Analysing Bovine sperm cells using Flow Cytometry

Irene Waters

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Discipline / School Plant & AgriBiosciences Research Centre (PABC) & School of Biological and Chemicals Sciences

Name of Supervisor(s) Prof. Charles Spillane, Dr Galina Brychkova, Dr Ciara O'Meara

Declaration

I, the candidate, certify that the Thesis is all my own work and that I have not obtained a degree in this University or elsewhere on the basis of any of this work.

Signed: _____ Date: _____

Dedication

I dedicate my thesis to my mother, Mary and my fiancé, Stephen, for their endless love, support and encouragement. Without their continuous support, I would not be where or who I am today.

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- Finally, to my family, who love and support me unconditionally.

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Chapter One

Literature Review

Abstract:
The aim of this review is to consider how flow cytometry can be used to assess the quality of semen used in artificial insemination. In recent years, flow cytometry has become the dominant approach in sperm evaluation and is increasingly being used for quality assessment and for research in veterinary science. The focus of this review will be in bovine andrology and the dominance that flow cytometry has over other methods in semen quality assessment. The connection between flow cytometry analysis and bull fertility are reviewed. The accurate assessment of bull fertility is hugely important to the beef and dairy industry. In this review it will look at how cryopreservation can affect sperm membranes; plasma membrane, acrosomal membrane and mitochondrial membrane. The review will look at methods to assess sperm quality using flow cytometry.

1.1.1 Introduction:

Artificial insemination (AI) is a method where male bull sperm is collected from selected sires and then artificially introduced into the female reproductive tract by a trained AI technician. Artificial insemination is a powerful tool for improved cattle breeding by using superior genetic sires. Semen from top genetic sires is used to improve the genetics in the national herd. *(*Al Makhzoomi, et al. 2008*)* The use of AI in the Diary herd has increased rapidly over recent years, which allows farmers to select semen from the top EBI sires. EBI is a single figure profit index, which helps identify the most profitable bulls for breeding dairy herd replacements. (ICBF, 2022)

Fertility can be defined as the ability to conceive offspring. Fertility traits have a major influence on production, these traits play a major role in the livestock industry. High fertility rates impact beef and dairy farmer's production and profits. Conception rates are influenced by the quality of semen, conception rates can be increased by determining a bull's fertility. Reproductive failure is a result of poor quality semen or low volume of semen. High conception rates in AI are crucial for famers, weather fresh semen or conventionally frozen semen is used for artificial insemination, the best quality sperm straws are required to obtain high conception rates. There are many aspects that can determine a bull's semen quality, injury, disease, nutrition, and heat stress can all decrease sperm quality. Bull fertility traits can be hereditary, which allows genetic selection to enhance bull fertility. *(*Sakase, M. et al. 2020*) (*Fuerst-Waltl, B., et al. 2006*)*

An evaluation of semen quality is essential in the AI industry, the evaluation of sperm (ejaculation) quality allows for the highest quality sperm being used in AI. Once the ejaculate is collected the sample is inspected. The following production data is collected for each ejaculation; volume of ejaculate, sperm concentration, visual evaluation of colour and density and visual evaluation of blood or dirt. Once the production data has been collected for the ejaculation then the of the sperm quality is evaluated; sperm viability, sperm mobility and sperm morphology is assessed. *(*Al Makhzoomi, et al. 2008*) (*Fuerst-Waltl, B., et al. 2006*)* Research by *Druet et al. (2009)* shows that, semen production traits were determined by genetic factors. Traits such as volume and sperm concentration were somewhat genetically heredity versus the semen quality traits, such as motility and abnormal sperm cells were

distinctly heredity. *(*Hering, D, et al. 2014*)* If the ejaculation can passed all aspects of the quality evaluation, it can then be processed into straws for frozen semen in AI. Sperm viability, sperm mobility and sperm morphology is assessed again after thawing to determine if the frozen semen meets the requirements to be used in AI. *(*Al Makhzoomi, et al. 2008*)*

Sperm viability is the percentage of live sperm in a sample, sperm viability is an indicator of sperm quality. Poor viability and high DNA fragmentation are linked to male infertility. Poor viability and DNA fragmentation are interconnected in where DNA fragmentation is one of the last steps before spermatozoa (sperm) death. *(*Samplaski, M., et al. 2015*)* Sperm motility is the capability of the sperm to move in a forward progress manor. Good sperm motility is a key factor in fertility, the sperm cells need to have good movement in a female reproductive tract to reach and fertilize the egg. Poor sperm motility is linked to male infertility. Sperm morphology relates to the shape, size and appearance of sperm cells. Where large quantities, roughly >30% of abnormal sperm cells can lead to fertility issues. With increased abnormalities to the sperm cells it becomes harder for the sperm to fertilize the egg. Abnormalities can be seen in misshaped heads, proximal droplets, distal droplets, bent or thick mid pieces, coiled tails and dag defect. These morphological defects in sperm cells are proven to show connection with reduced fertility. *(*Al Makhzoomi, et al. 2008*)*

Sperm morphology refers to the shape sperm; bull sperm have paddle-shaped heads and long tails called a flagellum. Under a microscope the shape of the sperm head and the size of the sperm can be analysed. Sperm morphology allows the percentages of sperm that are normal in size and shape to be determined. These sperm are the most likely to be able to fertilize an egg, Sperm morphology affects fertility because sperm have to be a certain shape to be able to penetrate an egg. Morphology plays a major role in bull fertility along with other factors. In all ejaculates there will be some abnormal cells, which when in low percentages it should not affect the sperm form fertilizing and egg. Sperm shapes can be classed into two categories; primary abnormalities and secondary abnormalities. Where primary abnormalities are defined as; significant defect which can hinder the sperm's potential to fertilise an egg.

Secondary abnormalities appear to have less effects on sperm cell and usually caused at the time of ejaculation.

Another factor that can affect sperm quality is bull age. In very young bulls immaturity plays a role in semen quality, high abnormalities in young sperm cells are common due to immaturity. Young bulls tend to produce low volumes of ejaculates when bulls are first jumped. These low volumes on ejaculates tend to have low sperm concentrations, with both low volume ejaculates and low sperm concentrations lead to poor quality ejaculates. To overcome this poor quality ejaculates in young bulls, studies show that collecting young bulls ejaculates regularly helps overcome these immaturities. *(*Taaffe, P, et al. 2020*)* Studies show as a bull gets older, the volume of the ejaculated by the bull increases and semen quality increases. A study carried out by *Hallap T. et al. (2004),* where sperm morphology was assessed post thaw. It was determined that sperm motility and membrane integrity improved with age but sperm morphology seemed to be unaffected with age. *(*Al Makhzoomi, et al. 2008*)*

1.1.2. The Sperm Cell

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Image 1. The sperm cell

The sperm cell is made up of, the head, the mid-piece and the tail. The sperm is enclosed by a plasma membrane. The head of the sperm contains the nucleus, this is the DNA/genetic material of the cell. The tip of the head is called the acrosome, the acrosome uses enzymes to assist the sperm in binding to the ovum (egg cell). The mid-piece of the sperm contains the mitochondria, the mitochondria produces the energy to the sperm cell tail, that it needs to move. The tail of the sperm is used to transport the sperm to the egg in the reproductive tract. The role of the sperm cell is to reach an ovum, then bind to the ovum and once attached the sperm can penetrated and fertilise the egg to produce new genetic material. The intact structure of the sperm cell is crucial in fertilization.

1.1.3. Semen Processing

Ejaculate arrives to the laboratory, quality checks are carried out on documentation. Once all documentation connects to the ejaculates, the ejaculates can enter production. Ejaculates are weighed to get the size (volume) of ejaculate, a raw semen sample is then tested for sperm concentration using an Accucell (IMV Technologies), quality checks are carried out under a microscope, assessing; total sperm viability, sperm motility (5 point scale: 1= sluggish/ twitching/ no forward progressive movement, 3= more curvilinear, some progression, 5 =excellent forward progression) and morphology . All ejaculates which meet the quality standards, where total sperm viability ≥70% and have a motility score of ≥3 and ≤30% abnormalities in sperm cells, are accepted into production, while samples which fail to meet these quality standards are rejected for production. *(*Murphy E, et al. 2018*)* Accepted ejaculates are then filled into straws and frozen and stored in liquid nitrogen (−196 °C). Straws are then reassessed to insure quality after semen has been frozen, freezing of straws may lead to loss of viability. *(*Murphy E, et al. 2018*)* The cold shock can also form functional changes in sperm cells, sperm plasma membrane can lead to changes in structure and mitochondria can lead to a calcium build up. *(*Jäkel, H., et al. 2021*)*

Four straws are tested from each ejaculate and assessed under a microscope where total sperm viability, sperm motility (5 point scale: 1= sluggish/ twitching/ no forward progressive movement, 5 =excellent forward progression) and morphology are assessed. All ejaculates which meet the quality standards, where total sperm viability ≥40% and have a motility score of ≥3 and ≤30% abnormalities in sperm cells, *(*Murphy E, et al. 2018*)*

Once frozen straws can be stored in liquid nitrogen (−196 °C) for long term, through cryopreservation, where the use of extreme low temperatures helps preserve living cells, cryopreservation stops the metabolic activities of the sperm cells and helps prevent loss of fertility. Semen quality can be affected by other factors, such as, type of extender, bacterial contamination. The presence of bacteria in sperm has adverse effects on sperm viability, motility and increase abnormalities in sperm cells. Bacteria from in the ejaculates can also have effects on the mitochondrial function which

causes formation of reactive oxygen species which leads to increasing DNA fragmentation. *(*Abadi A, et al. 2020*)* Studies show that bacteria can have huge negative influence on fertility.

Bull fertility is assessed using semen analysis, a visual assessment of viability, motility and morphology using a light microscope. Although visual assessment using a light microscope can help understand bull fertility, this visual technique cannot describe the whole biological properties of a sperm cell, properties that influence fertilization. This traditional method to assess sperm cells also is a subjective method, which can vary between analysts. Automated methods, such as flow cytometer can be used to help reduce variability that comes with the traditional method. Flow cytometer can be used to assess multiply sperm traits, such as sperm cell viability, DNA structure, acrosomal integrity and mitochondrial function. Flow cytometry is a valuable tool for andrology studies, being able to assess an individual bull to determine the fertility potential. Flow cytometry can measure one of more fluorescent stains, assessing multiply cell properties rapidly with precision, sensitivity and accuracy. *(*Cordelli, E, et al. 2005*) (*Harstine, B., et al. 2005*)*

1.1.4. Flow Cytomerty

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Image 2. Diagram of Flow Cytometry

Flow cytometry is based on the measurement of light scattered by particles, and the fluorescence observed when these particles are passed in a stream through a laser beam. Flow cytometry is used to identify and measure the physical and chemical characteristics of cells or particles. There are three systems that make up a flow cytometer; the fluidics system, optics system, and electronics system. The fluidics system transports a sample in sheath fluid stream to the laser beam. The sheath fluid restricts the sample as it passes through the laser beam, the cells pass through the laser beam in single file.

The optics system is comprised of an excitation optics and collection optics. The excitation optics is made up of lasers and lenses that help focus the laser beam on

the flow of sample. The collection optics contains a collection lens that collected the light that is emitted from the cells that have passed through the laser beam. The fluorescent signals are collected by detectors. The electronics system is translates the signals from the detectors to digital signals onto a computer. These signals can then be graphed and plotted and analysis carried out on the signals emitted by a sample. There are many advantages to flow cytometry, it allows for multiply analysis to be carried out simultaneously on samples. It can analyse many characteristics such as; partical size, granularity (complexity) and fluorescence intensity. (Flow cytometry, 2022)

Flow cytomerty has become an major step in sperm quality assessment in bovine andrology. Flow cytrometry can be used in the assessment many aspects of a sperm cell; plasma membrane, acrosome integrity, mitochondrial status, DNA fragmentation, intracellular Ca^{2+} levels and oxidative stress can all be assessed using flow cytrometery. Studies using flow cytometery have shown a relationship between bull fertility and sperm characteristics, plasma membrane and acrosome integrity, mitochondrial function, and Ca^{2+} levels. *(*Bucher, K, et al. 2019*)* Flow cytrometery using 3 or more colours (multicolour) have an edge against single colour assays in sperm assessment. The multicolour technique allows for the identification of sperm sub population, which gives more information into sperm functions. *(*Bucher, K, et al. 2019*)*

Flow cytometry is more popular in sperm analysis, replacing slower, less accurate methods. The capability of testing for multiply aspects of an ejaculate at once, has huge advantages over traditional methods. *(*Bucher, K, et al. 2019). Flow cytometry allows large scale repetition become feasible, thousands of cells can be analysed in seconds. Large number of data can be collected for each sample. The sample is placed in the flow cytometer where; the each cell passes through one or more lasers one by one. The emitted light is filtered and sent to the detectors where, the signals are amplified and data is plotted on a graph for interpretation. *(*Bucher, K, et al. 2019*)*)

1.1.5. Sperm Concentration

Flow cytometry can be used in sperm count, by using the grating based on the sperm cell size. The grating allows for all sperm population to determine by their size and all debris such as white blood cells, skin cells and bacteria be determined and then a true count of sperm population is determined. *(*Cordelli, E, et al. 2005*)* Flow cytometry analyses thousands of events in seconds, which allows for rapid and reliable results compared to other methods.

1.1.6. Viability

Flow cytometry is a rapid and accurate method to determine viable and dead sperm cells. Fluorescent staining of sperm can determine the viability of viable cells and non-viable cells. Non-viable sperm cells can be determined using Propidium iodide (PI) stain, PI is a florescent dye that stains DNA. The live sperm cells have an intact membrane which blocks the stain form penetrating the cell, where the dead sperm cells have damaged permeable membrane, which allows the PI stain to bind to the DNA in the dead cells. *(*Cordelli, E, et al. 2005*)*

1.1.7. Mitochondrial Membrane

Sperm motility is a fundamental when it comes to fertility. Progressive sperm motility is associated with high fertility. Adenosine triphosphate (ATP) is and energy carrying molecule that can be found in cells. ATP production occurs within the mitochondrial matrix, ATP is required for cells to have progressive motility. Studies show that mitochondria have a part to play in energy production which is necessary for cell survival. Mitochondrial function can be influenced by factors such as, diseases, environmental conditions, nutrition and injury. Mitochondrial function indicates the health of a cell and mitochondrial membrane potential (MMP) can help determine sperm motility, loss of MMP is an indicator of cell stress and can lead to cell death, for this reason MMP can be used in assessing sperm quality. *(*Cordelli, E, et al. 2005*)*

The probes that detect MMP are positively charged, cationic fluorescent stains are used to assess MMP, mitochondrial viability and function. Cationic dyes such as rhodamine-123 and 5,5,6,6’-tetrachloro-1,1’,3,3’ tetraethylbenzimi-

dazoylcarbocyanine iodide (JC-1). JC-1 is a green fluorescent dye, JC-1 advantages compare to other cationic dyes is that it can detect both healthy and unhealthy cells. When JC-1 dye enters the mitochondria it changes its fluorescence properties based on the aggression of the probe, in healthy cells with high MMP, a red fluorescence forms. But unhealthy cells with low MMP or apoptotic cells it remains in the original form, which exhibits a green florescence. Cells with high MMP allows the JC-1 dye to build up in the energy filled, negatively charged mitochondrion which on impulse produces a red fluorescence. Cells with low MMP or apoptotic cells do not have the same built up in the energy and have a less negative charged mitochondrion, thus the JC-1 dye does not build up enough to cause excitement and the impulse colour change does not occur, the dye stays the original green colour. *(*Farzane Sivandzade, et al. 2019*) (*Cordelli, E, et al. 2005*)(*Anzar, M, et al. 2011*)*

1.1.8. Acrosomal Status

The acrosome is very important in bull fertility; the acrosome is a cap that covers the head of the sperm cell. The acrosmal membrane assists the sperm head to stay attached to the egg and aids the sperm to fuse to the egg's plasma membrane by releasing a digestive enzyme called acrosine, which helps breaks down the zona pellucida. The zona pellucida is an extracellular coat that surrounds and protects the oocytes, eggs and embryos, it also helps regulates the exchanges between the ovulated egg and the sperm cell during fertilization. Abnormal acrosomes can prevent fertilization, the acrosomal status of sperm can be assessed by using probes that recognize targets inside the acrosome, identifying the spermatozoa with damaged or reacted acrosomes. Damaged acrosomes can hinder the acrosomal reaction, as there may not have enough acrosine to break down the zona pellucida. *(*Martínez-Pastor, F, et al. 2010*)*

Knobbed acrosome can be described as distorted, dark stained area or abnormal thickening which caused a beaded appearance at the tip of the sperm head. Knobbed acrosomes can be hereditary or can be caused by stress; this defect is usually seen in young bulls. Knobbed acrosomes are seen in the forms of; beaded, indented or flattened. The beaded form is hereditary from the autosomal recessive gene. Indented or flattened forms can differ in their effects on fertility. In significant volumes indented and flattened acrosomes will affect the sperms ability to attach to

the eggs plasma membrane. *(*Perry, V. E. A. 2021*)* Swollen acrosomes can lead to changes in distance between membranes within the sperm head, the swelling of the acrosome is typical during sperm aging. Swollen acrosomes are usually found occurring simultaneously with knobbed acrosomes, because knobbed acrosomes lead to early imitation of acrosome reaction. *(*Perry, V. E. A. 2021)

1.1.9. Oxidative Stress

Bull's fertility can also be influenced by oxidant species, they play a vital role in sperm physiology. Excessive reactive oxygen species (ROS) can overload the protective mechanism and influence changes in lipid/protein layers in sperm plasma membranes. Oxidative stress (OS) leads to a disproportion between ROS and antioxidants in the cell, which can cause damage to the sperm cells, which can influence a bulls fertility. Thus, to be able to determine ROS and OS in a sperm sample can help understand a bull's sperm quality. *(*Martínez-Pastor, F, et al. 2010*)*

1.1.10. Chromatin Status

The chromatin is; a combination of DNA and proteins that form the chromosomes found in the cell of organisms. These proteins have large amounts of DNA that are found in the cell nucleus. When the chromatin of sperm cells are damaged, this may affect the ability of the sperm to fertilise an egg, cause abortion or foetal abnormalities, which leads to a decrease in a bulls fertility. *(*Martínez-Pastor, F, et al. 2010*)* Sperm chromatin damage is linked to abortions. Apoptosis is associated with sperm chromatin damage. Apoptosis is a form of cell changes and cell death, where the cell contents breakdown. The changes that occur in apoptosis can be; cell shrinking, chromatin condensation and DNA fragmentation. *(*Kim, H. H, et al. 2013*)*

1.1.11. Debris (Non-sperm events)

A dominate problem in sperm quality assessment using flow cytometry is debris in the sperm sample. Debris can consist of; white blood cells, skin cells, bacteria and extender particles (Example: egg yolk). These debris can be seen as non-sperm events in flow cytometry. Using forward scatter (FSC) versus side scatter (SSC) on a dot plot and using the grating, cells of interest can be identified by cell size and cell granularity. The debris can also be determined by using a dot plot and grating using

both the forward scatter FSC versus SSC, where the FSC relates to cell size and SSC relates to the granularity of complexity of a cell. When debris in a sample are identified, they can then be discarded in assessment.

Debris can lead to overestimation in sperm populations which could lead to inaccurate results, debris can form auto fluorescence, where the debris may contain molecules which get excited by the UV radiation and form light fluorescence, which is then picked up by the detectors in the flow cytometer. Removing debris from flow cytometry data is required for accurate results for cells of interest. A study by *Petrunkina and Harrison (2009),* research showed how inaccurate sperm populations were being obtained due to debris in the sample. It was suggested by *Nagy et al. (2003)* that debris could be removed and separated from the sperm cells in samples by using a double stain SYBR-14/PI, because the probes can only label the nucleated events, while the debris were left unstained. Other research shows that using Hoechst 33342, it is a blue emitting fluorescence used in molecular staining, the nuclei is stains blue. *(*Martínez-Pastor, F, et al. 2010*)*

1.1.12. Flow cytometry in sperm evaluation

In sperm analysis, it is important to remember that only a small number of sperm are being analysed in the ejaculate, it is preferable to carry out larger analysis of an ejaculate to get a true representation of an ejaculate. In methods such as light microscopy and computer assisted sperm analysis, only a few hundred sperm are being analysed whereas flow cytometry can analyse thousands of sperm cells of one ejaculated in seconds, giving a greater representation of a sperm sample. Flow cytometry was first used in the 70s to analyse sperm, in the 90s flurosescent probes were established to detect; membrane integrity, mitochondrial membrane potential, DNA fragmentation and much more. *(*Peña, F, et al. 2016*)*

1.2.1 Methods

1.2.2. Assessment of Viability

To test sperm viability; propiduim iodide (PI) and ethidium homodimer (EH) stains are the most widely used, the live sperm cells have an intact membrane which blocks the stain form penetrating the cell, where the dead sperm cells have damaged permeable membrane, which allows the PI stain to bind to the DNA in the dead cells. PI can only bind to the DNA of cells with a damaged plasma membrane. When stained the sperm sample is excited at 488nm, the dead cells will emit red fluorescence. *(*Cordelli, E, et al. 2005*)*

SYBR-14 is used in sperm viability analysis to determine spermatozoa from the cellular debris in the sample, such as white blood cells and skin cells. SYBR-14 is membrane permeable, it stains green. When PI and SYBR-14 are used together, the PI stain will penetrate any cells with damaged plasma membrane and will replace the SYBR-14 fluorescence. *(*Martínez-Pastor, F, et al. 2010*) (*Kumaresan, A., et al. 2017*)*

1.2.3. Assessment of Mitochondrial Activity

Cationic dyes such as rhodamine-123 and 5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimi-dazoylcarbocyanine iodide (JC-1) are used for mitochondrial assessment. JC-1 is a green fluorescent dye, JC-1 dye enters the mitochondria it changes its fluorescence properties based on the aggression of the probe, in healthy cells with high MMP, a red fluorescence forms. But unhealthy cells with low MMP or apoptotic cells it remains in the original form, which exhibits a green florescence. Cells with high MMP allows the JC-1 dye to build up in the energy filled, negatively charged mitochondrion which on impulse produces a red fluorescence. Cells with low MMP or apoptotic cells do not have the same built up in the energy and have a less negative charged mitochondrion, thus the JC-1 dye does not build up enough to cause excitement and the impulse colour change does not occur, the dye stays the original green colour. *(*Farzane Sivandzade, et al. 2019*) (*Cordelli, E, et al. 2005*) (*Kumaresan, A., et al. 2017*) (*Anzar, M, et al. 2011*)*

$DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodide) is another mitochondrial probe that has been used for analysing bovine semen. *(*Martínez-Pastor, F, et al. 2010*)* Another method to assess mitochondria is; MitoTracker® dye. The dye stains active

mitochondria, they advantage of using the MitoTracker® dye is it's very specific with a broad range of fluorescence emissions. Both MitoTracker® Green FM and MitoTracker® Red CM-H_2TMRos have been used in bovine studies on the assessment of sperm *(*Martínez-Pastor, F, et al. 2010*) (*Anzar, M, et al. 2011*)*

1.2.4. Assessment of Acrosomal Status

Acrosomal status can be determined by using probes that can detect targets within the acrosome, abnormal, damaged or reacted acrosomes are all labelled. Flow cytometry can be used to quantify the acrosome reaction in sperm cells, using CD46 antibody binding versus lectin binding (Peanut agglutinin (PNA) and Pisum Sativum Agglutinin (PSA)), the anti-CD46 binds to the acrosomal matrix and lectins bind to the glucosdic residues in different parts of the acrosomal membrane. PNA and PSA are commonly used because of their specificity. PNA binds to the acromsome membrane of a reacted sperm and can then be used as the marker of the acrosome reaction by flow cytometry. *(*Martínez-Pastor, F, et al. 2010*)*

1.2.5. Assessment of Oxidative Stress

Oxidative stress can be detected using flow cytometry, reactive oxygen species can be detected using reagents that collect intracellulary and fluoresce when there is oxidation. 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) can be used as a generic reporter of intracellular *reactive oxygen species* in spermatozoa. H_2DCFDA enters the plasma membrane, where it is maintained after intracellular esterases, where the hydrolase enzyme divides esters into an acid and an alcohol and emits green fluorescent (504nm) upon oxidization. *(*Martínez-Pastor, F, et al. 2010*) (*Kumaresan, A., et al. 2017*)*

1.2.6. Assessment of Chromatin Status

In flow cytomerty, the assay used to assess sperm chromatin; The SCSA® (Sperm Chromatin Structure Assay), it is one of the most used assays for testing sperm chromatin. The sperm samples are go through a DNA denaturation step, where the denature step breaks the down the double stranded DNA into single stranded DNA and then acridine orange (AO) stain is added and analysed on a flow cytometer. AO is a fluorescent dye that inserts into nucleic acids and is used to identify RNA and DNA. When double stranded DNA is detected it will have a green fluorescence and when single strand DNA is detected it will have a red fluorescence. (Evenson, D. P. 2016)

Sperm integrity is altered with the freezing step in conventional straws for artificial insemination. The cooling, freezing and thawing steps in the conventional method resulting in damage to sperm cell structure and depletion of sperm cell function. This method can also create morphological changes in the sperm cells and damage to plasma membrane, acrosome and mitochondrial. These changes lead to a decrease in fertility potential. The sperm plasma membrane manages the calcium concentrations within the cell. Calcium allows for the growth in sperm cells and can influence sperm motility and acrosome reaction. Cryopreservation of bull sperm can alter sperm membrane integrity, resulting in reduced fertility. *(*Fernandes, G. H. C, et al. 2015*) (*Januskauskas, A., et al. 2003*)*

1.2.7. Triple stain

A triple stain method has been developed for sperm analysis using flow cytometry. Where cell viability, acrosomal intergrity and mitochondrial function are all analysed. Cell viability is analysed using a propidium iodine (PI) stain, acrosomal integrity is analysed using a pisum sativum agglutinin (PSA) stain and mitochondrial function is analysed using rhodamine 123. Using these three stains, three separate characteristics of the sperm cell can be analysed at one time, flow cytometry allows thousands of cells to be analysed with little preparation to the samples, allowing rapid, accurate results. *(*Graham, J., et al. 1990*)*

1.3.1 Discussion

Artificial insemination has become a powerful tool for cattle breeding, a bull's fertility is a key factor in cattle production. By using semen from superior genetics bull's allows the beef and dairy industry's reach higher levels of production while becoming more sustainable, while overall increasing the genetics in the national herd. Bull fertility can be assessed by multiply methods; light microscope, CASA and flow cytometry. While all can assess the quality of sperm, flow cytometry seems to have major advantages over other methods.

Traditionally the light microscope is used in the assessment of bovine sperm, where viability, motility and morphology can be determined. The CASA can also be used to determine viability, motility and morphology in a sperm sample. Research carried out in NCBC, showed that using a microscope and trained analyst was more accurate in the assessment of sperm quality compared to the CASA. While both methods can be used for viability, motility and morphology assessment, other quality assessments cannot be carried out using a microscope or CASA. For a long time, traditional methods for sperm assessment have been used, assessment of sperm count, motility and morphology is a key indicator in a bull's fertility, all these factors may pass all parts of sperm quality assessments and on occasion sperm quality assessments don't correspond with filed fertility. This is where flow cytometry methods are useful to further understand our sperm quality. Flow cytometry has allowed us to detect small changes in sperm physiology that can help determine a bull's fertility potential.

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Table 1. Comparison of methods for sperm analysis.

In Table 1. Microscope, CASA and Flow Cytometry methods can be seen where each method can detect and not detect in assessment of sperm cells. One area that flow cytometry cannot detect is motility, whereas the microscope and CASA methods can both determine sperm motility. Motility tells us how progressive the sperm cells are moving and is important when it comes to bull fertility. Microscope or CASA methods would still be required in conjunction with flow cytometry for the full

assessment of sperm cells. Other that motility, it can be seen in comparsion to other methods flow cytometry is far superior in the assays it can run and accuracy compared to microscope and CASA methods.

Flow cytomerty has been a major step in sperm quality assessment in bovine andrology, it can be used in the assessment many aspects of a sperm cell; plasma membrane, acrosome integrity, mitochondrial status, DNA fragmentation, intracellular Ca^{2+} levels and oxidative stress can all be assessed using flow cytrometery. Studies using flow cytometery have shown a relationship between bull fertility and sperm characteristics, plasma membrane and acrosome integrity mitochondrial function, and Ca^{2+} levels. *(*Bucher, K, et al. 2019*)*

Developments in understanding a bull's fertility are necessary to reach full potential in beef and dairy farming. Genetic selection and semen quality assessments allow improvement in bull fertility, which allows higher rates of conception and increasing production. Studies are looking for connections between bull fertility traits, such as, volume, sperm concentration, motility and sperm abnormalities. (Hering, D. 2014). Using these fertility traits can allow us to genetically reproduce the best offspring which will eventually become the sires used for artificial insemination..

Studies have shown that age does have an effect on fertility and as a bull age is increasing there can be seen a decrease in the quality of sperm. *(*Taaffe, P, et al. 2020*)* Using flow cytometry on older bulls can help us understand what is happening to the sperm to make the fertility decrease with age. It is known that sperm morphology or abnormalities have a direct connection with fertility, it is still unknown which abnormalities have the most negative impact when it comes to bull fertility. In recent years, flow cytometry has played a vital role in the study of sperm cells and is fast becoming the superior method in the assessment of bull sperm quality for use in artificial insemination. *(*Al Makhzoomi, et al. 2008*)*

Artificial insemination is usually carried out using frozen thawed semen. This process is known as cryopreservation, cryopreservation stops the metabolic activities of the sperm cells and helps prevent loss of fertility. The cold shock can form changes in sperm cells, sperm plasma membrane can lead to changes in structure and mitochondria can lead to a calcium build up. *(*Jäkel, H., et al. 2021*)* Cryopreservation can lead to stress on the sperm cells which can alter the sperm cells. Sperm integrity

is altered with the freezing step in conventional straws for artificial insemination. The cooling, freezing and thawing steps in the conventional method resulting in damage to sperm cell structure and depletion of sperm cell function. This method can also create morphological changes in the sperm cells and damage to plasma membrane, acrosome and mitochondrial. These changes lead to a decrease in fertility potential. . *(*Jäkel, H., et al. 2021*)*

Semen quality can be affected by other factors, such as, type of extender, bacterial contamination. The presence of bacteria in sperm has adverse effects on sperm viability, motility and increase abnormalities in sperm cells. Bacteria from in the ejaculates can also have effects on the mitochondrial function which causes formation of reactive oxygen species which leads to increasing DNA fragmentation. *(*Abadi A, et al. 2020*)*

In sperm analysis, it is important to remember that only a small number of sperm are being analysed in the ejaculate, it is preferable to carry out larger analysis of an ejaculate to get a true representation of an ejaculate. In methods such as light microscopy and CASA, only a few hundred sperm are being analysed whereas flow cytometry can analyse thousands of sperm cells of one ejaculated in seconds, giving a greater representation of a sperm sample. Flow cytometry advantages allows for many characteristics of cell structure and function to be assessed, simply, accurately and rapidly. It can be expected that with the use of flow cytometry and research a deeper understanding of sperm physiology can be obtained. In the future flow cytometry methods will hopefully open more doors to understand characteristics and functions of sperm cells, which will assist in the advancement of bull fertility potential. *(*Peña, F, et al. 2016*)*

To determine the viability, a sample can be stained with PI stain, where non-viable sperm cells can be determined. The live sperm cells have an intact membrane will stop the stain form penetrating the cell, where the dead sperm cells have damaged permeable membrane, which allows the PI stain to bind to the DNA in the dead cells. This viability method can be carried out on the microscope, CASA and flow cytometry. But flow cytometry has major advantages over the other two methods, in light microscopy and CASA methods only a few hundred sperm are being analysed whereas flow cytometry can analyse thousands of sperm cells of one ejaculated in

seconds, giving a greater representation of a sperm sample. *(*Peña, F, et al. 2016*)* Light microscopy is also a slow labour intensive process which would not be capable for every sample in a high production laboratory. Thus flow cytometry is superior due to being a rapid and more accurate method to determine viable and dead sperm cells compared to old methods which can be slow and labour intensive.

Flow cytometry can be used to determine sperm concentration, which is important in determining the quantity of straws witch can be produced based on each straw has 15 million cells per straw. A difficultly in sperm concentration assessment is debris in the sperm sample. Debris can consist of; white blood cells, skin cells, bacteria and extender particles. Debris can increase sperm concentrations, which leads to an inaccurate calculation, which then results to less than 15 million sperm cells per straw. Currently in AI straw production, a photometer called 'Accucell' is being used to determine sperm concentrations in raw ejaculates. The Accucell is a satisfactory method in determining sperm concentration but can be time consuming and cannot detect debris which can lead to overestimation in sperm populations which could lead to inaccurate results. Another method which sperm concentrations could be determined would be using a haemocytometer, which is deemed the gold standard in cell counting, unfortunately because this is a manual count method, it is extremely slow and labours and unsuitable for straw production, but an excellent method for validation of flow cytometer sperm concentrations.

Debris can lead to difficultly in assessing sperm concentrations, instruments such as the flow cytometer have allowed the identification of debris which allows them to be discarded in sperm concentrations, leaving with more accurate results. The debris can also be determined by using a dot plot and grating using both the forward scatter FSC versus SSC, where the FSC relates to cell size and SSC relates to the granularity of complexity of a cell. Flow cytometry is the superior method for sperm concentration, by accounting the debris, it allows for an accurate calculation for sperm concentration per straw, without accounting for debris, there are less cells per straw which could lead to a reduction in fertility because field studies have determined that 15 million cells per straw for dairy bulls is optimum for egg fertilization and 20 million cells per straw for beef bulls is optimum for egg fertilization.

Sperm motility is a fundamental when it comes to fertility. Progressive sperm motility is associated with high fertility. Although flow cytometers can't determine progressive sperm cell directly, it can look at the mitochondria in the cell and determine the energy and health of the cell. Studies show that mitochondria have a part to play in energy production which is necessary for cell survival. Mitochondrial function can be influenced by factors such as, diseases, environmental conditions, nutrition and injury. Mitochondrial function indicates the health of a cell and mitochondrial membrane potential can help determine sperm motility, loss of MMP is an indicator of cell stress and can lead to cell death, for this reason MMP can be used in assessing sperm quality. (Cordelli, E., 2005)

Mitochondrial assessment can be carried out using flow cytometry and JC-1 green fluorescent dye, JC-1 dye enters the mitochondria it changes its fluorescence properties based on the aggression of the probe. In healthy cells with high MMP, a red fluorescence forms. But unhealthy cells with low MMP or apoptotic cells it remains in the original form, which exhibits a green florescence. Samples that show the red fluorescence are identified as healthy cells with a high MMP and a sign that sperm motility should be progressive which is linked to high fertility. A green fluorescence shows unhealthy cells with low MMP, loss of MMP is an indicator of cell stress and can lead to cell death which can help understand why a bull may have poor fertility. Flow cytometry is the only method of the 3 mention which can assess mitochondrial potential, knowing if the majority of sperm cells in an ejaculate have high MMP, gives the information that sperm cells have enough energy to move progressively to reach an egg for fertilization. High MMP is linked to high fertility. *(*Farzane Sivandzade, et al. 2019*) (*Cordelli, E, et al. 2005*)(*Anzar, M, et al. 2011*)*

Flow cytometry can be used to quantify the acrosome reaction in sperm cells, the acrosome is a cap that covers the head of the sperm cell. The acrosmal membrane assists the sperm head to stay attached to the egg and aids the sperm to fuse to the egg's plasma. Abnormal acrosomes can prevent fertilization, the acrosomal status of sperm can be assessed by using probes that recognize targets inside the acrosome, identifying the spermatozoa with damaged or reacted acrosomes. Damaged acrosomes can hinder the acrosomal reaction, as there may not have enough acrosine to break down the zona pellucida. *(*Martínez-Pastor, F, et al. 2010*)* While

microscope and CASA methods can be used to detect acrosomes, they cannot be used to the same extent as flow cytometry. Clear deformed acrosomes can be determined under a microscope but dark stained areas and thickening of the acrosome cannot be determined under a microscope but can easily be determined using flow cytometry. Intact acrosomes are vital for fertilization, as they play a key role in the sperm entering the egg. The more intact sperm cell acrosomes the higher the fertility potential.

Oxidation has a vital role in sperm physiology, a bull's fertility can also be influenced by oxidant species. Excessive reactive oxygen species within the cell can overload the protective mechanism and influence changes in lipid/protein layers in sperm plasma membranes. Oxidative stress can cause damage to the sperm cells, which can influence bull fertility. *Flow cytometry can detect reactive oxygen species using reagents that collect intracellular and fluoresce when there is oxidation, while microscope and CASA methods cannot determine oxidative stress.* To be able to determine ROS and OS in a sperm sample can help understand a bull's sperm quality. *(*Martínez-Pastor, F, et al. 2010*) (*Kumaresan, A., et al. 2017*)*

The chromatin also plays a role in bull fertility. When the chromatin of sperm cells is damaged, this may affect the ability of the sperm to fertilise an egg, cause abortion or foetal abnormalities, which leads to a decrease in a bulls fertility. Due to the sensitivity and repeatability in flow cytometry, it can be used to help predict bull fertility; studies have shown correlations between bull fertility and DNA analysis using SCSA. SCSA is a method that can identify sperm samples that have a large amount of DNA fragmentation, DNA fragmentation is the breaks of DNA strands in sperm chromosomes, DNA fragmentation is the last step before cell death. High levels of DNA fragmentation are linked to poor fertility rates in bull semen which results in a reduction in pregnancies. *(*Kim, H. H, et al. 2013*)*

In this review microscope, CASA and flow cytometry methods have been discussed in how they're used for the assessment of sperm quality, the research shows that flow cytometry advances can be useful in helping to understand sperm cells and how they affect a bull fertility. Flow cytometry allows a better understanding of areas in sperm assessment where traditional methods cannot, these areas are; Debris, chromatin status, acrosome integrity, mitochondrial status, DNA fragmentation and

oxidative stress. As research on sperm quality increases the possibility for more targets to be identified are anticipated, which in the future will allow even greater understanding and predicating bull fertility. *(*Martínez-Pastor, F, et al. 2010*)*

1.4.1 Conclusion

Flow cytomerty has become an major step in sperm quality assessment in bovine andrology. Flow cytrometry can be used in the assessment many aspects of a sperm cell; plasma membrane, acrosome integrity, mitochondrial status, DNA fragmentation, intracellular Ca^{2+} levels and oxidative stress can all be assessed using flow cytrometery. Flow cytometry has allowed semen assessment to go into further depth of a sperm cell compared to traditional semen quality assessment. Flow cytometry is replacing slower, less accurate methods, the capability of testing for multiply aspects of an ejaculate at once, has huge advantages over traditional methods. Flow cytometry allows large scale repetition become feasible, thousands of cells can be analysed in seconds. Large number of data can be collected for each sample.

Chapter Two

Analysing Bovine sperm cells using Flow Cytometry

Abstract:
The accurate assessment of bull fertility is extremely important to the beef and dairy industry. In recent years, flow cytometry has become the dominant approach in sperm evaluation and is increasingly being used for quality assessment and for research in veterinary science. The aim of this research was to show how flow cytometry can be used to assess the quality of semen used in artificial insemination. The review focuses on the methodologies, currently used for bovine sperm concentration assessment and the advantage of flow cytometry has over other methods used for the semen concentration assessment. The research article investigates the technological differences between AccuCell Vs Flow Cytometry to determine semen concentration. It also accesses the reproducibility of results depending on the personnel's techniques. Based on the analysis of sample diluents' effect on the results generated with the flow cytometer and estimation of debris' presence effect in samples, the optimal parameters of ejaculates to determine semen concentration using the flow cytometer are presented. Finally, the sustainability assessment of novel flow cytometry methodology for the semen concentration analysis of an ejaculate demonstrated that flow cytometry is a more sustainable method to use to determine the concentration of an ejaculate.

2.1.1 Introduction

Artificial insemination is a powerful tool for improved cattle breeding by using superior genetic sires. Artificial insemination is a method where male bull sperm is collected from selected sires and then artificially introduced into the female reproductive tract by a trained AI technician. The National Cattle Breeding Centre (NCBC) operates Ireland's most successful dairy and beef breeding programs using artificial insemination (AI) and has delivered variety of genetics to improve the genotype of the Irish National Herd, which will result in increased profits for years to come for the Irish farming industry. Semen from top genetic sires is used to improve the genetics in the national herd. *(Dairy Gene Ireland programme 2023, ICBF 2023)* The use of AI in the Diary herd has increased rapidly over past 20 years, which allows farmers to select semen from the top EBI (Economic Breeding Index) sires (Fig. 1). EBI is a single-figure profit index designed to assist farmers in selecting the bulls and cows for dairy herd replacement breeding that will yield greater profits in milk production, fat %, protein% and increase in fertility. (Fig 2) The genetic selection allows farmers to get the most products out of each animal, improving farming sustainably. This results in the rapidly increased productivity and profits in Irish Agriculture (Fig. 1).

Figure 1. has been removed due to Copyright restrictions.

Fig 1. Genetic Trends in EBI, Average Milk and Average Fertility between 2002 and 2022 – *Agriland*

Figure 2. has been removed due to Copyright restrictions.

Fig 2. Traits which make up EBI - ICBF

Fertility plays a major role in the livestock industry and has the highest individual effect (33%) on Economic Breeding Index. Fertility can be defined as the ability to conceive offspring. In table 1. two bulls are compared, one with a high (FR6547) EBI and the other with a low (FR2239) EBI. As seen in the table FR6547 has an EBI score of 402 Vs FR2239 has an EBI score of 186. The EBI indicates the average

profit a farmer will have on each cow that has a calf. In the case of these two bulls if a famer was to use the higher EBI bull over the lower EBI bull, the famer would make 216 extra per cow that has a calf. Overall it can be seen that the higher EBI bull has greater; production, fertility, % fats and % protein compared to the lower EBI bull. FR2239 would be considered an old bull at age 8, whereas FR6547 would be younger at 3 years of age. As more and more Irish farmers continue to use AI straws to improve the national herd, the genetics will continue to improve and that will only allow the EBI, production, fertility, % fats and % protein continue to increase for Irish farmers and farmers across the globe.

Table 1. has been removed due to Copyright restrictions.

Table 1. Comparison of High Vs Low EBI Bulls. – *High EBI HOFR, Progressive Genetics*

High conception rates in AI are crucial for famers, weather fresh semen or conventionally frozen semen is used for artificial insemination, the best quality sperm straws are required to obtain high conception rates. Conception rates are influenced by the quality of semen, conception rates can be increased by determining a bull's fertility. One of the reasons of reproductive failure is a poor-quality semen or low volume of semen. Therefore, the evaluation of semen quality in ejaculate is essential in the AI industry, since it allows us to select the highest quality sperm for AI. The ejaculate should be inspected immediately upon collection. The following production data is collected for each ejaculation, volume of ejaculate, sperm concentration, visual evaluation of colour and density and visual evaluation of blood or dirt *(Edel M et al. 2018*, *Al Makhzoomi, et al. 2008, Fuerst-Waltl, B., et al. 2006).*

There are many aspects that can negatively affect a bull's semen quality, e.g. injury, disease, nutrition, and heat stress *(Jane M. Morrell, 2020, Yatta Linhares Boakari., et al. 2022*). Bull fertility traits can also be hereditary, which allows genetic selection to enhance bull fertility *(Sakase, M. et al. 2020, Fuerst-Waltl, B., et al. 2006).* Another factor that can affect sperm quality is bull age. The bull's immaturity (e.g. very young bulls) plays a role in semen quality, since high abnormalities in young sperm (examples of abnormalities; bent mid pieces, detached heads, coiled tails, proximal and distal droplets) cells are common due to immaturity. Young bulls tend to produce

low volumes of ejaculates when bulls are first jumped. These low volumes on ejaculates tend to have low sperm concentrations, with both low volume ejaculates and low sperm concentrations lead to poor quality ejaculates. To overcome these poor-quality ejaculates in young bulls, studies show that collecting young bulls ejaculates regularly helps overcome these problems (*Taaffe, P, et al. 2020*). Studies show as a bull gets older, the volume of the ejaculated by the bull increases and semen quality increases (*Taaffe, P, et al. 2020*).

The AccuCell (Photometer), where a pre-adjusted halogen bulb serves as the AccuCell's light source, determines the concentration of an ejaculate while reading the cell concentration based on size. Samples must be run using a single use plastic cuvette on the AccuCell, the cuvette is not reused to eliminate the chance of a false reading for concentration *(A. Camus , 2011)* The AccuCell method has low throughput, each sample must be ran in duplicate. On average between 40 – 50 bull ejaculates come in to be analysed, as all samples need to be ran in duplicate, 80 - 100 single use plastic cuvettes must be made up daily. On average it takes 60seconds to sample prep the cuvettes and each reading on the AccuCell takes 10 seconds. As each sample is ran in duplicate it takes 2 minute 20 seconds to get two concentrations of one bull which then needs to be averaged to get the final concentration.

The new method, Flow Cytometry is becoming more popular in sperm analysis, replacing slower, less accurate methods. The capability of testing for multiply aspects of an ejaculate at once has huge advantages over traditional methods. *(Bucher, K, et al. 2019)*. Flow cytometry allows large scale repetition to become feasible, thousands of cells can be analysed in seconds. Large number of data can be collected for each sample. The sample is placed in the flow cytometer where; each cell passes through one or more lasers one by one. The emitted light is filtered and sent to the detectors where, the signals are amplified, and data is plotted on a graph for interpretation. *(Bucher, K, et al. 2019)*

In sperm analysis, it is important to remember that only a small number of sperm are being analysed in the ejaculate, it is preferable to carry out larger analysis of an ejaculate to get a true representation of an ejaculate. In methods such as light microscopy, AccuCell and computer assisted sperm analysis, only a few hundred

sperm are being analysed whereas flow cytometry can analyse thousands of sperm cells of one ejaculated in seconds, giving a greater representation of a sperm sample. Flow cytometry was first used in the 70s to analyse sperm, in the 90s fluorescent probes were established to detect; membrane integrity, mitochondrial membrane potential, DNA fragmentation and much more (*Peña, et al. 2016*, *Martínez-Pastor, et al. 2010*)

2.1.2 Flow Cytometry

Image 1. has been removed due to Copyright restrictions.

Image 3. Schematic of flow cytometry system - *Cell Signaling Technology*

Flow cytometry can be used in sperm count, by using the grating based on the sperm cell size. The grating allows for all sperm population to determine by their size and all debris such as white blood cells, skin cells and bacteria are determined and then a true count of sperm population is determined. *(Cordelli, E, et al. 2005)* Flow cytometry analyses thousands of events in seconds, which allows for rapid and reliable results compared to other methods. A dominate problem in sperm quality assessment using flow cytometry is debris in the sperm sample; debris can be seen as non-sperm events in flow cytometry. Using forward scatter (FSC) versus side scatter (SSC) on a dot plot and using the grating, cells of interest can be identified by cell size and cell granularity. The debris can also be determined by using a dot plot and grating using both the forward scatter FSC versus SSC, where the FSC relates to cell size and SSC relates to the granularity of complexity of a cell. When debris in a sample are identified, they can then be discarded in assessment.

2.1.3 Sperm Concentration

Flow cytometry can be used in sperm count *(Huiping Yang, et al. 2015, Cordelli, E, et al. 2005),* by using the grating based on the sperm cell size. The grating allows for all sperm population to determine by their size and all debris such as white blood cells, skin cells and bacteria are determined and then a true count of sperm population is determined *(Cordelli, E, et al. 2005).* The sample is placed in the flow cytometer where; each cell passes through one or more lasers one by one. The emitted light is filtered and sent to the detectors where, the signals are amplified, and

data is plotted on a graph for interpretation *(Bucher, K, et al. 2019).* Flow cytometry allows large scale repetition to become feasible, thousands of cells can be analysed in seconds. Large number of data can be collected for each sample.

2.1.4 Debris (Non-sperm events)

A dominate problem in sperm quality assessment using flow cytometry is debris in the sperm sample. Debris can consist of; white blood cells, skin cells, bacteria and extender particles (Example: egg yolk). Debris can form auto fluorescence, since debris may contain molecules which get excited by the UV radiation, which is then picked up by the detectors in the flow cytometer and be seen as non-sperm events in flow cytometry. As a result, debris can lead to overestimation in sperm populations leading to inaccurate results and increased infertility rates. Using forward scatter (FSC) versus side scatter (SSC) on a dot plot and using the grating, cells of interest can be identified by cell size and cell granularity. The debris can also be determined by using a dot plot and grating using both the forward scatter FSC versus SSC, where the FSC relates to cell size and SSC relates to the granularity of complexity of a cell. When debris in a sample are identified, debris can then be discarded from assessment, resulting in more accurate results for cells of interest (*Petrunkina and Harrison (2009))*.

2.1.5 Bull Maturity

Bull age is another factor that can have an impact on sperm quality. In very young bulls immaturity plays a role in semen quality, high abnormalities in young sperm cells are common as a result of immaturity. (Taaffe, P. et al. 2020) When bulls are first jumped, young bulls frequently ejaculate in small amounts. Low sperm concentrations are often associated with low ejaculate volumes, which together with low sperm volumes result in ejaculates of poor quality. Studies have shown that routinely collecting the young bulls ejaculates can help young bulls overcome their low quality ejaculates and immaturities. (Taaffe, P. et al. 2020) According to studies, as a bull ages, both the quantity and quality of his ejaculates increase. *(Hallap T. et al. 2004)*

2.1.6 Sustainability

In recent years there has been a push in agriculture and other industries to use less plastic and to be more sustainable, NCBC alone generates 3 million doses of semen annually, which is packaged in single use plastic AI straws. IMV Technologies (France) "produces hundreds of millions of semen straws annually" and sells their products to AI firms all over the world. Moving towards Flow Cytometry allows the NCBC update the laboratory in areas where changes could be made to help bring the laboratory to a more ‘Green’ status. As NCBC works with AI in the cattle industry, insemination is a powerful tool for improved cattle breeding by using superior genetic sires, semen from top genetic sires is used to improve the genetics in the national herd. *(Diavão J, et al. 2023)* The use of AI allows the beef and dairy breeding to be more sustainable and the aim is to follow through that sustainability throughout the whole business starting with a more sustainable laboratory where the straws for AI are produced. *(Teagasc, 2022) (Brennan, A., 2023)*

Using Flow Cytometry to change the following assay ‘Determining the concentration of raw ejaculates, which allows the determination of quantity of straws produced in production’ by eliminating the use of single use plastics and move to reusable glassware*. (Evenson, D. P, et al. 1993)* The aim of the research was to determine if Flow Cytometry could be used in an AI laboratory to improved reproducibility of the semen counts results, and increased through put, in addition to this another objective of my research was to estimate if the new protocol for semen assessment could affect laboratory sustainability. *(Morrell J. M, 1991)* As the AI industry is helping the farming industry become more sustainable, NCBC would like to try updating the laboratory in areas where changes could be made to help bring the laboratory to a more ‘Green’ status. The use of AI allows the beef and dairy breeding to be more sustainable and the aim is to follow through that sustainability throughout the whole business starting with a more sustainable laboratory where the straws for AI are produced. (Teagasc, 2022)

2.2 Materials and Methods

2.2.1 Concentration measurement of Raw Ejaculates using Flow Cytometry – CytoFLEX ran at a slow flow rate of 10µL/ min and will automatically stop reading at 60 seconds

The CytoFLEX (Beckman Coulter) was set at the following settings: the flow rate was set to slow at 10µL/ min and it was set to stop reading after 60 seconds automatically. For sample preparation: 4.5g of Sodium Chloride (Sigma-Aldrich) was added to 500mL of dH_2O (ULTRAPURE Laboratories Ltd., Mayo, Ireland) to make NaCl dH_2O solution, once made keep refrigerated at 4°C. Add CytoFLEX sheath fluid (Beckman Coulter – Labplan, Kildare, Ireland) to the CytoFLEX, CytoFLEX sheath fluid can be kept at room temperature. 4mL +/- 0.02 µL of NaCl dH_2O solution was added to each test tube, then the sample was vortexed and 4 µL is of sample added to 4mL +/- 0.02 µL NaCl dH_2O in a labelled glass tube. The mixture is vortexed again and place test tube into CytoFLEX tube holder. Select record to run the CytoFlex and record results.

2.2.2 Concentration measurement of Raw Ejaculates using Flow Cytometry – CytoFLEX ran at a medium flow rate of 30µL/ min and will automatically stop reading at 20 seconds

The CytoFLEX (Beckman Coulter) was set at the following settings: the flow rate was set to slow at 30µL/ min and it was set to stop reading after 20 seconds automatically. Sample preparation was carried out the same as in section 2.1.

2.2.3 Concentration measurement of Raw Ejaculates using AccuCell

To determine the concentration of Raw Ejaculates, this allows the determination of quantity of straws to be produced in production. Set up and blank AccuCell (IMV Technologies, L'Aigle, France).

For sample preparation: 4.5g of Sodium Chloride (Sigma-Aldrich) was added to 500mL of dH_2O (ULTRAPURE Laboratories Ltd., Mayo, Ireland) to make NaCl dH_2O solution, once made keep refrigerated at 4°C. 4mL +/- 0.02 µL of NaCl dH_2O solution was added to each labelled cuvette, then the sample was vortexed and 4 µL is of sample added to 4mL +/- 0.02 µL NaCl dH_2O in a labelled cuvette. The mixture

inverted and place cuvette into AccuCell holder. Select ‘Val’ to start the analysis. Record result and then repeat, select ‘Val’ again to get a second concentration reading. The two readings must be within 10% of each other, if the results are not within 10% of each other, repeat the procedure from the start until two readings are within 10% of one and other.

2.2.4 Concentration measurement of Raw Ejaculates using Flow Cytometry – using dH_2O NaCl, at a flow rate of 30µL/ min for 20 seconds

To determine the concentration of Raw Ejaculates, this allows the determination of quantity of straws produced in production. Set up Flow Cytometer (Beckman Coulter – Labplan, Kildare, Ireland) and run daily Quality Control test using CytoFLEX Daily QC Fluorospheres (Beckman Coulter – Labplan, Kildare, Ireland), keep QC beads refrigerated at 4°C. Open the pre-developed template for determine the concentration of an ejaculate, ‘Sperm Concentration Template (Record)’. CytoFLEX will automatically run a flow rate of 30µL/ min and will automatically stop reading at 20 seconds using this pre-developed method. Sample preparation was carried out the same as in section 2.1.

2.2.5 Concentration measurement of Raw Ejaculates using Flow Cytometry – using Triton Isoton, at a flow rate of 30µL/ min for 20 seconds

To determine the concentration of Raw Ejaculates, this allows the determination of quantity of straws produced in production. Set up Flow Cytometer (CytoFLEX Beckman Coulter – Labplan, Kildare, Ireland) and run daily Quality Control test using CytoFLEX Daily QC Fluorospheres (Beckman Coulter – Labplan, Kildare, Ireland), keep QC beads refrigerated at 4°C. Open the pre-developed template for determine the concentration of an ejaculate, ‘Sperm Concentration Template (Record)’. CytoFLEX will automatically run a flow rate of 30µL/ min and will automatically stop reading at 20 seconds using this pre-developed method.

For sample preparation: 100mL of Triton (Sigma-Aldrich) was added to 900mL of Isoton (Beckman Coulter – Labplan, Kildare, Ireland) and mixed well to make a 10% Triton Isoton solution. 20mL of 10% Triton Isoton stock solution was then added to 980mL of Isoton solution and mixed well to make a 0.2% Triton Isoton solution. Keep 10% and 0.2% Triton Isoton solutions refrigerated at 4°C. Add CytoFLEX sheath fluid

(Beckman Coulter – Labplan, Kildare, Ireland) to the CytoFLEX, CytoFLEX sheath fluid can be kept at room temperature.

4mL +/- 0.02 µL of 0.2% Triton Isoton was added solution to each test tube, then vortex sample and add 4 µL of sample to 4mL +/- 0.02 µL 0.2%Triton Isoton in a labelled glass tube. Vortex mixture again and place glass tube into CytoFLEX tube holder. Select 'Record' (CytoFLEX will automatically run a flow rate of 30µL/ min and will automatically stop reading at 20 seconds) (Using a saved template to run samples).

2.2.6 Concentration measurement of Raw Ejaculates using a Hemocytometer (Manual Cell Count) – using dH_2O NaCl

Sample preparation was carried out the same as in section 2.1. The edges of the counting chamber were dampened with water and a cover slip was placed on the top of the chamber. Approximately 5 to 10 uL of the sample is added to either side of the chamber for analysing on an X20 magnification in a phase contrast microscope. The cells that fall within the grids are counted and recorded, tails without heads were not counted in the cell count. The cells count is then put into the following calculation to determine the concentration: *(Dr Ciara O' Meara, 2022)*

Total Count = Cell Count * Dilution Factor/0.0001 (Equation 1)

Million/mL = Total Count/ /1000000 (Equation 2)

Million/Straw = Million/mL * *0.225 (Equation 3)

2.2.7 Concentration measurement of Raw Ejaculates using a Hemocytometer (Manual Cell Count) – using Triton Isoton

Sample method and analysis were carried out the same as section 2.6 with the exception of the sample preparation was carried out the same as in section 2.5.

2.2.8 Statistical Analysis

The data was analysed using IBM SPSS statistics (Version 26). Ejaculate concentration, technician variation, sample matrix, materials (test tubes) and age were all analysed using ANOVA. Differences were considered statistically significant when $P < 0.05$

2.3 Results

The first objective was to determine the optimal parameters to use on the Flow Cytometer to determine the concentration of a raw ejaculate. In section 3.1, tests were carried out to determine the optimal flow rate and time (seconds), this is key to the research as the laboratory using the method is a high production laboratory which needs rapid and accurate results. The next objective was to determine could there be alternate chemicals use for the sample matrix in case of a supply chain issue. In section 3.2, NaCl dH2O and Triton Isoton are tested on the flow cytometer to determine the concentration of a raw ejaculate. In section 3.3, a hemocytometer was used to validate the results found in section 3.2.

The next aim was to determine if there is any difference in concentrations between three methods carried out the laboratory (Section 3.4). In section 3.4 it became apparent that the technicians in the laboratory were not getting similar results. This lead to the research in sections 3.5 and 3.6, where it looks at technician variation between different methods, a hemocytometer was used to validate the results found in sections 3.5 and 3.6.

In sections 3.7 and 3.8 the aim was to determine if the new validated method could be run on reusable glassware vs single use plastic. From the research it was determined that reusable glassware could substitute for single use plastic. In section 3.11 the reduction of single use plastic is discussed based off the findings in sections 3.7 and 3.8. After validation of determining the concentration of a raw ejaculate by Flow Cytometry. Research was carried out on the flow cytometer to determine if bull age would have an effect on the concentration of a raw ejaculate (Section 3.9) and research was carried out to determine if an injury to a bull such as a lame leg may affect a bulls raw ejaculate sperm concentration over a period of 60 days. (Section 10)

2.3.1 Comparison of different flow rates (uL) and run times (Seconds) on the CytoFLEX

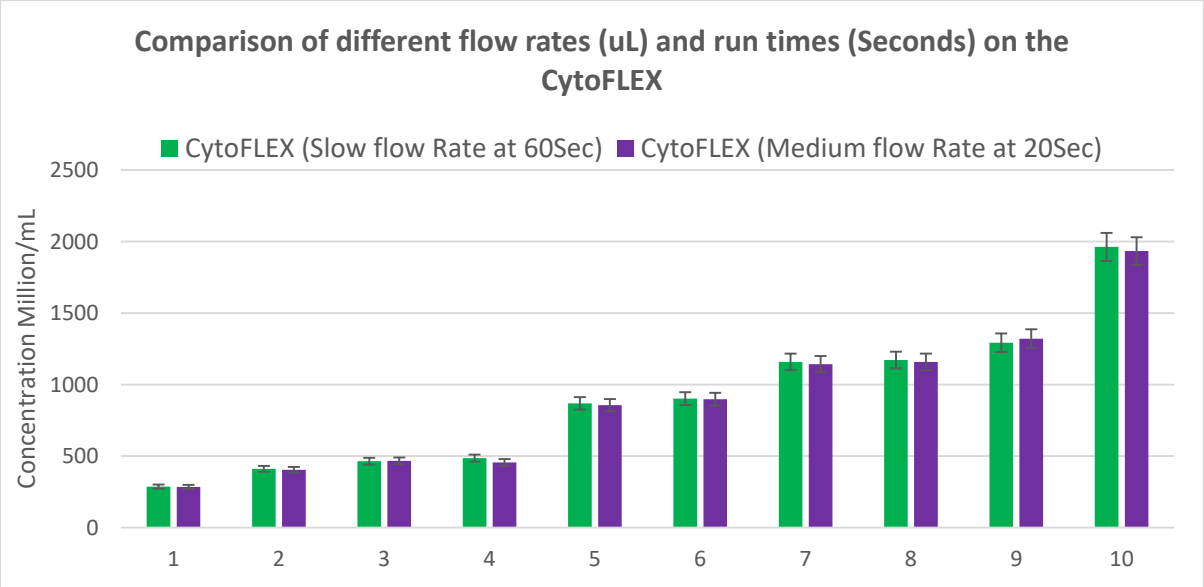

Fig 3. Comparison of different flow rates (uL) and run times (Seconds) on semen concentration estimated by the CytoFLEX. *(Error bars indicating standard error)*

The comparison of different flow rates (uL) and run times (Seconds) on semen concentration estimated by the CytoFLEX was to determine if the time in which each sample was ran was reduced and the flow rate increase would we get similar numbers to the original time and flow rate which the CytoFLEX was deemed to be successfully being able to determine the concentration of an ejaculate. The reason this was run as an experiment, as the laboratory is a high production laboratory and determining the fastest analysis is crucial to the business.

In Fig 3. The Comparison of different flow rates (uL) and run times (Seconds) on the CytoFLEX can be seen. The two flow rates tested were; Slow flow rate at a 60 second run time and a medium flow rate at a 20 second run time. As seen in Fig 3. it was determined that both setting was giving results within 5% of each other and both deemed acceptable to be used in the determination of concentration of a raw ejaculate. Due to the laboratory production being a high demand and fast-paced environment, it was determined that the medium flow rate (30uL) at 20 seconds was the optimal setting to use to determine of concentration of a raw ejaculate.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	352.8	1	352.8	0.001327	0.97134	4.413873
Within Groups	4784694	18	265816.3			
Total	4785047	19				

Table 2. ANOVA - Analysis of semen concentration estimated by the CytoFLEX at different flow rates (uL) and run times (Seconds)

Note, the results were found to be not statistically significant; the null hypothesis is accepted, $p > 0.05$.

2.3.2 Comparison of different sample matrixes on the CytoFLEX. NaCl dH2O Vs Triton Isoton

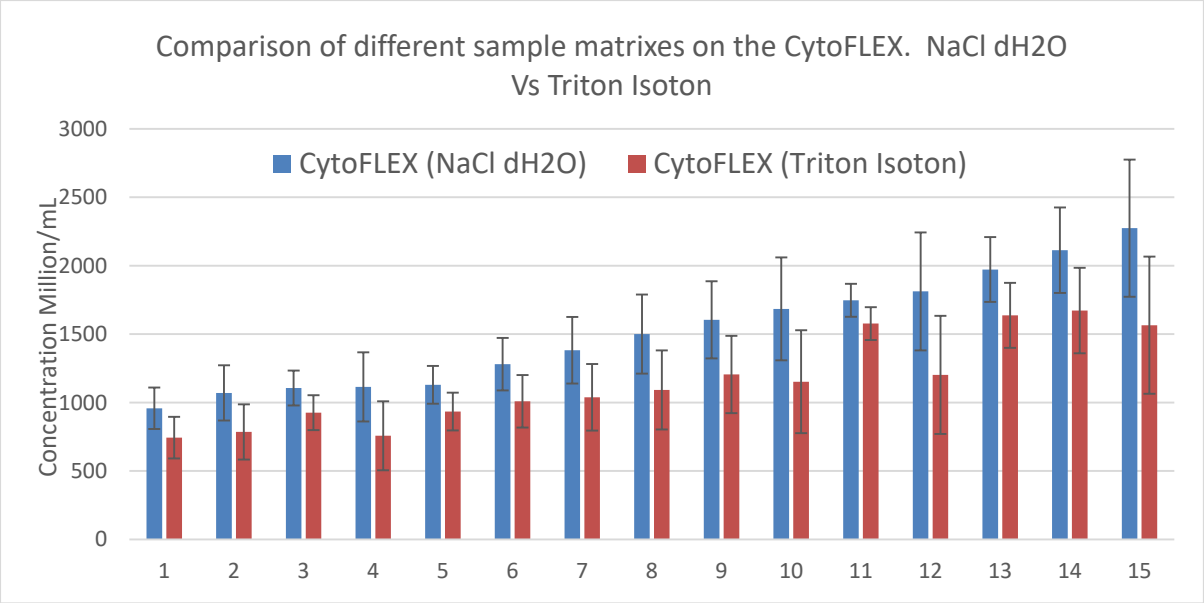

Fig 4. Comparison of different sample matrixes, (NaCl dH_2O Vs Triton Isoton) on semen concentration estimated by the CytoFLEX. *(Error bars indicating standard error)*

The comparison of different sample matrixes, (NaCl dH_2O Vs Triton) on semen concentration estimated by the CytoFLEX was carried out to determine if there was more than one method to run the sample, in case of supply chain issues of a reagent, there could be a potential back up method when needed. It was carried out using NaCl as that was currently being used in the old method (AccuCell) and Triton Isoton was used as there was excess of Triton in the laboratory from a previous method to determine semen concentration on a Coulter Counter. (Beckman coulter)

In Fig 4. It can be seen that the two sample matrixes tested on the CytoFLEX were differing significantly, with the samples ran on the Triton Isoton giving constantly lower readings than the NaCl dH_2O. It was determined that a Haemocytometer would be required to manually cell count to determine the accurate concentration readings.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	990446.7	1	990446.7	7.230917	0.011935	4.195972
Within Groups	3835268	28	136973.9			
Total	4825715	29				

Table 3. ANOVA - Analysis of semen concentration estimated by the CytoFLEX, for different sample matrixes, dH_2O Vs Triton Isoton

Note, the results were found to be statistically significant; the null hypothesis is rejected, $p < 0.05$. Thus, it was determined by ANOVA that the make-up of the sample matrix has a significant difference in results.

2.3.3 Comparison of concentration measurement of Raw Ejaculates using a Hemocytometer (Manual Cell Count)– NaCl dH2O Vs Triton Isoton

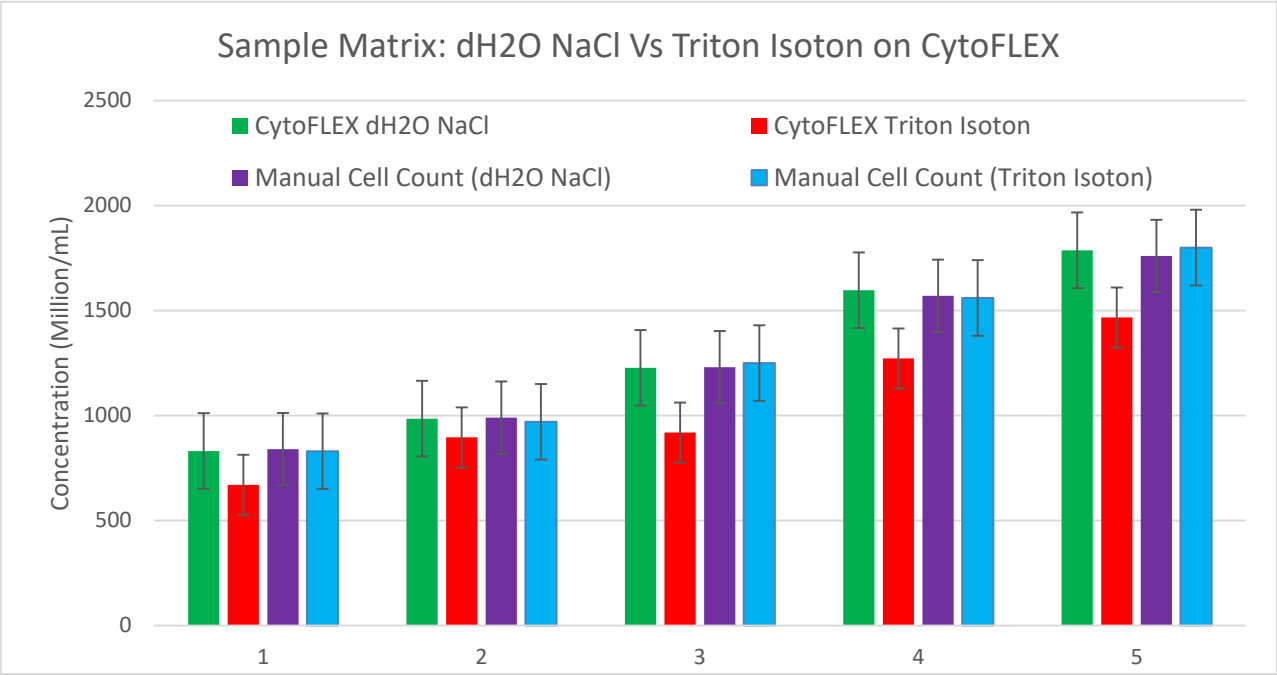

Fig 5. Comparison of different sample matrixes, (NaCl dH_2O Vs Triton) on semen concentration estimated by manual cell count. *(Error bars indicating standard error)*

From experiment in 3.2, It was determined that a Haemocytometer would be required to manually cell count to determine the accurate concentration readings. In Fig 5. It shows two different sample matrix's were tested on the CytoFLEX to determine the concentration of Raw Ejaculates, which allows the determination of quantity of straws produced in production. Both dH2O NaCl Vs Triton Isoton were tested on the CytoFLEX and then tested on a Hemocytometer (Manual Cell Count) to determine which or if both matrix's would be suitable to use on the Flow Cytometer. In Fig 5. It can be seen that dH2O NaCl (CytoFLEX), dH2O NaCl (Manual Cell Count) and Triton Isoton (Manual Cell Count) are all falling within the acceptable 10% range of each other, as seen on the bar chart all three are showing very similar results, which is what would be expected. It can be seen in the bar chart that Triton Isoton (CytoFLEX) is not giving the same results compare to the other three. The Triton Isoton matrix when ran on the CytoFLEX was giving significantly lower concentration readings than the dH2O NaCl (CytoFLEX), dH2O NaCl (Manual Cell Count) and Triton Isoton (Manual Cell Count).

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	210770.9	3	70256.98	0.488723	0.694964	3.238872
Within Groups	2300102	16	143756.4			
Total	2510873	19				

Table 4. ANOVA - Analysis of semen concentration estimated by the manual cell count, for different sample matrixes, dH_2O Vs Triton Isoton

Note, the results were found to be not statistically significant; the null hypothesis is accepted, $p > 0.05$.

2.3.4 Comparison of different concentration methods

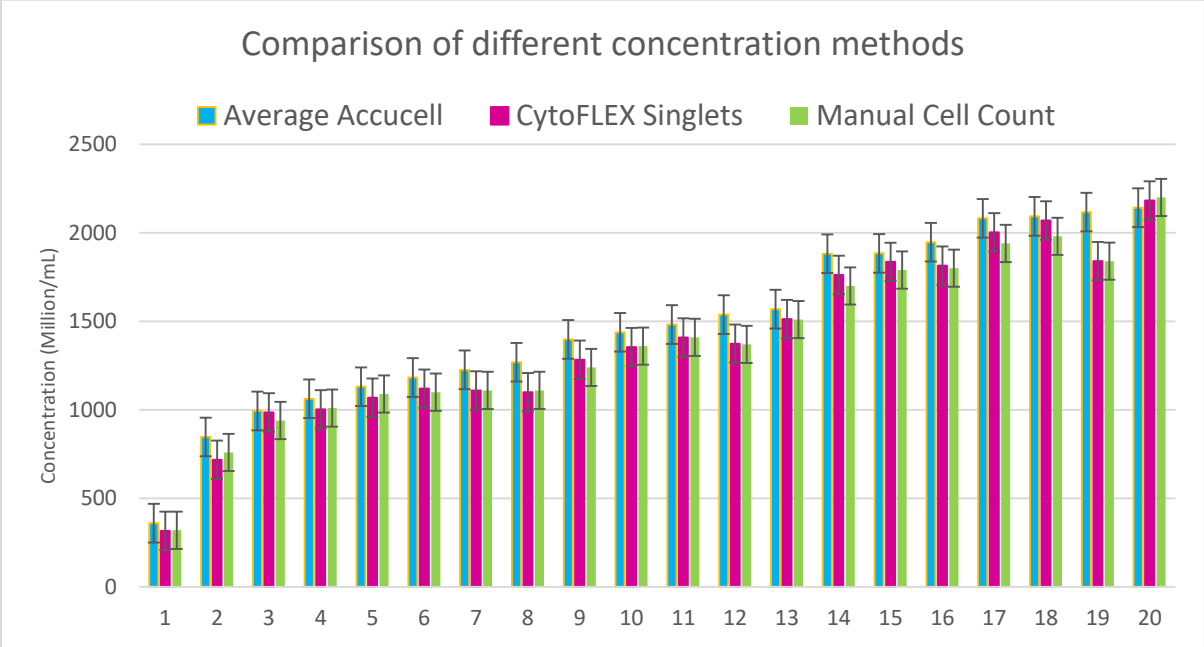

Fig 6. Comparison of different concentration methods for semen concentration estimation. *(Error bars indicating standard error)*

The comparison of different concentration methods for semen concentration estimation was carried out to determine whether the new or old method was more accurate in determination of semen concentration, both methods were compared to a Haemocytometer (Manual Cell Count) which is known as the gold standard for counting cells.

In Fig 6. It shows the comparison of different concentration methods, the methods compared were, AccuCell (Photometer) Flow cytometry (CytoFLEX) and Hemocytometer (Manual Cell Count). After running 100's of samples on the AccuCell concentration method and the new method using the Flow Cytometer (Singlets) and compared both with a Hemocytometer (gold standard in counting cells). It was found that the flow cytometer was closer (within 5%) to concentration reading than the old (AccuCell) method used (within 10%), as seen in Fig 6. The research shows that the CytoFLEX reading were more consistent with the manual cell count. The CytoFLEX readings compared to the manual cell count readings also allowed the CytoFLEX method that was developed to determine the concentration of a raw ejaculate to be validated.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	125181	2	62590.52	0.27052	0.763957	3.158843
Within Groups	13188156	57	231371.2			
Total	13313337	59				

Table 5. ANOVA - Analysis of semen concentration estimated by different concentration methods: AccuCell Vs CytoFLEX Vs Hemocytometer

Note, the results were found to be not statistically significant; the null hypothesis is accepted, $p > 0.05$.

2.3.5 Technician variation comparison on AccuCell

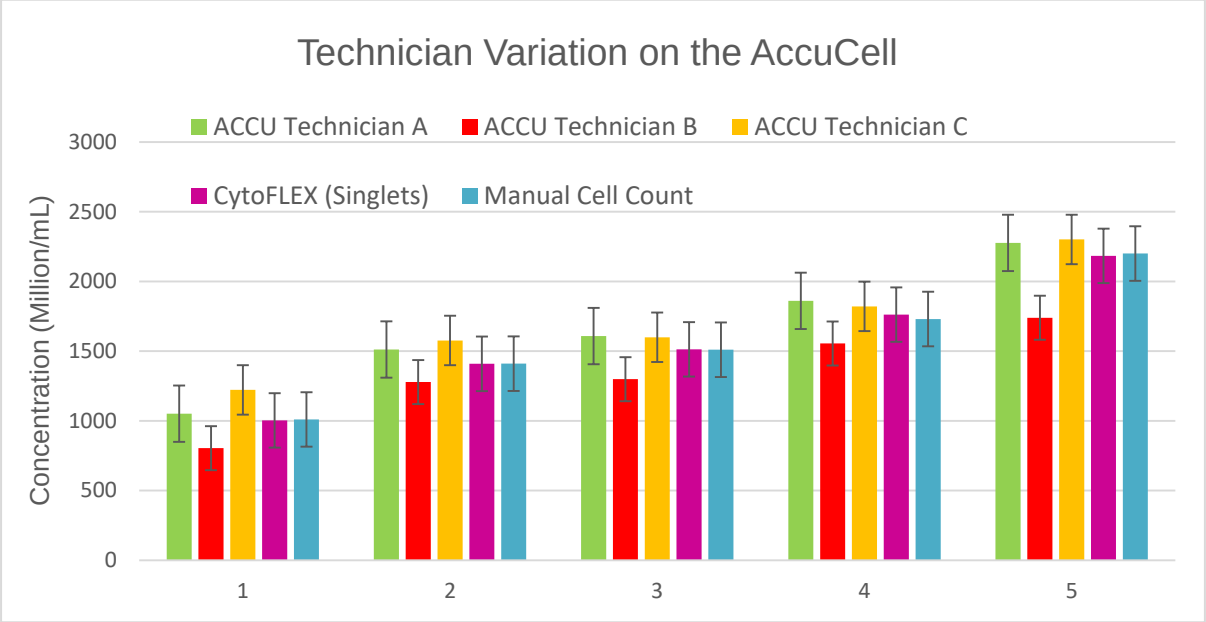

Fig 7. Comparison of different technicians on semen concentration estimated by the AccuCell. *(Error bars indicating standard error)*

The comparison of different technicians on semen concentration estimated by the AccuCell was carried out due to the inconsistent repeatability of a bulls sperm concentration over time. In Fig 7. Technician A, B and C all had varied results on the AccuCell for the same samples. The same samples when compared on the CytoFLEX and the Hemocytometer gave similar results to one and other, with the hemocytometer being the gold standard in counting cells. As seen in Fig 7. Technician C had significantly lower readings which would affect the final concentrations in each individual straw.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	407677	4	101919.3	0.58713	0.675692	2.866081
Within Groups	3471778	20	173588.9			
Total	3879455	24				

Table 6. ANOVA - Analysis of semen concentration estimated by the AccuCell by different technicians

Note, the results were found to be not statistically significant; the null hypothesis is accepted, $p > 0.05$.

2.3.6 Technician variation comparison on CytoFLEX

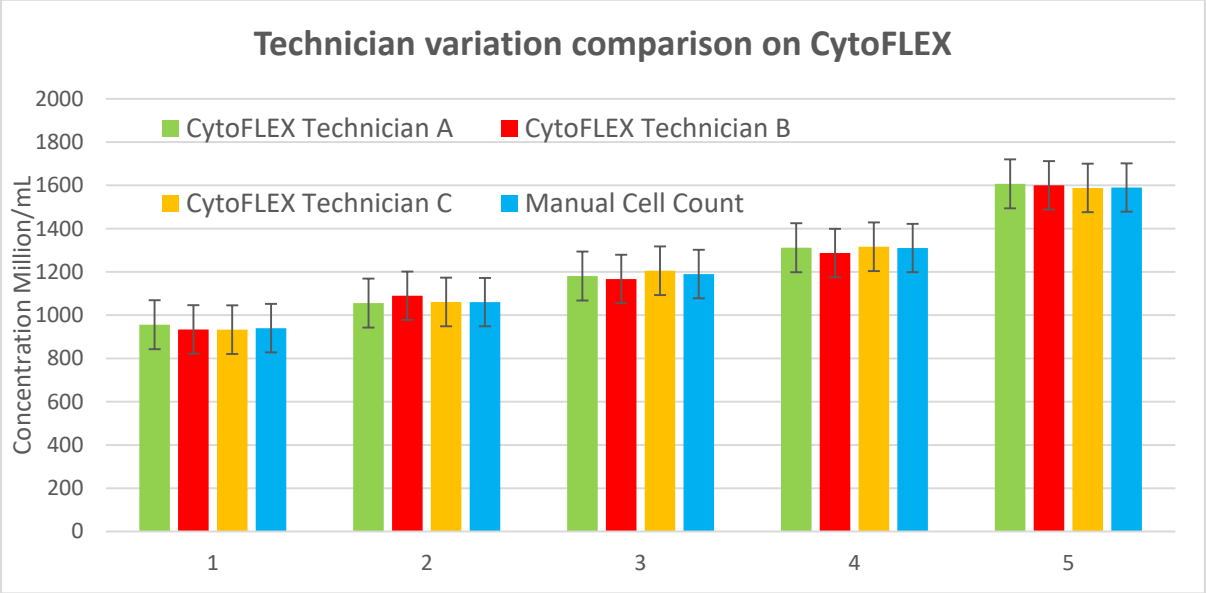

Fig 8. Comparison of different technicians on semen concentration estimated by the.CytoFLEX. *(Error bars indicating standard error)*

The comparison of different technicians on semen concentration estimated by the CytoFlex was to compare to the results of each technician AccuCell results. In Fig 8. Technician A, B and C all had consistent results on the CytoFLEX for the same samples. The same samples were then compared to the hemocytometer results and as seen in the bar chart all three technicians were getting very similar results to the gold standard for counting cells, all three technicians were within 5% of the hemocytometer result for the same sample. With the hemocytometer being the gold standard in counting cells and all three technicians falling within acceptable range of results, it was deemed that the CytoFLEX assay helped remove the technician variation which can be seen in Fig 8.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	132.95	3	44.31667	0.000703	0.999973	3.238872
Within Groups	1009008	16	63062.98			
Total	1009141	19				

Table 7. ANOVA - Analysis of semen concentration estimated by the CytoFLEX by different technicians

Note, the results were found to be not statistically significant; the null hypothesis is accepted, $p > 0.05$.

2.3.7 Comparison of Plastic test tubes Vs Glass test tubes for CytoFLEX – dH_2O NaCl

To help with the reduction in single use plastic in the laboratory, an assay was run using new (unwashed) glass test tubes, reused washed glass test tubes and plastic test tubes to determine if any populations would populate when ran on CytoFLEX using dH_2O NaCl. There were no cells found in any of the blank samples when comparing new Vs washed glassware and plastic tubes (dH_2O) on the CytoFLEX.

2.3.8 Comparison of Plastic test tubes Vs Glass test tubes for CytoFLEX – Raw Ejaculate

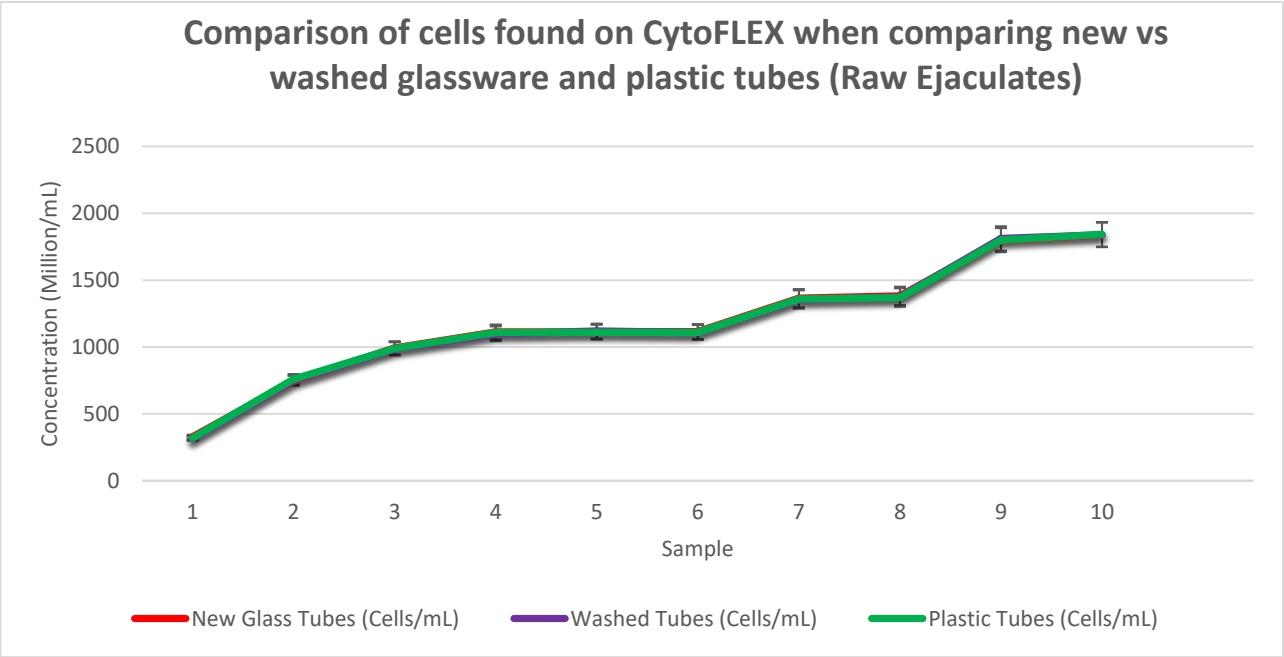

Fig 9. Comparison of different test tube materials on semen concentration estimated by the CytoFLEX.

After running the assay in 3.7 and it was deemed there were no cells found using water, the assay was then repeated but this time using semen samples to determine if what was seen in 3.7 could be replicated into the working laboratory. An assay was run using new (unwashed) glass test tubes, washed glass test tubes and plastic test tubes to determine if any difference in concentration reading with the three test tube types when ran on CytoFLEX using raw ejaculate, when comparing new Vs washed glassware and plastic tubes (Raw Ejaculates) between new, washed and plastic test tubes there was a standard deviation of < 10% in each case new glassware, washed glassware and plastic test tubes.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	67.2	2	33.6	0.000162	0.999838	3.354131
Within Groups	5598456	27	207350.2			
Total	5598523	29				

Table 8. ANOVA - Analysis of cells found by the CytoFLEX when comparing different test tube materials

Note, the results were found to be not statistically significant; the null hypothesis is accepted, $p > 0.05$.

2.3.9 Comparison of Immature (Young) Vs Mature (Older) Bulls

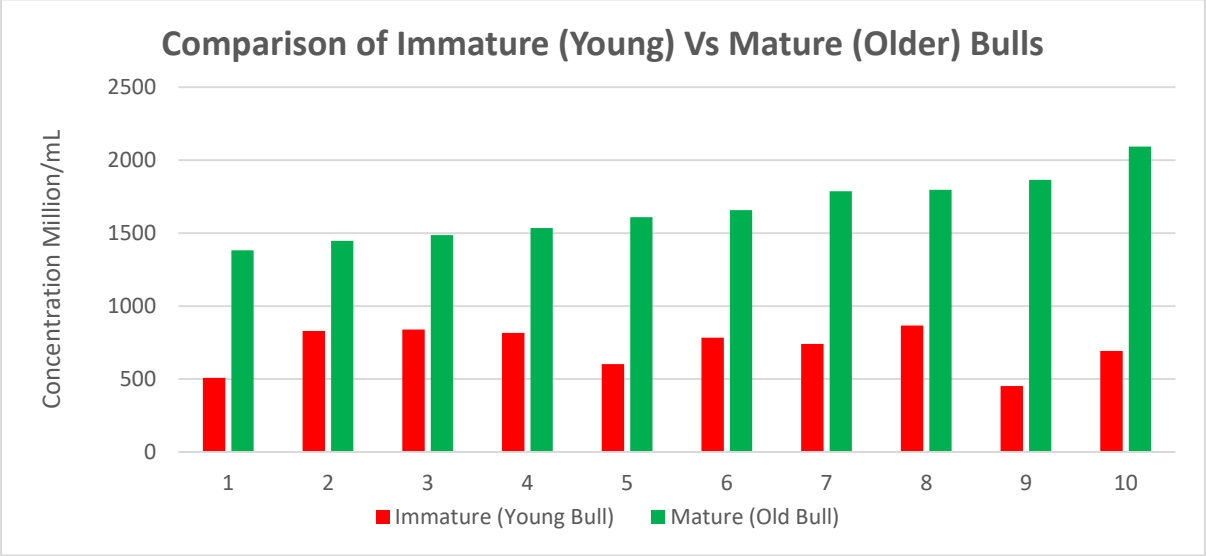

Fig 10. Comparison of Immature (Young) Vs Mature (Older) Bulls on semen concentration estimated by the CytoFLEX.

A factor that can affect sperm quality is bull age, young bulls tend to produce low volumes of ejaculates when bulls are first jumped. These low volumes on ejaculates tend to have low sperm concentrations, with both low volume ejaculates and low sperm concentrations lead to poor quality ejaculates. To overcome this poor quality ejaculates in young bulls, studies show that collecting young bulls ejaculates regularly helps overcome these immaturities. *(*Taaffe, P, et al. 2020*)* A study carried out by *Hallap T. et al. (2004),* shows as a bull gets older, the volume of the ejaculated by the bull increases and semen quality increases.

The Comparison of Immature (Young) Vs Mature (Older) Bulls on semen concentration estimated by the CytoFLEX was done to determine that what we expect to see from an immature and mature bull is captured on the CytoFlex. In Fig 10. A comparison of Immature (Young) Vs Mature (Older) bulls was analysed. It can be seen that the immature bulls are giving low concentrations of > 900 Cells/Million/mL and the mature bulls having higher concentrations averaging at 1666 Cells/Million/mL.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4538186	1	4538186	130.2074	0.0000000011	4.413873

Within Groups	627363.3	18	34853.52			
Total	5165550	19				

Table 9. ANOVA - Analysis of semen concentration estimated by the CytoFLEX using Immature (Young) Vs Mature (Older) Bulls

Note, the results were found to be statistically significant; the null hypothesis is rejected, $p < 0.05$. Thus, it was determined by ANOVA that the age of a bull (Maturity) has a significant difference in concentrations readings.

2.3.10 Concentration readings of a Bull suffering from injury (Lame Leg)

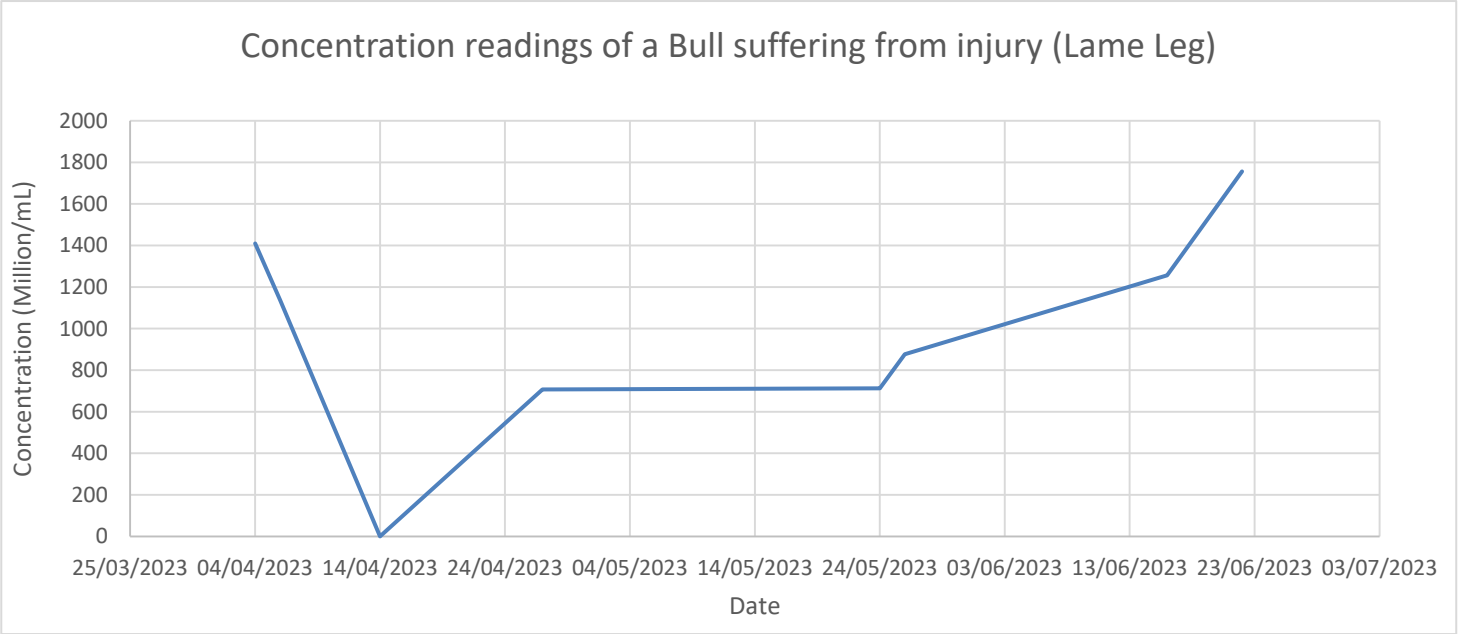

Fig 11. Comparison of a Bull suffering from an injury (Lame Leg) on semen concentration estimated by the CytoFLEX.

The comparison of a Bull suffering from an injury (Lame Leg) on semen concentration estimated by the CytoFLEX was analysed to show the effects on sperm concentration when a bull not at full health. In Fig 11. A single bull's concentrations were tracked over a 3 Month period, over the course of this period, a once healthy bull, suffered from an injury, in this case the bull had a lame leg which occurred on the 14th of April 2023. In Fig 11. It shows how that an injury can have significant effect on the concentration of an ejaculate. As seen in Fig 10. The concentration before injury is 1400 and 1200 Cells/Million/mL. The lame leg was noted on date 14/04/2023, from this date it can be seen that the bull's concentration of the bull's ejaculate begins to rapidly decline and these lower concentrations, to around 700-800 Cells/Million/mL, which can be seen over a 60 day period, which we can see in Fig 10. The concentrations begin to increase again after the 60-day period. Thus, it is determined that an injury such as a lame leg can greatly affect the production of AI straws.

2.3.11 Plastic Waste

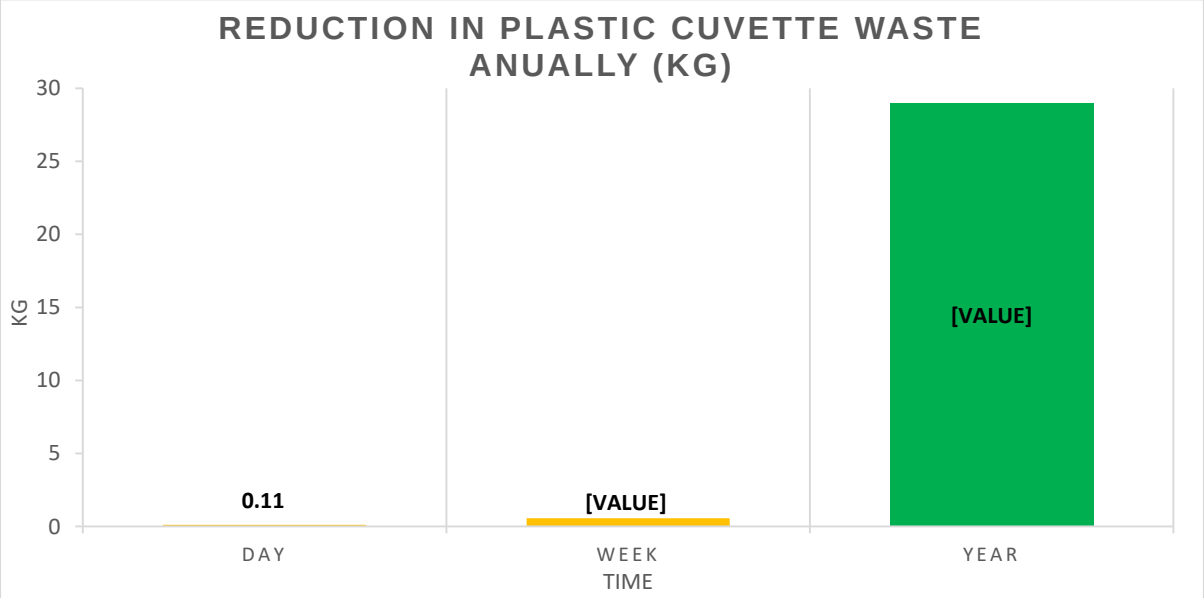

Fig 12. Annual Reduction in Plastic Cuvette waste (KG)

The reduction of single use plastic waste was looked at due to growing global sustainability crisis. As an AI company NCBC is helping contribute to a more sustainable farming for the future. Thus I wanted to determine in which areas the laboratory could potentially change materials from single use plastic to reusable materials such as glass. In Fig 12. The change in methods to determine the concentration of a raw ejaculate allows the laboratory to use less plastics in the new method. Using the AccuCell, plastic cuvettes were used to run a sample and it was determined 28.99KG of single use plastic was saved each year by changing to reusable glass test tubes on the CytoFLEX. One single use plastic cuvette weighs 2.23g with an average of 50 cuvettes used daily.

2.4 Discussion

2.4.1 Flow Cytometry

Artificial insemination has become a powerful tool for cattle breeding, a bull's fertility is a key factor in cattle production. By using semen from superior genetics bulls it allows the beef and dairy industry's reach higher levels of production while becoming more sustainable, while overall increasing the genetics in the national herd. Bull fertility can be assessed by multiply methods; light microscope, CASA and flow cytometry. While all can assess the quality of sperm, flow cytometry seems to have major advantages over other methods.

Traditionally the light microscope is used in the assessment of bovine sperm, where viability, motility and morphology can be determined. The CASA can also be used to determine viability, motility and morphology in a sperm sample. Research carried out by NCBC, Viking Genetics, CRV and IMV technologies, showed that using a microscope and trained analyst was more accurate in the assessment of sperm quality compared to the CASA. *(C O'Meara et al, 2022)*

Table 10. has been removed due to Copyright restrictions.

Table 10. Comparison of methods of how effective each method is to determine sperm concentration of an ejaculate, taking analysis speed and accuracy into account. -*IMV technologies*

*(*the '+' sign indicates how effective each method is for determining the concentration of a raw ejaculate, taking analysis speed and accuracy into account)*

In Table 10. Microscope, CASA and Flow Cytometry methods can be seen where each method can detect and not detect in assessment of sperm cells. Flow cytometry has major advantages over the other two methods, in light microscopy and CASA methods only a few hundred sperm are being analysed whereas flow cytometry can analyse thousands of sperm cells of one ejaculated in seconds, giving a greater representation of a sperm sample. *(*Peña, F, et al. 2016*)* Light microscopy is also a slow labour-intensive process which would not be capable for every sample

in a high production laboratory. Thus, flow cytometry is superior due to being a rapid and more accurate method to determine viable and dead sperm cells compared to old methods which can be slow and labour intensive. Flow cytometry allows a better understanding of areas in sperm assessment where traditional methods cannot, these areas are; Debris, chromatin status, acrosome integrity, mitochondrial status, DNA fragmentation and oxidative stress. As research on sperm quality increases the possibility for more targets to be identified are anticipated, which in the future will allow even greater understanding and predicating bull fertility. *(*Martínez-Pastor, F, et al. 2010*)*

Developments in understanding a bull's fertility are necessary to reach full potential in beef and dairy farming. Genetic selection and semen quality assessments allow improvement in bull fertility, which allows higher rates of conception and increasing production. Studies are looking for connections between bull fertility traits, such as, volume, sperm concentration, motility and sperm abnormalities. (Hering, D. 2014). Using these fertility traits can allow us to genetically reproduce the best offspring which will eventually become the sires used for artificial insemination. In sperm analysis, it is important to remember that only a small number of sperm are being analysed in the ejaculate, it is preferable to carry out larger analysis of an ejaculate to get a true representation of an ejaculate. In methods such as light microscopy and CASA, only a few hundred sperm are being analysed whereas flow cytometry can analyse thousands of sperm cells of one ejaculated in seconds, giving a greater representation of a sperm sample. Flow cytometry advantages allows for many characteristics of cell structure and function to be assessed, simply, accurately and rapidly. It can be expected that with the use of flow cytometry and research a deeper understanding of sperm physiology can be obtained. In the future flow cytometry methods will hopefully open more doors to understand characteristics and functions of sperm cells, which will assist in the advancement of bull fertility potential. *(*Peña, F, et al. 2016*)*

2.4.2 Concentration of Ejaculates

Flow cytometry can be used to determine sperm concentration, which is important in determining the quantity of straws which can be produced based on each straw has 15 million cells per straw. A difficultly in sperm concentration assessment is debris in

the sperm sample. Debris can consist of, white blood cells, skin cells, bacteria and extender particles. Debris can increase sperm concentrations, which leads to an inaccurate calculation, which then results to less than 15 million sperm cells per straw. Currently in AI straw production, a photometer called 'AccuCell' is being used to determine sperm concentrations in raw ejaculates. The AccuCell is a satisfactory method in determining sperm concentration but can be time consuming and cannot detect debris which can lead to overestimation in sperm populations which could lead to inaccurate results. Another method which sperm concentrations could be determined would be using a Hemocytometer, which is deemed the gold standard in cell counting, unfortunately because this is a manual count method, it is extremely slow and labours and unsuitable for straw production, but an excellent method for validation of flow cytometer sperm concentrations.

After running 100's of samples on the AccuCell concentration method and the new method using the Flow Cytometer (Singlets) and compared both with a hemocytometer (gold standard in counting cells). It was found that the flow cytometer was closer (within 5%) to concentration reading than the old (AccuCell) method used (within 10%), as seen in Fig 6. The research shows that the CytoFLEX readings were more consistent with the manual cell count. It was determined that the difference in the AccuCell results was due to debris in the sample, which cannot be identified by the AccuCell, instruments such as the flow cytometer have allowed the identification of debris which allows them to be discarded in sperm concentrations. By accounting for the debris, it allows for an accurate calculation for sperm concentration per straw, without accounting for debris, there are less cells per straw which could lead to a reduction in fertility because field studies have determined that 15 million cells per straw for dairy bulls is optimum for egg fertilization and 20 million cells per straw for beef bulls is optimum for egg fertilization.

2.4.3 Comparison of different Flow Rates on the CytoFLEX Vs AccuCell

As seen in Fig 3. it was determined that both setting was giving results within 5% of each other and in Table 2. The results were found to be not statistically significant; the null hypothesis is accepted, $p > 0.05$. Both flow rate settings deemed acceptable to be used in the determination of concentration of a raw ejaculate. Due to the laboratory production being a high demand and fast-paced environment, it was

determined that the medium flow rate (30uL) at 20 seconds was the optimal setting to use to determine of concentration of a raw ejaculate, as more samples could be analysed overtime.

An experiment using the AccuCell was done to replicate the readings that the CytoFLEX would make with one sample, one sample was ran on the CytoFLEX for 20 seconds and it took approximately 1 minute to set up the glass test tube and 20 seconds to analyse the sample. When repeating the same sample on the AccuCell, I ran 20 plastic cuvettes on the AccuCell, it took approximately 20 minutes to set up 20 plastic cuvettes and roughly another 20 minutes to run each cuvette in duplicate, my findings showed that when compared to the hemocytometer the one sample ran on the CytoFLEX was more accurate than running 20 samples in duplicate on the AccuCell. Thus, we can see the CytoFLEX method allows rapid analysis, which is vital to a high production laboratory.

2.4.4 Comparison of concentration measurement of Raw Ejaculates using a Hemocytometer (Manual Cell Count)– NaCl dH2O Vs Triton Isoton

In Fig 5. It shows two different sample matrixes were tested on the CytoFLEX to determine the concentration of Raw Ejaculates and then tested on a Hemocytometer (Manual Cell Count) to determine which or if both matrixes' would be suitable to use on the Flow Cytometer. In Fig 5. dH2O NaCl (CytoFLEX), dH2O NaCl (Manual Cell Count) and Triton Isoton (Manual Cell Count) are all falling within the acceptable 10% range of each other, as seen on Fig 5. all three are showing very similar results, which is what would be expected. It can be seen in Fig. 5 the Triton Isoton (CytoFLEX) is not giving the same results compare to the other three.

The Triton Isoton matrix when ran on the CytoFLEX was giving significantly lower concentration readings than the dH2O NaCl (CytoFLEX), dH2O NaCl (Manual Cell Count) and Triton Isoton (Manual Cell Count). It was determined that the issue with using Triton Isoton on the CytoFLEX is that the Triton was a thick liquid that was causing a viscosity issue when ran on the CytoFLEX. The samples fluid's viscosity gauges its flow resistance. The measurement of sperm motility and concentration may be affected by high viscosity. *(S. S. Vasan,2011)*

Thus it was determined that Triton Isoton solution combined with the semen sample that the viscosity was too high for it to flow correctly through the flow cytometer. To confirm this, manual cell counting using Triton Isoton was performed and the results were within range. It was determined that there was a viscosity issue while using the Triton Isoton and this sample matrix was then deemed not fit for purpose and dH_2O NaCl was determined to be the sample matrix for determining the concentration of Raw Ejaculates, which allows the determination of quantity of straws produced in production using Flow Cytometry. *(S. S. Vasan,2011)*

2.4.5 Comparison of different concentration methods

Determining the concentration of raw ejaculates, allows the determination of quantity of straws produced in production, getting the correct concentration reading is hugely important to the business regarding the profit for the company and keeping the laboratory as sustainable as possible. If a raw ejaculate concentration is incorrect and too high, the sample will be over diluted and thus the number of cells per straws will be incorrect (too low) and the straws will not pass Quality Control and will be discarded, which will add to plastic waste. If the concentration reading is too low, the raw ejaculate will not have reached its optimal straw potential, thus not making the most out of the product and being unsustainable. Thus, the concentration step in the production process is a major part of the process in the laboratory production to get the best quality and most sustainable product. After analysis of 100's of samples using AccuCell concentration method and the new method using the Flow Cytometer (Singlets) and compared both with a Hemocytometer, it was found that the flow cytometer was closer (within 5%) to concentration reading of the manual cell count than the old (AccuCell) method used (within 10%) (Fig 6). The obtained results show that the CytoFLEX readings were more consistent with the manual cell count.

The flow cytometer was set up based on the size of a bovine sperm cell which cuts out the readings for debris in a sample, giving a more accurate concentration of the sample. The AccuCell automatically calibrates and cannot distinguish the debris in the sample due to the size in which the AccuCell calibrates, which is likely leading to the differences in concentration readings between methods. The CytoFLEX readings compared to the manual cell count readings also allowed the CytoFLEX method that was developed to determine the concentration of a raw ejaculate to be validated.

2.4.6 Technician variation on the CytoFLEX Vs AccuCell

In Fig 7. Technician A, B and C all had varied results on the AccuCell for the same samples. The same samples when compared on the CytoFLEX and the Hemocytometer gave similar results to one and other, with the hemocytometer being the gold standard in counting cells. After watching how each technician carries out the assay, it was determined that the reasons for the variation in results were due to the different intensity of inverting the sample before it was read by the AccuCell. Technician B only lightly inverted each sample whereas technicians A and C inverted each sample with more force, helping provide a more accurate representation of the overall sample. As seen in Fig 7. Technician C had significantly lower readings which would affect the final concentrations in each individual straw. The cuvettes used in the AccuCell were not able to be vortexed (due to shape of cuvette) whereas the test tubes used for the CytoFLEX can be vortexed, which allowed more consistency between technicians in how well each sample was being mixed.

In Fig 8. Technician A, B and C all had consistent results on the CytoFLEX for the same samples. The same samples were then compared to the hemocytometer results and as seen in Fig 8. all three technicians were getting very similar results to the gold standard for counting cells, all three technicians were within 5% of the hemocytometer result for the same sample. With the hemocytometer being the gold standard in counting cells and all three technicians falling within acceptable range of results, it was deemed that the CytoFLEX assay helped remove the technician variation when determining the concentration of a raw ejaculate

Technician variation could be resulting in inaccurate concentration readings in the AccuCell, before the sample would run on the AccuCell each sample needed to be inverted to help mix the sample before readings took place, I found the variation in the inverting of the sample played a huge part in the inaccuracy of the sample concentrations. The samples had to be run in specific cuvettes that were plastic and these cuvettes were unable to be vortexed, so there was no consistency in inverting by laboratory technicians as seen in Fig 7. The new method using flow cytometry avoids this technician variation as the flow cytometer used glass/plastic test tubes which can be vortexed, allowing consistency throughout each laboratory technician

and every sample as seen in Fig 8.The flow cytometer was also set up based on the size of a bovine sperm cell which cuts out the readings for debris in a sample, giving a more accurate concentration of the sample

2.4.7 Comparison of Immature (Young) Vs Mature (Older) Bulls

Young bulls tend to produce low volumes of ejaculates when bulls are first jumped. These low volumes on ejaculates tend to have low sperm concentrations, with both low volume ejaculates and low sperm concentrations lead to poor quality ejaculates. A study carried out by P. Taaffe, 2020, "Increasing the frequency of ejaculate collection in young dairy bulls increases semen production and field fertility." The study shows that collecting young bulls ejaculates regularly helps overcome low volume ejaculates. Studies show as a bull gets older, the volume of the ejaculated by the bull increases and semen quality increases.

2.4.8 Concentration readings of a Bull suffering from injury (Lame Leg)

Each bull must be maintained in good physical condition to enable him to produce high volume ejaculates without becoming fatigued and to produce consistently high-quality semen. It entails preserving the health of the bull and lowering the possibility of infertility brought on by fever or injury. *. (Brian Cumming, 2007)* In Fig 10. A single bull's concentrations were tracked over a 3 Month period, over the course of this period, a once healthy bull, suffered from an injury, in this case the bull had a lame leg which occurred on the 14th of April 2023.

In Fig 11. It shows the concentration before injury is 1400 and 1200 Cells/Million/mL. The lame leg was noted on date 14/04/2023, from this date it can be seen that the bull's concentration of the bull's ejaculate begins to rapidly decline and these lower concentrations, to around 700-800 Cells/Million/mL, which can be seen over a 60 day period, which we can see in Fig 11. The concentrations begin to increase again after the 60-day period. , *(Tomaž Snoj. et al. 2020)* When lameness occurs in a bull, limping of the injured limb, lack of interest in mounting, low libido, slow thrust, all contribute to the concentration and volume of an ejaculate. It is determined that an injury such as a lame leg can greatly affect the production of AI straws. It takes a 60 day period for the spermatozoa in the scrotum to be fully removed from a bull. Thus,

it is determined that an injury such as a lame leg can greatly affect the production of AI straws. *(Chand N*, *et al. 2017*)

2.4.9 Sustainability

Temperatures are increasing globally, there has been a rise in greenhouse gases (GHG) in the atmosphere, primarily created when we burn fossil fuels and during industrial operations, as well as emissions linked to land use, are driving this trend. The main source of Ireland's GHG emissions is Irish agriculture. *(Teagasc, 2023)* Our world is changing due to the increase in climate change. As a result, the Irish government has set goals to achieve climate neutrality by 2050 and reduce greenhouse gas (GHG) emissions from agriculture by 25% by 2030. To reduce GHG emissions by 25% by 2030, Irish farmers need adoption technologies, such as Artificial Insemination. AI is one of the answers to be able to achieve the goal of reduce GHG emissions by 25% by 2030. AI's next big step is looking into breeding lower methane emitting animals. *(Teagasc, 2023)* Using AI can reduce methane emissions while increasing milk and beef production is Ireland's answer to become more sustainable in agriculture.

Determining the concentration of raw ejaculates, allows the determination of quantity of straws produced in production, getting the correct concentration reading is hugely important to the business regarding the profit for the company and keeping the laboratory as sustainable as possible. If a raw ejaculate concentration is incorrect and too high, the sample will be over diluted and thus the number of cells per straws will be incorrect (too low) and the straws will not pass Quality Control and will be discarded, which will add to plastic waste. If the concentration reading is too low, the raw ejaculate will not have reached its optimal straw potential, thus not making the most out of the product and being unsustainable. Thus, the concentration step in the production process is a major part of the process in the laboratory production to get the best quality and most sustainable product. In Fig 6. It can be seen the Average AccuCell readings for concentration were reading higher than CytoFLEX and the Hemocytometer and the CytoFLEX and the Hemocytometer were reading within 5% reading of each other. Thus, in my research I found the CytoFLEX method to be the more accurate method in determining the of quantity of straws produced in production.

In Fig 12. Shows using the AccuCell, plastic cuvettes are used to run a sample and it was determined 28.99KG of single use plastic was saved each year by changing to reusable glass test tubes on the CytoFLEX. One single use plastic cuvette weighs 2.23g with an average of 50 cuvettes used daily. The change in methods to determine the concentration of a raw ejaculate allows the laboratory to use less plastics in the new method, which helps the laboratory to become more of a Green Laboratory.

Thus, it was determined that the old method using an AccuCell, where only single plastic cuvettes could be used was less accurate than the new method Flow Cytometry where reusable glass test tubes can be used to determine the concentration of a raw ejaculates, which then allows determination of quantity of straws produced in production. Moving away from the AccuCell and moving towards the CytoFLEX it was seen the laboratory can reduce its single use plastic daily in the laboratory while also getting more accurate results to produce the optimal product for each batch of straws to be as sustainable as possible.

2.5.1 Conclusion

In conclusion, it was determined that the old method using an AccuCell, where only single plastic cuvettes could be used was less accurate than the new method Flow Cytometry where reusable glass test tubes can be used to determine the concentration of a raw ejaculates, which then allows determination of quantity of straws produced in production. Moving away from the AccuCell and moving towards the CytoFLEX it was seen the laboratory can reduce its single use plastic daily in the laboratory while also getting more accurate results to produce the optimal product for each batch of straws to be as sustainable as possible.

2.6.1 References

Abadi, A. R., G. Almaw, A. Solomon, W. Tadeg and B. Tadesse (2020). "Bacteriospermia and Sperm Quality of Cryopreserved Bull Semen Used in Artificial Insemination of Cows in South Wollo Zone, Ethiopia." Veterinary Medicine International 2020: 11.

Al Makhzoomi, A. K., N. Lundeheim, M. Håård and H. Rodríguez-Martínez (2008). "Sperm morphology and fertility of progeny-tested AI dairy bulls in Sweden." Theriogenology 70(4): 682-691.

Anzar, M., T. Kroetsch and L. Boswall (2011). "Cryopreservation of bull semen shipped overnight and its effect on post-thaw sperm motility, plasma membrane integrity, mitochondrial membrane potential and normal acrosomes." Animal Reproduction Science 126(1): 23-31.

Boakari, Y. L., M. F. Chamorro, L. Huber, J. G. Schnuelle, T. Passler, J. Stockler, J. Rush, B. W. Newcomer, K. Waters, J. Cowley, E. Malmstrom, D. Martinez, S. Kennedy and R. Hopper (2022). "Effect of lameness on breeding soundness examination results of beef bulls." Theriogenology 185: 134-139.

Brennan, A. (2023). "Breeding a key tool for sustainability."

Bucher, K., E. Malama, M. Siuda, F. Janett and H. Bollwein (2019). "Multicolor flow cytometric analysis of cryopreserved bovine sperm: A tool for the evaluation of bull fertility." Journal of Dairy Science 102(12): 11652-11669.

Camus A, C. S., Lévêque C, Schmitt E, Staub C. (2011). "Is photometry an accurate and reliable method to assess boar semen concentration?".

Chand N., S. A. S., Tyagi S., Sharma A. (2017). "Effect of lameness on semen quality in crossbred frieswal bulls."

Cordelli, E., P. Eleuteri, G. Leter, M. Rescia and M. Spanò (2005). "Flow cytometry applications in the evaluation of sperm quality: semen analysis, sperm function and DNA integrity." Contraception 72(4): 273-279.

Ciara O'Meara, E. H., Kasia Kupisiewicz, Catherine Latour, Marleen Broekhuijse, Agnes Camus, Lucie Gavin-Plagne, and Eli Sellem (2022). "The effect of adjusting settings within a Computer-Assisted Sperm Analysis (CASA) system on bovine sperm motility and morphology results."

Cumming, B. (2007). "Bull health."

Diavão J, S. A., Sguizzato ALL, da Silva CS, Tomich TR, Pereira LGR. (2023). "How does reproduction account for dairy farm sustainability?"

Druet T., S. F., E. Sellem,B. Basso,O. Gérard,L. Salas-Cortes,P. Humblot,X. Druart,A. Eggen (2009). "Estimation of genetic parameters and genome scan for 15 semen characteristics traits of Holstein bulls."

Evenson, D. P., J. E. Parks, M. T. Kaproth and L. K. Jost (1993). "Rapid Determination on Sperm Cell Concentration in Bovine Semen by Flow Cytometry1." Journal of Dairy Science 76(1): 86-94.

Evenson, D. P. (2016). "The Sperm Chromatin Structure Assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility." Animal Reproduction Science 169: 56-75.

Farzane Sivandzade, A. B., and Luca Cucullo (2019). "Analysis of the Mitochondrial Membrane Potential Using the Cationic JC-1 Dyeas a Sensitive Fluorescent Probe ".

Fernandes, G. H. C., C. Paulo de Tarso Camillo de, A. J. Serra, C. André Maciel, P. Jean Pierre Schatzman, C. Rossato, E. C. Pinto Leal-Junior and R. Albertini (2015). "The Effect of Low-Level Laser Irradiation on Sperm Motility, and Integrity of the Plasma Membrane and Acrosome in Cryopreserved Bovine Sperm." PLoS One 10(3).

Flow Cytometry-Definition, Principle, Parts, Steps, Types, Uses - https://microbenotes.com/flow-cytometry/

Fuerst-Waltl, B., H. Schwarzenbacher, C. Perner and J. Sölkner (2006). "Effects of age and environmental factors on semen production and semen quality of Austrian Simmental bulls." Animal Reproduction Science 95(1): 27-37.

Graham, J. K., E. Kunze and R. H. Hammerstedt (1990). "Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry." Biol Reprod 43(1): 55-64.

Hallap T., M. H., U. Jaakma, B. Larsson, H. Rodriguez-Martinez (2004). "Variations in quality of frozen-thawed semen from Swedish and white AI sires at 1 and 4 years of age."

Harstine, B., Utt, M., & DeJarnette, J. (2018). "Integrating a semen quality control program and sire fertility at a large artificial insemination organization.".

Hering, D. M., K. Olenski and S. Kaminski (2014). "Genome-wide association study for poor sperm motility in Holstein-Friesian bulls."

Huiping Yang, J. D., Terrence R Tiersch (2015). "Determination of sperm concentration using flow cytometry with simultaneous analysis of sperm plasma membrane integrity in zebrafish Danio rerio."

ICBF (2023). "Dairy Gene Ireland programme."

ICBF (2020). " https://www.icbf.com/wp-content/uploads/2020/02/Understanding-EBI-PTA-BV-Spring-2020.pdf."

IMV technologies. "Comparison of methods for sperm analysis."

Jäkel, H., Henning, H., Luther, A., Rohn, K., Waberski, D., (2021). "Assessment of chilling injury in hypothermic stored boar spermatozoa by multicolor flow cytometry. Cytometry Part A 99, 1033–1041.. doi:10.1002/cyto.a.24301."

Januskauskas, A., A. Johannisson and H. Rodriguez-Martinez (2003). "Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility." Theriogenology 60(4): 743-758.

Kim, H. H., M. Funaro, S. Mazel, M. Goldstein, P. N. Schlegel and D. A. Paduch (2013). "Flow cytometric characterization of apoptosis and chromatin damage in spermatozoa." Reproductive BioMedicine Online 26(4): 393-395.

Kumaresan, A., A. Johannisson, E. M. Al-Essawe and J. M. Morrell (2017). "Sperm viability, reactive oxygen species, and DNA fragmentation index combined can discriminate between above- and below-average fertility bulls." Journal of Dairy Science 100(7): 5824-5836.

Martínez-Pastor, F., Mata-Campuzano, M., Álvarez-Rodríguez, M., Álvarez, M., Anel, L., De Paz, P (2010). "Probes and Techniques for Sperm Evaluation by Flow Cytometry. " Reproduction in Domestic Animals 45, 67–78.. doi:10.1111/j.1439-0531.2010.01622.x

McDonnell, B. (2022). "Genetic Trends in EBI – Agriland,."

Meara, C. O. (2022). "Hemocytometer Calculation."

Morrell J. M. (1991). Applications of flow cytometry to artificial insemination: a review.

Morrell, J. M. (2020). "Heat stress and bull fertility." Theriogenology 153: 62-67.

Murphy, E. M., A. K. Kelly, C. O'Meara, B. Eivers, P. Lonergan and S. Fair (2018). "Influence of bull age, ejaculate number, and season of collection on semen production and sperm motility parameters in Holstein Friesian bulls in a commercial artificial insemination centre." Journal of Animal Science 96(6): 2408-2418.

Nagy, S., T. Hallap, A. Johannisson and H. Rodriguez-Martinez (2004). "Changes in plasma membrane and acrosome integrity of frozen-thawed bovine spermatozoa during a 4h incubation as measured by multicolor flow cytometry." Animal Reproduction Science 80(3): 225-235.

Peña, F. J., C. Ortega Ferrusola and P. Martín Muñoz (2016). "New flow cytometry approaches in equine andrology." Theriogenology 86(1): 366-372.

Perry, V. E. A. (2021). "The Role of Sperm Morphology Standards in the Laboratory Assessment of Bull Fertility in Australia." Frontiers in veterinary science 8: 672058-672058.

Petrunkina A.M. and Harrison R.A.P, (2009). "Systematic misestimation of cell subpopulations by flow cytometry: A mathematical analysis."

Progressive Genetics. (2022). "High EBI HOFR."

Sakase, M., Fukushima, M. (2020). "Necessity of exact examination of sperm characteristics to assess artificial insemination-subfertile bulls. Journal of Reproduction and Development " 66, 199–203.. doi:10.1262/jrd.2020-029.

Samplaski, M. K., Dimitromanolakis, A., Lo, K.C., Grober, E.D., Mullen, B., Garbens, A., Jarvi, K.A., (2015). "The relationship between sperm viability and DNA fragmentation rates. Reproductive Biology and Endocrinology." 13.. doi:10.1186/s12958-015-0035-y.

Snoj, T., K. Blažič, N. Šehić, T. Vake and G. Majdič (2020). "Calves' management conditions affect sperm count in adult bulls." Acta Veterinaria Scandinavica 62(1): 65.

Taaffe, P., C. M. O'Meara, M. Stiavnicka, C. J. Byrne, B. Eivers, P. Lonergan and S. Fair (2022). "Increasing the frequency of ejaculate collection in young dairy bulls increases semen production and field fertility." Theriogenology 182: 45-52.

Teagasc (2022). "Adoption of Breeding Technologies on Future Beef Farms."

Teagasc (2023). "Steering farmers on a sustainable journey."

Vasan S. S. (2011). "Semen analysis and sperm function tests: How much to test?"