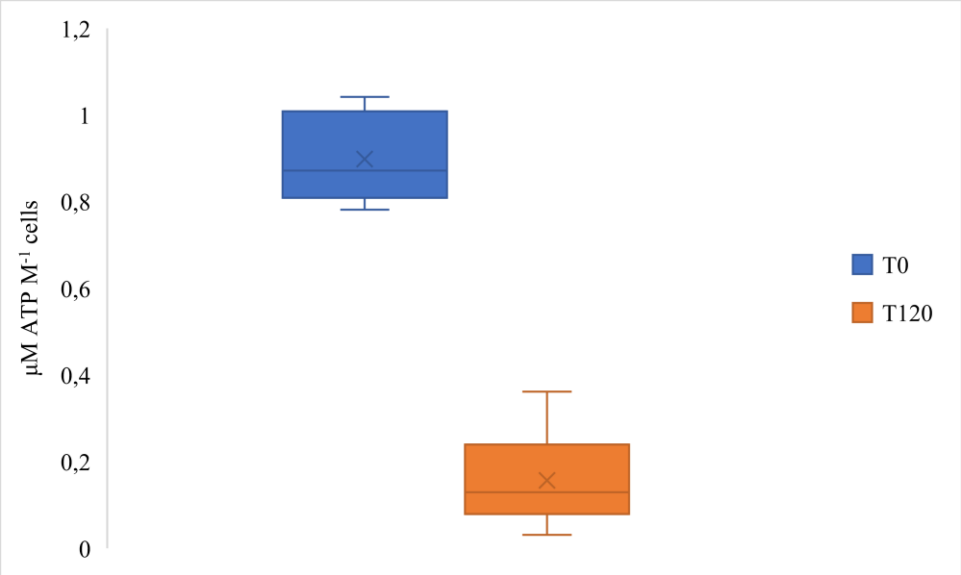

Figure 20: Boxplot representing ATP levels in sex-sorted sperm cell samples detected using CellTiter-Glo® luminescent cell viability assay measured with plate reader. The experiment was performed with semen from seven Norwegian Red bulls where one bull provided AI doses from two ejaculates, measured after T0 (0 minutes) and T120 (120 minutes) incubation at physiological temperature. For each measurement two straws were thawed and then pooled. Results shown as µM ATP million spermatozoa (SD).

Examining differences between individual bulls as seen in Figure 21a, bull nr 7.1 was found to ha the highest level of ATP both before and after incubation. Bull nr 3 had the lowest concentration of ATP at T120.

Figure 21b shows the individual bull ability to retain ATP after incubation as percentage. ATP assessment at T120 showed the percentual ATP retention in sample 7.1 at 35%, which is the highest of all bulls measured. In comparison bull nr 3 retained only 4% ATP.

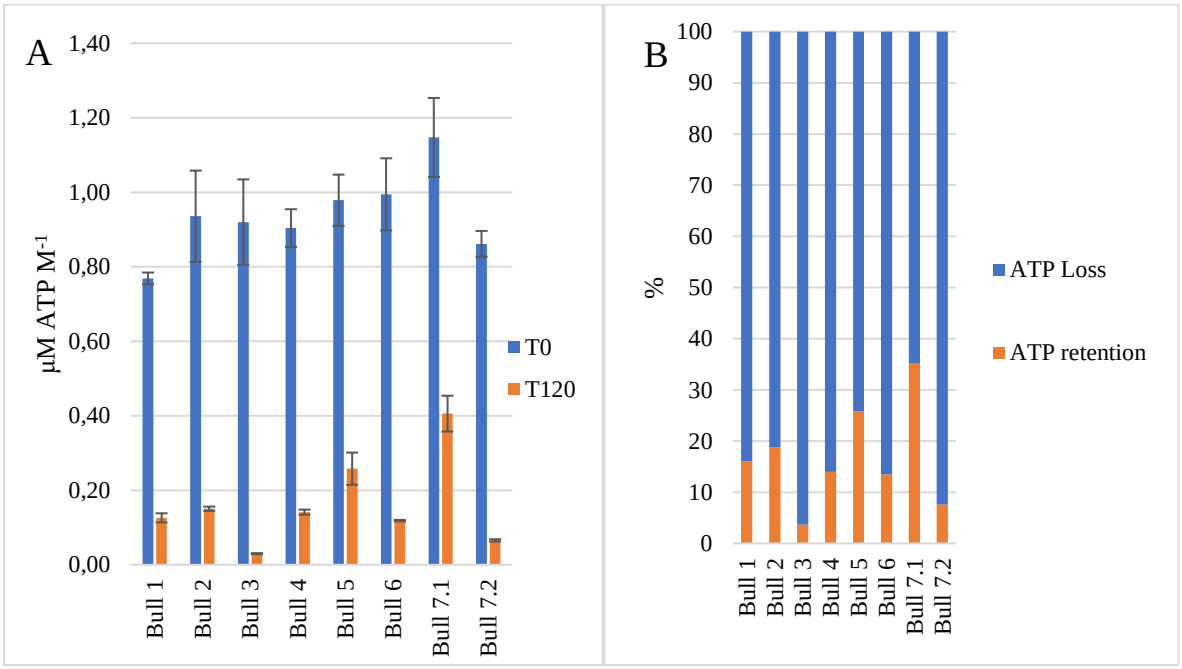

Figure 21: ATP levels detected using CellTiter-Glo® luminescent cell viability assay measured with plate reader. Eight ejaculates from seven sexed Norwegian Red bulls. The samples were incubated at physiological temperature and measured at T0 (0 minutes) and T120 (120 minutes). For each measurement two straws were thawed and then pooled from the same bull. Results shown as µM ATP per million spermatozoa (SD). A represents a bar plot showing the µM ATP per million (SD) per bull, B represents a percentage of ATP retention per bull.

3.6 Lipidperoxidation Assay For Sex-Sorted Spermatozoa

To measure the MDA level in sex-sorted sperm cells, a selection of high and low performers from the motility stress test was first done, the highest selected bulls were 2 and 7.1 and lowest selected bulls were 4 and 7.2. The determining factor from the motility stress test was the number of progressive motile spermatozoa after 120 minutes incubation at physiological temperature.

When comparing the high motility and low motility groups, the low motility group was found to have a had a higher concentration of MDA than the high motility group. There was not found any significant difference between the means of the high and low motility groups and there was not found any significant differences between T0 and T120 within the same group, shown in Figure 22.

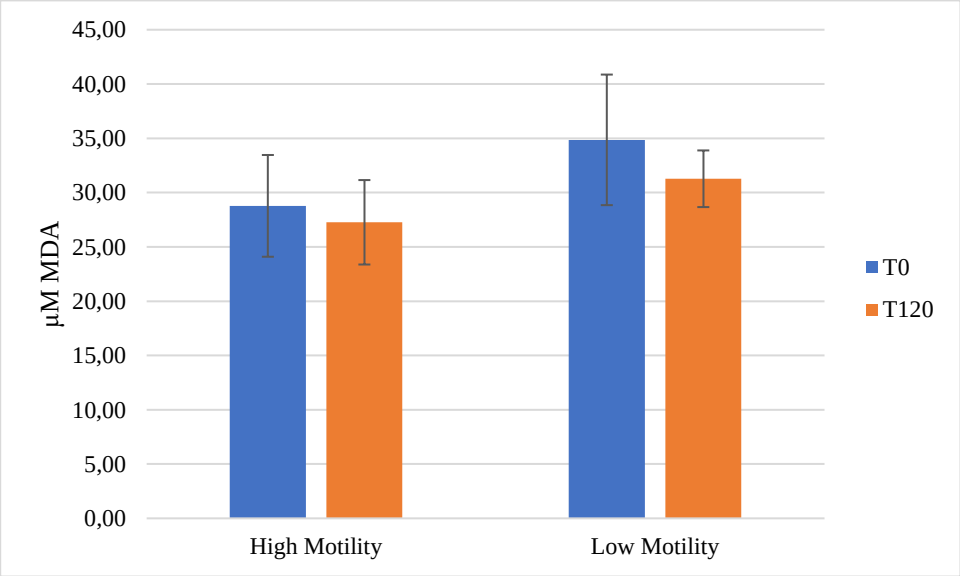

Figure 22: Malondialdehyde (MDA) measured on two high and two low motility Norwegian Red sexed bulls using lipid peroxidation assay detected using plate reader. The samples were incubated at physiological temperature and measured at T0 (0 minutes) and T120 (120 minutes). Two straws were thawed and then combined. Results shown as µM MDA (SD) per million.

When taking a closer look into the individual bull, we can see that overall was there only small differences in the MDA level both between bulls and post incubation. And as shown in Figure 23, at T0 bull 7.2 had the highest MDA level and bull 7.1 had the lowest. After

incubation at physiological temperatures, bull 7.1 had an increase in MDA concentration post incubation while the others had a decrease in MDA concentration at T120. Bull 7 provided AI doses from two ejaculates and was found to be one of the high and one of the low performers, illustrating differences between ejaculates of the same bull.

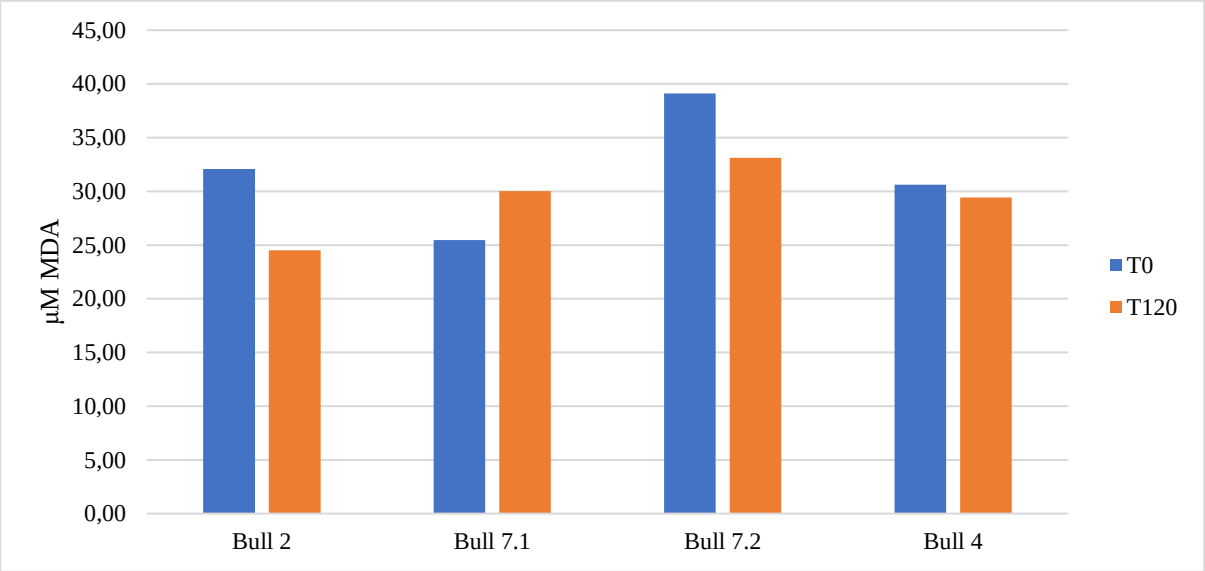

Figure 23: Malondialdehyde measured on Norwegian Red sexed samples using lipid peroxidation assay detected using plate reader. The samples were incubated at physiological temperature and measured at T0 (0 minutes) and T120 (120 minutes). Two straws were thawed and then combined. Results shown as µM MDA per million.

3.7 Analysis of Lipid Peroxidation In Relation To Fertility in Sex-Sorted Spermatozoa

Examining the MDA levels of bulls with known field fertility data shown in Figure 24, it was found that the high fertility group had a higher MDA concentration at T0 and T120 compared to the low fertility group at the same time points, however not significant. There was a significant decrease in MDA level for the high fertility group between T0 and T120 ($P<0,05$), but not for the low fertility group ($P>0,05$).

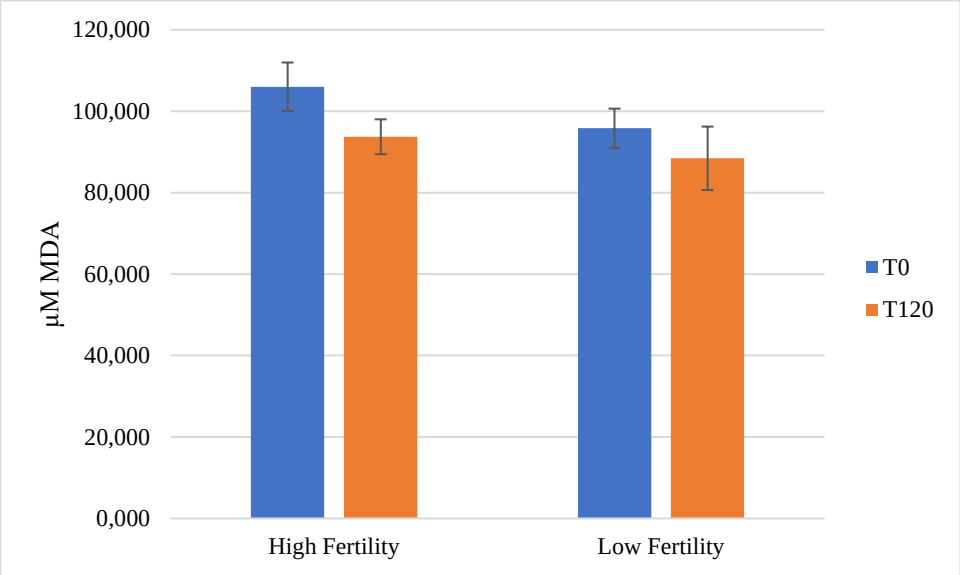

Figure 24: Malondialdehyde (MDA) concentrations measured on three high and three low fertility Norwegian Red sexed bulls using lipid peroxidation assay detected using plate reader. The samples were incubated at physiological temperature and measured at T0 (0 minutes) and T120 (120 minutes). Two straws were thawed and then combined. Results shown as µM MDA (SD) per million.

When we examine the individual bull to bull difference it was observed that bull nr 3 and nr 6 had the highest concentration of MDA, and the lowest concentration was detected in bull nr 5 at T0 (Figure 25). After 120 minutes incubation there was a decline in the concentration for most of the bulls except bull nr 5 that had a slight increase in MDA.

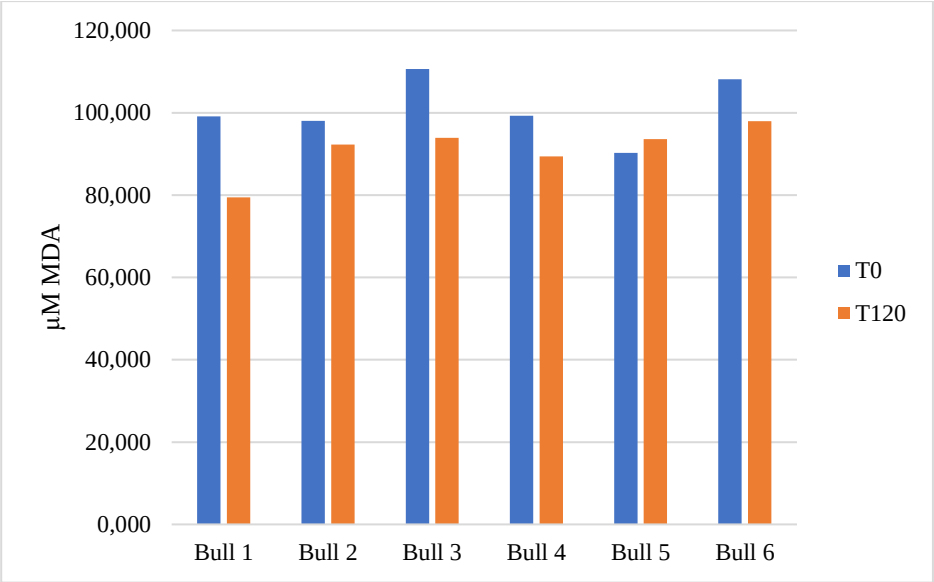

Figure 25: Malondialdehyde (MDA) concentrations measured on three high and three low fertility Norwegian Red sexed bulls using lipid peroxidation assay detected using plate reader. The samples were incubated at physiological temperature and measured at T0 (0 minutes) and T120 (120 minutes). Two straws were thawed and then combined. Results shown as µM MDA per million.

3.1 Viability and Acrosome Intactness

Since the motility and ATP level decreased over time, we wanted to examine whether the spermatozoa were alive or dead after incubation for 120 minutes. The acrosome intact live (AIL) spermatozoa measured with flowcytometry showed that about half of the sperm cells were still alive. More specifically, bull nr 3 contained a higher number of AIL sperm cells at T0 and at T120 than the other bull semen samples. Sex-sorted semen samples from bull 4, 5, 7.1, and 7.2 contained the lowest number of AIL spermatozoa at T120, as seen in Figure 26a.

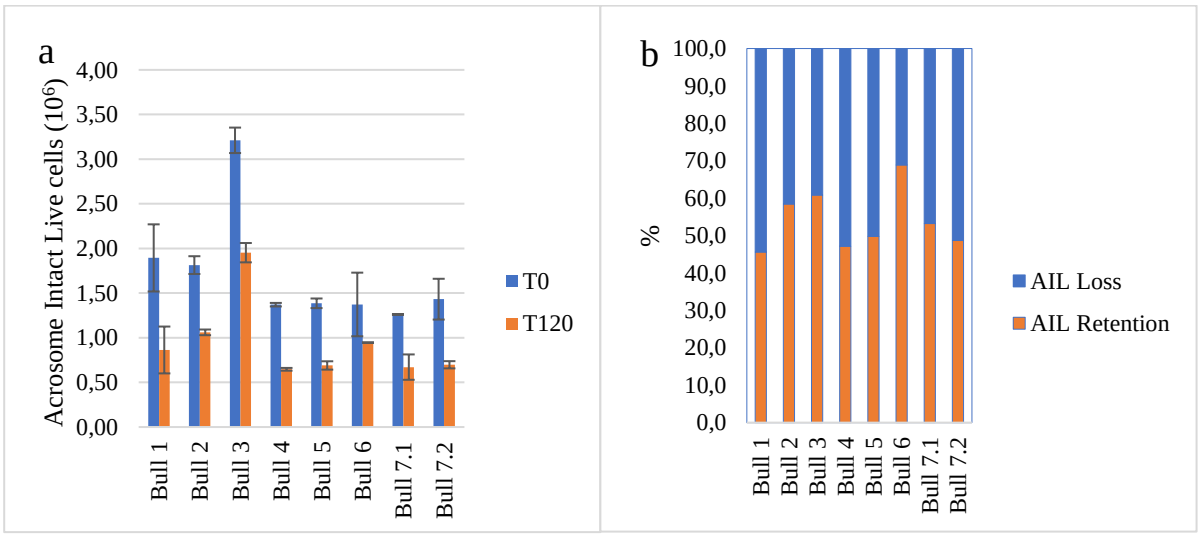

Figure 26: Acrosome Intact Live (AIL) Spermatozoa in sexed samples using flowcytometry with the fluorescent dyes: PNA conjugated Alexa 488, Propidium Iodine, and SYTO™ 60. Two straws for each bull were thawed at physiological temperature and then pooled. Samples were measured at T0 (0 minutes) and T120 (120 minutes). A represents a bar plot showing millions of AIL spermatozoa per bull, B represents a percentage of AIL retention per bull.

The ability to withstand the thermal stress from incubation at 120 minutes can be seen in Figure 26b. The range of retention was between 45,6%-68,9%. The bulls with the highest ability to retain AIL spermatozoa were bull nr 6 and then followed by bull nr 3 and bull nr 2. Even though bull nr 3 has a higher number of AIL spermatozoa then the rest of the group, the retention rate was high.

4. Discussion

4.1 Establishment of a Motility Stress Test For Sex-Sorted Spermatozoa

In creating the motility stress test, we saw a decline in motility of sexed Norwegian Red AI doses after incubation at physiological temperature, which have not been documented until now. The preliminary study showed that the spermatozoa still had progressive motility after T150 therefore the stress test was extended to T180. The samples were incubated at 38°C as to imitate physiological temperature *in vivo*. Incubation at physiological temperature prior to motility assessment has been done in other studies on Norwegian Red (Alm-Kristiansen et al., 2018; Berg et al., 2018). As for ordinary sperm cells, in this study there was a decrease in motility over time, being a significant decline of progressive motility between T60 to T120, The decrease in progressive motility indicates that there is a reduction in both velocity and direction, and progressive motility has shown to be an important factor in prediction of field fertility (Budworth et al., 1988; Oliveira et al., 2013), implying that a motility stress test decline as observed here could give additional information for approving or declining a production dose for sale. However, in this study we did not see a significant difference between bulls of high and low fertility. Thus, this can be caused by the limitation in the study having only six bulls available for experiments. In further studies, more sex-sorted samples from a higher number of bulls should be included, and ideally, with more contrast in fertility between the groups.

During the incubation period the cryopreserved spermatozoa were kept in the extender provided by the breeding company, for the purpose of simplicity and being readily able to be adapted into quality control assessment in production laboratory at the AI station. Another benefit of incubation in the extender would be to reduce additional steps that could potentially be harmful to the spermatozoa, such centrifugation and resuspending of the cell containing pellet (D. Mortimer, 2000).

In terms of optimalization for production perhaps an increase in temperature would decrease the time needed to conduct the motility stress test. Thus, a downside of increasing the temperature would be that the increase in temperature would make the motility stress test differ more from *in vivo* environments found in the female reproductive tract.

The motility stress test of the initial group was intended to provide insight into the differences between seven sexed Norwegian Red bulls and illuminate the decline of total motility, progressive motility, and velocity parameters (VCL and VAP), while the motility stress test for the secondary group consisted of six Norwegian Red bulls, which was intended to be compared to known fertility data and explore possible relationships between motility parameters and fertility groups. Noteworthy this test was conducted with six bulls and therefore the relationships that are found should be reconfirmed in a study with a higher sample size, which also enables a more robust statistical analysis that would allow for modeling of field fertility prediction. In studies where bulls are divided into different fertility potential groups it has been shown correlation between total motility and progressive motility towards prediction of field fertility (Puglisi et al., 2012).

There was a decline in the number of spermatozoa classified as motile and progressive, and a decrease in the velocity of individual spermatozoa measured as VCL and VAP. There were differences between bulls, implying that the stress test succeeded in providing multiple parameters highlighting the ability to withstand thermal stress over time for the individual bull. When determining bull fertility potential it has been shown that a combination of several parameters has been more effective in linking motility data towards field fertility (Farrell et al., 1998).

In the motility stress test of the seven Norwegian Red bulls, bull nr 2 had the highest total motility at T180 and Bull nr 1 had the highest progressive motility, VCL and VAP. It is noteworthy that bull nr 3 initially had the highest number of total motile and progressive motile spermatozoa at T0 and T10, then shortly afterwards went from having the highest number of progressive spermatozoa to being one of the lowest numbers of progressive spermatozoa in the group. One possible reason for this could be that the incubation was done in the extender, and then due to having a higher number of cells it also metabolizes the nutrients in the extender at a faster rate. Since sexed AI doses are prone to being diluted under processing, the nutrients do most likely not originate from seminal plasma (Klinc & Rath, 2007).

The NR56 range for the high fertility bulls was 66.9%-70.7% with an average of 69,33% and the low fertility group had a range of 59.5%-59.8% and an average of 59.7% using sexed AI doses in our study. Another study conducted on Norwegian Red cattle where they used high and low fertility groups had an mean NR56 for the low fertility group of 61% and the high fertility group with 76%, their data was collected from AI performed using conventional doses

and not sexed semen (Narud et al., 2020), giving higher contrast between the high and low fertility group also increasing the likelihood of finding relations between sperm parameters and fertility compared to our study. It is important to note that the fertility data we have on Norwegian Red cattle, only shows the higher end of the fertility range because the bull of with the lowest quality parameters would never reach the market forcing studies to determine what they define to be low and high fertility within the mid to high fertility range. Another effect of having a strict incoming quality control prior to semen processing is that we are creating an artificial selection towards an increase in sperm quality and a larger study in 20 generations from now might be able to find how strong that effect was.

In the motility stress test with the six bulls with known fertility was it found a significant difference between the high and low fertility group at T0 in total motility ($P< 0.001$), but we did not see any significant difference at T120. There was a significant difference between T0 and T120 for progressive, VAP and VCL. In this study there was a slightly higher mean total motility, progressive motility, VAP and VCL at T120 for the high fertility bulls compared to the low fertility group but not enough to determine a significant difference. Perhaps there could have been found a statistically significance difference if there would have been a larger sample size.

4.2 ATP in Relation to Motility of Sex-Sorted Spermatozoa Over Time

After observing the decline in motility from the stress tests we wanted to investigate what caused the decline in motility. Specifically, if there could be seen a similar effect on the amount of ATP, therefore ATP level for of the initial group (seven Norwegian Red bulls) were measured at T0 and T120, where incubation of 120 minutes was decided due to the significant motility decline found in the motility stress test. A correlation between motility and ATP levels had been found before with Norwegian Red AI doses extended with Biladyl and immobilized Spermvital doses (Alm-Kristiansen et al., 2018). It has also been found a correlation between field fertility and ATP before, where they used bulls with similar non-return rates and stipulates that the ATP formation might be an indicator of the fertility potential after cryopreservation (Garrett et al., 2008). Even if the membrane stays intact by cryopreservation techniques, the quality of the spermatozoa will degrade in several aspects, including possible DNA and mRNA damages, acrosomal damage, and changes to the membrane and metabolic

processes, which can cause a reduction of viable spermatozoa (Yánez-Ortiz et al., 2022). To provide the required energy, two metabolic pathways are used by spermatozoa to gain ATP which are oxidative phosphorylation facilitated in the midpiece and glycolysis in the principal piece (Amaral, 2022). The ATP serves another function other than providing energy by providing the phosphate group that has been shown necessary for the phosphorylation processes during the capacitation, where protein kinase A interacts with ATP forming cAMP in spermatozoa (O′Flaherty, 2015). Because of the variations in the location and initiation of the capacitation, the composition of the seminal and vaginal fluids as well as the different features of spermatozoa between species, it is not yet known whether these results are comparable with the capacitation of bull spermatozoa (Senger, 2012, pp. 256–260). The capacitation happens over a period of time and post capacitated spermatozoa initiates hyperactivation which has an increased ATP consumption (Sansegundo et al., 2022). Therefore, does it seem logical that a bull with a high ATP retention should do better at penetrating the cumulus matrix since it has more energy for hyperactivation.

The ATP assessment showed that there was a decline in ATP after incubation similar to the decline found in motility. The ATP levels prior to incubation showed that bull nr 7.1 had the highest levels of ATP as well as the highest retention of ATP at T120, and bull nr 7.1 was also one of the top performers at progressive motility measured at T120 from the motility stress test. The levels of ATP retained for bull nr 3 was also consistent with the motility stress test results where it was found initially a higher number of progressive spermatozoa and a rapid decline in motility over time, and it would be interesting to see if the same decline would have been measured if the samples were not incubated in the extender.

4.3 Lipid Peroxidation Assay

It is well established that oxidative stress may affect sperm pathology which cause lipid peroxidation, loss of motility, and viability (Bansal & Bilaspuri, 2011), and hence the decline of motility could be caused by increased oxidative stress. In this thesis we therefore attempted to establish a new method for measuring lipid peroxidation of AI doses containing sexed Norwegian Red spermatozoa. As we know the oxidative stress is caused by an imbalance of ROS where the rate of produced ROS are greater than the ability to remove ROS (Kurkowska et al., 2020), which in turn can cause lipid peroxidation and the end product MDA. The prosses

of sexing semen using flowcytometry has not been directly linked to inducing lipid peroxidation, yet the process of staining with the use of Hoechst 33342 requires a higher temperature which has shown an increase in lipid peroxidation in other species, mouse, and rabbit. The results showed that the levels of MDA detected were low and only minor differences between bulls were found, with a slight reduction after T120 incubation for some of the bulls. There was found a significant difference within the high fertility group between T0 an T120 ($P<0.05$), but there was not found any significant differences within the low fertility group and between the high fertility and low fertility groups ($P>0,05$). This is supported by another study where they did not find a link between the field fertility and ROS measured over time (Zoca et al., 2023).

Another consideration in regard to the levels of MDA are the possibility of extenders affecting the MDA formation during the incubation at physiological temperatures. The purpose of semen extenders are to provide spermatozoa protection from detrimental factors such as cooling, freezing, oxidative stress, and osmotic shock (Bustani & Baiee, 2021). Perhaps this has contributed to the reduced levels detected during the MDA assessment even more so for the samples measured at T120.

Due to its relatively new discovery, there is a need for research in regards of oxidative stress in sexed semen. The addition of antioxidants in extenders has shown to reduce oxidative stress, in an experiment that was done on sexed Holstein semen and not sexed Norwegian Red semen (Klinc & Rath, 2007). It showed a significant increase in motility, acrosome integrity and membrane integrity for samples with added pyruvate and catalase. Measurements were done post thermal stress test with incubation at physiological temperature for up to 24 hours (Klinc & Rath, 2007). It should be considered that mild exposure to ROS has been shown to be necessary for capacitation, which high amounts of antioxidants could affect. The study by Klinc and Rath (2007) did not provide any field fertility data regarding AI doses with additional antioxidants. Since some commercially available extenders contain compounds that potentially interact with ROS, this should be considered when applying our method (Stradaioli et al., 2007).

As reviewed by Sapanidou et al. (2023) high levels of antioxidants can also have detrimental effects, since an inadequate amount of ROS will impair normal spermatic functions, such as capacitation, acrosome reaction, and the sperm-oocyte interaction. In addition, possible

contributors for ROS resulting in oxidative stress are cryopreservation and thawing of spermatozoa, culture conditions, washing and centrifugation, and incubation and storage.

There is a possibility that lipid peroxidation might not be the optimal ROS damage to measure when examining sexed spermatozoa, a rapid form of oxidative damage might be better suited contrary to MDA, such as mitochondrial ROS. The mitochondrial ROS can be measured using mitochondrial superoxide indicators (Robinson et al., 2006), and perhaps future experiments can attempt to understand the relationship between oxidative stress and the decline of motility with the use of analytical tools measuring other forms of oxidative damage.

4.4 Viablity and Acrosome Status for Sex-Sorted Spermatozoa

To determine if the reduction observed in this study for the motility stress test could be explained by high level of dying sperm cells, the level of AIL spermatozoa after incubation at T120 was measured. The AIL assessment was conducted on sexed AI doses from seven Norwegian Red bulls. The assessment of viability and acrosome status on Norwegian Red has been done before using conventional AI doses extended with Biladyl (Alm-Kristiansen et al., 2018; Berg et al., 2018; Narud et al., 2020). The exocytotic acrosome reaction is an essential part of fertilization which releases the acrosomal contents responsible for enabling the penetration of the zona pellucida (Hasan et al., 2021). Both acrosome status and viability has shown a relationship with field fertility (Zoca et al., 2023). With research done on Norwegian Red there has been found a strong correlation between the viability, ATP and motility (Alm-Kristiansen et al., 2018). As mentioned in section 4.3 the cryopreservation is known to cause damage to the acrosome, and changes to the plasma membrane, which can cause a reduction of viable spermatozoa (Yánez-Ortiz et al., 2022). The AIL assessment after incubation succeeded in showing the individual differences between bulls, but the AIL spermatozoa post incubation does not seem similar to the decline we saw in motility and ATP, because bull nr 7.1 had one of the lowest retentions of AIL while bull nr 3 that had the lowest retention of ATP and motility, post incubation had one of the highest AIL spermatozoa. It is noteworthy that this was conducted on seven bull and should be reconfirmed using a higher sample size, this would also enable more robust statistical analysis. Another benefit of AIL assessment in terms of production, would be that the analysis is highly efficient in terms of time. However, this study showed that the sexed spermatozoa even though having reduced motility after 120 minutes, many of the sperm cells are still alive. These sperm cells would then most likely be

able to reactivate motility *in vivo*, being able to swim towards the oocyte for fertilization. In further studies, first of all, a higher number of sexed semen samples with known fertility should be analyzed for the percentage of AIL spermatozoa to examine whether this analysis could serve as a fertility predictor. If so, it would be valuable for the AI center to implement analysis of AIL in the approvement of sexed semen does for sales. Secondly, since more than half of the sperm cells are still alive after incubation over time, but does not swim, the motility assay can be further optimized by diluting the spermatozoa in different solutions with nutrients stimulating motility. In this scenario, possible differences in fertility between sexed semen samples might be detected.

4.5 Optimalization of the MDA assessment

Establishing a new assay for lipid peroxidation included several steps that needed optimalization, and several aspects might have influenced the results in this study and will be discussed here. At first, no MDA signal was detected, most likely since lysis of the sperm cells was incomplete. TCEP was therefore addend during the lysis step of the MDA assessment due to its ability to reduce disulfide bonds responsible for the chemical resistance of spermatozoa during lysis (Roszkowski & Mansuy, 2021). The use of TCEP has shown a dose dependent ability to induce/reduce oxidative stress (Mennillo et al., 2019), but it is noteworthy that the study of Mennillo and coworkers measured oxidative stress with the use of enzymatic activity of glutathione peroxidase and not the end product MDA. Measurement of glutathione peroxidase activity would have been interesting to include for sexed Norwegian Red AI doses also in future studies.

Another factor that might have impact on the assay is the substrate. TBA does not selectively react only with MDA, therefore the presence of certain substances can lead to false results. Therefore, it could be advantageous to verify the MDA levels using liquid or gas chromatography and mass spectroscopy (Ayala et al., 2014).

During the incubation period the AI doses were thawed and incubated without addition of any other influence as to not accidentally providing new factors to potentially induce oxidative stress, and therefore we did not apply a simple “washing step” prior to incubation. By “washing step” we mean centrifugation followed by a resuspension of the pellet containing spermatozoa with the use of buffers. Additional centrifugal resuspending should be avoided as it could cause potential harm, it is stated that it can cause irreversible damage, impair, and

destroy fertility capabilities of spermatozoa, yet it was stated that centrifugation with less than a force of g x 800 could be safe. There are also a list of alternative methods that could have been less detrimental, the list consist of the direct swim up method, and density gradients. (D. Mortimer, 2000), that can be tested in further studies to understand the functionality of sexed semen better.

In our study we measured the MDA-TBA adduct at 490 nm instead of the 532 nm recommended by the assay protocol provided by Sigma Aldrich (MAK085, Sigma Aldrich), since 532 nm was not available for analysis, while this may have an impact on the precision of our measurements, the results showed an improved R^2 in comparison to 560 nm, which was the other measurement wavelength available to us.

As stated above, TBA is not selective for MDA, but reacts with several other substances including aldehydes as well as "ketones, ketosteroids, acids, esters, sugars, imides and amides, amino acids, oxidized proteins, pyridines, pyrimidines, and vitamins" (Guillén-Sans & Guzmán-Chozas, 1998). An improved substitute for TBA could be 1-methyl-2-phenylindole (MPI) since it has been shown to be more effective in colorimetric detection of MDA. MPI has a detection peak at 594 nm. Another benefit of using the MPI-MDA adduct is the short reaction time at 3 minutes compared to the 60 minutes necessary for the TBA-MDA adduct to form (Islayem et al., 2022). Taking this method into a production context could mean an increase in efficiency and effectiveness compared to the TBA-MDA method. Therefore, it would have been of interest in future studies to compare the results of these two methods of measurements the MDA level.

5. Conclusion

In this study, several sperm quality parameters were evaluated for sex-sorted Norwegian Red spermatozoa. Specifically, a stress test for sexed semen was established, showing differences between bulls for total motility, progressive motility, and velocity. A higher number of bulls needs to be included in further studies to examine whether the relationship to fertility is significant. Establishment of the MDA assay optimized for sexed semen samples showed that the decrease in motility over time was not, at least not only, due to increased lipid peroxidation. In further studies other forms of oxidative damage could be better suited for evaluation of sex-sorted spermatozoa. The ATP measurements showed that the energy levels, as motility, decreased over time. Moreover, 120 minutes incubation proved efficient to capture individual ATP differences between bulls for sex-sorted AI doses. The assessment of AIL after 120 minutes thermal stress showed that immotile spermatozoa might still be live and therefore possibly could revert into motile spermatozoa, indicating a new quality parameter that could potentially be used in fertility prediction of sex-sorted spermatozoa. It would be interesting to perform a larger study to examine whether AIL would be valuable to include in approvement of sex-semen doses for sale. The assessment is highly efficient, and AI centers would be able to perform out-going quality control at a high pace. Altogether, the results from this thesis need further follow up studies both to get more knowledge and to confirm results but could implicate new procedures that be valuable to implement for AI centers worldwide.

6. Reference List

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