Linköping University | Department of Physics, Chemistry and Biology

Type of thesis, 60 hp | Educational Program: Physics, Chemistry and Biology

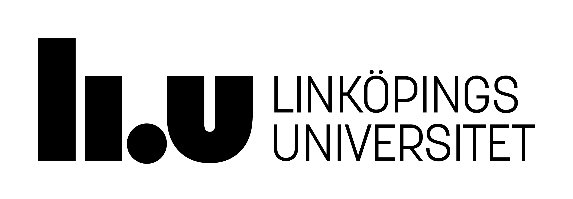
Spring or Autumn term 2022 | LITH-IFM-A-EX—22/4103--SE

Oxidative status and metabolomic profiling of seminal plasma and their association with semen motility and cryopreservation in Bulls

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1. **Abstract:**

Early detection of (in)fertility biomarkers is of great interest in the enhancement of artificial insemination programs, with the objective of providing equal access to food resources and potentially improving conservation strategies for endangered species. (In)Fertility is a complex trait governed by a multitude of physiological, molecular and external factors and has low heritability. Accurately assessing fertility markers *in vitro* and predicting reproduction outcomes *in vivo*, is thus, a difficult task. The current study aimed at evaluating the relationship between sperm motility parameters and seminal plasma composition (in both prooxidant-antioxidant and metabolites), in bulls, before and after cryopreservation. Advanced oxidative protein products (AOPPs) and protein carbonylation levels were found to be positively correlated with linearity in fresh semen and progressive and total motility in frozen/thawed semen. This observation gives an alternative perspective into the relationship between sperm motility and seminal plasma composition, whereby increased oxidative stress in seminal plasma does not necessarily, negatively affect sperm motility. The study of metabolomics in seminal plasma, using nuclear magnetic resonance (1 H NMR), resulted in 18 metabolites which explain the differences seen between low and high motility groups. There are still gaps in knowledge about the effect of cryopreservation on sperm motility, viability and the role of seminal plasma in the process. Nevertheless, sperm motility, oxidative stress parameters (AOPPs and thiols) as well as metabolomic profiling of seminal plasma can be considered as effective biomarkers of bull (in)fertility.

Keywords: Antioxidants, AOPP, artificial insemination, carbonyls, cryopreservation, 1 H NMR.

**List of abbreviations:**

**ALH**  Amplitude of lateral head displacement

**AOPP**  Advanced oxidative protein products

**ATP**  Adenosine triphosphate

**BCF** Beat cross frequency

**CASA**  Computer-assisted sperm motility analysis

**GHG**  Greenhouse gas

**GSH**  Glutathione

**HMF** High motility fresh (group)

**1H HMR** 1H Nuclear magnetic resonance

**HMT**  High motility thawed (group)

**HQB** High quality bulls

**LIN**  Linearity

**LMF** Low motility fresh

**LMT**  Low motility thawed

**LQB** Low quality bulls

**NAC**  N-acetyl cysteine

**PCA**  Principle component analysis

**PLSDA** Partial-Least Squares Discriminant Analysis

**PM**  Progressive motility

**ROS**  Reactive Oxygen Species

**TCA** Trichloric acid

**TM**  Total motility

**VAP**  Average path velocity

**VCL**  Curvilinear velocity

**VIP**  Variable importance projection value

**VSL** Straight-line velocity

**2. Introduction:**

Around 6000 years B.C., the transition from foraging to farming took place across the Mediterranean basin, where hunting for food/prey was replaced by securing and accumulating food sources, a process known as domestication (Crabtree, 1993). Domestication later developed into “selective breeding”, where individuals were bred based on their genetic background, with the aim of optimizing crop yields and livestock produce (Larson & Filler, 2014; Weigel et al., 2017). The rippling effects of such advances in human society, are obvious in today’s world, whereby the simplicity of acquiring and producing food led to a significant increase in population density.

In order to maintain such a rapidly growing and aging population, land and resources have been overexploited. Excessive emissions of greenhouse gas (GHG) from industries and animal condensed farms have participated majorly to global warming. Moreover, unsustainable food production systems and unequal distribution of food resources have caused “discriminatory” food security worldwide (Chiarelli, 2006; Larson & Filler, 2014; Memili et al., 2020; FAO, 2021). According to the United Nations (UN), population demographics and global temperatures are expected to continue increasing over the years. By 2050, the global population is predicted to reach 9.7 billion with an average temperature increase of + 2°C (UN, 2019). One of the main contributors of GHG emission and global warming, is the livestock production system, which accounts for about 14.5% of all anthropogenic GHG production (65% of which is caused by cattle) and requires large amounts of land used for feed, water and keeping of the animals (Memili et al., 2020; FAO 2021).

With time, the benefits accompanying genetic selection were impeded by a surge of infertility in cattle (Royal et al., 2000; Lucy, 2001). The root cause of this infertility has not yet been clearly defined. However, it is thought to involve a combination of factors ranging from intrinsic, physiological aspects of spermatozoa to the molecular components of the milieu these are maintained: seminal plasma and is a secondary effect of selective breeding (Lucy, 2001; Viana et al., 2018). This “inconvenience” is of major economic importance and has mobilized farmers, veterinarians and scientists to find solutions to the problem (Bruno, 2020).

Governments across the globe are now faced with two intertwined challenges: providing safe and abundant resources of food to accommodate a growing population, all whilst creating and maintaining new sustainable methods to do so (Gopalan, 2001; Godfray et al., 2010; Foley, 2011).

Thus, the prediction of bull fertility using innovative biotechnology, is of major economic and environmental importance in the sustainable, cattle breeding industry.

Artificial insemination has been most commonly used to counteract infertility in cattle (Memili et al., 2020). By selecting bull semen of high quality, copious amounts of breedings are possible. This type of reproductive biotechnology is unlimited by space and time, meaning that semen can be transported anywhere, and by methods of freezing (cryopreservation), can be preserved over time (Selvaraju et al., 2018; Memili et al., 2020). Artificial insemination thus contributes to bettering food safety and is considered a sustainable method in cattle farming (Selvaraju et al., 2018).

The main process associated with artificial insemination is known as cryopreservation: the preservation of biological material by freezing (Selvaraju et al., 2018). To most living systems, cold temperatures are lethal, causing the formation of ice within cells and/or halting most biological/enzymatic processes within them. The most common type of cryopreservation process is known as “slow freezing”. Slow freezing is characterized by the gradual cooling of samples (1°C/min) and is at low risk of (bacterial/fungus) contamination (Guven & Demirci, 2012; Yong et al., 2015). Nevertheless, the use of this method is disputed as it is commonly known to provoke extracellular ice formation causing restraint on the cell and damage to its structure (Li et al., 2010; Yong et al., 2015; Jang et al., 2017). However, with the addition of a cryoprotective agent, biological matter is maintained in a state of suspended animation allowing cells to survive for long periods of time at freezing temperatures (Jang et al., 2017). A cryoprotective agent is characterized by its low toxicity, capacity to tolerate very low temperatures, penetrate cells to regulate water transport, nucleation and ice crystal growth (Pegg, 2007).

Compared to other cells of the body, spermatozoa are found to be more “resilient” in facing the stresses of cryopreservation, due to their lower water content and high membrane permeability (Ugur et al., 2019). The damage degree endured by the cells is largely determined by the composition of sperm membranes and the rate of dehydration, cooling and thawing (Grötter et al., 2019). Inevitably, the process of sperm cell cryopreservation, regardless of the adjustments used is accompanied by certain deteriorations, namely, the loss of fertility-associated proteins and capacitation of spermatozoa, the generation of oxidative stress markers, carbonylation and changes in the metabolome (Jang et al., 2017; Ugur et al., 2019). Which will be discussed further throughout the report.

Male fertility is defined as the ability of sperm to reach, fuse, fertilize an egg and subsequently, maintain embryo development (Kumaresan et al., 2017). Fertility is considered a complex trait, governed by a multitude of factors, both morphological and molecular, and has low heritability (Özbek et al., 2021). Despite the advanced technologies used today, the multifactorial aspect of fertility and its low genetic component (heritability) makes it a tedious task to accurately determine or predict its outcome (Viana et al., 2018). The characteristics related to the evaluation of bull fertility are described below.

Motility is recognized as one of the most important and common factors predicting fertility. As, without active motility, spermatozoa cannot successfully move through the female reproductive tract and fertilize the egg. Computer-assisted sperm motility analysis (CASA) has been widely used to objectively study sperm motility, as opposed to the previously conventional/subjective microscope study. This method uses short video recordings to determine sperm concentration, viability, kinetics and morphology, and is specific to single cells (Lu et al., 2013; Kanno et al., 2017). The criteria evaluated are specified as: amplitude lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN), progressive motility (PM), total motility (TM), average path velocity (VAP), curvilinear velocity (VCL) and straight-line velocity (VSL) (See Table 1. Appendix). ALH is representative of the distance between the peak and low point of the centroid’s path (the centroid is the “mid-point” of the sperm head). BCF is the number of lateral oscillatory movements of the sperm head around the mean trajectory. LIN represents the linear or straight trajectory of a single spermatozoa per unit of time. PM is the percentage of spermatozoa that show a linear or directional movement. TM is the percentage of all motile spermatozoa in a sample. VAP is the mean trajectory of spermatozoa per unit of time (or mean velocity). VCL is specific to a single spermatozoon’s trajectory and represents the instantaneous recording of its sequential progression. Finally, VSL is the straight trajectory of all spermatozoa over time (Mortimer, 1997; Quintero-Moreno et al., 2003).

Generally, sperm motility is regarded as the principal factor determining semen quality, however, with recent advances in reproductive biotechnologies, seminal plasma has also proven to be involved in sperm metabolism, function and survival (Juyena & Stelletta, 2011). Seminal plasma is composed of a mixture of fluids originating from the testes, epididymis and accessory glands (such as the seminal vesicle, prostate and bulbo-urethral glands). This fluid in which sperm bathe, contains nutrients and proteins that take part in sperm metabolism, motility, capacitation, acrosome reaction and act in protecting spermatozoa from reactive oxygen species (Gomes et al., 2020). Still, knowledge of the relationship between seminal plasma components and spermatozoa remains unclear, as these parameters vary between ejaculates, individuals and species (Juyena & Stelletta, 2011).

Recently, with the development of high-throughput techniques (“the -omics”), proteomics have also shown to be potent in their analysis of bull fertility/infertility. Proteomics, as the name indicates, focuses on proteins, the main effectors of cellular function, metabolism and cell to cell communication (Mostek et al., 2017; Mostek et al., 2018).

Cryopreservation is faced with challenges, whereby the excessive production of reactive oxygen species, such as hydroxyl radicals, superoxide anion and hydrogen peroxide, leads to the deterioration of certain molecular structures and general physiology of spermatozoa. Reactive oxygen species (ROS) are known to react with DNA causing its methylation and changes in protein production; proteins suffer oxidation and lipids undergo peroxidation. These modifications engender changes in sperm motility and capacitation (Agarwal et al., 2014; Ugur et al., 2019).

Based on the study by Mostek et al. (2017), one method to measure oxidative stress in sperm proteins, is by quantifying protein carbonylation. Carbonyl groups, amongst other oxidation products, are more reliable in the study of oxi-proteomics of bull sperm, because of the stability and early development of such structures. These findings were further supported and further developed by Mostek et al. (2018), where a correlation between levels of reactive oxygen species, protein carbonylation and sperm quality was established. The study showed that increasing levels of reactive oxygen species are linked to an increase in protein carbonylation and thus poorer sperm quality. More specifically, the results demonstrated that most of the defect proteins were found intracellularly, within the inner and outer membranes of the mitochondria. Oxidative damage to these proteins is potentially linked to changes in adenosine triphosphate (ATP) production and by consequence, linked to impaired mitochondrial and flagellar function (Mostek et al., 2018). Thus, carbonyl groups are important proteomic biomarkers, in the classification and distinction between high- and low-quality sperm in bulls.

Although protein carbonylation has been widely used in detecting infertility, other oxidative stress markers such as advanced oxidation protein products (AOPP) have also shown to be crucial components in that matter. Advanced oxidation protein products are oxidative markers associated with the damage of plasma proteins. *In vivo*, AOPPs activate the oxidative metabolism of certain immune cells (neutrophils and monocytes), thus taking part in the inflammatory response. These markers are of particular interest in the study of numerous diseases and infertility, as they remain diffused in blood for prolonged periods of time, making them stable and more reliable (Celi & Gabai, 2015; Song et al., 2017). The study of AOPPs as infertility indicators has yet to be tested in cattle semen, nevertheless, based on different studies AOPPs have been found to be linked to infertility in both men and women (Kratz & Piwowar, 2017; Song et al., 2017).

Naturally, the generation of ROS species is stabilized by antioxidant molecules that counteract the notorious effects of excessive oxidation. One of the main defense mechanisms found in spermatozoa is the thiol group. Thiols are a group of cysteine residues containing sulfhydryl groups and are known as N-acetyl cysteine (NAC) with the most important one being glutathione (GSH). These antioxidants are synthesized in the cytoplasm of sperm cells as well as mitochondria. Intracellular thiol concentrations have been found to be strongly correlated with sperm motility parameters post- thaw. Additionally, studies report a depletion of intracellular thiols after thawing and an increase in sperm senescence (Bilodeau et al., 2001; Martin-Muñoz et al., 2015).

The impact of oxidative stress markers is intertwined with a variety of other processes, such as inflammation (immune response), intracellular signaling and successful sperm capacitation. However, in extreme cases, reactive oxygen species (ROS) could induce toxicity by causing DNA damage, lipid peroxidation and finally apoptosis (Ugur et al., 2019). Reactive oxygen species (superoxide anion and hydrogen peroxide) are usually generated by mitochondria and could be an indication of low antioxidant activity and a consequence of improper semen storage (Guthrie & Welch, 2012). They have been found to be associated with a decrease in sperm motility and correlated with lipid peroxidation. By interacting with polyunsaturated fatty acids, a chain of reactions is initiated and consequently provokes the lipid bilayer of sperm membrane to rigidify (Chatterjee & Gagnon, 2001). The created oxidative stress disrupts the fertilizing capacity of sperm as well as their ability to create a healthy embryo. In a study by Alyethodi et al. (2021), reactive oxygen species and the final break-down molecule of lipid peroxidation (malondialdehyde- MDA), negatively impacted sperm motility, function and the cryo-preservability of semen. These molecules (superoxide anion, hydrogen peroxide) and lipid peroxidation by-products are also regarded as important indicators of sperm incapacitation and infertility.

The mechanisms underlying infertility are not limited to the study of proteomics. Metabolites constitute the finer structures of a cell’s metabolome, such as amino acids, peptides, fatty acids and carbohydrates, which take part in important metabolic processes (Deepinder et al., 2007). Based on recent advances, both, metabolites of sperm cells and seminal plasma are important factors in the successful copulation and conception in cattle. More specifically, seminal plasma metabolites play a key role in regulating sperm function, motility, metabolic activity and aid in controlling pH values as well as the generation of reactive oxygen species (ROS). Thus, the study of metabolomics has been a promising technique in detecting fertility or infertility biomarkers in semen (Kovac et al., 2013; Ugur et al., 2019).

According to a study by Velho et al. (2018), sixty-three seminal plasma metabolites were considered as biomarkers in a group of bulls with distinct fertility rates. More specifically, fructose, was found to be the most prominent (highest in concentration) metabolite in seminal plasma, followed by citric, lactic and phosphoric acid. After assessing the determined biomarkers, a general trend was established: amino acids and peptides represented 33% of all biomarkers, organic compounds 31.4% and carbohydrates 1.58%. More importantly, each of these chemical compound plays a role in spermatozoid function. For instance, amino acids have a protective role during cryopreservation by decreasing lipid peroxidation and injury caused by free radicals. As for carbohydrates, these are essential for energy production, through processes such as glycolysis. Fructose, a carbohydrate, is the main compound for ATP production in sperm, or, in other words, supports sperm motility. By the use of multivariate statistics, fructose was recognized as the unique metabolite allowing the differentiation between high and low fertility groups. Thus, larger proportions of fructose in seminal plasma are indicative of better sperm motility and higher male fertility (Velho et al., 2018).

In a similar study by Kumar et al. (2015), seminal plasma metabolites of both high and low fertility bulls were compared using proton nuclear magnetic resonance (1H NMR). Nuclear magnetic resonance is described as the interaction of magnetic moments of nuclei (in this case 1H) and strong magnetic fields. This interaction is then translated into a spectrum with a variety of peaks. The peaks are representative of the atomic composition of molecules, they give information about adjacent molecules, molecular dynamics and quantitative measures of molecules present in a sample (Mlynárik, 2017). This innovative method would allow scientists to more accurately detect fertility metabolites without the need to handle sperm. Metabolites are considered good predictors of potential (in)fertility, as they are the end products of metabolic pathways.

Considering the above, the main aim of this project is to evaluate the relationship between seminal plasma composition and oxidative status of bull spermatozoa after cryopreservation. This will be performed by the use of an innovative technique, known as Nuclear Magnetic Resonance (NMR). The relationship between metabolic profiling (of seminal plasma) and oxidative stress markers of seminal plasma as well as, spermatozoa motility parameters will be studied. Quality parameters of bull ejaculates will be evaluated before and after freeze-thawing procedures. The hypothesis is that seminal plasma composition could affect fresh semen quality and could be related to different capabilities of spermatozoa to tolerate cryopreservation.

**3. Materials and Methods:**

**3.1 Sample collection and processing:**

Semen samples were obtained from 20 young Holstein Fireisan bulls ranging in age from 10 to 12 months at the Intermizoo A.I. station, located in Vallevecchia farm, Brussa, Carole (Venice).

Bull semen was collected using an artificial vagina and was diluted 1: 1 with the pre-warmed (+37 °C) extender. Sperm motility and morphology were assessed before cryopreservation procedure.

Seminal plasma (obtained after centrifugation of an aliquot of each ejaculate), was stored at -80 °C for the analyses performed in this study.

After dilution (with commercial Intermizoo media), semen samples were immediately cryopreserved. The diluted semen samples were packed into 0.5 mL labelled plastic straws (50-80x106 spermatozoa/mL). This procedure was performed at 4 °C.

Afterward, the straws were transferred to a programmable freezer. The freezing program consisted of the following rates: -4 °C/min from 4 °C to 0 °C, -1 °C/min from 0 °C to -4 °C, -12 °C/min from -4 °C to -40 °C, -30 °C/min from -40 °C to -140 °C. The straws were finally plunged into liquid N2 (at -196 °C) for further storage, until assessment of spermatozoa quality after thawing procedures, which is part of this project.

**3.2 Experiment 1: Fresh semen study**

**3.2.1 Motility of fresh semen**

Sperm motility will be determined using a computer-assisted sperm analysis system (CASA, Hamilton Thorne, IVOS Ver. 12); the standard bull setup was used (60 frames per sec; 30 n. of frames; min contrast 35; min cell size 8 pixels; progressive cells: VAP ≥25.0 µm s-1; straightness ≥75%; static cell cutoff: VAP = 24.9 µm s-1, VSL 20.0 µm s-1 (set up by commercially by Intermizoo and based on, Llavanera et al., 2022). Approximately one thousand cells at 30 x10 6 sperm/mL were evaluated for each sample using a fixed-height Leja Chamber SC 20-01-04-B (Leja, The Netherlands). Parameters assessed were percentages of total motile spermatozoa (TM), percentages of progressively motile spermatozoa (PM), curvilinear velocity (VCL mm s-1), average path velocity (VAP mm s-1), straight line velocity (VSL mm s-1), percentages of straightness (STR) and linearity (LIN), amplitude lateral head displacement (ALH mm) and beat cross frequency (BCF Hz).

**3.2.2 Oxidative stress biomarkers in Seminal Plasma**

Protein concentrations in in seminal plasma were measured by the BCA method (BCA Protein assay kit; Pierce Biotechnology, Rockford, IL, USA), following the manufacturer instructions. Carbonyl groups and advanced oxidation protein products (AOPP) concentrations were measured in seminal plasma, as biomarkers of protein oxidation. Thiol concentrations were considered as an indicator of antioxidant capacity of seminal plasma in response to ROS species.

The AOPP concentrations were measured spectrophotometrically (Witko-Sarsat et al., 1996). Samples (0.2 mL, diluted 1:5 or 1:20 in 20 mM phosphate buffer, pH 7.4) were placed in 96-well microplates (Perkin–Elmer) and mixed with 20 µL of acetic acid. A standard curve was prepared using 0.2 mL of a chloramine T solution (0–100 mol/L; Sigma–Aldrich) as the reference, 10 µL of 1.16 M potassium iodide and 20 µL of acetic acid. The absorbance of the reaction mixtures was immediately read at 340 nm in a microplate reader (Victor X4 2030 Multilabel Reader, Perkin El- mer) against blank (0.2 mL of 20 mM phosphate buffer, 10 µL of 1.16 M potassium iodide and 20 L of acetic acid). The AOPP content was expressed as chloramine T equivalents.

Protein carbonyls were measured following their derivatization with DNPH (2.4 dinitrophenylhydrazine; Sigma-Aldrich Co., St, Louis, MO). The seminal plasma samples (100 µL) were divided into two aliquots; one intended for negative control and the other intended to be treated with DNPH. After a first precipitation of the proteins with 10 % trichloroacetic acid (TCA), 0.5 mL of 10 mM DNPH solubilized in 2.5 N HCl were added in the series of samples to be derivatized and 0.5 ml of 2.5 N HCl respectively in the negative controls. After a second precipitation of the proteins with 20 % TCA, the supernatant is removed, the protein pellet is resolubilized and centrifuged (at 4 ˚C, for 10 minutes at 5000 g) three times with 1 mL of ethanol / ethyl acetate to remove excess DNPH. At the end of the washing the samples were taken up with 1 mL of Guanidine-HCl 6 M and left at 37 ° C for 15 minutes. The carbonyl content was determined with the spectrophotometer at an absorbance of 380 nm (Abs 380 nm), using the molar extinction coefficient (molar absorptivity, ε) of DNPH: 22,000 M-1 ∙ cm-1 (Reznick & Packer, 1994). The Carbonyl content was expressed as nmol/mL.

Thiol concentrations were determined according to the thiol/disulfide reaction of thiols and Ellman’s reagent (Hu, 1994). 200 µL of Seminal plasma diluted 1:100 and calibration curve are loaded respectively. Sulfhydryl groups are estimated against a standard cysteine curve (0.25-1.5 mM) after the addition of 3.5 µL of Ellman’s reagent (Sigma). The thiol concentration was measured by spectrophotometry at an optical density (OD) of 412 nm and expressed as nmol/mL.

* 1. **Experiment 2: Frozen/thawed semen study**

**3.3.1 Assessment of motility on frozen-thawed semen**

After at least a month of storage at -196 °C, three straws per ejaculate were taken and thawed in a water bath at 37 °C for thirty seconds and immediately evaluated for motility parameters by CASA flowcytometry. Motility was also assessed three hours after thawing. The setting used for motility analysis was the same as for fresh semen (paragraph 3.2.1).

* 1. **Experiment 3: Evaluation of seminal plasma metabolomics using 1H NMR**

The metabolome of the seminal plasma samples stored in liquid nitrogen will be assessed by 1H Nuclear Magnetic Resonance spectroscopy (1H NMR), a new explorative technique, which has not yet been deepened on bull sperm. Principal Component Analysis (PCA) followed by Partial-Least Squares Discriminant Analysis (PLSDA) will be used to reveal a separation between high quality bulls (HQB) and low-quality bulls (LQB) NMR spectra.

**3.5 Statistical analyses:**

Data were analysed using the R statistical environment v. 3.6.2 (The R Foundation for Statistical Computing, Vienna, Austria), except for NMR results which were analysed using the Amix 4.0.1 software, (Bruker Biospin GmbH, Germany). Unless otherwise stated, results are presented as the mean ± standard deviation. No multiple testing correction was performed.

**3.5.1 Experiment 1: Fresh semen study**

Principal component analysis (PCA) was performed on the motility parameters (VAP, VSL, VCL, ALH, BCF, TM and PM) of fresh semen and then the cluster analysis was done on the first two principal components (PCs), which explained more than 90 % of the variance. Thus, two clusters were obtained: high motility fresh (HMF) semen and low motility fresh (LMF) semen.

Correlation between motility variables and oxidative stress parameters was performed by Spearman test for non-parametric variables.

In all statistical analyses, the minimal level of significance was set at P < 0.05.

**3.5.2 Experiment 2: Frozen/ thawed semen study**

Principal component analysis (PCA) was performed on the motility parameters (VAP, VSL, VCL, ALH, BCF, TM and PM) of frozen/thawed semen and then the cluster analysis was done on the first two principal components (PCs), which explained more than 90 % of the variance. Two clusters were then obtained: high motility thawed (HMT) semen and low motility thawed (LMT) semen.

Correlation between motility variables and oxidative stress parameters were performed by Spearman test for non-parametric variables.

In all statistical analyses, the minimal level of significance was set at P < 0.05.

**3.5.3 Experiment 3: Evaluation of seminal plasma metabolomics using 1H NMR**

A total of 53 spectra (20 bulls, three replicates per bull, with seven missing replicates due to sampling problems or insufficient volume for 1H NMR analysis) were subjected to PCA multivariate analysis to highlight potential differences in the metabolic profile of seminal plasma from HQB and LQB.

The purpose of such a statistical processing, performed by using the AMIX software (Bruker Biospin), version 4.0.1, is to highlight whether the various metabolic profiles are all similar to each other or if they can be clustered into two or more groups of profiles, consisting each of profiles similar to each other but different from group to group. For this purpose, each NMR spectrum acquired (with the exclusion of the 4.50-5.10 ppm interval, corresponding to the spectral area containing the water signal) is divided into buckets, i.e. into rectangular intervals of the same size. The Principal Component Analysis is then applied to the numerical values contained in the Bucket Table, and produces two new tables, namely the Scores’ matrix and the Loadings’ matrix (See Figure 1).

Each point in the scores plot corresponds to a spectrum, i.e. to a sample, and its position, given by the coordinates PCi and PCj, indicates the statistical weight with which that spectrum contributes to the two Principal Components of the variance. The points on the plot can eventually cluster: if present, each cluster contains a set of samples similar to each other, and different from cluster to cluster, while the points external to any cluster (defined outliers) are actually spectra that, for some statistical variable, differ from the others.

Chart, scatter chart

Description automatically generated

Figure 1. Graphical representation of a scores plot and loadings plot. The values found on these plots are only used for visual purposes and are not specific to this study. Scores plot is found on the left and the loadings plot is found on the right.

Similarly, in the loadings plot each point corresponds to a bucket, and its position (coordinates PCi and PCj) indicates the statistical weight with which that bucket contributed to form the PCi and PCj. In other words, the points in the loadings plot provide the relationship between the statistical variables and the original variables (areas of the spectral peaks contained in the buckets). The overall interpretation of the PCA results arises by a combined reading of the scores and loadings plots, which shows which buckets are responsible for an outlier behavior of the samples.

Thus, since the signals of the various metabolites fall into different spectral areas (different buckets), it is possible to trace the metabolites responsible for the statistical differences between the various samples.

**4. Results**

**4.1 Experiment 1: Fresh semen study**

**4.1.1 Relation between High Motility Fresh (HMF) and Low Motility Fresh (LMF) semen groups and motility parameters.**

Using the CASA method to evaluate motility of spermatozoa in fresh semen samples, it was noted that all parameters studied showed significant differences between high and low motility groups. More specifically, the beat cross frequency (BCF), linearity (LIN), progressive motility (PM), straightness (STR), total motile spermatozoa (TM), average path velocity (VAP), curvilinear velocity (VCL) and straight-line velocity (VSL) were significantly higher in the high motility fresh (HMF) group compared to the low motility fresh (LMF) group. However, the amplitude lateral head displacement (ALH) was significantly lower (p<0.05) in the HMF group as compared to the LMF group (See Table 1, appendix Table 1).

Table 1. Motility parameter differences in fresh semen between low and high motility groups:

|  |  |  |
| --- | --- | --- |
|  | **LMF** | **HMF** |
| ALH \* | 5.88 ± 0.83 | 5.50 ± 0.75 |
| BCF \* | 29.93 ± 4.64 | 36.67 ± 3.23 |
| LIN \* | 52.82 ± 4.63 | 63.21 ± 5.38 |
| PM \* | 50.36 ± 12.32 | 68.53 ± 6.36 |
| STR \* | 82.12 ± 3.07 | 85.80 ± 2.76 |
| TM \* | 67.89 ± 17.40 | 87.34 ± 7.41 |
| VAP \* | 97.75 ± 13.85 | 124.12 ± 14.60 |
| VCL \* | 161.52 ± 24.67 | 175.83 ± 23.52 |
| VSL \* | 80.05 ± 10.44 | 107.85 ± 12.43 |

Values are expressed as mean ± standard deviation. The asterisk is representative of a significant difference (p<0.05) between the two groups LMF and HMF.

**4.1.2 Relation between oxidative stress parameters of seminal plasma and HMF and LMF semen groups**

After evaluating motility parameters of fresh spermatozoa, the effects of oxidative stress in seminal plasma were assessed in both high and low motility groups. The AOPP, carbonyl and thiol concentrations were compared between high and low motility groups (see Figures 2,3 and 4, respectively). All measured oxidative stress parameters (AOPP, carbonyls) and antioxidants (thiols) have been shown to be significantly higher (p<0.05) in the high motility group as compared to the low motility group (see Figures 2,3 and 4). The mean values of all parameters are shown in Table 2.

Chart, box and whisker chart

Description automatically generatedFigure 2. Box plot representation of the differences in advanced oxidative protein product concentrations of seminal plasma between high and low motility groups. The asterisk is representative of a significant difference (p<0.05) between the two groups LMF and HMF.

\*

**Chart, box and whisker chart

Description automatically generated**Figure 3. Box plot representation of the differences in carbonyl concentrations of seminal plasma between high and low motility groups. The asterisk is representative of a significant difference (p<0.05) between the two groups LMF and HMF.

\*

Chart, box and whisker chart

Description automatically generated

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Figure 4. Box plot representation of the differences in thiol concentrations of seminal plasma between high and low motility groups. The asterisk is representative of a significant difference (p<0.05) between the two groups LMF and HMF.

Table 2. Descriptive statistics of oxidative status parameters (AOPPs, Carbonyls, Thiols) in fresh semen samples:

|  |  |  |
| --- | --- | --- |
|  | **HMF** | **LMF** |
| AOPP (nmol/mL) | 106.90 ± 47.42 \* | 75.35 ± 33.85 |
| Carbonyls (nmol/mL) | 8.44 ± 6.08 \* | 6.57 ± 6.46 |
| Thiols (nmol/mL) | 143.74 ± 118.60 \* | 81.07 ± 92.35 |

Values are expressed as mean ± standard deviation. The asterisk is representative of a significant difference (p<0.05) between the two groups LMF and HMF.

**4.1.3 Relation between oxidative stress parameters of seminal plasma and sperm motility of fresh semen**

Finally, the relationship between oxidative stress parameters in seminal plasma and the motility of fresh spermatozoa was determined by Spearman correlation. As seen in Table 3., AOPP and thiol concentrations were significantly (p<0.05) positively correlated with linearity (r= 0.35 and r= 0.34, respectively) of spermatozoa, yet AOPP and thiol concentrations were found to be negatively correlated with ALH (r= -0.40 and r= -0.42, respectively) of spermatozoa in fresh semen samples (See Table 3).

|  |  |  |  |
| --- | --- | --- | --- |
|  | **AOPP (nmol/mL)** | **Thiols (nmol/mL)** | **Carbonyls (nmol/mL)** |
| **ALH** | -0.40\* | -0.42\* | -0.21 |
| **LIN** | 0.35\* | 0.34\* | 0.08 |
| **PM** | 0.17 | 0.13 | 0.05 |
| **STR** | 0.15 | 0.17 | 0.05 |
| **TM** | 0.18 | 0.11 | 0.07 |
| **VAP** | 0.11 | 0.05 | -0.08 |
| **VCL** | -0.08 | -0.13 | -0.05 |
| **VSL** | 0.16 | 0.14 | -0.03 |

Table 3. Correlation between oxidative stress parameters in seminal plasma and motility parameters of spermatozoa, in fresh semen samples:

Values are expressed as Spearman’s correlation coefficient r. The asterisk is representative of a significant correlation (p<0.05) between the oxidative stress parameters of seminal plasma and the motility parameters of spermatozoa.

**4.2 Experiment 2: Frozen/thawed semen study**

**4.2.1 Relation between semen groups (HMT and LMT) and motility parameters, 0 h and 3 h after cryopreservation.**

In order to understand the effects of cryopreservation on sperm motility, the samples were analyzed at zero hours/immediately (0 h) after thawing and three hours after thawing (3 h). LMT and HMT clusters were compared for each motility parameter. Firstly, by taking into consideration the effect of time within groups as well as the differences between groups at a specific time (either 0 h or 3 h). As seen in Table 4, ALH significantly increases between 0 h and 3 h in the LMT group (4.90 ± 0.61at 0 h and 5.53 ± 1.25at 3 h), however there is no significant change between 0 h and 3 h in the HMT group. In the LMT all motility parameters show a significant decrease between 0 h and 3 h after thawing, except for ALH and VCL. The latter does not present any significant change with time (113.77 ± 11.29at 0 h and 106.24 ± 39.35 at 3 h). In the HMT group, all motility parameters show a significant decrease between 0h and 3h after thawing, except ALH which does not show any significant change over time. As for the comparison of LMT and HMT at 0 h after thawing, ALH, PM, TM, VAP, VCL and VSL show significant differences between the groups. All these parameters are significantly higher in the HMT group. At 3 h after thawing, BCF, PM, TM and VCL show significant differences between the LMT and HMT groups. More specifically, BCF and VCL are significantly lower in the HMT group (19.83 ± 5.61 and 98.84 ± 22.23, respectively) as opposed to the LMT group (24.29 ± 8.25 and 106.24 ± 39.35, respectively).

Table 4. Motility parameters of LMT and HMT groups after thawing (0 h and 3 h incubation times):

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **LMT** | | **HMT** | |
|  | 0h | 3h | 0h | 3h |
| **ALH** | 4.90 ± 0.61a | 5.53 ± 1.25b | 5.56 ± 0.57b | 5.47 ± 0.91b |
| **BCF** | 30.19 ± 3.22b | 24.29 ± 8.25 c | 30.66 ± 2.41b | 19.83 ± 5.61a |
| **LIN** | 51.04 ± 4.89b | 42.83 ± 6.70a | 52.77 ± 4.56b | 43.34 ± 5.73a |
| **PM** | 18.28 ± 10.79b | 9.88 ± 8.71a | 31.29 ± 8.57c | 14.57 ± 8.57b |
| **STR** | 84.04 ± 5.48b | 74.33 ± 8.80a | 84.66 ± 3.22b | 75.11 ± 5.67a |
| **TM** | 36.60 ± 17.67b | 27.08 ± 17.17a | 57.26 ± 9.76c | 38.09 ± 14.84b |
| **VAP** | 64.93 ± 6.16b | 57.40 ± 17.84a | 79.78 ± 9.89c | 54.69 ± 11.10a |
| **VCL** | 113.77 ± 11.29b | 106.24 ± 39.35b | 133.64 ± 15.2c | 98.84 ± 22.23a |
| **VSL** | 54.42 ± 6.17b | 42.68 ± 13.74a | 68.14 ± 9.97c | 40.77 ± 9.57a |

Data are expressed as means ± standard deviation of means. Different superscript letters (a-b-c) represent significant interactions between groups (LMT and HMT) and incubation times (0 h and 3 h). More specifically the superscript letter “a” is representative of the smallest value found within a row.

**4.2.2 Relation between oxidative stress parameters of seminal plasma, HMT and LMT semen groups.**

As previously studied in the fresh semen samples, the differences in oxidative stress parameters between HMT and LMT groups were also determined. Similarly to fresh seminal plasma samples, AOPP, carbonyl and thiol concentrations were also found to be significantly higher in the HMT group as compared to the LMT group (Figures 5,6 and 7 respectively). The mean values of all parameters are shown in Table 5.

**Chart, box and whisker chart

Description automatically generated**

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Figure 5. Box plot representation of the differences in AOPP concentrations of seminal plasma between high and low motility groups after thawing. The asterisk is representative of a significant difference (p<0.05) between the two groups LMT and HMT.

Chart, box and whisker chart

Description automatically generated

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Figure 6. Box plot representation of the differences in carbonyl concentrations of seminal plasma between high and low motility groups after thawing. The asterisk is representative of a significant difference (p<0.05) between the two groups LMT and HMT.

**Chart, box and whisker chart

Description automatically generated**

\*

Figure 7. Box plot representation of the differences in thiol concentrations of seminal plasma between high and low motility groups after thawing. The asterisk is representative of a significant difference (p<0.05) between the two groups LMT and HMT.

Table 5. Descriptive statistics of oxidative status parameters (AOPPs, Carbonyls, Thiols) in frozen/thawed semen samples:

|  |  |  |
| --- | --- | --- |
|  | **HMT** | **LMT** |
| AOPP (nmol/mL) | 105.48 ± 47.24\* | 82.40 ± 39.96 |
| Carbonyls (nmol/mL) | 8.29 ± 6.02\* | 7.06 ± 6.56 |
| Thiols (nmol/mL) | 136.84 ± 124.70\* | 100.76 ± 93.80 |

Values are expressed as mean ± standard deviation. The asterisk is representative of a significant difference (p<0.05) between the two groups LMT and HMT.

**4.2.3 Relation between oxidative stress parameters of seminal plasma and sperm motility after cryopreservation.**

Following the independent study of sperm motility and oxidative stress parameters in frozen/thawed seminal plasma and the differences found between high and low motility groups, the relationship between both variables (sperm motility and oxidative stress parameters) was determined by Spearman correlation (see Table 6).

Table 6. Correlation between oxidative stress parameters in seminal plasma and motility parameters of spermatozoa, in frozen/thawed semen samples:

|  |  |  |  |
| --- | --- | --- | --- |
|  | **AOPP (nmol/mL)** | **Thiols (nmol/mL)** | **Carbonyls (nmol/mL)** |
| **ALH** | -0.03 | 0.01 | 0.004 |
| **BCF** | 0.03 | -0.003 | 0.03 |
| **LIN** | 0.14 | 0.12 | 0.04 |
| **PM** | 0.27 \* | 0.24 \* | 0.13 |
| **TM** | 0.33 \* | 0.30 \* | 0.22 |
| **VAP** | -0.04 | 0.02 | 0.004 |
| **VCL** | -0.16 | -0.10 | -0.07 |
| **VSL** | -0.04 | 0.01 | -0.003 |

Values are expressed as Spearman’s correlation coefficient r. The asterisk is representative of a significant correlation (p<0.05) between the oxidative stress parameters of seminal plasma and the motility parameters of spermatozoa.

Considering all oxidative stress parameters, known as AOPPs, thiols and carbonyls, solely the first two (AOPPs and thiols) showed a significant, positive correlation with TM (r= 0.27 and r= 0.24, respectively) and PM (r= 0.33 and r= 0.30, respectively) (See Table 6).

**4.3 Experiment 3: Evaluation of seminal plasma metabolomics using 1H NMR**

**4.3.1 Metabolomics of seminal plasma and relation with semen motility before and after cryopreservation**

The relationship between sperm motility and the ability to endure cryopreservation is related to the molecular composition of seminal plasma: the liquid environment that contains metabolites that are used and secreted by spermatozoa. By using NMR, the metabolomic profiles of all 52 samples were obtained and assessed by PCA statistical analysis, yielding scores and loadings plots. To better visualize the clustering of samples with different motility, in the scores plots (Figure 8 and Figure 9), the LMF and HMF groups are represented in two different colors (red and blue respectively). As seen in Figure 7, there is segregation between LMF (21 samples) and HMF clusters (31 samples). This indicates differences in metabolomic profiles, which could explain the differences in motility parameters between the groups.



Figure 8. Scores plot of metabolomic profiles of LMF and HMF groups. Each dot is representative of one sample. Red coloring is indicative of LMF samples and blue coloring is indicative of HMF samples. The dotted ellipse encloses three samples classified as HMF but resulting as outliers in metabolic profile.

A score plot was also generated based on the motility parameters of frozen semen. In Figure 9, the clustering of LMT and HMT groups is different compared to that seen previously in Figure 8. The number of HMT samples has also increased from 31 to 39.

Diagram, bubble chart

Description automatically generated

Figure 9. Scores plot of metabolomic profiles of LMT and HMT groups. Each dot is representative of one sample. Red coloring is indicative of LMT samples and blue coloring is indicative of HMT samples.

The clustering of LM and HM groups (by PCA analysis) is considered reliable as the total variance explained by the first four Principal Components is of 85.83 %, with PC1 explaining 49.12 % and PC2 15.04 %.

Following the score plot, the PCA analysis also generated a loadings plot (see Figure 10). The loadings plot below shows all buckets and their location along the x-axis of the NMR spectra. This is indicated by the number found above each dot, expressed in ppm, the unit of measure generally used for NMR spectra. The outliers or dots found furthest away from the main cloud and their “coordinates” are representative of specific metabolites in the area (or bucket). These metabolites could explain the differences seen in sperm motility between low and high motility groups. The loadings plot is representative of fresh seminal plasma metabolites.



Figure 10. Loadings plot of metabolomic profiles of LMF and HMF groups. Each dot is representative of one bucket area. The number found above each dot is the ppm value, or the location of the dot on the x-axis of the NMR spectra.

**5. Discussion:**

With the need to find sustainable ways of breeding cattle, as well as ensuring equal access and distribution of food resources, new scientific methods have been put into place to preserve semen samples and study their reproductive potential after handling (such as freezing and thawing). In this study, oxidative stress and metabolomic markers were assessed to determine their efficacy in evaluating differences in bull semen quality, before and after cryopreservation.

In fresh semen samples, all motility parameters were found to be significantly higher in the HMF group, with the exception of ALH. In fact, higher ALH levels are indicative of sperm hyperactivity (Mortimer et al., 1997), which is a feature that spermatozoa acquire in the female genital tract. *In vivo*, spermatozoa acquire fertilizing abilities along their trajectory, towards the oocyte. The main process associated with fertilization is capacitation, a process initiated in the female genital tract, in which spermatozoa acquire the ability to fertilize the oocyte by complex biochemical changes (Lessard et al., 2011; Suarez, 2016). The capacitation process involves sperm hyperactivation, which consists in drastic changes of motion patterns, that spermatozoa exhibit during their progress through the female oviduct (Suarez, 2008). The hyperactivated pattern is characterized by movements in a random path (described as “whiplash” or “figure-8” motions), which are usually associated with a decrease in LIN and STR. In addition, hyperactivation is characterized by an increase in VAP, VCL, BCF and ALH (Suarez, 2008). The process ends with the acrosome reaction of the sperm head, which occurs once the spermatozoa bind the oocyte zona pellucida. The acrosome reaction releases enzymes that create a “gateway” into the *zona pellucida* and the hyperactivation of the flagellum allows spermatozoa to penetrate the oocyte (Mortimer, 1997; Murase et al., 2010). Regardless of the biological function of hyperactivity *in vivo*, *in vitro*, the circular motion exhibited by hyperactivated spermatozoa is considered a marker of sub-fertility in bulls (Murase et al., 2010; Hyakutake et al., 2018). Additionally, in our study motility parameters LIN and PM were significantly lower in the LMF group, indicating a non-progressive motion of movement and potential sub-fertility (Hyakutake et al., 2018).

Oxidative stress parameters of seminal plasma, namely AOPPs and carbonyls were also found to significantly differ between HM and LM groups, more specifically these were in higher concentrations in the HMF group. Although excessive ROS production is usually associated with mitochondrial dysfunction, DNA alterations, membrane permeability and motility impairments, sufficient levels of ROS are required for hyperactivation, capacitation and oocyte-sperm fusion (Barranco et al., 2021). Oxidative stress is described as an imbalance between prooxidant and antioxidant components, which is ultimately translated into molecular and physiological damage of cells.

In numerous studies, ROS concentrations have shown to be negatively correlated with motility parameters and sperm viability (Takeshima et al., 2017; Homa et al., 2019; Al Smadi et al., 2021; Khoi et al., 2021). Few recent studies investigate the relationship between AOPPs concentrations in seminal plasma and male (in)fertility. In contrast with our results, a study by Kratz et al. (2016), observed that AOPP levels are significantly negatively correlated with male fertility. In the same study a significant decrease of melatonin, which acts as antioxidant, has been observed in infertile subjects. However, while the energy metabolism of human semen is dependent on glycolytic pathways (Nascimento et al., 2008), in other species such as equine, the main generation of ATP (required for movement) is reliant on intensive mitochondrial activity (Meyers et al., 2019; Giaretta et al. 2022;). Thus, considering that semen motility is dependent on energy production, increased oxidative phosphorylation of proteins (AOPPs and carbonyls) in bulls causes an increase in ROS generation, which could (paradoxically), be positively correlated to semen motility and fertility (Gibb et al., 2014; Meyers 2019). Regarding bull semen, a recent study elucidated the role of mitochondrial activity on specific sperm functions, suggesting that bovine sperm motility is impacted by mitochondrial functionality (Bulkeley, 2021). According to Bordignon et al. (2014), AOPPs, generated by bovine active neutrophils, inhibit free radical production *in vitro*, suggesting that AOPPs can act as an oxidative stress scavenger. Indeed, highly active spermatozoa produce more ROS, which leads to more oxidized seminal plasma proteins (AOPP), that in turn act as ROS scavengers. In this experiment, we cannot identify what proteins are involved in this phenomenon, but we can assert that in HM groups seminal plasma proteins may “protect” spermatozoa from ROS production.

In semen, the protective, antioxidant agents are found in seminal plasma. Antioxidant components are sourced under stressful conditions and are used as effective neutralizers against ROS products. There is a network of varying antioxidants, enzymatic, non-enzymatic and low molecular weight compounds that work together into maintaining prooxidant-antioxidant homeostasis within cells (Kratz & Piwowar, 2017). One of these important antioxidants is thiols. The ROS damage may lead to a decrease of sulfhydryl’s groups–SH groups (thiols), increasing the protein peroxidation and amino acid carbonyl derivatives (Domoslawska et al., 2018). In our experiment, the significant higher amounts of thiols and carbonyls in seminal plasma have been observed in both HMF and HMT groups. This may suggest that the production of ROS is being compensated by an equal production of antioxidants in seminal plasma.

Even though the general assumption on oxidative stress is that it is regulated by an even production of antioxidants, numerous studies have differing explanations on the interactions and relationship between prooxidant-antioxidant components. Based on a study by Kratz et al., (2016), increased levels of AOPPs are accompanied by an increase in total antioxidant capacity in spermatozoa. However, in their study, Kratz et al. (2016) concluded that the positive correlation found between AOPP concentrations and antioxidant capacity might not be enough to assume a causal relationship, where increased levels of antioxidants counteract/annul the negative effects of ROS on cells. Thus, the damage caused by ROS could be irreversible, regardless of the amount of antioxidants secreted. Making it a tedious task to accurately assess sperm quality and fertility capacities.

In this study, AOPP and thiols concentrations in fresh semen samples were found to be negatively correlated to ALH and positively correlated with LIN. As for frozen/thawed samples, AOPP and thiols were positively correlated with PM and TM. Therefore, the increase of AOPPs and carbonyls may not necessarily lead to a decrease of sperm quality. On the contrary, these results suggest that in HMF and HMT groups, the intense mitochondrial activity could be the major source of ROS. Higher amount of AOPP, carbonyls and thiols in seminal plasma could compensate for this increased ROS production on high motility semen. This is also supported by the changes in ROS and antioxidant levels seen three hours after thawing, whereby the HMT group maintained overall better motility parameters.

Based on these observations, it is assumed that seminal plasma may have an important protective function against ROS leakage from active sperms. The total antioxidant capacity of seminal plasma, which summarizes the overall activity of all types of antioxidants, may be modulate by the dynamic equilibrium between AOPP, carbonyls and thiols.

Traditionally, male fertility has been assessed based on motility, viability and morphology. However, these parameters have been shown to be limited in the accurate determination of fertility and cryopreservation capacity. This report is one of few to analyze the metabolomic profile of seminal plasma in bulls. Seminal plasma has been shown to regulate sperm motility, the conditions in the female reproductive tract as well as affect offspring health (Mateo-Otero et al., 2020).

1H NMR is most commonly used to detect and quantify numerous metabolites found in a mixed-composition fluid, such as seminal plasma (Mateo-Otero et al., 2020). In this study, the score plots revealed a clear clustering between high and low motility groups, demonstrating that there is a definite difference in metabolomic profiles between the two groups. In the loadings plot, 18 metabolites were found to explain the differences seen between both groups, however the specific metabolites responsible for this clustering were not specified in this study (and are the subject to further evaluation, in the continuation of this work). Nevertheless, according to Kumar et al. (2015), citric acid, taurine/tryptamine, leucine and isoleucine were recognized as the most significant seminal plasma metabolites. This was based on the Variable Importance Projection value (VIP), a scale from 2-5, which is a representative of the potential/significance of a given biomarker (Kumar et al., 2015).

By comparing biomarkers in both groups, high fertility bulls were shown to have low levels of citrate and isoleucine whilst having higher levels of taurine/tryptamine and leucine. These observations correlate with the cellular function of each of these components. Citrate (citric acid) is a chelator of calcium ions and hinders sperm capacitation as well as acrosome reactions. It has also been shown to play a role in coagulation, jellification of semen in other animal species (Hart, 1970; Kumar et al., 2015). On the other hand, tryptamine has been shown to support acrosomal reactions and sperm motility (Jiménez-Trejo et al., 2012; Kumar et al., 2015). These results depict the value of metabolite studies and their role in prematurely detecting bull (in)fertility.

In conclusion, bull (in)fertility, is a multifactorial trait, with low heritability and a large variation between individuals and species. This study demonstrated the importance of evaluating the composition of seminal plasma in relation to sperm motility and cryopreservation tolerance. As seminal plasma has both inhibitory and stimulatory roles in sperm function and cryopreservation, the identification of the critical role players of seminal plasma which affect sperm quality, could predict the cryopreservation potential of semen. Oxidative status parameters (AOPPs, carbonyls and thiols) and metabolomic profiling, could be, together, efficient (in)fertility biomarkers in bulls. Further studies, in understanding the causal relationship between sperm motility and oxidative stress (before and after cryopreservation), as well as the investigation on specific metabolite concentrations which and how they differ between HM and LM ejaculates, would aid in the better understanding and prediction of fertility in bulls.

**6. Societal and Ethical considerations**

With an ever-growing global population, there is an increasing demand in food resources and a dire need for improvement within the cattle breeding industry. The cattle breeding industry is largely threatened by an increasing rate of infertility, a lack of space for housing and growing feed, which could lead to great economic losses and an uneven repartition of resources (Salter, 2016).

The semen collected in this study complied with general animal welfare guidelines. Bulls were placed in a safe enclosed area, allowing them to move and mount the artificial cow freely. This method of semen collection is thought to reduce stress and the risks of injury on both the animals and the personnel.

Nowadays, artificial insemination has been widely implemented on farms, with the aim of maximizing reproductive potential, reducing disease spread (by minimizing physical contact) and impact of cattle breeding on global warming. This is accompanied by the use of cryopreservation, which allows samples to be preserved over time, transported readily to many locations and maximize genetic output. The livestock sector is responsible for emitting 14.5 percent of all human-induced GHG emissions, making it an important factor in climate change (FAO, 2021). Thus, understanding the underlying mechanisms of fertility, breeding and optimization of livestock produce, through artificial insemination, are of great importance, both economically and environmentally.

Advances in detecting and predicting (in)fertility in bulls, yield insights into understanding infertility related diseases found in other species. More specifically, for humans, these advances could lead to more efficient treatments for infertility as well as enhanced methods of in vitro fertilization (Verberckmoes et al., 2004).

Thus, the early detection of infertility in bulls is of great economic, environmental and conservational importance not only for the bettering of artificial insemination programs in cattle but also for the protection of other endangered species and the understanding of male infertility related diseases in other species.

**7. Acknowledgements**

I would like to personally thank Professor Gianfranco Gabai for his guidance and patience throughout the completion of this project, as well as his kind reception upon my arrival to Italy. I would also like to thank Professor Elisa Giaretta for her advice, for accompanying me every step of the way, and for sparking an interest in the study of fertility biomarkers within the animal world. Finally, I would like to thank Professor Lucio Zennaro for welcoming me in his laboratory and teaching me about the uses of NMR. It has been a very fulfilling experience with a lot of knowledge gained throughout.

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**Appendix:**

Table 1. Description of motility parameters:

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| --- | --- |
| **Motility parameters** | **Description** |
| ALH – Amplitude of Lateral Head Displacement | ALH is representative of the distance between the peak and low point of the centroid’s path (the centroid is the “mid-point” of the sperm head). |
| BCF – Beat Cross Frequency | BCF is the number of lateral oscillatory movements of the sperm head around the mean trajectory. |
| LIN – Linearity | LIN represents the linear or straight trajectory of a single spermatozoa per unit of time. |
| PM – Progressive Motility | PM is the percentage of spermatozoa that show a linear or directional movement. |
| TM – Total Motility | TM is the percentage of all motile spermatozoa in a sample. |
| VAP – Average Path Velocity | VAP is the mean trajectory of spermatozoa per unit of time (or mean velocity). |
| VCL – Curvilinear Velocity | VCL is specific to a single spermatozoon’s trajectory and represents the instantaneous recording of its sequential progression. |
| VSL – Straight-line Velocity | VSL is the straight trajectory of all spermatozoa over time. |