Determination of live human sperm metabolism during post-ejaculation life using ^{13}C-Magnetic Resonance Spectroscopy.

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Declaration

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

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Abbreviations

4-AP	4-aminopyridine
AAA	ATPase associated
ADP	Adenosine Diphosphate
ALH	Amplitude of Lateral Head Displacement
ATP	Adenosine Triphosphate
A.U.	Arbitrary Units
BSA	Bovine Serum Albumin
Bcl-2	B cell lymphoma 2
cAMP	Cyclic Adenosine Monophosphate
CASA	Computed Assisted Sperm Analysis
CO_2	Carbon Dioxide
CoA	Coenzyme A
DAPI	4',6-diamidino-2-phenylindole
DISC	Death Inducing Signalling Complex
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EBSS	Earle's Balanced Salt Solution
ERK	Extracellular Signal-regulated Kinase
ETC	Electron Transport Chain
FADH	Flavin Adenine Dinucleotide
FITC	Fluorescein Isothiocyanate Conjugated (FITC)
FRT	Female Reproductive Tract
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
HFF	Human Follicular Fluid
HSA	Human Serum Albumin
ICSI	Intracytoplasmic Sperm Injection
IP3	Inositol 1,4,5-trisphosphate
IVF	In Vitro Fertilisation
LDH	Lactate Dehydrogenase
LIN	Linearity
NADH	Nicotinamide Adenine Dinucleotide
NH_4Cl	Ammonium Chloride
NMR	Nuclear Magnetic Resonance
NSF	N-ethylmaleimide-sensitive fusion protein
ODF	Outer dense fibres
P4	Progesterone
PBS	Phosphate Buffered Saline
PDC	Pyruvate Dehydrogenase Complex
PESA	Percutaneous Epididymal Sperm Aspiration
PGE1	Prostaglandin E1
PKA	Protein Kinase A
PSA	*Pisum Sativum* Agglutin
P2X7	Purinoceptor 7
ROS	Reactive Oxygen Species
sAC	Soluble Adenylyl Cyclase
SCD	Sperm Chromatin Dispersion
SCSA	Sperm Chromatin Structure Assay
SEM	Standard Error of the Mean
STS	Staurosporine
SNAPS	Soluble NSF Attachment Protein

SNARE	Soluble NSF Attachment Protein Receptor
TBS	Tris Buffered Saline
TESA	Testicular Sperm Aspiration
TdT	Terminal Deoxynucleotidyl Transferase
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling
VAP	Average Path Velocity
VCL	Curvilinear Velocity
WHO	World Health Organisation
ZP3	Zona Pellucida Sperm-binding Protein 3

Abstract

The journey sperm make through the female reproductive tract (FRT) has two possible outcomes: fertilisation or death. During their journey three key events occur: (i) capacitation; (ii) hyperactivation; and (iii) the acrosome reaction. Whilst capacitation is a prerequisite for fertilisation to occur and the acrosome reaction allows sperm to penetrate the egg, the exact role of hyperactivation is debated. These key events likely use ATP generated by glycolysis or oxidative phosphorylation, but it is unclear what the exact ATP demands are. The aim of this research is to investigate live sperm metabolism during capacitation, hyperactivation, the acrosome reaction and apoptosis. Capacitation was successfully stimulated by HSA and bicarbonate in human and boar sperm, hyperactivation by caffeine, the acrosome reaction by progesterone, PGE1 and NH_4Cl, and apoptosis by staurosporine. Hyperactivation was detected by CASA, capacitation, acrosome reaction and apoptosis by fluorescent staining; tyrosine phosphorylation, FITC-PSA and TUNEL assay respectively. Metabolism was investigated using ^{13}C-NMR. There was a significant increase in lactate production during capacitation, hyperactivation and the acrosome reaction in human sperm and during capacitation and the acrosome reaction in boar sperm. A simultaneous decrease in bicarbonate production was observed during capacitation in human sperm and during the acrosome reaction in human and boar sperm. These results suggest that local glycolytic ATP generation in the head and principal piece support the capacitation, hyperactivation and acrosome reaction- associated events that occur in the head and tail, without a reliance on oxidative phosphorylation. An increased understanding of these essential events, and their underlying metabolic pathways, could lead to developments in assisted reproduction procedures, which could potentially impact the clinical outcome.

Chapter 1: Introduction and literature review

1.1 Introduction

In the UK, approximately 1 in 6 couples are faced with infertility and 30% of these cases are attributed to male factor (Cox *et al.*, 2022). Infertility is defined as the inability to achieve pregnancy after twelve months of regular, unprotected sex (Zegers-Hochschild *et al.*, 2017). Poor sperm quality can contribute to infertility amongst couples, and the energy metabolism pathways utilised by sperm to produce the adenosine triphosphate (ATP) required for sperm function could be an underlying cause. There has been little progress in understanding male infertility over the last 50 years despite the decline in male reproductive health becoming a global issue (Pacey, 2008; Barratt, De Jonge and Sharpe, 2018).

There are various contributing factors to male infertility with semen quality accounting for 30-50% of these cases (Pacey, 2008). A semen analysis is vital for the diagnosis of poor semen quality, and the sixth edition of the WHO manual has utilised semen analysis data from a reference population of approximately 3500 men worldwide, who had a natural conception within one year of trying, and suggested the lower fifth percentile of data can aid in the diagnosis of poor sperm quality. Examples of the data from the lower fifth percentile of the reference population includes: sperm concentration, ≤ 16 M/mL, (oligozoospermia), sperm progressive motility, ≤ 30%, (asthenozoospermia) and sperm morphology, ≤ 4%, (teratozoospermia) (World Health Organization, 2021). Men can also suffer from azoospermia, a condition in which the semen contains no sperm (World Health Organization, 2021). Assisted reproductive technologies are available for men suffering with the above conditions as well as to overcome other causes of infertility. Other causes of infertility include testicular disorders, genetic disorders, environment and lifestyle factors such as smoking, and sexual dysfunction (Leslie, Soon-Sutton and Khan, 2022). Despite the above known causes, infertility remains unexplained in 15-30% of cases (Gelbaya *et al.*, 2014).

The journey sperm make through the female reproductive tract (FRT) begins at ejaculation and ends at either fertilisation or death. There are three known key events which occur between these two time points. These are (i) capacitation; (ii) hyperactivation; and (iii) the acrosome reaction. After ejaculation, sperm are unable to fertilise the oocyte and must undergo physiological changes in the FRT to gain the ability to fertilise. This process is known as capacitation, and it is essential for fertilisation (Aitken and Nixon, 2013). During hyperactivation, sperm display non-progressive, vigorous, side to side movements. The exact function of hyperactivation remains unknown however it is thought to help with the penetration of the zona pellucida (Suarez, 2008a) and the detachment of sperm from the endosalpingeal epithelium prior to ovulation (Pacey et al., 1995). Prior to fertilisation sperm must also undergo the acrosome reaction to penetrate the zona pellucida of the oocyte (Jaiswal *et al.*, 1998). The underlying metabolic pathways utilised by sperm during capacitation, hyperactivation and the acrosome reaction, and whether these energy demands change during these processes, remains unknown and could be a contributing factor to poor sperm quality (Reynolds et al., 2017).

A deeper understanding of the metabolic pathways utilised by sperm as they prepare themselves for fertilisation could provide an insight into unexplained fertility, offering a potential explanation as to why some semen samples, despite having normal concentration, motility, and morphology, are unable to fertilise an oocyte. This could lead to developments in assisted reproduction procedures, both diagnostic and therapeutic, which could potentially impact the clinical outcome.

1.2 Literature review

1.2.1 Sperm structure

Sperm are highly specialised cells with the function of fertilising the ovum. Sperm are formed during spermatogenesis which occurs in the epithelium of the seminiferous tubules in the testes of the male reproductive tract (Griswold, 1995). This process begins at puberty and continues until death in a healthy male with no underlying testicular disorders. Initially, sperm are functionally immature and must undergo

initial maturation in the epididymis during which they acquire the ability to swim (Dacheux, Chevrier and Lanson, 1987). Sperm undergo further maturation in the FRT, following ejaculation, during processes known as capacitation (chapter 3), hyperactivation (chapter 4) and the acrosome reaction (chapter 5) which enable sperm to successfully fertilise an oocyte.

Sperm are comprised of a head, midpiece, principal piece and end piece enclosed by a plasma membrane, see Figure 1.1. The head contains a haploid nucleus which is covered by the acrosome. Between the head and the midpiece is the centriole, which is involved with the formation of the flagellum and subsequent sperm movement. The midpiece contains many mitochondria which are wrapped around the axoneme, the microtubule at the centre of the midpiece and principal piece of the sperm flagellum, which have been proposed to generate the ATP required for sperm function via the metabolic pathway oxidative phosphorylation (Hatefi, 1985). The principal piece contains cytosol where glycolysis takes place, thought to provide sperm with its motility, and the end piece contains the end of the axoneme (Amaral, Castillo, *et al.*, 2013).

The human sperm head is approximately 4 – 5.5 µm long, 3 µm wide and 1.5 µM thick and is surrounded by the inner acrosomal membrane, the outer acrosomal membrane and the plasma membrane (Mortimer, 2018). The haploid nucleus contains the paternal deoxyribonucleic acid (DNA) which is highly condensed during spermatogenesis and results in sperm being transcriptionally silent. During spermiogenesis, protamine's replace the histones bound to DNA making the DNA inaccessible to enzymes, protecting the genetic information (Dadoune, 2003). The acrosome covers around 40-60% of the sperm head, is located at the anterior portion of the sperm head and is divided into the apical and principal region (Mortimer, 2018). It is a secretory vesicle that contains proteolytic enzymes which digest the zona pellucida of the oocyte (Zaneveld *et al.*, 1991). The acrosome is essential for fertilisation as it is involved in the acrosome reaction which is discussed in detail in chapter 5.

The midpiece is approximately 1 – 1.5 times longer than the sperm head. The axoneme extends along the midpiece and contains nine outer doublet microtubules, connected to a central pair by radial spoke projections, see Figure 1.1. The radial spoke projections regulate the signal transfer between the central doublets and the dynein arms, producing the waveforms required for sperm propulsion (Fawcett, 1975; Linck, Chemes and Albertini, 2016). The dynein arms are motor proteins and coordinated movement of these proteins is responsible for flagella movement, with the inner dynein arms being responsible for flagella bending and the outer dynein arms being responsible for the increased flagellar beat and asymmetric waveforms (Inaba and Mizuno, 2016). Dynein consists of light, intermediate and heavy chains where ATP hydrolysis occurs, via an ATPase associated (AAA) ring, to release adenosine diphosphate (ADP) and an inorganic phosphate. The AAA ring consists of six domains, four of which can bind nucleotides. In the absence of a nucleotide, binding occurs between the microtubule and the AAA ring domain and in the presence of a nucleotide, a conformational change is induced, and detachment is observed (Burgess *et al.*, 2003). It is this process of binding and detachment that causes the adjacent microtubules to slide past one another, resulting in the flagella bending seen during sperm movement (Burgess *et al.*, 2003; Turner, 2003).

The outer microtubule doublets are surrounded by nine outer dense fibres (ODFs) that provide elasticity, flexibility, and structural support for sperm flagellum movement (Baltz, Oneeka Williams and Cone, 1990). Sperm contain approximately 50-75 mitochondria arranged in a helical pattern around the outer dense fibres in a structure known as the mitochondrial sheath (Hirata *et al.*, 2002; Sutovsky and Manandhar, 2006). Mitochondria are subcellular organelles, thought to be responsible for supplying ATP to the axoneme for sperm movement, derived from oxidative phosphorylation (Piomboni et al., 2012). Sperm mitochondria are different from somatic mitochondria in terms of their biochemistry. These differences are related to the specific protein isoforms and isoenzymes present, for example, cytochrome c and lactate dehydrogenase (LDH) (Piomboni et al., 2012).

The sperm flagellum, divided into the principal piece and the end piece, is approximately 45 – 50 µM, and is required to propel sperm through the FRT (Mortimer, 2018). It is surrounded by ODFs, which prevent damage to the sperm tail during ejaculation, and a fibrous sheath around the axoneme (Baltz, Oneeka Williams and Cone, 1990). The fibrous sheath is essential for sperm function as it not only provides structural support but also acts as a scaffold for glycolytic enzymes (Krisfalusi *et al.*, 2006). It also contains the sperm specific calcium channel CatSper, implying a role in calcium signalling (Qi *et al.*, 2007). The end piece contains the axoneme only (Bragina and Bocharova, 2017).

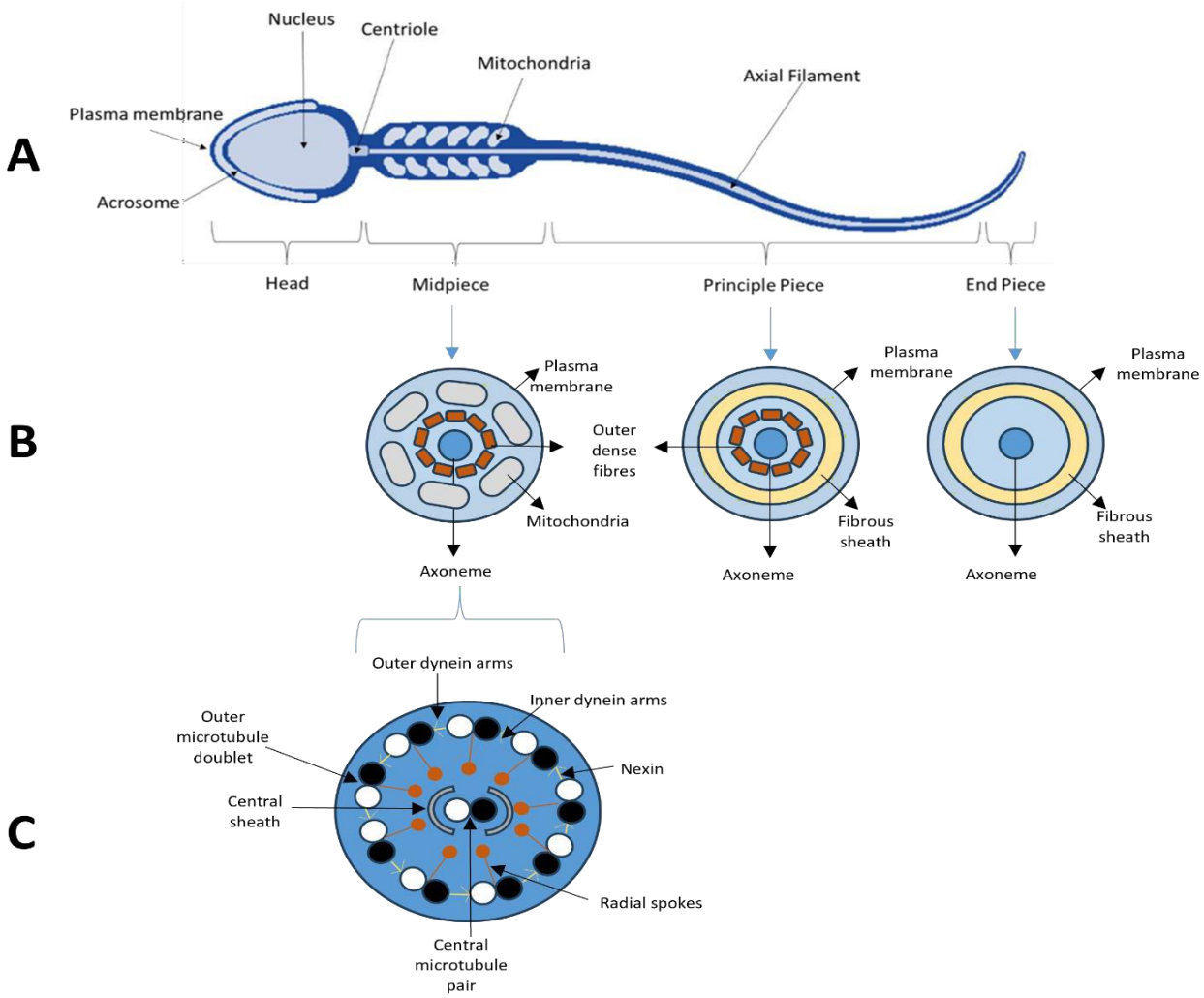


Figure 1.1: The overall structure of human sperm is shown in diagram (A). The human sperm consists of the following parts: the head, the midpiece, the principal piece and the end piece. Diagram **(B)** shows a cross section of the midpiece, principal piece and end piece. The midpiece contains mitochondria and the tail contains a fibrous sheath, with outer dense fibres present in the principal piece but absent in the end piece. Diagram **(C)** shows a cross section of the axoneme, which stretches across the midpiece, principal piece and end piece, and consists of nine outer doublet microtubules, connected to a central pair by radial spoke projections.

1.2.2 Sperm function

The ultimate function of sperm is to carry the male genome to allow it to fuse with a female genome to create life. Fertilisation occurs in the ampulla of the Fallopian tubes therefore following ejaculation, sperm must travel through the vagina, cervix, and uterus to fertilise the oocyte. An ejaculate contains seminal fluid as well as sperm, and the accessory proteins in the seminal fluid aid in the protection of sperm upon entering the FRT (Samanta *et al.*, 2018). For example, the alkaline pH of semen, pH 8, protects sperm from the acidic pH of the vagina, pH 4.5, and the presence of cytokines and other immunologic factors in the seminal fluid protects sperm from the female immune system (Robertson *et al.*, 2002; Politch *et al.*, 2007).

Ejaculated sperm display four typical patterns of movement: (i) rapid progressive; (ii) medium progressive; (iii) non-progressive; and (iv) immotile (World Health Organization, 2021). Progressive motility is the combination of both rapid and medium progressive sperm, which both move in a progressive direction, the difference being the speed at which they move. Non-progressive sperm display circular motions or alternatively twitch in the same location and immotile sperm do not show any movement. Motility has been shown to be dependent upon ATP, however, how they generate this ATP is not fully known (Ford, 2006; Turner, 2006). Differences in sperm motility have been suggested to be due to the different metabolic pathways utilised by sperm (Reynolds et al., 2017a). From a clinical perspective, progressive sperm are more likely to arrive at and fertilise the oocyte first hence are of more importance when considering sperm motility in relation to fertilisation (Comhaire *et al.*, 1988).

An average human ejaculate contains around 180 million sperm, however only an average of 50 sperm will reach the Fallopian tubes (Suarez, 2008b). The remining sperm will undergo cell death, presumably via apoptosis (Nakidkina and Kuzmina, 2019). The FRT, in which sperm can survive several days, is regulated to ensure only those sperm with the best motility and morphology will reach the oocyte. For example, sperm with poor motility and morphology are unable to pass through the cervical mucus (Suarez and Pacey, 2006). Sperm have been shown to survive in the FRT for up to five days prior to ovulation (Wilcox, Weinberg and Baird, 1995), where there is evidence that sperm, including human and boar, survive in a reservoir through the binding of their heads to the isthmic endosalpingeal epithelium (Hunter, Cook and Poyser, 1983; Pacey et al., 1995; Baillie et al., 1997; Reeve, Ledger and Pacey, 2003). It has been suggested that regulation of this sperm reservoir may have a role in selecting specific subpopulations of sperm for fertilisation, potentially those able to capacitate and undergo the acrosome reaction (Barratt and Cooke, 1991). It has been proposed that following ovulation, hyperactivation is required for the detachment of sperm from the isthmic endosalpingeal epithelium (Pacey et al., 1995).

1.2.3 The female reproductive tract

Physiological conditions in the FRT are tightly controlled with pH, ion concentration and O_2 tension varying throughout the different environments and throughout different stages of the menstrual cycle (Ng *et al.*, 2018). The different environments of the FRT contain fluids that are rich in metabolites, such as glucose, lactate, pyruvate, and amino acids, and these are available for sperm to utilise as they progress towards the Fallopian tubes (Gardner *et al.*, 1996; Leese *et al.*, 2007; Miller, 2018). The capability of sperm to metabolise a variety of substrates is fundamental for sperm survival in the various environments of the FRT. Fructose is present in high concentrations in the seminal plasma and this is the primary substrate metabolised by human sperm when in the seminal plasma, however, fructose is not present in high concentrations in the FRT (Mann, 1946). Glucose has been suggested to be preferably metabolised by sperm in the FRT, increasing the ATP concentration and motility of sperm (Williams and Ford, 2001). Pyruvate has also been shown to be present in the FRT and the ability of human sperm to convert pyruvate to lactate by lactate dehydrogenase following its transportation across the membrane has been noted (Reynolds et al., 2017b). The capacity of sperm to metabolise lactate has not been as well studied, despite its presence in the FRT (Boskey *et al.*, 2001).

The FRT also contains various ions that can influence sperm function. Calcium ions have been described as the key regulator of human sperm function, with an important role implicated in sperm motility, capacitation, hyperactivation and the acrosome reaction (Hong, Chiang and Turner, 1984). An increase in the concentration of intracellular calcium ions has been described during capacitation in human sperm (Dasgupta, Mills and Fraser, 1993). Intracellular calcium is modulated by the Na^+/Ca_2^+ antiporter and Ca_2^+- ATPase, which are both located in the sperm plasma membrane and control the influx/efflux of calcium ions (Lestari et al., 2018). Ca_2^+-ATPase is responsible for the removal of calcium ions and ATP is required for this process. The importance of intracellular calcium stores in mediating the calcium increase during capacitation and the acrosome reaction has been suggested following the identification of intracellular inositol-1,4,5-trisphosphate (IP3) receptor-gated calcium ion stores in human sperm (Ho and Suarez, 2001). This was supported by a study which associated calcium store defects with defective motility and hyperactivation

(Alasmari et al., 2013b). Extracellular calcium, in the FRT, has also been shown to be essential for the capacitation of human sperm in vitro (Luconi *et al.*, 1996). More recently, CatSper channels have also been shown to facilitate the influx of calcium ions across the sperm plasma membrane (Ren *et al.*, 2001). These are sperm specific, Ca^{2+} permeable, pH dependent ion channels, located on the sperm plasma membrane, and are responsible for regulating the influx of calcium ions from the FRT into sperm (Sun *et al.*, 2017). The CatSper channel was identified and described to be specific to sperm by Ren et al, (2001), and shown to be essential for fertilisation in a series of mice knockout experiments (Ren *et al.*, 2001; Qi *et al.*, 2007). The importance of CatSper channels has since been implicated for human sperm function and fertilisation potential (Carlson *et al.*, 2003; Li *et al.*, 2007). Calcium signalling has been shown to have a vital role in hyperactivation (Suarez and Ho, 2003; Suarez, 2008a), and has also been demonstrated to be the trigger of the acrosome reaction in human sperm (Baldi *et al.*, 1996; Michaut *et al.*, 2000).

The concentration of gases in the FRT are tightly controlled to provide optimum conditions for fertilisation and subsequent embryo development. The concentration of oxygen in the FRT is approximately 5%, hence in assisted reproductive procedures, gametes and subsequent embryos are cultured at the same concentration of oxygen to mimic the physiological environment. The concentration of carbon dioxide (CO_2) is also important as it can alter the pH of the cell/environment. The pH is highly regulated in sperm and the optimum pH for mammalian sperm function is approximately 7.4, required for maintenance of cell viability and homeostasis (Ng *et al.*, 2018). The pH in the uterus and in oviductal fluid in the human FRT is pH 7.3 – 7.7 which is optimum for sperm function (David, Serr and Czernobilsky, 1973). If changes in pH occur, it could hinder sperm function. It has been demonstrated that a reduction in intracellular pH, due to changes in CO_2, inhibits sperm motility in various species (Lodge, Graves and Salisbury, 1968; Bencic, Cloud and Ingermann, 2000; Ingermann *et al.*, 2002).

CO_2 and bicarbonate exist in equilibrium, see Equation 1 below. The concentration of bicarbonate in the oviduct, the site of capacitation, is > 20 mM compared to around 4 mM in the male reproductive tract and seminal plasma (Hereng et al., 2014). In an environment that contains CO_2, it will diffuse across the sperm plasma membrane providing an additional source of bicarbonate. Bicarbonate has been shown to induce sperm capacitation (Chen et al., 2000). Bicarbonate stimulates soluble adenylyl cyclase (sAC) which increases the product ion of the second messenger cyclic adenosine monophosphate (cAMP) from ATP, activating protein kinase A (PKA). This then results in the phosphorylation of serine, threonine, and tyrosine residues, indicative of sperm capacitation (Hess *et al.*, 2005). ATP is required to produce cAMP and subsequent activation of PKA. The stimulation of sAC by bicarbonate has also been suggested to increase the flagella beat frequency and support hyperactivation in sperm, demonstrating the importance of bicarbonate for sperm function and explaining its presence in the FRT (Xie et al., 2006; Hereng et al., 2014).

Equation 1: $CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$

1.2.4 Human vs boar sperm

Boar sperm are different in terms of their structure and motility compared to human sperm and other mammalian sperm. Whilst boar sperm have the same general structure and organelles that human sperm have, there are differences in sizes and shapes of the various regions. For example, boar sperm heads are roughly 7 µM long, 3.7 µM wide and 0.5 µM thick. The boar sperm head is also bilaterally flattened compared to human sperm, as noted by the difference of 1 µM in thickness. The midpiece is approximately 9 µM long and the flagellum, divided into the principal piece and the end piece, is 28 µM in length (Briz and Fàbrega, 2013). This makes the total length of boar sperm 43-45 µM compared to 50-65 µM seen for human sperm (Mortimer, 2018). It has previously been suggested that longer sea urchin sperm have an increased swimming velocity compared to shorter sea urchin sperm due to the increased flagella length providing greater forward propulsion (Fitzpatrick, Garcia-Gonzalez and Evans, 2010). This could explain the lower motility commonly reported for boar sperm, see below, which are shorter in length compared to human and other mammalian sperm (Briz and Fàbrega, 2013).

It has been well documented that boar sperm motility is much lower compared to other mammalian species and this has been suggested to be due to the site of semen deposition being closer to the oviduct compared to other species, combined with the contraction movements of the sow uterus during estrus, aiding sperm movement (Quintero-Moreno, Rigau and Rodrıǵ uez-Gil, 2004; Mogas, Álamo and Rodríguez-Gil, 2011). The site of deposition allows boar sperm to bypass the harmful areas of the FRT, such as the vagina and the cervix, in which there is a FRT immune response against sperm (Rodríguez-Gil and Bonet, 2016). Another key difference between human and boar sperm is that the boar sperm plasma membrane contains less cholesterol and glycolipids in comparison to human sperm as a result of their epididymal transit (Nikolopoulou, Soucek and Vary, 1985). Boar sperm contain higher levels of ether-linked phospholipids with around 70% of their membrane lipids being phospholipids, 25% neutral lipids and 5% glycolipids (Mann and Lutwak-Mann, 1981). This difference in lipid composition makes boar sperm extremely sensitive to cold shock, resulting in an irreversible decrease in motility and metabolic activity as well as a disrupted acrosome and plasma membrane (Simpson, Swan and White, 1987).

Despite these differences between human and boar sperm, they both must undergo capacitation, hyperactivation and the acrosome reaction in the FRT to successfully fertilise the oocyte and will require ATP to do so. How they generate this ATP and whether there are any differences in the metabolic pathways utilised by human and boar sperm remains unknown and will be investigated in this thesis.

1.2.5 Metabolism

Sperm require ATP which is utilised by the flagella protein dynein-ATPase to allow sperm to traverse the FRT (Lestari et al., 2018). Sperm generate ATP through two main metabolic pathways: glycolysis and oxidative phosphorylation (see Figure 1.2). However, whether sperm have preferred metabolic pathway to generate ATP remains unknown.

1.2.5.1 Glycolysis

Glycolysis occurs in the cytosol in the sperm flagellum, under anaerobic conditions and involves numerous enzyme-catalysed reactions that oxidise a six-carbon monosaccharide to produce two three-carbon compound pyruvate molecules and two ATP molecules. The oxidation of glucose to form pyruvate generates a net two molecules of ATP (Ford, 2006). Pyruvate can then either be further oxidised to acetyl-coenzyme A, producing CO_2 from the carboxyl group as a by-product, and enter the Krebs cycle (oxidative phosphorylation). Alternatively, pyruvate can be converted to lactate via the shuttling enzyme lactate dehydrogenase. The enzymatic reduction of pyruvate to lactate generally takes place in anaerobic conditions alongside a reduction of NADH to NAD+ which is recycled back into glycolysis (Piomboni et al., 2012). Following this reduction, sperm can re-oxidise lactate to pyruvate, by lactate dehydrogenase, where it can then enter the Krebs cycle to be metabolised further in aerobic conditions (Blanco and Zinkham, 1963; Goldberg, 1963). Numerous enzymes involved in glycolysis, such as glyceraldehyde 3-phosphate dehydrogenase (GADPH) and LDH, are bound to the fibrous sheath of the principal piece (Krisfalusi *et al.*, 2006). Sperm possess specific LDH isoforms and LDH-C4 has been shown to be crucial for sperm function as mutations in this enzyme reduce sperm fertilising capability (Odet *et al.*, 2008).

1.2.5.2 Oxidative phosphorylation

Oxidative phosphorylation occurs in the midpiece of sperm at the mitochondrial sheath and comprises of the Krebs cycle and the electron transport chain (ETC) (see Figure 1.2). It was at the University of Sheffield that Sir Hans Adolf Krebs published the work that led to the discovery of the Krebs cycle (Krebs, 1937, 1938; Krebs & Johnson, 1937; Krebs et al., 1938). Mature mammalian sperm contain around 80 mitochondria (Freitas, Vijayaraghavan and Fardilha, 2017). During oxidative phosphorylation, pyruvate is irreversibly oxidised to form Acetyl-Coenzyme A by the pyruvate dehydrogenase complex, producing CO_2 as a by-product, and Acetyl-coenzyme A enters the Krebs cycle (Wang *et al.*, 2021). This is oxidised further during the cycle in a series of reactions to release GTP, numerous cofactors, and additional CO_2 (Hatefi, 1985). Pyruvate is also capable of replenishing intermediates in the Krebs cycle, for example, pyruvate carboxylase catalyses the

carboxylation of pyruvate to oxaloacetate (Gray, Tompkins and Taylor, 2014a).

During these metabolic processes, both glycolysis and the Krebs cycle, numerous cofactors are generated, and these go on to drive the ETC. The transfer of electrons in the ETC from FADH2 and NADH to oxygen, which occurs via four protein complexes in the inner mitochondrial membrane, generates ATP. This transfer of electrons between these proteins causes protons to be pumped out of the mitochondrial membrane resulting in a proton motive force. As these protons are pumped back into the matrix, ATP is synthesised via ATP synthase. Oxidative phosphorylation generates approximately 30 molecules of ATP per molecule of glucose, hence is more efficient than glycolysis at generating ATP (du Plessis *et al.*, 2015).

1.2.5.3 Glycolysis vs oxidative phosphorylation: the debate

It remains unknown which is the preferred pathway for ATP generation in human sperm (Ford, 2006; Turner, 2006). Both pathways have been shown to be employed by human sperm through various experiments including the use of: (i) ^{14}C radiolabelled substrates (Ford and Harrison, 1981); (ii) metabolic inhibitors to measure the uptake of oxygen (Peterson and Freund, 1970); and (iii) identifying proteins involved in the regulation of sperm motility through the use of proteomics to identify the potential metabolic pathways and enzymes used by sperm (Amaral *et al.*, 2014).

Originally, sperm were thought to be exclusively glycolytic due to small respiration rates, however, through measurement of oxygen uptake, respiratory cytochrome enzymes were shown to produce ATP for sperm function (Macleod, 1943; Storey, 2008). Despite oxidative phosphorylation being active in sperm, glycolysis has been suggested to be the preferred metabolic pathway for the generation of ATP required for motility in human sperm by numerous studies (Peterson and Freund, 1974; Ford and Harrison, 1981; Lornage, Guerin and Czyba, 1986; Williams and Ford, 2001). Although there is a generally accepted idea that glycolysis powers sperm motility, oxidative phosphorylation is thought to support maturation and differentiation of sperm (Nakada *et al.*, 2006). One of the main reasons it is believed that glycolysis powers sperm motility is glycolytic enzymes have been identified in the fibrous sheath of boar sperm (Westhoff and Kamp, 1997). Additional studies have shown that when these glycolytic proteins on the fibrous sheath are knocked out, low sperm motility is observed (Miki *et al.*, 2004). In support of these findings, work by Mukai and Okuno (2004), showed that when glycolysis was inhibited and respiratory substrates supplied, low motility and lower levels of ATP were observed, suggesting oxidative phosphorylation alone is unable to support sperm motility (Mukai and Okuno, 2004). Similarly, the same study showed inhibitors of oxidative phosphorylation had no effect on motility. It must also be noted that there is a lack of mitochondria in the flagellum and the diffusion potential across the length of the flagellum is thought to be low. Work by Tombes and Shapiro (1987), shows insufficient diffusion capacity of ATP to the flagellum in sea urchin sperm when produced by mitochondria (Tombes and Shapiro, 1987). However, it remains unknown whether human sperm have a poor ATP diffusion capacity also (Turner, 2003).

The importance of glycolysis in generating the ATP required for human sperm capacitation has also been demonstrated by experiments that have highlighted the importance of glycolysable substrates, particularly glucose and pyruvate, for induction of capacitation (Ferrandi *et al.*, 1987; Rogers and Perreault, 1990; Hereng *et al.*, 2011). In support of the idea that glycolysis is the primary metabolic pathway in supporting human sperm motility, an association between a reduced expression of glycolytic enzymes and poor sperm quality, in particularly a reduced motility, has been reported (Liu *et al.*, 2019). Although there is a plethora of work showing glycolysis is responsible for the generation of ATP required for sperm function, the ability of glycolysis to meet the energy demands of motility remains disputed due to its significantly lower yield of ATP per glucose molecule when compared to oxidative phosphorylation.

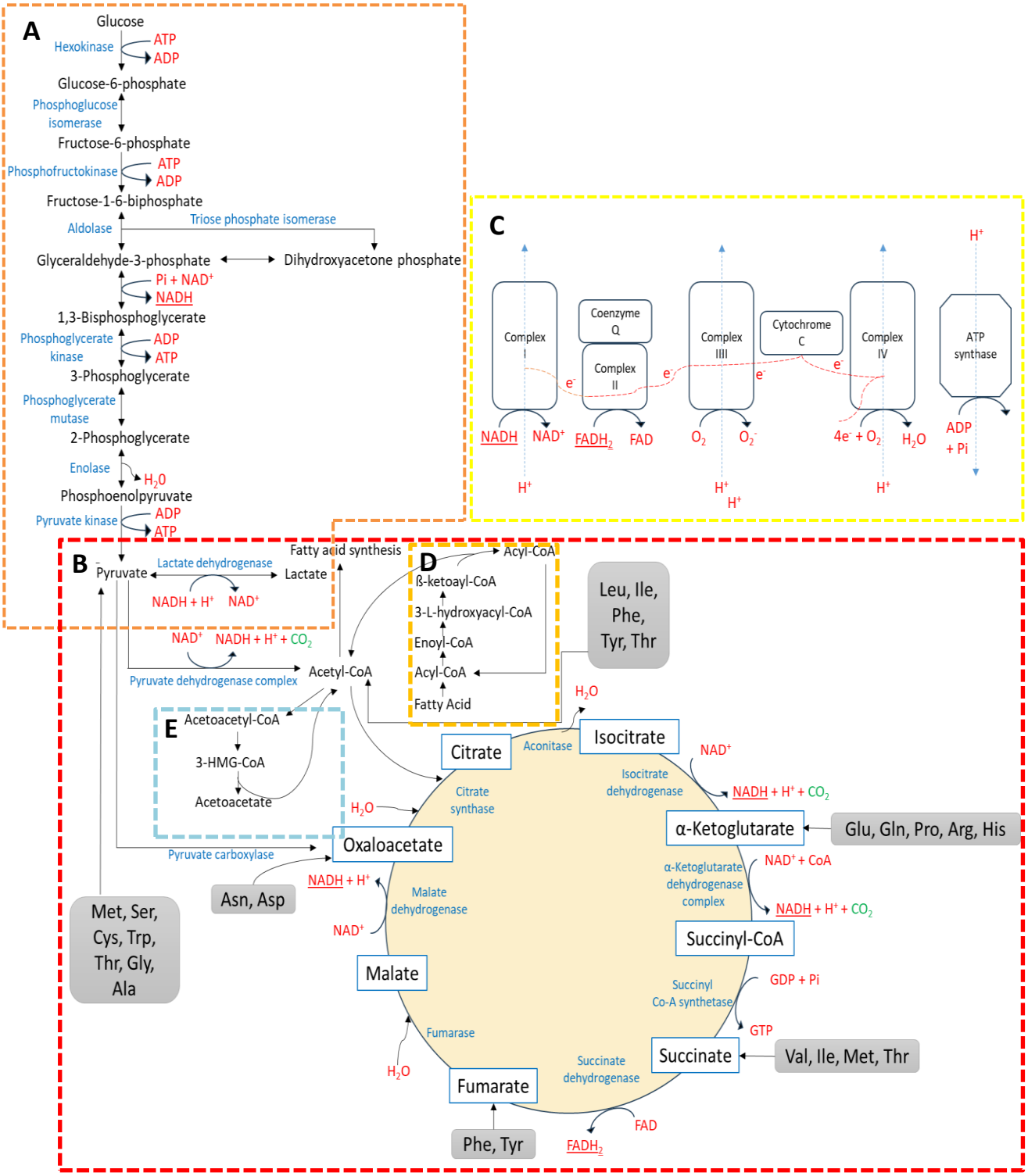


Figure 1.2: A diagram of the metabolic pathways utilised by human sperm. Diagram **(A)** is representative of glycolysis, which takes place in the cytosol, and converts one molecule of glucose to two molecules of pyruvate. Pyruvate is converted to lactate in anaerobic conditions, and this can occur in the cytosol via cytosolic LDH or the mitochondria via mitochondrial LDH. Diagram **(B)** is representative of the Krebs cycle, which occurs in the mitochondria, and consists of eight reactions which release cofactors that are fed into the electron transport chain. Pyruvate can be converted to Acetyl-CoA by the pyruvate dehydrogenase complex or into oxaloacetate by pyruvate carboxylase. Diagram **(C)** is representative of the electron transport chain which involves the transfer of electrons from the cofactors to reduce oxygen. This process generates a proton gradient across the membrane and as these protons are pumped back into the matrix, ATP is synthesised via ATP synthase. Enzymes are shown in blue text, and products of the reactions are shown in red text. Diagram (**D**) is representative of fatty acids beta-oxidation and diagram (**E**) is representative of ketogenesis. Blue boxes in diagram **B** show where amino acids enter the Krebs cycle.

There is a discord in the literature regarding the role of oxidative phosphorylation in producing ATP for sperm motility. On one hand, oxidative phosphorylation has been shown to be insufficient at producing enough ATP to sustain high motility in human sperm (Peterson and Freund, 1970; Makler et al., 1992; Nascimento et al., 2008). On the other hand, oxidative phosphorylation has been demonstrated to have an important role in the maintenance of motility (Ferramosca *et al.*, 2008, 2012; Panner Selvam *et al.*, 2020). In addition, when mitochondrial membrane potential drops, sperm motility has also been observed to decrease, implicating a key role of the mitochondria in sperm function (Troiano *et al.*, 1998). A downregulation of mitochondrial proteins involved in energy production are also seen in asthenozoospermic subjects, suggesting an important role for oxidative phosphorylation in maintenance of human sperm motility (Moscatelli *et al.*, 2019).

Numerous studies have demonstrated that both glycolysis and oxidative phosphorylation are necessary for sperm motility and function using inhibitors (Suter, Chow and Martin, 1979) and ^{13}C-NMR (Reynolds et al., 2017b). Other metabolic pathways involved in lipid metabolism have also been implicated to have a role in providing ATP for sperm motility (Amaral et al., 2013; Asghari, Marashi and Ansari-Pour, 2017). It has been suggested that sperm use different metabolic pathways depending upon their surrounding environment, for example, the metabolic substrates available and oxygen concentration (Piomboni et al., 2012). It has also been shown that when glycolytic substrates are low, sperm are capable of metabolising respiratory substrates and are also capable of gluconeogenesis (Mukai and Okuno, 2004). It is thought that the preference of substrates for ATP production also varies amongst species influencing the metabolic pathways used (Storey, 2008). Although previous work has investigated the metabolic pathways used to provide ATP for sperm motility, they have insufficiently investigated the metabolic pathways utilised during the various processes that take place in sperm prior to fertilisation, in particularly during hyperactivation, the acrosome reaction and apoptosis, hence this warrants further investigation.

In terms of boar sperm, it has been demonstrated they are able to metabolise similar substrates to human sperm, e.g., glucose and pyruvate, and adapt to their environment (Nichol *et al.*, 1992; Jones and Bubb, 2000). Boar sperm have also previously been shown to favour glycolysis over oxidative phosphorylation for sperm motility (Marin et al., 2003; Medrano et al., 2006; Rodriguez-Gil, 2006). The role of oxidative phosphorylation has still been shown to be of importance, like in human sperm. It has been suggested to be required for successful completion of capacitation and the acrosome reaction, through an observed increase in oxygen consumption and ATP levels during these processes, and by using inhibitors of oxidative phosphorylation (Ramió-Lluch et al., 2011, 2014). There are similarities between human and boar sperm in terms of energy production and the dispute in the literature, regarding the metabolic pathways utilised by human sperm for ATP production, is obvious for boar sperm too.

The ongoing debate regarding the preferred metabolic pathways utilised by sperm is complicated by different species being used in different studies. For example, studies have suggested that bull sperm require oxidative phosphorylation for successful capacitation whereas human sperm have been shown to depend upon glycolysis for this process (Williams and Ford, 2001). Differences in structure of sperm and in the FRT environment of the species could account for these discrepancies in the literature. Furthermore, when comparing studies, it is important to note at what stage metabolism is been measured as sperm undergoing capacitation, hyperactivation or the acrosome reaction may have differences in ATP demands, see chapters 3, 4 and 5, compared to freshly ejaculated sperm that have not been given the time, or conditions, to capacitate.

1.2.5.4 Measurement of metabolism

Various techniques can be used to measure metabolism, and these include ATP assays, enzyme assays, measurement of oxygen uptake, use of ^{14}C radiolabelled substrates, Seahorse technology and methods that detect metabolites such as mass spectrometry and ^{13}C- nuclear magnetic resonance (NMR) (TeSlaa and Teitell, 2014; Codella, 2021). ^{13}C-NMR has many advantages including the fact that ^{13}C labelled substrates are metabolised the same way as unlabelled versions in normal physiology (Calvert et al., 2018). In addition, NMR

can identify high numbers of metabolites simultaneously, including the source substrates as well as those arisen from metabolism, in live cells (Emwas, 2015). The high reproducibility of NMR and its ability to noninvasively and non-destructively identify numerous metabolic pathways simultaneously is the reason it was chosen to investigate sperm metabolism in this study.

1.2.5.6 Basic overview of NMR

NMR is a non-destructive method which detects the ^{13}C isotope of carbon in an organic molecule. By applying magnetic fields to the nucleus, ^{13}C-NMR detects substrates, for example glucose and lactate, that have been labelled in this way, hence providing an insight into the mechanisms of ATP production (Mlynárik, 2017).

Most of the atomic nuclei have intrinsic angular momentum, spin, which determines how the nucleus interacts with a magnetic field and remains constant for isotopes. Nuclei generate local magnetic fields, μ, because of their spin (Zia *et al.*, 2019). The ratio of a nuclei's local magnetic field to its angular momentum is known as the gyromagnetic ratio, γ, which is another property of the isotope. In the absence of an external magnetic field, the magnetic rotations are randomly distributed. In the presence of an applied magnetic field, these align, either parallel or anti-parallel to the external field and allows for different energy levels that can be measured and manipulated using radio waves. Radio receivers can detect this, generating an NMR signal (Neiss, 2006).

The magnetic field experienced by the nucleus depends upon the electronic structure of the molecule. Electrons magnetically shield the nuclei, reducing the strength of the main magnetic field experienced by the nucleus leading to the local magnetic field (Figure 1.3). Different frequencies of radio waves cause different nuclei to resonate, dependent upon the electron density in chemical bonds. This is known as the chemical shift and is specific to the structure and chemical environment of the molecules allowing for their identification (Zia *et al.*, 2019). To determine the resonance frequency of the nuclei, the Larmor equation is used:

Equation 2: $2\pi f = \gamma.(1-\sigma)B_0$

where f = frequency (Hz), γ = the gyromagnetic ratio, σ = the shielding constant and B_0 = the strength of the external magnetic field. The NMR signal can be translated to determine the molecule's structure using the Fourier Transform algorithm (Neiss, 2006; Mlynárik, 2017). NMR is a quantitative method that allows for the relation of the peak integrals to concentration, and the use of a reference compound of known concentration allows for the concentration of NMR signals to be determined (Mo and Raftery, 2008).

1.2.5.7 Previous work using NMR

^{13}C-NMR has been used to determine metabolic pathways in a variety of cell types and diseases (Kurhanewicz *et al.*, 2008; Buescher *et al.*, 2015). For example, during autophagy and in cancer (Glunde, Jie and Bhujwalla, 2004; Shestov *et al.*, 2016) and for research on brain metabolism and brain disorders such as Alzheimers (Morris and Bachelard, 2003; Mandal *et al.*, 2022). Previously, NMR has been used in Sheffield to investigate the metabolism of human sperm (Reynolds et al., 2017a; Reynolds et al., 2017b; Calvert et al., 2018). These studies showed evidence of glycolysis and oxidative phosphorylation in human sperm when supplied with a variety of substrates including glucose, fructose and pyruvate. Lactate, taken as a marker of glycolysis, was consistently detected when sperm were incubated with labelled glucose, fructose, and pyruvate, however bicarbonate, taken as a marker of oxidative phosphorylation, was intermittently detected, suggesting sperm predominantly use the glycolytic pathway to meet their energy needs (Calvert et al., 2018). Differences in metabolism were also identified between human sperm obtained from the 40/80% interface vs those obtained from the pellet following density gradient centrifugation (Reynolds et al., 2017; Calvert et al., 2018). As sperm collected from the interface typically have poor motility compared to the pellet, this suggests that there could be important metabolic differences between these sperm populations. However, whether there are any metabolic differences in sperm undergoing capacitation, hyperactivation and the acrosome reaction remains unknown and has not been investigated using ^{13}C-NMR in these studies. As these processes are vital

for successful fertilisation, whether sperm metabolism changes as they ready themselves for fertilisation warrants further investigation.

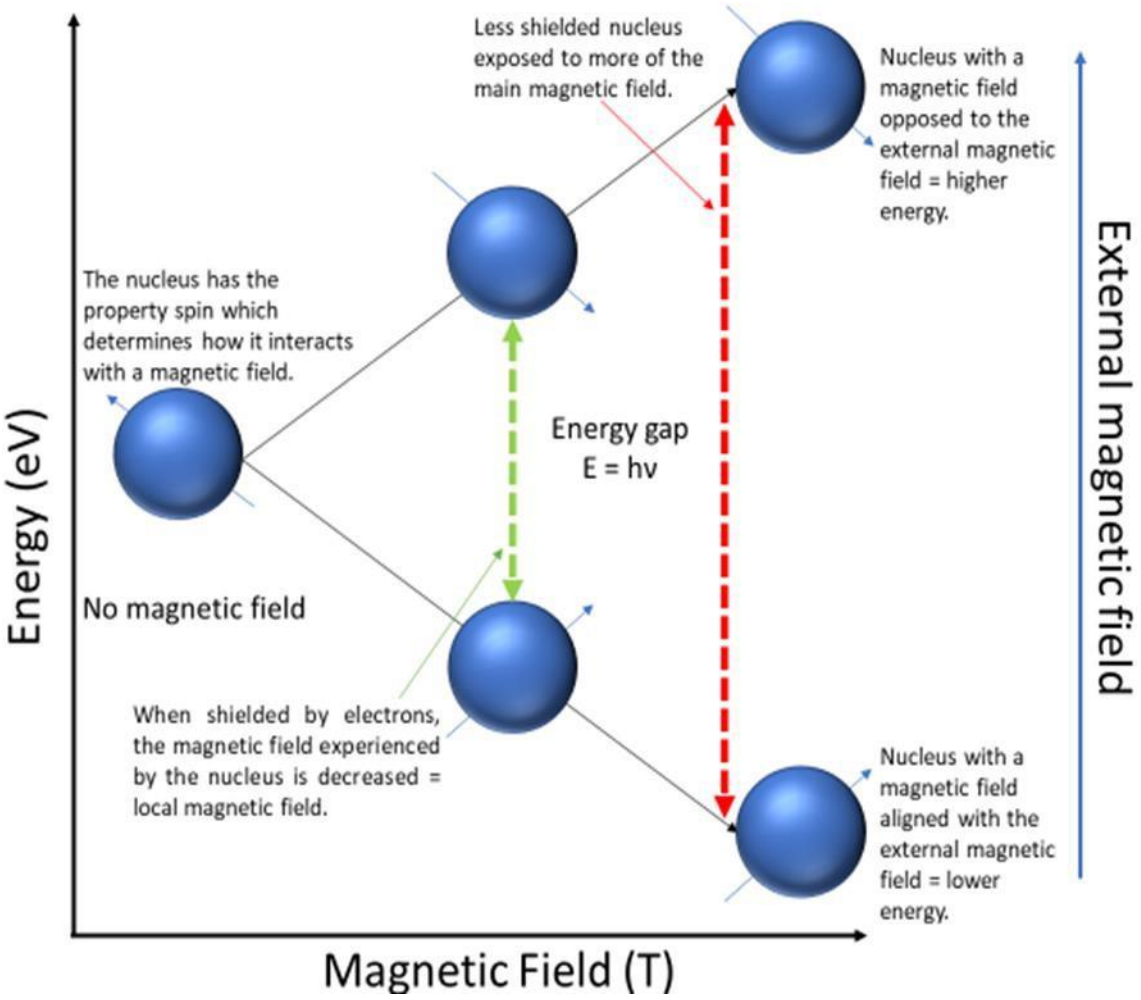


Figure 1.3: Diagram to show the effect of an applied external magnet field on the spin energy states of nuclei and the effect that electron shielding has on the strength of the magnetic field experienced by the nuclei. The blue arrows represent the direction of the magnetic field. The energy gap corresponds to the radio frequency which can be calculated using the equation E = hv, were E = energy, h = Planck's constant and v = frequency.

1.3 Aims and objectives

There is a gap in the knowledge regarding the preferred metabolic pathways utilised by sperm, particularly during capacitation, hyperactivation, the acrosome reaction and apoptosis. These key events are essential for sperm to gain the ability to fertilise the oocyte. It is understood that sperm function depends upon the generation of ATP, from the pathway's glycolysis and/or oxidative phosphorylation. However, whether sperm metabolism and energy demands change during capacitation, hyperactivation and the acrosome reaction remains unknown. There has been little progress on understanding these processes over the last decade and more work is needed to establish whether sperm metabolism changes as sperm ready themselves for fertilisation, and whether these changes can be detected, induced and/or inhibited. The first aim of this study is to determine and optimise the induction and measurement of these key processes in human and boar sperm. This will provide context to investigate the main aim of this thesis, which is to investigate sperm metabolism and determine a ranking of glycolysis and oxidative phosphorylation activity, for capacitation, hyperactivation, the acrosome reaction and apoptosis.

1.3.1 Specific objectives

1. To optimize the induction of capacitation, hyperactivation, the acrosome reaction and apoptosis in human and boar sperm.
2. To optimize the detection of capacitation, hyperactivation, the acrosome reaction and apoptosis in human and boar sperm.
3. To determine the metabolic pathways utilized by sperm during capacitation, hyperactivation, the acrosome reaction and apoptosis using ^{13}C labelled metabolites and NMR.
4. Identify any potential differences in metabolism between human and boar sperm.

Chapter 2: Materials and methods

2.1 Donor recruitment and ethics

For this research, human semen samples were obtained from an existing database of healthy volunteer donors, aged 18 - 65. They were recruited to participate in this study and gave written consent for their sample to be used in the study, which had ethical approval from the University of Sheffield Research Ethics Committee (Ref: SMBRER293 on 28/02/2014, 032045 on 24/02/2020 and 052011 on 24/03/2023). Volunteer donors were asked to abstain from sexual activity for a minimum of 2 days and the sample was produced at home in a sterile, plastic pot (Sarstedt, Leicester, UK). Each sample was analysed within an hour of production, following the guidelines from the World Health Organisation (World Health Organization, 2021). An aliquot of semen was taken for quality checks, involving measurement of concentration and motility using computer-assisted sperm analysis (CASA, see 2.3.2). Boar sperm samples were obtained from JSR genetics (breed GC400), supplied in semen extender, stored at 17 °C, and used within 5 days of production. Boar semen was used in addition to human semen in this thesis due to the restrictions during COVID-19 and the subsequent difficulties obtaining human semen samples between March 2020 and August 2021.

2.2 Buffers and reagents

The media used in this study, Earle's balanced salt solution (EBSS), contained 1.8 mM $CaCl_2$, 0.4 mM $MgSO_4$, 5.4 mM KCl, 116.4 mM NaCl, 1 mM NaH_2PO_4, 25 mM HEPES, adjusted to pH 7.4. EBSS was made, rather than purchased, as this study required media without the addition of energy substrates in order to track metabolism using added ^{13}C-labelled substrates. All other reagents were purchased from Sigma Aldrich, Gillingham, UK, unless stated otherwise.

2.3 Semen analysis and sperm function tests

2.3.1 Density gradient centrifugation

Sperm were isolated from semen using density gradient centrifugation (Figure 2.1). Briefly, 1.8 mL 40% Percoll (human) or 30% Percoll (boar), v/v, (Percoll, GE Healthcare Life Sciences, Little Chalfont, UK) was layered on top of 1.4 mL 80% percoll (human) or 70% percoll (boar), v/v, in 13 mL polypropylene tubes with ventilation caps (Sarstedt, Leicester, UK). Semen (~1 mL) was added on top of this density gradient. For samples with a semen volume greater than 1 mL, as estimated by mass, multiple tubes were prepared. The tubes were centrifuged at 300 g for 20 minutes, the supernatant was removed, and the 80% sperm pellets were collected and combined into a new tube, then resuspended in 12 mL of EBSS. This tube was centrifuged for 10 minutes at 500 g after which the supernatant was aspirated, and the pellet was resuspended in fresh EBSS until the concentration was approximately 50 million per mL. If upon analysis, the average concentration was below 20 million per mL or above 100 million per mL, it was excluded.

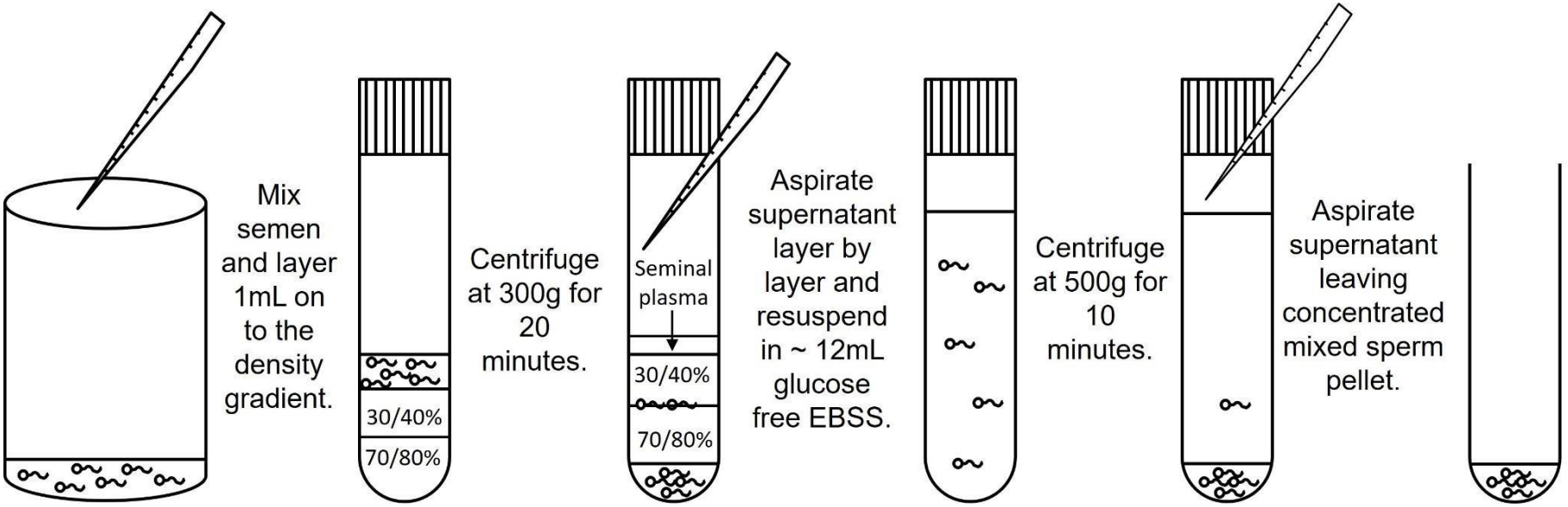


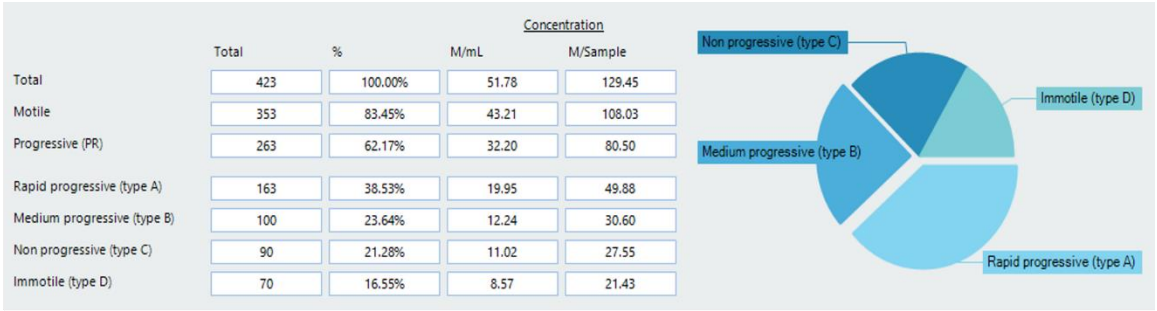
Figure 2.1: A diagram of density gradient centrifugation used for the washing of human and boar sperm samples. For boar sperm, 30/70% Percoll gradients (v/v) were used and for human sperm 40/80% Percoll gradients (v/v) were used.

2.3.2 Motility measurements

Sperm concentration and motility was measured by adding 2 µl of washed sperm preparation into a 10/20 µm depth sperm counting chamber (MicroCell, VitroLife, Goteborg, Sweden). The slides were placed on a heated plate at 37 °C (human) or 39 °C (boar) for 5 minutes and sperm concentration and motility were analysed in a randomised order using Sperm Class Analyzer, version 6 (Microptic SL, Barcelona, Spain). For human sperm, a Microtec LM-2 microscope (Mazurek Optical Services Ltd, Southam, UK) later changed to a Leica DM750 in March 2023 (Leica Microsystems, Wetzlar, Germany), both with x20 objective, and for boar sperm, a Nikon Labophot-2 (Nikon, Donnington, UK) with a x10 objective, were used with a Basler acA1300-200uc camera (Basler AG, Ahrensburg, Germany). One second clips from random locations within the chamber were obtained until either five fields of view had been recorded or 500 sperm had been identified (whichever came first). An average from the fields of view was recorded for sperm concentration, progressive motility, rapid progressive, medium progressive, non-progressive and immotile sperm (see section 1.2.2 for a description of these motility categories and Figure 2.2 for a representative image of the data obtained from motility analysis). A heated mat was purchased and added to the microscope stage in March 2023 and used to collect the human hyperactivation, boar hyperactivation and boar acrosome reaction data presented in this thesis.

A

	Concentration			
	Total	%	M/mL	M/Sample
Total	423	100.00%	51.78	129.45
Motile	353	83.45%	43.21	108.03
Progressive (PR)	263	62.17%	32.20	80.50
Rapid progressive (type A)	163	38.53%	19.95	49.88
Medium progressive (type B)	100	23.64%	12.24	30.60
Non progressive (type C)	90	21.28%	11.02	27.55
Immotile (type D)	70	16.55%	8.57	21.43



B

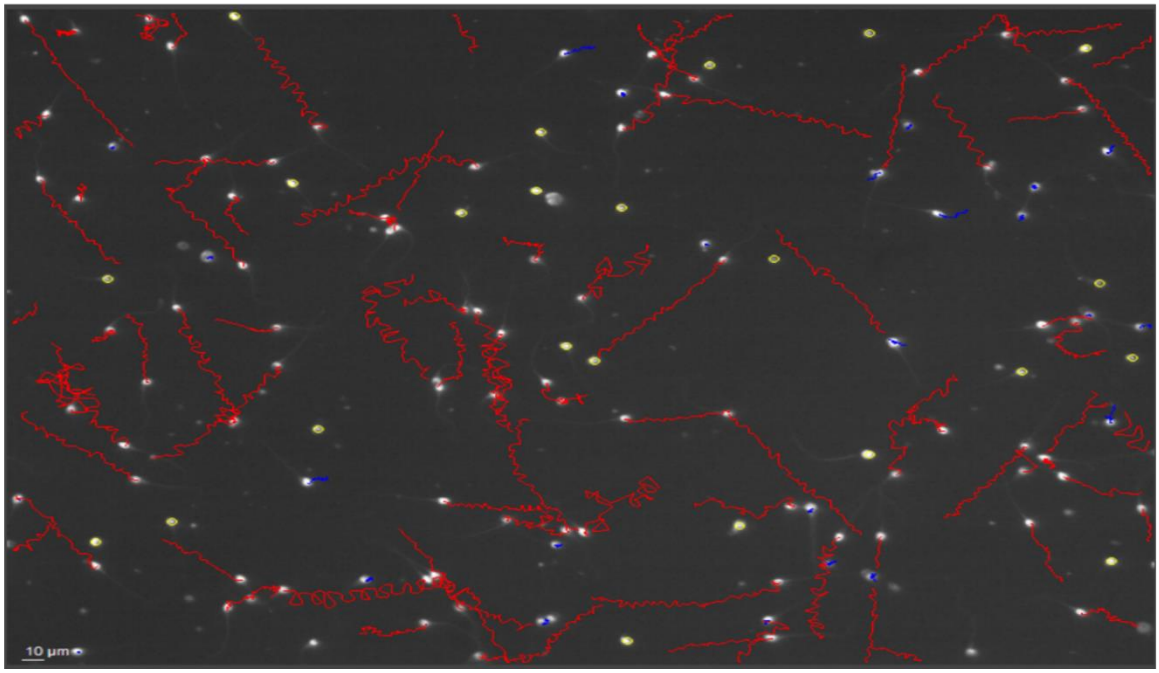


Figure 2.2: A representative image of the motility data obtained from CASA, including an example pie chart with data for rapid progressive, medium progressive, non-progressive and immotile sperm (A) and recorded sperm tracks (B). CASA was used to determine the concentration and motility of human and boar sperm and was carried out as outlined in 2.3.2. Red tracks represent progressive sperm, blue tracks represent non-progressive sperm and yellow dots represent immotile sperm. Scale bar shown = 10 µM.

2.3.3 Vitality assay

A LIVE/DEAD™ sperm viability kit was used to determine sperm vitality (Fisher Scientific, Loughborough, UK). Briefly, propidium iodide (2 µL) and Syber14 (1 µL) was added to 50 µl of washed sperm preparation and incubated at room temperature for 5 minutes. A 10 µl aliquot was placed on a glass slide, mounted, and observed in duplicate using an Olympus CKX41 fluorescence microscope (Olympus, London, UK). Live sperm appeared green and dead sperm appeared red, see Figure 2.2. A minimum of 200 sperm were recorded and the ratio of live (green) to the total number of sperm (green + red) was calculated and presented as a percentage. If the duplicate percentages were not within 10% of each other, the count was repeated using the remaining solution. If the duplicate percentages were not within 10% of each other, then an average of the four counts were used.

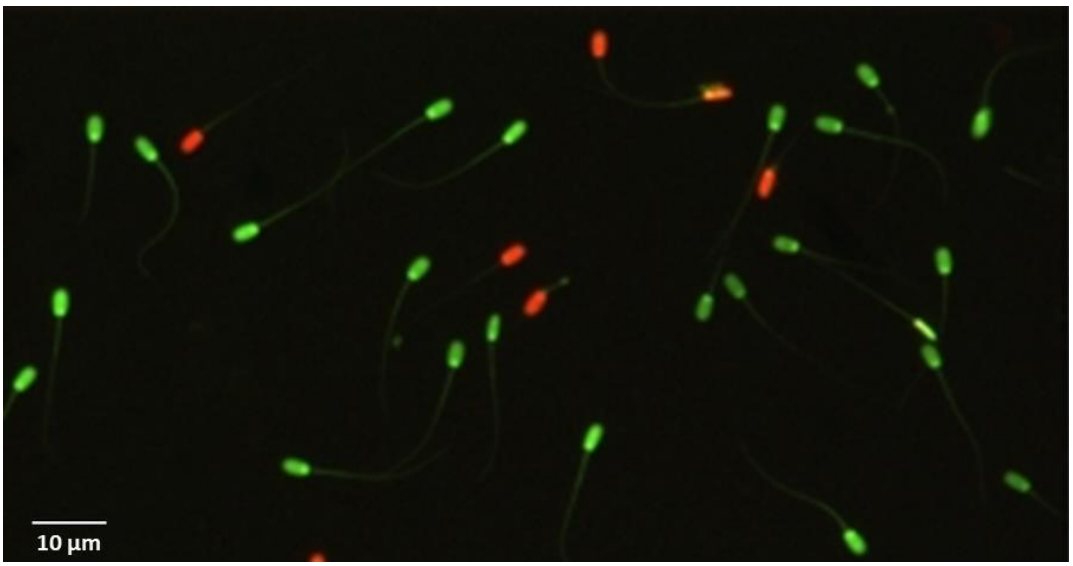


Figure 2.3: The vitality assay was used to determine the viability of human and boar sperm. The vitality assay was carried out using the LIVE/DEAD™ sperm viability kit as outlined in 2.3.3. Red sperm are representative of dead sperm and green sperm are representative of live sperm. Previous image from the SpermNMR group at the University of Sheffield. Viewed at x400 magnification. Scale bar shown = 10 µM.

2.3.4 Incubation with ^{13}C metabolites

Ventilation cap polystyrene round-bottom tubes, 5mL (Corning Falcon, Fisher Scientific), were used for sperm incubation with ^{13}C metabolites. Each tube contained 345 µL washed sperm preparation (suspended in EBSS (section 2.1) at a target average concentration of 50 million/mL), 15 µL antibiotics (10 000 units/mL penicillin and 10 mg/mL streptomycin diluted 1/3 with phosphate buffered saline (PBS) to reach final concentrations of 90 units/mL penicillin and 90 µg/mL streptomycin, Sigma Aldrich, Gillingham, UK), 40 µL 100 mM $^{13}C_6$ glucose and inducers of either capacitation, hyperactivation, acrosome reaction or apoptosis (see individual methods sections). The final volume of each tube was made up to 500 µL using EBSS. To identify any undesired metabolic activity (e.g. from bacterial contamination), a negative control tube (-ve) was prepared for each experiment, as above, containing EBSS media in place of the sperm. Each sample was used for one or more inducer experiments, depending upon the concentration of the washed sperm preparation, i.e. how many times the preparation could be split to achieve a sperm concentration of approximately 50 million/mL in each incubation tube. The tubes were incubated at either 37°C (human) or 39°C (boar) in a water bath incubator for 4 or 8 hours. After 4/8 hours (T4/T8), a 65 µL aliquot was taken from each incubation tube for motility analysis (section 2.3.2), the vitality assay (section 2.3.3) and to smear slides for fluorescent staining. The remaining sample was placed in an ultrasonic water bath for 10 minutes before being placed in the -80°C freezer until NMR analysis.

2.3.5 ^{13}C–Nuclear magnetic resonance

NMR metabolic analysis was conducted as described in Calvert et al., (2019). The incubation tubes, stored at -80°C, were thawed and 380 µL from each incubation tube was placed in a 5 mm NMR tube (Norell, Morganton, NC, USA), along with 20 µL of D_2O (Sigma Aldrich, Gillingham, UK) and 10 µL of 200 mM ^{13}C urea (Sigma Aldrich). The samples were scanned at room temperature using a 9.4T Bruker Avance III NMR spectrometer with a 5mm broadband observe probe, later changed to a 5 mm Quadnuclei probe in January 2023 (Bruker BioSpin GmbH, Karlsruhe, Germany). The broadband observe probe was used for capacitation and apoptosis experiments for human and boar sperm and the Quadnuclei probe was used for hyperactivation and acrosome reaction experiments for both human and boar sperm. The spectra were acquired using a $^{13}C\{^1H\}$ inverse-gated pulse sequence (Spectral Width = 239 ppm, Number of acquisitions = 4096, Acquisition Time = 0.5 s, Delay Time = 2 s, Time domain points = 24036, flip angle = 16°, total experiment time = 2.8 hours). The spectra were apodised using a 5 Hz exponential line broadening function and the phase and baseline were corrected using Bruker Topspin v3.5 software. The urea signal, which had a frequency offset δ = 165.5 ppm, was used as a reference.

2.3.6 Data and statistical analysis

2.3.6.1 Statistical analysis

All graphs and statistics were created/performed by GraphPad Prism 8 (Dotmatics, Boston, USA). All data were plotted as box and whisker plots showing the minimum and maximum values, the median value and the interquartile range. Where numbers are quoted, these are the mean ± standard error of the mean (SEM). The data was paired, as per individual experiment, the same sample was used and split into multiple tubes. The D'Agostino and Pearson test was used to test the data for normality. Data that was parametric and had more than 3 groups (physiological sperm data), was analysed using the RM one-way ANOVA, with the Geissser-Greenhouse correction and a Tukey multiple comparisons test. Where there were two groups, a paired t-test was used (comparison of normalised lactate/bicarbonate integrals). For data that was non-parametric, a Wilcoxon matched pairs signed rank test was performed for data where there were two groups and a Friedman test was performed with a Dunn's multiple comparisons test where there were more than two groups, $p < 0.05$ was classed as significant.

2.3.6.2 NMR data analysis

Qualitative analysis of peaks, identified by visual inspection of the spectra, obtained from ^{13}C-NMR, determined the metabolic products produced by sperm during the four/eight-hour incubations with $^{13}C_6$-glucose and a > 3:1 signal: noise ratio was defined as a peak. The spectrum integrals were determined using a custom peak fitting algorithm (Matlab R2018b, Mathworks, Natick, MA, USA), in specific chemical shifts ranges, shown in Table 2.1, for lactate (1, 2, 3 positions), pyruvate, glucose, bicarbonate, and CO_2. The integral for the last two of these were summed to account for their chemical equilibrium and lactate, 1 and 3 positions, were summed and plotted in the graphs shown in this thesis. See Figure 2.3 for a representative ^{13}C-NMR spectrum from incubation with and without sperm (negative control).

Table 2.1: The chemical shift integral ranges for the metabolites investigated in this study.

Metabolite	Chemical shift integral range
^{13}C glucose	99.6 – 94.0 ppm
1-^{13}C lactate	186.0 – 184.1 ppm
2-^{13}C lactate	71.8 – 70.5 ppm
3-^{13}C lactate	23.6 – 22.1 ppm
^{13}C bicarbonate	164.5 – 162.0 ppm
^{13}C urea	166.1 – 164.9 ppm
$^{13}CO_2$	127.9 – 127.3 ppm

2.3.6.3 Normalisation

Lactate and bicarbonate integrals (arbitrary units (A.U.)) were normalised by the total concentration, vital concentration, and motile sperm concentration. Total concentration was calculated using an average of the T0 and the T4 or T8 concentration values taken from all incubation tubes for each individual experiment. The vital concentration was calculated using an average of the T0 and the one T4/T8 vitality value for the incubation tube in question (+ve or inducer tube) per individual experiment, as the vitality changed depending on the treatment and it remains unknown at what point during the four-hour incubation sperm would have died. Sperm that are dead would not contribute to the lactate or bicarbonate integrals obtained from ^{13}C-NMR. The motile concentration used just the T4/T8 value for the one incubation tube in question (+ve or inducer tube) per individual experiment, as the motility changed depending on the treatment, and the T0 values were low due to sperm density gradient centrifugation occurring below 37°C and without glucose. The vital and motile concentration were calculated using the following equations, where Vit = vitality and Mot = motility.

Equation 3: Vital concentration = $$\frac{Total\ Concentration \times \frac{VitT0+VitT4\text{or}8}{2}}{100}$$

Equation 4: Motile concentration = $$\frac{Total\ Concentration \times MotT4\text{or}8}{100}$$

Integrals were normalised by sperm concentration as the higher the concentration of sperm, the more glucose consumed, and the more lactate/bicarbonate and other metabolites produced. The correlation between sperm concentration and raw lactate and bicarbonate integrals in this thesis showed a significant positive correlation for human sperm, determined by Pearson linear regression, appendix A6 and A7. Previous work has also shown that as the concentration of human sperm increases, the production of lactate generally increases (Reynolds et al., 2017).

In cases where bicarbonate was added as an inducer of capacitation (all except for T4 control for capacitation), corrected bicarbonate integrals were calculated by subtracting the average bicarbonate integral in the negative controls per each event (n=11), from the bicarbonate integrals in all spectra prior to normalisation, to account for the bicarbonate already present in the sample. This allowed for the identification of bicarbonate produced during oxidative phosphorylation.

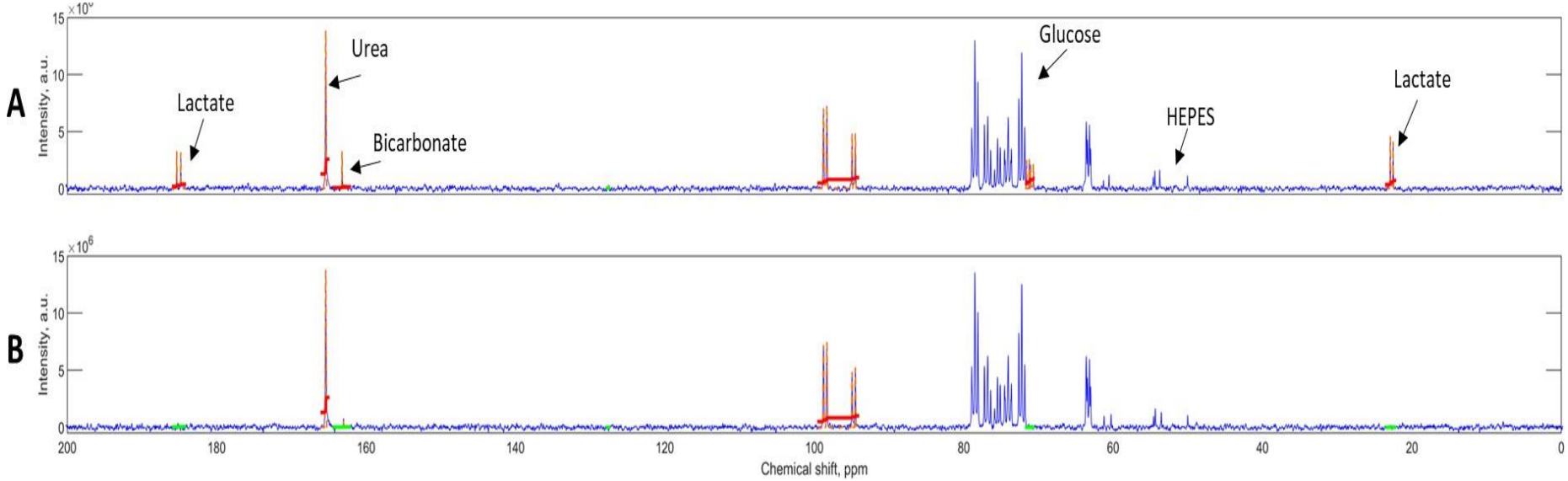


Figure 2.4: Representative ^{13}C-NMR spectre. Representative ^{13}C-NMR spectre from incubation with **(A)** and without **(B)** sperm. The incubation without sperm **(B)**, the negative control, contained $^{13}C_6$-glucose, EBSS media and inducers of the various processes. The incubation with sperm **(A)**, contained $^{13}C_6$-glucose, inducers of the various processes and human sperm at a concentration of approximately 50 M/mL. Both were incubated for either hfour or eight-hours at 37 or 39°C. The lack of lactate in the negative control suggests there is no contamination present.

Chapter 3: The induction and measurement of capacitation and the underlying metabolic pathways utilised in this process.

3.1 Introduction

3.1.1 The discovery of capacitation

Capacitation was first described by Austin and Chang in 1951, when initial work with rats and rabbits suggested that after ejaculation, sperm needed time in the FRT before fertilisation could occur (Hammond, 1934; Austin, 1951; Chang, 1951). They discovered this by placing sperm into the periovarian sac of the rat and the Fallopian tube of the rabbit and showed that several hours passed until fertilisation occurred. Chang (1951) demonstrated that when sperm, which had spent 5 hours in a rabbit's uterus, were moved to the Fallopian tube of a different rabbit, fertilisation occurred more rapidly. This led them to conclude that sperm are physiologically modified in the FRT and gain the ability to fertilise. These results were confirmed following the natural insemination of rats (Austin, 1952). It has since been suggested that all sperm require at least four hours for capacitation to occur *in vitro* (Barros and Austin, 1967). This led to the use of capacitating media; containing calcium and bicarbonate ions, energy substrates and a cholesterol acceptor, to culture sperm *in vitro* (Calvo *et al.*, 1993). The process of physiological modification of sperm in the FRT was named capacitation, and its discovery has been vital in the development of assisted reproductive technologies, particularly *in vitro* fertilisation (IVF) (Pacey, 2008).

3.1.2 Function of capacitation

Freshly ejaculated sperm, although motile, remain functionally immature and require further maturation in the FRT, by responding to various stimuli to capacitate (Austin and Bishop, 1958). Capacitation must occur to allow for sperm to undergo hyperactivation (chapter 4) and the acrosome reaction (chapter 5). In particular, the tyrosine phosphorylation of key proteins during capacitation allows for successful fertilisation to occur. This is because sperm are transcriptionally inactive because of the condensation of the nucleus during initial maturation in the epididymis (Grunewald *et al.*, 2005). As a result, post translational modifications, including tyrosine phosphorylation, are responsible for altering protein functions to allow for hyperactivation, the acrosome reaction and subsequent fertilisation to take place (Mostek-Majewska *et al.*, 2023).

Prior to ejaculation, capacitation is actively prevented by various factors, including the low bicarbonate concentration in the male reproductive tract. After ejaculation, the seminal plasma proteins also have a role in preventing capacitation, however these proteins detach during the remodelling of the plasma membrane that occurs during capacitation and this allows for hyperactivation and the acrosome reaction to occur (Ou *et al.*, 2012). Capacitation must occur in the female reproductive tract around the time of ovulation for successful fertilisation to take place, therefore premature capacitation, for example in the male reproductive tract, would negatively impact a sperms ability to fertilise an oocyte (Fraser, 1984).

3.1.3 Cholesterol efflux

The process of capacitation involves the remodelling of the plasma membrane and changes in intracellular ion concentrations (Buffone *et al.*, 2004, 2009). An increase in cAMP followed by the efflux of cholesterol from the plasma membrane occurs during capacitation. This efflux of cholesterol results in membrane fluidity causing an influx of bicarbonate and calcium ions (Baldi et al., 1991; Marıń -Briggiler et al., 2002). Cholesterol is initially deposited during epididymal maturation to provide stability to the plasma membrane (Langlais *et al.*, 1981). An increase in intracellular bicarbonate activates the enzyme scramblase, which is ATP-independent, and reduces phospholipid asymmetry by moving phospholipids across the plasma membrane bilayer (Harrison and Gadella, 2005). It is this loss of phospholipid asymmetry that allows for cholesterol efflux, giving the membrane fluidity, which is essential for the acrosome reaction and sperm- oocyte binding. Albumin, a cholesterol acceptor, is present in high concentrations in the FRT to facilitate the removal of cholesterol from the sperm plasma membrane (Ehrenwald, Foote and Parks, 1990).

3.1.4 Influx of calcium and bicarbonate ions

Calcium ions are important during capacitation as many of the functional changes that take place during capacitation, and subsequent hyperactivation and the acrosome reaction, are reliant on calcium dependant signalling pathways (Baldi *et al.*, 1991). Calcium enters sperm via the CatSper channel, located in the membrane of the principal piece. This channel is activated by intracellular alkalinization and membrane depolarisation which occurs as a result of the simultaneous influx of bicarbonate ions (Kirichok, Navarro and Clapham, 2006). In the oviduct, the concentration of bicarbonate is > 20 mM, compared to approximately 4 mM in the epididymis. This allows for the influx of bicarbonate ions via Na^+/HCO_3^- co-transporters, which in turn causes intracellular alkalization. The influx of bicarbonate activates soluble adenyl cyclase (sAC), which further increases the production of cAMP from ATP which then goes on to activate PKA (Hess et al., 2005; Molina et al., 2018).

3.1.5 Tyrosine phosphorylation

During capacitation, tyrosine phosphorylation of 22 proteins, including the fibrous sheath proteins, p80 and p105, has been shown to occur (Baldi, 2000; Visconti *et al.*, 2002; Urner and Sakkas, 2003). PKA signalling pathways have been shown to influence tyrosine phosphorylation of sperm proteins which is considered to have an important role in fertilisation, as the proteins that are phosphorylated have been shown to remodel the sperm surface prior to fertilisation, allowing for recognition of the zona pellucida (Visconti *et al.*, 1995, 2011; Asquith *et al.*, 2004). The PKA signalling pathway is activated by an increase in cAMP, resulting in the activation of tyrosine kinases which results in the tyrosine phosphorylation of sperm proteins. Physiological concentrations of reactive oxygen species (ROS) have also been shown to initiate tyrosine phosphorylation in human sperm via the PKA signalling pathway and via the extracellular signal-related protein kinase (ERK) pathways (Aitken *et al.*, 1998).

The 22 proteins that are tyrosine phosphorylated during capacitation are essential for hyperactivation, the acrosome reaction and sperm-oocyte binding. These proteins include ion channels, metabolic enzymes, and structural proteins (Nassar *et al.*, 1999). When tyrosine phosphorylation is inhibited, a reduction in hyperactivation and the acrosome reaction is observed (Brener *et al.*, 2003; Vizel *et al.*, 2015). Various metabolic enzymes have been shown to be tyrosine phosphorylated during capacitation in mouse sperm, including aldolase, a glycolytic enzyme, as well as pyruvate dehydrogenase, dihydrolipoamide dehydrogenase and the ETC enzyme NADH dehydrogenase (Arcelay *et al.*, 2008; Kumar, Kota and Shivaji, 2008). This suggests that there is a change in the ATP demands of sperm during capacitation.

3.1.6 ATP demands of capacitation

The ATP demand of sperm during capacitation could potentially increase to facilitate the increased production of cAMP required for capacitation. Membrane lipid remodelling, plasma membrane hyperpolarization and an increase in protein kinase A (PKA)-dependant protein tyrosine phosphorylation are features of capacitation regulated by cAMP (Allouche-Fitoussi, Bakhshi and Breitbart, 2018). The process of phosphorylation itself, which alters the structure of a protein and can activate, deactivate or modify the protein function, also requires ATP. For the protein to receive a phosphate group from ATP, ATP hydrolysis and kinase activity is required (Fukami and Lipmann, 1983; Brady and Lau, 2012). The influx of bicarbonate via the Na^+/HCO_3- co-transporters is also powered by the hydrolysis of ATP, however, the calcium influx via CatSper channels does not require ATP and is instead activated by progesterone (Lishko, Botchkina and Kirichok, 2011).

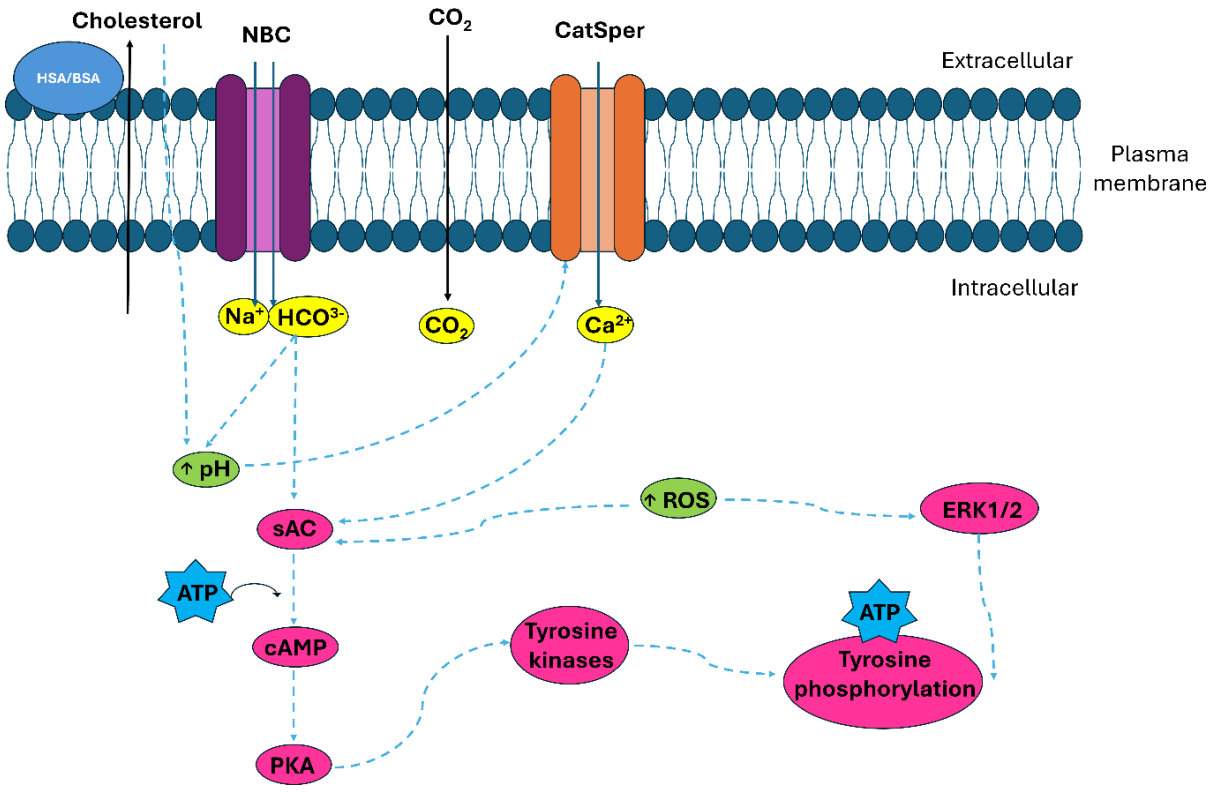


Figure 3.1: Molecular changes during capacitation. During capacitation, cholesterol efflux occurs via HSA/BSA. This alongside an increase in intracellular bicarbonate, via sodium bicarbonate cotransporters (NBCs), increases intracellular pH. This increase in pH stimulates CatSper channels leading to an increase in intracellular calcium. Calcium and bicarbonate activate sAC, which stimulates the production of cAMP from ATP, and this activates PKA. PKA phosphorylates tyrosine kinases, leading to the phosphorylation of tyrosine residues, a process which also requires ATP. Tyrosine phosphorylation can also be induced by an increase in intracellular ROS, which activates the ERK1/2 pathways leading to phosphorylation of tyrosine residues.

3.1.7 The induction of capacitation

One of the first inducers of capacitation discovered was the globular protein serum albumin; human serum albumin (HSA) and bovine serum albumin (BSA) (Yanagimachi, 1970). The authors of this study suggested that albumin must be present in the media for efficient induction of capacitation. The main role of serum albumin is to act as a cholesterol acceptor (Suzuki and Yanagimachi, 1989). This function allows albumin to remove cholesterol from the sperm plasma membrane during the essential cholesterol efflux seen in capacitation (Visconti *et al.*, 2002). The cholesterol efflux subsequently allows for the influx of calcium ions via calcium channels, particularly CatSper channels (Xia and Ren, 2009). Bicarbonate is also thought to induce capacitation due to its role in activating sAC, further increasing the production of cAMP from ATP (Marıń - Briggiler et al., 2002).

3.1.8 The detection of capacitation

Capacitation can be detected by numerous methods including chlortetracycline staining, anti-acrosin antibody assay, anti-phosphotyrosine antibody assay and fluorescein isothiocyanate-conjugated phalloidin (FITC-phall) assay (Ded *et al.*, 2019). One of the more common methods is detecting changes in sperm Tyr phosphorylation using immunofluorescence. The immunofluorescence technique uses sperm suspensions that are fixed and permeabilised prior to incubation with an anti-phosphotyrosine antibody followed by a fluorescent secondary antibody. Sperm displaying fluorescence in their tails is equivalent to the capacitation status of sperm (Figure 3.2). Although protein tyrosine phosphorylation is an indicator of capacitation, it must be noted that it is a continuous process that is regulated by phosphatases and kinases, therefore the images of the tyrosine phosphorylated proteins reflect just one point in time.

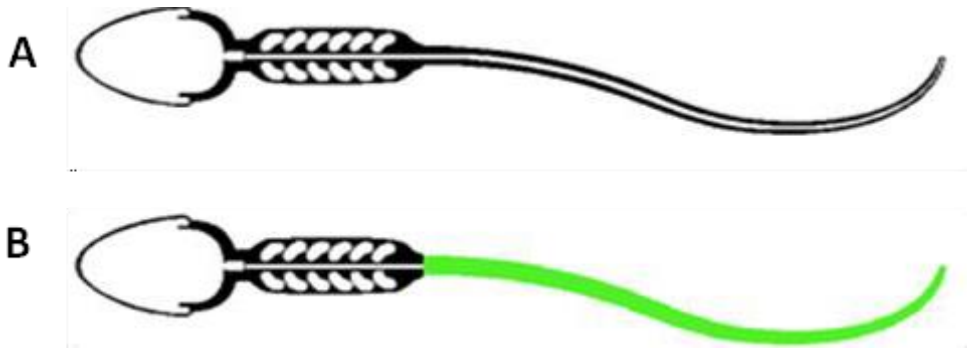


Figure 3.2: Tyrosine phosphorylation immunofluorescence patterns. A diagram displaying the pattern of fluorescent staining with tyrosine phosphorylation. **(A)** No fluorescence indicative of uncapacitated sperm and **(B)** Intense fluorescence over the sperm tail indicative of capacitated sperm.

3.2 Rationale

The aim of this chapter was to investigate live human and boar sperm metabolism during capacitation using ^{13}C-NMR. Since ATP is required to support the various physiological changes that take place during capacitation, it was hypothesised that there would be an increased demand for ATP during capacitation, thus an increase in glycolysis and/or oxidative phosphorylation would be observed.

3.2.1 Specific objectives

1. Induce capacitation in human and boar sperm using HSA and bicarbonate.
1. Detect capacitation in human and boar sperm using tyrosine phosphorylation immunofluorescence.
2. Investigate the effects of capacitation on motility and vitality of human and boar sperm.
3. Investigate the metabolic pathways utilised by human and boar sperm during capacitation.

3.3 Methods

3.3.1 Induction of capacitation

Capacitation was induced by incubating washed sperm (2.3.1) at 37°C (human) or 39°C (boar) for 4 hours in the presence of 5 mg/mL HSA and 25 mM bicarbonate. The sperm were split between two tubes, a positive control containing no HSA or bicarbonate (+ ve) and one containing HSA and bicarbonate (inducer), see Table 3.1. Sperm motility (2.3.2), sperm vitality (2.3.3) and capacitation status (3.3.2) were measured before and after incubation and the metabolic pathways utilised by sperm during the incubation were analysed using ^{13}C-NMR (2.3.5).

Table 3.1: The contents of the tubes prepared for the induction of capacitation. Each tube contained a final volume of 500 µl. All components added in µL. Final concentrations are: 8 mM $^{13}C_6$-glucose, 5 mg/mL HSA and 25 mM $NaHCO_3$. The -ve contains no sperm.

Added at the start of the incubation (T0), (µL)						
Tubes	**Sperm (50 M/mL)**	**EBSS**	**1/3 Pen/strep**	**100 mM $^{13}C_6$-glucose**	**50 mg/mL HSA**	**1 M $NaHCO_3$**
-VE	0	382.5	15	40	50	12.5
+VE	345	100	15	40	0	0
Inducer	345	37.5	15	40	50	12.5

3.3.2 Measurement of capacitation: tyrosine phosphorylation immunofluorescence

Levels of tyrosine phosphorylation were measured by immunofluorescence using an α-phosphotyrosine mouse monoclonal antibody (clone 4G10) (Merck Millipore, California, USA), as outlined in Hosseinzadeh et al., (2000).

Briefly, ~2x10^6/mL sperm in a volume of 10 µl, was added to a polysine slide (Epredia, Breda, Netherlands), smeared and left to air dry overnight at room temperature. The slides were fixed in methanol for 1 minute before storing at -20°C until analysis. Slides were warmed to room temperature and re-hydrated using 1 mL TBS. The primary α-phosphotyrosine mouse monoclonal antibody (clone 4G10) was diluted 1:500 in antibody diluent (final concentration 2 µg/mL) and 100µl was added to each slide. The slides were incubated at 37°C for 1 hour in a humidified chamber. The slides were washed twice with TBS and 100 µl of secondary antibody (rabbit α-mouse IgG-fluorescin isothiocynate conjugated, diluted 1:100 with PBS) was added to each slide and incubated at 37°C for 1 hour. Each slide was washed twice with TBS and the back of the slide dried. Slides were mounted using a drop of prolong antifade mounting media (Thermo Fisher Scientific, Massachusetts, USA) and left overnight at 4°C in the dark. Slides were analysed on an Olympus IX73 at x600 magnification (UV filter 492 nm). For each incubate, 200 sperm were counted, in duplicate (two smears per slide), and the level of tyrosine phosphorylation fluorescence assessed. If the duplicate percentages were not within 10% of each other, the count was repeated. Sperm displaying tyrosine phosphorylation exhibit fluorescence along the entire length of the tail (Figure 3.2 and 3.3). Only full tail fluorescence was observed and sperm displaying dull or bright fluorescence were characterized as capacitated.

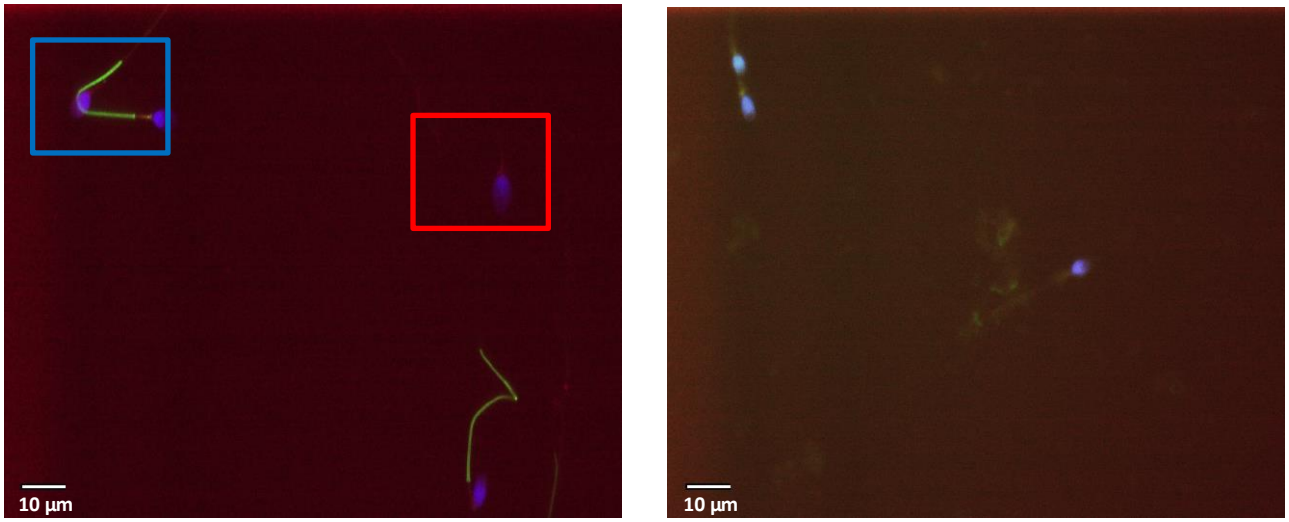


Figure 3.3: Tyrosine phosphorylation immunofluorescence to determine the capacitation status of human and boar sperm. Tyrosine phosphorylation immunofluorescence was carried out as outlined in 3.3.2. The panel on the left is the patterns of fluorescence sperm display after incubation with both primary and secondary antibody; fluorescence across the entire tail shows a capacitated sperm and no fluorescence is an uncapacitated sperm. The panel on the right is the patterns of fluorescence sperm display after incubation without the primary antibody and just the secondary. No green fluorescence across the length of the tail is identified. A blue box is an example of a capacitated sperm, and a red box is an example of an uncapacitated sperm. Viewed at x600 magnification. Scale bar shown = 10 µM.

3.4 Results

3.4.1 Total and progressive motility of human and boar sperm incubated with or without inducers of capacitation.

CASA was used to observe whether HSA and bicarbonate had any effect on total and progressive sperm motility. Figure 3.4A shows that total motility significantly increased for human sperm, after a four-hour incubation with capacitation inducers compared to time zero, but did not in the absence of inducers (T0, 37.6 ± 7.2%; T4 HSA + bicarb, 52.0 ± 4.9%; P = 0.008; T4 control, 37.2 ± 6.1%). At 4 hours (T4) the total motility for the capacitated group was also significantly different from the non-capacitated group, P = 0.006. Figure 3.4B shows that for boar sperm, there was also a significant increase in total motility after a four-hour incubation with capacitation inducers compared to time zero (T0, 2.0 ± 0.54%; T4 HSA + bicarb, 8.41 ± 1.33%; P= 0.04).

In terms of progressive motility, figure 3.4C shows that for human sperm, after 4 hours, there was a significant increase as a result of capacitation inducers compared to time zero but did not in the absence of inducers (T0, 17.8 ± 6.6%; T4 HSA + bicarb, 36.3 ± 3.3%; P = 0.002; T4 control, 21.4 ± 5.2%). At 4 hours (T4) the progressive motility for the capacitated group was also significantly different from the non-capacitated group, P = 0.003. For boar sperm, there were no significant differences in progressive motility between the three groups: T0, T4 control and T4 HSA and bicarb, as shown in figure 3.4D.

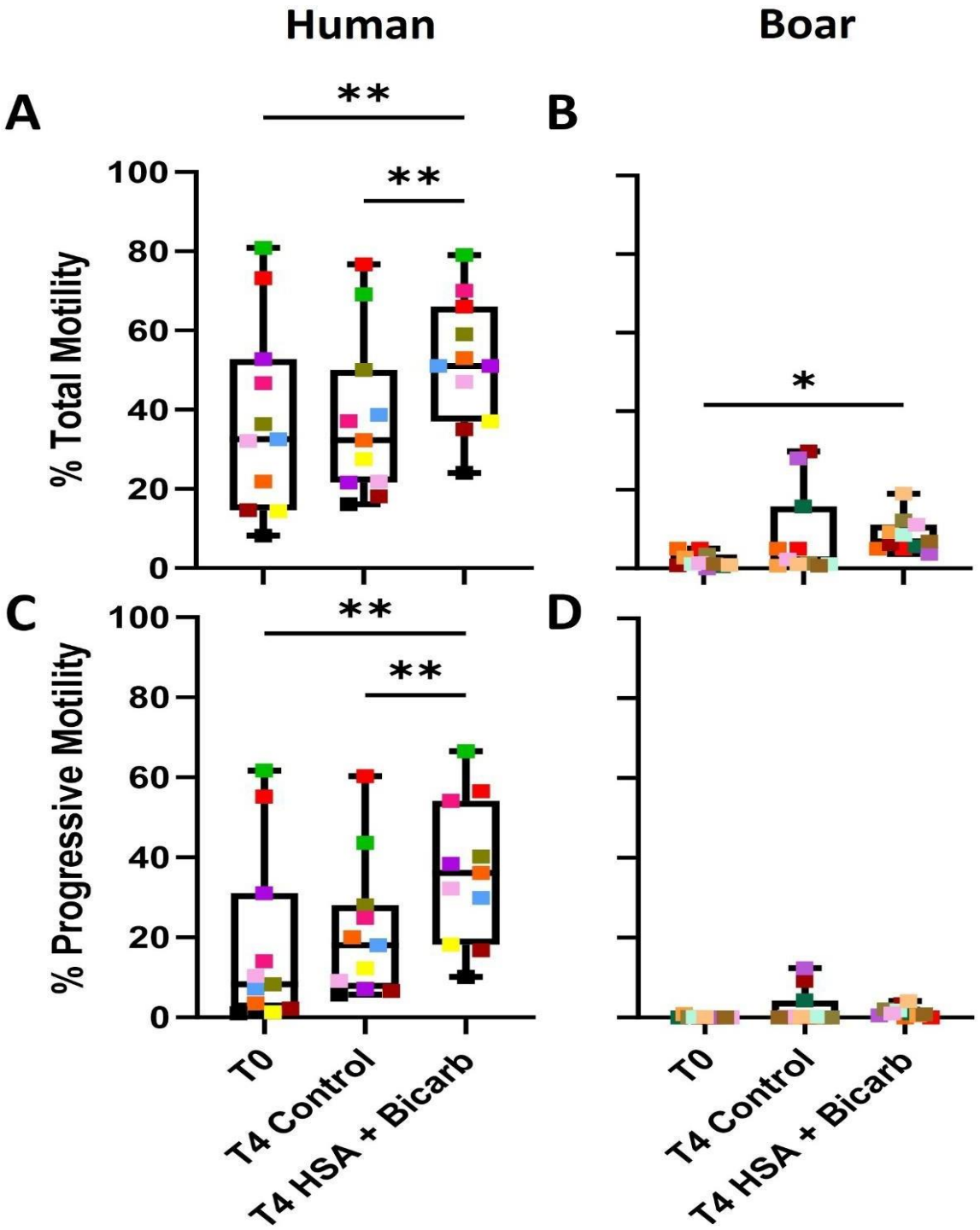


Figure 3.4: Human sperm total (A) and progressive (C) motility and boar sperm total (B) and progressive (D) motility (%) prior to (T0) and following (T4) a four-hour incubation with or without 5 mg/mL HSA and 25 mM bicarbonate. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance for boar sperm was analysed using a Friedman test with a Dunn's multiple comparisons test and for human sperm, a RM one-way ANOVA was used with a Tukey's multiple comparisons test. * = $P < 0.05$ and ** = $P < 0.01$, n = 11. Points colour coded per individual donor/boar.

3.4.2 Vitality of human and boar sperm incubated with or without inducers of capacitation.

To observe whether a four-hour incubation with 5 mg/mL HSA and 25 mM bicarbonate influenced the vitality of human and boar sperm, the LIVE/DEAD™ sperm viability kit was used. Figure 3.5A shows that for human sperm, after 4 hours, the percentage of live human sperm significantly decreased in the absence of capacitation inducers but did not in the presence of the inducers (T0, 58.8 ± 3.5%; T4 control, 48.1 ± 2.7%; P = 0.0009). For boar sperm, figure 3.5B shows the converse was true and the significant decrease in sperm vitality was seen in the presence of the capacitation inducers (T0, 55.6 ± 3.5%; T4 HSA + bicarb, 40.3 ± 1.5%; P = 0.002).

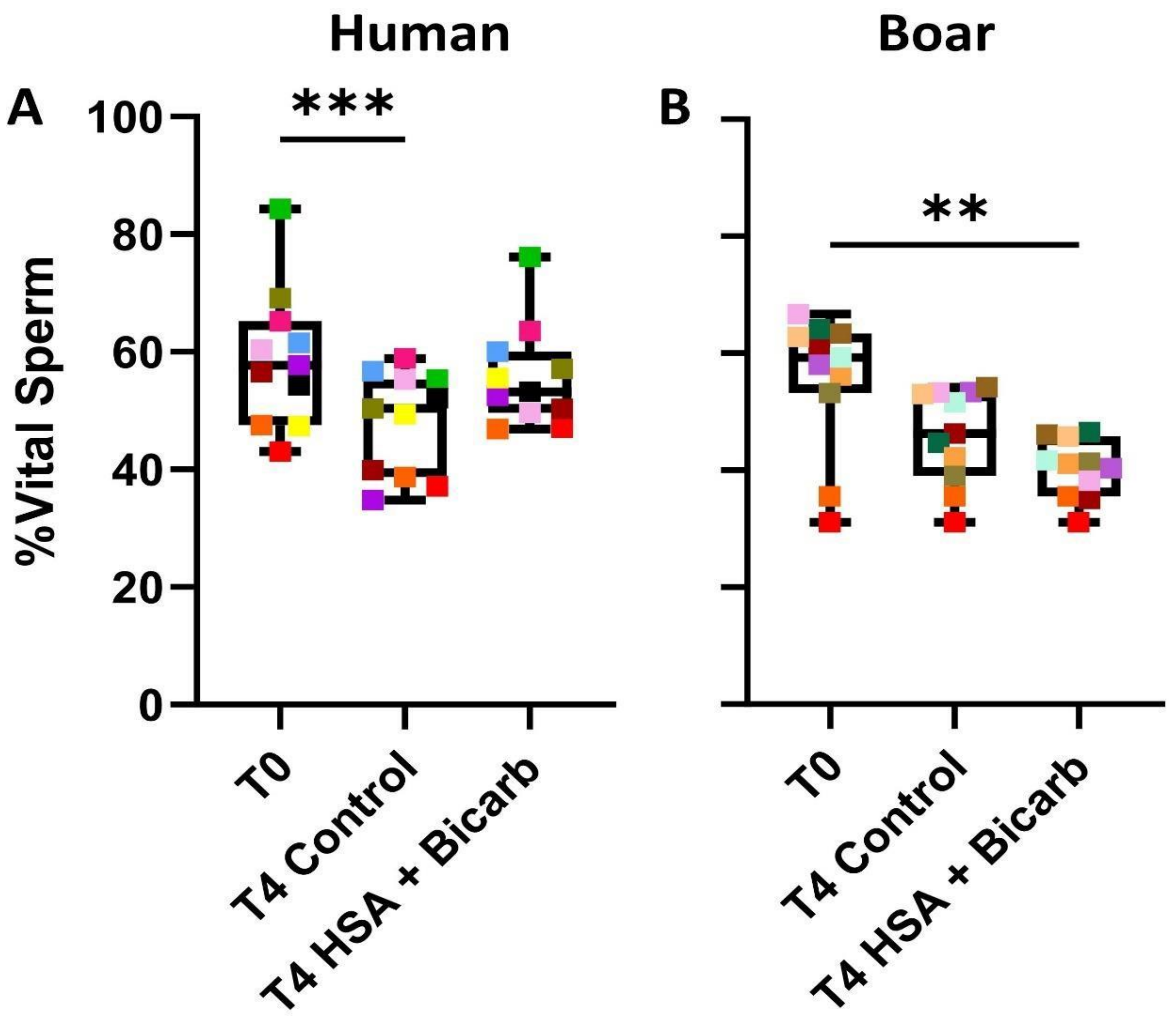


Figure 3.5: Human (A) and Boar (B) sperm vitality (%) prior to (T0) and following (T4) a four-hour incubation with or without 5 mg/mL HSA and 25 mM bicarbonate. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a Friedman test with a Dunn's multiple comparisons test. ** = P <0.01 and *** = P <0.001, n = 11. Points colour coded per individual donor/boar.

3.4.3 Tyrosine Phosphorylation immunofluorescence of human and boar sperm incubated with or without inducers of capacitation.

The capacitation status of human and boar sperm was observed by fluorescent staining of phosphorylated tyrosine residues in the sperm flagellum, a hallmark of sperm capacitation, as seen in section 3.3.2. For human sperm, figure 3.6A shows that after 4 hours, the percentage of capacitated sperm significantly increased both in the presence and absence of inducers (T0, 7.8 ± 1.6%; T4 HSA + bicarb, 46.2 ± 3.1%; P < 0.0001; T4 Control, 23.9 ± 2.4%, P = 0.0001). A significant increase was also seen in figure 3.6A when incubated for four-hours with inducers compared to without, P = 0.0002. An increase in the percentage of capacitated boar sperm was also seen after a four-hour incubation with capacitation inducers compared to time zero (T0, 0.5 ± 0.2%; T4 HSA + bicarb, 42.5 ± 6.6%; P < 0.0001).

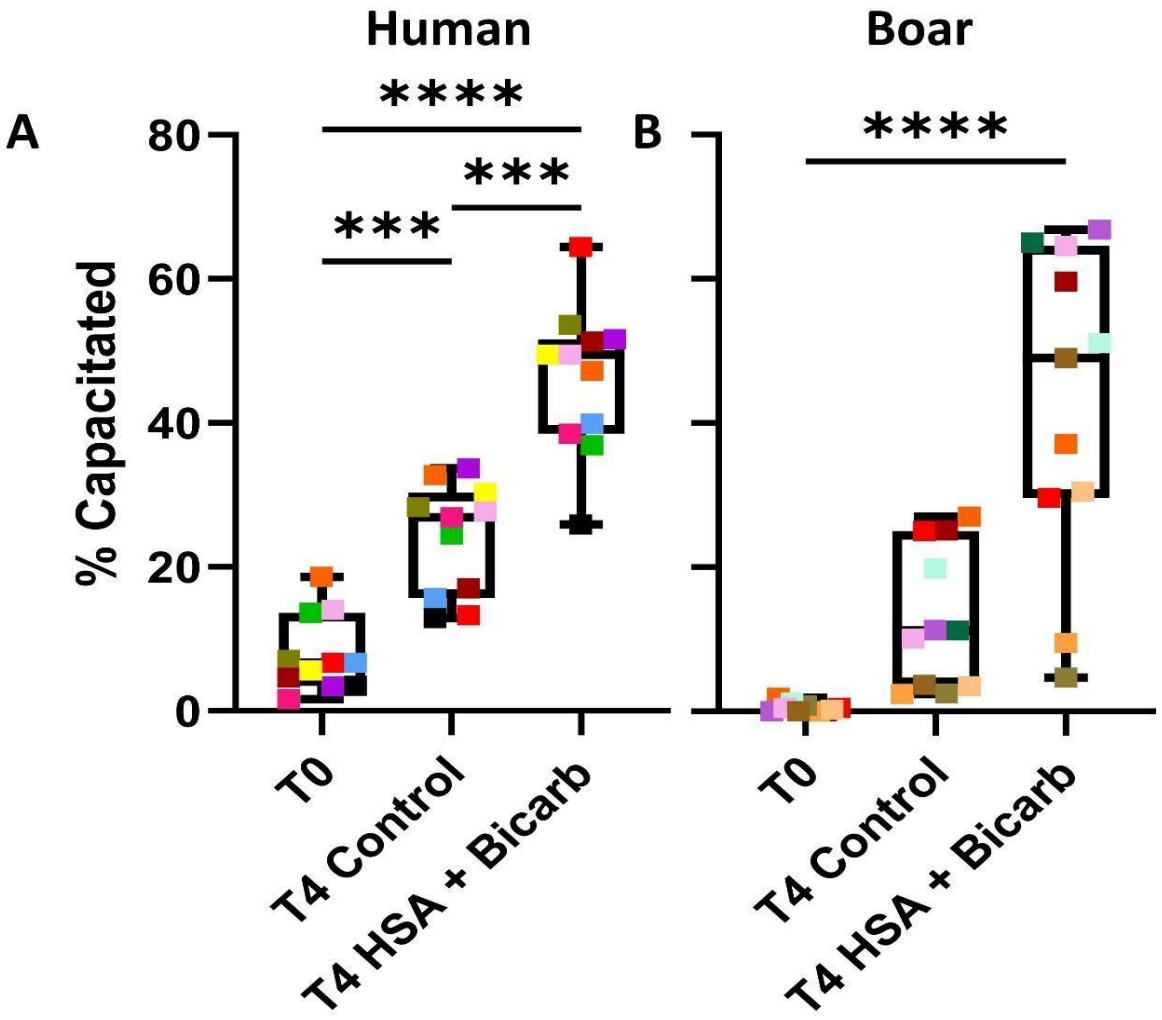


Figure 3.6: Percentage of capacitated human (A) and Boar (B) sperm prior to (T0) and following (T4) a four-hour incubation with or without 5 mg/mL HSA and 25 mM bicarbonate. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. For boar sperm, statistical significance was analysed using a Friedman test with a Dunn's multiple comparisons test and for human sperm, a RM one-way ANOVA was used with a Tukey's multiple comparisons test. *** = P < 0.001 and **** = P <0.0001, n = 11. Points colour coded per individual donor/boar.

3.4.5 NMR

^{13}C-NMR was used to investigate whether human (n=11) and boar (n=11) sperm, incubated with HSA and bicarbonate to induce capacitation, showed differences in metabolism compared to those incubated without HSA and bicarbonate. The production of lactate is indicative of glycolysis and the production of bicarbonate is indicative of oxidative phosphorylation. The lactate and bicarbonate integral values were normalised by sperm total, vital and motile concentration, see section 2.3.6. No lactate peaks were recorded for the negative control samples suggesting no metabolism occurs in the absence of sperm (appendix A7).

3.4.5.1 Lactate production by human and boar sperm incubated with or without inducers of capacitation.

Figure 3.7A shows that for human sperm, after 4 hours, there was a significant increase in lactate production, normalised by total human sperm concentration, when incubated withcccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccc ccccccccccccccccccccccccccccccccccccccccccccc capacitation inducers compared to those incubated in the absence of inducers (T4 control, 3.1 ± 0.4 x 10^6 A.U.; T4 HSA + bicarb, 4.1 ± 0.5 x 10^6 A.U.; P = 0.003). The same was true when normalised by vital human sperm concentration (T4 control, 5.7 ± 0.5 x 10^6 A.U.; T4 HSA + bicarb, 7.0 ± 0.7 x 10^6 A.U.; P = 0.01). Figure 3.7B shows the same pattern was observed for boar sperm when normalised by total boar sperm concentration (T4 control, 3.2 ± 0.3 x 10^5 A.U.; T4 HSA + bicarb, 6.8 ± 1.1 x 10^5 A.U.; P = 0.004) and vital boar sperm concentration (T4 control, 6.2 ± 0.4 x 10^5 A.U.; T4 HSA + bicarb, 1.4 ± 0.2 x 10^6 A.U.; P = 0.002). No differences were observed when lactate integrals were normalised by motile concentrations for human and boar sperm.

3.4.5.2 Bicarbonate production by human and boar sperm incubated with or without inducers of capacitation.

Figure 3.8A shows that for human sperm, after 4 hours, there was a significant decrease in bicarbonate production, normalised by total human sperm concentration, when incubated in the presence of capacitation inducers compared to those incubated in the absence of inducers (T4 control, 2.4 ± 0.7 x 10^5 A.U.; T4 HSA + bicarb, 6.3 ± 4.5 x 10^4 A.U.; P = 0.024). The same was true when normalised by vital human sperm concentration (T4 control, 4.6 ± 1.1 x 10^5 A.U.; T4 HSA + bicarb, 1.1 ± 0.8 x 10^5 A.U.; P = 0.014) and motile human sperm concentration (T4 control, 9.4 ± 3.3 x 10^5 A.U.; T4 HSA + bicarb, 1.4 ± 0.9 x 10^5 A.U.; P = 0.005). No significant differences in bicarbonate production were observed for boar sperm incubations with or without HSA and bicarbonate, figure 3.8B.

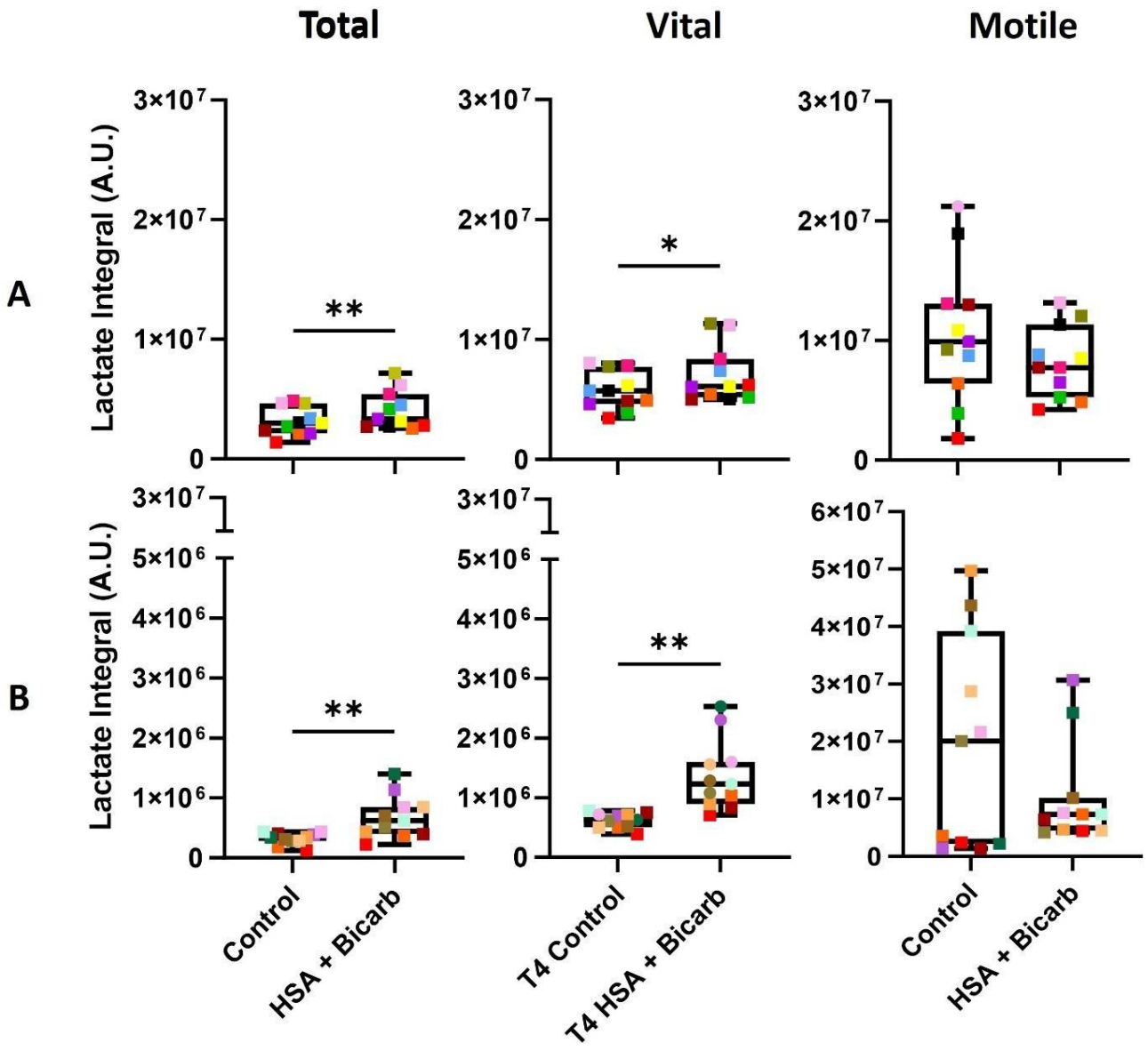


Figure 3.7: Lactate production by human (A) and boar (B) sperm incubated for four-hours with or without HSA and bicarbonate. Lactate integrals are normalised by total, vital and motile human and boar sperm concentrations. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a Wilcoxon test for boar sperm where the lactate integral was normalised by motile concentration and a paired t-test was used for the other five. * = P <0.05 and ** = P <0.01, n = 11. Points colour coded per individual donor/boar.

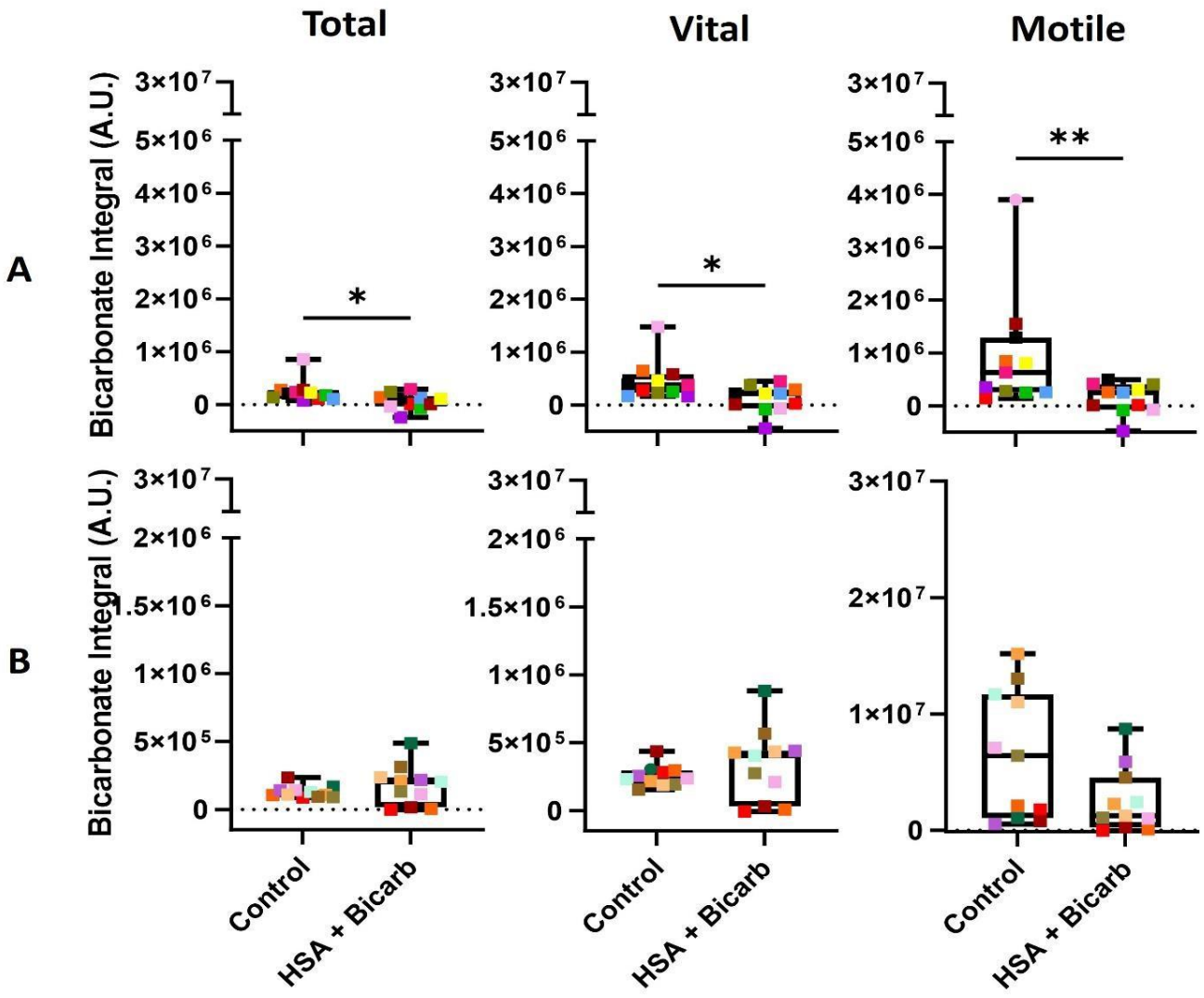


Figure 3.8: Bicarbonate production by human (A) and boar (B) sperm incubated for four-hours with or without HSA and bicarbonate. Bicarbonate integrals are normalised by total, vital and motile human and boar sperm concentrations. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a Wilcoxon test for all, except boar sperm where the bicarbonate integrals were normalised by motile concentration, where a paired t-test was used. * = P <0.05 and ** = P <0.01, n = 11. Points colour coded per individual donor/boar.

3.5 Discussion

It has previously been demonstrated that ATP consumption is higher during capacitation in numerous species, including human, and it has been suggested that glycolysis supplies the ATP that is responsible for this increase (Hicks *et al.*, 1972; Ferrandi *et al.*, 1987; Rogers and Perreault, 1990; Williams and Ford, 2001; Hereng *et al.*, 2011). ATP is required for the activation of intracellular signalling pathways such as cAMP and subsequent activation of PKA, ion channel function and the phosphorylation of tyrosine residues (Sansegundo, Tourmente and Roldan, 2022). LDH-C gene knockout in mouse models prevented tyrosine phosphorylation suggesting sperm- specific LDH-C is responsible for regenerating NAD+ that is required for glycolysis to continue functioning, allowing continuous glycolytic ATP generation during capacitation (Odet *et al.*, 2008). The role of glycolysis in capacitation has also been shown to be important in boar sperm, as capacitation was inhibited when an inhibitor of LDH, oxamic acid, was used (Rodriguez *et al.*, 2020). However, the role of oxidative phosphorylation during capacitation has also been suggested to be of importance, as an increased oxygen uptake was observed when human sperm were incubated under capacitating conditions (Stendardi et al., 2011). This chapter has used ^{13}C-NMR to determine whether glycolysis and/or oxidative phosphorylation are responsible for the increase in ATP required during capacitation.

To be confident that any increase in metabolism is a result of an increase in capacitation, the effects of HSA and bicarbonate on motility and vitality of sperm were analysed. A significant increase in total and progressive motility was identified when human sperm were incubated with HSA and bicarbonate for four- hours compared to those incubated without inducers and compared to before incubation. An increase in total motility was observed for boar sperm incubated with HSA and bicarbonate for four-hours compared to before incubation, however, no differences in progressive motility were identified for boar sperm, suggesting an increase in non-progressive sperm were responsible for the increase in total motility. This can be explained by the general low progressive motility observed for boar sperm samples. The concentration of glucose (8 mM) and bicarbonate (25 mM) used are physiologically representative of the FRT and it has previously been shown that 8 mM glucose is sufficient to support human sperm motility (Gardner *et al.*, 1996; Williams and Ford, 2001). Bicarbonate has long been known to influence motility of sperm, through the activation of sAC and subsequent production of cAMP from ATP, and this could be a potential explanation for the increase in total and progressive motility seen in human sperm when incubated with HSA and bicarbonate (Tajima, Okamura and Sugita, 1987).

No significant differences in vitality were identified between human and boar sperm incubated for four-hours with or without capacitation inducers, only when comparing to before incubation. The decrease in percentage of live sperm after a four-hour incubation could in part be due to length of time of the incubation as sperm survival rates naturally decreases over time. Sperm can survive for up to five days in the FRT, thought to be aided by interactions with the isthmic endosalpingeal epithelium (Wilcox, Weinberg and Baird, 1995; Reeve, Ledger and Pacey, 2003), however, the model in this thesis does not allow for these interactions, therefore potentially reducing the time sperm would be able to survive. Despite this, it has been shown that sperm are able to survive and continue metabolising over a few days *in vitro* (Calvert et al., 2018). Lactate and bicarbonate NMR integrals were normalised by sperm vital concentration and the proportion of live sperm contained both motile and immotile sperm, both of which require ATP. For those sperm which are motile, ATP would be required for motility, but it is also required for various signalling pathways that both motile (progressive and non-progressive) and immotile sperm will undergo. For example, sperm are still able to capacitate, therefore require ATP despite being immotile, and are still capable of fertilising an oocyte (World Health Organization, 2021). Immotile sperm are selected during intracytoplasmic sperm injection (ICSI) procedures and during testicular sperm aspiration (TESA) and percutaneous epididymal aspiration (PESA), where sperm have not yet acquired motility (Oseguera-López *et al.*, 2019).

An aim of this chapter was to induce and detect capacitation. A significant increase in the percentage of capacitated human and boar sperm was identified after a four-hour incubation with HSA and bicarbonate compared to before as determined using tyrosine phosphorylation immunofluorescence, supporting

previous work that demonstrated that HSA and bicarbonate are successful inducers of capacitation (Yanagimachi, 1970; Marıń -Briggiler et al., 2002). A significant increase in the percentage of capacitated human sperm was also seen after a four-hour incubation without HSA and bicarbonate compared to T0 and after a four-hour incubation with capacitation inducers compared to without inducers. This suggests that capacitation is a time-dependent process, that is enhanced by the presence of HSA and bicarbonate. Tyrosine phosphorylation is a hallmark of capacitation and has previously been shown to require ATP (Fukami and Lipmann, 1983; Brady and Lau, 2012). It has been shown that tyrosine phosphorylation increased when sperm, incubated with HSA and bicarbonate, were treated with exogenous pyruvate, alongside an observed increase in progressive motility and intracellular ATP that was shown to be a result of glycolytic flux (Hereng *et al.*, 2011). Their study demonstrated that when human sperm are supplemented with exogenous pyruvate, it is converted to lactate, rather than being oxidised as part of the Krebs cycle. The data presented in this thesis suggests that pyruvate is predominantly converted to lactate, however bicarbonate was still observed suggesting some pyruvate may also enter the Krebs cycle.

To investigate whether there was an increase in glycolysis or oxidative phosphorylation during capacitation in this thesis, NMR data was analysed. Lactate integrals seen in NMR are indicative of glycolysis and suggests that the $^{13}C_6$-glucose added prior to incubation is metabolised to pyruvate by sperm and then converted to lactate, by lactate dehydrogenase. Alongside, NADH is oxidised to NAD+ which is fed back into glycolysis, generating ATP (Odet et al., 2011). The production of lactate following human sperm incubation with $^{13}C_6$-glucose has previously been noted using ^{13}C-NMR (Calvert et al., 2018). An increase in lactate production, normalised by total and vital concentration, was seen when both human and boar sperm were incubated with HSA and bicarbonate compared to those incubated without. This suggests that human and boar sperm rely on glycolysis to produce the ATP required for capacitation related changes, agreeing with previous literature, that has used various methods to detect metabolism, including mass spectrometry, liquid chromatography and enzyme assays (Hicks *et al.*, 1972; Ferrandi *et al.*, 1987; Rogers and Perreault, 1990; Williams and Ford, 2001; Hereng *et al.*, 2011).

However, when looking at the motility data, it must be considered that the increase in lactate production seen for human sperm incubated with capacitation inducers, could be due to the increase in total and progressive motility seen for this group, rather than an effect of the increase in the percentage of capacitated sperm. Despite this, the increase in motility between the two groups was not noted for boar sperm and a significant increase in lactate production was still observed. This suggests that human and boar sperm undergoing capacitation utilise glycolysis to produce the ATP required to complete the process. As there were no significant differences in vitality between those that had been incubated with inducers compared to without, only when compared to T0, this provides evidence that this increase in lactate production seen for sperm incubated with capacitation inducers is not influenced by the percentage of vital sperm.

Bicarbonate integrals seen in NMR are indicative of oxidative phosphorylation and suggests that the $^{13}C_6$-glucose added prior to incubation is metabolised to pyruvate and then fed into the Krebs cycle. No significant difference was identified when boar sperm were incubated with or without HSA and bicarbonate, however, a decrease in bicarbonate production was identified when human sperm were incubated with HSA and bicarbonate compared to those incubated without. This suggests that both human and boar sperm do not rely on glucose oxidation for ATP generation during capacitation. Oxygen is required for oxidative phosphorylation and a previous study using rat liver cells has suggested that as pH increases, the oxygen dependence for oxidative phosphorylation increases (Wilson *et al.*, 1988). The addition of 25 mM bicarbonate as a capacitation inducer raised the pH of the media to approximately pH 8, despite 25 mM HEPES being used as a pH buffer, compared to pH 7.4 in the absence of bicarbonate. This could have potentially raised the oxygen dependence for oxidative phosphorylation. In the FRT, oxygen concentration is approximately 5% hence IVF clinics tend to incubate sperm, oocytes and embryos at this level (Ng *et al.*, 2018). With atmospheric oxygen concentrations used in this study (~21%), it was expected to see evidence of consistent aerobic oxidative phosphorylation, however, this was not the case (Fischer and Bavister, 1993). This supports the

argument that glycolysis is the predominant pathway for ATP production in human sperm (Hereng *et al.*, 2011).

It must be noted that bicarbonate is added to sperm as an inducer of capacitation, and to the negative controls used for NMR. As stated in 2.3.6, the average of the 11 bicarbonate integrals in the negative controls were subtracted from the bicarbonate integral values, for only the group that was incubated with HSA and bicarbonate before normalisation. As bicarbonate was added in small quantities, 12.5 µL, pipetting error could have accounted for subtle differences in the final concentration of bicarbonate added. Consequently, some of the bicarbonate integrals in this group are negative values and this could have contributed to the significant decrease in bicarbonate production seen for human sperm incubated with HSA and bicarbonate. Despite this, a significant increase in bicarbonate production was seen in all the positive controls in this thesis containing sperm (both human and boar) and bicarbonate, compared to the negative controls which also contained bicarbonate but no sperm, Appendix A9. This suggests that sperm metabolism contributed to the bicarbonate signals observed by NMR and that oxidative phosphorylation is occurring in both human and boar sperm in this study.

For human sperm, an increase in lactate production was observed alongside a decrease in bicarbonate production. This raises the question of why sperm utilise the inefficient metabolic pathway glycolysis, to generate ATP per unit of glucose, compared to oxidative phosphorylation (Locasale and Cantley, 2011). The Warburg effect, which is a key feature of cancer cells, is the observation that cells produce energy through the less efficient process of glycolysis as opposed to oxidative phosphorylation in normal cells, even in the presence of high oxygen and active mitochondria (Warburg, 1925). Sperm seem to display a similar behaviour during capacitation according to the data in this thesis. It has been suggested that whether glycolysis or oxidative phosphorylation occurs, the same amount if ATP is synthesised over any given time and this is because the production of lactate from glucose, that occurs during glycolysis, occurs 10-100 times quicker than the oxidation of glucose in the mitochondria of cancer cells (Shestov *et al.*, 2014). It has been proposed that these cells, that have a low yield but higher rate of ATP production, may have an advantage when competing for energy resources (Pfeiffer, Schuster and Bonhoeffer, 2001). This could be true for sperm, as an average of 180 million sperm are present in an ejaculate therefore will be competing for the shared, resources available in the FRT, to produce the ATP required for motility and capacitation so they are able to fertilise the oocyte, hence a higher rate of ATP production may be beneficial.

A further explanation as to why sperm utilise glycolysis as opposed to oxidative phosphorylation during capacitation is because sperm energy metabolism is compartmentalised, as glycolysis mostly occurs in the sperm head and the fibrous sheath of the principal piece, whereas oxidative phosphorylation occurs in the mitochondria, in the midpiece of sperm (Hereng *et al.*, 2011). Various glycolytic enzymes have been shown to be localised in the head and on the fibrous sheath of the principal piece (Kim *et al.*, 2007; Kichine *et al.*, 2013). The data in this study suggests that the ATP generated by glycolysis is sufficient at supporting capacitation and this could be due to sperm energy compartmentalisation. For example, glycolysis that occurs in the sperm head could support cholesterol efflux and tyrosine phosphorylation of proteins in the sperm head, and glycolysis occurring in the principal piece supports tyrosine phosphorylation of the proteins in the sperm flagellum. Despite the fact oxidative phosphorylation is more efficient in terms of ATP production, it remains disputed whether ATP diffusion from the mitochondria in the midpiece is sufficient to support capacitation, where most of the changes occur in the head and flagellum (Nevo and Rikmenspoel, 1970; Travis *et al.*, 2001; Mukai and Okuno, 2004; Takei *et al.*, 2014).

In conclusion, the reliance on glycolysis as opposed to oxidative phosphorylation during capacitation, as shown by the data in this chapter, suggests that local glycolytic ATP generation in the head and principal piece support the capacitation-associated events that occur in the head and tail. In the next chapter, hyperactivation, another key process a sperm must undergo in order to successfully fertilise the oocyte, is discussed and the metabolic pathways utilised during this process are investigated.

Chapter 4: The induction and measurement of hyperactivation and the underlying metabolic pathways utilised in this process.

4.1 Introduction

4.1.1 Discovery of hyperactivation

Hyperactivated sperm motility was first described in mammals in 1970 when a different motility pattern of golden hamster sperm was identified after capacitation compared to before (Yanagimachi, 1970). This study observed that after incubation with media containing oviduct fluids, follicular fluids and detoxified blood sera of the hamster and other species, hamster sperm showed vigorous motility and displayed high amplitude, asymmetrical beating of the flagella. Yanagimachi termed this state "hyperactivation" and claimed that sperm exhibited this behaviour prior to the acrosome reaction. Hyperactivation was subsequently studied and described in human sperm in 1984 (Burkman, 1984; Mortimer *et al.*, 1984).

4.1.2 Function of hyperactivation

A variety of functions of hyperactivation have been proposed including the detachment of capacitated sperm from the endosalpingeal epithelium prior to ovulation (Pacey et al., 1995) and the penetration of the zona pellucida (Stauss, Votta and Suarez, 1995). Through a series of experiments, Strauss et al (1995), demonstrated that hyperactivated sperm were more effective at penetrating the zona pellucida compared to those which had been treated with verapamil, an inhibitor of hyperactivation, suggesting that hyperactivation has an essential role in fertilisation.

4.1.3 Features of hyperactivation

Hyperactivation is characterised by a reduction in flagella beat frequency and a high amplitude flagellum bending which results in non-progressive, vigorous, side to side movements. There are different states of motility, and these include:

I. the activated state which describes the point where immature sperm acquire motility following interaction with seminal plasma;
II. the transitional state which refers to the transition towards the hyperactivated motion;
III. the hyperactivated state which refers to sperm assumed to demonstrate the hyperactivated motion (Suarez *et al.*, 1991).

Furthermore, four motility patterns associated with the hyperactivated state have previously been described, see Figure 4.1 (Burkman, 1991). These include:

A. Circling, high-curvature pattern
B. Thrashing pattern
C. Helical pattern and
D. Star-spin pattern (Burkman, 1991).

Sperm are known to switch between these motility states and patterns of hyperactivation (Burkman, 1984; Alasmari et al., 2013a). It has been reported that sperm switch between different motility patterns an average of six times a minute in approximately 90% of cells (Achikanu *et al.*, 2019). This continuous behavioural switching seen in sperm is thought to be a result of calcium oscillations affecting signalling activity and changes in flagella beat and this is a potential explanation for sperm that display random bursts of the hyperactivated motion (Harper, Barratt and Publicover, 2004; Bedu-Addo *et al.*, 2007).

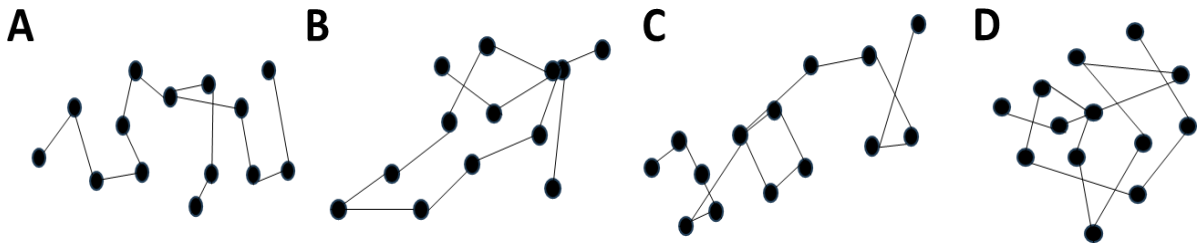


Figure 4.1: Hyperactivated motility patterns. The four motility patterns associated with the hyperactivated state: **(A)** Circling, **(B)** Thrashing, **(C)** Helical and **(D)** Star-spin. Filled circles represent the trajectory of the sperm head.

4.1.4 ATP demands of hyperactivation

Hyperactivation has been shown to be mediated by calcium signalling pathways and an increase in intracellular pH is essential for the induction of hyperactivation (Zeng, Oberdorf and Florman, 1996; Marquez and Suarez, 2004, Marquez and Suarez, 2007). This increase in pH stimulates the calcium channels responsible for the calcium influx as well as the bending at the axoneme, which is seen during hyperactivation (Ho, Granish and Suarez, 2002). The influx of calcium ions through the CatSper channel is thought to be the trigger of hyperactivation in sperm (Alasmari et al., 2013). This channel does not require ATP to function as it is activated by intracellular alkalisation that occurs as a result of the influx of bicarbonate ions seen during capacitation. It is also stimulated by progesterone, which is present in the FRT (Lishko, Botchkina and Kirichok, 2011). Testosterone has been shown to bind to and inhibit CatSper suggesting that hyperactivation is prevented in the male reproductive tract by the high levels of testosterone and activated once in the FRT to provide sperm with the ability to fertilise the oocyte (Mannowetz, Miller and Lishko, 2017).

The mobilisation of internal calcium stores has also been suggested to contribute to hyperactivation of sperm as receptors for IP3, a signalling molecule that releases calcium from intracellular stores, have been localised in the flagellum, as well as plasma membrane Ca^{2+}-ATPase, which may have a role in the regulation of hyperactivation, see Figure 4.2 (Armon and Eisenbach, 2011). IP3 receptors have been shown to be potentiated by ATP suggesting that although the CatSper channels do not require ATP to function, ATP is required for release and modulation of calcium from intracellular stores during hyperactivation (Harper and Publicover, 2005; Da Costa *et al.*, 2016; Schmitz, Takahashi and Karakas, 2022). In addition, to achieve the high amplitude flagellum bending seen in hyperactivation, higher levels of ATP have been shown to be required by the axoneme (Ho, Granish and Suarez, 2002).

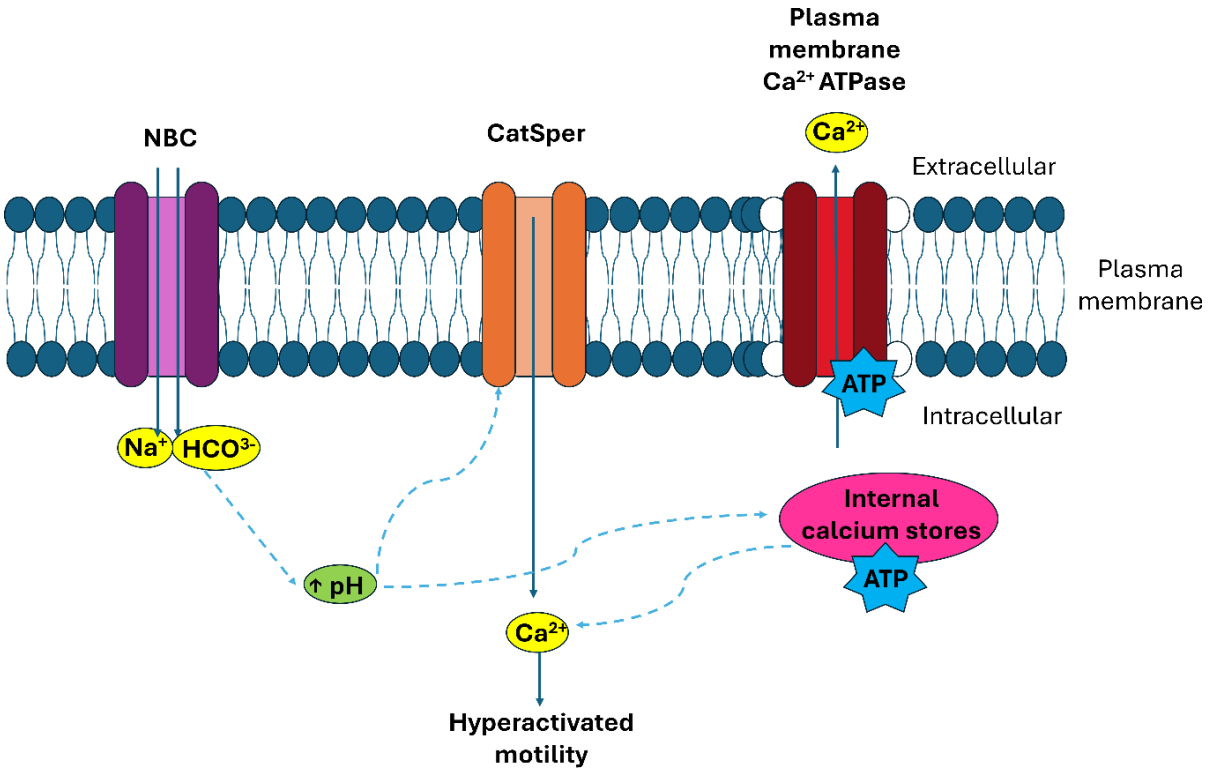


Figure 4.2: Molecular changes during hyperactivation. During hyperactivation, the increase in intracellular bicarbonate that occurs during capacitation, via sodium bicarbonate cotransporters (NBCs), increases intracellular pH. This increase in pH stimulates CatSper channels leading to an increase in intracellular calcium. The increase in intracellular pH also contributes to the release of calcium from internal calcium stores. The increase in calcium stimulates hyperactivation.

4.1.5 The induction of hyperactivation

Various molecules have been proposed as inducers of hyperactivation in numerous species of sperm, including human. These inducers include P4 (Sueldo *et al.*, 1993), myo-inositol (Keyser, van der Horst and Maree, 2021), procaine hydrochloride (Keyser, van der Horst and Maree, 2022), 4-ap (Achikanu *et al.*, 2019), caffeine, thimerosal and thapsigargin (Ho and Suarez, 2001). Their mode of action is via increasing intracellular calcium by either the mobilisation of intracellular stores or the influx of calcium ions via the CatSper channel. In this study, 4-ap and caffeine were chosen to induce hyperactivation in boar and human sperm due to success in recent publications.

4-aminopyridine has been shown to induce hyperactivation in human sperm (Achikanu *et al.*, 2019). It is known that 4-aminopyridine increases intracellular calcium through the mobilisation of internal stores and subsequently induces hyperactivation in human sperm (Alasmari et al., 2013; Kasatkina, 2016). In the study by Achikanu et al, (2019), hyperactivation was assessed based upon the differing motility patterns and kinematic criteria that had previously been described by Mortimer, (2000). The authors found that 4-aminopyridine increases the amount of hyperactivated sperm and the length of time sperm displayed hyperactivated motility (Achikanu *et al.*, 2019).

Caffeine was chosen to induce hyperactivation in this study, as it is widely used for inducing intracellular calcium release (Ho and Suarez, 2001). Caffeine is an antagonist of adenosine receptors and adenosine has a role in modulating intracellular calcium levels (Minelli *et al.*, 2008; Ribeiro and Sebastião, 2010). Caffeine triggers the mobilisation of calcium from intracellular calcium stores and encourages calcium entry via voltage-operated calcium channels (Karhapää and Törnquist, 1997). A range of concentrations from 5 mM to 10 mM have been used to successfully induce caffeine in human and ram sperm (Colás, Cebrián-Pérez and Muiño-Blanco, 2010; Keyer, Van der Horst and Maree, 2022). Caffeine has also been shown to induce

fluctuations in calcium ions in other cell types as well as in sperm, demonstrating its suitability for use in this research (Nehlig, Daval and Debry, 1992; Chirasani *et al.*, 2019).

4.1.6 The detection of hyperactivation

The hyperactivation status of sperm is commonly assessed using specific kinematic criteria and computer assisted semen analysis (CASA) software. Kinematic criteria refer to the motion and movement of sperm by analysing the trajectories of points and lines. This information can determine various aspects of movement, for example, acceleration, displacement, and velocity (Mortimer, 1997). Previously, different studies and research groups have used various kinematic criteria to determine whether a sperm is hyperactivated, however, the kinematic criteria commonly used to detect hyperactivation include a high curvilinear velocity along actual swimming path (VCL), a low linearity of a curvilinear path (LIN), which is calculated using equation 5 below, and a high amplitude of lateral head displacement (ALH), see Figure 4.3 (Alipour *et al.*, 2017). The most commonly referenced criteria that defines a sperm as hyperactivated is VCL ≥ 150 µm/s, LIN <50% and ALH ≥7 µm, however various CASA systems use different criteria, and it still remains unstandardized (Suarez, 2008).

Equation 5: $Lin = \frac{VSL}{VCL} X\ 100$

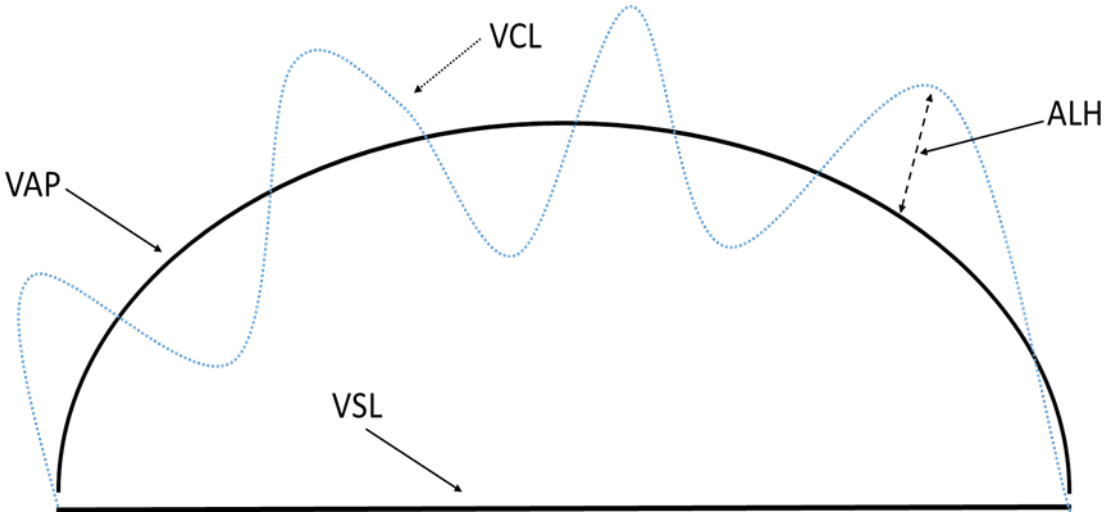


Figure 4.3: Kinematic criteria used to determine hyperactivation. Diagram to show the kinematic criteria of sperm measured by CASA, usually over the period of one second at 50 fps; VSL = Straight Line Velocity, the straight line distance travelled between the first and last point of the trajectory, VCL = Curvilinear Velocity, the distance travelled along the curvilinear path, VAP = Average Path Velocity, the average trajectory of sperm and ALH = Amplitude of Lateral Head Displacement, how far the sperm head deviates from the VAP.

CASA is regularly used to analyse hyperactivated sperm within a population as it can study the movement of several hundred sperm in a very short space of time hence making it a practical approach (Mortimer, 2000; Suarez, 2008). Despite CASA being commonly used to detect hyperactivation, it has many disadvantages that affect its accuracy and reliability when comparing studies. For example, different studies use different CASA systems which have differences in the image sampling frequency during the recordings, known as the frame rate (Mortimer and Swan, 1995a; Mortimer, 1997). As certain kinematic parameters are frame rate dependant, a lack of consistency between CASA systems suggests that results are not always reliable and comparable. Further work has also concluded that the detection of transitional hyperactivated cells by CASA is also a potential problem when determining the population of hyperactivated cells in a sample (Mortimer, Swan and Mortimer, 1996; Pacey *et al.*, 1997). Despite this, objective analysis using CASA remains the most

common method in the detection of hyperactivated sperm and the use of playback features allow the user to confirm the accuracy of the measurement.

An alternative method to measure hyperactivation assesses flagellar waveforms by measuring curvature at points along the sperm flagella and plotting this against the distance from the base of the flagella (Ishijima *et al.*, 2006). This method remains unpopular due to the time-consuming nature of this process and the limited number of sperm analysed per sample when compared to CASA, making the measurements less representative of the heterogenous human sperm population (Suarez, 2008)

4.2 Rationale

The aim of this chapter was to attempt to induce hyperactivation in human and boar sperm and then investigate the underlying metabolism during hyperactivation using ^{13}C-NMR. An increased demand for ATP, in addition to the ATP requirement of motility as discussed in chapter 1.2, is hypothesised to be required for the vigorous movement and increased flagella bending seen in hyperactivation. This increase in ATP has previously been demonstrated for bull sperm (Ho, Granish and Suarez, 2002). It is therefore postulated that the increased ATP demand during hyperactivation in human and boar sperm will be met by an increase in both glycolysis and oxidative phosphorylation.

4.2.1 Specific objectives

1. Induce hyperactivation in human and boar sperm.
2. Detect hyperactivation in human and boar sperm using CASA.
3. Investigate the effects of hyperactivation on motility and vitality of human and boar sperm.
4. Investigate the metabolic pathways utilised by human and boar sperm during hyperactivation.

4.3 Methods

4.3.1 Induction of hyperactivation

Capacitation was first induced as described in 3.3.1. Following on from this, hyperactivation was induced by adding either 8 mM caffeine or 2 mM 4-AP to one of the tubes, and to the other tube, DMSO (solvent) was added as a control (+ve). The inducers were present for four-hours. Hyperactivation was measured after an eight-minute incubation with caffeine (4 hours + 8 minutes), as communicated by Professor Gerhard van der Horst, or after a five-minute incubation with 4-ap (4 hours + 5 minutes), based upon recommendations in previous literature (Achikanu *et al.*, 2019). After measurement of hyperactivation (4.3.2), sperm were left to incubate for eight-hours in total. Sperm motility (2.3.2), sperm vitality (2.3.3) and hyperactivation status (4.3.2) were measured before (T0) and after (T8) incubation and the metabolic pathways utilised by sperm during the incubation were analysed using ^{13}C-NMR (2.3.5).

Table 4.1: The contents of the tubes prepared for the induction of hyperactivation. Each tube contained a final volume of 500 µl. All components added in µL. Final concentrations are: 8 mM $^{13}C_6$-glucose, 5 mg/mL HSA, 25 mM $NaHCO_3$, 8 mM caffeine or 2 mM 4-ap.

Added at the start of the incubation (T0), (µL)						
Tubes	**Sperm (50 M/mL)**	**EBSS**	**1/3 Pen/strep**	**100 mM $^{13}C_6$-glucose**	**50 mg/mL HSA**	**1 M $NaHCO_3$**
-VE	0	382.5	15	40	50	12.5
+VE	345	37.5	15	40	50	12.5
Inducer	345	37.5	15	40	50	12.5
Added at T4, (µL)						
Tubes	**EBSS**	**100 mM caffeine**	**500 mM 4-ap**			
-VE	0	40	2			
+VE	40	0	0			
Inducer	0	40	0			
Inducer	0	0	2			

4.3.2 Measurement of hyperactivation - CASA

A 5 µL aliquot of the sperm sample was added to a pre warmed 10 µM depth sperm counting chamber and placed on a heated mat at 37°C for 5 minutes. Hyperactivation was then detected using Sperm Class Analyzer, version 6, as described in 2.3.2, with a frame rate of 50 fps. Random locations within the chamber were imaged until either 500 sperm had been identified or five fields of view had been examined. For each sperm, a minimum of 13 track points were required for inclusion. The kinematic criteria used to define hyperactivated sperm are: VCL ≥ 150 µM/s, LIN < 50% and ALH ≥ 7 µm (Mortimer, 2000). The videos for each field of view were used to confirm the accuracy of the system.

4.4 Results

Both 4-ap and caffeine were used with the aim of inducing hyperactivation, however, 2 mM 4-ap was unsuccessful (n=7) at inducing hyperactivation in both human and boar sperm, Appendix A8. As hyperactivation was not induced in either species, no differences in metabolism were identified (data not shown). For this reason, 8 mM caffeine was used as an inducer of hyperactivation with some success (data shown below).

4.4.1 Motility of human and boar sperm incubated with or without inducers of hyperactivation.

CASA was used before (T0) and after (T4 control, T4 caffeine, T8 control and T8 caffeine) incubations to observe whether caffeine had any effect on human and boar sperm motility respectively. As seen in figure 4.4A, for human sperm, the percentage of sperm total motility increased following an eight-minute incubation with caffeine (following the four-hour capacitation incubation), compared to time zero, but did not in the absence of caffeine (T0, 61.5 ± 4.3%; T4 caffeine, 79.2 ± 3.2%; P = 0.0001; T4 control, 66.5 ± 4.7%). A significant increase in total motility was also seen after incubation with caffeine compared to without, P = 0.006. Over the following four-hour incubation (bringing the total to eight-hours), a significant decrease in human sperm total motility was observed for those incubated with and without caffeine, compared to T4 caffeine (T8 control, 64.9 ± 4.8%; P = 0.008; T8 caffeine, 61.5 ± 4.5%; P = 0.0003).

In terms of progressive motility, for human sperm, there was a significant increase after a four-hour incubation both with and without the addition of caffeine for eight minutes compared to time zero (T0, 34.2 ± 5%; T4 control, 45.6 ± 4.9%; P = 0.02; T4 caffeine, 60.5 ± 4.2%; P <0.0001), figure 4.4C. Over the following four-hour incubation (bringing the total to eight-hours), a significant decrease in human sperm progressive motility was observed for those incubated with and without caffeine, compared to T4 caffeine (T8 control, 42.7 ± 5.1%; P = 0.02; T8 caffeine, 39.9 ± 4.8%; P = 0.0009).

For boar sperm, a significant decrease in the total motility of sperm was observed, as seen in figure 4.4B, after an eight-hour incubation, with caffeine added after four-hours, compared to an eight-hour incubation without caffeine (T8 caffeine, 34.1 ± 4.7%; T8 control, 41.5 ± 6.2%; P = 0.02), and when compared to a four-hour incubation with caffeine added for eight-minutes (T4 caffeine, 48.2 ± 5.4%; P = 0.01), and when compared to a four-hour incubation without the addition of caffeine (T4 control, 56.3 ± 7.5%; P = 0.003). A significant decrease in the total motility of boar sperm was also observed after an eight-hour incubation without caffeine compared to a four-hour incubation without caffeine, P = 0.01.

In terms of boar sperm progressive motility, figure 4.4D, the same pattern seen for boar sperm total motility was observed. There was a significant decrease after an eight-hour incubation, with caffeine added after four-hours, compared to an eight-hour incubation without caffeine (T8 caffeine, 11.7 ± 2.1%; T8 control, 19.0 ± 3.7%; P = 0.049), and when compared to a four-hour incubation with caffeine added after eight-minutes (T4 caffeine, 23.4 ± 4.2%; P = 0.02), and when compared to a four-hour incubation without the addition of caffeine (T4 control, 30.0 ± 5.6%; P = 0.0008). A significant decrease in the progressive motility of boar sperm was also observed after an eight-hour incubation without caffeine compared to a four-hour incubation without caffeine, P = 0.01.

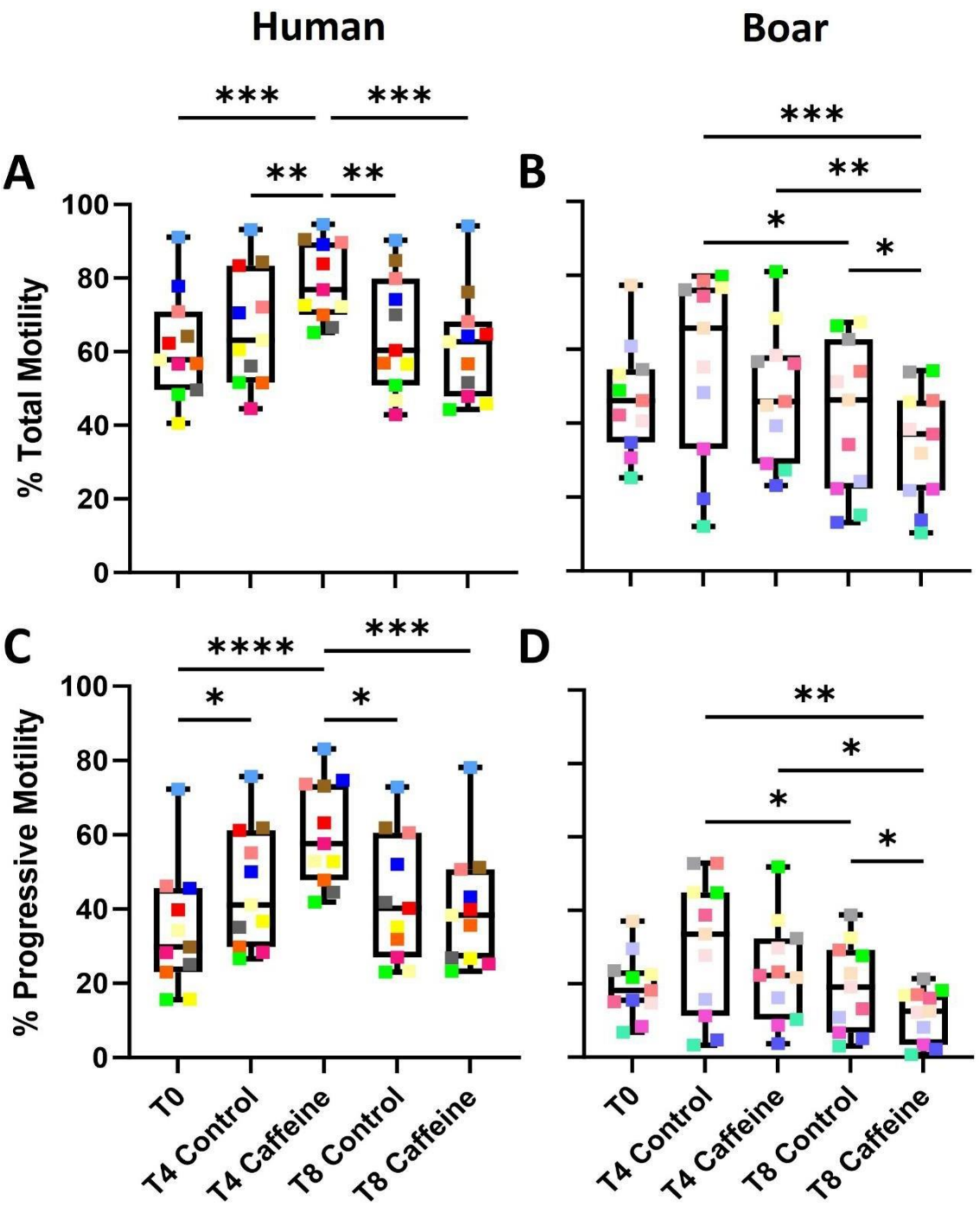


Figure 4.4: Human sperm total (A) and progressive (C) motility and boar sperm total (B) and progressive (D) motility (%) prior to (T0) and following either a four-hour plus eight minutes (T4) or an eight-hour (T8) incubation with or without 8 mM caffeine. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a Friedman test with a Dunn's multiple comparisons test for all except human sperm total motility (A), where a RM one-way ANOVA with a Tukey's multiple comparisons test was used. * = P <0.05, ** = P <0.01, *** = P <0.001 and **** = P <0.0001, n = 11. Points colour coded per individual donor/boar.

4.4.2 Vitality of human and boar sperm incubated with or without inducers of hyperactivation.

To observe whether an incubation with 8 mM caffeine influenced the vitality of human and boar sperm, the LIVE/DEAD™ sperm viability kit was used, see 2.3.3. For boar sperm, figure 4.5B shows that after 8 hours, the percentage of live human sperm significantly decreased both in the presence and absence of caffeine (added after 4 hours of incubation), compared to time zero (T0, 51.6 ± 3.4%; T8 control, 28.5 ± 2.3%; $P < 0.0001$; T8 caffeine, 25.2 ± 2.3%; $P < 0.0001$). There were no significant differences in the percentage of live human sperm over the eight-hour incubations, figure 4.5A.

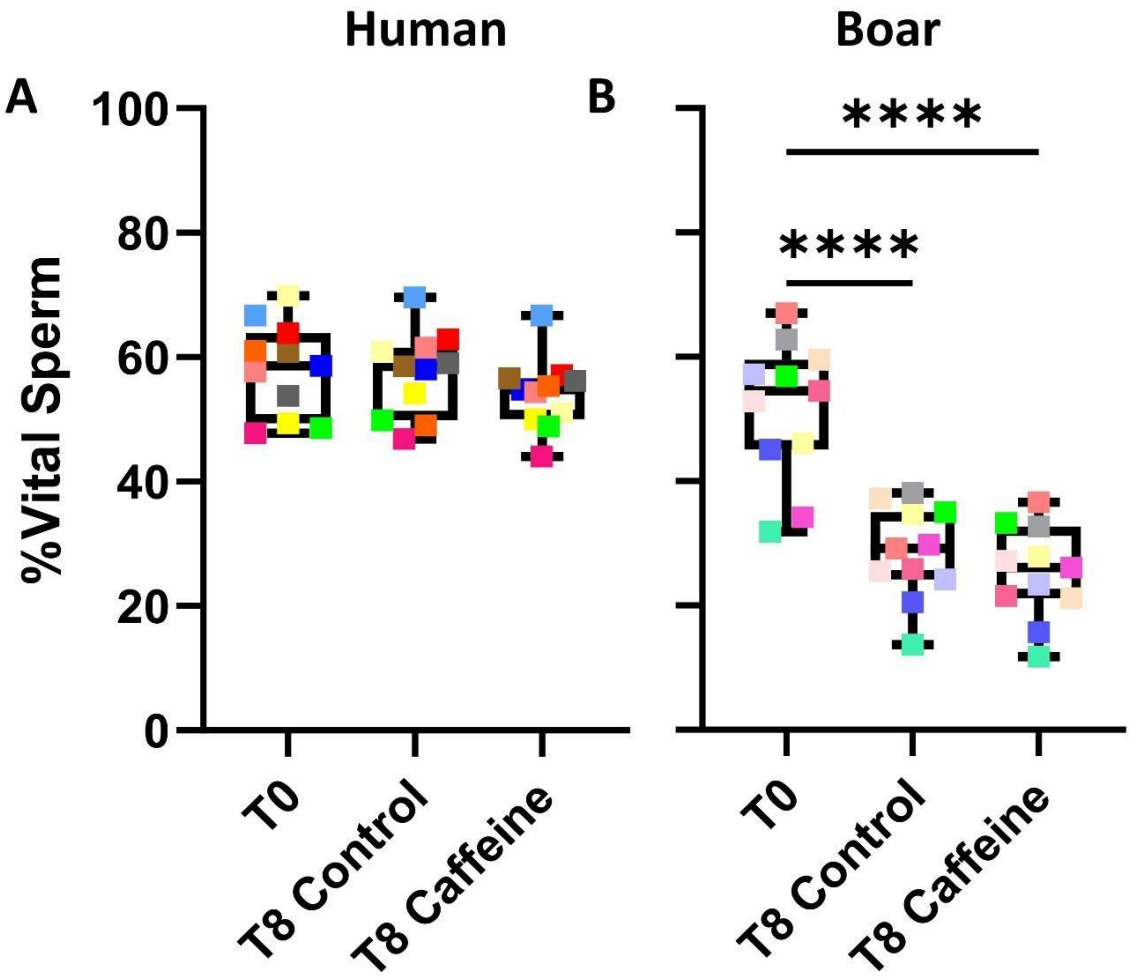


Figure 4.5: Human (A) and boar (B) sperm vitality (%) prior to (T0) and following (T8) an eight-hour incubation with or without 8 mM caffeine. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a RM one-way Anova with a Tukey's multiple comparisons test. **** = $P < 0.0001$, n = 11. Points colour coded per individual donor/boar.

4.4.3 Hyperactivation data for human and boar sperm incubated with or without inducers of hyperactivation.

Figure 4.6A shows that for human sperm, there was a significant increase in the percentage of hyperactivated sperm after a four-hour capacitation incubation followed by an eight-minute caffeine incubation, compared to time zero and compared to a four-hour incubation without caffeine (T4 caffeine, 28.1 ± 5.9%; T0, 0.12 ± 0.06%; P < 0.0001; T4 control, 4.7 ± 1.9%; P = 0.004). A significant increase was also identified following a further four-hour incubation with caffeine (T8 caffeine, 15.6 ± 4.9%) compared to T0 (P < 0.0001) and T4 control (P = 0.02).

For boar sperm, a significant decrease in hyperactivated sperm was observed after an eight-hour incubation, with caffeine added after four-hours, compared to T0 (T8 caffeine, 0.4 ± 0.1%; T0, 2.1 ± 0.6%; P = 0.006). This decrease in the percentage of hyperactivated sperm after an eight hour-incubation, with caffeine added after four-hours, was also observed when compared to a four-hour incubation without the addition of caffeine (T4 control, 3.1 ± 1.1%; P = 0.012) and when compared to a four-hour incubation followed by an eight-minute caffeine incubation (T4 caffeine, 1.7 ± 0.5%; P = 0.03).

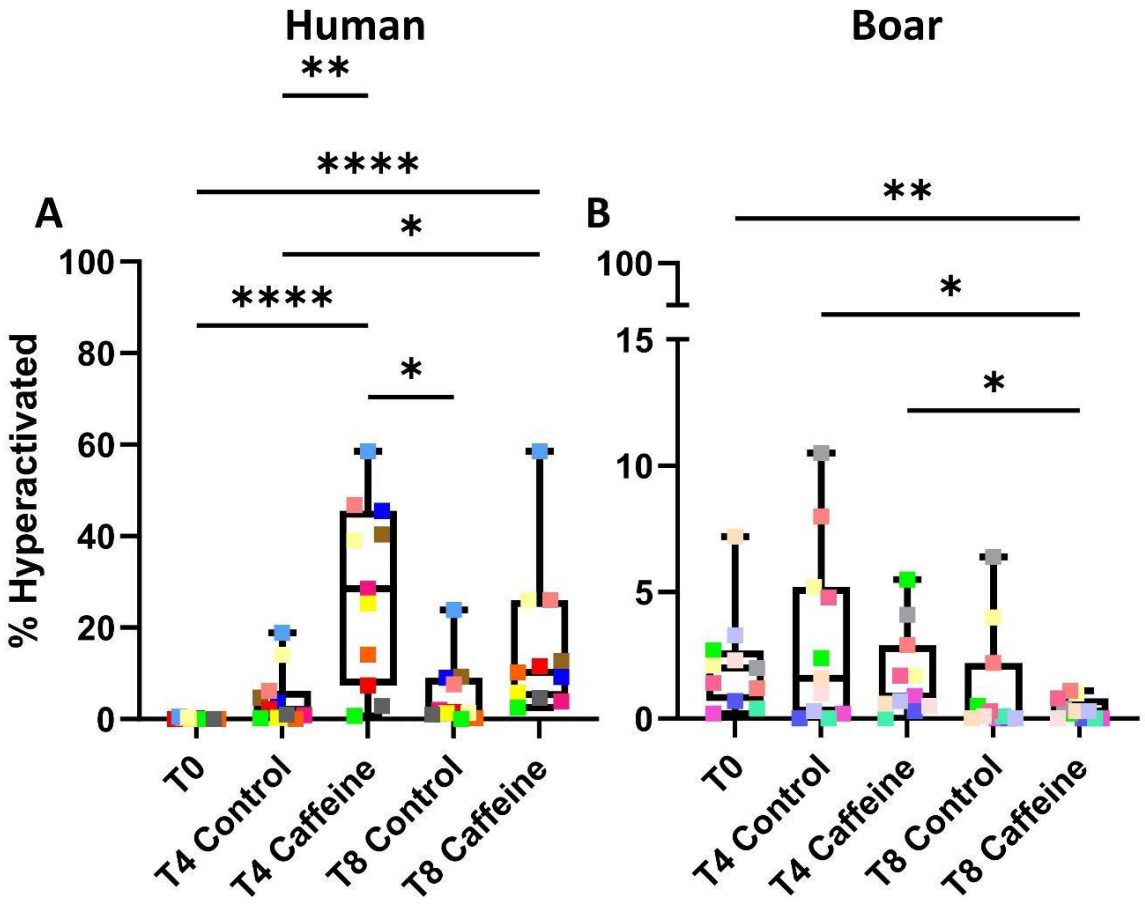


Figure 4.6: Percentage of hyperactivated human (A) and boar (B) sperm prior to (T0) and following either a four-hour (T4) or eight-hour (T8) incubation with or without the addition of 8 mM caffeine. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a Friedman test with a Dunn's multiple comparisons test for human and boar sperm. * = P <0.05, ** = P <0.01 and **** = P <0.0001, n = 11. Points colour coded per individual donor/boar.

4.4.5 NMR

^{13}C-NMR was used to investigate whether human (n=11) and boar (n=11) sperm, incubated with caffeine to induce hyperactivation, showed differences in metabolism compared to those incubated without caffeine. The production of lactate is indicative of glycolysis and the production of bicarbonate is indicative of oxidative phosphorylation. The lactate and bicarbonate integral values were normalised by sperm total, vital and motile concentration, see section 2.3.6. No lactate peaks were recorded for the negative control samples suggesting no metabolism occurs in the absence of sperm (appendix A7).

4.4.5.1 Lactate production by human and boar sperm incubated with or without inducers of hyperactivation.

For human sperm, figure 4.7A shows that after 8 hours, there was a significant increase in lactate production, normalised by total human sperm concentration, when human sperm were incubated with caffeine compared to those incubated in the absence of caffeine (T8 control, 7.3 ± 0.8 x 10^6 A.U.; T8 caffeine, 1.1 ± 0.1 x 10^7 A.U.; $P < 0.0001$). The same was observed when normalised by vital human sperm concentration (T8 control, 1.3 ± 0.1 x 10^7 A.U.; T8 caffeine, 1.9 ± 0.2 x 10^7 A.U.; $P < 0.0001$), and when normalised by motile human sperm concentration, (T8 control, 1.1 ± 0.08 x 10^7 A.U.; T8 caffeine, 1.8 ± 0.2 x 10^7 A.U.; $P = 0.0002$). No significant differences in lactate production were observed for boar sperm incubations with or without caffeine, figure 4.7B.

4.4.5.2 Bicarbonate production by human and boar sperm incubated with or without inducers of hyperactivation.

No significant differences in bicarbonate production were observed for human or boar sperm incubations with or without caffeine, as seen in figure 4.8.

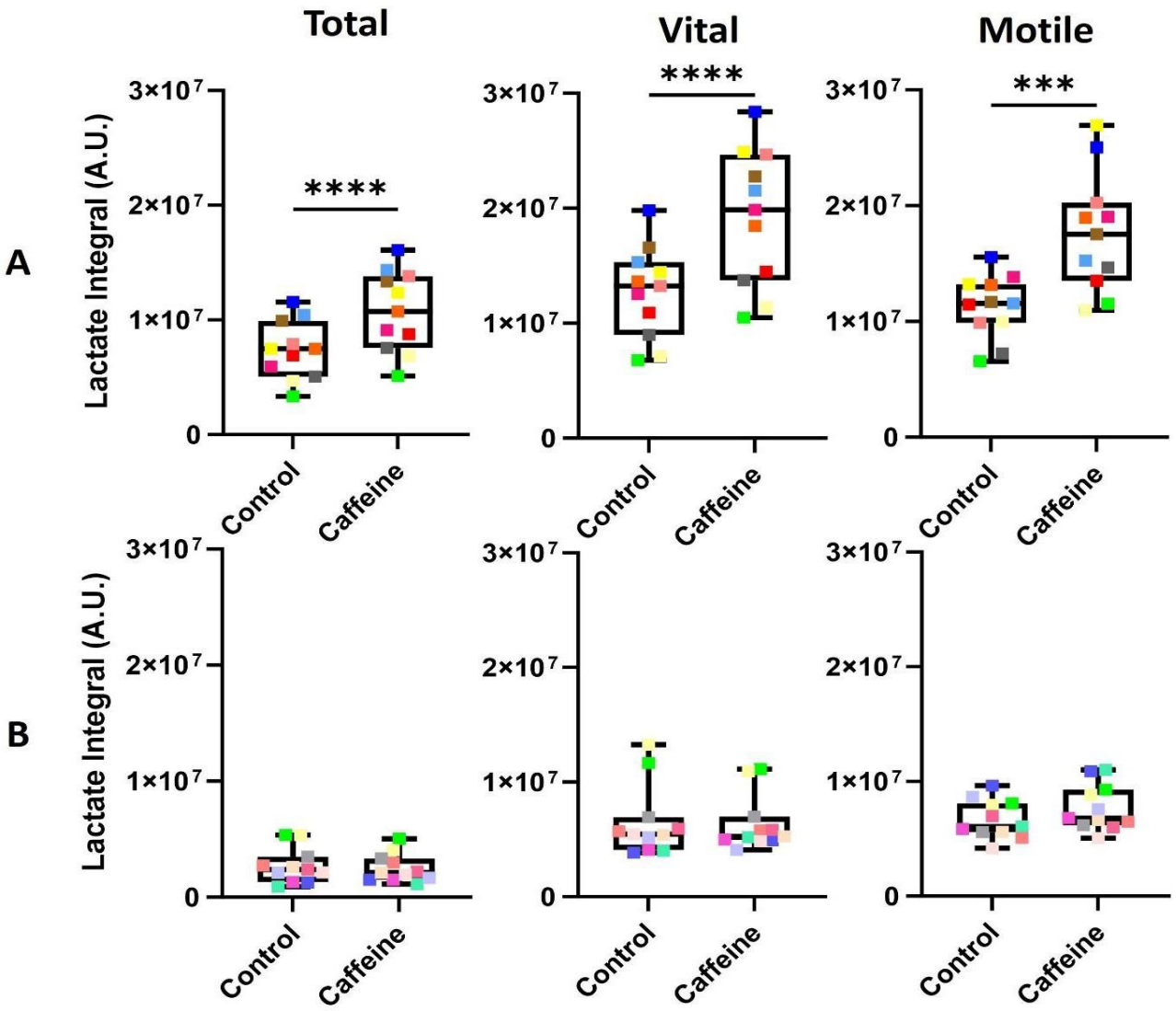


Figure 4.7: Lactate production by human (A) and boar (B) sperm incubated with or without caffeine. Lactate integrals are normalised by total, vital and motile human and boar sperm concentrations. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a paired t-test for all graphs except the lactate integrals for boar sperm normalised by vital concentration which was analysed using the wilcoxon test. *** = P <0.001 and **** = P < 0.0001, n = 11. Points colour coded per individual donor/boar.

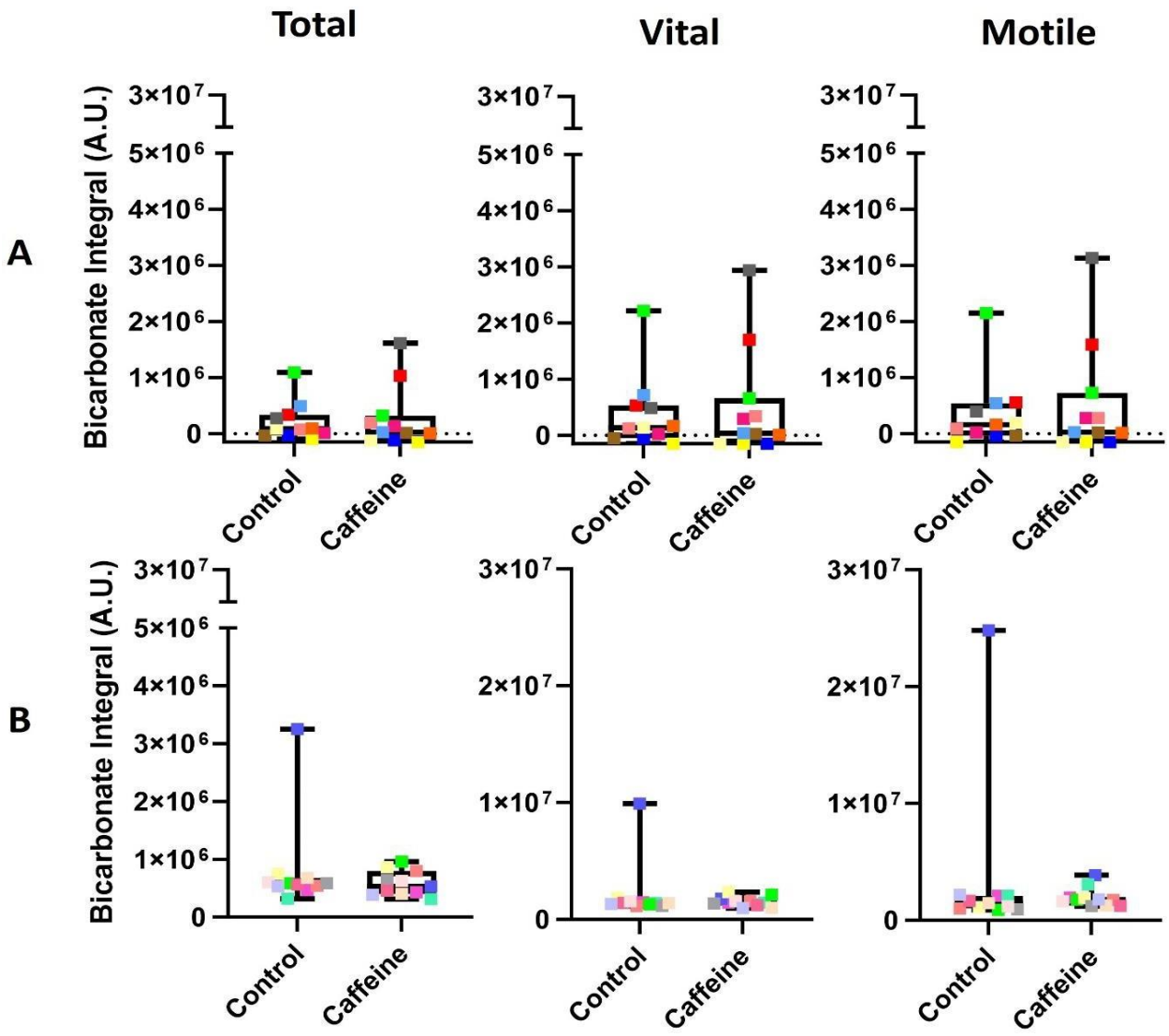


Figure 4.8: Bicarbonate production by human (A) and boar (B) sperm incubated with or without caffeine. Bicarbonate integrals are normalised by total, vital and motile human and boar sperm concentrations. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a Wilcoxon test for all datasets. No significant differences were identified, n = 11. Points colour coded per individual donor/boar.

4.5 Discussion

Hyperactivation has been suggested to be an ATP demanding process due to the deep flagella bending seen during hyperactivation and the mobilisation of intracellular calcium stores. An increase in ATP usage during hyperactivation has previously been observed in bull sperm (Ho, Granish and Suarez, 2002) and in mouse sperm (Sansegundo, Tourmente and Roldan, 2022). The latter study in mice postulated that the increased demand in ATP could also be linked to the higher velocity and lateral head displacement seen during hyperactivation (Sansegundo, Tourmente and Roldan, 2022). It has previously been suggested that this ATP is a result of glycolysis as mice sperm that lack sperm specific lactate dehydrogenase are unable to hyperactivate (Odet *et al.*, 2013). However, there has been little research, particularly in human sperm, regarding whether glycolysis or oxidative phosphorylation is responsible for generating the ATP required for hyperactivation. Therefore, this chapter has utilised ^{13}C-NMR to determine whether glycolysis, oxidative phosphorylation or both are responsible for the increase in ATP required during hyperactivation.

Initially, 4-ap was hypothesised to be suitable choice for the induction of hyperactivation in both human and boar sperm due to its ability to modulate the release of intracellular Ca^{2+} and stimulate CatSper channels (Achikanu *et al.*, 2018). However, it was unsuccessful at inducing hyperactivation in this thesis, despite previous research suggesting 4-ap was a potent inducer of hyperactivation in human and bovine sperm (Alasmari et al., 2013; Achikanu et al., 2019; Zaferani, Suarez and Abbaspourrad, 2021). Studies in human sperm that reported success with 2 mM 4-ap used individual sperm tracking to evaluate hyperactivation compared to CASA used in this chapter, hence there may have been some selection bias towards only progressively motile sperm. As 4-ap was unsuccessful at inducing hyperactivation in this thesis, caffeine was used to induce hyperactivation based on previous success in other studies (Johnson *et al.*, 2017; Zaferani, Suarez and Abbaspourrad, 2021; Keyser, van der Horst and Maree, 2022).

The effects of caffeine on the motility and vitality of human and boar sperm were analysed to observe whether caffeine had any detrimental effects on sperm physiology. In addition, this was of interest because motility is closely linked to hyperactivation, and a decrease in the percentage of progressive sperm would be expected if the percentage of hyperactivated sperm were to increase. This is because hyperactive sperm typically display non-progressive, vigorous, side to side movements (Suarez, 2008). Therefore, both total and progressive motility were analysed before and after incubation with or without caffeine. There was an increase in total and progressive motility over the first four-hours of the incubation for human sperm incubated with and without caffeine, and when comparing the two groups, T4 control and T4 caffeine, there was a significant increase in total motility (approximately 13%) when incubated with caffeine and a similar increase in progressive motility (approximately 15%), however this was not significant. This suggests that caffeine stimulates progressive motility, which is a combination of both rapid and medium progressive sperm, rather than non-progressive. Upon visual observation of the CASA analysis, of the sperm that were classified as hyperactivated, the majority of these seemed to display patterns that reflected transitional hyperactivation, rather than the typical star-spin pattern which was less frequently observed. Previous studies have noted that sperm displaying these transitional patterns can be identified as both progressive and hyperactivated by CASA systems which rely on standard thresholds for kinematic criteria, used in this thesis, explaining why an increase in progressive motility may have been seen (Goodson *et al.*, 2017). For both human and boar sperm, significant decreases in total and progressive motility were observed after eight-hours of incubation both with and without caffeine compared to T4 suggesting this is a time-dependent decrease in motility and not an effect of caffeine. Specifically for boar sperm, this decrease is also likely to be an effect of the simultaneous decrease observed in vitality.

A significant decrease in the percentage of vital boar sperm was observed when incubated both with and without caffeine compared to before incubation, and no differences in human sperm vitality were observed, suggesting caffeine is not a toxic molecule to sperm (Keyser, van der Horst and Maree, 2022). As dead sperm would not be capable of metabolising the glucose provided, lactate and bicarbonate NMR integrals were

normalised by sperm vital concentration. The significant decrease in percentage of live boar sperm after an eight-hour incubation is probably due to length of time of the incubation, suggesting boar sperm are not as adaptable to *in vitro* conditions compared to human sperm for which no significant decrease in vitality was observed. This could be due to the composition of the boar sperm plasma membrane, which makes them more vulnerable to the detrimental effects of ROS during the eight-hour incubation, alongside a lack of antioxidants in the media used to culture sperm (Awda, Mackenzie-Bell and Buhr, 2009a; Jawad *et al.*, 2023).

An aim of this chapter was to optimise the induction of hyperactivation in human and boar sperm. To investigate whether caffeine was a successful inducer of hyperactivation, CASA was used. A significant increase in the percentage of hyperactivated human sperm was identified after a four-hour capacitation incubation plus an eight-minute incubation with 8 mM caffeine, compared to before incubation and compared to sperm incubated without caffeine. There remained significantly more hyperactivated sperm after an eight-hour incubation, with caffeine added after four-hours, compared to T0 and T4 control. This suggests that not only is caffeine able to induce hyperactivation in human sperm, but it continues to induce hyperactivation for a significant period after its addition. This supports the idea that sperm can switch between different motility states and repeatedly display brief bursts of hyperactivation as there are no significant differences in the percentage of hyperactivated sperm after an eight minute, T4, and four-hour, T8, incubation with caffeine (Burkman, 1984; Harper, Barratt and Publicover, 2004; Bedu-Addo et al., 2007; Alasmari et al., 2013; Achikanu et al., 2019). However, it must be noted that the same individual sperm was not assessed at T4 and T8 therefore it cannot be confirmed that the same sperm is repeatedly hyperactivating in this thesis. Previous studies that have used sperm tracking have observed the ability of sperm to repeatedly hyperactivate (Achikanu *et al.*, 2019).

The ability of sperm to repeatedly display bursts of hyperactivation is probably due to sperm being exposed to an increase in pH and progesterone in the FRT for long periods of time, as bicarbonate and progesterone are present in oviductal fluids in the FRT, where sperm have been shown to survive several days (Suarez and Pacey, 2006). However, sperm responding to these stimuli and displaying brief periods of hyperactivation over prolonged periods of time prior to fertilisation would be presumed to be an energy-wasting process in the absence of ovulation. The binding of sperm to the endosalpingeal epithelium in the FRT prior to ovulation has been shown to prevent calcium influx, hence preventing capacitation and hyperactivation (Saint-Dizier *et al.*, 2020; Mahé *et al.*, 2021). It has been suggested that heparin in the oviductal fluids around the time of ovulation could facilitate this detachment, as heparin binding sites have been identified on sperm heads and the addition of heparin has previously been shown to aid in the detachment of bull sperm from the epithelium (Ardón *et al.*, 2016). In addition, hormonal changes around the time of ovulation could promote detachment, particularly the increase in progesterone following ovulation, a known inducer of hyperactivation. Hyperactivation is thought to aid in the detachment from the endosalpingeal epithelium prior to ovulation (Suarez, 2008). However, the exact mechanisms of these interactions and sperm detachment from the epithelium remains unknown due to the limited access to live mammal oviducts and the difficulties in maintaining the morphological and molecular characteristics of oviductal epithelial cells *in vitro* (Mahé *et al.*, 2021).

No significant increase in hyperactivation was observed for boar sperm suggesting that caffeine is not a successful inducer of hyperactivation in boar sperm under the *in vitro* conditions used in this research. There were similar percentages of hyperactivated sperm after four hours for boar sperm which had been stimulated with caffeine and those which had not. Previously, studies have shown that incubation of sperm under capacitating conditions can induce human sperm hyperactivation levels, up to around 20% in some cases, explaining how sperm still hyperactivated without the addition of caffeine in this thesis (Morales, Overstreet and Katz, 1988; Mortimer and Swan, 1995b). This is most likely due to the increase in pH seen during capacitation, where 25 mM bicarbonate was added as an inducer, stimulating the CatSper channels (Zeng, Oberdorf and Florman, 1996; Marquez and Suarez, 2007). A potential explanation as to why caffeine

induced hyperactivation in human sperm, but not in boar could be due to the poor motility observed for boar sperm in general as well as the significant decrease in the percentage of vital boar sperm over the eight-hour incubation, that was not observed for human sperm. There was only approximately 25-30% of boar sperm alive after the incubation in both groups therefore high levels of hyperactivation were not expected at T8, although it remains unknown at what point during the eight-hour incubation sperm died. Based upon data in chapter 3, following a four-hour incubation sperm vitality decreased by approximately 15% from 55% at T0 to 40% at T4. For hyperactivation, sperm vitality started at 51% at T0 and decreased to 25-30% at T8. This suggests that sperm vitality decreases gradually throughout incubation.

To investigate whether there was an increase in glycolysis or oxidative phosphorylation during human sperm hyperactivation, NMR data was analysed. An increase in lactate production was seen when human sperm were incubated with caffeine for four hours, in addition to a previous four-hour capacitation incubation, compared to those incubated without caffeine, when the lactate integral was normalised by total, vital and motile concentration. This suggests that human sperm rely on glycolysis to produce the ATP required for hyperactivation agreeing with previous work in mice (Odet *et al.*, 2013). The fact there were no significant differences in total motility, progressive motility and vitality between the two groups after an eight-hour incubation, T8 control and T8 caffeine, suggests this increase in lactate production is due to the effects of caffeine on hyperactivation of human sperm and supports the idea that hyperactivation has an increased energy demand compared to that of motility (Ho, Granish and Suarez, 2002; Odet *et al.*, 2013). No significant differences in lactate production were identified for boar sperm and this was expected as caffeine was unable to induce hyperactivation in boar sperm.

Bicarbonate integrals seen in NMR are indicative of oxidative phosphorylation and suggests that the $^{13}C_6$-glucose added prior to incubation is metabolised to pyruvate and then fed into the Krebs cycle. The fact that no significant differences were identified for bicarbonate production in human and boar sperm, suggests that sperm hyperactivation does not rely on oxidative phosphorylation to generate the ATP required for this process. These results were expected for boar sperm as hyperactivation was not induced and no significant differences in progressive motility or vitality were identified between the two groups at T8. The fact no significant differences in bicarbonate production were observed for human sperm suggests glycolysis is the main ATP-producing pathway utilised by human sperm during hyperactivation. This supports the conclusion in chapter 3, as most ATP is required in the flagellum for hyperactivation therefore supporting the idea that local ATP generation to the site of ATP utilisation is crucial for the various processes that take place in sperm prior to fertilisation.

In conclusion, 8 mM caffeine is capable of inducing hyperactivation in human sperm, agreeing with previous literature, but not in boar sperm. Furthermore, 2 mM 4-ap is unable to stimulate hyperactivation in human and boar sperm in contrast to published literature, and this is most likely due to the difference in methods used to assess hyperactivation. The reliance on glycolysis as opposed to oxidative phosphorylation during hyperactivation, as shown by the data in this chapter, suggests that local glycolytic ATP generation in the flagellum supports the deep flagella bending, higher velocity and lateral head displacement that occur during hyperactivation. In the next chapter, the acrosome reaction, which is essential for successful fertilisation of the oocyte, is discussed and the metabolic pathways utilised during the acrosome reaction are investigated.

Chapter 5: The induction and measurement of the acrosome reaction and the underlying metabolic pathways utilised in this process.

5.1 Introduction

5.1.1 Discovery of the acrosome reaction

The role of the acrosome in mammalian sperm was first studied using phase contrast microscopy (Austin and Bishop, 1958). The data from this study shows that the acrosome is modified as it passes through the FRT and is no longer attached when the sperm penetrates the zona pellucida. They concluded that the acrosome reaction was necessary for sperm penetration of the zona pellucida prior to fertilisation. Later studies have shown that the percentage of acrosome reacted sperm can be an indirect indicator of sperm capacitation as capacitation is thought to be a prerequisite for the acrosome reaction (Jaiswal *et al.*, 1998; Fabro *et al.*, 2002).

5.1.2 Function of the acrosome reaction

The acrosome reaction involves the fusion of the sperm plasma membrane with the outer acrosomal membrane, and it occurs prior to the penetration of the zona pellucida and subsequent fertilisation of the oocyte (Figure 5.1). Exocytosis occurs upon interaction with the zona pellucida sperm-binding protein 3 (ZP3) (Baldi *et al.*, 1996). This fusion of the sperm plasma membrane with the outer acrosomal membrane releases the acrosomal content, which contains numerous polysaccharides, proteins, and enzymes, and exposes inner acrosomal membrane proteins that allow for oocyte membrane fusion (Zaneveld *et al.*, 1991). These inner acrosomal membrane proteins are involved with recognition of the oocyte.

The timing of the acrosome reaction is extremely important in relation to fertilisation as sperm that acrosome react prior to the ovulation of the oocyte, known as a premature acrosome reaction, are unable to penetrate the zona pellucida hence unable to fertilise the oocyte (Brown et al., 1989; Tesarik, 1989; Yanagimachi, 2011). Therefore, the acrosome reaction is tightly controlled and mediated by the secretion of progesterone from the cumulous oophorous cells following ovulation (Breitbart and Spungin, 1997; Gómez-Torres *et al.*, 2015).

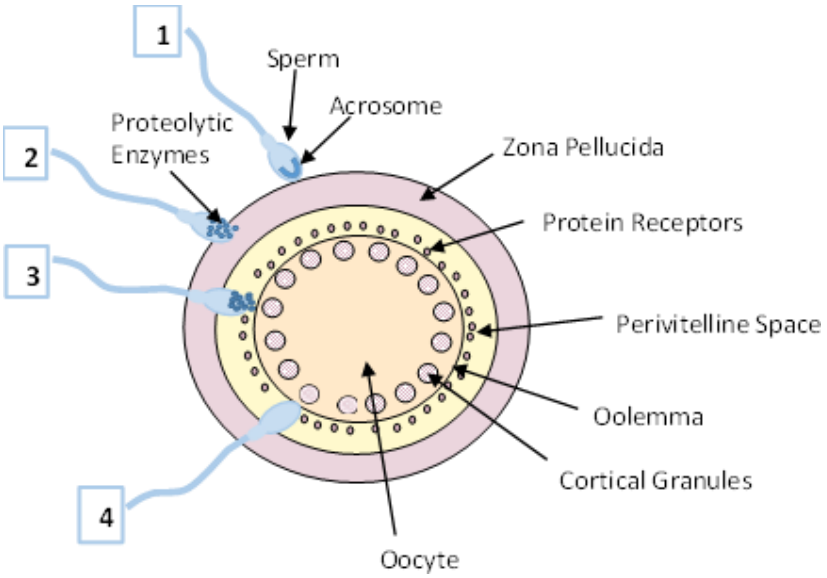


Figure 5.1: Diagrammatic representation of the acrosome reaction. (1) Sperm arrives at the oocyte and binds to the zona pellucida. **(2)** The acrosome undergoes exocytosis and releases the contents of the acrosome (proteolytic enzymes). **(3)** The proteolytic enzymes digest the zona pellucida and sperm enters the perivitelline space. **(4)** Sperm plasma membrane fuses with the oocyte plasma membrane (oolemma) and then goes on to deliver the paternal genome, contained within the nucleus, to the oocyte.

5.1.3 Features of the acrosome reaction and their ATP requirements

Numerous processes require exocytosis for the release of intracellular granules, including the acrosome reaction. There are three stages to the acrosome reaction: priming, triggering and exocytosis (Simons and Fauci, 2018). Several proteins are involved in exocytosis, importantly N-ethylmaleimide-sensitive fusion protein (NSF), soluble3` NSF attachment proteins (SNAPS) and soluble NSF attachment protein receptors (SNARES) (Sutton *et al.*, 1998). During the priming stage, the acrosome is tethered to the plasma membrane via SNARE proteins on the two joining membranes. These SNARE proteins form a complex and NSF binds to snare complexes in the membrane, facilitated by SNAPS, forming the 20 S fusion particle (Whiteheart *et al.*, 1994). The triggering stage involves the influx of Ca^{2+} into the sperm head, thought to be mediated by CatSper channels, which do not require ATP and are instead activated by intracellular alkalisation (Lishko, Botchkina and Kirichok, 2011). The exocytosis stage involves the fusion of the acrosomal membrane with the sperm plasma membrane, releasing the acrosomal contents. These steps have been suggested to be ATP independent and it is thought ATP is only required following fusion for the disassembly of the 20 s fusion particle and subsequent recycling of the SNARE proteins (Vilmart-Seuwen *et al.*, 1986; Ramalho-Santos, Schatten and Moreno, 2002; Baker and Hughson, 2016; Sengupta, Durairajanayagam and Agarwal, 2020; Kim *et al.*, 2021).

Studies using bovine neuroendocrine cells and mouse pancreatic B-cells were some of the first to reveal ATP-independent exocytosis can take place (Parsons *et al.*, 1995; Eliasson *et al.*, 1997). The ATP-independent exocytosis is hypothesised to occur in response to an increase in calcium (Vilmart-Seuwen *et al.*, 1986). This is supported by work in neuronal and neuroendocrine cells that suggest that ATP is required to prepare vesicles for exocytosis however the actual release of these vesicles is thought to be due to a calcium influx via voltage-gated calcium channels (Theander, Lew and Nüsse, 2002). CatSper channels contribute to this calcium influx in sperm and these channels are stimulated by intracellular alkalization, which occurs because of the bicarbonate influx seen in capacitation, see Figure 5.2 (Kirichok, Navarro and Clapham, 2006). Vilmart and Seuwen, (1986), proposed that other studies which may report ATP consumption during exocytosis could be due to events post- exocytosis such as the disassembly of the SNARE complex and potentially bringing calcium back to basal levels. After exocytosis, it has been suggested that ATP is responsible for the removal of calcium from the cytosol via Ca^{2+}-ATPases (Morimoto and Ogihara, 1996).

NSF is responsible for the disassembly of the 20 S fusion particle following the fusion of the outer acrosomal membrane and the sperm plasma membrane (Gerst, 1999). This disassembly requires ATP, which is hydrolysed by NSF, which contains two ATP-binding domains (Söllner *et al.*, 1993; Whiteheart *et al.*, 1994). Previous work has identified the presence of NSF in the acrosome of mammalian sperm and has observed a reduction in sperm exocytosis when ATP was depleted from the media and when agonists were added to the media to prevent the hydrolysis of ATP (Michaut *et al.*, 2000). This work suggests that ATP hydrolysis is required for acrosomal exocytosis suggesting that there may be an increased demand for ATP during the acrosome reaction. However, the amount of ATP required for the acrosome reaction remains unknown as other proteins have been suggested to be involved in exocytosis as opposed to just NSF, including protein kinases, which are activated when the zona pelluicda protein binds to receptors in sperm (Breitbart and Spungin, 1997; Klenchin and Martin, 2000). Despite their activation during the acrosome reaction, their role is not fully understood (Harrison, Carr and Meizel, 2000).

Interestingly, it has been suggested that ATP is an effective inducer of the acrosome reaction in rat, human and bovine sperm (Foresta, Rossato and Di Virgilio, 1992; Luria *et al.*, 2002; Torres-Fuentes, Rios and Moreno, 2015). These studies suggest a minimum of 500 µM ATP is required to stimulate the acrosome reaction, mediated through the ATP gated ion channel P2X7 receptor. Extracellular ATP has been shown to increase intracellular calcium levels in numerous cell types including sperm and this is most likely the mechanism by which the acrosome reaction is induced by ATP in the above studies (Xia and Ren, 2009; Ito, Ruegg and Takeda, 2018; Gao et al., 2023). However, it is suggested that ATP is not the physiological trigger of the acrosome reaction, but rather progesterone and ZP3 are (Okabe, 2016; Simons and Fauci, 2018). Both

glycolysis and oxidative phosphorylation have been suggested to generate the ATP required for the disassembly of the 20 S fusion particle and other features of the acrosome reaction in bovine sperm, however a debate remains within the literature regarding whether one energy pathway is preferred by sperm, in particular human sperm (Misro and Ramya, 2012; Dahan and Breitbart, 2022).

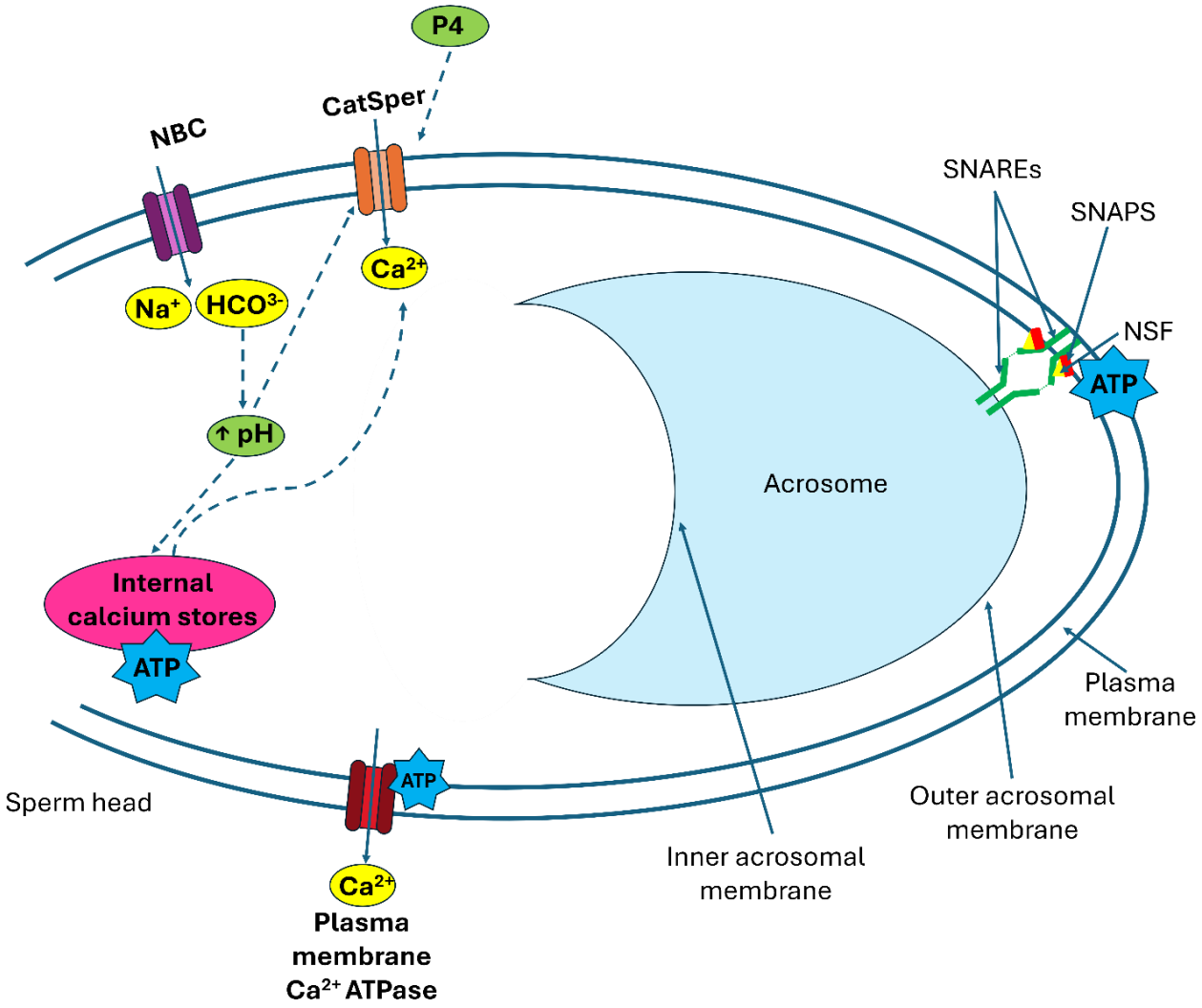


Figure 5.2: Molecular changes during the acrosome reaction. During the acrosome reaction, the outer acrosomal membrane is tethered to the plasma membrane via SNARE proteins on the two joining membranes. These SNARE proteins form a complex and NSF binds to snare complexes in the membrane, facilitated by SNAPS. The acrosome reaction is triggered by the influx of calcium, which occurs via CatSper channel. The CatSper channels are stimulated by P4 and the increase in intracellular pH that occurs due to the bicarbonate influx during capacitation, via sodium bicarbonate cotransporters (NBCs). The increase in intracellular pH also contributes to the release of calcium from internal calcium stores. Following fusion of the acrosomal membrane with the sperm plasma membrane, releasing the acrosomal contents, NSF is responsible for the recycling of the SNARE proteins and ATP is responsible for the removal of calcium from the cytosol via Ca^{2+}-ATPases.

5.1.4 The induction of acrosome reaction

Human follicular fluid (HFF) has been shown to induce the acrosome reaction *in vitro*, due to its physiological presence in the FRT (Siegel, Paulson and Graczykowski, 1990; Mariń -Briggiler et al., 2002; Burrello et al., 2004). These studies suggest previously capacitated sperm require a short incubation with HFF to induce the acrosome reaction. Progesterone is the active component in HFF and is assumed to be responsible for the induction of the acrosome reaction in human sperm due to its involvement in the induction of capacitation and hyperactivation (Foresta *et al.*, 1992; Uhler *et al.*, 1992). Progesterone is a steroid hormone which is secreted by cumulus cells at ovulation and has been shown to induce capacitation and hyperactivation, as

well as the acrosome reaction in human sperm at a variety of concentrations (Foresta et al., 1992; de Lamirande, Harakat and Gagnon, 1998; Gatica et al., 2013). Progesterone promotes the influx of calcium ions, via CatSper channels which is a necessary process during capacitation following the cholesterol efflux (De Jonge, 2005; Lishko, Botchkina and Kirichok, 2011).

It has recently been suggested that a combination of progesterone (P4), prostaglandin E1 (PGE1) and ammonium chloride (NH_4Cl) is most effective at inducing the acrosome reaction (Prajapati *et al.*, 2022). PGE1, in addition to P4, can stimulate CatSper channels leading to the calcium influx which is required for exocytosis (Aitken, Irvine and Kelly, 1986; Lishko, Botchkina and Kirichok, 2011; Mannowetz, Miller and Lishko, 2017). It has also been demonstrated that PGE1 is able to bind to human sperm membranes and induce a pertussis toxin-insensitive increase in intracellular calcium concentration (Schaefer et al., 1998). The role of NH_4Cl is to facilitate the increase the pH which is necessary to stimulate the CatSper channels and it has been used in previous studies for this reason (Marquez and Suarez, 2007; Prajapati *et al.*, 2022). As calcium is the trigger for exocytosis, calcium ionophore is also an inducer of the acrosome reaction, albeit non-physiological.

Table 5.1. The contents of the tubes prepared for the induction of the acrosome reaction. Each tube contained a final volume of 500 µl. All components added in µL. Final concentrations are: 8 mM $^{13}C_6$-glucose, 5 mg/mL HSA, 25 mM $NaHCO_3$ and 8 mM caffeine.

Added at the start of the incubation (T0), (µL)						
Tubes	**Sperm (50 M/mL)**	**EBSS**	**1/3 Pen/strep**	**100 mM $^{13}C_6$-glucose**	**50 mg/mL HSA**	**1 M $NaHCO_3$**
-VE	0	382.5	15	40	50	12.5
+VE	345	37.5	15	40	50	12.5
Inducer	345	37.5	15	40	50	12.5
Added at T4, (µL)						
Tubes	**DMSO**	**1 mM P4**	**500 µM PGE1**	**3 M NH_4Cl**		
-VE	0	5	5	5		
+VE	5	0	0	0		
Inducer	0	5	5	5		

5.1.5 The detection of acrosome reaction

A common method used to detect acrosome reacted sperm uses fluorescein labelled Pisum sativum Agglutin (PSA), which targets acrosomal vesicles (Cross *et al.*, 1986). After exocytosis, it has been shown that parts of the acrosomal contents remains associated with sperm, localised to the equatorial segment, for prolonged periods of time (Kim, Foster and Gerton, 2001). Sperm suspensions are incubated with PSA-FITC solution then fixed and viewed on an epifluorescence microscope. Different staining patterns have been reported to correlate to different stages of the acrosome reaction. However, the most accepted patterns are (Figure 5.3):

A. non-reacted if the acrosomal cap is fully labelled;
B. reacted if only the equatorial segment is labelled with PSA-FITC solution;
C. reacted if there is no fluorescence (Köhn *et al.*, 1997).

Detecting inner acrosomal membrane proteins using immunofluorescence, such as CD46 and IZUMO, are also thought to be useful indicators of the acrosome reaction and the technique is similar to that of FITC-PSA (Jaiswal, Eisenbach and Tur-Kaspa, 1999; Zeginiadou, Papadimas and Mantalenakis, 2000; Satouh *et al.*, 2012; Frolikova *et al.*, 2016).

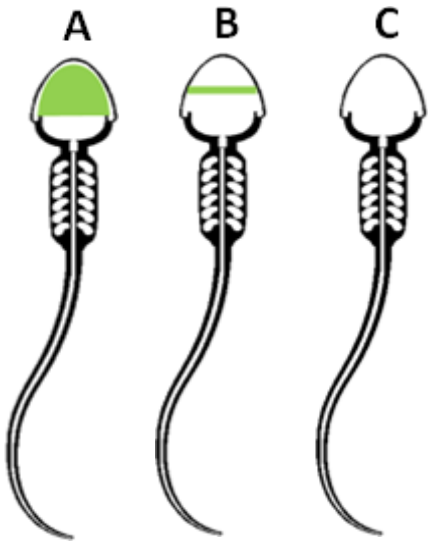


Figure 5.3: A cartoon to show the different staining patterns of the acrosome observed with FITC-PSA. (A) Fluorescence in the acrosome indicative of an intact acrosome, **(B)** Fluorescence in the equatorial segment and **(C)** Absence of fluorescence in the head indicative of acrosome reacted sperm.

5.2 Rationale

The aim of this chapter was to attempt to induce the acrosome reaction in human and boar sperm and then investigate the underlying metabolism during this process using ^{13}C-NMR. An increased demand for ATP is hypothesised to be required for the fusion of the outer acrosomal membrane with the sperm plasma membrane. To meet this increased demand for ATP, it was expected that an increase in glycolysis and/or oxidative phosphorylation would be observed.

5.2.1 Specific objectives

1. Induce the acrosome reaction in human and boar sperm using p4, PGE1 and NH_4Cl.
2. Detect the acrosome reaction in human and boar sperm using FITC-PSA fluorescence.
3. Investigate the effects of the acrosome reaction on motility and vitality of human and boar sperm.
4. Investigate the metabolic pathways utilised by human and boar sperm during the acrosome reaction.

5.3 Methods

5.3.1 Induction of the acrosome reaction

Capacitation was first induced as described in 3.3.1. Following on from this, the acrosome reaction was induced by adding 10 µM P4 in DMSO, 5 µM PGE1 in DMSO and 30 mM NH_4Cl to one of the tubes, and to the other tube, DMSO (solvent) was added as a control (+ve). Sperm were left to incubate for a further four-hours bringing the total to eight hours in total. Sperm motility (2.3.2), sperm vitality (2.3.3) and acrosomal status (5.3.2) were measured before and after incubation and the metabolic pathways utilised by sperm during the incubation were analysed using ^{13}C-NMR (2.3.5).

5.3.2 Measurement of the acrosome reaction - Fluorescein labelled Pisum sativum Agglutin

The method chosen to detect acrosome reacted sperm used fluorescein labelled Pisum sativum Agglutin (PSA), which targets acrosomal vesicles (Cross *et al.*, 1986).

Briefly, ~2x10^6/mL sperm in a volume of 10 µL, was added to a polysine slide, in duplicate, smeared, and left to air dry overnight at room temperature. The slides were fixed in methanol for 1 minute before storing at -20°C until analysis. Slides were warmed to room temperature and re-hydrated using 1ml TBS. Slides were then incubated with 0.5 µg/mL PSA-FITC solution for 2 hours at 4°C. Slides were rinsed with PBS twice and the back of the slide dried. Slides were mounted using prolong antifade mounting media and left overnight at 4°C in the dark. Slides were analysed on an Olympus IX73 at x600 magnification (UV filter 492 nm). For each incubate, 200 sperm were counted. Acrosomal status was recorded using the criteria stated in 5.1.5 and these are shown in Figures 5.3 and 5.4. In intermediate cases, where the equatorial line was visible as well as fluorescence in other regions of the head, these were classed as non-reacted (A).

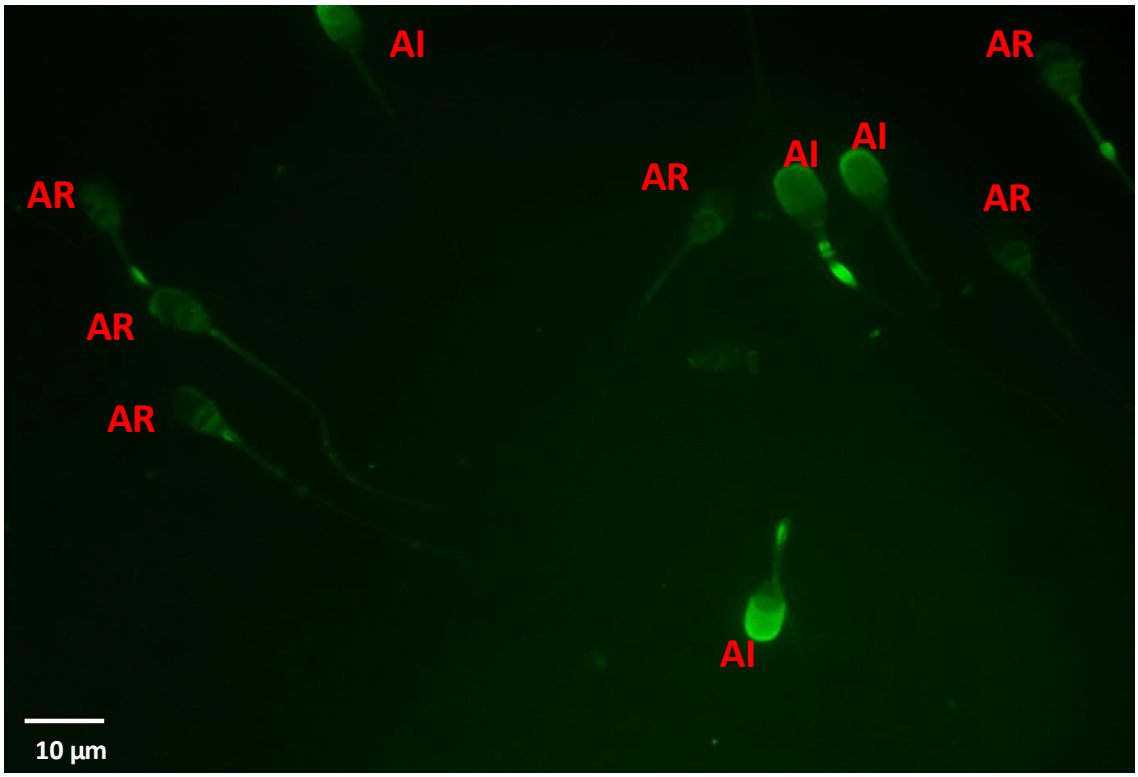


Figure 5.4: FITC-PSA fluorescence. FITC-PSA was used to determine the acrosomal status of human and boar sperm. FITC-PSA staining was carried out as outlined in 5.3.2. The figure shows the patterns of fluorescence sperm display after incubation with FITC-PSA; AR = acrosome reacted and AI = acrosome intact. Viewed at x600 magnification. Scale bar shown = 10 µM.

5.4 Results

5.4.1 Motility of human and boar sperm incubated with or without inducers of the acrosome reaction.

CASA was used before (T0) and after (T8 control and T8 P4 + PGE1 + NH_4Cl) incubations to observe whether P4, PGE1 and NH_4Cl in combination had any effect on human and boar sperm motility respectively. Figure 5.5 A shows that for human sperm, there was a significant increase in total motility after the eight-hour incubation without the addition of P4, PGE1 and NH_4Cl compared to time zero (T0, 37.2 ± 6.9%; T8 control, 49.3 ± 6.9%; P = 0.002). The same pattern was also observed for human sperm progressive motility, figure 5.5C, (T0, 14.9 ± 6.0%; T8 control, 31.1 ± 6.2%; P = 0.004). For boar sperm, there were no significant differences in total or progressive motility between the three groups: T0, T8 control and T8 P4 + PGE1 + NH_4Cl, figure 5.5B/5.5D.

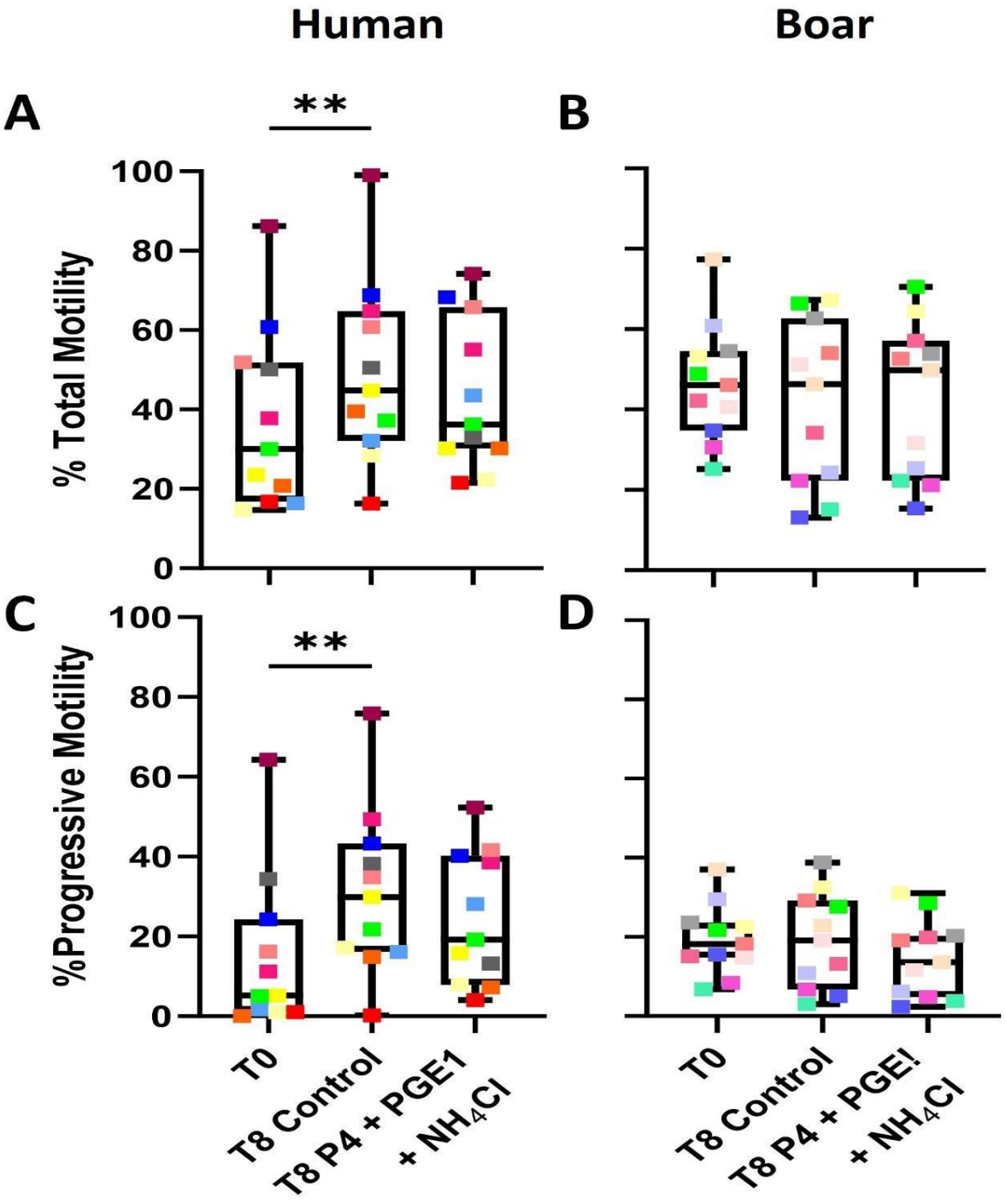


Figure 5.5: Human sperm total (A) and progressive (C) motility and boar sperm total (B) and progressive (D) motility (%) prior to (T0) and following (T8) an eight-hour incubation with or without 10 µM P4, 5 µM PGE1 and 30 mM NH_4Cl. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a RM one-way ANOVA with a Tukey's multiple comparisons test for all except human sperm progressive motility where a Friedman test with a Dunn's multiple comparisons test was used (C). ** = P <0.01, n = 11. Points colour coded per individual donor/boar.

5.4.2 Vitality of human and boar sperm incubated with or without inducers of the acrosome reaction.

To observe whether an incubation with 10 µM P4, 5 µM PGE1 and 30 mM NH_4Cl in combination influenced the vitality of human and boar sperm, the LIVE/DEAD™ sperm viability kit was used. Figure 5.6A shows that for human sperm, a significant decrease in vitality was observed when sperm were incubated for eight hours with the addition of acrosome reaction inducers compared to an incubation in the absence of acrosome reaction inducers (T8 control, 57.6 ± 4.1%; T8 P4 + PGE1 + NH_2Cl, 51.1 ± 3.6%; P = 0.02). For boar sperm, figure 5.6B shows the percentage of live human sperm significantly decreased after an eight-hour incubation both with and without the addition of inducers of the acrosome reaction, compared to time zero (T0, 51.7 ± 3.4%; T8 control, 28.5 ± 2.3%; P < 0.0001; T8 P4 + PGE1 + NH_2Cl, 25.9 ± 2.2%; P < 0.0001).

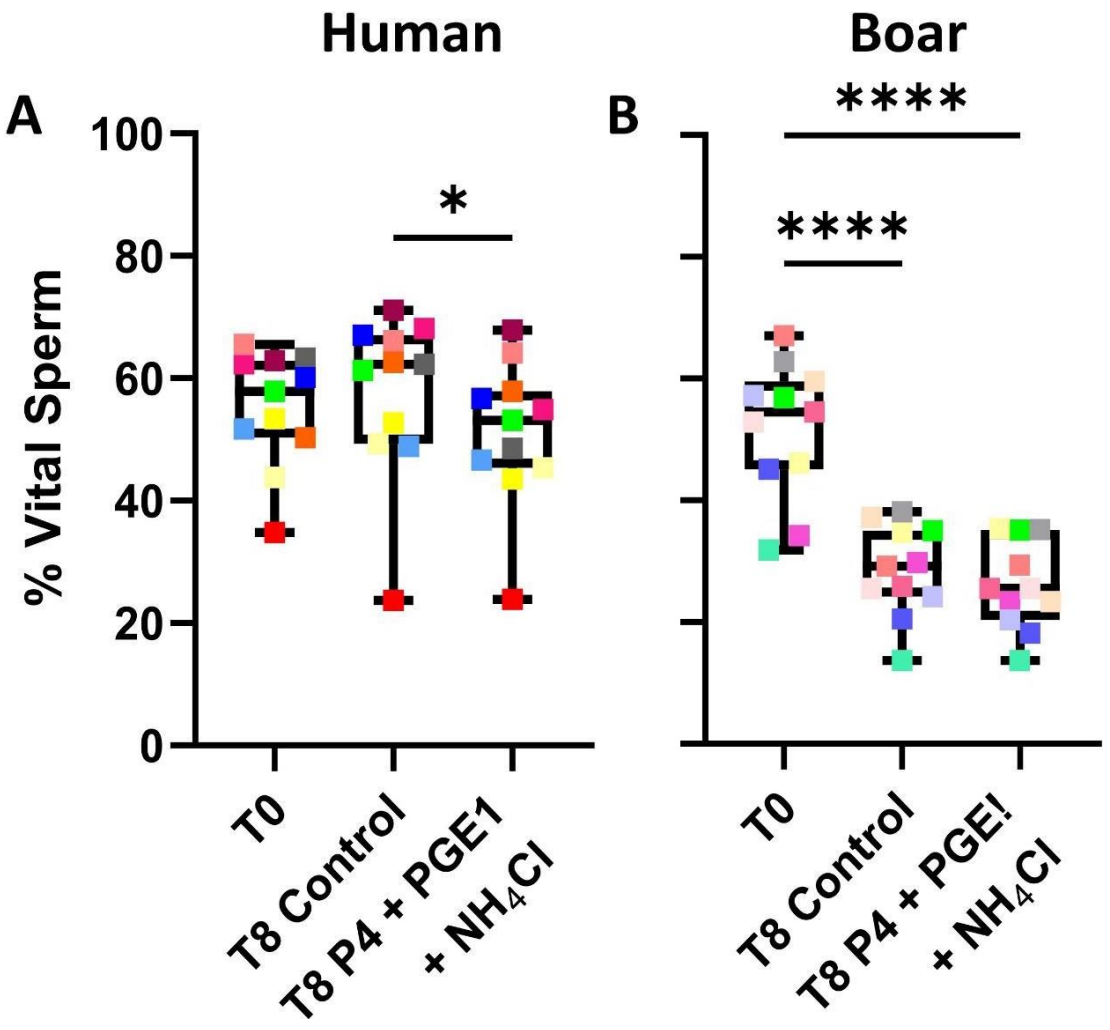


Figure 5.6: Human (A) and Boar (B) sperm vitality (%) prior to (T0) and following (T8) an eight-hour incubation with or without 10 µM P4, 5 µM PGE1 and 30 mM NH_4Cl. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a Friedman test with a Dunn's multiple comparisons test for human sperm and a RM one-way ANOVA with a Tukey's multiple comparisons test for boar sperm. * = P < 0.05 and **** = P < 0.0001, n = 11. Points colour coded per individual donor/boar.

5.4.3 FITC-PSA immunofluorescence of human and boar sperm incubated with or without inducers of the acrosome reaction.

To determine the percentage of acrosome reacted human and boar sperm, fluorescent staining was used: FITC-PSA. For human sperm, as shown in figure 5.7A, after a four-hour capacitation incubation followed by a four-hour incubation with P4, PGE1 and NH_4Cl, the percentage of acrosome reacted sperm increased compared to time zero but did not in the absence of inducers (T0, 6.0 ± 1.4%; T8 P4 + PGE1 +NH_4Cl, 9.7 ± 1.1%; P = 0.017; T8 control, 6.3 ± 1.2%). After eight-hours, the percentage of acrosome reacted sperm for those incubated without inducers was also significantly different from those that had been incubated with inducers, P = 0.009. Figure 5.7B shows that for boar sperm, there was a significant increase in the percentage of acrosome reacted sperm after an eight-hour incubation, with and without the addition of inducers of the acrosome reaction, compared to time zero (T0, 13.4 ± 1.6%; T8 control, 38.0 ± 3.9%; P = 0.0005; T8 P4 + PGE1 + NH_2Cl; 44.3 ± 3.0%; P < 0.0001). A significant increase was also seen when boar sperm were incubated with inducers of the acrosome reaction compared to without, P = 0.007.

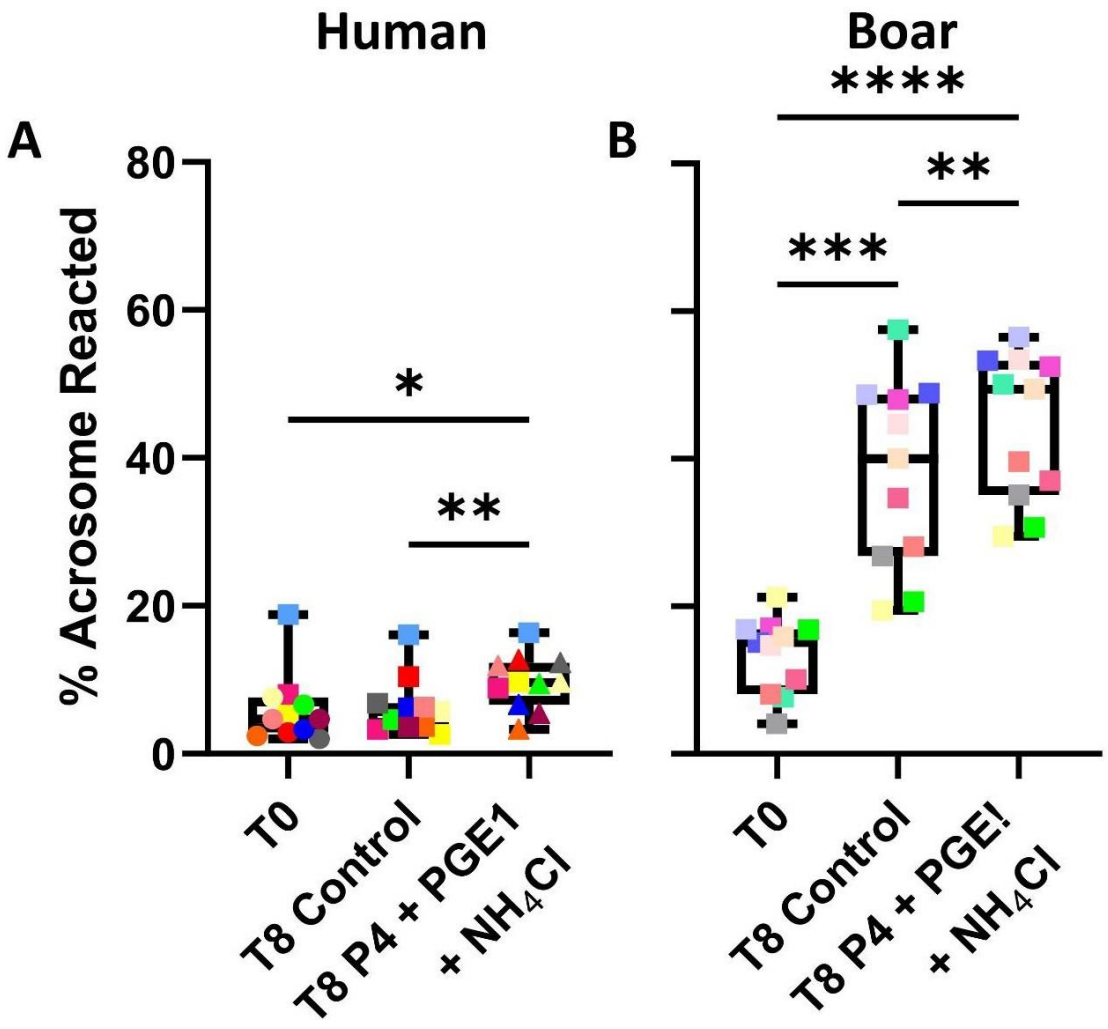


Figure 5.7: The percentage of acrosome reacted human (A) and boar (B) sperm prior to (T0) and following (T8) an eight-hour incubation with or without 10 µM P4, 5 µM PGE1 and 30 mM NH_4Cl. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a Friedman test with a Dunn's multiple comparisons test for human sperm and a RM one-way ANOVA with a Tukey's multiple comparisons test for boar sperm. * = P <0.05, ** = P <0.01, *** = P <0.001 and **** = P <0.0001, n = 11. Points colour coded per individual donor/boar.

5.4.4 NMR

^{13}C-NMR was used to investigate whether human (n=11) and boar (n=11) sperm, incubated with P4, PGE1 and NH_4Cl in combination to induce the acrosome reaction, showed differences in metabolism compared to those incubated without the inducers. The production of lactate is indicative of glycolysis and the production of bicarbonate is indicative of oxidative phosphorylation. The lactate and bicarbonate integral values were normalised by sperm total, vital and motile concentration, see section 2.3.6. No lactate peaks were recorded for the negative control samples suggesting no metabolism occurs in the absence of sperm (appendix A7).

5.4.4.1 Lactate production by human and boar sperm incubated with or without inducers of the acrosome reaction.

As seen in figure 5.8A For human sperm, after 8 hours, there was a significant increase in lactate production, normalised by total human sperm concentration, when human sperm were incubated with P4, PGE1 and NH_4Cl compared to those incubated in the absence of P4, PGE1 and NH_4Cl (T8 control, 5.7 ± 0.7 x 10^6 A.U.; T8 P4 + PGE1 + NH_4Cl, 6.7 ± 0.8 x 10^6 A.U.; P = 0.0003). The same was observed when normalised by vital human sperm concentration (T8 control, 9.8 ± 0.8 x 10^6 A.U.; T8 P4 + PGE1 + NH_4Cl, 1.2 ± 0.1 x 10^7 A.U.; P < 0.0001), and when normalised by motile human sperm concentration (T8 control, 1.2 ± 0.1 x 10^7 A.U.; T8 P4 + PGE1 + NH_4Cl, 1.6 ± 0.2 x 10^7 A.U.; P = 0.004).

Figure 5.8B shows that for boar sperm, after 8 hours, there was a significant increase in lactate production, normalised by total boar sperm concentration, when incubated with P4, PGE1 and NH_4Cl compared to those incubated in the absence of P4, PGE1 and NH_4Cl (T8 control, 2.7 ± 0.5 x 10^6 A.U.; T8 P4 + PGE1 + NH_4Cl, 3.2 ± 0.5 x 10^6 A.U.; P = 0.0006). The same was true when normalised by vital human sperm concentration (T8 control, 6.5 ± 0.9 x 10^6 A.U.; T8 P4 + PGE1 + NH_4Cl, 8.0 ± 0.9 x 10^6 A.U.; P = 0.003), and when normalised by motile human sperm concentration (T8 control, 6.7 ± 0.5 x 10^6 A.U.; T8 P4 + PGE1 + NH_4Cl, 8.0 ± 0.8 x 10^6 A.U.; P = 0.01).

5.4.4.2 Bicarbonate production

For human sperm, after 8 hours, there was a significant decrease in bicarbonate production, normalised by total human sperm concentration, when incubated in the presence of acrosome reaction inducers compared to those incubated in the absence of inducers (T8 control, 1.7 ± 0.3 x 10^5 A.U.; T8 P4 + PGE1 + NH_4Cl, 8.9 ± 3.3 x 10^4 A.U.; P = 0.014), figure 5.9A. The same was observed when normalised by vital human sperm concentration (T8 control, 3.1 ± 0.5 x 10^5 A.U.; T8 P4 + PGE1 + NH_4Cl, 1.6 ± 0.6 x 10^5 A.U.; P = 0.032).

The same pattern was observed for boar sperm, see figure 5.9B, when normalised by total boar sperm concentration (T8 control, 8.1 ± 2.5 x 10^5 A.U.; T8 P4 + PGE1 + NH_4Cl, 3.8 ± 0.4 x 10^5 A.U.; P = 0.002), vital boar sperm concentration (T8 control, 2.2 ± 0.8 x 10^6 A.U.; T8 P4 + PGE1 + NH_4Cl, 9.6 ± 0.9 x 10^5 A.U.; P = 0.003) and motile boar sperm concentration (T8 control, 3.6 ± 2.1 x 10^6 A.U.; T8 P4 + PGE1 + NH_4Cl, 1.1 ± 0.2 x 10^6 A.U.; P = 0.005).

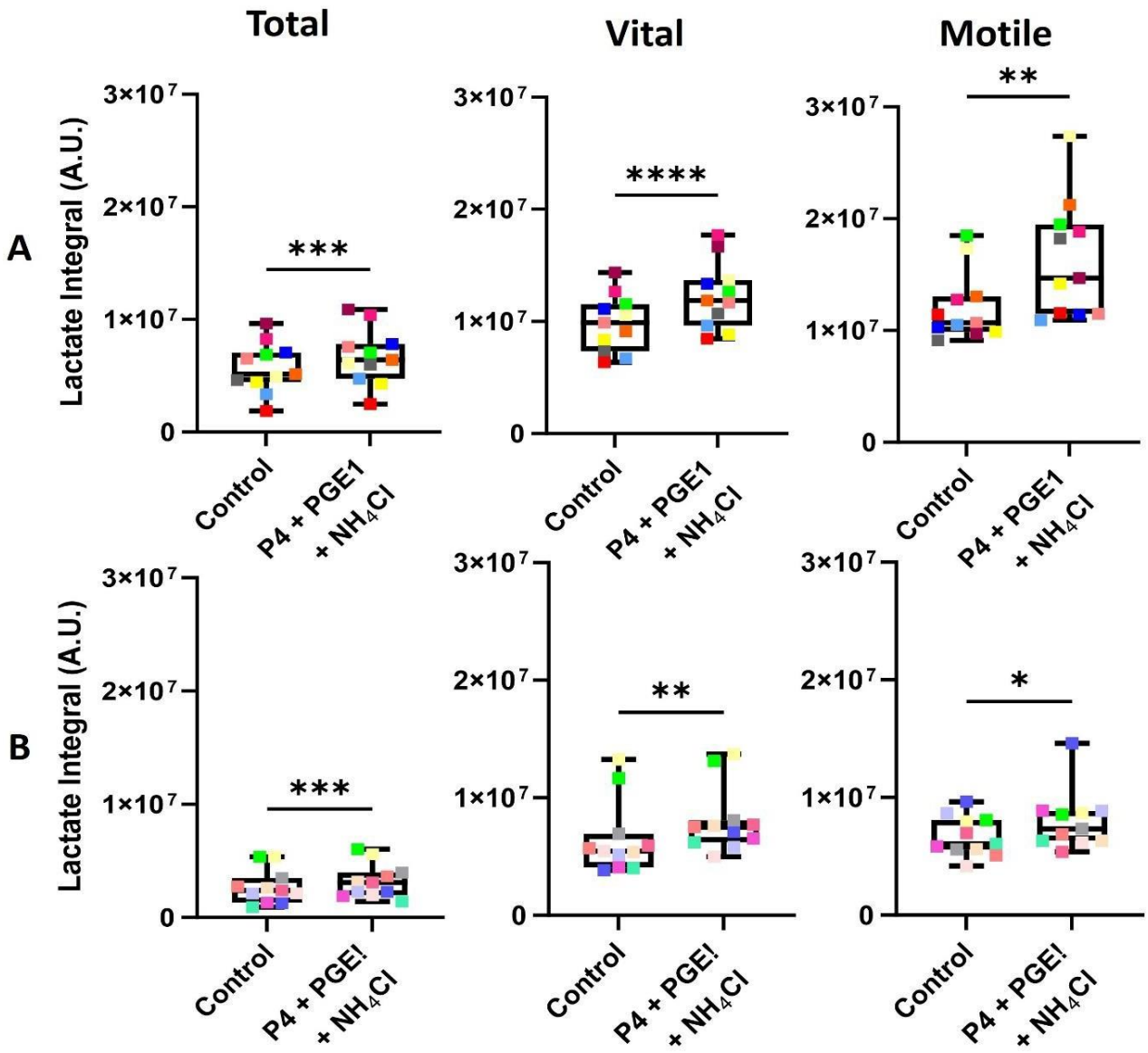


Figure 5.8: Lactate production by human (A) and boar (B) sperm incubated with or without P4, PGE1 and NH_4Cl in combination. Lactate integrals are normalised by total, vital and motile human and boar sperm concentrations. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a paired t test for all except boar sperm lactate integrals normalised by vital and motile concentration where a Wilcoxon test was used. * = P <0.05, ** = P <0.01, *** = P <0.001 and **** = P <0.0001, n = 11. Points colour coded per individual donor/boar.

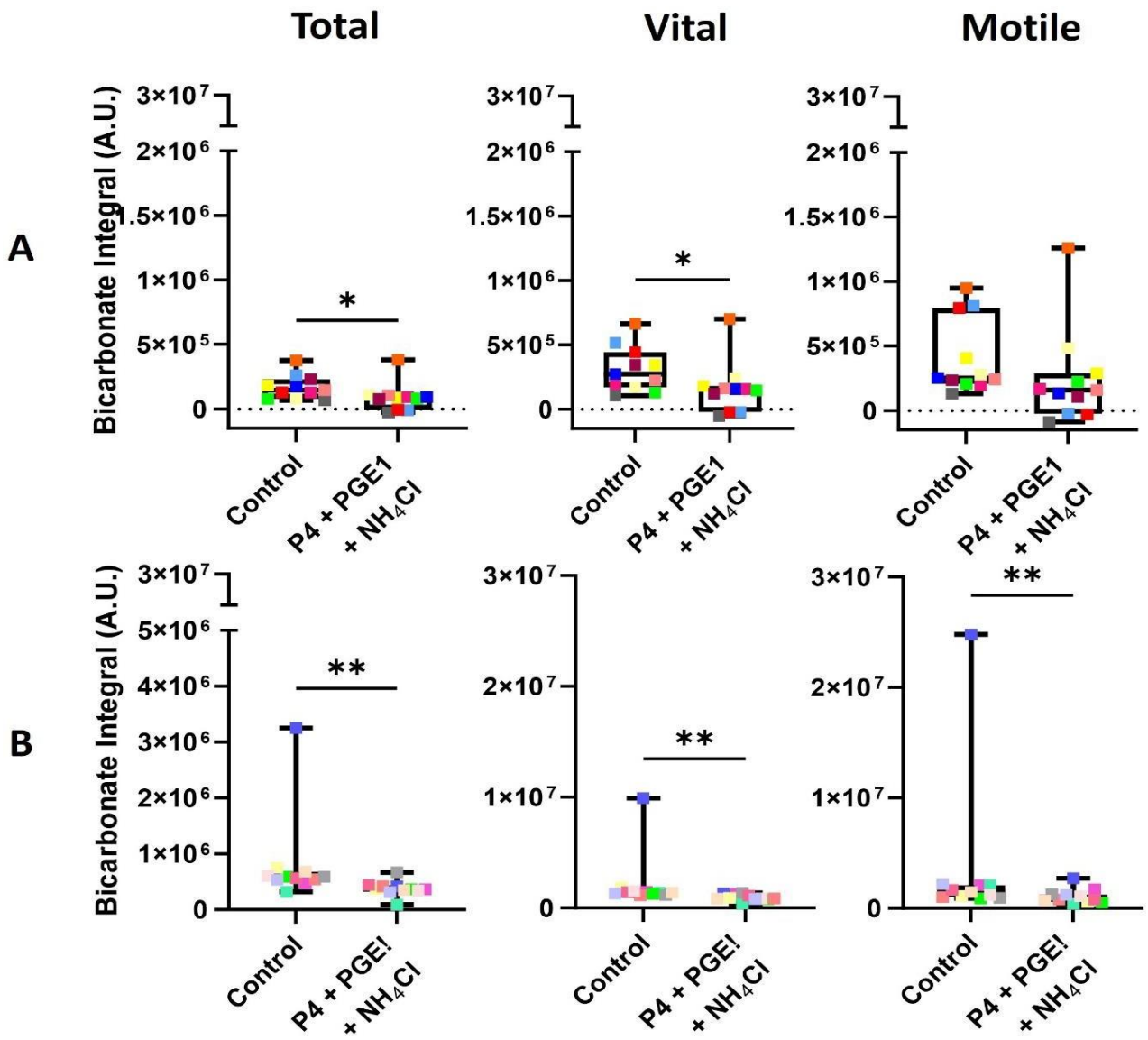


Figure 5.9: Bicarbonate production by human (A) and boar (B) sperm incubated with or without P4, PGE1 and NH_4Cl in combination. Bicarbonate integrals are normalised by total, vital and motile human and boar sperm concentrations. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a Wilcoxon test for all datasets. * = P <0.05 and ** = P <0.01, n = 11. Points colour coded per individual donor/boar.

5.5 Discussion

The acrosome reaction involves the exocytosis of hydrolytic enzymes, contained within the acrosome, that are responsible for the digestion of the oocyte prior to fertilisation. The release of these enzymes is stimulated by an influx of calcium, via CatSper channels (Vilmart-Seuwen *et al.*, 1986; Breitbart, 2002; Theander, Lew and Nüsse, 2002). These CatSper channels are activated by elevation of intracellular pH and extracellular progesterone and do not require ATP (Lishko, Botchkina and Kirichok, 2011). Despite this, an increased ATP demand has been observed during the acrosome reaction (Foresta *et al.*, 1996; Luria *et al.*, 2002; Torres-Fuentes, Rios and Moreno, 2015). This ATP is suggested to be required for the function of NSF as well as for the regulation of protein kinases involved in the acrosome reaction (Söllner *et al.*, 1993; Whiteheart *et al.*, 1994; Morimoto and Ogihara, 1996; Harrison, Carr and Meizel, 2000). However, the source of this ATP is unclear and there has been little research investigating this. This chapter has used ^{13}C-NMR to determine whether glycolysis, oxidative phosphorylation or both are responsible for the increase in ATP required during the acrosome reaction.

A combination of P4, PGE1 and NH_4Cl was used to induce the acrosome reaction in human and boar sperm based upon previous research (Prajapati *et al.*, 2022). The total and progressive motility of sperm was measured using CASA and the vitality of sperm was investigated to observe whether these inducers had any detrimental effects on sperm physiology. No significant differences in total or progressive motility were observed when both human and boar sperm were treated with inducers of the acrosome reaction suggesting these inducers do not impair sperm motility. This was expected as P4 and PGE1 are found within the FRT and previous studies have observed no detrimental effects of these inducers when used at similar concentrations (Foresta et al., 1992; de Lamirande, Harakat and Gagnon, 1998; Gatica et al., 2013; Okabe, 2016; Simons and Fauci, 2018).

In terms of sperm vitality, a significant decrease was observed for boar sperm incubated both with and without inducers of the acrosome reaction compared to before incubation. A similar observation was made for boar sperm treated with and without caffeine, and as previously discussed in chapter 4, this is likely due to boar sperm being vulnerable to the external environment due to the composition of their plasma membrane and the lack of antioxidants used to culture sperm (Awda, Mackenzie-Bell and Buhr, 2009; Achikanu et al., 2018; Jawad et al., 2023). For human sperm, the significant decrease in vitality was observed when sperm were incubated with inducers of the acrosome reaction compared to those without, approximately by 6.5%. The percentage of acrosome reacted sperm increased by roughly 3.5% for sperm incubated with inducers and it has previously been suggested that the increase in intracellular calcium observed during the acrosome reaction may trigger apoptosis (Engel, Springsguth and Grunewald, 2018). Calcium is known to stimulate caspase 9 in the apoptosis cascade, which is discussed further in chapter 6 (Hengartner, 2000). However, based on the data in this thesis, it remains unknown whether the decrease in vitality observed for human sperm incubated with inducers of the acrosome reaction, included sperm that had acrosome reacted, as the vitality assay does not provide data regarding the acrosomal status of sperm. It also remains unknown the proportion of acrosome reacted sperm at T8 that were alive vs dead, as no viability dyes were used alongside the fluorescent staining of the acrosome. Therefore, further work would be required to investigate a potential correlation between the decrease in vitality and increase in acrosome reacted sperm seen in this thesis when human sperm were incubated with inducers of the acrosome reaction.

P4, PGE1 and NH_4Cl used in combination were successful at inducing the acrosome reaction in both human and boar sperm, supporting previous literature (Prajapati *et al.*, 2022). Interestingly, for boar sperm, around 44% acrosome reacted over the eight-hour incubation in comparison to less than a quarter of this for human sperm, ~10%. This could be explained by the plasma membrane composition of boar sperm making them more vulnerable to ROS compared to other species, including human. ROS has been shown to stimulate a premature acrosome reaction in boar sperm via membrane lipid peroxidation (Awda, Mackenzie-Bell and Buhr, 2009). Whilst ROS have been shown to be important for the acrosome reaction, due to their role in

regulating the protein kinases and the modulation of CatSper channels, excessive ROS have detrimental effects on sperm function, including the induction of a premature acrosome reaction (Brown et al., 1989; Tafuri et al., 2015; Dutta et al., 2020).

To investigate whether there was an increase in glycolysis or oxidative phosphorylation during human and boar sperm acrosome reaction, NMR data was analysed. The increase in lactate production, normalised by total, vital and motile concentration, seen when human and boar sperm were incubated with inducers of the acrosome reaction compared to without, suggests that both human and boar sperm rely on glycolysis to produce the ATP required for the acrosome reaction. Interestingly, the increase in lactate production was seen despite the decrease in vitality seen for human and boar sperm incubated with inducers of the acrosome reaction. This, along with the fact no differences in motility between the two groups were observed, suggests that the increase in lactate, representative of glycolysis, is to fulfil the ATP requirements of the acrosome reaction and not to maintain or increase cell viability and motility respectively.

The significant decrease in bicarbonate production identified for human and boar sperm incubated with P4, PGE1 and NH_4Cl, suggests there is no reliance on oxidative phosphorylation to generate ATP during the acrosome reaction. This is the same pattern that was identified for capacitated human sperm in chapter 3. It must be noted that one sample had a very large bicarbonate response in the control group. As bicarbonate was added in small quantities, 12.5 µL, as an inducer of capacitation, it is likely that pipetting or human error occurred in this case. However, when this value was removed for statistical analysis, data not shown, the significant decrease in bicarbonate was still observed.

This is in contrast to a study that measured oxygen consumption in boar sperm during the induction of the acrosome reaction (Ramió-Lluch et al., 2011). They found that following the addition of progesterone in pre-capacitated sperm, a rapid and significant increase in oxygen consumption was observed after five minutes of incubation, suggesting an increase in oxidative phosphorylation may be required for the initiation of the acrosome reaction. Following on from this, a time dependant decrease in oxygen consumption was then observed over a further 60 minutes, with oxygen consumption decreasing to below pre-induction levels. However, this study did not measure glycolytic activity hence it is unknown whether glycolysis would have also increased at a similar, or perhaps more rapid rate. The authors also acknowledged that the oxygen consumption levels observed in their study were very low in comparison to other cell types and suggest that glycolysis is therefore most likely to generate the ATP required for capacitation and acrosome reaction in boar sperm (Ramió-Lluch et al., 2011). Nonetheless, the study observed an increase in oxidative phosphorylation whereas this thesis observed a decrease in oxidative phosphorylation when boar sperm were treated with inducers of the acrosome reaction. It must be noted different methods were used to measure oxidative phosphorylation, and this could explain the differences observed. The method in this thesis used a time average measurement of the accumulation of bicarbonate over an eight-hour period, compared to the real time oxygen consumption measurement used in the above-mentioned study (Ramió-Lluch et al., 2011). Therefore, an increase in oxidative phosphorylation may have occurred, following the addition of acrosome reaction inducers after five minutes, in the experiments presented in this thesis, however, this would not be seen when an average of the bicarbonate accumulation over an eight-hour incubation is presented.

This increase in lactate production alongside a decrease in bicarbonate production, observed for both human and boar sperm during the acrosome reaction, could potentially be due to sperm requiring an increased rate of ATP production for completion of the acrosome reaction. As the acrosome reaction must take place at a specific time for fertilisation to occur, and as all the sperm that reached the ampulla of the fallopian tubes will be competing to fertilise the egg, a faster rate of ATP production to meet the requirements of the acrosome reaction would seem practical (Breitbart and Spungin, 1997; Gómez-Torres *et al.*, 2015). Glycolysis allows for a faster rate of ATP production albeit has a lower yield (Pfeiffer, Schuster and Bonhoeffer, 2001; Shestov *et al.*, 2014). In addition, as discussed in chapter 3 and 4, local ATP generation to the site of ATP is

seen in sperm (Hereng *et al.*, 2011). As the acrosome reaction related changes occur in the sperm head, this would explain the observation of an increased lactate production alongside a decreased bicarbonate production, as oxidative phosphorylation occurs at a distal site, suggesting glycolysis in the sperm head is the main ATP-producing pathway utilised during this process.

In conclusion, 10 µM P4, 5 µM PGE1 and 30 mM NH_4Cl can induce the acrosome reaction in human and boar sperm. The reliance on glycolysis as opposed to oxidative phosphorylation during the acrosome reaction in human sperm, as shown by the data in this chapter, suggests that local glycolytic ATP generation in the sperm head supports the numerous ATP requiring events of the acrosome reaction. A reason glycolysis could be preferred by sperm during the acrosome reaction could be because sperm require a faster rate of ATP production, via glycolysis, to undergo the acrosome reaction and successfully fertilise the oocyte. In the next chapter, apoptosis of sperm in the FRT, presumed to occur in sperm that have not successfully fertilised the oocyte, is discussed, and the metabolic pathways utilised during sperm apoptosis are investigated.

Chapter 6: The induction and measurement of apoptosis and the underlying metabolic pathways utilised in this process.

6.1 Introduction

6.1.1 Discovery of apoptosis

The process of programmed cell death was first described in 1842 by Carl Vogt, however, it wasn't until the twentieth century that the importance of apoptosis was recognised and since then it has become a focus of research amongst the scientific community (Glucksmann, 1951; Clarke and Clarke, 1996; Elmore, 2007; Singh and Lim, 2022). Apoptosis in mature sperm, including in human, wasn't described until the end of the twentieth century (Stephan *et al.*, 1996; Maione *et al.*, 1997). Originally, although apoptosis was described for sperm cell death during spermatogenesis (Miething, 1992; Bartke, 1995), the ability of mature sperm to undergo apoptosis was controversial with studies suggesting that mature sperm were unable to undergo apoptosis due to their transcriptionally inactive nucleus, and that apoptosis observed in sperm may be a side effect of incomplete spermatogenesis (Weil, Jacobson and Raff, 1998; Sakkas *et al.*, 1999). However, sperm have since been shown to possess the proteins involved in the apoptotic pathway, such as caspase 3, and the functionality of the apoptotic signalling pathways in human sperm has been demonstrated and widely accepted by the scientific community (Grunewald, Paasch and Glander, 2001; Weng *et al.*, 2002; Wündrich *et al.*, 2006; Doğan *et al.*, 2012).

6.1.2 Function of apoptosis

A primary function of apoptosis is the programmed death of cells that are no longer needed or are damaged and pose a threat to the organism (Singh, Letai and Sarosiek, 2019). Apoptosis has been implicated to have a role in embryogenesis, preventing cancer, immune system regulation and maintenance of tissue homeostasis (Norbury and Hickson, 2001; Elmore, 2007). In mature sperm, it is proposed the function of apoptosis, and subsequent phagocytosis in the FRT, is to prevent an inflammatory response by the FRT, which would negatively impact fertilisation and subsequent embryo development (Nakidkina and Kuzmina, 2019).

6.1.3 Features of apoptosis

Cell death has been shown to occur by two major mechanisms, necrosis, and apoptosis. Classical necrotic cell death occurs due to noxious injury or trauma while apoptosis takes place during normal cell development, regulating cellular differentiation and number (Kerr, Wyllie and Currie, 1972; Elmore, 2007). While necrotic cell death results in cell lysis, cellular apoptosis is characterized morphologically by cell shrinkage, nuclear pyknosis, chromatin condensation, and blebbing of the plasma membrane (Renehan, Booth and Potten, 2001). During later stages of apoptosis, apoptotic bodies are formed from cell fragments, and these are engulfed by phagocytes to prevent the release of their contents and subsequent inflammation (Elmore, 2007). Causes of apoptosis include oxidative stress amongst other factors, and the mitochondria is the key organelle involved in apoptosis (Wolvetang *et al.*, 1994).

Apoptosis can occur via two pathways, the intrinsic and extrinsic pathway, and involves the activation of caspases, a group of cysteine proteins, see Figure 6.1 (Sahoo *et al.*, 2023). The intrinsic pathway is triggered by stress and damage to the cell and is regulated by the protein family B cell lymphoma 2 (Bcl-2). Briefly, during this pathway, cytochrome C is released from the mitochondria and forms apoptosomes which activate caspase 9 (Chinnaiyan, 1999). The extrinsic pathway is triggered by the binding of extracellular ligands to the death receptors on the cell surface. This pathway is regulated by caspase 8 and relies upon the formation of the death inducing signalling complex (DISC) (Xu and Shi, 2007). Once caspase 8 or 9 have been activated in either pathway, they cleave and activate effector caspases downstream, caspase 3, 6 and 7, which ultimately lead to cell death via a cascade of molecular and biochemical events including activation of an endogenous endonuclease that cleaves DNA into oligonucleosomes (Sahoo *et al.*, 2023). It has been suggested that sperm undergo the intrinsic pathway of apoptosis and the major cause of apoptosis in sperm is oxidative stress, as

sperm are especially vulnerable to ROS (Sakkas, Mariethoz and St. John, 1999; Ricci *et al.*, 2002; Aitken and Koppers, 2011; Nakidkina and Kuzmina, 2019).

6.1.4 ATP demands of apoptosis

Initially it remained unknown whether the overall decrease in ATP concentration, characteristic of cell death, was a cause or consequence of cell death (Richter et al., 1996). Intracellular ATP levels are thought to be a determinant in cell death by apoptosis or necrosis, with a baseline level of ATP appearing to be required for the events of apoptosis (Eguchi, Shimizu and Tsujimoto, 1997). This study demonstrated that when levels of ATP decline, necrosis occurs in place of apoptosis, as necrosis does not require ATP. This is supported by a further study that showed T cells switched between apoptosis to necrosis when emptied of ATP (Nicotera, Leist and Ferrando-May, 1998). When T cells were predepleted of ATP, no DNA fragmentation or nuclear condensation was identified when treated with an inducer of apoptosis. The opposite was observed in cells that were not predepleted of ATP. The authors also attempted to define the ATP threshold required for apoptosis by clamping the ATP concentration of oligomycin treated cells by altering glucose concentration. Cells were then treated with staurosporine (STS) and those with a higher ATP concentration favoured apoptosis over necrosis. The authors also confirmed that the ATP produced by glycolysis was sufficient to drive cells down the apoptotic route as opposed to necrotic, by using the glycolytic substrates glucose and fructose (Nicotera, Leist and Ferrando-May, 1998).

A further study seemed to confirm the above by investigating intracellular ATP levels and whether they influence hypoxic cardiac myocyte death i.e. by apoptosis or necrosis. The authors state that in ischemic myocardium, it is typically seen that cell apoptosis appears early followed later by necrosis, supporting the idea that a minimum level of ATP is needed for apoptosis (Tatsumi *et al.*, 2003). This study used rat myocytes and when they deprived these of glucose, they observed necrosis. When the concentration of glucose was increased, ATP increased along with levels of apoptosis. A lack of effect of oligomycin during apoptosis also suggests that the level of intracellular ATP required for apoptosis is derived from glycolysis. Previously, a study using rat pheochromocytoma and rat hepatoma cell lines treated with respiratory chain inhibitors, concluded that cells which depend on oxidative phosphorylation to produce ATP die by necrosis whereas cells which rely on glycolysis over oxidative phosphorylation die by apoptosis (Shimizu *et al.*, 1996).

Based up on these studies it can be argued that when there is a decline in ATP concentration, apoptosis occurs only if there is enough ATP to fulfil the energy requirements of apoptosis. This seems to imply that ATP is required during apoptosis and is derived from glycolysis, as the mitochondrial membrane potential is known to decline during apoptosis, therefore compromising its ability to generate ATP (Zorova *et al.*, 2018). As sperm have been suggested to prefer glycolysis in this thesis and in previous studies, it could be surmised they would preferentially undergo apoptosis over necrosis (Peterson and Freund, 1974; Ford and Harrison, 1981; Lornage, Guerin and Czyba, 1986; Williams and Ford, 2001). During apoptosis, this ATP is thought to be required for the formation of the apoptosome, which occurs as part of the intrinsic pathway, Figure 6.1 (Eguchi *et al.*, 1999). Active nuclear transport has also shown to be required during apoptosis and this process has been shown to require ATP hydrolysis (Melchior and Gerace, 1995; Yasuhara *et al.*, 1997). Previous research has also proposed that the function of hydrolytic enzymes, vesicle formation and chromatin condensation during apoptosis would likely require ATP (Richter et al., 1996; Eguchi, Shimizu and Tsujimoto, 1997).

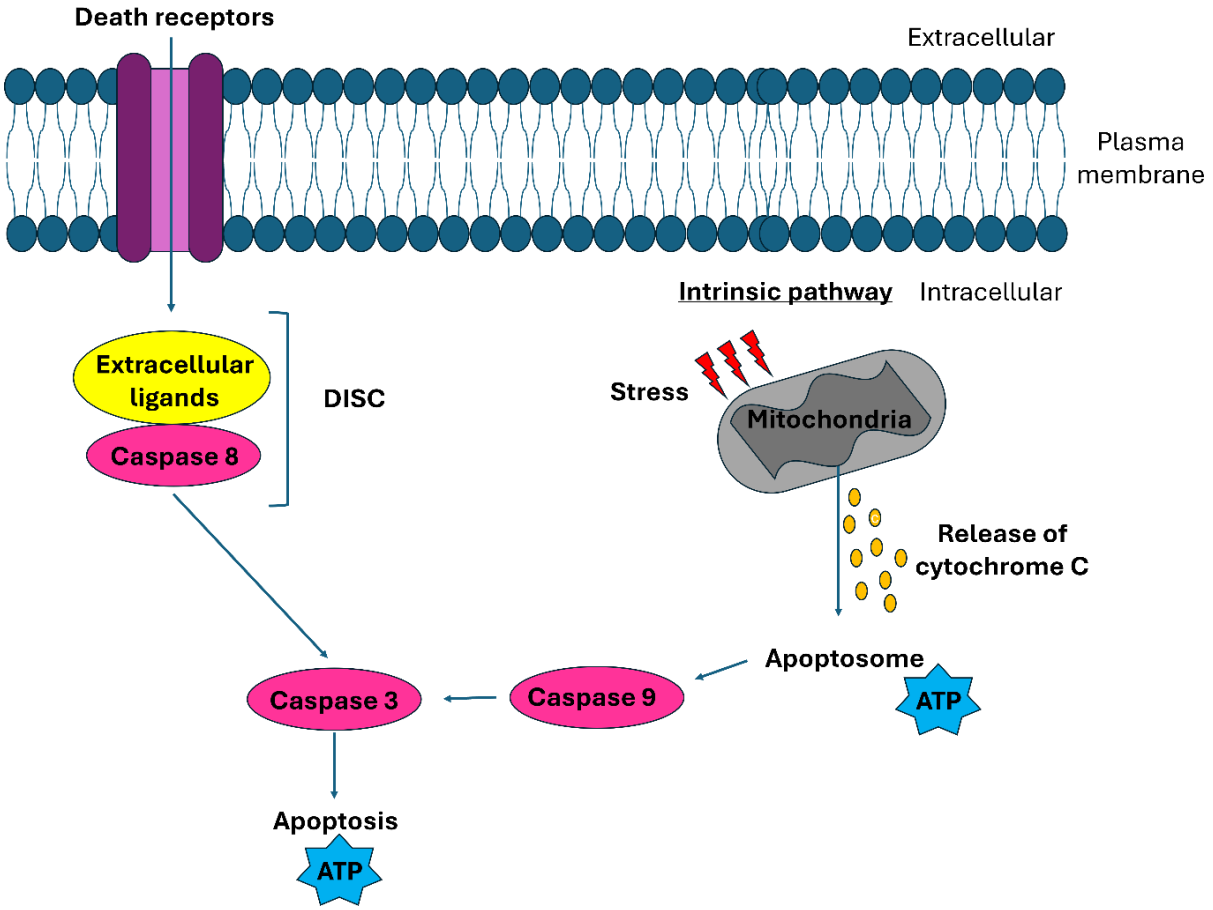


Figure 6.1 The intrinsic and extrinsic apoptotic pathways. The intrinsic pathway is triggered by stress to the cell. This causes the release of cytochrome C from the mitochondria and the formation of the apoptosome. The apoptosome activates caspase 9, which goes on to activate caspase 3, leading to cell death. The extrinsic pathway is triggered by the binding of extracellular ligands to the death receptors on the cell surface. Along with caspase 8, these form the death inducing signalling complex (DISC), which activates caspase 3, leading to cell death.

6.1.5 The induction of apoptosis

Staurosporine (STS) has been shown to induce apoptosis in a wide range of cell types (Belmokhtar, Hillion and Ségal-Bendirdjian, 2001; Feng and Kaplowitz, 2002; Šimenc and Lipnik-Štangelj, 2012). Crucially, STS has been suggested to be an effective inducer of apoptosis in sperm (Taylor *et al.*, 2004; Eley *et al.*, 2005). STS is a natural product isolated from the bacterium Streptomyces staurosporeus (Omura *et al.*, 1977). It is an ATP-competitive kinase inhibitor therefore inhibits protein kinase activity preventing many aspects of cell signalling (Karaman *et al.*, 2008). The mechanism by which STS mediates apoptosis is not fully understood, however it has been suggested that it can activate caspase 3 of the apoptosis cascade (Chae *et al.*, 2000). Other inducers of apoptosis include various small molecule drug treatments such as 2- deoxyglucose and thapsigargin, ultraviolet B irradiation and death receptor ligation (Roberts and Rosen, 2004).

6.1.6 The detection of apoptosis

Apoptosis has been suggested to be the main cause of DNA fragmentation in sperm therefore DNA fragmentation was taken as a marker of apoptosis in this chapter (Nakidkina and Kuzmina, 2019). There are four commonly used assays that quantify DNA damage; sperm chromatin structure assay (SCSA), sperm chromatin dispersion (SCD), the Comet assay and Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL) (Dada, 2017). These are direct and indirect assays which quantify different aspects of DNA damage, and the advantages and disadvantages of each assay have been previously discussed (Agarwal *et al.*, 2016). It remains disputed which assay has the highest diagnostic value hence there is lack of

standardization of these techniques. DNA fragmentation can be either single or double strand breaks and the TUNEL and comet assays assess both types of breaks. The TUNEL assay, was utilised in this chapter in this chapter due to previous studies reporting it as a reliable and reproducible method for the identification of DNA fragmentation in sperm (Sharma *et al.*, 2010; Ribeiro *et al.*, 2017). The TUNEL assay is also outlined in the sixth edition of the WHO manual (World Health Organization, 2021).

6.2 Rationale

An aim of this research was to induce apoptosis in human and boar sperm and then investigate the underlying metabolism during apoptosis using ^{13}C-NMR. Based on previous literature, is anticipated that ATP derived from glycolysis is required for apoptosis, to facilitate the various processes characteristic of apoptosis. Therefore, the hypothesis of this research is that during apoptosis, an increase in glycolysis alongside a simultaneous decrease in oxidative phosphorylation will be observed.

6.2.1 Specific objectives

1. Induce apoptosis in human and boar sperm using STS.
2. Detect apoptotic human and boar sperm using the TUNEL assay.
3. Investigate the effects of staurosporine on motility and vitality of human and boar sperm.
4. Investigate the metabolic pathways utilised by human and boar sperm during apoptosis.

6.3 Methods

6.3.1 Induction of apoptosis

Capacitation was first induced as described in 3.3.1. Following on from this, apoptosis was induced by adding 100 µM STS for a further four-hours, to one of the tubes, and to the other tube, DMSO (solvent) was added as a positive control (+ve). After the eight-hour incubation, sperm motility (2.3.2), sperm vitality (2.3.3) and DNA fragmentation (6.3.2) were measured before (T0) and after (T8) incubation and the metabolic pathways utilised by sperm during the incubation were analysed using ^{13}C-NMR (2.3.5).

Table 6.1. The contents of the tubes prepared for the induction of apoptosis. Each tube contained a final volume of 500 µl. All components added in µL. Final concentrations are: 8 mM $^{13}C_6$-glucose, 5 mg/mL HSA, 25 mM $NaHCO_3$ and 100 µM STS.

Added at the start of the incubation (T0), (µL)						
Tubes	**Sperm (50 M/mL)**	**EBSS**	**1/3 Pen/strep**	**100 mM $^{13}C_6$-glucose**	**50 mg/mL HSA**	**1 M $NaHCO_3$**
-VE	0	382.5	15	40	50	12.5
+VE	345	37.5	15	40	50	12.5
Inducer	345	37.5	15	40	50	12.5

Added at T4, (µL)		
Tubes	**DMSO**	**10 mM STS**
-VE	0	5
+VE	5	0
Inducer	0	5

6.3.2 Measurement of apoptosis

Apoptosis was assessed via the TUNEL assay (Merck Millipore, California, USA), using the Terminal deoxynucelotidyl transferase (TdT) enzyme. DNA fragmentation was taken as a marker of apotosis. Briefly, ~$2x10^6$ /mL sperm in a volume of 10 µl, was added to a polylysine slide and left to air dry overnight at room temperature. The slides were fixed in methanol for 1 minute before storing at -20°C until staining. Slides were warmed to room temperature and re-hydrated using 1ml TBS for 15 minutes. Slides were permeabilised by adding 100 µl of 20 µg/ml Proteinase K (2 mg/ml Proteinase K was diluted 1:100 with 10 mM Tris pH8) and incubating at room temperature for 5 minutes. This was tapped off and slides were washed 3 times with 1x TBS and excess liquid removed. The equilibration and labelling reaction was then induced by adding 100 µL 5x TdT equilibration buffer diluted 1 in 5 with distilled water to each slide. The slides were incubated 30 minutes at room temperature. During this time, the TdT labelling reaction mix was prepared on ice by diluting TdT enzyme 1 in 20 with Fluorescein FragEL TdT labelling reaction mix. The TdT equilibration buffer was tapped off and 60 µL TdT labelling reaction mix was added to the slide, covered with parafilm and incubated at 37°C for 90 minutes in a humidified chamber. After the incubation, the parafilm was removed and the slides were washed three times with 1 X TBS and excess liquid tapped off. The slides were mounted using Fluorescein-Frag-EL mounting media and the edges were sealed with clear nail varnish. Slides were kept in the dark at 4°C and analysed on an Olympus IX73 with x600 magnification (UV filter 330 – 380 nm for DAPI and 465 – 495 nm for labelled nuclei). For each incubate, the number of sperm with blue fluorescence (no DNA damage), green fluorescence (DNA damage) were counted (Figure 6.2). 500 sperm per incubate was counted and the number of sperm in each category expressed as a percentage.

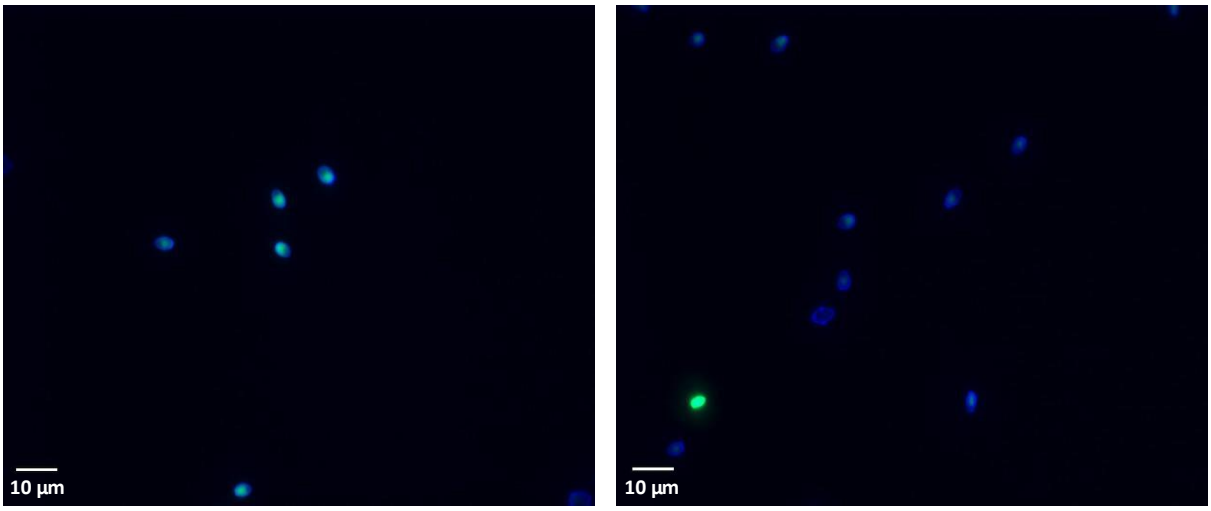


Figure 6.2: TUNEL staining. TUNEL was used to determine levels of DNA fragmentation in human and boar sperm. TUNEL staining was carried out as outlined in 6.3.2. The panel on the left **(A)** is sperm incubated without TdT enzyme (negative control) whereas the panel on the right **(B)** is sperm incubated with the TdT enzyme. When TdT enzyme is present, DNA fragmented sperm fluoresce green as seen in the panel on the right, however in the absence of TdT enzyme, or in cases where there is no DNA fragmentation, sperm fluoresce blue. Viewed at x600 magnification.

6.4 Results

6.4.1 Motility of human and boar sperm incubated with or without inducers of apoptosis.

CASA was used before (T0) and after (T8 control and T8 STS) incubations to observe whether STS had any effect on human and boar sperm total and progressive motility. Figure 6.3A shows that for human sperm, after an eight-hour incubation without the addition of STS, the total motility increased compared to time zero (T0, 24.3 ± 5.2%; T8 control, 35.3 ± 5.0%; P = 0.02). However, total motility decreased after an eight- hour incubation with the addition of STS (T8 STS, 4.8 ± 0.9%) compared to T0 (P = 0.009) and T8 control (P = 0.0002). The percentage of progressively motile human sperm displayed the same pattern, as seen in figure 6.3C. There was an increase in progressive motility after an eight-hour incubation without the addition of STS compared to time zero (T0, 6.8 ± 2.0%; T8 control, 19.9 ± 3.4%; P < 0.0001). However, progressive motility decreased after an eight-hour incubation with the addition of STS (T8 STS, 0.8 ± 0.21%) compared to T0 (P = 0.03) and T8 control (P = 0.0006).

For boar sperm, figure 6.3B shows that there was a significant decrease in total motility when incubated for eight-hours with the addition of STS compared to those that had been incubated for eight-hours without the addition of STS (T8 control, 13.5 ± 3%; T8 STS, 6.6 ± 1.7%; P = 0.02). A significant decrease in progressive motility was also observed, as seen in figure 6.3D, when boar sperm were incubated for eight-hours with the addition of STS compared to time zero and compared to incubation without the addition of STS (T8 STS, 1.2 ± 0.6%; T0, 7.5 ± 2.6%; P = 0.046; T8 control, 3.7 ± 1.2%; P = 0.04).

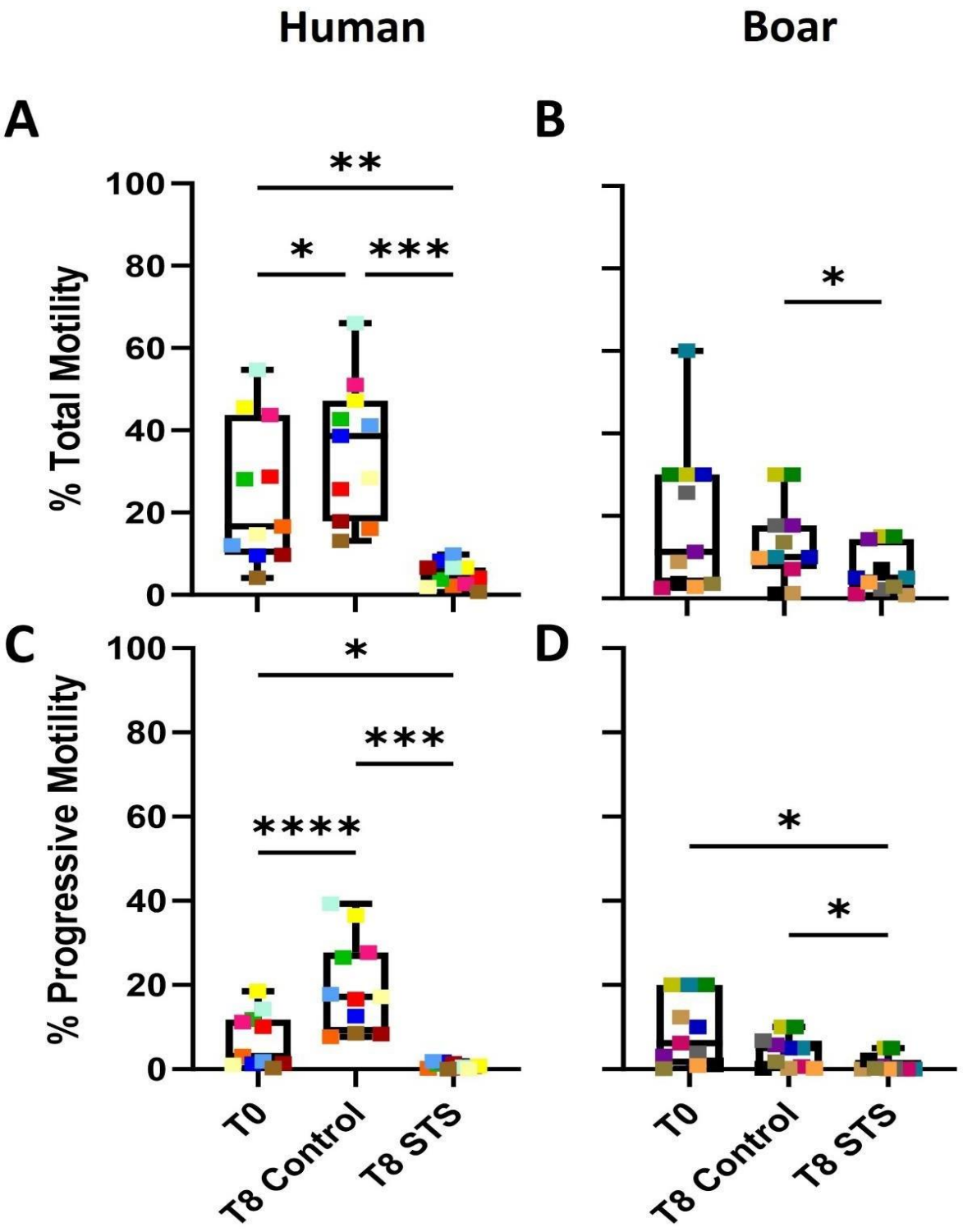


Figure 6.3: Human sperm total (A) and progressive (C) motility and boar sperm total (B) and progressive (D) motility (%) prior to (T0) and following (T8) an eight-hour incubation with or without 100 µM staurosporine. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a RM one-way ANOVA with a Tukey's multiple comparisons test. * = P <0.05, ** = P <0.01, *** = P <0.001 and **** = P <0.0001, n = 11. Points colour coded per individual donor/boar.

6.4.2 Vitality of human and boar sperm incubated with or without inducers of apoptosis.

To observe whether an eight-hour incubation, with 100 µM STS added after four-hours, influenced the vitality of human and boar sperm, the LIVE/DEAD™ sperm viability kit was used. Figure 6.4A shows that for human sperm, a decrease in vitality was observed after an eight-hour incubation with the addition of STS, compared to time zero and compared to an eight-hour incubation without the addition of STS (T8 STS, 41.6 ± 3.3%; T0, 54.6 ± 3.0%; P = 0.004; T8 control, 48.8 ± 3.7%; P = 0.03). For boar sperm, the percentage of live sperm significantly decreased after the eight-hour incubation both with and without the addition of STS, compared to time zero (T0, 57.7 ± 2.5%; T8 control, 33.7 ± 2.1%; P < 0.0001; T8 STS, 30.2 ± 1.6%; P < 0.0001), figure 6.4B.

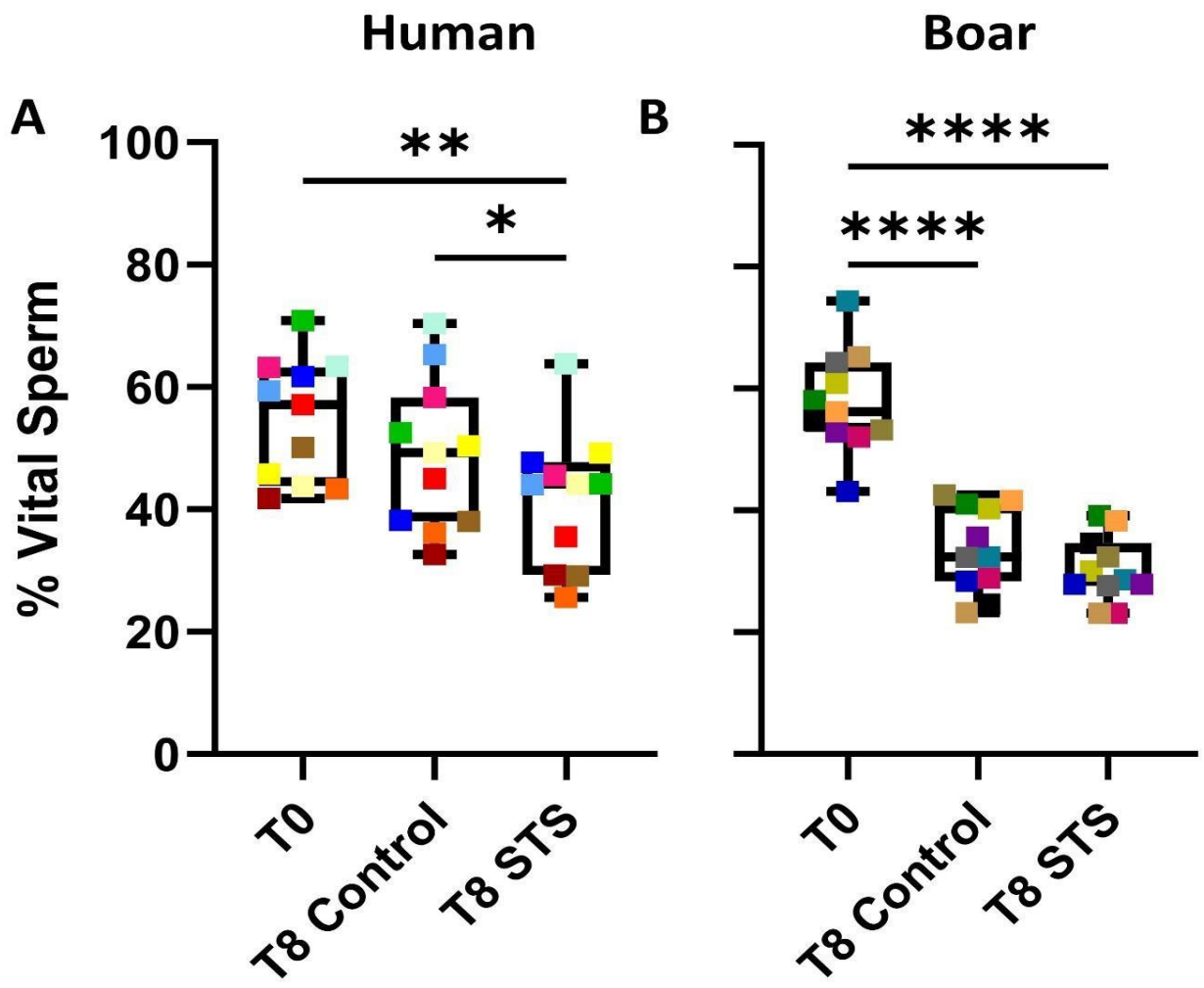


Figure 6.4: Human (A) and boar (B) sperm vitality (%) prior to (T0) and following (T8) an eight-hour incubation with or without the addition of 100 µM staurosporine. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a RM one-way ANOVA with a Tukey's multiple comparisons test. * = P <0.05, ** = P <0.01 and **** = P <0.0001, n = 11. Points colour coded per individual donor/boar.

6.4.3 TUNEL of human and boar sperm incubated with or without inducers of apoptosis.

To determine the percentage of apoptotic human and boar sperm, the TUNEL assay was used. For human sperm, figure 6.5A shows that after a four-hour capacitation incubation followed by a four-hour incubation with STS, the percentage of DNA fragmented sperm increased compared to time zero (T0, 3.4 ± 0.9%; T8 STS, 7.6 ± 1.2%; P = 0.0004. The same pattern for boar sperm was observed (T0, 0.1 ± 0.05%; T8 STS, 0.8 ± 0.2%; P = 0.03), as seen in figure 6.5B.

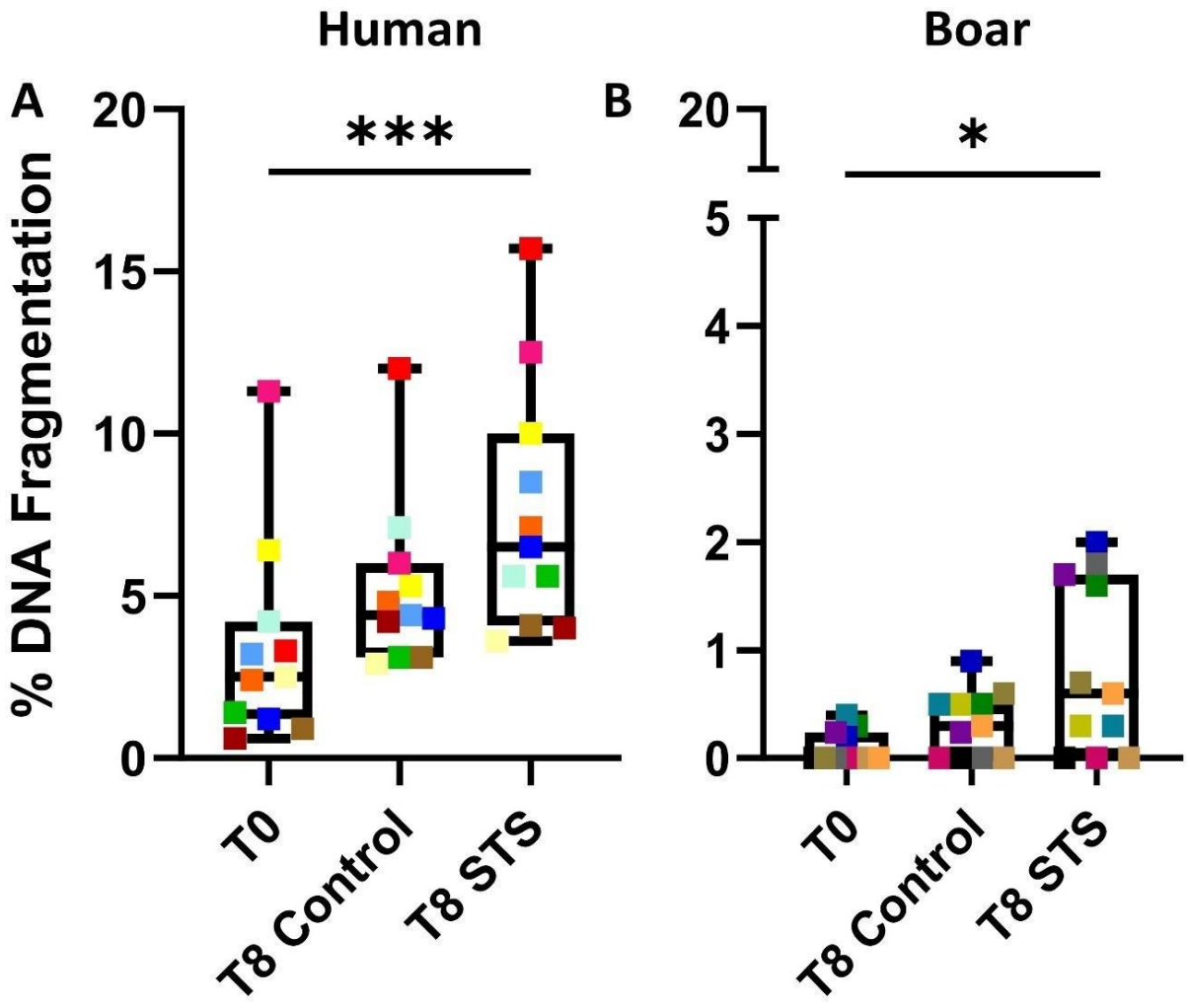


Figure 6.5: The percentage of DNA fragmented human (A) and boar (B) sperm prior to (T0) and following (T8) an eight-hour incubation with or without 100 µM staurosporine. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a Friedman test with a Dunn's multiple comparisons test for human sperm and a RM one-way ANOVA with a Tukey's multiple comparisons test for boar sperm. * = P <0.05 and *** = P <0.001, n = 11. Points colour coded per individual donor/boar.

6.4.4 NMR

^{13}C-NMR was used to investigate whether human (n=11) and boar (n=11) sperm, incubated with STS to induce apoptosis, showed differences in metabolism compared to those incubated without STS. The production of lactate is indicative of glycolysis and the production of bicarbonate is indicative of oxidative phosphorylation. The lactate and bicarbonate integral values were normalised by sperm total, vital and motile concentration, see section 2.3.6. No lactate peaks were recorded for the negative control samples suggesting no metabolism occurs in the absence of sperm (appendix A7).

6.4.4.1 Lactate production by human and boar sperm incubated with or without inducers of apoptosis.

For human sperm, as seen in figure 6.6A, after 8 hours, there was a significant decrease in lactate production, normalised by total human sperm concentration, when human sperm were incubated with STS compared to those incubated in the absence of STS (T8 control, 5.2 ± 0.6 x 10^6 A.U.; T8 STS, 3.6 ± 0.5 x 10^6 A.U.; P = 0.0003). The same was true when normalised by vital human sperm concentration (T8 control, 9.9 ± 0.9 x 10^6 A.U.; T8 STS, 7.8 ± 0.8 x 10^6 A.U.; P = 0.002).No significant differences in lactate production were observed for boar sperm incubations with or without STS, figure 6.6B.

6.4.4.2 Bicarbonate production

Figure 6.7 shows that no significant differences in bicarbonate production were observed for human or boar sperm incubations with or without STS when normalised by total or vital sperm concentrations.

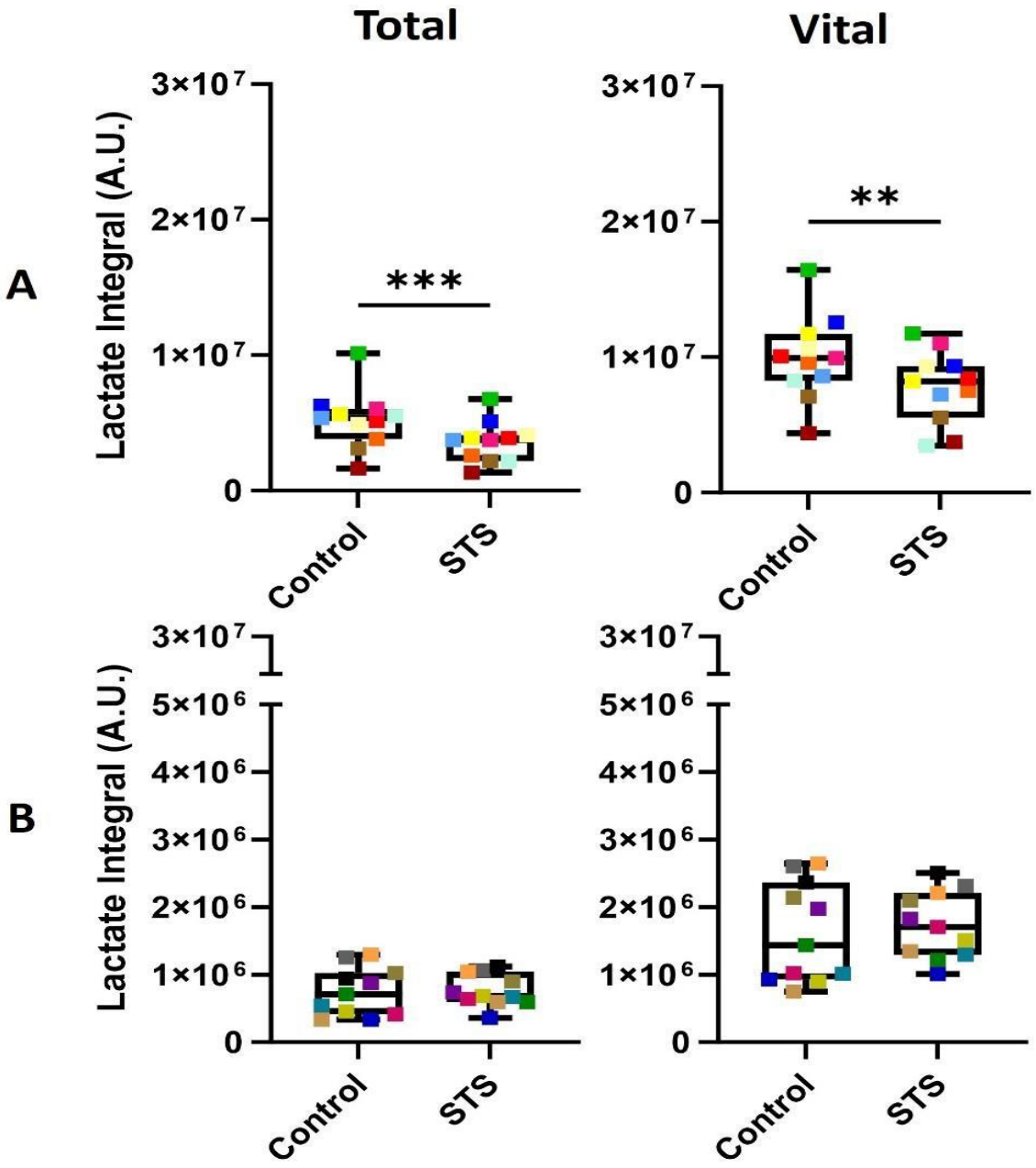


Figure 6.6: Lactate production by human (A) and boar (B) sperm incubated with or without STS. Lactate integrals are normalised by total and vital human and boar sperm concentrations. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a paired t test. ** = P <0.01 and *** = P <0.001, n = 11. Points colour coded per individual donor/boar.

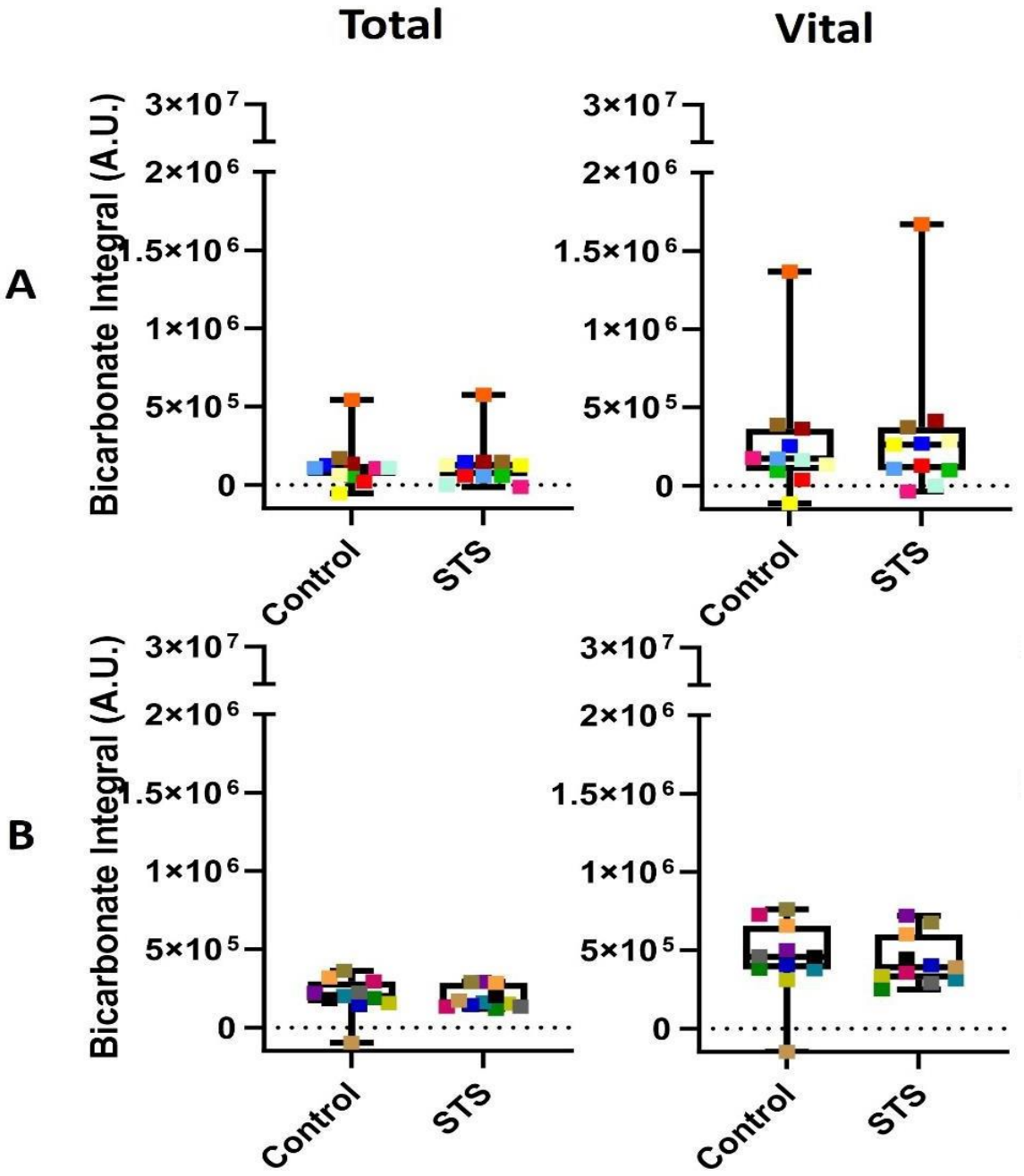


Figure 6.7: Bicarbonate production by human and boar sperm incubated with or without STS. Bicarbonate integrals are normalised by total and vital human and boar sperm concentrations. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. Statistical significance was analysed using a Wilcoxon test. No significant differences were identified. Points colour coded per individual donor/boar.

6.5 Discussion

A reliance on ATP for the induction of apoptosis has previously been demonstrated (Eguchi, Shimizu and Tsujimoto, 1997; Nicotera, Leist and Ferrando-May, 1998; Tatsumi *et al.*, 2003). This ATP has been suggested to be required for various processes including the function of caspases, hydrolytic enzymes, vesicle formation and chromatin condensation (Melchior and Gerace, 1995; Richter et al., 1996; Yasuhara et al., 1997; Eguchi et al., 1999). It is hypothesised that this ATP is generated by glycolysis, most likely due to the decrease in mitochondrial membrane potential seen in apoptosis (Shimizu *et al.*, 1996; Nicotera, Leist and Ferrando-May, 1998; Tatsumi *et al.*, 2003). This chapter has used STS to induce apoptosis and ^{13}C-NMR to determine whether glycolysis, oxidative phosphorylation or both supply the ATP required for apoptosis to confirm the above findings.

As an extensively used inducer of apoptosis, STS was expected to decrease the motility and vitality of sperm (Ahmed Belmokhtar, Hillion and Seâ Gal-Bendirdjian, 2001; Feng and Kaplowitz, 2002; Šimenc and Lipnik-Štangelj, 2012). A decrease in total and progressive sperm motility was seen when human and boar sperm were incubated with STS compared to those incubated without STS after an eight-hour incubation. This observation suggests that staurosporine is responsible for this decrease, potentially via the activation of caspase 3 of the apoptotic process or disruption of protein kinases, which are known to be involved in the initiation and regulation of sperm motility (Chae *et al.*, 2000; Karaman *et al.*, 2008; Swain *et al.*, 2022).

A simultaneous significant decrease in the percentage of vital human sperm was observed when incubated with STS compared to those incubated without, however whether this decreased linearly with time or immediately after the addition of STS remains unknown. For boar sperm the reduction in vitality was observed for sperm treated with and without STS compared to time zero. The decreases in vitality following incubation with STS were expected, however, approximately 30% of boar sperm and 40% of human sperm remained viable, in comparison to less than 5% sperm that remained motile. The sperm viability kit stains live sperm using sybr 14 and dead sperm using propidium iodide. Sybr 14 is a membrane permeable nuclear stain whereas propidium iodide is a membrane impermeable nuclear dye and is therefore only able to stain cells, including sperm, that have a disrupted plasma membrane (Kumar, Saneja and Panda, 2021). Early apoptotic cells do not have a disrupted membrane therefore would not take up propidium iodide (Patel *et al.*, 2006). Only late apoptotic cells, necrotic cells or cells with a compromised membrane would be able to take up the dye. This could be a potential explanation as to why sperm were rendered virtually immotile by STS despite the vitality remaining at 30/40%. The length of time it takes for completion of apoptosis is debated in the literature however has been suggested to occur anywhere between 2-3 hours to 72 hours depending on the cell type and inducer used, including for STS (Belmokhtar, Hillion and Ségal-Bendirdjian, 2001; Sundquist *et al.*, 2006). Therefore, the remaining live sperm, as determined by the sperm viability kit following a four-hour incubation with STS, could potentially still be in the early phases of apoptosis and therefore have an intact plasma membrane.

STS increased DNA fragmentation in human and boar sperm, however only compared to before incubation, T0, and not compared to those incubated without STS. Furthermore, low levels of DNA fragmentation were observed in human and boar sperm when treated with STS, an average of 7.6% and 0.8% respectively. A previous study which used 1 mM STS to induce apoptosis in human sperm found levels of apoptosis between 35 and 50%, following a six-hour incubation, using Annexin V binding and flow cytometry (Eley *et al.*, 2005). As this thesis used a lower concentration 100 µM, it might explain the lower percentages of DNA fragmentation identified. It is also important to consider that the methods of detection of apoptosis can also influence the percentage of apoptotic cells identified (McCarthy and Evan, 1997; Guo *et al.*, 2021). STS has been reported to induce apoptosis in various cell types, including sperm, at concentrations between 1 µM and 10 mM, and is often used as a positive control in the induction of apoptosis (Belmokhtar, Hillion and Ségal-Bendirdjian, 2001; Feng and Kaplowitz, 2002; Taylor *et al.*, 2004; Eley *et al.*, 2005; Šimenc and Lipnik-Štangelj, 2012; Malsy *et al.*, 2019). However, the recommended working concentration for the induction of

apoptosis by suppliers such as Sigma Aldrich is 1-10 µM, and this is the range most used amongst literature. Therefore, 100 µM STS was chosen, based on the concentrations used in these studies/recommendations from suppliers, and due to its observed ability to significantly reduce sperm motility and vitality in initial experiments.

STS certainly influenced sperm function due to its observed reduction in motility however the motility, vitality and DNA fragmentation data do not complement each other. There could be two possible explanations for this. Firstly, 100 µM STS may not have induced apoptosis, hence low levels of DNA fragmentation and 30/40% of viable boar/human sperm were observed. In this scenario, STS possibly affected motility via the inhibition of kinase activity (Swain, 2022). Alternatively, STS did induce apoptosis in sperm but following the four-hour incubation, the majority remained in earlier stages of apoptosis rather than later stages where DNA fragmentation and a disrupted plasma membrane would be observed (Patel *et al.*, 2006). In future work, an alternative method to detect apoptosis could be employed to gain a deeper understanding of these observations. For example, a cleaved caspase assay or Annexin V staining could be used as a direct measure of apoptosis. This thesis used DNA-fragmentation as a marker of apoptosis and the TUNEL assay is not a direct measure of apoptosis. Furthermore, whilst the TUNEL assay is one of the most standardized methods to detect DNA damage and cell death, the steric impediment for the terminal nucleotidyl transferase enzyme to access the highly condensed sperm nucleus, which results in highly condensed chromosomes that form hair pin structures, may decrease the ability of this test to detect internal DNA breaks in sperm (Ribas- Maynou *et al.*, 2021). This could be a potential explanation as to why low levels of DNA fragmentation were observed in this chapter.

A significant decrease in lactate production was seen when human sperm were incubated with STS compared to those without, when the lactate integral was normalised by total and vital concentration. As no significant difference in DNA fragmentation was observed between the two groups, this is unlikely to be an effect of apoptosis and sperm no longer requiring ATP. Although it could be considered that the TUNEL assay underestimated the amount of DNA-fragmented sperm, due to the vitality values remaining at 30-40%, the most plausible reason behind this decrease in lactate is the significant decrease in motility seen when human and boar sperm were treated with STS. This explanation would support the literature that claims sperm motility is reliant upon the ATP generated by glycolysis (see chapter 1). However, it remains unknown whether STS rendered sperm immotile immediately or whether this decreased linearly over the four-hour incubation with STS, hence, whether the decrease in lactate production was immediate or gradual is not known based on the data presented in this chapter.

Normalisation by the motile concentration was not included in this chapter as STS significantly reduced the progressive motility to <1%. Motile concentrations were calculated using the T8 value only due to the justifications explained in chapter 2. Therefore, this method of normalisation would assume sperm were immotile throughout the entire eight-hour incubation which was not true as STS was added after a four-hour pre- capacitation incubation, where sperm would have been motile (confirmed by the data for the positive control). For this reason, only normalisation by the total and vital concentration are shown in this chapter.

No significant differences in lactate production were identified for boar sperm and this was, in part, expected as STS only induced DNA fragmentation, indicative of apoptosis, by less than 1% in boar sperm and no differences in vitality were observed between those treated with and without STS. However, STS significantly reduced boar sperm total and progressive motility, as seen for human sperm, so a similar effect on lactate production would have expected to have been seen if the decrease in lactate production seen for human sperm was solely a consequence of the reduction in motility. Although when comparing the motility values in the control group, human sperm progressive motility was approximately 20%, whereas boar was only 4%, meaning the effect of STS on reducing motility was not as drastic in boar sperm.

No significant differences in bicarbonate production were identified for human or boar sperm when normalised by vital or total concentration. These results were expected as there was no significant difference

in DNA fragmentation between sperm treated with or without STS. It could be noted that the decrease in progressive motility seen when human and boar sperm were treated with STS, does not affect bicarbonate production. From this, it could be postulated that oxidative phosphorylation does not support human sperm motility, when glucose is the sole energy substrate, and that oxidative phosphorylation is potentially just occurring at a constant, background level.

In conclusion, STS did not significantly induce DNA fragmentation in human or boar sperm compared to the control but significantly reduced sperm motility. The decrease in lactate production seen is therefore most likely a result of the reduced motility in human and boar sperm, suggesting that glycolysis is the main ATP producing pathway that powers motility as no differences in bicarbonate production were observed.

Chapter 7: Discussion

7.1 Summary of study aims and main findings

The main aim of this thesis was to determine whether the energy demands of human and boar sperm change prior to fertilisation by investigating the metabolic pathways utilised by sperm during capacitation, hyperactivation, the acrosome reaction and apoptosis. There remains considerable debate in the literature regarding the preferred metabolic pathways utilised for sperm function, particularly motility. Glycolysis has been suggested to be the main pathway by various studies, even in the presence of mitochondria and oxygen (Peterson and Freund, 1974; Ford and Harrison, 1981; Lornage, Guerin and Czyba, 1986; Williams and Ford, 2001), with glycolytic enzymes been localised to the fibrous sheath of human sperm (Miki *et al.*, 2004). However, the role of oxidative phosphorylation has been implicated due to its efficiency of ATP production and early studies using inhibitors of oxidative phosphorylation, show inhibition of human sperm motility (Suter, Chow and Martin, 1979). There has been less research focusing on the energy requirements of capacitation, hyperactivation and the acrosome reaction, despite their importance for successful fertilisation of the oocyte (Sutovsky, 2010). Therefore, this thesis utilised ^{13}C-NMR to investigate the relative contribution of glycolysis and oxidative phosphorylation during these events.

The increase in lactate production observed during capacitation, hyperactivation and the acrosome reaction for human sperm, alongside a decrease in bicarbonate production during capacitation and the acrosome reaction, suggest glycolysis is the predominant pathway utilised during these processes in agreement with previous literature (Ferrandi *et al.*, 1987; Rogers and Perreault, 1990; Ho, Granish and Suarez, 2002; Hereng *et al.*, 2011). An increase in lactate production was also observed for boar sperm during capacitation and the acrosome reaction, with a decrease in bicarbonate production been observed in the latter, suggesting that this preference of glycolysis is conserved amongst these two species. As apoptosis was not significantly induced in either species when compared to the positive control, the decrease in lactate production is most likely an effect of the decrease in motility, as discussed in chapter 6. The increase in lactate production in the other processes was shown to be due to sperm metabolism as there was a significant positive correlation between sperm concentration and lactate production for human sperm (appendix A6), and no lactate was observed in the negative controls. Oxidative phosphorylation seems to still be occurring in sperm, potentially at a background level. This is supported by the increase in bicarbonate production, when the raw bicarbonate integrals in the positive controls containing sperm, were compared to the negative controls containing no sperm (Appendix A9), and the significant positive correlation between sperm concentration and bicarbonate production for human sperm (appendix A7). However, it does not appear to be responsible for the increased demand for ATP required during capacitation, hyperactivation and the acrosome reaction, as noted by the absence of a significant increase in bicarbonate production during these processes, and the observed decrease during capacitation in human sperm and the acrosome reaction in both human and boar sperm.

7.1.1 Sperm preference of glycolysis

For human sperm, the increase in lactate production observed alongside a decrease in bicarbonate production raises the question of why sperm utilise the inefficient metabolic pathway glycolysis, to generate ATP per unit of glucose (Locasale and Cantley, 2011). As discussed briefly in chapter 3.5, this has been shown to be true for cancer cells, even in the presence of oxygen despite their active mitochondria (Warburg, 1925). Numerous benefits of the use of glycolysis have been proposed for cancer cells including the production of biosynthetic precursors, which allow for proliferation and division seen in tumour growth, and the lowering of pH via the production of lactic acid to cause immunosuppression (Lin *et al.*, 2020; Zhao *et al.*, 2022; Zhou *et al.*, 2022).

Sperm display similar behaviour according to this thesis and although they don't require proliferation or division, other benefits of favouring glycolysis could be important for sperm function. The increased rate of glycolysis, despite its lower ATP yield, could offer an advantage during situations where sperm have a high energy demand but have competition for the energy resources (Pfeiffer, Schuster and Bonhoeffer, 2001;

Shestov *et al.*, 2014). As hyperactivation and the acrosome reaction must occur at specific times following ovulation, presumably mediated by chemoattractants, a faster rate of ATP production would also seem practical to reach and successfully fertilise the oocyte (Suarez, 2008). Furthermore, oxidative phosphorylation produces more harmful byproducts, namely ROS, and sperm are extremely vulnerable to high levels of ROS in comparison to somatic cells, due to the composition of their plasma membrane. ROS interacts with various cell components and can cause peroxidation of lipids, damage to nucleic acids, inhibition of enzymes and DNA damage (Sharma *et al.*, 2012). As sperm are thought to have reduced DNA repair mechanisms, the effects of ROS would compromise their ability to fertilise the egg hence the transmission of genetic information across generations (Baarends, van der Laan and Grootegoed, 2001; González-Marín, Gosálvez and Roy, 2012). Therefore, the preference for glycolysis by human and boar sperm could also be a protective function.

In addition, a common theme in this thesis has been sperm energy compartmentalization, with glycolysis occurring in the head and principal piece and oxidative phosphorylation occurring in the midpiece (Hereng *et al.*, 2011). This is in contrast to other cell types where glycolysis is coupled to the Krebs cycle. There is a debate in the literature regarding whether pyruvate, produced in the principal piece, is able to support oxidative phosphorylation in the midpiece, and whether the ATP produced by oxidative in the midpiece is then able to diffuse along the length of the flagellum to support the energy demanding processes at distal sites (Nevo and Rikmenspoel, 1970; Travis et al., 2001; Turner, 2003; Mukai and Okuno, 2004; Ford, 2006; Takei et al., 2014). The authors in the above studies have acknowledged there are differences in the ATP diffusion potential between different species, partly due to variations in the length of the flagellum. Based upon the data in this thesis, it can be concluded that the energy demands of capacitation, hyperactivation and the acrosome reaction, in which the changes occur in either the head or flagellum of sperm, is supported by a local, increased glycolytic rate.

The data in this thesis suggests sperm dependence on glycolysis/increased glycolytic rate is at the expense of oxidative phosphorylation during capacitation and the acrosome reaction, as seen by the increase in lactate production and decrease in bicarbonate production during these processes for human and boar sperm. The increased lactate production may reflect an increase in the amount of pyruvate that is reduced to lactate as opposed to being transported into the mitochondria for oxidation in the Krebs cycle. The reduction of pyruvate to lactate would regenerate NAD+ to maintain the high glycolytic rate human and boar sperm seem to rely on during these processes. Previous work has also identified a switch from oxidative phosphorylation to glycolysis during capacitation in murine sperm (Tourmente *et al.*, 2022). The authors propose the function of this switch is most likely to be to sustain the increased ATP demands of capacitation in the sperm head and distal flagellar. This supports the theory that ATP produced during oxidative phosphorylation may not diffuse along the flagellum at a sufficient rate, hence local ATP production to the site of ATP utilisation is essential for sperm.

7.1.2 The role of the mitochondria

As this thesis suggests glycolysis is the preferred pathway for ATP production in human and boar sperm, this raises the question of why sperm have mitochondria. Despite not being relied upon to produce ATP during motility, capacitation, hyperactivation and the acrosome reaction, as determined by the results in this thesis and in agreement with previous literature, the importance of the mitochondria in sperm is undeniable (Rodríguez-Gil and Bonet, 2016). This has been demonstrated by various studies which have shown that structural and functional alterations in the mitochondria are present in asthenozoospermic patients as well as studies which have demonstrated a decrease in human sperm motility and capacitation when inhibitors of oxidative phosphorylation were used (Piomboni et al., 2012). Oxidative phosphorylation has still been shown to be active in human sperm in this thesis and in other studies utilising NMR, as when they were supplemented with $^{13}C_6$-glucose, bicarbonate responses were observed (Reynolds et al., 2017; Calvert et al., 2018). In addition, energy pathways utilised during other processes in sperm, such as fusion of sperm and egg pronuclei, were not investigated in this study, and oxidative phosphorylation could have a role there.

Furthermore, sperm are highly specialised and compact cells therefore it would not seem logical for them to retain an organelle that was redundant. The mitochondria are generally known as the powerhouse of the cell due to their ability to efficiently generate ATP via oxidative phosphorylation. However, there are many other important roles of mitochondria that would explain their presence in cells, such as sperm, that do not seem to rely upon oxidative phosphorylation for the majority of ATP generation (McBride, Neuspiel and Wasiak, 2006). As sperm motility has been shown to be maintained in the absence of glycolytic and Krebs cycle metabolites, this suggests sperm are able to utilise different pathways for survival in the FRT depending on their environment and the variations in the availability and concentrations of substrates (Marín-Briggiler *et al.*, 2021). Hu and Yu, (2017), demonstrated that the ability of sperm to utilise different substrates and activate different metabolic pathways to adapt to their external environment appears to be crucial in ensuring successful fertilisation and conserving the paternal genome (Amaral et al., 2013). For example, sperm use alternative metabolic pathways in the mitochondria, to remain motile in the absence of glucose and pyruvate, by metabolising lipids using fatty acid beta-oxidation, amino acid metabolism and oxidative carboxylation of alpha ketoacids. Previous work has also provided evidence for butyrate and hydroxybutyrate metabolism in human sperm (Calvert et al., 2018). As sperm possess the oxidative phosphorylation machinery and have been shown to generate ATP via this pathway, it is most likely still of importance for sperm function, potentially in cases where there is a lack of glycolytic substrates.

The mitochondria also have key roles in apoptosis, calcium signalling and ROS generation (Kroemer, Zamzami and Susin, 1997; Hirata *et al.*, 2002; Patergnani *et al.*, 2011; Tait and Green, 2012). Sperm apoptosis has suggested to be important for sperm to prevent immune response in the FRT which would negatively impact fertilisation success and subsequent embryo development, see chapter 6. The importance of the regulation of ROS and calcium for capacitation, hyperactivation and the acrosome reaction have also been implicated, suggesting alternative roles of the mitochondria for sperm function as opposed to just the generation of ATP (Brown et al., 1989; Tafuri et al., 2015; Dutta et al., 2020).

7.2 Human vs boar sperm

Boar sperm are similar in structure to human sperm, however there are some fundamental differences between the two that could affect their function, which were discussed in chapter 1 (Rodríguez-Gil and Bonet, 2016). Although boar and human sperm responded similarly to the inducers of the various processes, discussed in detail in chapters 3, 4, 5 and 6, throughout this thesis boar sperm motility and vitality were consistently lower than human sperm. The lactate peaks observed were also consistently smaller than those observed for human sperm, suggesting boar sperm have a lower metabolic rate compared to human sperm, and this is most likely an effect of the consistent reduced motility and vitality identified. Only a significant decrease in bicarbonate production during the acrosome reaction was observed for boar sperm, however evidence of oxidative phosphorylation in boar sperm was seen when comparing raw bicarbonate integrals between the negative and positive controls, Appendix A9. This suggests oxidative phosphorylation is potentially just occurring at a constant, background level in boar sperm.

The low vitality for boar sperm could be a result of them being stored for 5 days in semen extender at 17°C. The low motility can potentially be explained by their site of deposition in vivo being closer to the oviduct, hence boar sperm may display a naturally lower rate of motility (Quintero-Moreno, Rigau and Rodrıǵ uez-Gil, 2004; Mogas, Álamo and Rodríguez-Gil, 2011). Furthermore, boar sperm are extremely sensitive to ROS due to the lipid composition of their plasma membrane, and this could contribute to the lower motility and vitality observed for boar sperm throughout the eight-hour incubations in comparison to human sperm (Simpson, Swan and White, 1987). Prior to incubation, sperm were subject to density gradient centrifugation in the absence of metabolic substrates and were not maintained at 39 degrees at all stages due to the transfer of tubes to and from the centrifuge and the efforts to prevent capacitation and other events until the addition of $^{13}C_6$-glucose. As cold shock is irreversible in boar sperm, this could also explain the consistent low motility observed for this species, hence the low levels of hyperactivation when stimulated with caffeine.

Boar sperm were supplied in semen extender, in which they appeared hyperactivated. This remains elusive as the role of semen extender is to protect sperm from cold shock during the chilling and shipping process, which is supposed to reduce the metabolism. Semen extender typically contains antibiotics and antioxidants as a protective role as well as sugars for metabolism however the exact composition of the extender boar semen in this study were supplied in remains unknown (Bustani and Baiee, 2021). Following density gradient centrifugation and suspensions in EBSS, boar motility was drastically lower than what was observed in the extender, presumably due to the reasons discussed previously.

7.3 Use of NMR

Various techniques can be used to measure metabolism, and these include ATP assays, enzyme assays, measurement of oxygen uptake, use of ^{14}C radiolabelled substrates, Seahorse technology and methods that detect metabolites such as mass spectrometry and ^{13}C- nuclear magnetic resonance (NMR) (TeSlaa and Teitell, 2014; Codella, 2021). The aim of this thesis was to determine the relative contribution of glycolysis and oxidative phosphorylation to the generation of ATP required for various processes, therefore a method that measured both glycolytic and oxidative phosphorylation activity was required.

Both NMR and mass spectrometry allow for metabolite detection and are the most common analytical methods used in metabolomics research (Emwas, 2015). Although mass spectrometry has high sensitivity, the high reproducibility of NMR and its ability to noninvasively and non-destructively identify numerous metabolic pathways simultaneously is the reason it was chosen to investigate sperm metabolism in this study. Specifically, ^{13}C-NMR was chosen as the chemical shifts cover a large range of values hence can monitor the metabolism of a range of substrates through various metabolic pathways (Dona *et al.*, 2016).

Lactate and bicarbonate integrals obtained from NMR were normalised by the total concentration, vital concentration, and motile sperm concentration. These normalisation methods provide different information on the lactate and bicarbonate integrals. Whereas normalisation by the total concentration assumes that all sperm present in the sample are contributing to the lactate and bicarbonate signal, normalisation by the vital and motile concentration assumes only the live or motile sperm respectively, are contributing to signals, thus removing motility/vitality as factors. There are limitations to each of these methods. Firstly, normalisation by total concentration does not account for sperm which were already dead at T0. These sperm would not contribute to the ^{13}C-NMR signal. Normalisation by motile concentration was based upon the T4/T8 value only, and in cases where motility was significantly affected, e.g. by staurosporine in chapter 6, the normalisation value was not representative of the entire incubation. Finally, normalisation by the vital concentration uses an average of sperm vitality across T0 and T4/T8 measurements, and this method assumes vitality declines linearly over the eight-hour incubation, which may not be true, particularly where sperm were treated with staurosporine. Furthermore, normalisation by the vital concentration includes both motile and immotile sperm, both of which require ATP, however presumably different amounts. Nonetheless, normalising by sperm concentration was appropriate due to work in this thesis, Appendix A6 and A7, and previous work, that has shown that as the concentration of human sperm increases, the production of lactate and bicarbonate generally increases (Reynolds et al., 2017).

7.4 Wider implications of findings

Male infertility is currently diagnosed based upon a patient's medical history and a diagnostic semen analysis. The latter investigates sperm concentration, motility and morphology however fails to investigate the potential causes of low concentration, motility, or morphology as well as a sperms ability to undergo fundamental processes required for fertilisation; capacitation, hyperactivation and the acrosome reaction. This could account for the unexplained fertility seen in 15-30% of cases (Gelbaya *et al.*, 2014). How sperm generate the ATP required for sperm function remains unknown and could aid in the diagnosis of male factor infertility and offer an insight into potential treatments.

In addition, an increased understanding of the pathways of ATP generation in sperm prior to fertilisation could have a role in the development of safe, effective, non-hormonal male contraceptives, which has become a major focus of research in the field of reproduction (Barratt, Andrews and Gruber, 2019; Agarwal, Panner Selvam and Baskaran, 2020; Norcross *et al.*, 2022). If the energy pathways that generate ATP were disrupted, and motility was hindered as well as the ability for sperm to undergo capacitation, hyperactivation and the acrosome reaction, then sperm would be unable to fertilise the egg. Previous work investigating the role of EPP055, a small organic molecule that has been shown to reduce sperm progressive motility in non-human primates, has shown that via a reduction in intracellular pH and calcium levels alongside an inhibition of cAMP and subsequent ATP production, capacitation and hyperactivation were prevented (O'Rand, Silva and Hamil, 2016; O'Rand *et al.*, 2018a). The development of male contraceptives is currently a focus of reproduction related research and targeting energy pathways seems a promising option.

7.5 Limitations

There are numerous limitations in this thesis. Firstly, the addition of bicarbonate as an inducer of capacitation was observed using NMR. A volume of 12.5 µL was added making a final concentration of 25 mM in 500 µL of sperm suspension, therefore it wasn't originally anticipated that the natural abundance of bicarbonate would be NMR observable. As stated in 2.3.6, to overcome this, the average of the 11 bicarbonate integrals in the negative controls were subtracted from the bicarbonate integral values, to provide a common baseline for removing the bicarbonate signal.

Furthermore, pyruvate nor intermediates of the Krebs cycle were identified in this study, which would have increased the reliability of the findings presented in this thesis, agreeing with previous literature that was unable to detect them also (Calvert et al., 2018). This is because for ^{13}C-labelled metabolites to be detectable by NMR, they must accumulate to high concentrations. Therefore, intermediate metabolites are difficult to observe without the utilisation of inhibitors, such as of the Krebs cycle, to allow accumulation at a particular step. Despite this, the use of mass spectrometry and western blot analysis has confirmed their presence during sperm metabolism (Marin et al., 2003; Medrano et al., 2006). The lack of peaks observed for pyruvate could be due to a variety of reasons which include: the insensitivity of NMR and the fact that pyruvate doesn't accumulate within the cell in high quantities (Gray, Tompkins and Taylor, 2014). Further work, mentioned below in chapter 7.6, could overcome these limitations.

7.6 Recommendations for future work

Based upon the data collected in this thesis, there are numerous recommendations for future work. Many of these suggestions for future work were based upon the original aims of this thesis, before the pandemic and its subsequent effects. Firstly, in relation to the lack of Krebs cycle intermediates mentioned above, the use of inhibitors at certain stages would allow potential metabolites to accumulate there should they be present. This could be extended to use inhibitors of glycolysis or oxidative phosphorylation, the latter of which would help determine the pathways to bicarbonate production, providing a greater insight into sperm metabolism. Although it has been concluded that spermatozoa rely on glycolysis during capacitation in this thesis, inhibiting oxidative phosphorylation and observing the effects on capacitation would further increase the confidence in these results. Alternative methods of measuring metabolism, including Seahorse XF technology, which was an original aim of this thesis prior to the pandemic, would also increase confidence in the results presented in this thesis to give a more absolute answer on whether sperm prefer glycolysis or oxidative phosphorylation to produce the ATP required for capacitation, hyperactivation and the acrosome reaction. The use of Seahorse XF technology for metabolic characterisation of human sperm has previously been demonstrated, however human sperm metabolism during capacitation, hyperactivation and the acrosome reaction has not (Freitas-Martins *et al.*, 2023).

In addition, the experiments in this current study involved incubating sperm with a single substrate; glucose, however *in vivo* numerous substrates would be available to the sperm. Sperm are exposed to fructose,

citrate, lipids, and galactose in the seminal fluid, and primarily metabolise fructose which has a concentration roughly 3-fold higher than the glucose concentration in seminal fluid. A review of the properties of human semen reported the mean average values of the following metabolites found in human semen from literature: citrate (5.3 mg/mL), fructose (15 mM), glucose (5.6 mM), lactic acid (0.6 g/mL) and urea (0.5 g/mL) (Owen & Katz, 2005).

In contrast, in the female reproductive tract, glucose, pyruvate, and lactate are the primary energy sources for sperm, the oocyte and for the embryo (Dean, 2019). For this reason, most media for the culture of sperm, oocytes and embryos typically contain glucose, pyruvate, and lactate as metabolites. The concentrations of glucose and lactate vary between the oviductal and uterine fluids and oviductal fluids vary further depending on the different phases of menstrual cycle (Gardner et al., 1996). In human, the glucose concentrations in the oviduct are between 0.5 and 3.1 mM depending on the cycle whereas in the uterus it is 3.1 mM. Lactate concentration in the oviduct varies between 4.87 mM to 10.5 mM depending on the cycle whereas in the uterus in remains at 5.87 mM. Pyruvate does not vary with the menstrual cycle and an average concentration of 0.24 mM was identified in the oviduct, compared to 0.1 mM in the uterus. The lower glucose concentration in the oviduct serves to protect the early embryo which is known to preferentially metabolise lactate and pyruvate in the zygote and morula stage, and then the uptake of glucose is known to significantly increase once the embryo reaches blastocyst stage (Dean, 2019).

Furthermore, the incubation tubes used in this study for the incubation of sperm over a four-hour period are not representative of the *in vivo* environments of the FRT that vary in terms of pH, substrates, and ion concentrations within the different regions and throughout the different stages of the menstrual cycle (Ng et al., 2017). More importantly, sperm communicate with the FRT cells as they move through the tract and this is thought to influence their function (Pacey et al., 1995). Therefore, future work could stimulate the environment of the FRT using multiple ^{13}C-molecules known to be present in the FRT, such as pyruvate and lactate. This basic model of sperm FRT interactions would address how sperm metabolism behaves in a more realistic setting of multiple substrate availability in the FRT and would identify whether multiple metabolites have a synergistic effect on human sperm metabolism.

The ATP requirements of sperm motility and its subsequent contribution to the NMR spectrum was not investigated in this study, therefore it remains unknown exactly how much the various processes contributed to the overall observed energy usage. Although normalisation of the lactate and bicarbonate integrals from NMR by vital and motile concentrations were performed, in an attempt to account for changes in vitality and motility, it would be interesting to measure the basal metabolic rate of human sperm. This could be achieved by inhibiting sperm flagella movement, to estimate how much ATP is required for sperm motility. For example, inhibitors of dynein or alternative molecules, such as the small organic molecules EPP055, have been shown to reduce sperm motility (Bouchard et al., 1981; O'Rand, Silva and Hamil, 2016; O'Rand et al., 2018). This could then be used as a control to allow for the ATP demands during capacitation, hyperactivation and the acrosome reaction to be more accurately determined. Alternatively, multiple linear regression analysis could be performed to provide an insight into the relative contributions of vitality, motility, and the various investigated processes, to the NMR spectrum.

7.7 Conclusion

In conclusion, during capacitation, hyperactivation and the acrosome reaction, human and boar sperm preferentially use glycolysis to generate the ATP required. This could be due to numerous reasons discussed in this thesis, including the increased rate of glycolysis and sperm energy compartmentalization. Despite this, the mitochondria are important for sperm function and oxidative phosphorylation still occurs, albeit at a background level. Understanding the key physiological changes that take place in sperm, and the underlying energy requirements, is crucial in determining new diagnostic tests for male factor fertility, and could lead to developments in assisted reproduction procedures, which could potentially impact the clinical outcome.

Chapter 8: References

Achikanu, C. *et al.* (2018) 'Effects of pH manipulation, CatSper stimulation and Ca2+-store mobilization on [Ca2+]i and behaviour of human sperm', *Human Reproduction*, 33(10), pp. 1802–1811. Available at: https://doi.org/10.1093/humrep/dey280.

Achikanu, C. *et al.* (2019) 'Continuous behavioural "switching" in human spermatozoa and its regulation by Ca2+-mobilising stimuli', *Molecular Human Reproduction*, 25(8), pp. 423–432. Available at: https://doi.org/10.1093/molehr/gaz034.

Agarwal, A. *et al.* (2016) 'Clinical utility of sperm DNA fragmentation testing: practice recommendations based on clinical scenarios', *Translational andrology and urology*, 5(6), pp. 935–950. Available at: https://doi.org/10.21037/tau.2016.10.03.

Agarwal, A., Panner Selvam, M.K. and Baskaran, S. (2020) 'Proteomic Analyses of Human Sperm Cells: Understanding the Role of Proteins and Molecular Pathways Affecting Male Reproductive Health', *International Journal of Molecular Sciences*, 21(5). Available at: https://doi.org/10.3390/ijms21051621.

Ahmed Belmokhtar, C., Hillion, J. and Seâ Gal-Bendirdjian, E. (2001) *Staurosporine induces apoptosis through both caspase-dependent and caspase-independent mechanisms*. Available at: www.nature.com/onc.

Aitken, R. and Koppers, A. (2011) 'Apoptosis and DNA damage in human spermatozoa', *Asian journal of andrology*, 13, pp. 36–42. Available at: https://doi.org/10.1038/aja.2010.68.

Aitken, R.J. *et al.* (1998) 'A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation', *Journal of Cell Science*, 111(5), pp. 645–656. Available at: https://doi.org/10.1242/jcs.111.5.645.

Aitken, R.J., Irvine, S. and Kelly, R.W. (1986) 'Significance of intracellular calcium and cyclic adenosine 3',5'-monophosphate in the mechanisms by which prostaglandins influence human sperm function', *Reproduction*, 77(2), pp. 451–462. Available at: https://doi.org/10.1530/jrf.0.0770451.

Aitken, R.J. and Nixon, B. (2013) 'Sperm capacitation: A distant landscape glimpsed but unexplored', *Molecular Human Reproduction*, 19(12), pp. 785–793. Available at: https://doi.org/10.1093/molehr/gat067.

Alasmari, W., Costello, S., *et al.* (2013a) 'Ca^{2+} Signals Generated by CatSper and Ca^{2+} Stores Regulate Different Behaviors in Human Sperm* [S]', *Journal of Biological Chemistry*, 288(9), pp. 6248–6258. Available at: https://doi.org/10.1074/jbc.M112.439356.

Alasmari, W., Barratt, C.L.R., *et al.* (2013b) 'The clinical significance of calcium-signalling pathways mediating human sperm hyperactivation', *Human Reproduction*, 28(4), pp. 866–876. Available at: https://doi.org/10.1093/humrep/des467.

Alipour, H. *et al.* (2017) 'Improved sperm kinematics in semen samples collected after 2 h versus 4-7 days of ejaculation abstinence', *Human reproduction (Oxford, England)*, 32(7), pp. 1364–1372. Available at: https://doi.org/10.1093/humrep/dex101.

Allouche-Fitoussi, D., Bakhshi, D. and Breitbart, H. (2018) 'Signaling pathways involved in human sperm hyperactivated motility stimulated by Zn2', *Molecular reproduction and development*, 85(6), pp. 543–556. Available at: https://doi.org/10.1002/mrd.22996.

Amaral, A., Castillo, J., *et al.* (2013a) 'Human sperm tail proteome suggests new endogenous metabolic pathways.', *Molecular & cellular proteomics : MCP*, 12(2), pp. 330–342. Available at: https://doi.org/10.1074/mcp.M112.020552.

Amaral, A., Lourenço, B., *et al.* (2013b) 'Mitochondria functionality and sperm quality', *Reproduction*. Available at: https://doi.org/10.1530/REP-13-0178.

Amaral, A. *et al.* (2014) 'Identification of proteins involved in human sperm motility using high-throughput differential proteomics', *Journal of Proteome Research*, 13(12), pp. 5670–5684. Available at: https://doi.org/10.1021/pr500652y.

A.P. Harrison, R. and Gadella, B.M. (2005) 'Bicarbonate-induced membrane processing in sperm capacitation', *Theriogenology*, 63(2), pp. 342–351. Available at: https://doi.org/https://doi.org/10.1016/j.theriogenology.2004.09.016.

Arcelay, E. *et al.* (2008) 'Identification of proteins undergoing tyrosine phosphorylation during mouse sperm capacitation', *The International journal of developmental biology*, 52(5–6), pp. 463–472. Available at: https://doi.org/10.1387/ijdb.072555ea.

Ardón, F. *et al.* (2016) 'Dynamics of Bovine Sperm Interaction with Epithelium Differ Between Oviductal Isthmus and Ampulla', *Biology of Reproduction*, 95. Available at: https://doi.org/10.1095/biolreprod.116.140632.

Armon, L. and Eisenbach, M. (2011) 'Behavioral Mechanism during Human Sperm Chemotaxis: Involvement of Hyperactivation', *PLoS ONE*, 6. Available at: https://api.semanticscholar.org/CorpusID:1367538.

Asghari, A., Marashi, S.A. and Ansari-Pour, N. (2017) 'A sperm-specific proteome-scale metabolic network model identifies non-glycolytic genes for energy deficiency in asthenozoospermia', *Systems Biology in Reproductive Medicine*, 63(2), pp. 100–112. Available at: https://doi.org/10.1080/19396368.2016.1263367.

Asquith, K.L. *et al.* (2004) 'Tyrosine phosphorylation activates surface chaperones facilitating sperm-zona recognition', *Journal of Cell Science*, 117(16), pp. 3645–3657. Available at: https://doi.org/10.1242/jcs.01214.

AUSTIN, C.R. (1951) 'Observations on the penetration of the sperm in the mammalian egg', *Australian journal of scientific research. Ser. B: Biological sciences*, 4(4), pp. 581–596. Available at: https://doi.org/10.1071/bi9510581.

AUSTIN, C.R. (1952) 'The "Capacitation" of the Mammalian Sperm', *Nature*, 170(4321), p. 326. Available at: https://doi.org/10.1038/170326a0.

AUSTIN, C.R. and BISHOP, M.W. (1958) 'Role of the rodent acrosome and perforatorium in fertilization', *Proceedings of the Royal Society of London. Series B, Biological sciences*, 149(935), pp. 241–248. Available at: https://doi.org/10.1098/rspb.1958.0066.

Awda, B.J., Mackenzie-Bell, M. and Buhr, M.M. (2009) 'Reactive Oxygen Species and Boar Sperm Function1', *Biology of Reproduction*, 81(3), pp. 553–561. Available at: https://doi.org/10.1095/biolreprod.109.076471.

Baarends, W.M., van der Laan, R. and Grootegoed, J.A. (2001) 'DNA repair mechanisms and gametogenesis', *Reproduction (Cambridge, England)*, 121(1), pp. 31–39. Available at: https://doi.org/10.1530/rep.0.1210031.

Baillie, H.S. *et al.* (1997) 'Greater numbers of human spermatozoa associate with endosalpingeal cells derived from the isthmus compared with those from the ampulla.', *Human Reproduction*, 12(9), pp. 1985–1992. Available at: https://doi.org/10.1093/humrep/12.9.1985.

Baker, R. and Hughson, F. (2016) 'Chaperoning SNARE assembly and disassembly', *Nature Reviews Molecular Cell Biology*, 17. Available at: https://doi.org/10.1038/nrm.2016.65.

Baldi, E. *et al.* (1991) 'Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa', *Journal of andrology*, 12(5), pp. 323–330. Available at: http://europepmc.org/abstract/MED/1765568.

Baldi, E. *et al.* (1996) 'Human sperm activation during capacitation and acrosome reaction: role of calcium, protein phosphorylation and lipid remodelling pathways.', *Frontiers in bioscience : a journal and virtual library*. Available at: https://doi.org/10.2741/A125.

Baldi, E. (2000) 'Intracellular events and signaling pathways involved in sperm acquisition of fertilizing capacity and acrosome reaction', *Frontiers in Bioscience*, 5, pp. e110-123. Available at: https://doi.org/10.2741/A572.

Baltz, J.M., Oneeka Williams, P. and Cone, R.A. (1990) 'Dense Fibers Protect Mammalian Sperm Against Damage1', *Biology of Reproduction*, 43(3), pp. 485–491. Available at: https://doi.org/10.1095/biolreprod43.3.485.

Barratt, C., Andrews, P. and Gruber, F. (2019) *A phenotypic screening platform utilising human spermatozoa identifies new compounds with contraceptive activity*. Available at: https://doi.org/10.1101/808105.

BARRATT, C.L.R. and COOKE, I.D. (1991) 'Review Sperm transport in the human female reproductive tract—a dynamic interaction', *International Journal of Andrology*, pp. 394–411. Available at: https://doi.org/10.1111/j.1365-2605.1991.tb01268.x.

Barratt, C.L.R., De Jonge, C.J. and Sharpe, R.M. (2018) '"Man Up": the importance and strategy for placing male reproductive health centre stage in the political and research agenda', *Human Reproduction*, 33(4), pp. 541–545. Available at: https://doi.org/10.1093/humrep/dey020.

Barros, C. and Austin, C.R. (1967) 'In vitro fertilization and the sperm acrosome reaction in the hamster', *The Journal of experimental zoology*, 166(3), pp. 317–323. Available at: https://doi.org/10.1002/jez.1401660304.

Bartke, A. (1995) 'Apoptosis of male germ cells, a generalized or a cell type-specific phenomenon?', *Endocrinology*, 136(1), pp. 3–4. Available at: https://doi.org/10.1210/endo.136.1.7828545.

Bedu-Addo, K. *et al.* (2007) 'Patterns of [Ca2+]i mobilization and cell response in human spermatozoa exposed to progesterone', *Developmental Biology*, 302(1), pp. 324–332. Available at: https://doi.org/https://doi.org/10.1016/j.ydbio.2006.09.040.

Belmokhtar, C.A., Hillion, J. and Ségal-Bendirdjian, E. (2001) 'Staurosporine induces apoptosis through both caspase-dependent and caspase-independent mechanisms', *Oncogene*, 20(26), pp. 3354–3362. Available at: https://doi.org/10.1038/sj.onc.1204436.

Bencic, D.C., Cloud, J.G. and Ingermann, R.L. (2000) *Carbon dioxide reversibly inhibits sperm motility and fertilizing ability in steelhead (Oncorhynchus mykiss)*, *Fish Physiology and Biochemistry*.

Blanco, A. and Zinkham, W.H. (1963) 'Lactate dehydrogenases in human testes', *Science*, 139(3555), pp. 601–602. Available at: https://doi.org/10.1126/science.139.3555.601.

Boskey, E.R. *et al.* (2001) *Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source*, *Human Reproduction*.

Bouchard, P. *et al.* (1981) 'erythro-9-[3-(2-Hydroxynonyl)]adenine is an inhibitor of sperm motility that blocks dynein ATPase and protein carboxylmethylase activities', *Proceedings of the National Academy of Sciences of the United States of America*, 78(2), pp. 1033–1036. Available at: https://doi.org/10.1073/pnas.78.2.1033.

Brady, S.T. and Lau, L.-F. (2012) ‘Chapter 26 - Tyrosine Phosphorylation’, in S.T. Brady et al. (eds) *Basic Neurochemistry (Eighth Edition)*. New York: Academic Press, pp. 493–513. Available at: https://doi.org/https://doi.org/10.1016/B978-0-12-374947-5.00026-2.

Bragina, E.E. and Bocharova, E.N. (2017) ‘Ultrastructure of Spermatozoa from Infertility Patients’, in R. Meccariello and R. Chianese (eds) *Spermatozoa*. Rijeka: IntechOpen, p. Ch. 5. Available at: https://doi.org/10.5772/intechopen.71596.

Breitbart, H. (2002) ‘Role and regulation of intracellular calcium in acrosomal exocytosis’, *Journal of Reproductive Immunology*, 53(1), pp. 151–159. Available at: https://doi.org/https://doi.org/10.1016/S0165-0378(01)00085-7.

Breitbart, H. and Spungin, B. (1997) ‘The biochemistry of the acrosome reaction.’, *Molecular Human Reproduction*, 3(3), pp. 195–202. Available at: https://doi.org/10.1093/molehr/3.3.195.

Brener, E. *et al.* (2003) ‘Remodeling of the actin cytoskeleton during mammalian sperm capacitation and acrosome reaction’, *Biology of reproduction*, 68(3), pp. 837–845. Available at: https://doi.org/10.1095/biolreprod.102.009233.

Briz, M. and Fàbrega, A. (2013) ‘The Boar Spermatozoon’, in S. Bonet et al. (eds) *Boar Reproduction: Fundamentals and New Biotechnological Trends*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 3–47. Available at: https://doi.org/10.1007/978-3-642-35049-8_1.

Brown, J. *et al.* (1989) ‘A premature acrosome reaction is programmed by mouse t haplotypes during sperm differentiation and could play a role in transmission ratio distortion’, *Development*, 106(4), pp. 769–773. Available at: https://doi.org/10.1242/dev.106.4.769.

Buescher, J.M. *et al.* (2015) ‘A roadmap for interpreting 13C metabolite labeling patterns from cells’, *Current Opinion in Biotechnology*, 34, pp. 189–201. Available at: https://doi.org/https://doi.org/10.1016/j.copbio.2015.02.003.

Buffone, M.G. *et al.* (2004) ‘Human sperm subpopulations: relationship between functional quality and protein tyrosine phosphorylation’, *Human Reproduction*, 19(1), pp. 139–146. Available at: https://doi.org/10.1093/humrep/deh040.

Buffone, M.G. *et al.* (2009) ‘Capacitation-associated changes in membrane fluidity in asthenozoospermic human spermatozoa.’, *International journal of andrology*, 32 4, pp. 360–75. Available at: https://api.semanticscholar.org/CorpusID:44539787.

Burgess, S.A. *et al.* (2003) ‘Dynein structure and power stroke’, *Nature*, 421(6924), pp. 715–718. Available at: https://doi.org/10.1038/nature01377.

Burkman, L.J. (1984) ‘Characterization of Hyperactivated Motility by Human Spermatozoa During Capacitation: Comparison of Fertile and Oligozoospermic Sperm Populations’, *Archives of Andrology*, 13(2–3), pp. 153–165. Available at: https://doi.org/10.3109/01485018408987514.

Burkman, L.J. (1991) ‘Discrimination between nonhyperactivated and classical hyperactivated motility patterns in human spermatozoa using computerized analysis**Presented in part at the Serono Symposium on Fertilization in Mammals, Boston, Massachusetts, August 1 to 5, 1989; and at the 45th Annual Meeting of The American Fertility Society, San Francisco, California, November 13 to 16, 1989.’, *Fertility and Sterility*, 55(2), pp. 363–371. Available at: https://doi.org/https://doi.org/10.1016/S0015-0282(16)54131-4.

Burrello, N. *et al.* (2004) ‘Human follicular fluid stimulates the sperm acrosome reaction by interacting with the γ-aminobutyric acid receptors’, *Fertility and Sterility*, 82, pp. 1086–1090. Available at: https://doi.org/https://doi.org/10.1016/j.fertnstert.2004.04.028.

Bustani, G. and Baiee, F. (2021) 'Semen extenders: An evaluative overview of preservative mechanisms of semen and semen extenders', *Veterinary World*, 14, pp. 1220–1233. Available at: https://doi.org/10.14202/vetworld.2021.1220-1233.

Calvert, S.J. *et al.* (2018) 'Probing human sperm metabolism using 13C-magnetic resonance spectroscopy', *Molecular Human Reproduction*, 25(1), pp. 30–41. Available at: https://doi.org/10.1093/molehr/gay046.

Calvo, L. *et al.* (1993) 'Chemical composition and protein source in the capacitation medium significantly affect the ability of human spermatozoa to undergo follicular fluid induced acrosome reaction*', *Human Reproduction*, 8(4), pp. 575–580. Available at: https://doi.org/10.1093/oxfordjournals.humrep.a138099.

Carlson, A.E. *et al.* (2003) 'CatSper1 required for evoked Ca2+ entry and control of flagellar function in sperm', *Proceedings of the National Academy of Sciences of the United States of America*, 100(25), pp. 14864–14868. Available at: https://doi.org/10.1073/pnas.2536658100.

Chae, H.-J. *et al.* (2000) 'Molecular mechanism of staurosporine-induced apoptosis in osteoblasts', *Pharmacological Research*, 42(4), pp. 373–381. Available at: https://doi.org/https://doi.org/10.1006/phrs.2000.0700.

CHANG, M.C. (1951) 'Fertilizing Capacity of Spermatozoa deposited into the Fallopian Tubes', *Nature*, 168(4277), pp. 697–698. Available at: https://doi.org/10.1038/168697b0.

Chen, Y. *et al.* (2000) *Soluble Adenylyl Cyclase as an Evolutionarily Conserved Bicarbonate Sensor*, *Cold Spring Harbor Symp. Quant. Biol*. Available at: https://www.science.org.

Chinnaiyan, A.M. (1999) 'The Apoptosome: Heart and Soul of the Cell Death Machine', *Neoplasia*, 1(1), pp. 5–15. Available at: https://doi.org/https://doi.org/10.1038/sj.neo.7900003.

Chirasani, V. *et al.* (2019) 'A central core disease mutation in the Ca 2+ binding site of skeletal muscle ryanodine receptor impairs single channel regulation', *American Journal of Physiology-Cell Physiology*, 317. Available at: https://doi.org/10.1152/ajpcell.00052.2019.

Clarke, P.G.H. and Clarke, S. (1996) 'Nineteenth century research on naturally occurring cell death and related phenomena', *Anatomy and Embryology*, 193(2), pp. 81–99. Available at: https://doi.org/10.1007/BF00214700.

Codella, R. (2021) 'Techniques to Study Metabolism', in L. Luzi (ed.) *Thyroid, Obesity and Metabolism: Exploring Links Between Thyroid Function, Obesity, Metabolism and Lifestyle*. Cham: Springer International Publishing, pp. 117–129. Available at: https://doi.org/10.1007/978-3-030-80267-7_8.

Colás, C., Cebrián-Pérez, J.A. and Muiño-Blanco, T. (2010) 'Caffeine induces ram sperm hyperactivation independent of cAMP-dependent protein kinase', *International Journal of Andrology*, 33(1), pp. e187–e197. Available at: https://doi.org/https://doi.org/10.1111/j.1365-2605.2009.00991.x.

Comhaire, F.H. *et al.* (1988) *Accuracy of Sperm Characteristics in Predicting the in Vitro Fertilizing Capacity of Semen*, *Journal of in Vitro Fertilization and Embryo Transfer*.

Da Costa, R. *et al.* (2016) 'Cadmium inhibits motility, activities of plasma membrane Ca2+-ATPase and axonemal dynein-ATPase of human spermatozoa', *Andrologia*, 48(4), pp. 464–469. Available at: https://doi.org/https://doi.org/10.1111/and.12466.

Cox, C.M. *et al.* (2022) 'Infertility prevalence and the methods of estimation from 1990 to 2021: a systematic review and meta-analysis', *Human Reproduction Open*, 2022(4), p. hoac051. Available at: https://doi.org/10.1093/hropen/hoac051.

Cross, N.L. *et al.* (1986) 'Two simple methods for detecting acrosome-reacted human sperm', *Gamete Research*, 15(3), pp. 213–226. Available at: https://doi.org/10.1002/mrd.1120150303.

Dacheux, J.L., Chevrier, C. and Lanson, Y. (1987) 'Motility and Surface Transformations of Human Spermatozoa during Epidiymal Transita', *Annals of the New York Academy of Sciences*, 513(1), pp. 560–563. Available at: https://doi.org/https://doi.org/10.1111/j.1749-6632.1987.tb25107.x.

Dada, R. (2017) 'Sperm DNA damage diagnostics: when and why', *Translational andrology and urology*, 6(Suppl 4), pp. S691–S694. Available at: https://doi.org/10.21037/tau.2017.05.26.

Dadoune, J.-P. (2003) 'Expression of mammalian spermatozoal nucleoproteins', *Microscopy Research and Technique*, 61(1), pp. 56–75. Available at: https://doi.org/https://doi.org/10.1002/jemt.10317.

Dahan, T. and Breitbart, H. (2022) 'Involvement of metabolic pathway in the sperm spontaneous acrosome reaction', *Theriogenology*, 192, pp. 38–44. Available at: https://doi.org/https://doi.org/10.1016/j.theriogenology.2022.08.018.

Dasgupta, S., Mills, C.L. and Fraser, L.R. (1993) *Ca2+-related changes in the capacitation state of human spermatozoa assessed by a chlortetracycline fluorescence assay*.

David, A., Serr, D.M. and Czernobilsky, B. (1973) 'Chemical Composition of Human Oviduct Fluid', *Fertility and Sterility*, 24(6), pp. 435–439. Available at: https://doi.org/10.1016/S0015-0282(16)39731-X.

Ded, L. *et al.* (2019) 'Fluorescent analysis of boar sperm capacitation process in vitro', *Reproductive Biology and Endocrinology*, 17(1), p. 109. Available at: https://doi.org/10.1186/s12958-019-0554-z.

Doğan, Ş. *et al.* (2012) 'Interrelationships Between Apoptosis and Fertility in Bull Sperm', *The Journal of reproduction and development*, 59. Available at: https://doi.org/10.1262/jrd.2012-068.

Dona, A.C. *et al.* (2016) 'A guide to the identification of metabolites in NMR-based metabonomics/metabolomics experiments', *Computational and Structural Biotechnology Journal*, 14, pp. 135–153. Available at: https://doi.org/https://doi.org/10.1016/j.csbj.2016.02.005.

Dean, M. (2019). Glycogen in the uterus and fallopian tubes is an important source of glucose during early pregnancy†. Biology of Reproduction, 101(2), 297–305. https://doi.org/10.1093/biolre/ioz102

Dutta, S. *et al.* (2020) 'Physiological Role of ROS in Sperm Function', in S.J. Parekattil, S.C. Esteves, and A. Agarwal (eds) *Male Infertility: Contemporary Clinical Approaches, Andrology, ART and Antioxidants*. Cham: Springer International Publishing, pp. 337–345. Available at: https://doi.org/10.1007/978-3-030-32300-4_27.

Eguchi, Y. *et al.* (1999) 'ATP-dependent Steps in Apoptotic Signal Transduction1', *Cancer Research*, 59(9), pp. 2174–2181.

Eguchi, Y., Shimizu, S. and Tsujimoto, Y. (1997) 'Intracellular ATP Levels Determine Cell Death Fate by Apoptosis or Necrosis1', *Cancer Research*, 57(10), pp. 1835–1840.

Ehrenwald, E., Foote, R.H. and Parks, J.E. (1990) 'Bovine oviductal fluid components and their potential role in sperm cholesterol efflux', *Molecular Reproduction and Development*, 25. Available at: https://api.semanticscholar.org/CorpusID:25863949.

Eley, A. *et al.* (2005) 'Apoptosis of ejaculated human sperm is induced by co-incubation with Chlamydia trachomatis lipopolysaccharide', *Human Reproduction*, 20(9), pp. 2601–2607. Available at: https://doi.org/10.1093/humrep/dei082.

Eliasson, L. *et al.* (1997) 'Rapid ATP-dependent priming of secretory granules precedes Ca(2+)-induced exocytosis in mouse pancreatic B-cells', *The Journal of physiology*, 503 (Pt 2), pp. 399–412. Available at:

https://doi.org/10.1111/j.1469-7793.1997.399bh.x.

Elmore, S. (2007) *Apoptosis: A Review of Programmed Cell Death*.

Emwas, A.-H.M. (2015) 'The Strengths and Weaknesses of NMR Spectroscopy and Mass Spectrometry with Particular Focus on Metabolomics Research', in J.T. Bjerrum (ed.) *Metabonomics: Methods and Protocols*. New York, NY: Springer New York, pp. 161–193. Available at: https://doi.org/10.1007/978-1-4939-2377-9_13.

Engel, K.M., Springsguth, C.H. and Grunewald, S. (2018) 'What happens to the unsuccessful spermatozoa?', *Andrology*, 6(2), pp. 335–344. Available at: https://doi.org/10.1111/andr.12467.

Fabro, G. *et al.* (2002) 'Chemotaxis of capacitated rabbit spermatozoa to follicular fluid revealed by a novel directionality-based assay', *Biology of reproduction*, 67(5), pp. 1565–1571. Available at: https://doi.org/10.1095/biolreprod.102.006395.

Fawcett, D.W. (1975) 'The mammalian spermatozoon', *Developmental Biology*, 44(2), pp. 394–436. Available at: https://doi.org/https://doi.org/10.1016/0012-1606(75)90411-X.

Feng, G. and Kaplowitz, N. (2002) 'Mechanism of staurosporine-induced apoptosis in murine hepatocytes'. Available at: https://doi.org/10.1152/ajpgi.00467.

Ferramosca, A. *et al.* (2008) 'Oxygen uptake by mitochondria in demembranated human spermatozoa: A reliable tool for the evaluation of sperm respiratory efficiency', *International Journal of Andrology*, 31(3), pp. 337–345. Available at: https://doi.org/10.1111/j.1365-2605.2007.00775.x.

Ferramosca, A. *et al.* (2012) 'Mitochondrial respiratory efficiency is positively correlated with human sperm motility', *Urology*, 79(4), pp. 809–814. Available at: https://doi.org/10.1016/j.urology.2011.12.042.

Ferrandi, B. *et al.* (1987) 'Cytochemical study on human spermatozoa metabolism during in vitro capacitation.', *Andrologia*, 19 Spec No, pp. 278–283. Available at: https://doi.org/10.1111/j.1439-0272.1987.tb02346.x.

Fischer, B. and Bavister, B.D. (1993) 'Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits', *Reproduction*, 99(2), pp. 673–679. Available at: https://doi.org/10.1530/jrf.0.0990673.

Fitzpatrick, J., Garcia-Gonzalez, F. and Evans, J. (2010) 'Linking sperm length and velocity: The importance of intramale variation', *Biology letters*, 6, pp. 797–799. Available at: https://doi.org/10.1098/rsbl.2010.0231.

Ford, W.C.L. (2006) 'Glycolysis and sperm motility: Does a spoonful of sugar help the flagellum go round?', *Human Reproduction Update*, pp. 269–274. Available at: https://doi.org/10.1093/humupd/dmi053.

Ford, W.C.L. and Harrison, A. (1981) 'The role of oxidative phosphorylation in the generation of ATP in human spermatozoa', *Journal of Reproduction and Fertility*, 63(1), pp. 271–278. Available at: https://doi.org/10.1530/jrf.0.0630271.

Foresta, C. *et al.* (1992) 'Progesterone induces capacitation in human spermatozoa', *Andrologia*, 24(1), pp. 33–35. Available at: https://doi.org/https://doi.org/10.1111/j.1439-0272.1992.tb02605.x.

Foresta, C. *et al.* (1996) 'Mechanism of human sperm activation by extracellular ATP', *American Journal of Physiology-Cell Physiology*, 270(6), pp. C1709–C1714. Available at: https://doi.org/10.1152/ajpcell.1996.270.6.C1709.

Foresta, C., Rossato, M. and Di Virgilio, F. (1992) 'Extracellular ATP is a trigger for the acrosome reaction in human spermatozoa.', *Journal of Biological Chemistry*, 267(27), pp. 19443–19447. Available at: https://doi.org/10.1016/S0021-9258(18)41795-4.

Fraser, L.R. (1984) 'Mouse sperm capacitation in vitro involves loss of a surface-associated inhibitory

component', *Journal of reproduction and fertility*, 72(2), pp. 373–384. Available at: https://doi.org/10.1530/jrf.0.0720373.

Freitas, M.J., Vijayaraghavan, S. and Fardilha, M. (2017) 'Signaling mechanisms in mammalian sperm motility', *Biology of Reproduction*. Society for the Study of Reproduction, pp. 2–12. Available at: https://doi.org/10.1095/biolreprod.116.144337.

Freitas-Martins, A. *et al.* (2023) 'Metabolic characterization of human sperm cells using the Seahorse metabolic flux analyzer', *Andrology*, n/a(n/a). Available at: https://doi.org/https://doi.org/10.1111/andr.13486.

Frolikova, M. *et al.* (2016) 'Characterization of CD46 and β1 integrin dynamics during sperm acrosome reaction', *Scientific Reports*, 6. Available at: https://api.semanticscholar.org/CorpusID:15844256.

Fukami, Y. and Lipmann, F. (1983) 'Reversal of Rous sarcoma-specific immunoglobulin phosphorylation on tyrosine (ADP as phosphate acceptor) catalyzed by the src gene kinase.', *Proceedings of the National Academy of Sciences*, 80(7), pp. 1872–1876. Available at: https://doi.org/10.1073/pnas.80.7.1872.

Gao, X. *et al.* (2023) 'Extracellular ATP-induced calcium oscillations regulating the differentiation of osteoblasts through aerobic oxidation metabolism pathways', *Journal of Bone and Mineral Metabolism*, 41(5), pp. 606–620. Available at: https://doi.org/10.1007/s00774-023-01449-4.

Gardner, D.K. *et al.* (1996) 'Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells**Supported by IVF America Inc., Greenwich, Connecticut and Monash IVF Pty. Ltd., Melbourne, Victoria, Australia.', *Fertility and Sterility*, 65(2), pp. 349–353. Available at: https://doi.org/https://doi.org/10.1016/S0015-0282(16)58097-2.

Gatica, L. V *et al.* (2013) 'Picomolar gradients of progesterone select functional human sperm even in subfertile samples', *Molecular human reproduction*, 19(9), pp. 559–569. Available at: https://doi.org/10.1093/molehr/gat037.

Gelbaya, T.A. *et al.* (2014) 'Definition and Epidemiology of Unexplained Infertility', *Obstetrical & Gynecological Survey*, 69(2). Available at: https://journals.lww.com/obgynsurvey/fulltext/2014/02000/definition_and_epidemiology_of_unexplained.17.aspx.

Gerst, J.E. (1999) 'SNAREs and SNARE regulators in membrane fusion and exocytosis', *Cellular and Molecular Life Sciences CMLS*, 55(5), pp. 707–734. Available at: https://doi.org/10.1007/s000180050328.

GLUCKSMANN, A. (1951) 'Cell deaths in normal vertebrate ontogeny', *Biological reviews of the Cambridge Philosophical Society*, 26(1), p. 59—86. Available at: https://doi.org/10.1111/j.1469-185x.1951.tb00774.x.

Glunde, K., Jie, C. and Bhujwalla, Z.M. (2004) 'Molecular causes of the aberrant choline phospholipid metabolism in breast cancer', *Cancer research*, 64(12), pp. 4270–4276. Available at: https://doi.org/10.1158/0008-5472.can-03-3829.

Goldberg, E. (1963) *Lactic and Malic Dehydrogenases in Human Spermatozoa*, *Source: Science, New Series*.

Gómez-Torres, M.J. *et al.* (2015) 'Metabolites involved in cellular communication among human cumulus-oocyte-complex and sperm during in vitro fertilization', *Reproductive biology and endocrinology : RB&E*, 13, p. 123. Available at: https://doi.org/10.1186/s12958-015-0118-9.

González-Marín, C., Gosálvez, J. and Roy, R. (2012) 'Types, Causes, Detection and Repair of DNA Fragmentation in Animal and Human Sperm Cells', *International Journal of Molecular Sciences*, 13(11), pp. 14026–14052. Available at: https://doi.org/10.3390/ijms131114026.

Goodson, S.G. *et al.* (2017) 'CASAnova: a multiclass support vector machine model for the classification of

human sperm motility patterns†', *Biology of Reproduction*, 97(5), pp. 698–708. Available at: https://doi.org/10.1093/biolre/iox120.

Gray, L.R., Tompkins, S.C. and Taylor, E.B. (2014) 'Regulation of pyruvate metabolism and human disease', *Cellular and Molecular Life Sciences*, 71(14), pp. 2577–2604. Available at: https://doi.org/10.1007/s00018-013-1539-2.

Griswold, M.D. (1995) 'Interactions Between Germ Cells and Sertoli Cells in the Testis', *Biology of Reproduction*, 52(2), pp. 211–216. Available at: https://doi.org/10.1095/biolreprod52.2.211.

Grunewald, S. *et al.* (2005) 'Mature human spermatozoa do not transcribe novel RNA', *Andrologia*, 37(2–3), pp. 69–71. Available at: https://doi.org/https://doi.org/10.1111/j.1439-0272.2005.00656.x.

Grunewald, S., Paasch, U. and Glander, H.-J. (2001) 'Enrichment of non–apoptotic human spermatozoa after cryopreservation by immunomagnetic cell sorting', *Cell and Tissue Banking*, 2(3), pp. 127–133. Available at: https://doi.org/10.1023/A:1020188913551.

Guo, M. *et al.* (2021) 'Apoptosis detection: a purpose-dependent approach selection', *Cell cycle (Georgetown, Tex.)*, 20(11), pp. 1033–1040. Available at: https://doi.org/10.1080/15384101.2021.1919830.

Hammond, J. (1934) 'The Fertilisation of Rabbit Ova in Relation to Time: A Method of Controlling the Litter Size, the Duration of Pregnancy and the Weight of the Young at Birth', *Journal of Experimental Biology*, 11(2), pp. 140–161. Available at: https://doi.org/10.1242/jeb.11.2.140.

Harper, C. V, Barratt, C.L.R. and Publicover, S.J. (2004) 'Stimulation of human spermatozoa with progesterone gradients to simulate approach to the oocyte. Induction of [Ca(2+)](i) oscillations and cyclical transitions in flagellar beating', *The Journal of biological chemistry*, 279(44), pp. 46315–46325. Available at: https://doi.org/10.1074/jbc.m401194200.

Harper, C. V and Publicover, S.J. (2005) 'Reassessing the role of progesterone in fertilization—compartmentalized calcium signalling in human spermatozoa?', *Human Reproduction*, 20(10), pp. 2675–2680. Available at: https://doi.org/10.1093/humrep/dei158.

Harrison, D.A., Carr, D.W. and Meizel, S. (2000) 'Involvement of Protein Kinase A and A Kinase Anchoring Protein in the Progesterone-Initiated Human Sperm Acrosome Reaction1', *Biology of Reproduction*, 62(3), pp. 811–820. Available at: https://doi.org/10.1095/biolreprod62.3.811.

Hatefi, Y. (1985) 'The mitochondrial electron transport and oxidative phosphorylation system'.

Hengartner, M.O. (2000) 'The biochemistry of apoptosis', *Nature*, 407(6805), pp. 770–776. Available at: https://doi.org/10.1038/35037710.

Hereng, T.H. *et al.* (2011) 'Exogenous pyruvate accelerates glycolysis and promotes capacitation in human spermatozoa', *Human Reproduction*, 26(12), pp. 3249–3263. Available at: https://doi.org/10.1093/humrep/der317.

Hereng, T H *et al.* (2014) 'Serum albumin and HCO3- regulate separate pools of ATP in human spermatozoa.', *Human reproduction (Oxford, England)*, 29(5), pp. 918–930. Available at: https://doi.org/10.1093/humrep/deu028.

Hess, K.C. *et al.* (2005) 'The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization', *Developmental Cell*, 9(2), pp. 249–259. Available at: https://doi.org/10.1016/j.devcel.2005.06.007.

Hicks, J.J. *et al.* (1972) 'Metabolic changes in human spermatozoa related to capacitation.', *Fertility and sterility*, 23(3), pp. 172–179. Available at: https://doi.org/10.1016/s0015-0282(16)38822-7.

Hirata, S. *et al.* (2002) 'Spermatozoon and mitochondrial DNA', *Reproductive Medicine and Biology*, 1(2), pp. 41–47. Available at: https://doi.org/10.1046/j.1445-5781.2002.00007.x.

Ho, H.C., Granish, K.A. and Suarez, S.S. (2002) 'Hyperactivated Motility of Bull Sperm Is Triggered at the Axoneme by Ca2+ and Not cAMP', *Developmental Biology*, 250(1), pp. 208–217. Available at: https://doi.org/10.1006/DBIO.2002.0797.

Ho, H.-C. and Suarez, S.S. (2001) 'An Inositol 1,4,5-Trisphosphate Receptor-Gated Intracellular Ca2+ Store Is Involved in Regulating Sperm Hyperactivated Motility', *Biology of Reproduction*, 65(5), pp. 1606–1615. Available at: https://doi.org/10.1095/biolreprod65.5.1606.

Hong, C.Y., Chiang, B.N. and Turner, P. (1984) 'Calcium ion is the key regulator of human sperm function', *The Lancet*, 324(8417–8418), pp. 1449–1451. Available at: https://doi.org/10.1016/S0140- 6736(84)91634-9.

Hosseinzadeh, S. *et al.* (2000) 'Coincubation of human spermatozoa with Chlamydia trachomatis in vitro causes increased tyrosine phosphorylation of sperm proteins', *Infection and immunity*, 68(9), pp. 4872–4876. Available at: https://doi.org/10.1128/iai.68.9.4872-4876.2000.

Hu, K. and Yu, Y. (2017) 'Metabolite availability as a window to view the early embryo microenvironment in vivo', *Molecular reproduction and development*, 84(10), pp. 1027–1038. Available at: https://doi.org/10.1002/mrd.22868.

Hunter, R.H.F., Cook, B. and Poyser, N.L. (1983) 'Regulation of oviduct function in pigs by local transfer of ovarian steroids and prostaglandins: a mechanism to influence sperm transport', *European Journal of Obstetrics & Gynecology and Reproductive Biology*, 14(4), pp. 225–232. Available at: https://doi.org/https://doi.org/10.1016/0028-2243(83)90264-2.

Inaba, K. and Mizuno, K. (2016) 'Sperm dysfunction and ciliopathy', *Reproductive Medicine and Biology*, 15(2), pp. 77–94. Available at: https://doi.org/10.1007/s12522-015-0225-5.

Ingermann, R. *et al.* (2002) 'Carbon dioxide and pH affect sperm motility of white sturgeon (Acipenser transmontanus)', *The Journal of experimental biology*, 205, pp. 2885–2890. Available at: https://doi.org/10.1242/jeb.205.18.2885.

Ishijima, S. *et al.* (2006) 'Hyperactivation of monkey spermatozoa is triggered by Ca2+ and completed by cAMP', *Molecular Reproduction and Development*, 73(9), pp. 1129–1139. Available at: https://doi.org/https://doi.org/10.1002/mrd.20420.

Ito, N., Ruegg, U.T. and Takeda, S. (2018) 'ATP-Induced Increase in Intracellular Calcium Levels and Subsequent Activation of mTOR as Regulators of Skeletal Muscle Hypertrophy', *International Journal of Molecular Sciences*, 19(9). Available at: https://doi.org/10.3390/ijms19092804.

Jaiswal, B.S. *et al.* (1998) 'Sperm capacitation is, after all, a prerequisite for both partial and complete acrosome reaction', *FEBS Letters*, 427(2), pp. 309–313. Available at: https://doi.org/10.1016/S0014-5793(98)00455-4.

Jaiswal, B.S., Eisenbach, M. and Tur-Kaspa, I. (1999) 'Detection of partial and complete acrosome reaction in human spermatozoa: which inducers and probes to use?', *Molecular Human Reproduction*, 5(3), pp. 214–219. Available at: https://doi.org/10.1093/molehr/5.3.214.

Jawad, A. *et al.* (2023) 'Myo-inositol improves the viability of boar sperm during liquid storage', *Frontiers in Veterinary Science*, 10. Available at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1150984.

Johnson, G.P. *et al.* (2017) 'Genomic identification, expression profiling, and functional characterization of CatSper channels in the bovine†', *Biology of Reproduction*, 97(2), pp. 302–312. Available at: https://doi.org/10.1093/biolre/iox082.

Jones, A.R. and Bubb, W.A. (2000) 'Substrates for endogenous metabolism by mature boar spermatozoa', *Journal of reproduction and fertility*, 119(1), pp. 129–135. Available at: https://doi.org/10.1530/jrf.0.1190129.

De Jonge, C. (2005) 'Biological basis for human capacitation', *Human Reproduction Update*, 11(3), pp. 205–214. Available at: https://doi.org/10.1093/humupd/dmi010.

Karaman, M.W. *et al.* (2008) 'A quantitative analysis of kinase inhibitor selectivity', *Nature Biotechnology*, 26(1), pp. 127–132. Available at: https://doi.org/10.1038/nbt1358.

Karhapää, L. and Törnquist, K. (1997) 'Effects of caffeine on the influx of extracellular calcium in GH4C1 pituitary cells', *Journal of Cellular Physiology*, 171(1), pp. 52–60. Available at: https://doi.org/https://doi.org/10.1002/(SICI)1097-4652(199704)171:1<52::AID-JCP7>3.0.CO;2-G.

Kasatkina, L.A. (2016) '4-Aminopyridine sequesters intracellular Ca2+ which triggers exocytosis in excitable and non-excitable cells', *Scientific Reports*, 6. Available at: https://api.semanticscholar.org/CorpusID:1066142.

Kerr, J.F.R., Wyllie, A.H. and Currie, A.R. (1972) 'Apoptosis: A Basic Biological Phenomenon with Wideranging Implications in Tissue Kinetics', *British Journal of Cancer*, 26(4), pp. 239–257. Available at: https://doi.org/10.1038/bjc.1972.33.

Keyser, S., van der Horst, G. and Maree, L. (2021) 'Progesterone, Myo-Inositol, Dopamine and Prolactin Present in Follicular Fluid Have Differential Effects on Sperm Motility Subpopulations', *Life*, 11(11). Available at: https://doi.org/10.3390/life11111250.

Keyser, S., van der Horst, G. and Maree, L. (2022) 'New Approaches to Define The Functional Competency of Human Sperm Subpopulations and Its Relationship to Semen Quality', *International Journal of Fertility & Sterility*, 16, pp. 140–151. Available at: https://api.semanticscholar.org/CorpusID:251866178.

Kichine, E. *et al.* (2013) 'Analysis of the sperm head protein profiles in fertile men: consistency across time in the levels of expression of heat shock proteins and peroxiredoxins', *PloS one*, 8(10), p. e77471. Available at: https://doi.org/10.1371/journal.pone.0077471.

Kim, C. *et al.* (2021) 'Extreme parsimony in ATP consumption by 20S complexes in the global disassembly of single SNARE complexes', *Nature Communications*, 12. Available at: https://api.semanticscholar.org/CorpusID:235241539.

Kim, K.S., Foster, J.A. and Gerton, G.L. (2001) 'Differential release of guinea pig sperm acrosomal components during exocytosis', *Biology of reproduction*, 64(1), pp. 148–156. Available at: https://doi.org/10.1095/biolreprod64.1.148.

Kim, Y.-H. *et al.* (2007) 'Compartmentalization of a unique ADP/ATP carrier protein SFEC (Sperm Flagellar Energy Carrier, AAC4) with glycolytic enzymes in the fibrous sheath of the human sperm flagellar principal piece', *Developmental biology*, 302(2), pp. 463–476. Available at: https://doi.org/10.1016/j.ydbio.2006.10.004.

Kirichok, Y., Navarro, B. and Clapham, D.E. (2006) 'Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca2+ channel', *Nature*, 439(7077), pp. 737–740. Available at: https://doi.org/10.1038/nature04417.

Klenchin, V. and Martin, T. (2000) 'Klenchin, V. A. & Martin, T. F. Priming in exocytosis: attaining fusion-competence after vesicle docking. Biochimie 82, 399-407', *Biochimie*, 82, pp. 399–407. Available at: https://doi.org/10.1016/S0300-9084(00)00208-X.

Köhn, F.M. *et al.* (1997) 'Detection of human sperm acrosome reaction: comparison between methods using double staining, Pisum sativum agglutinin, concanavalin A and transmission electron microscopy', *Human reproduction (Oxford, England)*, 12(4), pp. 714–721. Available at: https://doi.org/10.1093/humrep/12.4.714.

Krisfalusi, M. *et al.* (2006) 'Multiple Glycolytic Enzymes Are Tightly Bound to the Fibrous Sheath of Mouse Spermatozoa', *Biology of Reproduction*, 75(2), pp. 270–278. Available at: https://doi.org/10.1095/biolreprod.105.049684.

Kroemer, G., Zamzami, N. and Susin, S.A. (1997) 'Mitochondrial control of apoptosis', *Immunology Today*, 18(1), pp. 44–51. Available at: https://doi.org/10.1016/S0167-5699(97)80014-X.

Kumar, R., Saneja, A. and Panda, A.K. (2021) 'An Annexin V-FITC—Propidium Iodide-Based Method for Detecting Apoptosis in a Non-Small Cell Lung Cancer Cell Line', in P.G. Santiago-Cardona (ed.) *Lung Cancer: Methods and Protocols*. New York, NY: Springer US, pp. 213–223. Available at: https://doi.org/10.1007/978-1-0716-1278-1_17.

Kumar, V., Kota, V. and Shivaji, S. (2008) 'Hamster sperm capacitation: role of pyruvate dehydrogenase A and dihydrolipoamide dehydrogenase', *Biology of reproduction*, 79(2), pp. 190–199. Available at: https://doi.org/10.1095/biolreprod.107.066704.

Kurhanewicz, J. *et al.* (2008) 'Current and potential applications of clinical 13C MR spectroscopy', *Journal of nuclear medicine : official publication, Society of Nuclear Medicine*, 49(3), pp. 341–344. Available at: https://doi.org/10.2967/jnumed.107.045112.

De Lamirande, E. and O'Flaherty, C. (2012) 'Sperm capacitation as an oxidative event', in *Studies on Men's Health and Fertility*. Humana Press Inc., pp. 57–94. Available at: https://doi.org/10.1007/978-1-61779-776-7_4.

de LAMIRANDE, E.V.E., HARAKAT, A. and GAGNON, C. (1998) 'Human Sperm Capacitation Induced by Biological Fluids and Progesterone, but Not by NADH or NADPH, Is Associated With the Production of Superoxide Anion', *Journal of Andrology*, 19(2), pp. 215–225. Available at: https://doi.org/https://doi.org/10.1002/j.1939-4640.1998.tb01991.x.

Langlais, J. *et al.* (1981) 'Localization of cholesteryl sulfate in human spermatozoa in support of a hypothesis for the mechanism of capacitation.', *Proceedings of the National Academy of Sciences*, 78(12), pp. 7266–7270. Available at: https://doi.org/10.1073/pnas.78.12.7266.

Leese, H.J. *et al.* (2007) 'Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease', *Reproduction, Fertility and Development*, 20(1), pp. 1–8. Available at: https://doi.org/10.1071/RD07153.

Leslie, S.W., Soon-Sutton, T.L. and Khan, M.A.B. (2022) *Male Infertility*. StatPearls Publishing, Treasure Island (FL). Available at: http://europepmc.org/abstract/MED/32965929.

Lestari, Silvia Werdhy *et al.* (2018) 'Sperm Na+ K+-ATPase and Ca2+-ATPase Activities: A Potential Predictive Parameter of Sperm Motility Disorder in Infertile Men', *Biomedical and Pharmacology Journal*, 11, pp. 411–416. Available at: https://api.semanticscholar.org/CorpusID:51759549.

Lestari, Silvia W. *et al.* (2018) 'Sperm Na + ,K + -ATPase and dynein ATPase activity: A study of embryo development in in vitro fertilization (IVF)', in *AIP Conference Proceedings*. American Institute of Physics Inc. Available at: https://doi.org/10.1063/1.5096752.

Li, H.-G. *et al.* (2007) 'Expression of CatSper family transcripts in the mouse testis during post-natal development and human ejaculated spermatozoa: relationship to sperm motility', *Molecular human reproduction*, 13(5), pp. 299–306. Available at: https://doi.org/10.1093/molehr/gam009.

Li, Y. *et al.* (2023) 'IKBA phosphorylation governs human sperm motility through ACC-mediated fatty acid beta-oxidation', *Communications Biology*, 6(1), p. 323. Available at: https://doi.org/10.1038/s42003-023-04693-6.

Lin, X. *et al.* (2020) 'Glucose Metabolism on Tumor Plasticity, Diagnosis, and Treatment', *Frontiers in Oncology*, 10. Available at: https://doi.org/10.3389/fonc.2020.00317.

Linck, R., Chemes, H. and Albertini, D. (2016) 'The axoneme: the propulsive engine of spermatozoa and cilia and associated ciliopathies leading to infertility', *Journal of Assisted Reproduction and Genetics*, 33. Available at: https://doi.org/10.1007/s10815-016-0652-1.

Lishko, P. V, Botchkina, I.L. and Kirichok, Y. (2011) 'Progesterone activates the principal Ca2+ channel of human sperm', *Nature*, 471(7338), pp. 387–391. Available at: https://doi.org/10.1038/nature09767.

Liu, X. *et al.* (2019) 'Aberrant expression of sperm-specific glycolytic enzymes are associated with poor sperm quality.', *Molecular medicine reports*, 19(4), pp. 2471–2478. Available at: https://doi.org/10.3892/mmr.2019.9926.

Locasale, J.W. and Cantley, L.C. (2011) 'Metabolic Flux and the Regulation of Mammalian Cell Growth', *Cell Metabolism*, 14(4), pp. 443–451. Available at: https://doi.org/https://doi.org/10.1016/j.cmet.2011.07.014.

Lodge, J.R., Graves, C.N. and Salisbury, G.W. (1968) 'Carbon Dioxide in Anaerobic Spermatozoan Metabolism', *Journal of Dairy Science*, 51(1), pp. 96–103. Available at: https://doi.org/10.3168/JDS.S0022-0302(68)86927-9.

Lornage, J., Guerin, J.F. and Czyba, J.C. (1986) 'Glycolytic activity of human spermatozoa in normospermic men and in men with abnormal spermograms', *Systems Biology in Reproductive Medicine*, 16(1), pp. 81–88. Available at: https://doi.org/10.3109/01485018608986926.

Luconi, M. *et al.* (1996) 'Extracellular Calcium Negatively Modulates Tyrosine Phosphorylation and Tyrosine Kinase Activity during Capacitation of Human Spermatozoa1', *Biology of Reproduction*, 55(1), pp. 207–216. Available at: https://doi.org/10.1095/biolreprod55.1.207.

Luria, A. *et al.* (2002) 'Extracellular Adenosine Triphosphate Stimulates Acrosomal Exocytosis in Bovine Spermatozoa via P2 Purinoceptor', *Biology of Reproduction*, 66(2), pp. 429–437. Available at: https://doi.org/10.1095/biolreprod66.2.429.

Macleod˜, J. (1943) 'The role of oxygen in the metabolism and motility of human spermatozoa' Available at: http://ajplegacy.physiology.org/.

Mahé, C. *et al.* (2021) 'Sperm migration, selection, survival, and fertilizing ability in the mammalian oviduct†', *Biology of Reproduction*, 105(2), pp. 317–331. Available at: https://doi.org/10.1093/biolre/ioab105.

Maione, B. *et al.* (1997) 'Activation of endogenous nucleases in mature sperm cells upon interaction with exogenous DNA', *DNA and cell biology*, 16(9), p. 1087—1097. Available at: https://doi.org/10.1089/dna.1997.16.1087.

Makler, A. *et al.* (1992) 'Use of a sealed mini-chamber to investigate human sperm motility in real time under aerobic and anaerobic conditions', *Systems Biology in Reproductive Medicine*, 29(3), pp. 255–261. Available at: https://doi.org/10.3109/01485019208987733.

Malsy, M. *et al.* (2019) 'Staurosporine induces apoptosis in pancreatic carcinoma cells PaTu 8988t and Panc-1 via the intrinsic signaling pathway', *European Journal of Medical Research*, 24(1), p. 5. Available at: https://doi.org/10.1186/s40001-019-0365-x.

Mandal, P.K. *et al.* (2022) 'In Vivo 13C Magnetic Resonance Spectroscopy for Assessing Brain Biochemistry in Health and Disease', *Neurochemical Research*, 47(5), pp. 1183–1201. Available at: https://doi.org/10.1007/s11064-022-03538-8.

MANN, T. (1946) 'Studies on the metabolism of semen; fructose as a normal constituent of seminal plasma; site of formation and function of fructose in semen', *The Biochemical journal*, 40(4), pp. 481–491. Available at: https://doi.org/10.1042/bj0400481.

Mann, T.R.R. and Lutwak-Mann, C. (1981) 'Male Reproductive Function and Semen: Themes and Trends in Physiology, Biochemistry and Investigative Andrology', in. Available at: https://api.semanticscholar.org/CorpusID:83032772.

Mannowetz, N., Miller, M.R. and Lishko, P. V (2017) 'Regulation of the sperm calcium channel CatSper by endogenous steroids and plant triterpenoids', *Proceedings of the National Academy of Sciences*, 114(22), pp. 5743–5748. Available at: https://doi.org/10.1073/pnas.1700367114.

Marin, S., Chiang, K., Bassilian, S., Lee, W.-N.P., *et al.* (2003) 'Metabolic strategy of boar spermatozoa revealed by a metabolomic characterization', *FEBS Letters*, 554(3), pp. 342–346. Available at: https://doi.org/https://doi.org/10.1016/S0014-5793(03)01185-2.

Marín-Briggiler, C.I. *et al.* (2021) 'Human Sperm Remain Motile After a Temporary Energy Restriction but do Not Undergo Capacitation-Related Events', *Frontiers in Cell and Developmental Biology*, 9. Available at: https://doi.org/10.3389/fcell.2021.777086.

Marıń -Briggiler, C.I. *et al.* (2002) 'Effect of incubating human sperm at room temperature on capacitation-related events', *Fertility and Sterility*, 77(2), pp. 252–259. Available at: https://doi.org/https://doi.org/10.1016/S0015-0282(01)02982-X.

Marquez, B. and Suarez, S.S. (2004) 'Different Signaling Pathways in Bovine Sperm Regulate Capacitation and Hyperactivation1', *Biology of Reproduction*, 70(6), pp. 1626–1633. Available at: https://doi.org/10.1095/biolreprod.103.026476.

Marquez, B. and Suarez, S.S. (2007) 'Bovine Sperm Hyperactivation Is Promoted by Alkaline-Stimulated Ca2+ Influx1', *Biology of Reproduction*, 76(4), pp. 660–665. Available at: https://doi.org/10.1095/biolreprod.106.055038.

McBride, H.M., Neuspiel, M. and Wasiak, S. (2006) 'Mitochondria: More Than Just a Powerhouse', *Current Biology*, 16(14), pp. R551–R560. Available at: https://doi.org/https://doi.org/10.1016/j.cub.2006.06.054.

McCarthy, N.J. and Evan, G.I. (1997) '15 Methods for Detecting and Quantifying Apoptosis', in F. de Pablo, A. Ferrús, and C.D. Stern (eds) *Current Topics in Developmental Biology*. Academic Press, pp. 259–278. Available at: https://doi.org/https://doi.org/10.1016/S0070-2153(08)60507-4.

Medrano, A. *et al.* (2006) 'Utilization of citrate and lactate through a lactate dehydrogenase and ATP-regulated pathway in boar spermatozoa', *Molecular reproduction and development*, 73, pp. 369–378. Available at: https://doi.org/10.1002/mrd.20414.

Mejia, E.M. and Hatch, G.M. (2016) 'Mitochondrial phospholipids: role in mitochondrial function', *Journal of Bioenergetics and Biomembranes*, 48(2), pp. 99–112. Available at: https://doi.org/10.1007/s10863-015-9601-4.

Melchior, F. and Gerace, L. (1995) 'Mechanisms of nuclear protein import', *Current Opinion in Cell Biology*, 7(3), pp. 310–318. Available at: https://doi.org/https://doi.org/10.1016/0955-0674(95)80084-0.

Michaut, M. *et al.* (2000) *Calcium-triggered acrosomal exocytosis in human spermatozoa requires the coordinated activation of Rab3A and N-ethylmaleimide-sensitive factor*. Available at: www.pnas.orgcgidoi10.1073pnas.180206197.

Miething, A. (1992) 'Germ-cell death during prespermatogenesis in the testis of the golden hamster', *Cell and Tissue Research*, 267(3), pp. 583–590. Available at: https://doi.org/10.1007/BF00319381.

Miki, K. *et al.* (2004) 'Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility'. Available at: www.pnas.orgcgidoi10.1073pnas.0407708101.

Miller, D.J. (2018) 'Review: The epic journey of sperm through the female reproductive tract', *Animal*. Cambridge University Press, pp. s110–s120. Available at: https://doi.org/10.1017/S1751731118000526.

Miller, E.A. *et al.* (2016) 'Lactobacilli dominance and vaginal pH: Why is the human vaginal microbiome unique?', *Frontiers in Microbiology*, 7(DEC). Available at: https://doi.org/10.3389/fmicb.2016.01936.

Minelli, A. *et al.* (2008) 'Promiscuous coupling and involvement of protein kinase C and extracellular signal-regulated kinase 1/2 in the adenosine A1 receptor signalling in mammalian spermatozoa', *Biochemical Pharmacology*, 75(4), pp. 931–941. Available at: https://doi.org/https://doi.org/10.1016/j.bcp.2007.10.024.

Misro, M.M. and Ramya, T. (2012) 'Fuel/Energy Sources of Spermatozoa', in S.J. Parekattil and A. Agarwal (eds) *Male Infertility: Contemporary Clinical Approaches, Andrology, ART & Antioxidants*. New York, NY: Springer New York, pp. 209–223. Available at: https://doi.org/10.1007/978-1-4614-3335-4_21.

Mlynárik, V. (2017) 'Introduction to nuclear magnetic resonance', *Analytical Biochemistry*, 529, pp. 4–9. Available at: https://doi.org/https://doi.org/10.1016/j.ab.2016.05.006.

Mo, H. and Raftery, D. (2008) 'Solvent Signal is a NMR Concentration Reference', *Analytical chemistry*, 80, pp. 9835–9839. Available at: https://doi.org/10.1021/ac801938j.

Mogas, M.T., Álamo, M.R. del and Rodríguez-Gil, J.E. (2011) 'Roles of Na+/K+-dependent ATPase, Na+/H+ antiporter and GLUT hexose transporters in the cryosurvival of dog spermatozoa: Effects on viability, acrosome state and motile sperm subpopulation structure', *Theriogenology*, 75(9), pp. 1669–1681. Available at: https://doi.org/https://doi.org/10.1016/j.theriogenology.2010.12.030.

Morales, P., Overstreet, J.W. and Katz, D.F. (1988) 'Changes in human sperm motion during capacitation in vitro', *Reproduction*, 83(1), pp. 119–128. Available at: https://doi.org/10.1530/jrf.0.0830119.

Morimoto, T. and Ogihara, S. (1996) 'ATP is required in platelet serotonin exocytosis for protein phosphorylation and priming of secretory vesicles docked on the plasma membrane', *Journal of cell science*, 109 (Pt 1), pp. 113–118. Available at: https://doi.org/10.1242/jcs.109.1.113.

Morris, P. and Bachelard, H. (2003) 'Reflections on the application of 13C-MRS to research on brain metabolism', *NMR in Biomedicine*, 16(6–7), pp. 303–312. Available at: https://doi.org/https://doi.org/10.1002/nbm.844.

Mortimer, D. *et al.* (1984) 'Human sperm motility after migration into, and incubation in, synthetic media', *Gamete Research*, 9, pp. 131–144. Available at: https://api.semanticscholar.org/CorpusID:85918886.

Mortimer, D. (2018) 'The functional anatomy of the human spermatozoon: relating ultrastructure and function', *Molecular Human Reproduction*, 24(12), pp. 567–592. Available at: https://doi.org/10.1093/molehr/gay040.

Mortimer, S.T. (1997) 'A critical review of the physiological importance and analysis of sperm movement in mammals', *Human Reproduction Update*, 3(5), pp. 403–439. Available at: https://doi.org/10.1093/humupd/3.5.403.

MORTIMER, S.T. (2000) 'CASA—Practical Aspects', *Journal of Andrology*, 21(4), pp. 515–524. Available at: https://doi.org/https://doi.org/10.1002/j.1939-4640.2000.tb02116.x.

Mortimer, S.T. and Swan, M.A. (1995a) 'Andrology: Kinematics of capacitating human spermatozoa analysed at 60 Hz', *Human Reproduction*, 10(4), pp. 873–879. Available at: https://doi.org/10.1093/oxfordjournals.humrep.a136053.

Mortimer, S.T. and Swan, M.A. (1995b) 'Variable kinematics of capacitating human spermatozoa*', *Human Reproduction*, 10(12), pp. 3178–3182. Available at: https://doi.org/10.1093/oxfordjournals.humrep.a135882.

Mortimer, S.T., Swan, M.A. and Mortimer, D. (1996) 'Fractal analysis of capacitating human spermatozoa*', *Human Reproduction*, 11(5), pp. 1049–1054. Available at: https://doi.org/10.1093/oxfordjournals.humrep.a019295.

Moscatelli, N. *et al.* (2019) 'Comparative proteomic analysis of proteins involved in bioenergetics pathways associated with human sperm motility', *International Journal of Molecular Sciences*, 20(12). Available at: https://doi.org/10.3390/ijms20123000.

Mostek-Majewska, A. *et al.* (2023) 'New insights into posttranslational modifications of proteins during bull sperm capacitation', *Cell Communication and Signaling : CCS*, 21. Available at: https://api.semanticscholar.org/CorpusID:258064687.

Mukai, C. and Okuno, M. (2004) 'Glycolysis Plays a Major Role for Adenosine Triphosphate Supplementation in Mouse Sperm Flagellar Movement', *Biology of Reproduction*, 71(2), pp. 540–547. Available at: https://doi.org/10.1095/biolreprod.103.026054.

Nakada, K. *et al.* (2006) 'Mitochondria-related male infertility', *Proceedings of the National Academy of Sciences*, 103(41), pp. 15148–15153. Available at: https://doi.org/10.1073/pnas.0604641103.

Nakidkina, A.N. and Kuzmina, T.I. (2019) 'Apoptosis in Spermatozoa and Its Role in Deteriorating Semen Quality', *Russian Journal of Developmental Biology*, 50(4), pp. 165–172. Available at: https://doi.org/10.1134/S1062360419040064.

Nascimento, J.M. *et al.* (2008) 'Comparison of glycolysis and oxidative phosphorylation as energy sources for mammalian sperm motility, using the combination of fluorescence imaging, laser tweezers, and real-time automated tracking and trapping', *Journal of Cellular Physiology*, 217(3), pp. 745–751. Available at: https://doi.org/10.1002/jcp.21549.

Nassar, A. *et al.* (1999) 'Modulation of sperm tail protein tyrosine phosphorylation by pentoxifylline and its correlation with hyperactivated motility', *Fertility and sterility*, 71(5), pp. 919–923. Available at: https://doi.org/10.1016/s0015-0282(99)00013-8.

Nehlig, A., Daval, J.L. and Debry, G. (1992) 'Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects', *Brain research. Brain research reviews*, 17(2), pp. 139–170. Available at: https://doi.org/10.1016/0165-0173(92)90012-b.

Neiss, T.G. (2006) ‘Carbon-13 Nuclear Magnetic Resonance Spectroscopy’, in *Encyclopedia of Analytical Chemistry*. Available at: https://doi.org/https://doi.org/10.1002/9780470027318.a6102.

Nevo, Abraham C. and Rikmenspoel, R. (1970) ‘Diffusion of ATP in sperm flagella’, *Journal of Theoretical Biology*, 26(1), pp. 11–18. Available at: https://doi.org/10.1016/S0022-5193(70)80027-3.

Ng, K.Y.B. *et al.* (2018) ‘In vivo oxygen, temperature and pH dynamics in the female reproductive tract and their importance in human conception: A systematic review’, *Human Reproduction Update*, 24(1), pp. 15–34. Available at: https://doi.org/10.1093/HUMUPD/DMX028.

Nichol, R.W. *et al.* (1992) ‘Concentrations of energy substrates in oviductal fluid and blood plasma of pigs during the peri-ovulatory period.’, *Journal of reproduction and fertility*, 96 2, pp. 699–707. Available at: https://api.semanticscholar.org/CorpusID:26015479.

Nicotera, P., Leist, M. and Ferrando-May, E. (1998) ‘Intracellular ATP, a switch in the decision between apoptosis and necrosis’, *Toxicology Letters*, 102–103, pp. 139–142. Available at: https://doi.org/https://doi.org/10.1016/S0378-4274(98)00298-7.

Nikolopoulou, M., Soucek, D.A. and Vary, J.C. (1985) ‘Changes in the lipid content of boar sperm plasma membranes during epididymal maturation’, *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 815(3), pp. 486–498. Available at: https://doi.org/https://doi.org/10.1016/0005-2736(85)90377-3.

Norbury, C. and Hickson, I. (2001) ‘Cellular Responses to DNA damage’, *Annual review of pharmacology and toxicology*, 41, pp. 367–401. Available at: https://doi.org/10.1146/annurev.pharmtox.41.1.367.

Norcross, N. *et al.* (2022) ‘Male contraceptive development: A medicinal chemistry perspective’, *European Journal of Medicinal Chemistry*, 243, p. 114709. Available at: https://doi.org/10.1016/j.ejmech.2022.114709.

Odet, F. *et al.* (2008) ‘Expression of the Gene for Mouse Lactate Dehydrogenase C (Ldhc) Is Required for Male Fertility1’, *Biology of Reproduction*, 79(1), pp. 26–34. Available at: https://doi.org/10.1095/biolreprod.108.068353.

Odet, F. *et al.* (2011) ‘Lactate dehydrogenase C and energy metabolism in mouse sperm’, *Biology of Reproduction*, 85(3), pp. 556–564. Available at: https://doi.org/10.1095/biolreprod.111.091546.

Odet, F. *et al.* (2013) ‘Glycolysis and mitochondrial respiration in mouse LDHC-Null sperm’, *Biology of Reproduction*, 88(4). Available at: https://doi.org/10.1095/biolreprod.113.108530.

Okabe, M. (2016) ‘The Acrosome Reaction: A Historical Perspective’, in M.G. Buffone (ed.) *Sperm Acrosome Biogenesis and Function During Fertilization*. Cham: Springer International Publishing, pp. 1–13. Available at: https://doi.org/10.1007/978-3-319-30567-7_1.

Omura, S. *et al.* (1977) ‘A new alkaloid AM-2282 OF Streptomyces origin. Taxonomy, fermentation, isolation and preliminary characterization’, *The Journal of antibiotics*, 30(4), p. 275—282. Available at: https://doi.org/10.7164/antibiotics.30.275.

O’Rand, M.G. *et al.* (2018) ‘Inhibition of sperm motility in male macaques with EP055, a potential non-hormonal male contraceptive’, *PLOS ONE*, 13(4), pp. e0195953-. Available at: https://doi.org/10.1371/journal.pone.0195953.

O’Rand, M.G., Silva, E.J.R. and Hamil, K.G. (2016) ‘Non-hormonal male contraception: A review and development of an Eppin based contraceptive’, *Pharmacology & Therapeutics*, 157, pp. 105–111. Available at: https://doi.org/https://doi.org/10.1016/j.pharmthera.2015.11.004.

Oseguera-López, I. *et al.* (2019) 'Novel Techniques of Sperm Selection for Improving IVF and ICSI Outcomes', *Frontiers in Cell and Developmental Biology*, 7. Available at: https://doi.org/10.3389/fcell.2019.00298.

Ou, C.-M. *et al.* (2012) 'The mode of reproductive-derived Spink (serine protease inhibitor Kazal-type) action in the modulation of mammalian sperm activity', *International journal of andrology*, 35(1), pp. 52–62. Available at: https://doi.org/10.1111/j.1365-2605.2011.01159.x

Owen, D. H., & Katz, D. F. (2005). A Review of the Physical and Chemical Properties of Human Semen and the Formulation of a Semen Simulant. Journal of Andrology, 26(4), 459–469. https://doi.org/https://doi.org/10.2164/jandrol.04104

Pacey, A. A. *et al.* (1995) 'Hyperactivation may assist human spermatozoa to detach from intimate association with the endosalpinx', *Human Reproduction*, 10(10), pp. 2603–2609. Available at: https://doi.org/10.1093/oxfordjournals.humrep.a135754.

Pacey, A.A. *et al.* (1997) 'The potential shortcomings of measuring hyperactivated motility by computer-aided sperm analysis when sperm motion is multiphasic.', *Human reproduction update*, 3 3, pp. 185–93. Available at: https://api.semanticscholar.org/CorpusID:10329000.

Pacey, A.A. (2008) 'Sperm, human fertility and society', in *Sperm Biology: An Evolutionary Perspective*. Elsevier, pp. 565–597. Available at: https://doi.org/10.1016/B978-0-12-372568-4.00015-X.

Panner Selvam, M.K. *et al.* (2020) 'The effect of oxidative and reductive stress on semen parameters and functions of physiologically normal human spermatozoa', *Free Radical Biology and Medicine*, 152, pp. 375–385. Available at: https://doi.org/10.1016/j.freeradbiomed.2020.03.008.

Parsons, T.D. *et al.* (1995) 'Docked granules, the exocytic burst, and the need for ATP hydrolysis in endocrine cells', *Neuron*, 15(5), pp. 1085–1096. Available at: https://doi.org/https://doi.org/10.1016/0896-6273(95)90097-7.

Patel, V.A. *et al.* (2006) 'Apoptotic Cells, at All Stages of the Death Process, Trigger Characteristic Signaling Events That Are Divergent from and Dominant over Those Triggered by Necrotic Cells: IMPLICATIONS FOR THE DELAYED CLEARANCE MODEL OF AUTOIMMUNITY*', *Journal of Biological Chemistry*, 281(8), pp. 4663–4670. Available at: https://doi.org/https://doi.org/10.1074/jbc.M508342200.

Patergnani, S. *et al.* (2011) 'Calcium signaling around Mitochondria Associated Membranes (MAMs)', *Cell Communication and Signaling*, 9(1), p. 19. Available at: https://doi.org/10.1186/1478-811X-9-19.

Peterson, R.N. and Freund, M. (1970) 'ATP synthesis and oxidative metabolism in human spermatozoa.', *Biology of reproduction*, 3(1), pp. 47–54. Available at: https://doi.org/10.1093/biolreprod/3.1.47.

Peterson, R.N. and Freund, M. (1974) 'Citrate formation from exogenous substrates by washed human spermatozoa', *Journal of Reproduction and Fertility*, 38(1), pp. 73–79. Available at: https://doi.org/10.1530/jrf.0.0380073.

Pfeiffer, T., Schuster, S. and Bonhoeffer, S. (2001) 'Cooperation and Competition in the Evolution of ATP-Producing Pathways', *Science*, 292(5516), pp. 504–507. Available at: https://doi.org/10.1126/science.1058079.

Piomboni, P. *et al.* (2012) 'The role of mitochondria in energy production for human sperm motility', *International Journal of Andrology*, pp. 109–124. Available at: https://doi.org/10.1111/j.1365-2605.2011.01218.x.

du Plessis, S.S. *et al.* (2015) 'Oxidative phosphorylation versus glycolysis: what fuel do spermatozoa use?', *Asian journal of andrology*, 17(2), pp. 230–235. Available at: https://doi.org/10.4103/1008-682X.135123.

Politch, J.A. *et al.* (2007) 'Concentrations and significance of cytokines and other immunologic factors in semen of healthy fertile men', *Human Reproduction*, 22(11), pp. 2928–2935. Available at: https://doi.org/10.1093/humrep/dem281.

Prajapati, P. *et al.* (2022) 'Elevated and Sustained Intracellular Calcium Signalling Is Necessary for Efficacious Induction of the Human Sperm Acrosome Reaction', *International Journal of Molecular Sciences*, 23(19). Available at: https://doi.org/10.3390/ijms231911253.

Puga Molina, L. *et al.* (2018) 'Molecular Basis of Human Sperm Capacitation', *Frontiers in Cell and Developmental Biology*, 6. Available at: https://doi.org/10.3389/fcell.2018.00072.

Qi, H. *et al.* (2007) 'All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility', *Proceedings of the National Academy of Sciences*, 104(4), pp. 1219–1223. Available at: https://doi.org/10.1073/pnas.0610286104.

Quintero-Moreno, A., Rigau, T. and Rodrıǵ uez-Gil, J.E. (2004) 'Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis', *Theriogenology*, 61(4), pp. 673–690. Available at: https://doi.org/https://doi.org/10.1016/S0093-691X(03)00248-6.

Ramalho-Santos, J., Schatten, G. and Moreno, R.D. (2002) 'Control of Membrane Fusion During Spermiogenesis and the Acrosome Reaction1', *Biology of Reproduction*, 67(4), pp. 1043–1051. Available at: https://doi.org/10.1095/biolreprod67.4.1043.

Ramió-Lluch, L. *et al.* (2011) '"In Vitro" Capacitation and Acrosome Reaction are Concomitant with Specific Changes in Mitochondrial Activity in Boar Sperm: Evidence for a Nucleated Mitochondrial Activation and for the Existence of a Capacitation-Sensitive Subpopulational Structure', *Reproduction in Domestic Animals*, 46(4), pp. 664–673. Available at: https://doi.org/10.1111/j.1439-0531.2010.01725.x.

Ramió-Lluch, L. *et al.* (2014) 'Oligomycin A-induced inhibition of mitochondrial ATP-synthase activity suppresses boar sperm motility and *in vitro* capacitation achievement without modifying overall sperm energy levels', *Reproduction, Fertility and Development*, 26(6), pp. 883–897. Available at: https://doi.org/10.1071/RD13145.

Reeve, L., Ledger, W.L. and Pacey, A.A. (2003) 'Does the Arg-Gly-Asp (RGD) adhesion sequence play a role in mediating sperm interaction with the human endosalpinx?', *Human Reproduction*, 18(7), pp. 1461–1468. Available at: https://doi.org/10.1093/humrep/deg296.

Ren, D. *et al.* (2001) 'A sperm ion channel required for sperm motility and male fertility', *Nature*, 413(6856), pp. 603–609. Available at: https://doi.org/10.1038/35098027.

Renehan, A.G., Booth, C. and Potten, C.S. (2001) 'What is apoptosis, and why is it important?', *BMJ (Clinical research ed.)*, 322(7301), pp. 1536–1538. Available at: https://doi.org/10.1136/bmj.322.7301.1536.

Reynolds, S. *et al.* (2017a) '1H Magnetic Resonance Spectroscopy of live human sperm', *Molecular Human Reproduction*, 23(7), pp. 441–451. Available at: https://doi.org/10.1093/molehr/gax025.

Reynolds, Steven, Ismail, N.F.B., *et al.* (2017b) 'Evidence for Rapid Oxidative Phosphorylation and Lactate Fermentation in Motile Human Sperm by Hyperpolarized (13)C Magnetic Resonance Spectroscopy.', *Scientific reports*, 7(1), p. 4322. Available at: https://doi.org/10.1038/s41598-017-04146-1.

Ribas-Maynou, J. *et al.* (2021) 'The TUNEL assay underestimates the incidence of DNA damage in pig sperm due to chromatin condensation', *Theriogenology*, 174, pp. 94–101. Available at: https://doi.org/https://doi.org/10.1016/j.theriogenology.2021.08.024.

Ribeiro, J.A. and Sebastião, A.M. (2010) 'Caffeine and adenosine', *Journal of Alzheimer's disease : JAD*, 20 Suppl 1, pp. S3-15. Available at: https://doi.org/10.3233/jad-2010-1379.

Ribeiro, S. *et al.* (2017) 'Inter- and intra-laboratory standardization of TUNEL assay for assessment of sperm DNA fragmentation', *Andrology*, 5(3), p. 477—485. Available at: https://doi.org/10.1111/andr.12334.

Ricci, G. *et al.* (2002) 'Apoptosis in human sperm: its correlation with semen quality and the presence of leukocytes', *Human Reproduction*, 17(10), pp. 2665–2672. Available at: https://doi.org/10.1093/humrep/17.10.2665.

Richter, C. *et al.* (1996) 'Control of apoptosis by the cellular ATP level', *FEBS Letters*, 378(2), pp. 107–110. Available at: https://doi.org/https://doi.org/10.1016/0014-5793(95)01431-4.

Roberts Kathryn M. and Rosen, A. and C.-R.L.A. (2004) 'Methods for Inducing Apoptosis', in A. Perl (ed.) *Autoimmunity: Methods and Protocols*. Totowa, NJ: Humana Press, pp. 115–128. Available at: https://doi.org/10.1385/1-59259-805-6:115.

Robertson, S.A. *et al.* (2002) 'Transforming growth factor β—a mediator of immune deviation in seminal plasma', *Journal of Reproductive Immunology*, 57(1), pp. 109–128. Available at: https://doi.org/https://doi.org/10.1016/S0165-0378(02)00015-3.

Rodriguez, P.C. *et al.* (2020) 'Participation of lactate dehydrogenase in capacitation and acrosome reaction of fresh and cryopreserved, with or without α-tocopherol, boar sperm.', in. Available at: https://api.semanticscholar.org/CorpusID:226627826.

Rodriguez-Gil, J.E. (2006) 'Mammalian Sperm Energy Resources Management and Survival during Conservation in Refrigeration', *Reproduction in Domestic Animals*, 41(s2), pp. 11–20. Available at: https://doi.org/https://doi.org/10.1111/j.1439-0531.2006.00765.x.

Rodríguez-Gil, J.E. and Bonet, S. (2016) 'Current knowledge on boar sperm metabolism: Comparison with other mammalian species', *Theriogenology*, 85(1), pp. 4–11. Available at: https://doi.org/https://doi.org/10.1016/j.theriogenology.2015.05.005.

Rogers, B.J. and Perreault, S.D. (1990) 'Importance of glycolysable substrates for in vitro capacitation of human spermatozoa', *Biology of Reproduction*, 43(6), pp. 1064–1069. Available at: https://doi.org/10.1095/biolreprod43.6.1064.

Sahoo, G. *et al.* (2023) 'A Review on Caspases: Key Regulators of Biological Activities and Apoptosis', *Molecular Neurobiology*, 60(10), pp. 5805–5837. Available at: https://doi.org/10.1007/s12035-023-03433-5.

Saint-Dizier, M. *et al.* (2020) 'Sperm interactions with the female reproductive tract: A key for successful fertilization in mammals', *Molecular and Cellular Endocrinology*, 516, p. 110956. Available at: https://doi.org/https://doi.org/10.1016/j.mce.2020.110956.

Sakkas, D. *et al.* (1999) 'Origin of DNA damage in ejaculated human spermatozoa', *Reviews of reproduction*, 4, pp. 31–37. Available at: https://doi.org/10.1530/revreprod/4.1.31.

Sakkas, D., Mariethoz, E. and St. John, J.C. (1999) 'Abnormal Sperm Parameters in Humans Are Indicative of an Abortive Apoptotic Mechanism Linked to the Fas-Mediated Pathway', *Experimental Cell Research*, 251(2), pp. 350–355. Available at: https://doi.org/https://doi.org/10.1006/excr.1999.4586.

Samanta, L. *et al.* (2018) 'The enigmatic seminal plasma: a proteomics insight from ejaculation to fertilization', *Reproductive Biology and Endocrinology*, 16(1), p. 41. Available at: https://doi.org/10.1186/s12958-018-0358-6.

Sansegundo, E., Tourmente, M. and Roldan, E. (2022) 'Energy Metabolism and Hyperactivation of Spermatozoa from Three Mouse Species under Capacitating Conditions', *Cells*, 11, p. 220. Available at: https://doi.org/10.3390/cells11020220.

Satouh, Y. *et al.* (2012) 'Visualization of the moment of mouse sperm-egg fusion and dynamic localization of IZUMO1', *Journal of cell science*, 125. Available at: https://doi.org/10.1242/jcs.100867.

Schaefer, M. *et al.* (1998) 'A new prostaglandin E receptor mediates calcium influx and acrosome reaction in human spermatozoa', *Proceedings of the National Academy of Sciences*, 95(6), pp. 3008–3013. Available at: https://doi.org/10.1073/pnas.95.6.3008.

Schmitz, E.A., Takahashi, H. and Karakas, E. (2022) 'Structural basis for activation and gating of IP3 receptors', *Nature Communications*, 13(1), p. 1408. Available at: https://doi.org/10.1038/s41467-022-29073-2.

Sengupta, P., Durairajanayagam, D. and Agarwal, A. (2020) 'Fuel/Energy Sources of Spermatozoa', in S.J. Parekattil, S.C. Esteves, and A. Agarwal (eds) *Male Infertility: Contemporary Clinical Approaches, Andrology, ART and Antioxidants*. Cham: Springer International Publishing, pp. 323–335. Available at: https://doi.org/10.1007/978-3-030-32300-4_26.

Sharma, P. *et al.* (2012) 'Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions', *Journal of Botany*. Edited by A. Polle, 2012, p. 217037. Available at: https://doi.org/10.1155/2012/217037.

Sharma, R.K. *et al.* (2010) 'TUNEL as a Test for Sperm DNA Damage in the Evaluation of Male Infertility', *Urology*, 76(6), pp. 1380–1386. Available at: https://doi.org/https://doi.org/10.1016/j.urology.2010.04.036.

Shestov, A.A. *et al.* (2014) 'Quantitative determinants of aerobic glycolysis identify flux through the enzyme GAPDH as a limiting step', *eLife*. Edited by D.M. Sabatini, 3, p. e03342. Available at: https://doi.org/10.7554/eLife.03342.

Shestov, A.A. *et al.* (2016) '13C MRS and LC–MS Flux Analysis of Tumor Intermediary Metabolism', *Frontiers in Oncology*, 6. Available at: https://doi.org/10.3389/fonc.2016.00135.

Shimizu, S. *et al.* (1996) 'Induction of Apoptosis as well as Necrosis by Hypoxia and Predominant Prevention of Apoptosis by Bcl-2 and Bcl-XL1', *Cancer Research*, 56(9), pp. 2161–2166.

Siegel, M.S., Paulson, R.J. and Graczykowski, J.W. (1990) 'The influence of human follicular fluid on the acrosome reaction, fertilizing capacity and proteinase activity of human spermatozoa', *Human reproduction (Oxford, England)*, 5(8), pp. 975–980. Available at: https://doi.org/10.1093/oxfordjournals.humrep.a137231.

Šimenc, J. and Lipnik-Štangelj, M. (2012) 'Staurosporine induces apoptosis and necroptosis in cultured rat astrocytes', *Drug and Chemical Toxicology*, 35(4), pp. 399–405. Available at: https://doi.org/10.3109/01480545.2011.633087.

Simons, J. and Fauci, L. (2018) 'A Model for the Acrosome Reaction in Mammalian Sperm', *Bulletin of Mathematical Biology*, 80(9), pp. 2481–2501. Available at: https://doi.org/10.1007/s11538-018-0478-3.

Simpson, A.M., Swan, M.A. and White, I.G. (1987) 'Susceptibility of epididymal boar sperm to cold shock and protective action of phosphatidylcholine', *Gamete Research*, 17(4), pp. 355–373. Available at: https://doi.org/https://doi.org/10.1002/mrd.1120170408.

Singh, P. and Lim, B. (2022) 'Targeting Apoptosis in Cancer', *Current Oncology Reports*, 24(3), pp. 273–284. Available at: https://doi.org/10.1007/s11912-022-01199-y.

Singh, R., Letai, A. and Sarosiek, K. (2019) 'Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins', *Nature Reviews Molecular Cell Biology*, 20(3), pp. 175–193. Available at: https://doi.org/10.1038/s41580-018-0089-8.

Söllner, T. *et al.* (1993) 'SNAP receptors implicated in vesicle targeting and fusion', *Nature*, 362(6418), pp. 318–324. Available at: https://doi.org/10.1038/362318a0.

Stauss, C.R., Votta, T.J. and Suarez, S.S. (1995) 'Sperm motility hyperactivation facilitates penetration of the hamster zona pellucida', *Biology of reproduction*, 53(6), pp. 1280–1285. Available at: https://doi.org/10.1095/biolreprod53.6.1280.

Stendardi, A. *et al.* (2011) 'Evaluation of mitochondrial respiratory efficiency during in vitro capacitation of human spermatozoa', *International Journal of Andrology*, 34(3), pp. 247–255. Available at: https://doi.org/10.1111/j.1365-2605.2010.01078.x.

Stephan, H. *et al.* (1996) 'Distribution of deoxyribonuclease I (DNase I) and p53 in rat testis and their correlation with apoptosis', *Histochemistry and Cell Biology*, 106(4), pp. 383–393. Available at: https://doi.org/10.1007/BF02473297.

Storey, Bayard T (2008) 'Mammalian sperm metabolism: oxygen and sugar, friend and foe.', *The International journal of developmental biology*, 52(5–6), pp. 427–437. Available at: https://doi.org/10.1387/ijdb.072522bs.

Suarez, S.S. *et al.* (1991) 'Evidence for the function of hyperactivated motility in sperm', *Biology of reproduction*, 44(2), pp. 375–381. Available at: https://doi.org/10.1095/biolreprod44.2.375.

Suarez, Susan S. (2008a) 'Control of hyperactivation in sperm', *Human Reproduction Update*, pp. 647–657. Available at: https://doi.org/10.1093/humupd/dmn029.

Suarez, Susan S. (2008b) 'Regulation of sperm storage and movement in the mammalian oviduct', *International Journal of Developmental Biology*, 52(5–6), pp. 455–462. Available at: https://doi.org/10.1387/ijdb.072527ss.

Suarez, S.S. and Ho, H.C. (2003) 'Hyperactivated motility in sperm', *Reproduction in Domestic Animals*, 38(2), pp. 119–124. Available at: https://doi.org/10.1046/j.1439-0531.2003.00397.x.

Suarez, S.S. and Pacey, A.A. (2006) 'Sperm transport in the female reproductive tract', *Human Reproduction Update*, pp. 23–37. Available at: https://doi.org/10.1093/humupd/dmi047.

Sueldo, C.E. *et al.* (1993) 'Effect of progesterone on human zona pellucida sperm binding and oocyte penetrating capacity', *Fertility and Sterility*, 60(1), pp. 137–140. Available at: https://doi.org/https://doi.org/10.1016/S0015-0282(16)56051-8.

Sun, X. *et al.* (2017) 'The Catsper channel and its roles in male fertility: a systematic review', *Reproductive Biology and Endocrinology*, 15(1), p. 65. Available at: https://doi.org/10.1186/s12958-017-0281-2.

Sundquist, T. *et al.* (2006) 'Timing your apoptosis assays', *Cell Notes*, 16, pp. 18–21.

Suter, D., Chow, P.Y.W. and Martin, I.C.A. (1979) 'Maintenance of motility in human spermatozoa by energy derived through oxidative phosphorylation and addition of albumin', *Biology of Reproduction*, 20(3), pp. 505–510. Available at: https://doi.org/10.1095/biolreprod20.3.505.

Sutovsky, P. (2010) 'Sperm Capacitation, the Acrosome Reaction, and Fertilization', in D.T. Carrell and C.M. Peterson (eds) *Reproductive Endocrinology and Infertility: Integrating Modern Clinical and Laboratory Practice*. New York, NY: Springer New York, pp. 389–421. Available at: https://doi.org/10.1007/978-1-4419-1436-1_25.

Sutovsky, P. and Manandhar, G. (2006) 'Mammalian spermatogenesis and sperm structure: anatomical and compartmental analysis', in C. Barratt and C.J. De Jonge (eds) *The Sperm Cell: Production, Maturation, Fertilization, Regeneration*. Cambridge: Cambridge University Press, pp. 1–30. Available at: https://doi.org/DOI: 10.1017/CBO9780511545115.002.

Sutton, R.B. *et al.* (1998) 'Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution', *Nature*, 395(6700), pp. 347–353. Available at: https://doi.org/10.1038/26412.

Suzuki, F. and Yanagimachi, R. (1989) 'Changes in the distribution of intramembranous particles and filipin-reactive membrane sterols during in vitro capacitation of golden hamster spermatozoa', *Gamete research*, 23(3), pp. 335–347. Available at: https://doi.org/10.1002/mrd.1120230310.

Swain, D.K. *et al.* (2022) 'Introduction to the pathways involved in the activation and regulation of sperm motility: A review of the relevance of ion channels', *Animal Reproduction Science*, 246, p. 107052. Available at: https://doi.org/https://doi.org/10.1016/j.anireprosci.2022.107052.

Tafuri, S. *et al.* (2015) 'Reactive Oxygen Species (ROS) and Male Fertility', in B. Wu (ed.) *New Discoveries in Embryology*. Rijeka: IntechOpen, p. Ch. 2. Available at: https://doi.org/10.5772/60632.

Tait, S.W.G. and Green, D.R. (2012) 'Mitochondria and cell signalling', *Journal of Cell Science*, 125(4), pp. 807–815. Available at: https://doi.org/10.1242/jcs.099234.

Takei, G.L. *et al.* (2014) 'Glycolysis plays an important role in energy transfer from the base to the distal end of the flagellum in mouse sperm', *Journal of Experimental Biology*, 217(11), pp. 1876–1886. Available at: https://doi.org/10.1242/jeb.090985.

Tatsumi, T. *et al.* (2003) 'Intracellular ATP is required for mitochondrial apoptotic pathways in isolated hypoxic rat cardiac myocytes', *Cardiovascular Research*, 59(2), pp. 428–440. Available at: https://doi.org/10.1016/S0008-6363(03)00391-2.

Taylor, S.L. *et al.* (2004) 'Somatic cell apoptosis markers and pathways in human ejaculated sperm: Potential utility as indicators of sperm quality', *Molecular Human Reproduction*, 10(11), pp. 825–834. Available at: https://doi.org/10.1093/molehr/gah099.

Tesarik, J. (1989) 'Appropriate timing of the acrosome reaction is a major requirement for the fertilizing spermatozoon', *Human reproduction (Oxford, England)*, 4(8), pp. 957–961. Available at: https://doi.org/10.1093/oxfordjournals.humrep.a137020.

TeSlaa, T. and Teitell, M.A. (2014) 'Techniques to monitor glycolysis', *Methods in enzymology*, 542, pp. 91–114. Available at: https://doi.org/10.1016/b978-0-12-416618-9.00005-4.

Theander, S., Lew, D.P. and Nüsse, O. (2002) 'Granule-specific ATP requirements for Ca2+-induced exocytosis in human neutrophils. Evidence for substantial ATP-independent release', *Journal of cell science*, 115(Pt 14), pp. 2975–2983. Available at: https://doi.org/10.1242/jcs.115.14.2975.

Tombes, R.M. and Shapiro, B.M. (1987) 'Enzyme termini of a phosphocreatine shuttle. Purification and characterization of two creatine kinase isozymes from sea urchin sperm.', *Journal of Biological Chemistry*, 262(33), pp. 16011–16019. Available at: https://doi.org/10.1016/S0021-9258(18)47689-2.

Torres-Fuentes, J.L., Rios, M. and Moreno, R.D. (2015) 'Involvement of a P2X7 Receptor in the Acrosome Reaction Induced by ATP in Rat Spermatozoa', *Journal of Cellular Physiology*, 230(12), pp. 3068–3075. Available at: https://doi.org/https://doi.org/10.1002/jcp.25044.

Tourmente, M. *et al.* (2022) 'Capacitation promotes a shift in the energy metabolism in murine sperm', *bioRxiv*, p. 2022.05.08.490683. Available at: https://doi.org/10.1101/2022.05.08.490683.

Travis, A.J. *et al.* (2001) 'Functional relationships between capacitation-dependent cell signaling and compartmentalized metabolic pathways in murine spermatozoa', *The Journal of biological chemistry*, 276(10), pp. 7630–7636. Available at: https://doi.org/10.1074/jbc.m006217200.

Troiano, L. *et al.* (1998) 'Mitochondrial Membrane Potential and DNA Stainability in Human Sperm Cells: A Flow Cytometry Analysis with Implications for Male Infertility', *Experimental Cell Research*, 241(2), pp. 384–393. Available at: https://doi.org/10.1006/EXCR.1998.4064.

Turner, R. (2003) 'Tales from the Tail: What Do We Really Know about Sperm Motility?', *Journal of andrology*, 24, pp. 790–803. Available at: https://doi.org/10.1002/j.1939-4640.2003.tb03123.x.

Turner, R.M. (2006) 'Moving to the beat: A review of mammalian sperm motility regulation', *Reproduction, Fertility and Development*, pp. 25–38. Available at: https://doi.org/10.1071/RD05120.

Uhler, M.L. *et al.* (1992) 'Direct effects of progesterone and antiprogesterone on human sperm hyperactivated motility and acrosome reaction**Presented in part at the 39th Annual Meeting of the Society of Gynecologic Investigation, San Antonio, Texas, March 18 to 21, 1992.', *Fertility and Sterility*, 58(6), pp. 1191–1198. Available at: https://doi.org/https://doi.org/10.1016/S0015-0282(16)55568-X.

Urner, F. and Sakkas, D. (2003) 'Protein phosphorylation in mammalian spermatozoa', *Reproduction (Cambridge, England)*, 125(1), pp. 17–26. Available at: https://doi.org/10.1530/rep.0.1250017.

Vilmart-Seuwen, J. *et al.* (1986) *ATP Keeps Exocytosis Sites in a Primed State But Is Not Required for Membrane Fusion: An Analysis with Paramecium Cells In Vivo and In Vitro*. Available at: http://rupress.org/jcb/article-pdf/103/4/1279/1464715/1279.pdf.

Visconti, P. *et al.* (2011) 'Ion channels, phosphorylation and mammalian sperm capacitation', *Asian journal of andrology*, 13, pp. 395–405. Available at: https://doi.org/10.1038/aja.2010.69.

Visconti, P.E. *et al.* (1995) 'Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation', *Development (Cambridge, England)*, 121(4), pp. 1129–1137. Available at: https://doi.org/10.1242/dev.121.4.1129.

Visconti, P.E. *et al.* (2002) 'Novel signaling pathways involved in sperm acquisition of fertilizing capacity', *Journal of Reproductive Immunology*, 53(1), pp. 133–150. Available at: https://doi.org/https://doi.org/10.1016/S0165-0378(01)00103-6.

Vizel, R. *et al.* (2015) 'AKAP3 degradation in sperm capacitation is regulated by its tyrosine phosphorylation', *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1850(9), pp. 1912–1920. Available at: https://doi.org/https://doi.org/10.1016/j.bbagen.2015.06.005.

Wang, X. *et al.* (2021) 'Pyruvate dehydrogenase kinases (PDKs): an overview toward clinical applications', *Bioscience Reports*, 41(4), p. BSR20204402. Available at: https://doi.org/10.1042/BSR20204402.

Warburg, O. (1925) 'The Metabolism of Carcinoma Cells1', *The Journal of Cancer Research*, 9(1), pp. 148–163. Available at: https://doi.org/10.1158/jcr.1925.148.

Weil, M., Jacobson, M.D. and Raff, M.C. (1998) 'Are caspases involved in the death of cells with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes', *Journal of Cell Science*, 111(18), pp. 2707–2715. Available at: https://doi.org/10.1242/jcs.111.18.2707.

Weng, S.-L. *et al.* (2002) 'Caspase activity and apoptotic markers in ejaculated human sperm', *Molecular Human Reproduction*, 8(11), pp. 984–991. Available at: https://doi.org/10.1093/molehr/8.11.984.

Westhoff, D. and Kamp, G. (1997) 'Glyceraldehyde 3-phosphate dehydrogenase is bound to the fibrous sheath of mammalian spermatozoa.', *Journal of cell science*, 110 (Pt 1, pp. 1821–1829.

Whiteheart, S.W. *et al.* (1994) 'N-ethylmaleimide-sensitive fusion protein: a trimeric ATPase whose hydrolysis of ATP is required for membrane fusion', *The Journal of cell biology*, 126(4), pp. 945–954. Available at: https://doi.org/10.1083/jcb.126.4.945.

Wilcox, A.J., Weinberg, C.R. and Baird, D.D. (1995) 'Timing of sexual intercourse in relation to ovulation'. *Effects on the Probability of Conception, Survival of the Pregnancy, and Sex of the Baby*.

Williams, A.C. and Ford, W.C. (2001) 'The role of glucose in supporting motility and capacitation in human spermatozoa.', *Journal of andrology*, 22(4), pp. 680–695.

Wilson, D.F. *et al.* (1988) 'The oxygen dependence of mitochondrial oxidative phosphorylation measured by a new optical method for measuring oxygen concentration', *The Journal of biological chemistry*, 263(6), p. 2712—2718. Available at: https://doi.org/10.1016/s0021-9258(18)69126-4.

Wolvetang, E.J. *et al.* (1994) 'Mitochondrial respiratory chain inhibitors induce apoptosis', *FEBS Letters*, 339(1–2), pp. 40–44. Available at: https://doi.org/https://doi.org/10.1016/0014-5793(94)80380-3.

World Health Organization (2021) *WHO laboratory manual for the examination and processing of human semen Sixth Edition*, *World Health Organization*. Available at: http://whqlibdoc.who.int/publications/2010/9789241547789_eng.pdf.

Wündrich, K. *et al.* (2006) 'Activation of Caspases in Human Spermatozoa during Cryopreservation – An Immunoblot Study', *Cell and Tissue Banking*, 7(2), pp. 81–90. Available at: https://doi.org/10.1007/s10561-005-0276-7.

Xia, J. and Ren, D. (2009) 'The BSA-induced Ca(2+) influx during sperm capacitation is CATSPER channel-dependent', *Reproductive Biology and Endocrinology*, 7(1), p. 119. Available at: https://doi.org/10.1186/1477-7827-7-119.

Xie, F. *et al.* (2006) 'Soluble adenylyl cyclase (sAC) is indispensable for sperm function and fertilization', *Developmental Biology*, 296(2), pp. 353–362. Available at: https://doi.org/10.1016/J.YDBIO.2006.05.038.

Xu, G. and Shi, Y. (2007) 'Apoptosis signaling pathways and lymphocyte homeostasis', *Cell Research*, 17(9), pp. 759–771. Available at: https://doi.org/10.1038/cr.2007.52.

Yanagimachi, R. (1970) 'The movement of golden hamster spermatozoa before and after capacitation', *Journal of reproduction and fertility*, 23(1), pp. 193–196. Available at: https://doi.org/10.1530/jrf.0.0230193.

Yanagimachi, R. (2011) 'Mammalian Sperm Acrosome Reaction: Where Does It Begin Before Fertilization?', *Biology of Reproduction*, 85(1), pp. 4–5. Available at: https://doi.org/10.1095/biolreprod.111.092601.

Yasuhara, N. *et al.* (1997) 'Essential role of active nuclear transport in apoptosis', *Genes to cells : devoted to molecular & cellular mechanisms*, 2(1), pp. 55–64. Available at: https://doi.org/10.1046/j.1365-2443.1997.1010302.x.

Yutaka Tajima, Naomichi Okamura and Yoshiki Sugita (1987) 'The activating effects of bicarbonate on sperm motility and respiration at ejaculation', *Biochimica et Biophysica Acta (BBA) - General Subjects*, 924(3), pp. 519–529. Available at: https://doi.org/https://doi.org/10.1016/0304-4165(87)90168-1.

Zaferani, M., Suarez, S.S. and Abbaspourrad, A. (2021) 'Mammalian sperm hyperactivation regulates navigation via physical boundaries and promotes pseudo-chemotaxis', *Proceedings of the National Academy of Sciences*, 118(44), p. e2107500118. Available at: https://doi.org/10.1073/pnas.2107500118.

Zaneveld, L.J.D. *et al.* (1991) 'Human sperm capacitation and the acrosome reaction'. *Human Reproduction*, 6(9), pp. 1265–1274. Available at: https://doi.org/10.1093/oxfordjournals.humrep.a137524.

Zegers-Hochschild, F. *et al.* (2017) 'The International Glossary on Infertility and Fertility Care, 2017†‡§', *Human Reproduction*, 32(9), pp. 1786–1801. Available at: https://doi.org/10.1093/humrep/dex234.

Zeginiadou, T., Papadimas, J. and Mantalenakis, S. (2000) 'Acrosome reaction: methods for detection and clinical significance', *Andrologia*, 32(6), pp. 335–343. Available at: https://doi.org/10.1046/j.1439-0272.2000.00359.x.

Zeng, Y., Oberdorf, J.A. and Florman, H.M. (1996) 'pH Regulation in Mouse Sperm: Identification of Na+-, Cl−-, and HCO_3^- Dependent and Arylaminobenzoate-Dependent Regulatory Mechanisms and Characterization of Their Roles in Sperm Capacitation', *Developmental Biology*, 173(2), pp. 510–520. Available at: https://doi.org/https://doi.org/10.1006/dbio.1996.0044.

Zhao, Y. *et al.* (2022) 'Natural products targeting glycolysis in cancer', *Frontiers in Pharmacology*, 13. Available at: https://doi.org/10.3389/fphar.2022.1036502.

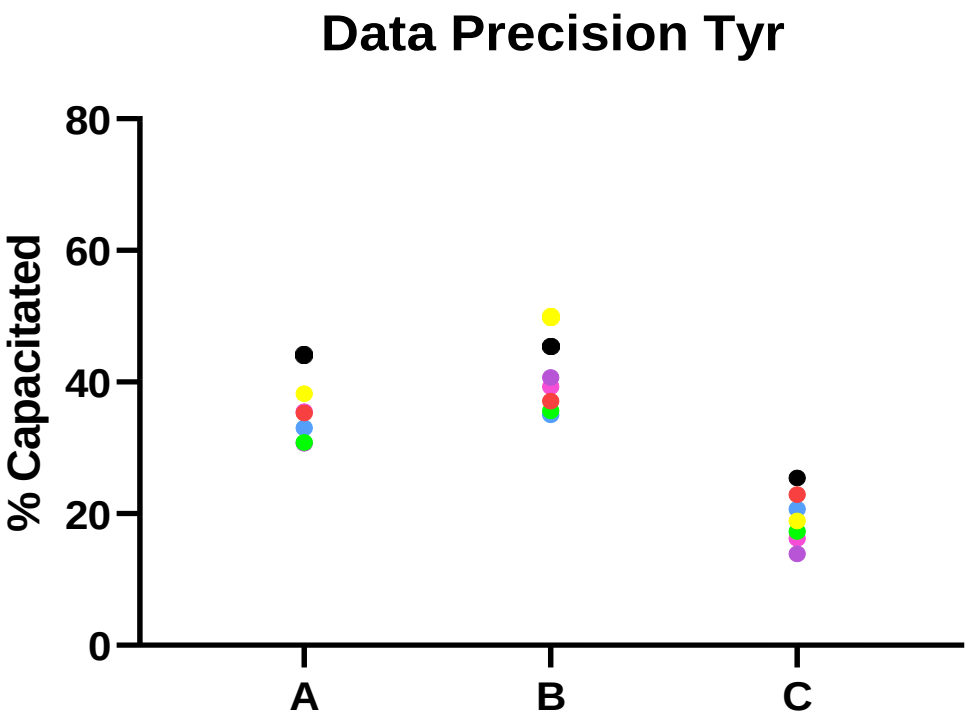
Zhou, D. *et al.* (2022) 'The significance of glycolysis in tumor progression and its relationship with the tumor microenvironment', *Frontiers in Pharmacology*, 13. Available at: https://doi.org/10.3389/fphar.2022.1091779.

Zia, K. *et al.* (2019) 'Nuclear Magnetic Resonance Spectroscopy for Medical and Dental Applications: A Comprehensive Review', *European Journal of Dentistry*, 13, pp. 124–128. Available at: https://api.semanticscholar.org/CorpusID:174811755.

Zorova, L.D. *et al.* (2018) 'Mitochondrial membrane potential', *Analytical Biochemistry*, 552, pp. 50–59. Available at: https://doi.org/https://doi.org/10.1016/j.ab.2017.07.009.

Appendices

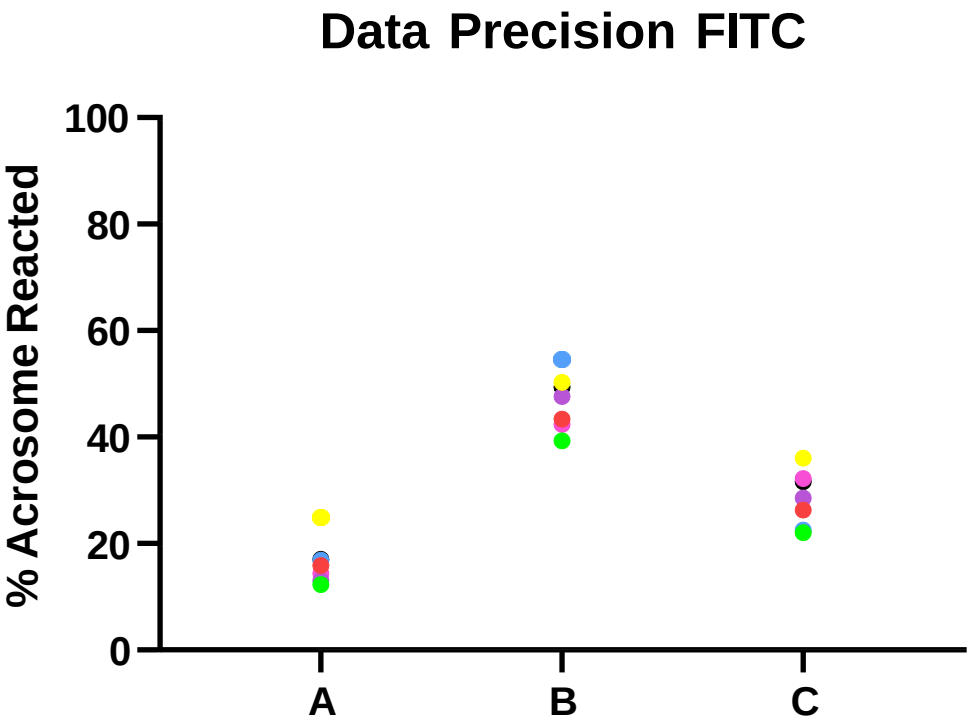
A1 Repeated measurements of tyrosine phosphorylation-stained slides and the calculated coefficient of variance.



	Repeat 1	Repeat 2	Repeat 3	Repeat 4	Repeat 5	Repeat 6	Repeat 7	CV
A	35.3	38.2	30.8	33	30.7	35.5	44.1	13.3%
B	37.1	49.9	35.6	35	40.7	39.3	45.4	13.5%
C	22.9	18.9	17.3	20.7	13.9	16.2	25.4	20.6%

Figure A1: Repeated measurements of tyrosine phosphorylation-stained slides. The 7 repeated measurements of 3 slides stained using the tyrosine phosphorylation method described in 2.6 to determine the accuracy of assessment of these slides. The coefficient of variance was calculated for each set of repeats per slide.

A2 Repeated measurements of FITC-PSA-stained slides and the calculated coefficient of variance.



	Repeat 1	Repeat 2	Repeat 3	Repeat 4	Repeat 5	Repeat 6	Repeat 7	CV
A	15.9	24.9	12.3	16.9	13	14.4	17	25.6%
B	43.4	50.3	39.3	54.5	47.6	42.4	49.4	11.3%
C	26.3	36	22	22.5	28.5	32.2	31.6	18.3%

Figure A2: Repeated measurements of FITC-PSA-stained slides. The 7 repeated measurements of 3 slides stained using the FITC-PSA method described in 2.8 to determine the accuracy of assessment of these slides. The coefficient of variance was calculated for each set of repeats per slide.

A3 Experimental workflow

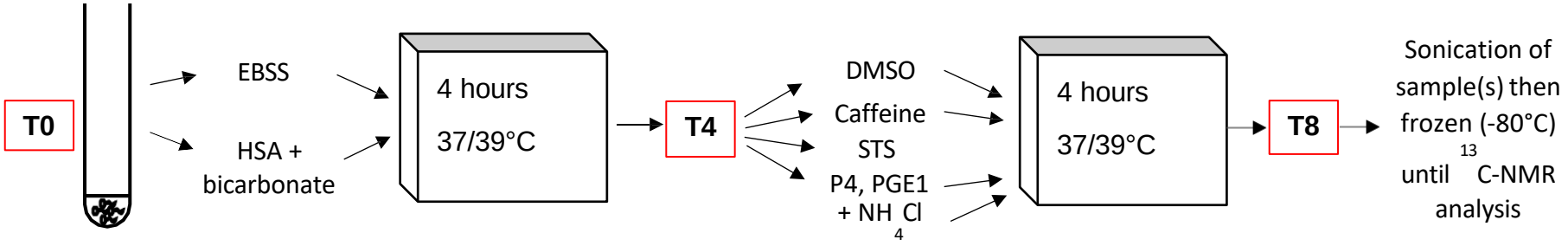


Figure A3: A schematic of methods used to induce capacitation, hyperactivation, acrosome reaction and apoptosis in an isolated sperm preparation. Initially capacitation was induced over a four-hour period followed by the addition of inducers of either hyperactivation, the acrosome reaction or apoptosis for a further four-hours. At T0, T4 and T8, sperm motility and vitality were measured alongside the percentage of capacitated, hyperactivated, acrosome reacted or DNA fragmented sperm. After incubation samples were frozen until ^{13}C-NMR analysis.

A4 Human sperm colour-code

Donor	Colour on graphs	Capacitation	Hyperactivation	Acrosome Reaction	Apoptosis
SR065	E4	☑	☑	☑	☑
SR071	D9		☑	☑	☑
SR072	E3	☑	☑	☑	☑
SR129	D6	☑			☑
SR234	D10	☑			
SR235	G1	☑			
SR238	D12	☑	☑	☑	☑
SR239	F5	☑			
SR241	C8	☑	☑	☑	☑
SR244	F3	☑			☑
SR245	D5	☑	☑	☑	☑
SR249	F12			☑	
SR251	B7				☑
SR252	C3		☑	☑	
SR256	F7				
SR257	C6		☑	☑	
SR266	F2		☑		☑
SR272	E1		☑	☑	
SR275	C5		☑	☑	☑
SR278	B11	☑			
Total	-	11	11	11	11

Figure A4: A table to show which experiments human semen samples were used for in chapter 3/4/5/6 and the corresponding colour points on the graphs shown in this chapter.

A5 Boar sperm colour-code

SAMPLE	COLOUR ON GRAPHS	CAPACITATION	HYPERACTIVATION	ACROSOME REACTION	APOPTOSIS	METHOD DEVELOPMENT / NOT INCLUDED IN ANALYSIS
BOAR 1	-					☑
BOAR 2	-					☑
BOAR 3	-					☑
BOAR 4	-					☑
BOAR 5	-					☑
BOAR 6	-					☑
BOAR 7	-					☑
BOAR 8	-					☑
BOAR 9	-					☑
BOAR 10	-					☑
BOAR 11	-					☑
BOAR 12	-					☑
BOAR 13	-					☑
BOAR 14	-					☑
BOAR 15	-					☑
BOAR 16	E3	☑				
BOAR 17	E4	☑				
BOAR 18	-					☑
BOAR 19	-					☑
BOAR 20	-					☑
BOAR 21	-					☑
BOAR 22	-					☑
BOAR 23	-					☑
BOAR 24	E5				☑	
BOAR 25	E6				☑	

BOAR 26	**E8**				☑	
BOAR 27	**E9**				☑	
BOAR 28	-					☑
BOAR 29	-					☑
BOAR 30	-					☑
BOAR 31	**E10**				☑	
BOAR 32	-					☑
BOAR 33	-					☑
BOAR 34	**E12**				☑	
BOAR 35	**E2**				☑	
BOAR 36	**E1**				☑	
BOAR 37	**G1**				☑	
BOAR 38	**F3**	☑				
BOAR 39	**F7**	☑				
BOAR 40	**C10**	☑				
BOAR 41	-					☑
BOAR 42	-					☑
BOAR 43	-					☑
BOAR 44	-					☑
BOAR 45	-					☑
BOAR 46	-					☑
BOAR 47	**C4**	☑			☑	
BOAR 48	**C2**	☑			☑	
BOAR 49	**B7**	☑				
BOAR 50	**B11**	☑				
BOAR 51	**F2**	☑				
BOAR 52	**B4**	☑				
BOAR 53						☑
BOAR 54						☑

BOAR 55						☑
BOAR 56	**C11**		☑	☑		
BOAR 57	**C9**		☑	☑		
BOAR 58	**C7**		☑	☑		
BOAR 59	**C6**		☑	☑		
BOAR 60	**C5**		☑	☑		
BOAR 61	**-**					☑
BOAR 62	**C3**		☑	☑		
BOAR 63	**C1**		☑	☑		
BOAR 64	**C12**		☑	☑		
BOAR 65	**A3**		☑	☑		
BOAR 66	**A4**		☑	☑		
BOAR 67	**A9**		☑	☑		
TOTAL	**-**	**11**	**11**	**11**	**11**	**36**

Figure A5: A table to show which experiments boar semen samples were used for in chapter 3, 4, 5 and 6, and the corresponding colour points on the graphs shown in this thesis. In total, 67 boar ejaculates were used, however 36 of these were used for method development and optimisation hence do not have a corresponding colour.

A6 Lactate vs concentration

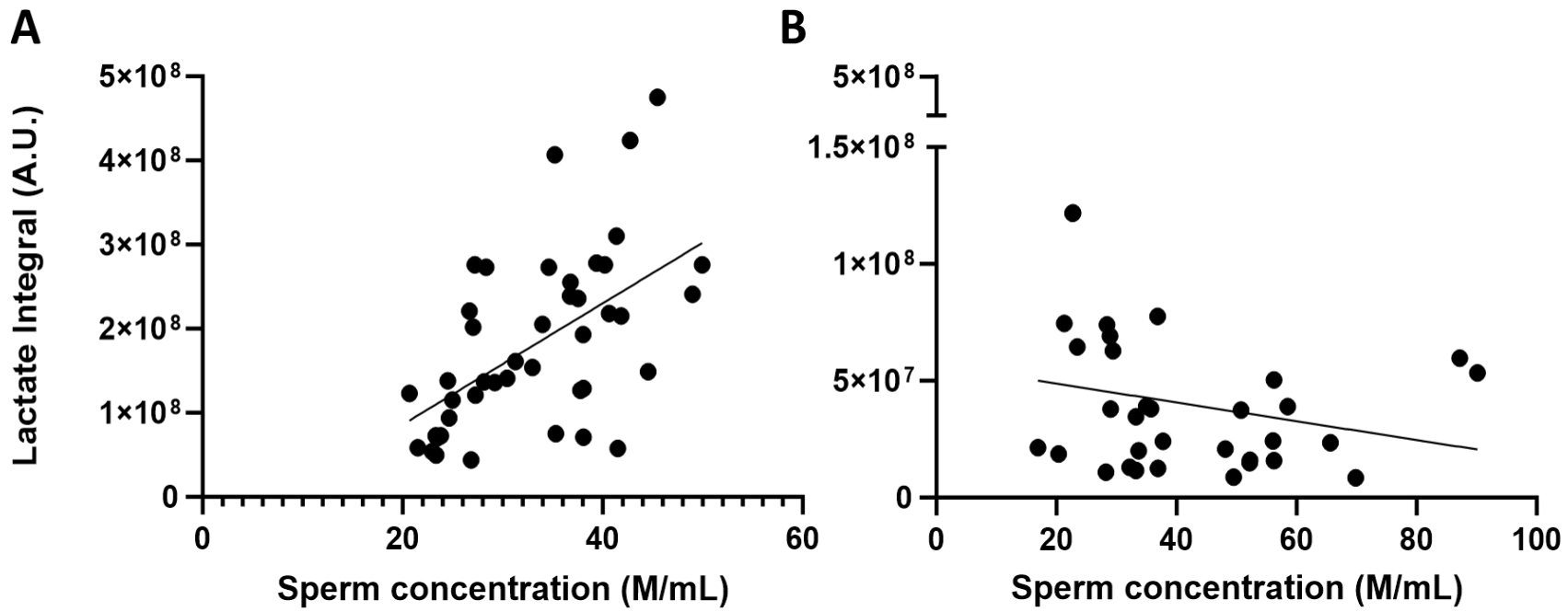


Figure A6: Raw lactate integrals from the positive control samples shown in this thesis vs total human (A) and boar (B) sperm concentration. For human sperm n = 44 and for boar sperm n = 33 as the same positive controls were used for the hyperactivation and acrosome reaction experiments. For human sperm (A), $P < 0.0001$ and $R^2 = 0.32$. For boar (B), $P = 0.17$ and $R^2 = 0.06$.

A7 Bicarbonate vs concentration

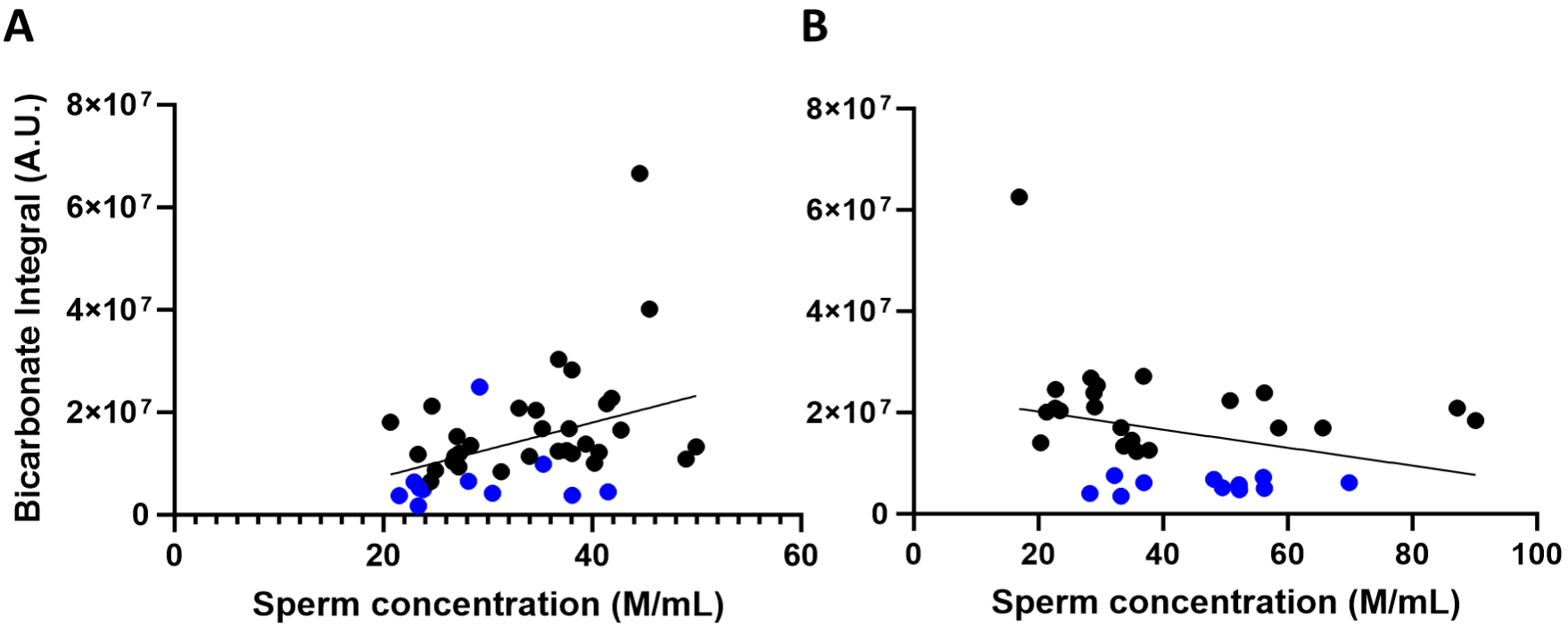


Figure A7: Raw bicarbonate integrals from the positive control samples shown in this thesis vs total human (A) and boar (B) sperm concentration. For human sperm n = 44 and for boar sperm n = 33 as the same positive controls were used for the hyperactivation and acrosome reaction experiments. Blue dots are indicative of the positive controls in capacitation where bicarbonate was not added as an inducer of capacitation. For human (A), P = 0.01 and R^2 = 0.15. For boar (B), P =0.10 and R2 = 0.08.

A8 The induction of hyperactivation in human and boar sperm using 4-aminopyridine.

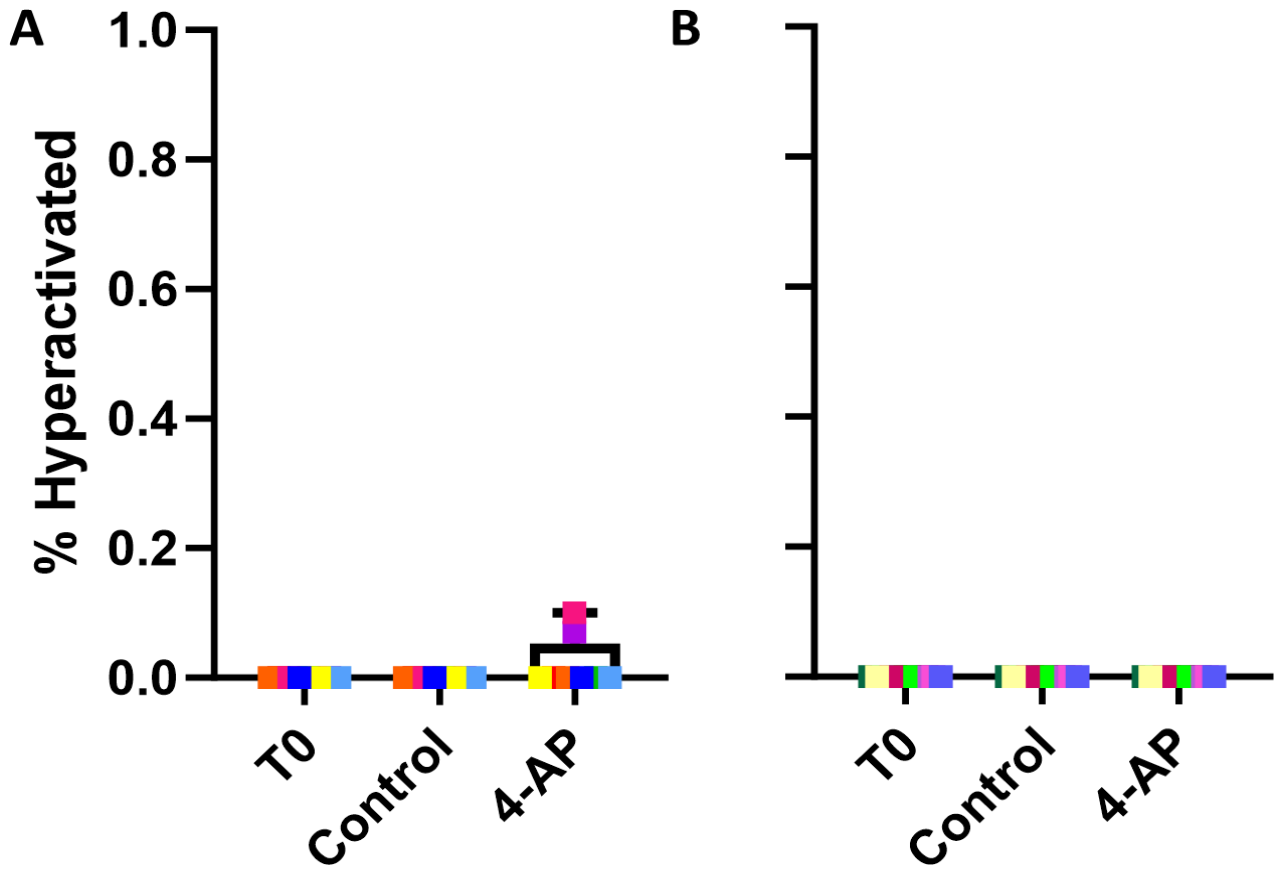


Figure A8: Percentage of hyperactivated human (A) and boar (B) sperm prior to (T0) and following a four-hour capacitation incubation and a five-minute incubation with or without the addition of 2 mM 4-AP. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. No significant differences were identified. Points colour coded per individual donor/boar, n = 8 human, and n = 11 boar.

A9 Raw bicarbonate integrals in the negative vs positive controls

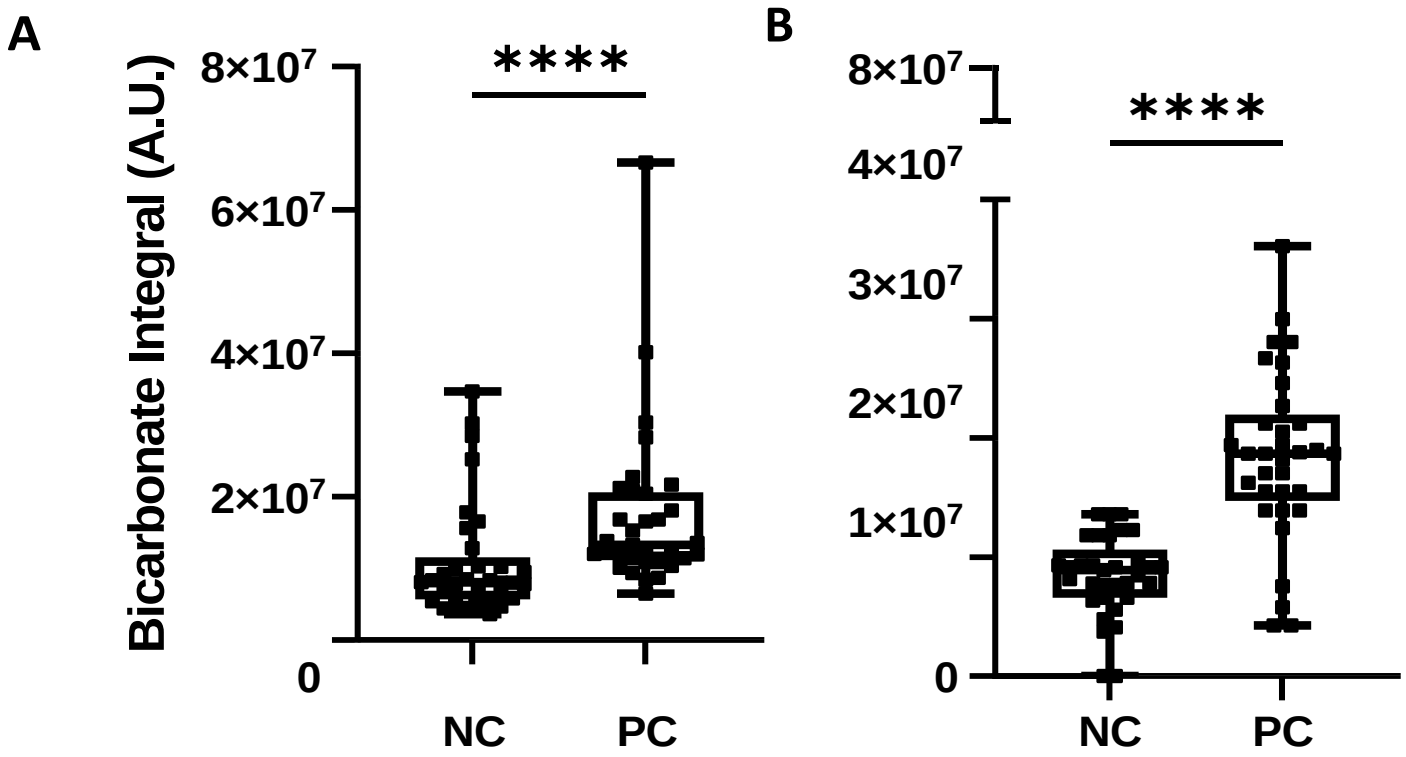


Figure A9: Raw bicarbonate integrals from NMR in negative controls (NC) containing no sperm vs positive controls (PC) containing either human (A) or boar (B) sperm. Data displayed as box and whisker plots showing the minimum and maximum values, the median value, and the interquartile range. A Wilcoxon test was used for human sperm (A) and a paired t test was used for boar sperm (B) based upon results from normality tests, **** = $p < 0.0001$. For both human and boar, n = 33 as all positive controls which contained bicarbonate were included.