EVALUATION OF SPERM FUNCTIONALITY IN NON-HUMAN PRIMATES, FOCUSSING ON SPERM CAPACITATION

Luke Allen Mabotha

Thesis submitted in partial fulfillment of the requirements for the degree *Master of Science* in Medical Bioscience at the University of the Western Cape

Supervisor: **Dr Retha Kotze**
Co-Supervisor: **Dr Liana Maree**
Dr Charon de Villiers

May 2019

Evaluation of sperm functionality in non-human primates, focussing on sperm capacitation

Luke Allen Mabotha

KEYWORDS

Infertility

Non-human primates

Rhesus monkey (*Macaca mulatta*)

Vervet monkey (*Chlorocebus aethiops*)

Sperm functionality

Sperm motility

Sperm kinematics

Sperm capacitation

Sperm hyperactivation

Sperm acrosome reaction

Mitochondrial membrane potential

Mitochondrial membrane inhibitor

Sperm vitality

Sperm functional tests

Physiological media

ABSTRACT

EVALUATION OF SPERM FUNCTIONALITY IN NON-HUMAN PRIMATES, FOCUSSING ON SPERM CAPACITATION

L. A. Mabotha

MSc thesis, Department of Medical Bioscience, University of the Western Cape

The incidence of male infertility is increasing, with up to 50% of infertile males having "unexplained" (idiopathic) infertility. Newly developed molecular techniques have great value in detecting subtle causes of male infertility, as compared to idiopathic infertility which may be explained by standardizing and optimizing sperm functional and structural tests in non-human primate (NHP) sperm. The aim of the study was to evaluate sperm functionality utilizing the sperm of two NHP species, i.e.1) the rhesus monkey (*Macaca mulatta*) and 2) the vervet monkey (*Chlorocebus aethiops*), and further evaluate the effect of physiological media (including commonly used, and newly formulated sperm wash and sperm capacitating media) on NHP sperm functionality. Sperm functionality was evaluated by investigating the following sperm functions i.e.: sperm motility, vitality, acrosome reaction (AR), hyperactivation, and mitochondrial membrane potential (MMP). Sperm functional tests included computer-aided semen analysis (CASA), motility analysis, BrightVit staining for sperm vitality, flourescenin isothiocyanate (FITC)- conjugated peanut agglutinin (PNA) staining for sperm acrosome integrity, induction of hyperactivation by stimulants (sperm preparation media containing capacitating ingredients), and mitochondrial inhibitor (Oligomycin-A) for testing MMP. All functional and structural tests were investigated in both species, except for acrosome integrity, mitochondrial inhibition and functional tests compared over time that could not be successfully completed and investigated in the rhesus species. Motility analysis tests proved that within the vervet species, the use of different physiological media results in statistically significant differences in motility and kinematic parameters over a 1 hour time period. Hyperactivation tests proved that capacitating physiological media produced significantly higher percentages hyperactivation when compared to sperm wash media within the vervet species over a 1 hour time period. Furthermore, within both NHP species, sperm structural analysis (vitality and acrosome integrity) results showed that no significant differences are present when making use of different physiological media over a period of 1 hour incubation. The incubation of vervet sperm with different concentrations of mitochondrial inhibitor, Oligomycin-A (0 μM, 5 μM, and 25 μM), resulted in motility inhibition over a 1 hour incubation period. By the evaluation of these tests it was found that the use of different sperm wash [Human tubal fluid (HTF), Ham's F-10® and HD Sperm Wash Plus (HDSWP)] and sperm capacitation media [Human tubal fluid with added caffeine (HTFC) and HD Sperm Capacitating Plus (HDSCP)] resulted in significantly different results within sperm functional tests as compared to sperm structural tests. The study indicates that the composition of media, varying from simple to more complex, used for semen

preparation plays an important role in determining NHP sperm functionality. Based on these findings further investigation in larger NHP sample groups and human sperm are required to evaluate the role of certain ingredients in the development of more cost-effective media producing satisfactory results in terms of sperm functionality for artificial reproductive technologies (ART).

May 2019

DECLARATION

I declare that **"Evaluation of Sperm Functionality in Non-human Primates, Focussing on Sperm Capacitation**" is my own work, that it has not been submitted before for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Luke Allen Mabotha

May 2019

Signed: 

ACKNOWLEDGEMENTS

I would like to recognise the following persons and institutions:

- The Comparative Spermatology Group from the University of the Western Cape
- Dr Retha Kotze for aiding as my Supervisor
- Dr Liana Maree for aiding as my Co-supervisor
- Dr Charon De Villiers for aiding as my Co-supervisor and staff of the Medical Research Council
- National Research Foundation
- My family for their support

LIST OF ABBREVIATIONS AND SYMBOLS

°C	Degree Celsius
β	Beta
γ	Gamma
δ	Delta
%	Percentage
<	Less than
>	Greater than
≥	Greater than or equal to
±	Standard deviation
10^6/ml	Million per millilitre
µg	Microgram
µl	Microliter
µm	Micrometre
µm/s	Micrometre per second
µM	Micromolar
ADP	Adenosine diphosphate
AG	Accessory sex gland
AI	Artificial insemination
ALH	Amplitude of lateral head displacement
AR	Acrosome reaction
ART	Assisted reproductive technologies
ATP	Adenosine triphosphate
BCF	Beat cross frequency
C3	Three–carbon molecule
C6	Six-carbon molecule

Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CASA	Computer-aided sperm analysis
CatSper	Cation channel of sperm
CCP	Carbonyl cyanide m-chlorophenylhydrazone
CHO	Chinese hamster ovary
Cl^-	Chloride
CNG	Cyclic nucleotide-gated channel
CO_2	Carbon dioxide
Cyt C	Cytochrome-C
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
ECRA	Ethics committee for research on animals
ETC	Electron transport chain
F_0	Oligomycin-sensitive sub-unit of the mitochondrial ATP-synthase complex in the mitochondria
F_1	Oligomycin-sensitive sub-unit of the mitochondrial ATP-synthase complex in the mitochondria
FAD	Flavin adenine dinucleotide
$FADH^2$	Flavin adenine dinucleotide (reduced form)
FITC	Flourescenin isothiocyanate
FSH	Follicle stimulating hormone
g	Gram
H^+	Hydrogen
H_2O	Water

H_2O_2	Hydrogen peroxide
HCL	Hydrochloric acid
HCO_3^-	Bicarbonate
HDSCP	HD Sperm Capacitation Plus
HDSWP	HD Sperm Wash Plus
HTF	Human tubal fluid
HTFC	Human tubal fluid with caffeine
HV1	Voltage-gated H^+ channel 1
Hz	Hertz
IAM	Inner acrosomal membrane
ICSI	Intracytoplasmic sperm injection
i.e.	In other words
IVF	In-vitro fertilization
K^+	Potassium
kg	Kilogram
LIN	Linearity
MAN	Mean angular displacement
mg	Milligram
Mg^+	Magnesium
MI	Male infertility
Mil/ml	Million per millilitre
Min	Minute (s)
ml	Millilitre
mm	Millimetre
MMP	Mitochondrial membrane potential
n	Number
Na^+	Sodium

Na^+/Ca^{2+} exchanger	Sodium-calcium ion channel protein
NAD	Nicotinamide adenine
NADH	Nicotinamide adenine (reduced)
NE	Nigrosin eosin
NHP	Non-human primate
NP	Non-progressive
O_2	Oxygen
O_2^-	Superoxide
OAM	Outer acrosomal membrane
OAT	Oligoasthenoteratozoospermia
ODF	Outer dense fibres
OWM	Old world monkey
OXPHOS	Oxidative phosphorylation
P-value	Calculated probability
PDE	Phosphodiesterase inhibitor
PGC	Primordial germ cell
pH	Expression of acidity or alkalinity
pH^-	Negative phase contrast
Pi	Inorganic phosphate
PM	Progressive motility
PNA	Peanut agglutinin
PUFA	Polyunsaturated fatty acids
Q	Ubiquinone
ROS	Reactive oxygen species
RPE	Rectal probe electro-ejaculation
sAC	Soluble adenylate cyclase
SANS	South Africa National Standard

SCA®	Sperm Class Analyser
SD	Standard deviation
SLO3	pH- and voltage-gated potassium channel
sNHE	Sperm-specific sodium-hydrogen exchanger
SP	Seminal plasma
STR	Straightness
T0	Baseline time point
T15	15-minute time point
T30	30-minute time point
T45	45-minute time point
T60	60-minute time point
TCA	Tricarboxylic acid
TM	Total motility
TRP	Transient receptor potential channel
UMI	Unexplained male infertility
V	Volt
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity
WHO	World Health Organisation
WOB	Wobble
Zn^+	Zinc
ZP	Zona pellucida

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

Chapter 1: Introduction

1.1 Introduction

Human semen quality has shown a decline over the last decades and is partly attributed to the increase of male infertility factor, and has subsequently brought about an increase in infertility treatment, including various artificial reproductive techniques (ART) (McSwiggin and O'Doherty, 2018). Infertility can be ascribed to a decrease in semen quality, testicular dysfunction, and genital tract infections. However, many male patients suffer from a condition known as unexplained male infertility (UMI) or idiopathic infertility (McSwiggin and O'Doherty, 2018), which is a major challenge in reproductive medicine and studies. Idiopathic infertility may be explained by standardizing and optimizing sperm functional and structural tests in non-human primate (NHP) sperm. Sperm are affected by various materials present in seminal plasma (SP). These materials can produce bioactive and toxic substances (decapacitation factors or free radicals) that can alter the capacity of the sperm to fertilize the oocyte (Rozeboom *et al*., 2000; Henkel, 2005). Abnormal sperm parameters such as low sperm concentration, poor sperm motility, and abnormal morphology contribute to low fertilization rates (Larson-Cook *et al*., 2003). The separation of sperm from the entire semen sample is important in ART. Various semen preparation techniques have been developed in ART programs to isolate motile, morphologically normal, and healthy functioning sperm for use in in-vitro fertilization (IVF). For example sperm washing techniques can be used to separate motile sperm from non-motile or dead sperm, as well as other debris that could cause impaired sperm functionality. The most commonly used methods are the simple washing of sperm for use in IVF. Sperm washing encompasses the selection of a suitable physiological medium for washing the whole semen sample before use in ART (Kim et al., 2015).

Media used for semen preparation plays an important role in ART as their composition can influence sperm motility. However, there remains a lack of studies comparing the effects of commonly used physiological media in semen preparation.

1.2 Aims and objectives

Aim:

The aim of this study was to evaluate two non-human primate species' sperm functionality with selected sperm parameters and tests when exposed to different physiological media over time.

Objectives:

1. Assess current or standard sperm functional tests in NHPs, and improve on the techniques
2. Develop alternative or new sperm functional tests by focussing on
    a. Sperm hyperactive motility
    b. Sperm mitochondrial functions
3. Analyse the effect of different physiological media on sperm functional characteristics:
    a. Sperm motility
    b. Sperm hyperactive motility
    c. Sperm vitality
    d. Sperm mitochondrial functions
4. Analyse the effect of different concentrations of mitochondrial inhibitor on NHP sperm functionality

1.3 Structure of thesis

This thesis consists of five chapters. Chapter 1 is the introductory chapter. Chapter 2 contains the literature review, reviewing relevant topics including male infertility, the use of NHP for reproductive research and sperm functionality, with emphasis on capacitation, which is the focus of this study.

Chapter 3 explains the methods and materials of this study, and protocols for the techniques used to evaluate sperm function, namely, sperm motility, vitality, acrosome integrity and hyperactivation. The evaluation of the primate sperm functionality incubated with the mitochondrial inhibitor is also included.

Chapter 4 presents the results obtained in this study. Sperm functional (motility parameters, kinematic parameters, hyperactivation, and mitochondrial activity) and structural characteristics (vitality and acrosome integrity) of the NHP species are provided. Furthermore, radar graphs, bar graphs and images from CASA, SCA® analysis are presented to depict the results obtained in the study. Motility analysis consisted of motility and kinematic analysis, whereas structural analysis consisted of vitality and acrosome integrity analysis. Mitochondrial inhibition was performed only on vervet species in this study.

Chapter 5 contains the discussion of the results obtained for this study. The chapter further discusses the limitations and recommendations for future studies, followed by a conclusion.

Chapter 2: Literature review

2.1 Male infertility

The decline in human semen quality over the last five decades is partly attributed to the increase of male infertility factor and has subsequently brought about an increase in infertility treatment, including various artificial reproductive techniques (ART). Despite extensive research, male infertility remains poorly understood. Infertility can be ascribed to a decrease in semen quality, testicular dysfunction, and genital tract infections. Many male patients suffer from a condition known as unexplained male infertility (UMI) or idiopathic infertility, (McSwiggin and O'Doherty, 2018) which is a major challenge in reproductive medicine and studies. In UMI, studies focus on the fertility potential of a sperm population, thus sperm functional and structural parameters play an important role in this field (Prag, 2017; McSwiggin and O'Doherty, 2018). However, ethical constraints often prevent the pursuit of many basic research questions in humans and consequently non-human primates (NHP) are utilised as alternative research models (Seier *et al.,* 1989; van der Horst *et al.,* 1999; VandeVoort, 2004; van der Horst, 2005; Wolf, 2009; Dancet *et al.,* 2011; Prag, 2017).

The most commonly used NHPs in reproductive research are the Old World monkeys (OWM). As compared to humans, these monkeys show a close relationship in terms of structural features of the male reproductive tract, hormonal control of reproduction, spermatogenesis and most aspects of fertilization (VandeVoort, 2004; van der Horst, 2005, Wolf, 2009; Dancet *et al.,* 2011; Maree, 2011; Thomsen, 2013). It is due to these close relationships that NHP's are often used in studies on male fertility or infertility, *in vitro* fertilization (IVF) or ART, male contraception and reproductive toxicology (van der Horst, 2005).

In NHPs, specifically vervet and rhesus monkeys, large variations in sperm parameters, with a notable difference between species, and samples were previously reported (de Villiers, 2018). Additionally, observing the sperm concentrations and percentage motility provides crucial baseline information aiding in selection criteria of individuals and application of reproductive study protocols in NHPs (Seier *et al.,* 1989; van der Horst *et al.,* 1999; Dancet *et al.,* 2011; Maree, 2011). The similarities noted between NHPs and humans, when referring to anatomy, endocrinology, and physiology, is key when appropriately examining the effects of experimental manipulation in reproductive biology (Seier *et al.,* 1989; van der Horst *et al.,* 1999; de Villiers, 2006; Maree, 2011; Prag, 2017).

To date, various studies on NHPs have concluded the significance of use in reproductive technologies (Seier *et al.,* 1989; van der Horst *et al.,* 1999; Mdhluli *et al.,* 2004; van der

Horst, 2005; Maree, 2011; Prag, 2017; de Villiers, 2018). For the most part, sperm functional studies focus on identifying and counteracting sperm dysfunction cellularly and molecularly. These studies work around finding reasons for this happening and ways to counteract it to be able to make use of ARTs and IVF (Kizilay and Altay, 2017). These sperm tests include motility, vitality, hyperactivation, AR, deoxyribonucleic acid (DNA) integrity, morphology testing and the use of different substances and chemicals in different sperm species. Although some of these studies have focussed on the use of OWM, as stated, the use of specific OWM species has not yet been tested in the comparison of different physiological media' effect on the sperm capacitating processes.

The following section describes the selection of NHP species for reproductive research; rhesus and vervet monkey species; an overview of fertilization and sperm characteristics, including sperm structure and functionality; and sperm structural and functional testing using computer-aided sperm analysis (CASA).

2.2 Selection of NHP species for reproductive research

NHP are valuable animal models that have significantly advanced our understanding of numerous biological phenomena in human reproduction. The value of NHP as models of human biological processes derives from their common ancestry and is evident in the unique characteristics (structural and functional) they possess in comparison to non-primate mammals. Asian and African OWM share 92% of their genetic material with humans (Sibley and Ahlquist, 1987). Most commonly used species of OWM in reproductive research, include rhesus monkeys (*Macaca mulatta*), vervet monkeys (*Chlorocebus aethiops*), cynomolgus monkeys (*Macaca fascicularis*) and bonnet monkeys (*Macaca radiata*) (Seier et al., 1989; van der Horst et al., 1999; VandeVoort, 2004; van der Horst, 2005; Wolf, 2009; Dancet et al., 2011; Prag, 2017Seier) to name a few.

The NHP species used in this study include the rhesus monkey and vervet monkey.

2.2.1 Rhesus monkey (*Macaca mulatta*)

With the widest geographical distribution, the rhesus monkey is the most adaptable of all the NHP species, and originates from the rugged mountains of north-central China, in Asia. These highly social animals live in groups of 10-60 individuals or more, exhibiting a multiple-male mating system (de Villiers, 2006; Maree, 2011; Massen *et al.,* 2011). They are seasonal breeders and in the Southern hemisphere, most births occur in October to January as compared to the Northern hemisphere where most births occur in April to September (Bielert and Vandenbergh, 1981). Males reach maturity between four to five years of age and denote sexual "activation" by displaying a dark reddish scrotum, penis and sex skin folds on their rump. The use of the rhesus monkey in reproductive studies has long been standing

with reference to different studies including, multiple follicle stimulation, and oocyte fertilization by intracytoplasmic sperm injection (ICSI), toxicology studies, contraceptive studies and cryopreservation of sperm to name a few (Hewitson *et al.,* 1998; Wolf, 2004; Nichols and Bavister, 2006, Hung, 2008; Maree, 2011; Prag, 2017; de Villiers, 2018).

2.2.2 Vervet monkey (*Chlorocebus aethiops*)

The vervet monkey (also referred to as the African green monkey) is considered the most widely distributed and most abundant monkeys in the world. The male vervet monkey averages with a mass of 5.5 kg and exhibit a red penis and a blue scrotum to signal sexual prestige (Mdhluli *et al.,* 2004; Maree, 2011; Ramulondi, 2018). In captivity, these monkeys breed all year round and are not affected by seasonal variation, rendering them as an appropriate animal model for ART studies. Additionally, another contributing factor to their use in ART is their reported human-like sperm characteristics (e.g. concentration, motility, pH of seminal fluid, and acrosomal integrity) in reproductive studies (Seier *et al.,* 1989; van der Horst *et al.,* 1999; Mdhluli *et al.,* 2004).

The rhesus and vervet monkey have been selected for this study due to their continuous availability, a successful captive breeding program, and effective semen collection protocols due to its reported human-like sperm characteristics.

2.3 Overview: Fertilization and sperm characteristics

2.3.1 Fertilization

Fertilization is the process by which the male gamete (sperm) and female gamete (oocyte) meet, fuse and form a zygote. In mammals, this fusion occurs in the female reproductive tract (Martini *et al.,* 2012). Figure 2.1 illustrates a sperm's path from insemination up until zona penetration within the female reproductive tract. Although ejaculated mammalian sperm are motile and morphologically normal, they are still unable to fertilize an oocyte. This is due to the fact that they undergo physiological changes and maturation, to become activated while traveling through the female reproductive tract towards the oocyte (Visconti, 2009; Gervasi and Visconti, 2016).

After ejaculation, the sperm seek to reach the oocyte first and capacitate, a process that includes acquiring of hyperactivated motility and the AR. Achievement of the latter processes requires sperm to first undergo capacitation, defined as a series of biochemical modifications of sperm to bind and penetrate the oocyte for fertilization to occur (Austin, 1952; Gervasi and Visconi, 2016; Rahman *et al.,* 2017).

Following the deposit of sperm in the vagina, the sperm move out of the SP and enter the highly hydrated cervical mucus. Within the cervical mucus sperm selection for morphology

and motility occurs. These selected sperm are characterized by low plasma membrane content of polyunsaturated fatty acids (PUFA), good motility and morphology. Selected sperm are transported through the uterus where further selection, by motility and capacitation, take place. After transport through the uterotubal junction, sperm enter the isthmus of the fallopian tube, a storage reservoir for maintaining sperm up to five days. The final selection of capacitated sperm, thermo-responsive, chemotaxis-responsive sperm and sperm that have good DNA integrity takes place here. Despatching of sperm through the cumulus oophorous indicates the final stages before fertilization, which includes sperm and zona pellucida (ZP) binding (Henkel, 2012).

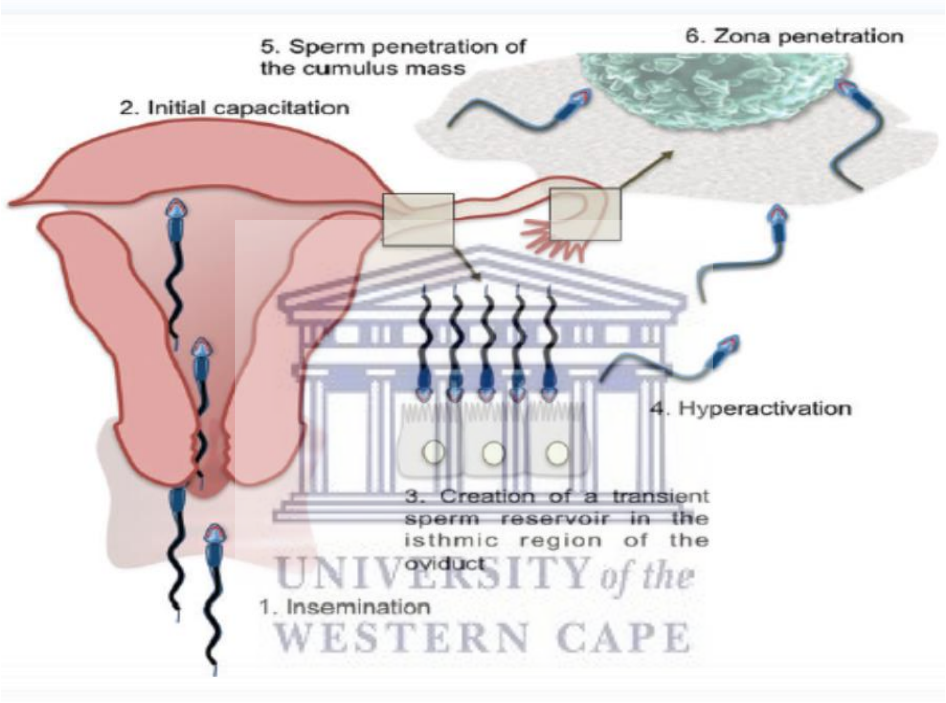


Figure 2.1 Path of mammalian sperm from insemination up to zona penetration in the female reproductive tract [Adopted from Martini *et al*. (2009)].

Sperm are “multi-compartmental cells” with a diverse array of physiological and biological properties. In order for a sperm to fertilize an oocyte, all structural compartments of the sperm, need to be functional and intact for the cell to serve its purpose (Ramalho-Santos *et al.,* 2002; Pesch and Bergmann, 2006; Martini *et al.,* 2009 Freitas *et al.,* 2017; McSwiggin and O’Doherty, 2018).

2.3.2 Mammalian sperm structural characteristics

The mammalian sperm is a differentiated cell (Ramalho-Santos *et al.,* 2002). Sperm become terminally differentiated through spermatogenesis taking place in the male testis, during which a large number of normal, motile sperm is produced. Spermatogenesis is

defined as the production of haploid sperm from diploid spermatogonia within the seminiferous tubules of the testes (Wilson and Hunt, 2002) and is initiated by the increased levels of testosterone at puberty. The three main stages of sperm production are comprised of 1) spermatocytogenesis (mitosis and meiosis of stem cells), 2) spermatidogenesis and finally, 3) spermiogenesis, which are all regulated by Sertoli cells (Wilson and Hunt, 2002; Sharpe, 2010). Figure 2.2 illustrates the various stages of cell division and differentiation during spermatogenesis.

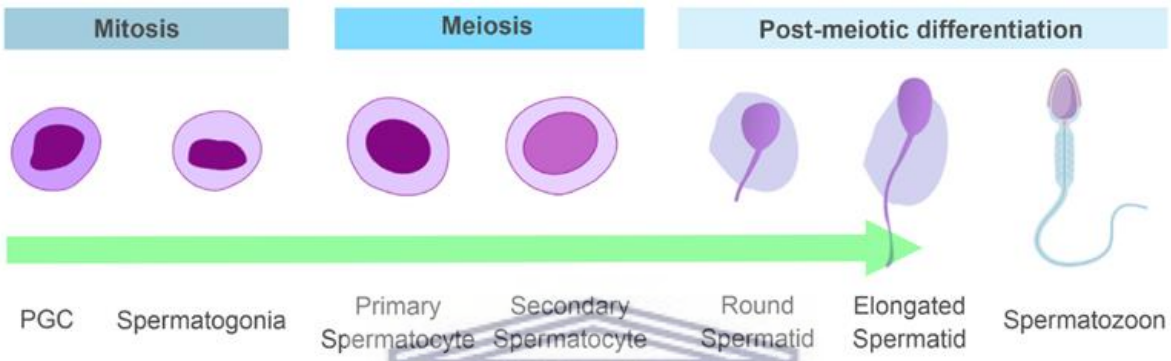


Figure 2.2 Process of spermatogenesis [Adopted from McSwiggin and O'Doherty, (2018)].

In mammals, immature germ cells, termed spermatogonia, are located on the outer edge of the seminiferous tubules next to the basal lamina where continuous mitotic proliferation takes place. Primary spermatocytes are produced from non-proliferating daughter cells. Two secondary spermatocytes are produced by division-I of meiosis. Following this, two secondary spermatocytes derived from each primary spermatocyte proceed through meiotic division-II to produce spermatids. The spermatids undergo morphological differentiation into sperm which escape into the lumen of the seminiferous tubules. The newly formed sperm are passed into the epididymis where they then undergo further maturation and are stored (Ramalho-Santos *et al.,* 2002; Wilson and Hunt, 2002; Pesch and Bergmann, 2006; Martini *et al.,* 2009; McSwiggin and O'Doherty, 2018).

A functional mammalian sperm consists of a head, neck, midpiece, and tail; its total length varies but is species-specific (Pesch and Bergmann, 2006) (Figure 2.3).

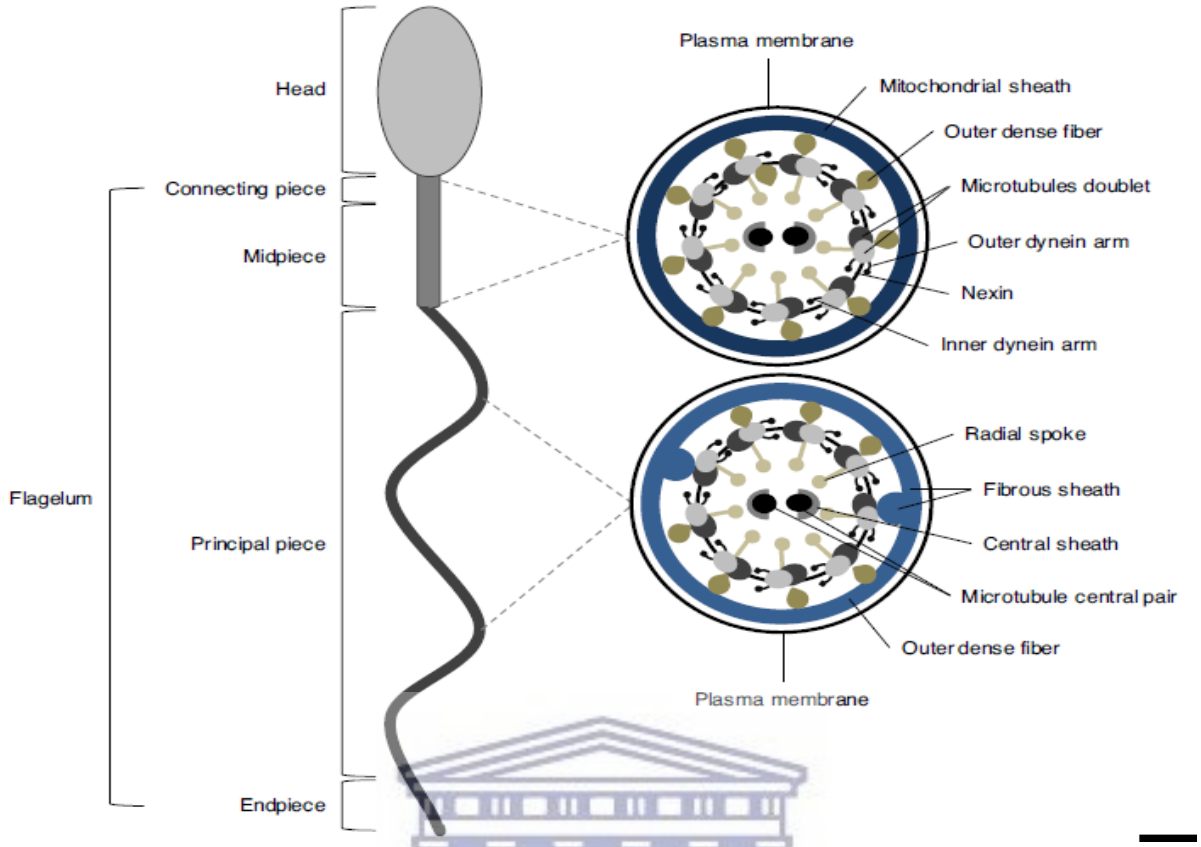


Figure 2.3 Structural diagram of a mammalian sperm [Adopted from Freitas *et al.* (2017)].

Head

The sperm head contains the nucleus with protamines, a sperm-specific DNA-binding protein (Fawcett, 1975). Protamines permit higher chromatin packaging than histones and play a role in reducing sperm volume, thereby increasing aerodynamic properties of sperm and facilitating fertilization. The sperm head is divided into two regions namely, the acrosomal (anterior head) and post-acrosomal regions (Abou-Haila, Bendahmane, and Tulsiani, 2014). The acrosome overlaying the nucleus is a large secretory vesicle containing hydrolytic enzymes, such as acrosin and hyaluronidase. Acrosomal enzymes, released during the AR, play a role in a sperm's penetration of the protective, glycoprotein based ZP of the oocyte (Henkel, 2012). The mature sperm is surrounded by a plasma membrane, which is characterized by variations in regional glycoprotein and lipid makeup. These surface domains are important for the specific function of certain membrane areas (Topfer-Petersen and Waberski, 2001), such as the equatorial segment of the sperm head's membrane which is responsible for the contact with the oocyte membrane during fertilization (Rovan, 2001). It has been suggested that sperm possess surface receptors that recognize and bind to units on the ZP, known as an extracellular glycocalyx surrounding the egg (Tulsiani and Abou-Haila, 2012; Abou-Haila, Bendahmane and Tulsiani, 2014). These receptors function to

recognize terminal sugar residues on the ZP aiding in the fertilization process in mammalian sperm. The ZP functions in species-specific sperm-egg recognition and binding; induction of the AR; the blocking of polyspermy (penetration of the oocyte cytoplasm by more than one sperm); and the protection of the growing embryo following fertilization up to implantation (Abou-Haila, Bendahmane and Tulsiani, 2014).

Neck

The neck or connecting piece of sperm is approximately 1 µm long and attached anteriorly to the basal plate of the flagellum and posteriorly to the outer dense fibers (ODF) of the flagellum, and serves as a connecting, articular piece (Pesch and Bergmann, 2006). The connecting piece is a short linking segment between the flagellum and the sperm head, composed of segmented columns and a dense fibrous structure, the capitulum, with the proximal centriole located next to it (Pesch and Bergmann, 2006).

Tail

The tail is the largest part of the sperm and consists of four major parts: the connecting piece, the midpiece, the principal piece, and the end piece (Figure 2.3) (Pesch and Bergmann, 2006; Lehti and Sironen, 2017). The connecting piece connects the head and tail together and contains the basal body. The midpiece of human sperm contain the mitochondria and is about 5 µm long and is characterized by the mitochondrial sheath that surrounds the axonemal complex and the nine ODF (Pesch and Bergmann, 2006; Lehti and Sironen, 2017; Freitas *et al.,* 2017). A mature mammalian sperm cell contains 22 - 75 mitochondria arranged around the flagellar portion of the midpiece in a helix formation (May-Panloup *et al.,* 2003).

There is a positive correlation between survivability and the number of mitochondria; sperm with a higher number of mitochondria have higher survivability (Rovan, 2001) and therefore a higher chance of fertilization potential. Thus, mitochondria present in mammalian sperm are necessary for motility (Freitas *et al.,* 2017).

The principal piece is the longest segment and is enclosed by fibrous sheaths made of two longitudinal columns (Fawcett, 1961; Eddy *et al.,* 2003). The sheath ends 9-10 µm from the tip of the tail where the principal piece merges into the end piece (Pesch and Bergmann, 2006). The fibrous sheath also works as a scaffold for proteins in signaling pathways; therefore it might be involved in regulating sperm maturation, motility, capacitation, hyperactivation, and AR as well as adding both strength and resistance to the tail (Eddy *et al.,* 2003). The end piece contains the axoneme that is surrounded by the plasma membrane. The mammalian mitochondrial and fibrous sheath encircling most of the axoneme and the ODFs add mechanical rigidity to the sperm tail creating an increased bend

wavelength which further aids sperm forward movement. These specialized anatomical features in the mammalian sperm tail are crucial for effective interaction of the sperm with the female oocyte, by providing forward swimming movement (Lindemann and Lesich, 2016).

2.3.3 Vitality

Sperm vitality is the percentage of sperm which are alive, not to be confused with the percentage sperm which are motile, since non-motile sperm may or may not be alive (Prag, 2017). Immotile sperm are still able to fertilize an oocyte, with the help of ART and ICSI, if their structural components are still intact and the cell thus still viable. Vitality testing is important when a sample presents with less than 40% progressively motile sperm; the test can verify the percentage dead cells and should not exceed the percentage immotile cells (WHO, 2010; Maree, 2011; Prag, 2017). The recommended vitality percentage for a sample to be deemed acceptable by the World Health Organization (WHO) is a lower reference limit of 58% (WHO, 2010).

Sperm structural characteristics including sperm acrosome intactness, morphology, DNA and membrane integrity are important in reproductive studies. In this study, the focus is aimed at sperm acrosome integrity as well as sperm vitality.

2.3.4 Seminal plasma

Semen consists of sperm suspended in a fluid medium called SP, which mediates the chemical function of the ejaculate. SP is a nutritive-proactive medium for sperm and some SP components are important for sperm metabolism as well as sperm function, survival, and transport in the female reproductive tract. Biochemical components of SP are secreted from the rete testis, epididymis, and accessory sex glands (AG) of the male reproductive tract (Mann and Lutwak-mann, 1981). The AGs consist of the seminal vesicles, prostate, and bulbourethral glands which collectively contribute to majority of the ejaculates volume. The secretion from the seminal vesicles constitutes the major portion of SP at ejaculation (Metafora *et al.,* 1989). SP contains organic compounds (citric acid, amino acids, peptides, proteins, lipids, and hormones cytokines), ions (Na^+, K^+, Zn^+, Ca^{2+}, Mg^{2+}, Cl^-), and energy substrates (fructose, sorbitol, and glycerylphosphocholine). Several factors in SP, including proteins, cytokines, sex hormones, and prostaglandins, accompany the migration of sperm to the female reproductive tract (Matousek, 1985) and possess potential biological capabilities to protect sperm from different pathogens, both in the male and female reproductive tract (Maegawa *et al.,* 2002).

Sperm function is known to be adversely affected by extended exposure of sperm to SP (Marti *et al.,* 2006). SP is known to have stimulatory as well as inhibitory effects on sperm

(Marti *et al.,* 2006; Maxwell *et al.,* 2007). Ejaculated semen contain senescent sperm and debris that negatively affect other sperm's survival rate (Marti *et al.,* 2006), and at ejaculation decapacitating factors (inhibitory factors) sourced from the AG bind to the sperm surface. Therefore, *in vitro* handling of sperm in preparation for artificial insemination (AI) aims to remove the SP and thereby the decapacitating factors. The removal of decapacitating factors is known to cause pre-capacitation of the sperm which could lead to unsuccessful fertilization of the oocyte (Bailey, 2010). Therefore, the use of sperm preparation medium is imperative when performing *in vitro* testing to aid sperm survival and sperm motility.

2.4 Sperm functionality

Sperm functionality can be defined as the capacity of sperm to successfully cross the different barriers in the female reproductive tract and become capable of eventually fertilizing the female oocyte (Marti *et al.,* 2006; Baily, 2010, WHO, 2010).

2.4.1 Motility

Sperm motility is an important feature of sperm to enable them to reach and fertilise the oocyte. Within the epididymis, the sperm become motile once they have matured and undergone several morphological and physical changes (Suarez, 2008). The flagellum produces the driving force of sperm. It contains microtubular-based machinery that generates the flagellar beat needed for motility, enabling a sperm to travel the long distance to the oocyte. In a freshly ejaculated semen sample, human sperm are said to be actively motile and display symmetrical movement, assisting the sperm in a straight line (Henkel *et al.,* 2005; Maree, 2011). The sperm axoneme is made up of nine microtubule doublets and a central pair in a 9+2 structure. These 9+2 structures are connected to each other through nexin links and radial spokes that connect the central pair. The radial spokes are responsible for positioning and spacing ensuring a circular arrangement of the microtubules around the circular pair. The two proteins playing a key role in sperm motility are the inner and outer axonemal arms that promote sliding of microtubule doublets. The flagellar beat is based on an on-and-off switch of the axonemal dynein arms within the axoneme (Freitas *et al.,* 2017). Once sperm are deposited in the female reproductive tract, they seek to reach the oocyte and simultaneously develop an asymmetrical flagellar movement, known as hyperactivation (active movement) upon arrival at the oviduct (Yanagimachi, 1970; Suarez, 2008; Bennet *et al.,* 2015).This movement is facilitated by the use of biochemical energy known as adenosine triphosphate (ATP). Once ejaculated, the sperm are exposed to many different environments and need to be "versatile" to maximize their chances of reaching the ovum (Ruiz-Pesini *et al.,* 2007). This motility is accomplished by interchanging between mechanisms for the production of ATP (Maree, 2011). The process and role of ATP production in sperm functionality will be discussed in section 2.4.5.

Role of calcium (Ca^{2+}) in sperm motility

Differentiated mammalian sperm do not make use of transcriptional or translational physiological processes for fertilization, thus, all cell activities are carried out through protein pathways present in sperm. These pathways are accomplished through the use of second messengers. The most common and essential second messenger present in sperm is calcium (Ca^{2+}), which is known to modulate nearly every aspect of cellular function in bacteria, protists, plants, fungi and animals (Cai *et al.,* 2015). In mammalian sperm, Ca^{2+} is known to regulate sperm motility, hyperactivation, chemotaxis, AR, and overall capacitation (Jimenez-Gonzalez *et al.,* 2005).

Mammalian sperm have to take part in various physiological changes, *in vivo* and *in vitro*, before achieving fertilization competency. Ca^{2+} acts as a central signaling molecule when inside mammalian sperm by exerting allosteric regulatory effects on enzymes and many proteins (Rahman, Kwon and Pang, 2014). *In vivo*, Ca^{2+} is obtained from the fallopian tubes in the female reproductive tract whereas, *in vitro*, it is resourced from the culture medium. Therefore, the mechanism of how Ca^{2+} crosses the sperm plasma membrane and initiates various physiological events is important to understand.

The influx of Ca^{2+} is achieved and facilitated by Ca^{2+} permeable ion channel proteins, located on or within the sperm plasma membrane. Examples of these proteins include voltage-gated (high/low) Ca^{2+} channels, transient receptor potential channels (TRP), and cyclic nucleotide-gated channels (CNG). These Ca^{2+} channel proteins have been demonstrated to play a vital role in numerous cellular processes via regulation of membrane potential and intracellular ionic balance. An increase in infertility or subfertility with the complete or partial absence of single or multiple Ca^{2+} channels has been seen, but no results have been shown for the cascading events (Zhang *et al.,* 2016). Ca^{2+} binds to the calmodulin in the 9+2 axoneme structure which generates sperm motion. Majority of the sperm activities include Ca^{2+} in their signaling pathway. Although other molecules and ions are also involved in active intracellular signaling, activities of these molecules or ions are always followed by the fluctuation of Ca^{2+} concentrations within the sperm (Zhang *et al.,* 2016). It has been shown that the main Ca^{2+} channel in mammalian sperm is the pH-sensitive voltage-gated calcium channel, cation channel of sperm (CatSper). CatSper consists of 4 α-subunits (CatSper 1-4) and 3 auxiliaries (β- beta, γ-gamma, and δ-delta) subunits. Sun *et al.* (2017) have shown that sperm selected through the swim-up method presented mostly with sperm that possessed CatSper 1 compared to the other CatSper subunits, also no hyperactivation was present with CatSper null sperm. The increase of intracellular Ca^{2+} coupled with the lowering of H^{+} increases pH, which is known to induce sperm hyperactivation (Sun *et al.,* 2017; Mishra *et al.,* 2018). Within the sperm cell CatSper is confined to the principle piece of the sperm

flagellum and functions to increase: 1) intracellular Ca^{2+} (Ca^{2+}-influx) 2) vigorous and fast sperm movements, 3) sperm hyperactivated motility (as stated), 4) sperm detachment from epithelial cells of female reproductive tract, and 5) female egg coat penetration and fertility (Tamburrino *et al.,* 2014; Sun *et al.,* 2017; Puga Molina *et al.,* 2018). Mammalian sperm hyperactivation is dependent on Ca^{2+} influx into the sperm cytoplasm. Figure 2.4 presents the regulation of the CatSper channel in mammalian sperm. The CatSper channel is opened by alkalization (mainly in mouse sperm), progesterone and prostaglandins (Puga Molina *et al.,* 2018), generating different effects on mammalian sperm. Different signaling channels, present in mammalian sperm, are presented in Table 2.1 coupled with their different effects on sperm.

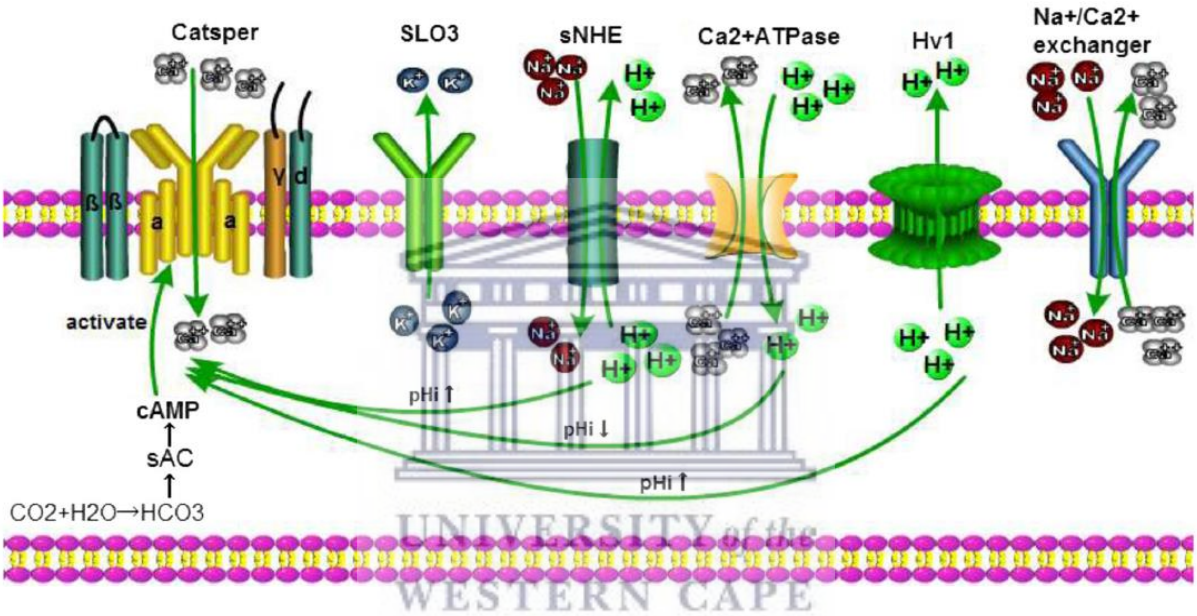


Figure 2.4 The regulation of mammalian CatSper channel [Adopted from Sun *et al.* (2017)].

Table 2.1 Effects of different ion channels in sperm [Adopted from Sun *et al*., (2017) and Mishra *et al*., (2018)].

Signaling channel	Channel name	Channel function	Channel effect on sperm
H^+	Na^+/H^+ exchangers (NHEs)	• Exchange of Na^+ and H^+ • Maintains intracellular alkalization	• Plays a role in sperm fertility
	Voltage-gated H+ channel 1 (HV1)	• Maintains intracellular alkalinization by removing H^+	• Associated with sperm capacitation
K^+	pH- and voltage-gated potassium channel (SLO3)	• Helps maintain sperm flagellar membrane potential balance	• Strongly related to sperm hyperactivation and motility
Ca^{2+}	The cation of sperm channel (CatSper)	• Accelerates Ca^{2+} influx	• Increased sperm motility) • Increased vigorous, fast sperm movements • Hyperactivated motility • Sperm detachment from epithelial cells of the female reproductive tract • Egg coat penetration and fertility. • Maintains sperm pH
	Ca^{2+} adenosine triphosphatase pump (Ca^{2+}ATPase)	• Removes intracellular Ca^{2+} and permits H^+ entry	
	Na^+/Ca^{2+} exchanger	• Negatively regulates CatSper channel • Intracellular Ca^{2+} balance	
HCO_3^-	Soluble adenylate cyclase (sAC)	• Activates and increases cAMP pathways • Activates CatSper channel	• Increases flagellar beat frequency

2.4.2 Capacitation

Whilst traveling through the female reproductive tract, sperm undergo alterations in their membrane composition, resulting in a change in sperm motility and becoming hyperactivated, a preparatory step essential for sperm to bind and penetrate the oocyte. These alterations involve the sperm head to undergo capacitation and the AR. Capacitation is defined as a fundamental maturational cascade of physiological processes by which sperm become capable of fertilization (Gadella, 2008), and involves a sequence of structural and functional changes of the sperm membrane, modulation of their enzyme activities, and protein phosphorylation (Abou-haila and Tulsiani, 2009).

Furthermore, as previously mentioned, Ca^{2+} plays a dynamic role in these processes as an intracellular second messenger (Rahman, Kwon and Pang, 2014).

Mammalian sperm capacitation, illustrated in Figure 2.5, involves the removal of adsorbed SP proteins (glycoproteins) from the sperm surface via female reproductive proteins (albumin) as well as the modification or reorganization of sperm surface molecules (Abou-haila and Tulsiani, 2009). Furthermore, the efflux of sterols (cholesterol) from the sperm

plasma membrane takes place, leading to a change in sperm membrane permeability and fluidity, and in turn, resulting in the influx of Ca^{2+} and HCO_3^- into the sperm head. Upon influx of Ca^{2+} and HCO_3^-, second messenger pathways are activated increasing cyclic adenosine monophosphate (cAMP) levels, followed by the activation of protein kinases. Protein kinases finally stimulate protein tyrosine phosphorylation and sperm hyperactivation (Abou-haila and Tulsiani, 2009).

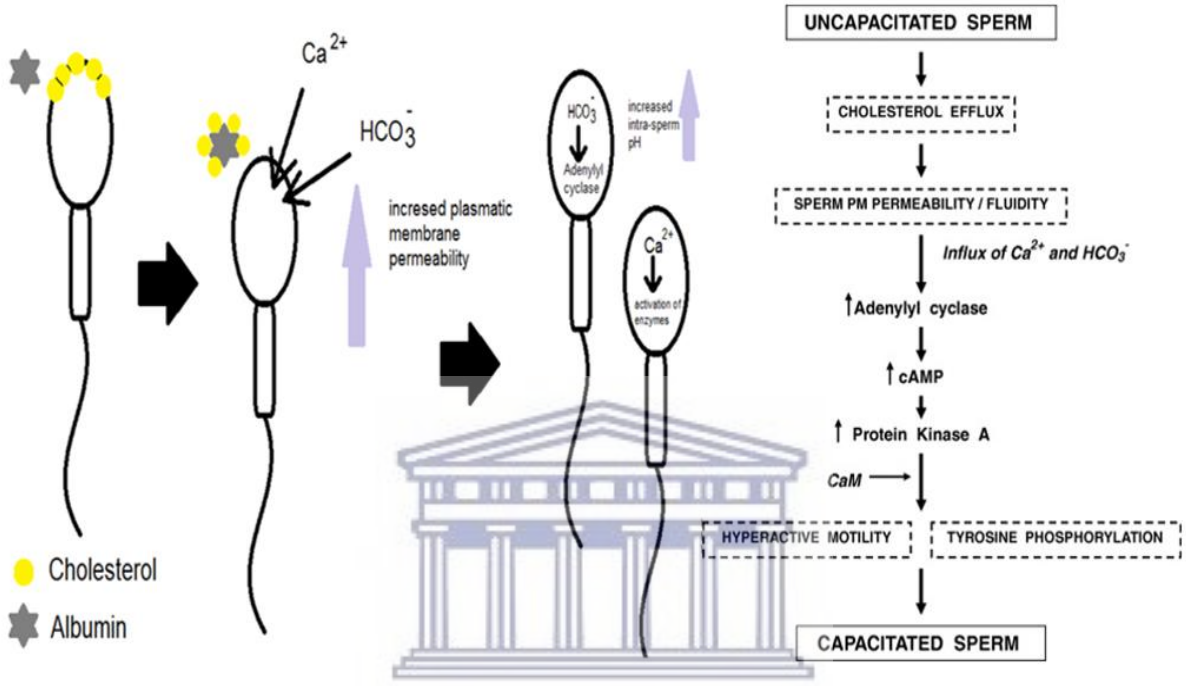


Figure 2.5 Mammalian sperm modifications during capacitation and the proposed physiological events taking place during capacitation [Adapted from Fertilitypedia.org, (2018); Abou-haila and Tulsiani, (2009)].

2.4.3 Hyperactivation

In addition to capacitation, sperm need to undergo hyperactivation, in preparation for fertilization (Jones, 2009). Hyperactivation is an important landmark for the final stages of capacitation and is essential for sperm to be able to detach from the isthmic 'reservoir' in the female reproductive tract and to speed up its movement to the ampulla of the fallopian tube for fertilization of an oocyte (Mújica *et al.,* 1994; Baumber and Meyers, 2006; Suarez, 2008; van der Horst, 2014; Ntanjana, 2015; Goodson *et al.,* 2017). Hyperactivation involves changes in the sperm flagellar beat, typically resulting in an increase in flagellar bend amplitude, thereby providing the needed force to overcome the attraction between sperm and epithelium within the fallopian tube. Hyperactivated sperm has a typical star-spin pattern as shown by van der Horst *et al.,* (2014) and Goodson *et al.,* (2017) (Figure 2.6). It was revealed that human semen samples with 20% hyperactivation poses the ability to fertilise the female oocyte (Mortimer and Mortimer, 1999; van der Horst *et al.,* 2018).

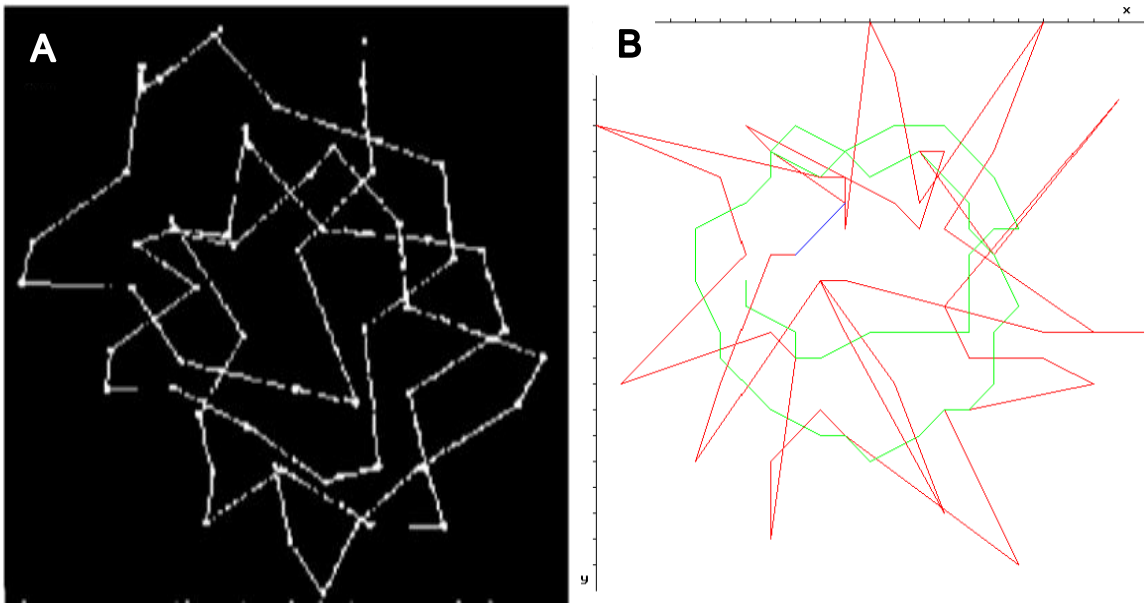


Figure 2.6 Imaging of hyperactivated sperm (star- spin) swimming patterns using SCA® [A. Adapted from Goodson *et al.* (2017); B. Adapted from van der Horst *et al.* (2014)].

Numerous physiological factors such as Ca^{2+}, cAMP, HCO_3^-, and metabolic substrates have been found to be essential for the initiation of maintaining hyperactivated motility in sperm *in vitro (*Nishigaki *et al.,* 2014*)*. Experimental evidence suggests that hyperactivated motility has four major roles in the process of fertilization (Figure 2.7).

Furthermore, hyperactivation assists sperm with flexibility, by navigation through the increasingly complex tubal maze (Jones, 2009; Lu *et al.,* 2014). High-quality sperm are hypothesized to display a high percentage of hyperactivation.

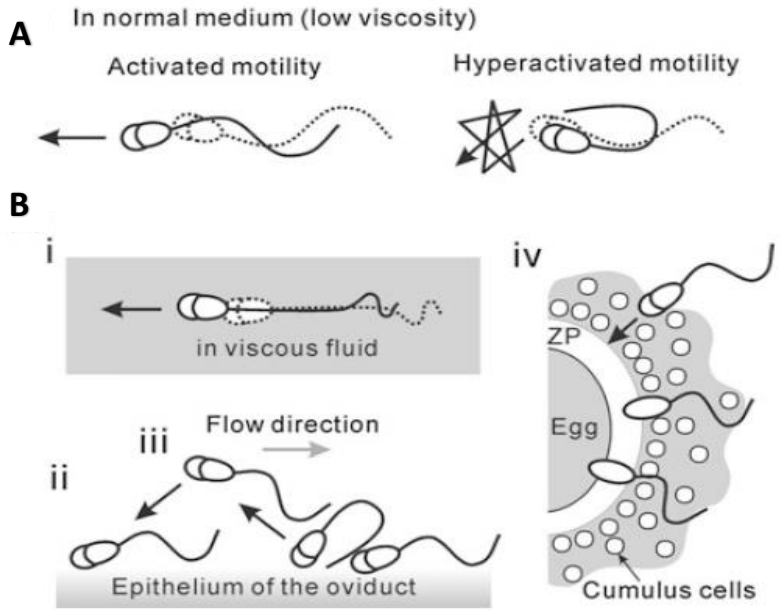


Figure 2.7 Roles of hyperactivated motility in fertilization. A) Sperm immediately after ejaculation, B) Hyperactivated sperm, i) maintained progressive motility within the viscous environment, ii) detachment from the sperm reservoir,iii) rheotaxis, iv) penetrate the extracellular matrix of zona pellucida. [Adopted from Nishigaki *et al.* (2014)].

2.4.4 Acrosome reaction

The final maturation step, known as the AR, occurs once the sperm have reached the oocyte (Baumber and Meyer, 2006) and the process is induced by the binding of the sperm to the ZP (Rovan, 2001; Ramalho-Santos *et al.,* 2007; Abou-haila and Tulsiani, 2009; Bedford, 2011). The AR enables the sperm to fuse with the oocyte by penetrating the ZP (Ickowicz *et al.,* 2012). Only hyperactivated, capacitated sperm will bind to the ZP and further penetrate the oocyte (van der Horst *et al.,* 2014; Goodson *et al.,* 2017; van der Horst *et al.,* 2018). The AR is defined as the Ca^{2+}- dependent exocytosis of the acrosome, requiring multiple fusions between the outer acrosomal membrane (OAM) and the plasma membrane of the sperm. The AR includes the release of the proteolytic enzymes (hyaluronidase and acrosin) within the sperm head region, which facilitates the penetration of the ZP. Another aspect of the AR is the exposure and formation of a new surface membrane, the inner acrosomal membrane, which is required for fertilization (Figure 2.8 illustrates the sequence of events taking place during the AR, leading to acrosomal exocytosis and release of acrosomal contents). Therefore, the lack of an acrosome in any condition is an indication that the sperm will likely not be fully functional (Tollner *et al.,* 2003, de Villiers, 2006; Wolf, 2009; Ickowicz *et al.,* 2012; Prag, 2017).

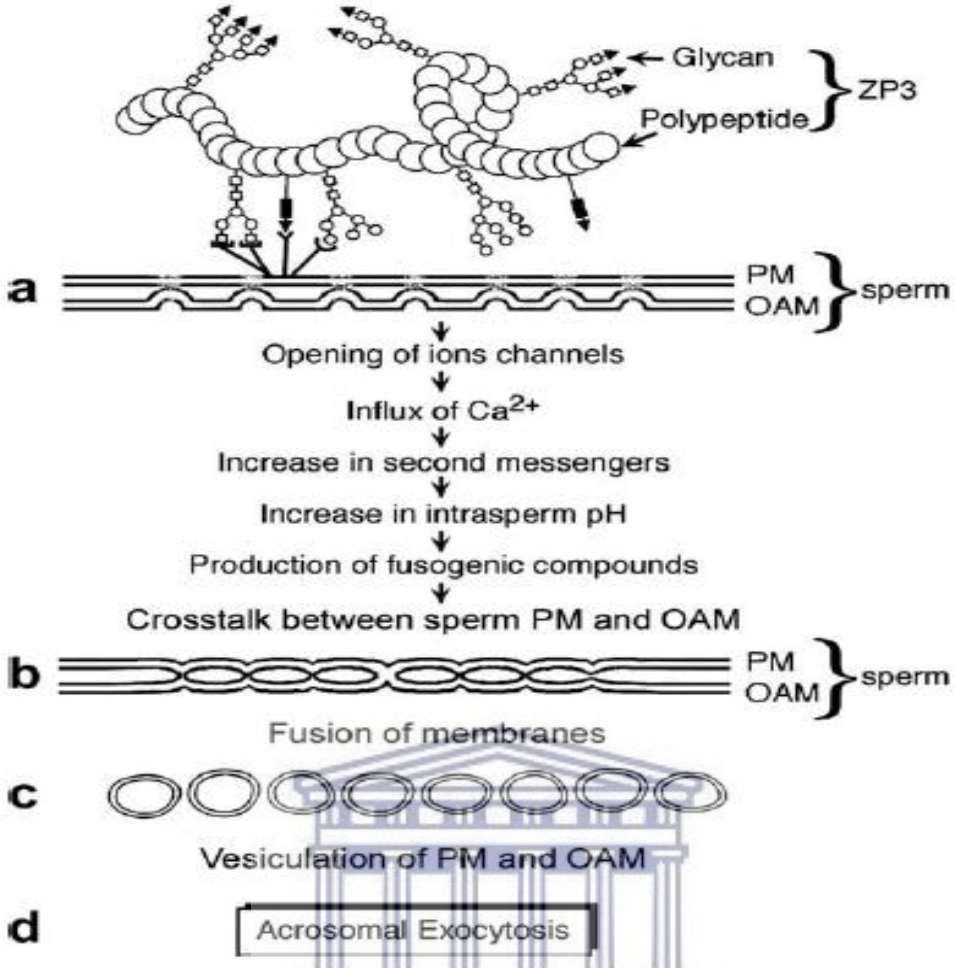


Figure 2.8 Model illustrating a possible sequence of events leading to acrosomal exocytosis and release of acrosomal contents a-d = Sperm plasma membrane and sperm outer acrosomal membrane changes leading up to acrosomal exocytosis [Adopted from Abou-haila and Tulsiani, (2009)]. PM = plasma membrane, OAM = outer acrosomal membrane.

The phases of the AR are initiated by the fusion of the sperm plasma membrane and the underlying OAM at multiple sites. This binding causes the increase of Ca^{2+} channels to open and the influx of Ca^{2+}. The increase in Ca^{2+} ions results in the increase of second messengers (cAMP), sequentially causing an increase in pH. Fusion results in the formation of hybrid vesicles and the release of acrosomal contents (hydrolytic enzymes). The release of hydrolytic enzymes causes the vesiculation of the sperm plasma membrane and OAM (Abou-haila and Tulsiani, 2009; Ickowicz *et al.,* 2012).

2.4.5 ATP production in sperm

In mammalian sperm, ATP is specifically used for maintenance of its intracellular environment and cellular processes comprising of motility, capacitation, hyperactivation and the AR (du Plessis *et al.,* 2015). Sperm can activate different metabolic pathways, depending on the availability of substrates and conditions they are exposed to; this trait is critical to ensure motility and finally fertilisation (Piomboni *et al.,* 2012).

In mammalian sperm, ATP must be delivered along the entire length of the flagellum, meaning sperm must adopt a variety of metabolic strategies to generate ATP. The two major mechanisms that have been considered for ATP production in sperm are 1) oxidative phosphorylation (OXHPOS) and 2) glycolysis. Figure 2.9 illustrates these two mechanisms of ATP production at varying sites in the sperm. OXHPOS is defined as the process in which ATP is formed as a result of the transfer of electrons by a series of electron carriers inside the mitochondria (Berg *et al.,* 2002), and originates from the midpiece (from the mitochondria) (du Plessis *et al.,* 2015).

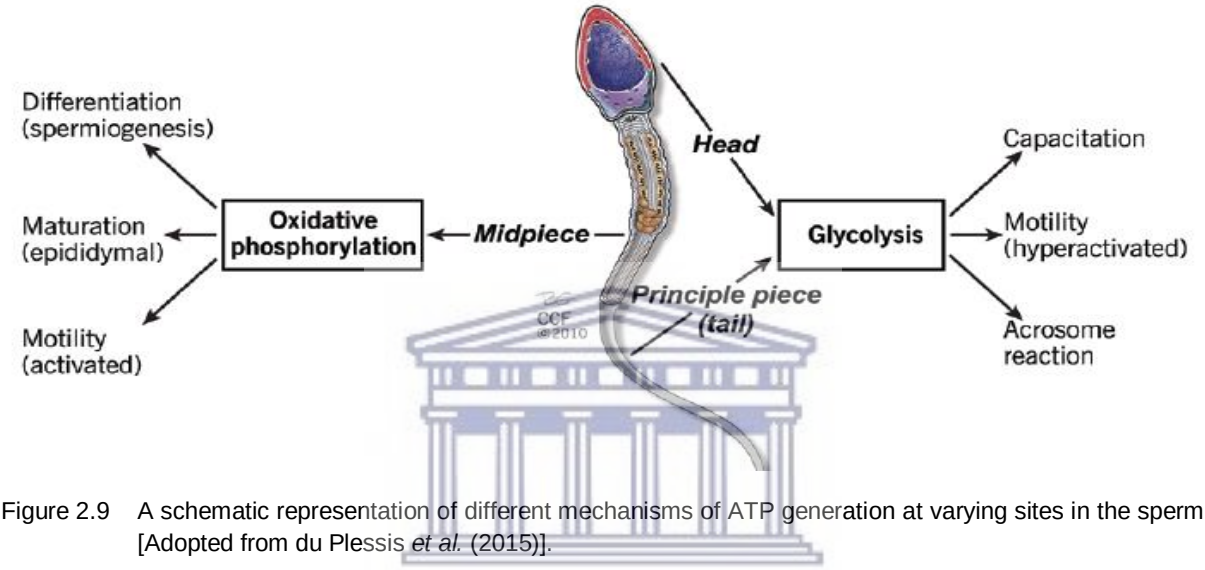


Figure 2.9 A schematic representation of different mechanisms of ATP generation at varying sites in the sperm [Adopted from du Plessis *et al.* (2015)].

Glycolysis is defined as an anaerobic (without O_2 present) set of reactions that converts one glucose molecule into two ATP molecules (by catabolising glucose) through ten cascading reactions and originates within the head and principal piece of the sperm flagellum (Hung *et al.,* 2008). These newly formed ATP molecules can, among its role in other processes, be used for sperm motility. The sperm's head and principle piece do not contain respiratory enzymes, thus, ATP production can only occur through the process of glycolysis in this area (du Plessis *et al.,* 2015).

After ejaculation, mammalian sperm acquire energy from molecules present in SP and the female reproductive tract. These molecules are converted into ATP and high-energy compounds used in the cell (Visconti, 2012). ATP is produced in a three-step process including glycolysis, tricarboxylic acid (TCA) cycle and electron transport chain (ETC/ OXPHOS), as illustrated in Figure 2.10 below.

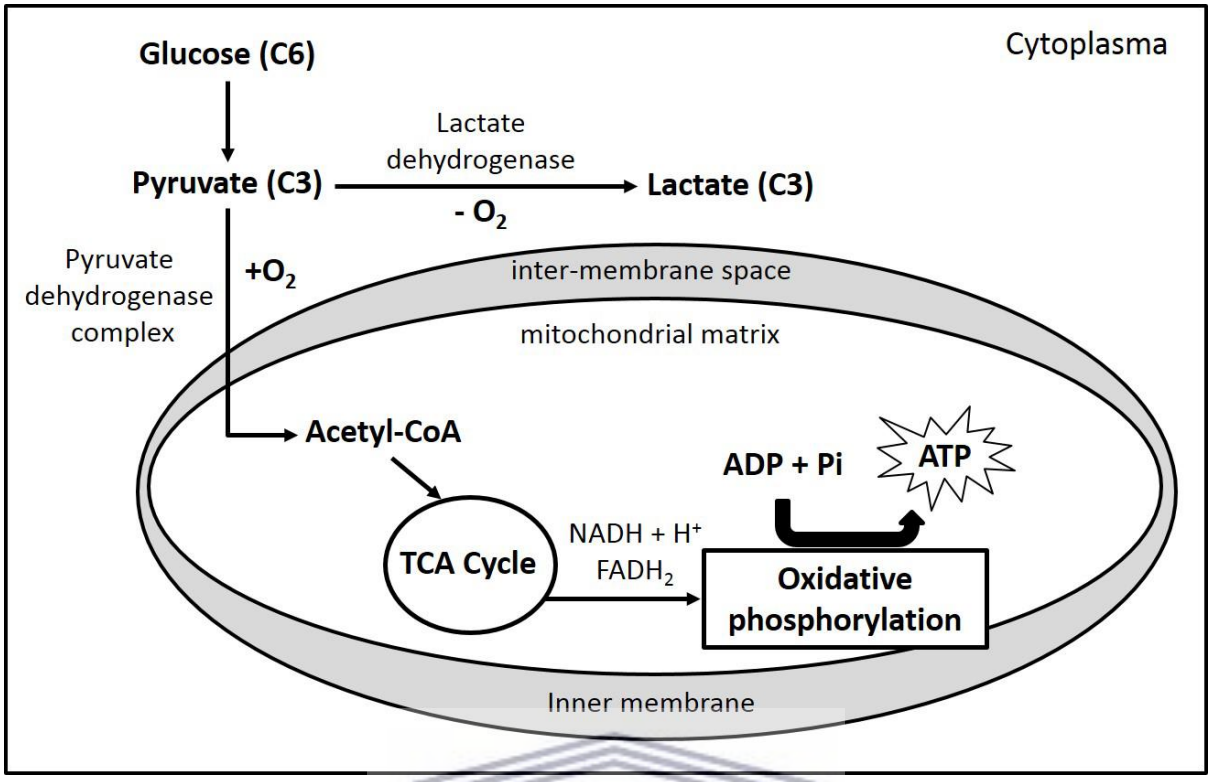


Figure 2.10 ATP production processes in animal cells [Adopted from Thiago *et al. (*2015)]. ADP = adenosine diphosphate, ATP = adenosine triphosphate, CoA = coenzyme A, TCA = tricarboxylic acid, FADH = flavin adenine dinucleotide (reduced form), NADH = nicotinamide adenine (reduced).

Under anaerobic conditions pyruvate is reduced by lactate dehydrogenase into lactate, generating two molecules of ATP per molecule of glucose. More often, under aerobic conditions (with O_2), pyruvate is transported into the mitochondria matrix, where it is oxidatively decarboxylated to acetyl-coenzyme A (CoA) by pyruvate dehydrogenase complex. The TCA cycle is a sequence of reactions that occur in the mitochondrial matrix and is always followed by OXPHOS. In the TCA cycle, Acetyl-CoA is oxidized and nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) are reduced, respectively, to NADH and $FADH_2$. These coenzymes are subsequently reoxidized in the respiratory chain, coupled with ATP production (Thiago *et al*., 2015).

2.4.6 Role of mitochondria in ATP production and sperm functionality

During sperm maturation, the mitochondrion is one of the structures that stay prominent to aid in sperm functionality. The purpose of the mitochondria, throughout its diverse locations within different cells, is for the production of chemical energy (ATP) for various purposes (Hung *et al.,* 2008; Maree, 2011; Piomboni *et al.,* 2012; Losano *et al.,* 2017). Thus, in order to understand the role of mitochondria in ATP production and subsequently sperm functionality, the steps involved in ATP synthesis within mitochondria will be explained below. Once glycolysis (in the cytoplasm) and the TCA cycle (in the mitochondria) has been

completed, the next step of ATP production in the mitochondria is OXPHOS. During OXPHOS electrons from NADH-H^+ and $FADH_2$ (produced from glycolysis and TCA cycle) are transferred through an ETC consisting of five protein complexes (complex I-V) embedded within the inner mitochondrial membrane (Figure 2.11). For OXPHOS to take place O_2 must be present, as opposed to glycolysis that is an anaerobic process. At complex-I, electrons are transferred from NADH-H^+ to NADH-Q oxidoreductase coupled with 4 H^+ molecules being transferred to the intermembrane space. Simultaneously, electrons are transferred from $FADH_2$ to succinate-Q reductase/complex- II. The next step in the electron transport chain involves the reduction of ubiquinone (coenzyme Q) to ubiquinol (QH_2, dihydroquinone), followed by a donation of electrons from ubiquinol to Q- cytochrome C oxidoreductase/complex-III, and termed the Q-cycle (4 additional H^+ transferred to the intermembrane space). Once reduced, cytochrome C functions as a shuttle for electrons from complex-III to cytochrome C oxidase/complex-IV. Simultaneously, O_2 is reduced to H_2O and $2H^+$ are transferred to the intermembrane space. The production of ATP is achieved in the final step of OXPHOS. ATP synthase/complex-V drives the synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate, coupled with the release of energy from the passage of H^+ back within the mitochondrial matrix (Piomboni *et al*., 2012).

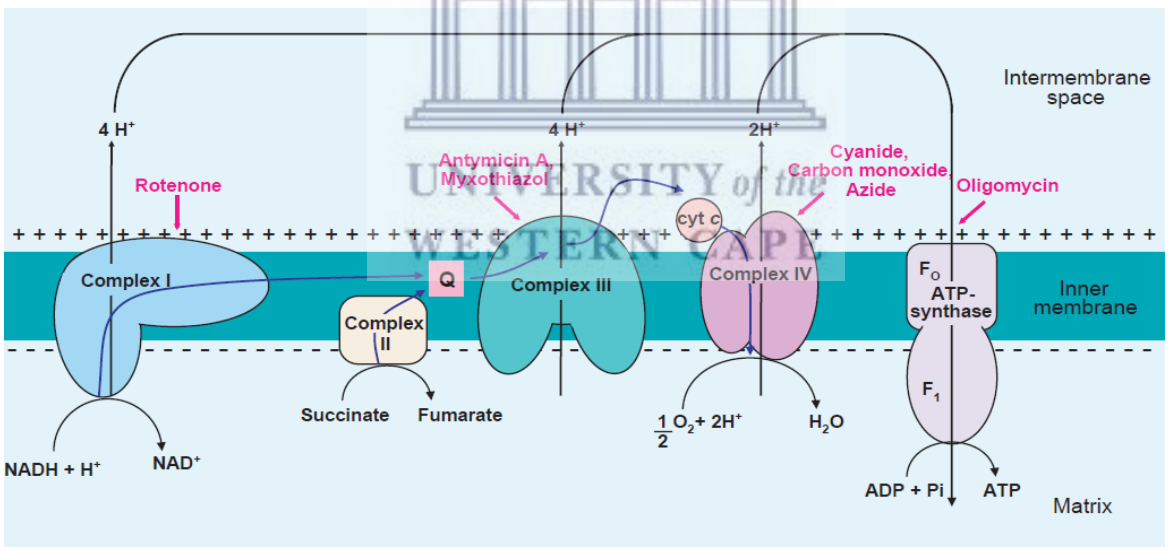


Figure 2.11 Electron transport system in the inner mitochondrial membrane and common mitochondrial respiratory chain inhibitors. ADP = adenosine diphosphate, ATP = adenosine triphosphate, Cyt c = cytochrome c; Q = ubiquinone; NAD, NADH, Complex- I-IV = protein complexes [Adopted from Piomboni *et al*. (2012)].

The midpiece, and therefore the mitochondria, is believed to be the driving force of ATP production over the sperm head (glycolysis), owing to studies reporting midpiece irregularities of asthenozoospermic males producing sperm with decreased motility and studies noting the decrease in sperm motility due to sperm mitochondrial defects (Maree,

2011; Amaral *et al.,* 2013). Furthermore, the inhibition of various OXPHOS complexes, via specific complex inhibitors/uncouplers e.g. rotenone, antimycin-A, cyanide, and oligomycin, also demonstrated a decrease in sperm motility with the use of glucose-containing media (Maree, 2011).

Mitochondria contain a double membrane, and during OXPHOS the protons are pumped across the inner mitochondrial membrane and into the mitochondrial intermembrane space, creating an electrochemical gradient termed the mitochondrial membrane potential (MMP) (Wang *et al.,* 2003; Zhang *et al.,* 2016). MMP is an important indicator of mitochondrial function and therefore sperm functionality, with a clear correlation to sperm motility and fertilization ability. A decrease in MMP is correlated to the increase in reactive oxygen species (ROS) in human sperm. ROS are normally produced by the mitochondria and are regulated by anti-oxidants for sperm functions (capacitation, hyperactivation, AR, and sperm-oocyte fusion), however, once the elimination by anti-oxidants is disrupted or an increase of ROS occurs, the result is oxidative stress (Wang *et al.,* 2003; Maree, 2011; Zang *et al.,* 2016). The main way of ROS-mediated sperm damage is peroxidative damage to the cell membrane, impairment of sperm motility, and oxidative damage to DNA (Wang *et al.,* 2003; Tremellen, 2008). Furthermore, Wang and colleagues (2003) concluded that measurement of MMP in sperm provided useful information about fertility potential and that an increase in ROS production by sperm was associated with a decrease in MMP. In this study, the main focus is on NHP sperm. The lack of research on these species with regards to evidence to which pathway is the main driver for sperm motility is marked. A study performed by Hung and co-workers (2008) on rhesus monkey sperm, suggesting glycolysis was essential to support sperm motility, hyperactivity, and protein tyrosine phosphorylation, whereas energy from OXPHOS was not necessary for hyperactivated sperm motility, tyrosine phosphorylation, sperm-zona binding, and AR in this species.

2.5 Sperm preparation and physiological media

Physiological media used for sperm preparation is designed to maintain sperm structural and functional qualities. The ideal sperm preparation medium ensures the maintenance of a stable pH and osmolality while providing the proper physiological environment for cell survival (Okamura *et al.,* 1985; Quinn, 2000; Zollner, 2001; Gadella and van Gestel, 2004; Gadella, 2005; Witte and Schafer-Somi, 2007; Murase *et al.,* 2010). It has been shown that dilution of a semen sample with a medium can improve sperm functionality and enhance the chances of fertilization (Alvarez *et al.,* 2007; Lanzafame *et al.,* 2009). Established criteria for "good" sperm selection procedure or techniques include the following: elimination of SP, de-capacitating factors, and debris; elimination or reduction of dysfunctional and ROS-producing sperm, leukocytes, and bacteria; supporting of functional sperm in terms of

motility, DNA integrity, AR, and normal sperm morphology; cost-effectiveness; easy and quick procedures; and the processing of large volumes of ejaculates (Henkel *et al.,* 2005; Maree, 2011; Prag, 2017; de Villiers, 2018; van der Horst *et al.,* 2018).

The nature of sperm preparation media affects sperm structural and functional characteristics (Khalili *et al.,* 2016). For example, sperm washing media may contain several chemicals that function in enhancing the competence of sperm. Studies have suggested that media composed of 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), bicarbonate (HCO_3^-), Ca^{2+}, and magnesium (Mg^{2+}) may increase the chances of successful fertilization (Okamura *et al.,* 1985; Wong *et al.,* 2001; Zollner, 2001; Gadella and Van Geste, 2004; Gadella, 2005; Witte and Schafer-Somi, 2007; Murase *et al.,* 2010). In particular, HCO^-_3 in SP stimulates adenylate cyclase in sperm, and the HCO_3^--sensitive adenylate cyclase system has been shown to regulate sperm motility, as well as serum albumin and myo-inositol (Ashrafi *et al.,* 2013; Pamieri, 2016). HEPES is a buffer that is used in combination with sperm physiological media. Studies show that HEPES aids with protection against free oxygen radicals known to cause sperm membrane and DNA damage in oligoasthenoteratozoospermic (OAT) samples (Zollner, 2001). Mg^{2+}, an important cation present in the majority of enzymatic systems, plays an important role in energy metabolism (ATP) into spermatogenesis and sperm motility (Wong *et al.,* 2001).

Different physiological media are commercially available and are manipulated to mimic SP and aid sperm survival for use in ART and IVF techniques (Quinn, 2000; Kim, 2015). However, despite the variety of physiological media used for sperm preparation very few studies have reported data comparing the outcomes of different sperm preparation (semen washing) media on semen parameters.

Although there are a variety of media used for sperm preparation, for the purpose of this study only media commonly used for sperm preparation i.e. human tubal fluid (HTF) and Ham's F-10® (WHO, 2010) will be briefly discussed. Table 3.1 (chapter 3) presents the nutritional makeup of the different physiological media used in this study.

2.5.1 Commonly used physiological media

Human tubal fluid (HTF)

HTF is a defined medium used in human ART procedures. It has been formulated to mimic the composition of the fluid present in human fallopian tubes (Lippes *et al.,* 1972; Borland *et al.,* 1980). HTF makes use of a sodium HCO_3^- buffering system and is appropriate for procedures requiring the use of a carbon dioxide atmosphere during incubation (Quinn *et al.,* 1985). Therefore, HTF is intended for the retrieval, culture, transport, storage, and transfer of sperm *in vivo* and *in vitro* (Quinn *et al.,* 1985).

Ham's F-10®

Ham's F-10® was originally designed for serum-free growth of Chinese hamster ovary (CHO) cells and has been developed specifically to support the growth of various cells with specific nutritional requirements. Ham's F-10® has been used in the growth of mammalian cells, and as a sperm washing medium (van der Horst *et al.,* 1999; Quinn, 2000; de Villiers, 2006; Maree, 2011; Kim *et al.,* 2015). Ham's F-10® has been proven to sustain sperm functions for long periods of time and has been used in various NHP species (van der Horst *et al.,* 1999; de Villiers, 2006; Maree, 2011). Ham's F-10® function's to extend sperm motility and imitate the environment of the female reproductive tract and is widely used for the improvement of motility of ejaculated sperm (Quinn, 2000; Kim *et al.,* 2015). The presence of various nutritional substrates, (Table 3.1) in Ham's F-10® justifies the use of this physiological medium in reproductive studies focussing on sperm preservation.

2.5.2 Ingredients associated with sperm functionality

To reach their full fertilization capacity, mammalian sperm must undergo a series of physiological changes termed capacitation. It is well known that the use of different capacitating agonists inducing hyperactivation is common practice within reproductive research (van der Horst *et al.,* 2018). Therefore the following section briefly describes the ingredients of physiological media used/known to induce mammalian sperm capacitation as well as hyperactivation. These ingredients include caffeine, progesterone, myo-inositol and procaine hydrochloride.

Caffeine

Caffeine is a naturally occurring chemical present in various species of plants and is known for its stimulatory effects on alertness, decreased sleepiness and fatigue. Caffeine is structurally similar to adenosine and therefore binds adenosine receptors causing an antagonistic effect which subsequently stimulates physiological activity. The stimulatory effects on sperm cells have been demonstrated showing the increase of cAMP levels (Ntanjana, 2014) and an increase in hyperactivated sperm in different species (Mbizvo *et al.,* 1993; Marquez and Suarez, 2004; Baumber and Meyers, 2006; Nyacheio *et al.,* 2010; Vande Voort, 2014; Jin and Yang, 2016). Furthermore, it was demonstrated that higher concentrations of caffeine resulted in the release of Ca^{2+} from Ca^{2+} stores internally in humans and in the rhesus monkey (Hong *et al.,* 1985; Bavister *et al.,* 1984).

Progesterone

Active synthesis of progesterone by the cumulus oophorous of the female oocyte affects sperm almost immediately due to progesterone receptors present on the cell membrane (Publicover and Barratt, 2011; López-Torres and Chirinos, 2016). The effects of

progesterone binding to its receptor on the sperm include: increased Ca^{2+} influx into the cell, CatSper channel activation, increase in cAMP pathway, alterations in flagellar movement patterns, induction of hyperactivated motility, heightened responsiveness to ZP binding, as well as the AR (Calogero, *et al.,* 2000; Colas, 2010; Publicover and Barratt, 2011; Alasmari *et al.,* 2013; López-Torres and Chirinos, 2016).

Myo-inositol

Myo-inositol is known for its therapeutic use in female infertility; however, its use in male infertility has been increasing (Condorelli *et al.,* 2017). In males, myo-inositol is present in the seminiferous tubules, produced by the Sertoli cells in response to follicle stimulating hormone (FSH). The Sertoli cells aid the development of sperm by providing nutrients to the growing spermatogonia (França *et al.,* 2016). Within the female reproductive tract, myo-inositol is present in follicular fluid, in high concentrations (Carlomagno *et al.,* 2011). Myo-inositol is involved in the regulation of sperm motility, capacitation and the AR in ejaculated sperm (Condorelli *et al.,* 2017). Furthermore, it is known to act directly on the mitochondria by increasing the membrane potential of the cell. MMP is related to increased functional parameters of sperm and thus fertilization capacity of a sperm (Condorelli *et al.,* 2012; Oliva *et al.,* 2016; Palmieri *et al.,* 2016; Condorelli *et al.,* 2017; Korosi *et al.,* 2017). Similar to the aforementioned capacitating molecules, the increase in Ca^{2+} has been reported within sperm when in contact with myo-inositol (Korosi *et al.,* 2017). It has been shown that sperm functionality (progressive motility) increases with myo-inositol when used in semen samples with high viscosity *in vitro* (Scarselli *et al.,* 2016).

Procaine hydrochloride

Forming part of the phosphodiesterase inhibitors (PDE), procaine hydrochloride is generally known to inhibit the conduction in nerve fibers, as a regional nerve blocking anaesthetic, and is commonly used as a pain killer (Ntanjana, 2014). With regard to reproductive studies, procaine has been reported to stimulate sperm of different mammalian species in terms of hyperactivation, and also decrease sperm swimming patterns, particularly linearity (LIN) and straightness (STR), and straight line velocity (VSL) (Ortgies *et al.,* 2012, Ntanjana, 2014). Similar to caffeine, procaine plays a role in the increase of the plasma membrane permeability to Ca^{2+} as reported for stallion sperm (Ortgies *et al.,* 2010; Ntanjana, 2014).

2.6 Sperm analyses and CASA

The evaluation of various sperm structural and functional parameters (e.g. sperm motility, vitality, and hyperactivation) is essential, all of which need to be measured accurately and be reproducible. Furthermore, the techniques used for semen analysis should be objective, standardised and sensitive in order to recognize any deviations from the normal reference

ranges (Maree, 2011). Modern CASA systems are known to automatically evaluate multiple fields to provide a summary for each sperm and sperm population. CASA cannot accurately predict 'fertility', however, the system provides important information on the quality of semen and the understanding of the sperms response to changes in the microenvironment in research (Amann and Waberski, 2014; van der Horst *et al.,* 2018).

2.6.1 Standard semen analysis

The analysis of semen is one of the most important parts of detecting or diagnosing male factor infertility. Abstinence of 2-5 days is recommended before a semen sample can be collected, usually by masturbation. This sample is then examined in the laboratory within an hour of collection. The examination includes analysis of the volume, the total number of sperm, sperm concentration, total motility, progressive motility, vitality, and morphology of the sperm present. The assessment is done according to the WHO lower reference levels updated in 2010 (WHO, 2010) presented in Table 2.2.

Table 2.2 Lower reference levels proposed by WHO 2010 semen analysis manual for various human sperm and semen parameters [Adopted from WHO (2010)].

Parameters	Cut-off values (WHO 5th addition 2010)
Volume (ml)	1.5 (1.4 - 1.7)
Total sperm number (mil/ ejaculate volume)	39 (33 - 46)
Sperm concentration (mil/ml)	15 (12 - 16)
Total motility (PR+NP, %)	40 (38 - 42)
Progressive motility (PR, %)	32 (31 - 34)
Vitality (Live sperm, %)	58 (55 - 63)
Sperm morphology (Normal forms, %)	4 (3.0 - 4.0)

Computer-aided semen analysis (CASA)

CASA allows for measurement of baseline semen parameters (semen volume, total sperm number, sperm concentration, total sperm motility, sperm vitality, and sperm morphology) and sperm functionality, e.g. motility characteristics (e.g. progressive motility, non-progressive motility, rapid, medium and slow swimming sperm), including various kinematic parameters, and hyperactivation. In addition, CASA is useful to determine sperm structural characteristics, such as vitality and sperm acrosomal integrity or AR accurately and in a short period of time. In order to ensure accurate analysis with CASA, the correct settings must be applied and the evaluations of captured fields need to be done accurately (Nieschlag *et al.,* 1998; Larsen *et al.,* 2000; Maree, 2011; van der Horst, 2014; Amann and

Waberski, 2014; van der Horst., 2018). CASA relies on the detection and tracking of the sperm head as a dense sequence of points along the sperm path recorded on the system. These successive captured locations are used to calculate the various motility and kinematic parameters (Dunson *et al.,* 1999; Maree, 2011; Amann and Waberski, 2014; van der Horst., 2018) and assist to determine the fertilization potential of a specific sample.

2.6.2. Sperm structural analyses

Vitality

The proportion of live sperm can be deducted from the percentage total motility, whereas distinguishing live and immotile sperm from dead sperm requires specific techniques. Vitality is measured as a percentage and mainly involves examining the permeability and integrity of the cell membrane of sperm (Björndahl *et al.,* 2003; Cooper and Hellenkemper, 2009). Vitality tests are especially important when the sample contains very low motility (less than 40% progressive motility) (WHO, 2010). For this reason, the percentage of viable cells should exceed that of motile cells with 58% viability set as the lower reference limit (Cooper and Hellenkemper, 2009; Björndahl *et al.,* 2003; WHO, 2010; Van Der Horst, 2014). To measure vitality a nigrosin and eosin (NE) - based solution is used and dead cells or sperm with compromised cell membranes will allow eosin (vital dye) to enter the cell and stain it pink. Live cells will remain white and can easily be detected against a dark background and contrast provided by nigrosin. The ratio of live and dead sperm can be accurately determined either manually, by counting compromised sperm (pink) and live sperm (white) by eye using a bright field microscope or automatically, using a CASA system (Figure 2.12).

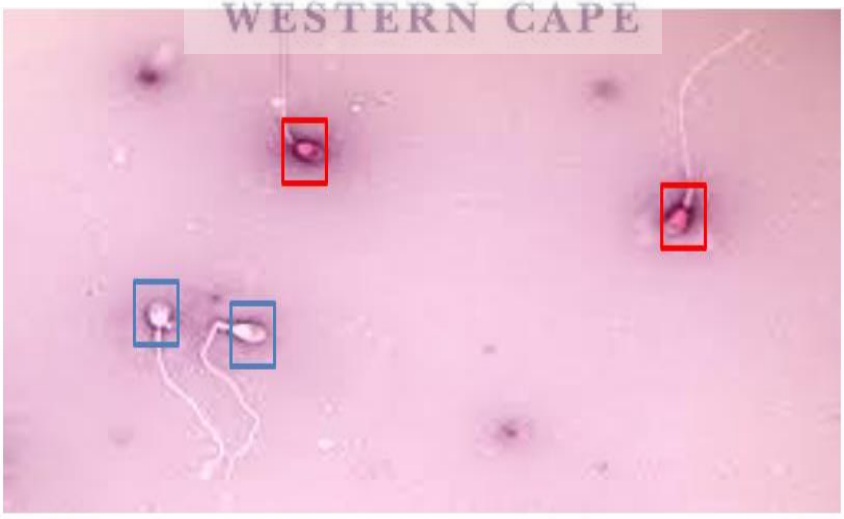


Figure 2.12 BrightVit stain recognized with CASA, SCA® (Saeednia *et al.,* 2016). SCA® recognizes and indicates the “dead” sperm with a red block and the “live” sperm with a blue block.

2.6.3. Sperm functionality analyses

Motility and kinematics

Figure 2.13 presents the different types of sperm motility parameters and their tracks read by CASA, SCA® and Figure 2.14 illustrates CASA's detection and identification of the sperm head as a dense sequence of points along its path. These successive captured locations are used to calculate the various motility and kinematic parameters (listed in Table 2.3) as indicated.

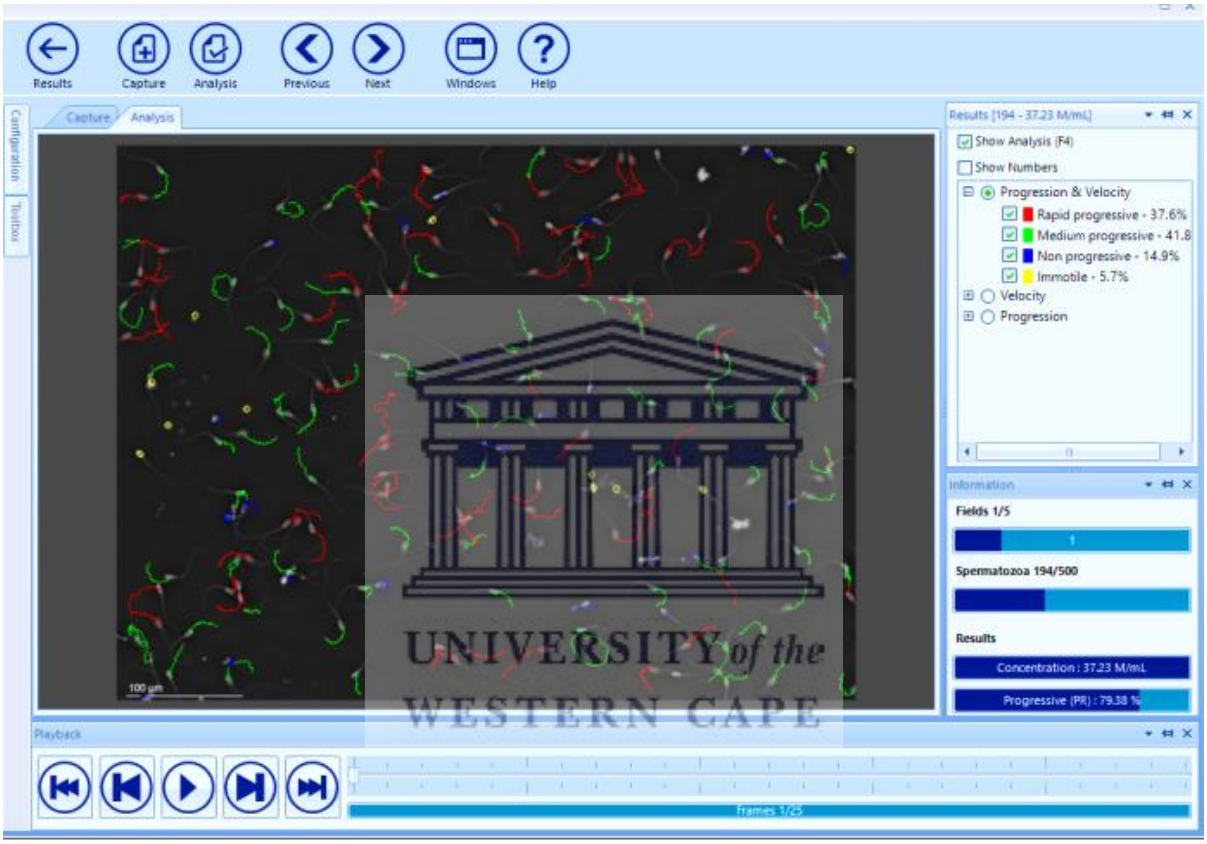


Figure 2.13 Type a-d classification of sperm motility parameters and their tracks read by CASA, SCA® (Microptic, 2015). Type "a" (red) = sperm with rapid progressive motility; Type "b" (green) = medium progressive motility; Type "c" (blue) = non-progressive motility; Type "d" (yellow) = immotile sperm (WHO, 1999).

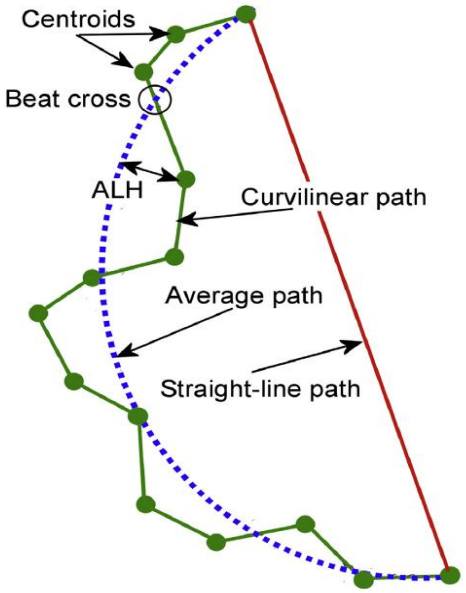


Figure 2.14 Schematic presentation of the various sperm motility parameters determined by CASA [Adapted from Amann and Waberski (2014)].VCL = curvilinear path velocity, VAP = average path velocity, ALH = amplitude of later head displacement, VSL= straight-line velocity, VAP = average path velocity.

Table 2.3 Motility and kinematic parameters of human sperm typically determined by CASA, SCA® [Adapted from WHO, (1999); Maree, (2011) and Mortimer et al., (2015)]

Motility parameters	Definition
Motile (%)/ (type a+b+c)	Tailing beating observed
Immotile (%)/ (type d)	No tail beating observed
Progressively motile (%) / (type a+b)	Space gain of ≥5µm/s-1 but <25 µm/s-1
Non-Progressive motility (%)	Tail beating seen but no net space gain (movement <5 µm/s-1)
Rapid (µm/s)	Space gain, > 80 µm/s-1
Medium (µm/s)	Space gain but >50 µm/s-1 and < 80 µm/s-1
Slow (µm/s)	Space gain but > 20 µm/s-1 and < 50 µm/s-1
VCL (µm/s) (*curvilinear velocity)*	Time-averaged velocity of a sperm head along its actual curvilinear path
VSL (µm/s) (*straight-line velocity)*	Time-averaged velocity of a sperm head along the straight line between its first detected position and its last
VAP (µm/s) (*average path velocity)*	Time-averaged velocity of a sperm head along its average path
LIN (%) (*linearity)*	Linearity of the curvilinear path = VSL/VCL

STR (%) (*straightness)*	Linearity of the average path = VSL/VAP
WOB (%) (*wobble)*	Measure of oscillation of the actual path about the average path = VAP/VCL
BCF (Hz) (*beat cross frequency)*	Average rate at which the curvilinear path crosses the average path
ALH (µm) (*amplitude of lateral head displacement)*	Magnitude of lateral displacement of a sperm head about its average path

Progressively motile sperm are classified as rapid, medium or slow, based on either curvilinear path velocity (VCL) or average path velocity (VAP). In the field of andrology, the percentage of rapid, medium and slow swimming sperm are considered to be of great importance, but primarily that of rapid swimming sperm as it indicates a positive correlation with fertility (Mortimer *et al.,* 2015).

Furthermore, connected points are used to calculate the various motion parameters, which are composed of three variables reflecting sperm motion velocity, three variables of ratio velocity and finally two variables reflecting wobble characteristics. Sperm motion velocity variables include VCL, straight-line velocity (VSL), VAP (Lu *et al.,* 2014) (Figure 2.14). VCL, the average speed between adjacent time points, refers to the time-average velocity of sperm head along its actual curvilinear path and is always the highest compared to the other sperm motion velocity values (Dunson *et al.,* 1999; Lu *et al.,* 2014). VSL, the distance from the first point/time, refers to the time-averaged velocity of the sperm head along a straight line between its first and last detected positions; thus, reflecting the net space of forward motility during the observed time and will always be the lowest value compared to the other sperm motion values (Dunson *et al.,* 1999; Lu *et al.,* 2014). VAP refers to the time-averaged velocity of the sperm head along its average path. This average path is determined by the smoothing of the curvilinear trajectory in the CASA instrumentation. Therefore, if the sperm motion trajectory is very regular and linear, VAP will be almost identical to VSL, but if irregular VAP will be much higher than VSL (Dunson *et al.,* 1999; Lu *et al.,* 2014).

The three velocity ratios include linearity of a curvilinear path (LIN), straightness (STR) and wobble (WOB). LIN, expressed as VSL/VCL, refers to the linearity of a curvilinear path, whereas STR, expressed as VSL/VAP, refers to the linearity of the average path. Furthermore, WOB refers to the oscillation of the actual path about the average path and is, therefore, expressed as VAP/VCL (Dunson *et al.,* 1999; Lu *et al.,* 2014).

The parameters representing the sperm wobble characteristics include the amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). The ALH refers to the

magnitude of lateral head displacement of the sperm head, thus the deviation of the track about its average path, and can, therefore, be expressed either as a maximum or an average of such displacements (Dunson *et al.,* 1999; Lu *et al.,* 2014). It is important to obtain accurate measurements of ALH, as this kinematic parameter has been correlated with both IVF success and the ability of the human sperm to penetrate cervical mucus and fuse with the oocyte. The importance of ALH is contributed to the fact that ALH indicates the force of flagellar beating along with the frequency of cell rotation (Kraemer *et al.,* 1998). The BCF refers to the average rate at which the curvilinear path crosses the average path, and is a useful parameter when assessing the changes in the wobble of the sperm flagellum (Dunson *et al.,* 1999; Lu *et al.,* 2014).

Hyperactivation

Sperm hyperactivation can be measured with great accuracy using CASA which assists in rapid and objective analysis of hyperactivation and kinematic parameters (Nieschlag *et al.,* 1998). Hyperactivated sperm are very different from those of fresh ejaculate and non-capacitated sperm. To determine and detect hyperactivation, the frame rate for CASA should be set relatively high to accurately detect the true track of the hyperactivated sperm (Lu *et al.,* 2014). Detection of the sperm head trajectory to determine the flagellar movement and the occurrence of hyperactivation are analysed according to the cut-off values for a combination of kinematic parameters (Mortimer *et al.,* 2015). Hyperactivated sperm typically, but not in all cases, show a star-spin pattern that is easily detected with the assistance of CASA (Figure 2.15). The induction of hyperactivation, *in vitro*, is accomplished by incubation of sperm with the use of specific chemicals (e.g. caffeine, progesterone, myo-inositol and procaine) acting to open cation channels of sperm (CatSper) resulting in the release of internally stored Ca^{2+} or increase intracellular pH (van der Horst *et al.,* 2018).

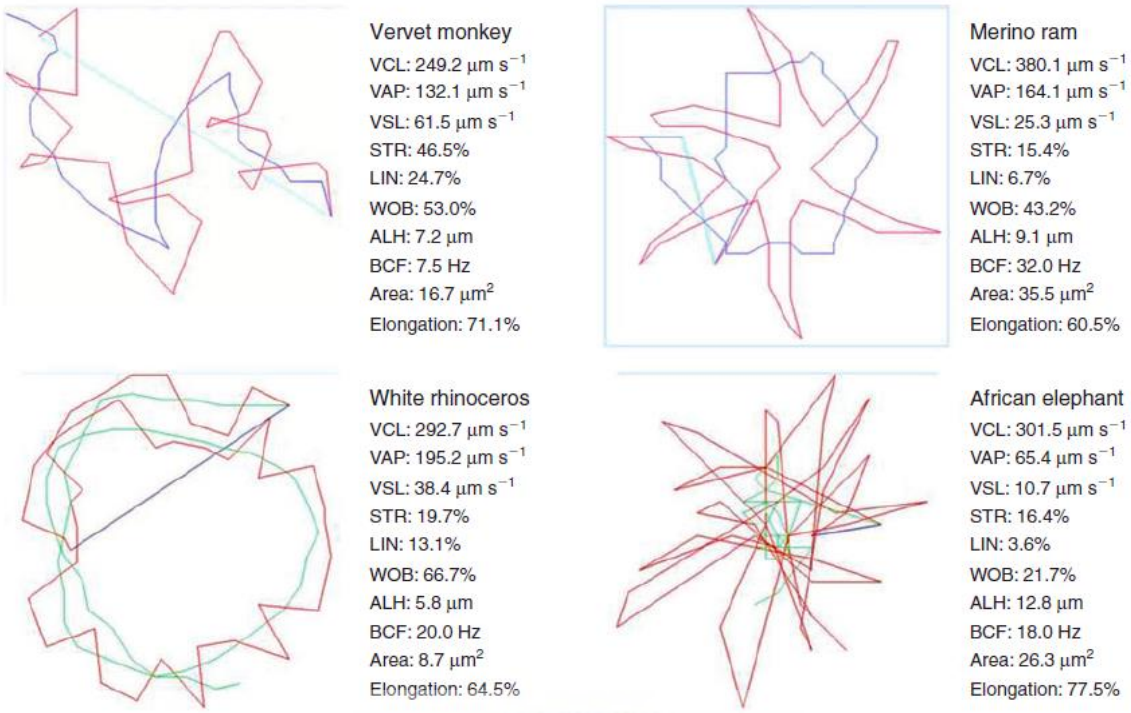


Figure 2.15 Examples of individual hyperactivated sperm tracks of four mammalian species with detailed kinematics to the right. Tracks were captured at 50 frames per second (f.p.s.), except for Merino ram, with a frame rate of 100 f.p.s [Adapted from van der Horst et al. (2018)]. VCL, curvilinear velocity (µm/s) (red line); VAP, average path velocity (µm/s) [blue (monkey, ram) or green (rhinoceros, elephant) lines) VSL, straight-line velocity (µm/s) (turquoise (monkey, ram) or blue (rhinoceros, elephant) lines]; STR, straightness (%); LIN, linearity (%); WOB, wobble (%); ALH, amplitude of lateral head displacement (µm); BCF, beat cross frequency (Hz).

AR and acrosome integrity

Acrosome integrity can be assessed using CASA and manually. In the SCA® system, AR is a module that automatically provides the percentage of sperm with intact acrosomes and or acrosomes that reacted in a given semen sample. The AR is one of the most important sperm functional tests. The test measures the percentage of intact acrosomes of washed or diluted sperm samples. Another optional step is the use of a biological substitute to induce the AR such as Ca^{2+} ionophore, to test the occurrence of the AR. Detection of the acrosomes is done by the use of lectins as a label. Specifically, PNA is used in the labeling of acrosomes by binding to residues located on the OAM (Lybaert *et al.,* 2009; Prag, 2017). With the use of PNA, the OAM can be detected by producing a bright green acrosome when viewed with fluorescence microscopy. A counterstain (Hoechst stain) is used as contrast and to visualize intact acrosomes (green fluorescence), and reacted acrosomes (blue fluorescence) of the sperm heads (Figure 2.16) (Microptic, 2018).

Figure 2.16 Acrosome reaction evaluation using PNA and Hoescht stain coupled with a CASA, SCA®. Blue boxes = Reacted acrosome sperm, Green boxes = Intact acrosome sperm (Microptic, 2016).

2.7 Conclusion

From reproductive studies, mainly conducted in humans, several structural and functional aspects of sperm have been investigated and linked with male infertility. However, male infertility remains largely unexplained, and warrants further research, particularly with the use of NHP models, to aid in determining contributing factors. From reproductive studies in NHP, it is clear that there is a general lack in studies reporting on the effects of different physiological media focussing on the physiological events forming part of sperm capacitation.

It was, therefore, the aim of this study to determine essential baseline data for many aspects of sperm of two NHP species, i.e. rhesus and vervet monkeys, by evaluation of sperm functionality with emphasis on sperm capacitation (hyperactivation and AR), and to determine hyperactivation cut-off values to select for vervet monkey analysis when using CASA for human reproductive studies. In addition, the study aims to determine the role of mitochondria in NHP sperm by inhibiting mitochondrial function and analysing the effects on sperm motility with the use of CASA. Furthermore, this study tested newly formulated sperm washing (HDSWP) and capacitating media (HDSCP) as an alternative to commonly used physiological media in reproductive studies.

From the above-mentioned data, it will be possible to gain a better understanding of sperm functionality and relevant tests in NHPs when exposed to different physiological media to help aid in reproductive studies aiming to shed light on UMI. The experimental protocols used in this study could be used in prospective studies to evaluate similar sperm functional and structural characteristics in other mammalian species and humans.

Chapter 3: Materials and Methods

3.1 Introduction

The comparative nature of this study required the use of standardized protocols to ensure that possible relationships reported among sperm parameters and primate species would not be due to bias, subjective or inaccurate measurements. In this regard, the use of a CASA system for the evaluation and analysis of numerous sperm characteristics was conducted.

3.2 Ethical approval

Ethical authorization for this study was obtained from the Ethics Committee of the University of the Western Cape (Ethical clearance number: 13/10/91) as well as the Ethics Committee for Research on Animals (ECRA) of the South African Medical Research Council (Project number: 11/13). All procedures for sample collection were in accordance with the ethical guidelines of the Medical Research Council and the University of the Western Cape.

3.3 Primate housing and care

The two non-human primate (NHP) species used in this study were the vervet monkey (*Chlorocebus aethiops*) and rhesus monkey (*Macaca mulatta*). The animals were maintained at the Primate Unit and Delft Animal Centre of the South African Medical Research Council (Cape Town, South Africa). The primates were housed in accordance with the national guidelines of the revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386).

The description of materials and methods in this chapter comprises both species unless stated otherwise.

3.3.1 Vervet monkey (*Chlorocebus aethiops*)

Vervet monkeys breed successfully in a closed environment throughout the year, and thus, semen samples were frequently available. The sampling period for this study was from October 2017 to November 2017 and January 2018 to February 2018. Adult male vervet monkeys were selected from an indoor breeding colony at the Primate Unit, South African Medical Research Council (Cape Town, South Africa). The colony included 46 adult males (breeders and non-breeders) with a 6th generation history of successful breeding and use in reproductive studies. In order to select suitable males for the study, semen samples of all males were screened prior to the study, using a standard semen analysis. Inclusion criteria for semen samples were based on sperm concentrations of 25-30 x 10^6 /ml and total motility > 40% (Maree, 2011). The conditions in the closed indoor environment were maintained at a room temperature of 25-27°C, humidity of 45%, about 15 air changes/ hour, and a photoperiod of 12 hours. A total of 7 vervet monkeys were used throughout the study period,

the animals remained in good health with an average body weight of 5.62 kg and presented semen samples with varying sperm concentrations and motility, which is comparable to humans.

3.3.2 Rhesus monkey (*Macaca mulatta*)

Rhesus monkeys are seasonal breeders, and semen samples are only available for approximately four months of the year from May until August. Six adult rhesus males were selected from an outdoor breeding colony at the Delft Animal Centre, South African Medical Research Council (Cape Town, South Africa). All the selected males were previously used in non-invasive *in vitro* reproductive studies. The original breeding stock is of Chinese origin, and the colony has a ten-year history of successful second generation reproductive performance. The rhesus male housing conditions comprised of an outdoor free-ranging harem breeding group with some males used in reproductive studies being housed indoors paired with a breeding female in rooms with a temperature of 25-27°C and a 12-hour light/dark controls. During the course of the study period, the animals were kept in good health with an average body weight of 8.63 kg. Semen samples produced varying sperm concentrations and motility.

3.4 Experimental outline

Non-human primate sperm functionality and structural tests performed included the assessment of sperm motility, vitality, acrosome integrity, hyperactivation, and mitochondrial activity. The detailed methods used are described in the remainder of this chapter. Figure 3.1 provides a basic outline of the experimental procedures of this study.

Semen Collection

Standard semen analysis

Selection of motile sperm
- 1) Motility
- 2) Hyperactivation

1) Motility (T0-T60)
- Direct swim up (5 physiological media)
- Functional test
  - Motility analysys (SCA)
- Structural tests
  - Vitality analysis (SCA)
  - Acrosome activity analysis (SCA)

2) Hyperactivation (T0-T60)
- Flush technique
- Functional test
  - Hyperactivation analysis (SCA)

Mitochondrial activity (T0-T30)
- Sperm preparation (Centrifugation)
- Physiological medium wash
- Flush technique
- Functional test
  - Motility module analysis (SCA)

Semen Collection

Standard semen analysis

Selection of motile sperm
- 1) Motility
- 2) Hyperactivation

1) Motility (T0-T60)
- Sperm preparation (Centrifugation)
- Direct swim up (5 physiological media)
- Functional test
  - Motility analysys (SCA)
- Structural tests
  - Vitality analysis (SCA)
  - Acrosome activity analysis (SCA)

2) Hyperactivation (T0-T60)
- Flush technique
- Functional test
  - Hyperactivation analysis (SCA)

Figure 3.1 Outline of experimental procedures for each primate species with functional and structural tests applied (T0-T60 = different time intervals of analysis; SCA® = Sperm class analyser)

3.4.1 Semen collection

Semen samples were collected from both the rhesus monkey and the vervet monkey by rectal probe electro-ejaculation (RPE). The electro-ejaculation equipment consisted of a transformer (Eloff Transformers, Montague Gardens, Cape Town, South Africa) connected to a rectal probe. The latter was 15.4 mm in diameter with two brass bands (4.2 mm x 4.0 mm) embedded in the tip and 10 mm apart (Figure 3.2). The transformer allowed various current settings (2.5 V, 3.5 V, 4.5 V, 5.5 V and 8 V), of which the position was manually controlled by one operator whilst another operated the placement of the rectal probe. The same RPE equipment and operators were used throughout the study to ensure consistency.

The animals were immobilized with an intramuscular injection of ketamine hydrochloride (Anaket V, Cape Medical Supplies, Cape Town) at 10 mg/kg body weight and, depending on the primate, was placed in either dorsal (vervet monkey) or lateral (rhesus monkey) recumbence. The penis was cleaned with a mild detergent, then rinsed thoroughly with water and dried with a sterile gauze.

The rectal probe was coated with a lubricating gel (Kyrogel, Kyron Laboratories, Benrose, South Africa) and gently inserted into the rectum of the animal, with the brass bands positioned at the level of the prostate gland. Stimulation started at 2.5 V, while the probe was adjusted inside the rectum to obtain an erection. The erection would cease after 15-30 seconds and the probe had to be gently withdrawn a short distance and then re-positioned to re-establish the erection. The same current was applied for 1-2 minutes, followed by a 15-20 second rest period (no stimulation) and an increase in the voltage thereafter. The same procedure and higher voltages were applied until ejaculation was achieved (Seier *et al.,* 1989; Cseh *et al.,* 2000, Maree and van der Horst, 2013). During the procedure, the penis was milked to get the maximal emission of semen from the urethra. Ejaculates were collected directly into pre-warmed 15 ml wide-mouthed graded plastic tubes (188261) (Greiner Bio-one, Germany).

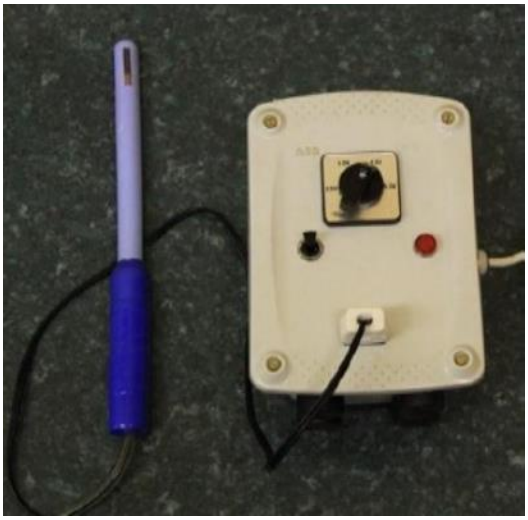


Figure 3.2 Rectal probe and transformer used for electro-ejaculation

3.4.2 Standard semen analysis

Standard semen analysis (volume, sperm concentration, total motility, and progressive motility) was performed on ejaculates to assess the semen quality. After collection, the semen was allowed to liquefy in an incubator at 37 °C and 5% CO_2. The ejaculated semen volume were measured with a graded plastic test tube. Sperm concentration and total motility analysis was assessed by placing 3 µl of semen into a Leja 4 chamber slide (Leja Products B.V., Nieuw- Vennep, The Netherlands) using a positive displacement pipette. This semen aliquot was assessed using the Motility/Concentration module of the Sperm Class Analyser® (SCA®) (Microptic S.L., Barcelona, Spain) CASA system, Version 6.2, in combination with a Nikon Eclipse 50i Phase Contrast Microscope (IMP, Cape Town, South Africa) equipped with a digital camera (Basler ace acA 1300-200uc colour USB 3.0) (Microptic S.L., Barcelona, Spain) and a heated stage (37 °C) (Figure 3.3). The properties of the SCA® system was set as follows for the two species: Frame rate = 50 images/sec; Optics = pH- (negative phase contrast); Chamber = Leja 20; Species = Human; Particle area = no less than 5 and no more than 80 μm^2; VCL intervals vervet = 60<slow<164<medium<264<rapid (µm/s) (initial) and 50<slow<70<medium<150<rapid (µm/s) (adjusted); VCL intervals rhesus= 80<slow<164<medium<240<rapid ; VAP points = 7 and Connectivity = 12.

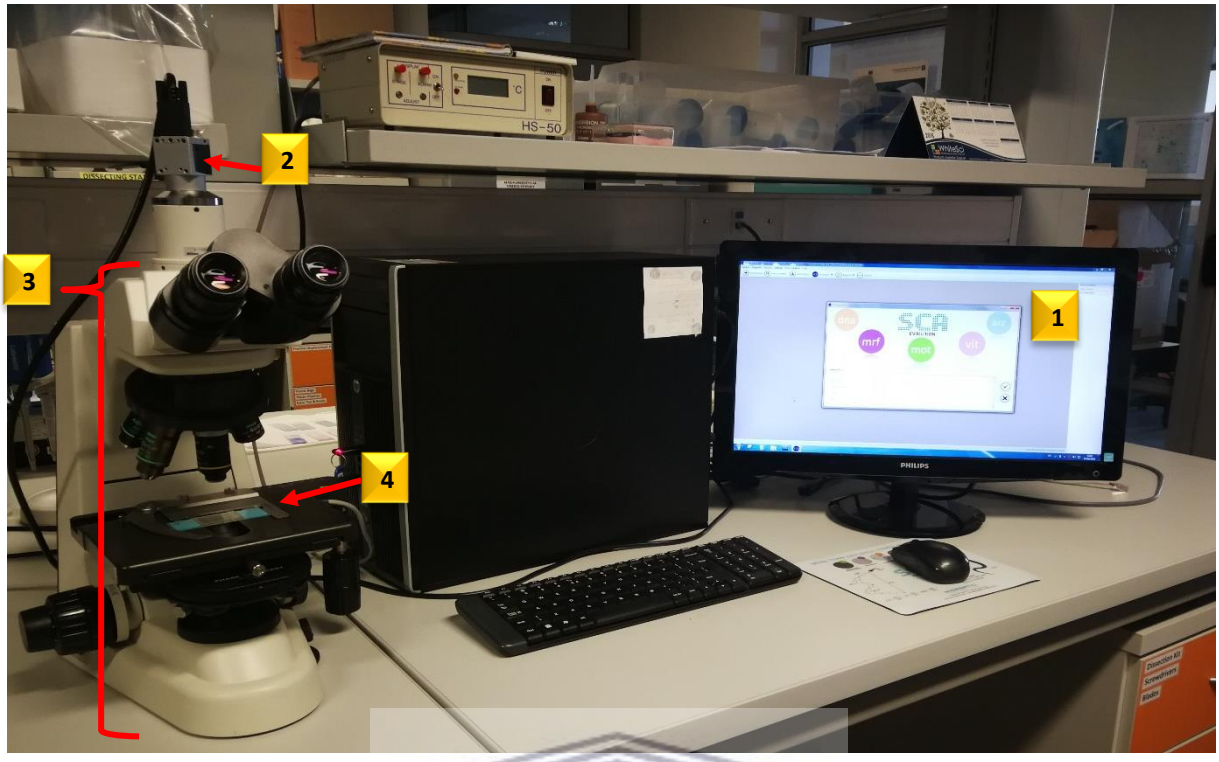


Figure 3.3 Equipment used for the analysis of semen parameters by CASA. 1) Computer with SCA® software for CASA analysis, 2) Digital camera (Basler ace acA 1300-200uc colour USB 3.0), 3) Nikon Eclipse 50i microscope, 4) Heated stage

For rhesus monkey semen samples presenting with very high sperm density/ concentration (>50 x 10^6 /mL), a centrifugation step was added to ensure the most accurate analysis was obtained. The entire semen sample was centrifuged at 300 *g* for 5 minutes. Once the centrifugation step was concluded, the supernatant was removed and physiological media was added to the pellet to ensure a final concentration ranging between 15-30 x 10^6/ml sperm.

3.5 Sperm processing and motile sperm selection

3.5.1 Direct sperm swim-up

Sperm were selected for further experimental procedures by their ability to swim out of SP and into physiological media. This method was used to separate motile sperm from semen. The swim-up technique was performed by layering liquefied semen under physiological media, a modification of the “direct swim up” preparation technique recommended by WHO (WHO, 2010) as performed by Maree (2011). This sperm preparation step was performed for both vervet and rhesus samples, as described below.

After semen liquefaction, the sample was mixed well to allow equal distribution of sperm throughout the sample. A volume ranging from 70-100 µl of five different pre-warmed physiological media was pipetted into individual 2 ml Eppendorf tubes. A final volume range

(70-100 µl) of the semen sample was pipetted underneath the physiological media, as stated in the previous page, for all five Eppendorf tubes. The amount of semen pipetted depended on the original concentration of the semen sample to ensure a final concentration of between 15 and 30 x 10^6/ ml sperm. Care was taken to avoid disturbance of the two liquid fractions and a clear interface between the semen (bottom) and physiological medium (top) was evident afterward. Figure 3.4 illustrates the sperm swim-up principle. The eppendorf tubes were then incubated at 37°C and 5% CO_2 for 5 minutes, to allow the motile sperm in the semen to swim up into the physiological media.

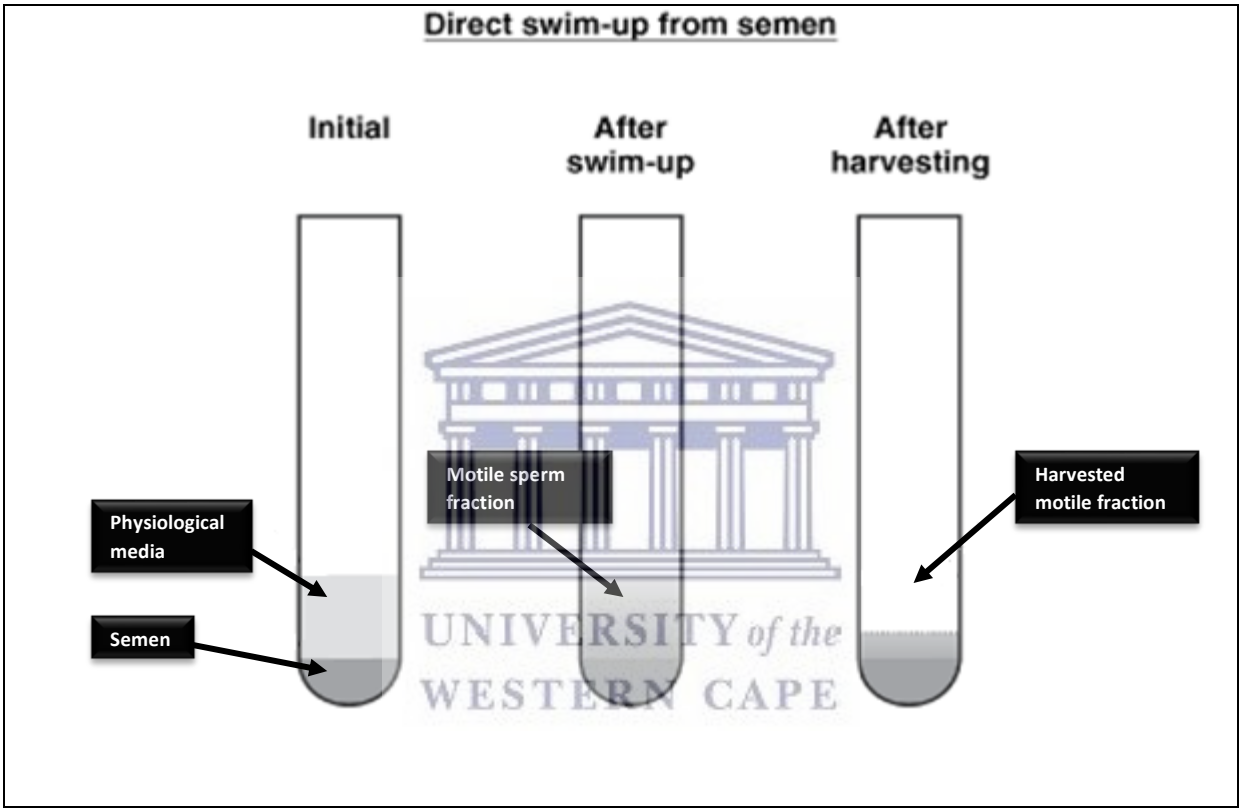


Figure 3.4 Annotated illustration of the swim-up method [Adopted from Bjorndahl *et al.* (2003)].

3.5.2 Exposure of motile sperm fractions to different physiological media

The five different physiological media used for sperm selection and incubation was: 1) HTF (90125) (Irvine Scientific), 2) Ham's F-10® (MFCD00217607) (Sigma, Cape Town, South Africa), 3) Human tubal fluid containing 10 mM caffeine (HTF-C), 4) a newly developed test wash medium, HD Sperm Wash Plus (HDSWP), and 5) a newly developed capacitation medium, HD Sperm Capacitation Plus (HDSCP). Media was used in a randomised manner for each sample in order to avoid bias. Table 3.1 presents the chemical components of the different physiological media used throughout this study.

Table 3. 1 Chemical components of five different physiological media used

HTF	HTFC	Ham's F-10®				HDSWP	HDSCP
Sodium chloride	Sodium chloride	Sodium chloride	L-Glutamine	Vitamin B-12	Thimidine	Sodium chloride	Sodium chloride
Potassium chloride	Potassium chloride	Potassium chloride	L-Isoleucine	Pantothenic acid, Ca salt	Zinc sulfate	Potassium chloride	Potassium chloride
Magnesium Sulfate, Anhydrous	Magnesium Sulfate, Anhydrous	Choline chloride	L-Methionine	Riboflavin	Thiamine HCl	Sodium hydrogen phosphate	Sodium hydrogen phosphate
Potassium Phosphate	Potassium Phosphate	Sodium phosphate, anhydrous	L-Serine	Thimidine	Hepoxanthine	Sodium hydrogen carbonate	Sodium bicarbonate
Calcium Chloride, Anhydrous	Calcium Chloride, Anhydrous	Glucose	L-Tyrosine	Nicotinic acid amide	Pyruvic acid	Glucose	Glucose
Sodium Bicarbonate	Sodium Bicarbonate	L-Asparagine	L-Arginine HCl	Thioctic acid, dihydrochloride	Magnesium	Myo-inositol	Myo-inositol
HEPES	HEPES	L-Glutamic acid	L-Cysteine	Ferrous sulphate	Thimidine	HEPES (free acid)	Procaine hydrochloride
Glucose	Glucose	L-Histidine	L-Leucine	d-Biotin	Zinc sulfate	Calcium Chloride	Calcium Chloride
Sodium Pyruvate	Sodium Pyruvate	L-Lysine HCl	L-Phenylalanine	Pyridoxine	Thiamine HCl		Potassium phosphate Progesterone
Phenol Red	Phenol Red	L-Proline	L-Threonine	Cupric sulphate	Hepoxanthine		
Gentamicin	Gentamicin	L-Tryptophan	L-Valine	Thiamine	Pyruvic acid		
	Caffeine	L-Aspartic acid	Folic acid	Riboflavin	Magnesium		

After a baseline incubation period of 5 minutes (T0), each swim-up sample was subjected to an SCA® Motility evaluation (baseline time point). Thereafter, approximately 70 µL of the uppermost physiological medium layer was removed (see Figure 3.4) and incubated as previously described. The incubated aliquots were exposed for a further 60 minutes at 37 °C and 5% CO_2. Various sperm functional and structural parameters (described below) were then evaluated at different time points within the 60 minutes (baseline, 30 minutes and 60 minutes) for each physiological medium.

3.7 Sperm functional tests

3.7.1 Analysis of sperm motility

As mentioned above, sperm motility was assessed at three different time points, i.e. baseline, 30 minutes, and 60 minutes for all five media. The Motility module of SCA® (Microptic S.L., Barcelona, Spain) CASA system, version 6.2, was used to determine sperm motility with the equipment setup as described in section 3.4.2 above. Apart from the percentage total motility, the percentages rapid, medium and slow swimming sperm, as well as detailed kinematic parameters (Chapter 2, Table 2.3) were also measured at the three-time intervals. Based on the low motility values obtained from vervet monkeys by means of CASA, cut-offs for motility and kinematic analyses were adjusted from: Rapid (µm/s) >280, Slow-Medium (µm/s) = 164, Static (µm/s) <64 to Rapid (µm/s) >150, Slow-Medium (µm/s) = 70, Static (µm/s) <50. With regard to the rhesus samples the cut-off values used were: Rapid (µm/s) >120, Slow-Medium (µm/s) = 80, Static (µm/s) <50.

3.7.2 Analysis of sperm hyperactivation

Chemically induced sperm hyperactivation was evaluated for specific media. HTF was used as a medium for control samples. HTF modified as capacitating medium (addition of 10 mM caffeine) (HTFC) was used to induce "natural" hyperactivation. A newly developed wash medium (HDSWP) and capacitating medium (HDSCP) were also used to test the ability of these media to induce hyperactivation. The percentage hyperactivated sperm were determined at four different time intervals (baseline, 15 minutes, 30 minutes, 45 minutes and 60 minutes) by using a flush technique (van der Horst, 2014, Boshoff *et al.,* 2018) and the Motility module of SCA®, version 6.2. The flush technique was performed by pipetting 1 µl of undiluted semen sample into a chamber of a 4 chamber Leja slide. An aliquot of 2 µl of the selected media was pipetted/ flushed into the same chamber containing 1 µl semen.

The selection criteria for classifying sperm comprising of hyperactivated swimming tracks using various kinematic parameters are indicated in Table 3.2 and were set according to restrictions indicated by Baumber and Meyers (2006) for rhesus monkeys. Initially, one of the aims of this study was to produce new cut-off values for hyperactivation for the analysis of vervet samples. Providentially the analysis of these cut-off values used for rhesus samples proved to be appropriate for the assessment of hyperactivation in the vervet.

Table 3.2 Selection criteria for measurement of hyperactivation using various kinematic parameters

Parameters	Restriction setting
VCL (µm/s)	≥130
LIN (%)	≤69
ALH (µm)	3.75
VCL= Curvilinear velocity, LIN= Linearity, ALH= Amplitude of lateral head displacement	

3.8 Sperm structural tests

3.8.1 Analysis of sperm vitality

Vitality was determined using the BrightVit nigrosin-eosin staining technique (Delfran, Johannesburg, South Africa). This staining technique is used to assess the percentage of dead and alive sperm. Due to their compromised plasma membrane, dead sperm will allow eosin to enter the cell and thus are stained pink, while viable sperm remain colourless and appear white. The nigrosin functions as a background (purple) stain to provide contrast.

For the staining procedure (Figure 3.5), BrightVit stain was pre-heated to 37 °C and mixed with the previously incubated swim up sperm aliquots at a ratio 5:1 (stain: semen) for 7-10 minutes at 37 °C for all the respective media. After 7-10 minutes a smear was made on a microscopic slide and left to dry in the dark. A coverslip was mounted, once dried, using DPX mounting medium (06522) (Sigma, Cape Town, South Africa).

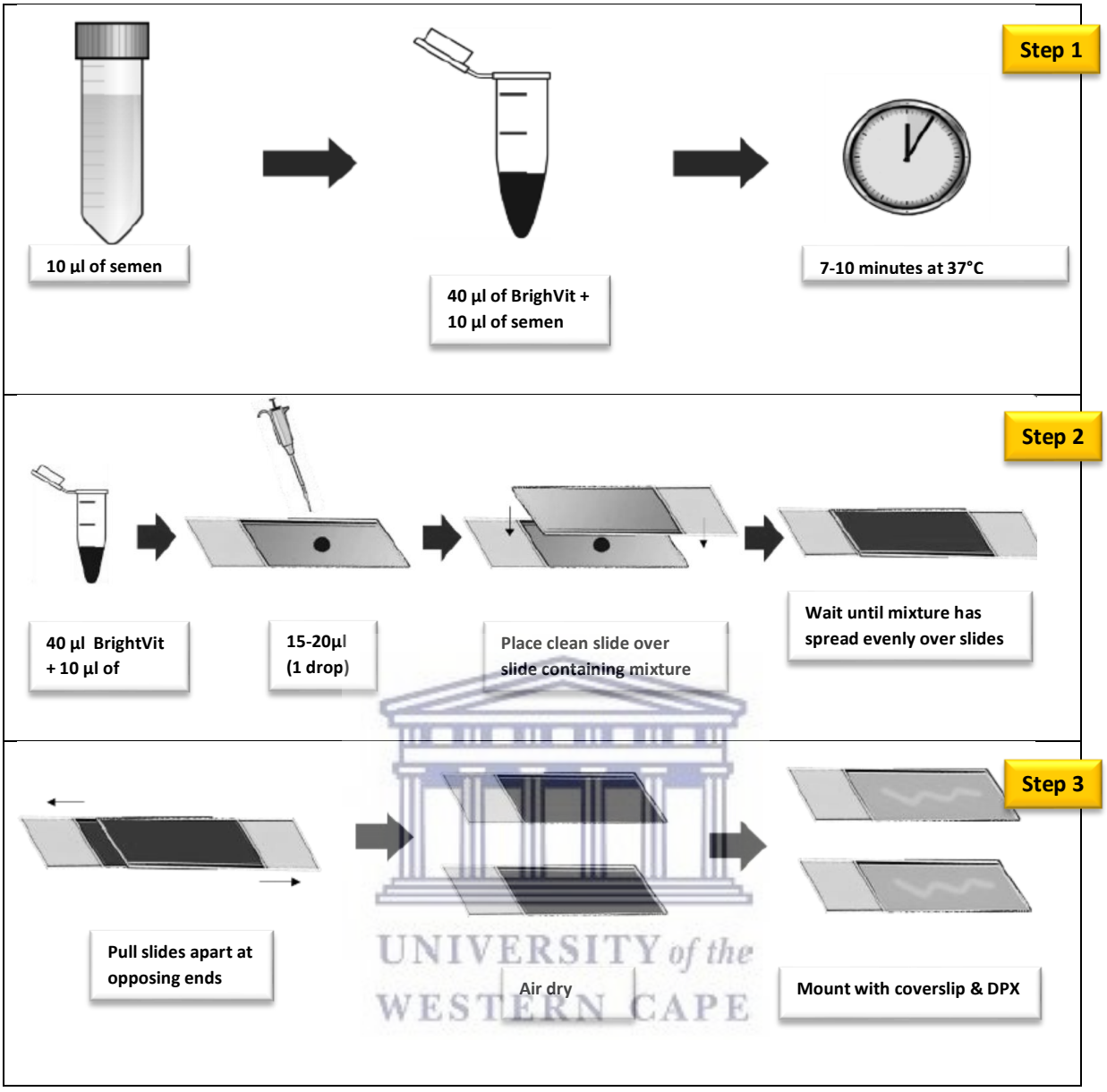


Figure 3.5 Annotated diagram illustrating BrightVit protocol [Adopted from Microptic (2016)].

Vitality was analysed at three time points (baseline, 30 minutes, and 60 minutes) as performed in the Motility analysis section, 3.7.1. All samples were viewed under a 40X objective using bright field optics on a Nikon Eclipse 50i microscope (IMP, South Africa), with an attached digital camera (Basler ace acA 1300-200uc colour USB 3.0; Microptic S.L., Barcelona, Spain). Percentage vitality of a minimum of 200 sperm was counted manually with the Vitality module of SCA® (version 6.2) and Nikon Eclipse 50i microscope and recorded accordingly. Images were visually evaluated to ensure that viable and dead sperm were correctly assessed as seen in Figure 3.6 (pink= dead sperm; white= alive sperm).

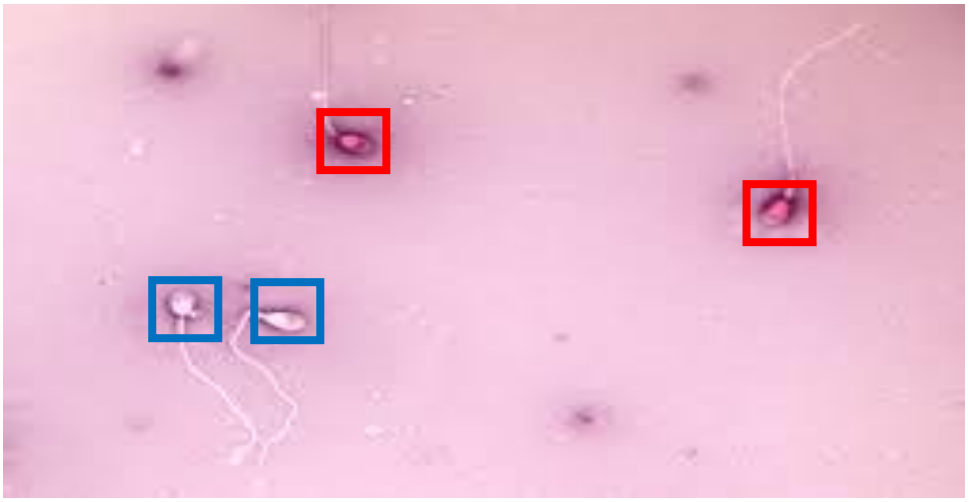


Figure 3.6 BrightVit stain recognized with CASA, SCA® [Adopted from Saeednia *et al.*, (2016)].

3.8.2 Analysis of acrosome integrity

The presence or absence of the acrosome on the sperm head was determined by staining sperm fractions with PNA (L7381) (Sigma, Cape Town, South Africa) and Hoechst solution (33258) (Sigma, Cape Town, South Africa), and slides were analysed with the use of fluorescence microscopy to determine acrosome integrity.

An aliquot of 5 µl swim-up sperm was used to make smears on microscopic slides and left to air-dry. Dried, smears were fixated by immersion in pre-cooled 95% ethanol at 4 °C for 30 minutes, whereafter the slides were rinsed with distilled water (dH_2O). Slides were then left to dry at room temperature. Dried slides were stained with Hoechst for 7 minutes in a darkroom and thereafter rinsed with dH_2O and allowed to dry in the darkroom. Once dried, to prevent dehydration, the slides were stained using PNA in a damp slide holder for 60 minutes. The slides were then washed with dH_2O and left to dry. A coverslip was fixed onto slides with Dako fluorescent mounting medium (Diagnostech, Johannesburg, South Africa) and viewed under a Nikon Eclipse 50i fluorescence microscope (IMP, Cape Town, South Africa) using the 100X oil immersion objective and Triband fluorescence filter. A total of at least 100 sperm were counted and the percentage reacted acrosomes were calculated using the Acrosome module of SCA®, version 6.2. Green fluorescence indicated the presence of an intact acrosome on the sperm head and blue fluorescence indicated acrosome-reacted sperm (Figure 3.7).

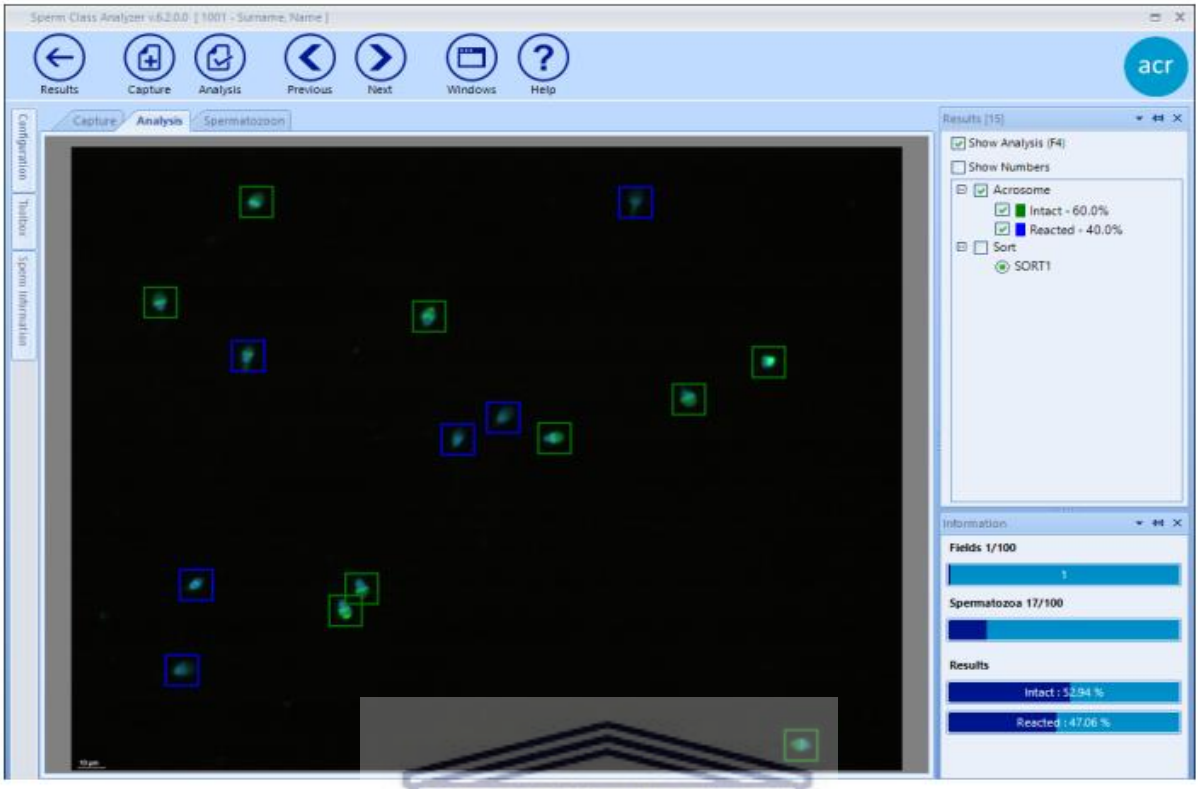


Figure 3.7 Acrosome reaction evaluation using PNA and Hoescht stain coupled with a CSA (SCA®) system. Blue boxes= Reacted acrosome sperm, Green boxes = Intact acrosome sperm (Microptic, 2015).

Acrosome integrity was analysed at all three time points (baseline, 30 minutes, and 60 minutes) as done for the motility and vitality analysis for all physiological media used.

3.8.3 Analysis of mitochondrial activity

Due to low sample numbers obtained from the rhesus monkeys, mitochondrial activity was not performed and therefore only determined in vervet monkey samples. The mitochondrial activity of vervet sperm was evaluated by use of a mitochondrial inhibitor, Oligomycin-A (579-13-5) (Sigma, Cape Town, South Africa) and analysed using a CASA system, to determine the effect of mitochondrial inhibition on sperm motility. Oligomycin-A, specifically inhibits the F_0 , oligomycin-sensitive sub-unit of the mitochondrial ATP- synthase complex in the mitochondria, by blocking its proton channel necessary for oxidative phosphorylation (OXPHOS) and thus ATP production (Ramió-Lluch *et al.,* 2014). A control sperm wash medium sample (Oligomycin-A not present) was compared to two test samples exposed to different concentrations of Oligomycin-A, 5 µM and 25 µM, to determine whether sperm mitochondria are essential for NHP sperm motility. Two media were used in this part of the experiment, namely, HTF and HDSWP. The Motility module of SCA® (version 6.2) was used to evaluate sperm motility parameters at two time points, namely after 5 minutes (baseline/ T0) and 30 minutes (T30).

A double density gradient centrifugation technique was used to select sperm for mitochondrial inhibition. This technique involved the layering 100 µl of AllGrad® 90% (AG90-050) (at the bottom) and 100 µl AllGrad® 45% (AG45-050) (on top) (Delfran Pharmaceuticals) in a 2 ml Eppendorf tube. This was followed by layering 100 µl of undiluted semen on top of the AllGrad® medium and centrifuging the preparations at 300 *g* for 15 minutes. The top layers were removed after centrifugation to ensure that only the pellet (motile fraction) remained in the Eppendorf tube. The pellet was washed with 100 µl of HTF and centrifuged for 8 minutes at 300 *g*. After centrifugation, the top layer was removed once again to ensure only the pellet remained at the bottom of the tube. The pellet was then used to perform a flush technique, using two different concentrations of Oligomycin-A i.e. 5 µM and 25 µM with the selected sperm wash medium. Motility analysis was performed as described in the previous sections.

3.9 Statistical analysis

All captured data were collected and stored electronically on CASA, SCA®, and further transferred onto MedCalc software (version 16.8.4; MedCalc software, Mariakerke, Belgium). Summary statistics were performed to determine the mean and standard deviation (SD) for the standard semen analysis, functional and structural parameters for both NHP species studied. Tests were performed for normality of distribution and most data sets represented normal distributions. One-way ANOVA's were performed on data pertaining to physiological media, sperm motility parameters, kinematic parameters, vitality percentage, hyperactivation, acrosome integrity and mitochondrial activity analysis, for all time intervals, respectively. Data presenting with a $P < 0.05$, for the Levene's test for equality of variables, were not normally distributed and were analysed using the Kruskal-Wallis test to determine statistical significance. Bar graphs and radar graphs were constructed for the comparison of the different physiological media's effect on sperm motility parameters, sperm kinematic parameters, vitality, hyperactivation, acrosome integrity and mitochondrial inhibition analysis. A P-value of 0.05 was used as the cut-off point for statistical significance in this study.

Chapter 4: Results

4.1 Introduction

This study investigated sperm structural and functionality parameters, including motility, hyperactivation, vitality, acrosome integrity and mitochondrial function in two NHP species, the vervet and the rhesus monkey. Concurrently the effect of selected physiological media on the above-mentioned parameters was also examined. During the study, only a small number of rhesus semen samples were obtained and the measured functional parameters presented unreliable results. Subsequently, sperm mitochondrial function could not be assessed and was achieved in the vervet monkey only.

This chapter will describe the statistically analysed results as obtained from the study and are presented separately for vervet and rhesus monkeys. Error bars present on Figures depict SDs obtained. Figures lacking error bars are due to one sample being presented in bar graphs. Chapter 5 will discuss the results presented in this chapter as well as the conclusions drawn based on the results obtained.

4.2 Vervet monkey

4.2.1 Baseline semen sample characteristics

The baseline semen sample characteristics for vervet monkeys are presented in Table 4.1. Baseline motility parameters, reflected overall low values for samples, with a mean total motility (TM) of 20.3 ± 16.8%, of which the mean progressive motile (PM) sperm represented 4.1 ± 1.9%, and non-progressive (NP) 16.2 ± 15%, respectively.

Table 4. 1 Baseline vervet semen sample characteristics (mean ± SD)

Characteristics	Range (min - max)	Total sample distribution (n=17)
Semen volume (µl)	2 - 4000	696.7 ± 513.5
Sperm concentration (mil/ml)	0.2 - 201.7	62.9 ± 50.7
Total motility (PR+ NP, %)	0 - 70	20.3 ± 16.8
Progressive motility (PR, %)	0 – 11.2	4.1 ± 1.9
Non-progressive (NP, %)	0 - 62.3	16.2 ± 15
Immotile (%)	37.9 – 94.2	79.6 ± 22.9

4.2.2 Sperm functionality characteristics

Motility and Kinematic parameters

Motility parameters

Based on the low motility values obtained from vervet monkeys by means of CASA, cut-offs for motility and kinematic analyses were adjusted from: Rapid (µm/s) >280, Slow-Medium (µm/s) = 164, Static (µm/s) <64 to Rapid (µm/s) >150, Slow-Medium (µm/s) = 70, Static (µm/s) <50. Therefore, only data obtained from the adjusted cut-off values used are presented in this section. Data obtained from initial cut-off values used are presented in Appendix A (Table 4.A) and B (Table 4.B).

Figure 4.1 illustrates the effects of different media on motility parameters by means of radar plots and Figures 4.2 and 4.3 present sperm swimming tracks for media that significantly differed at time points. Appendix C (Table 4.2) presents the complete set of results on the effects of different physiological media on sperm motility parameters at baseline (T0), 30 minute (T30), and 60 minute (T60) time intervals, as obtained from using adjusted cut-off values.

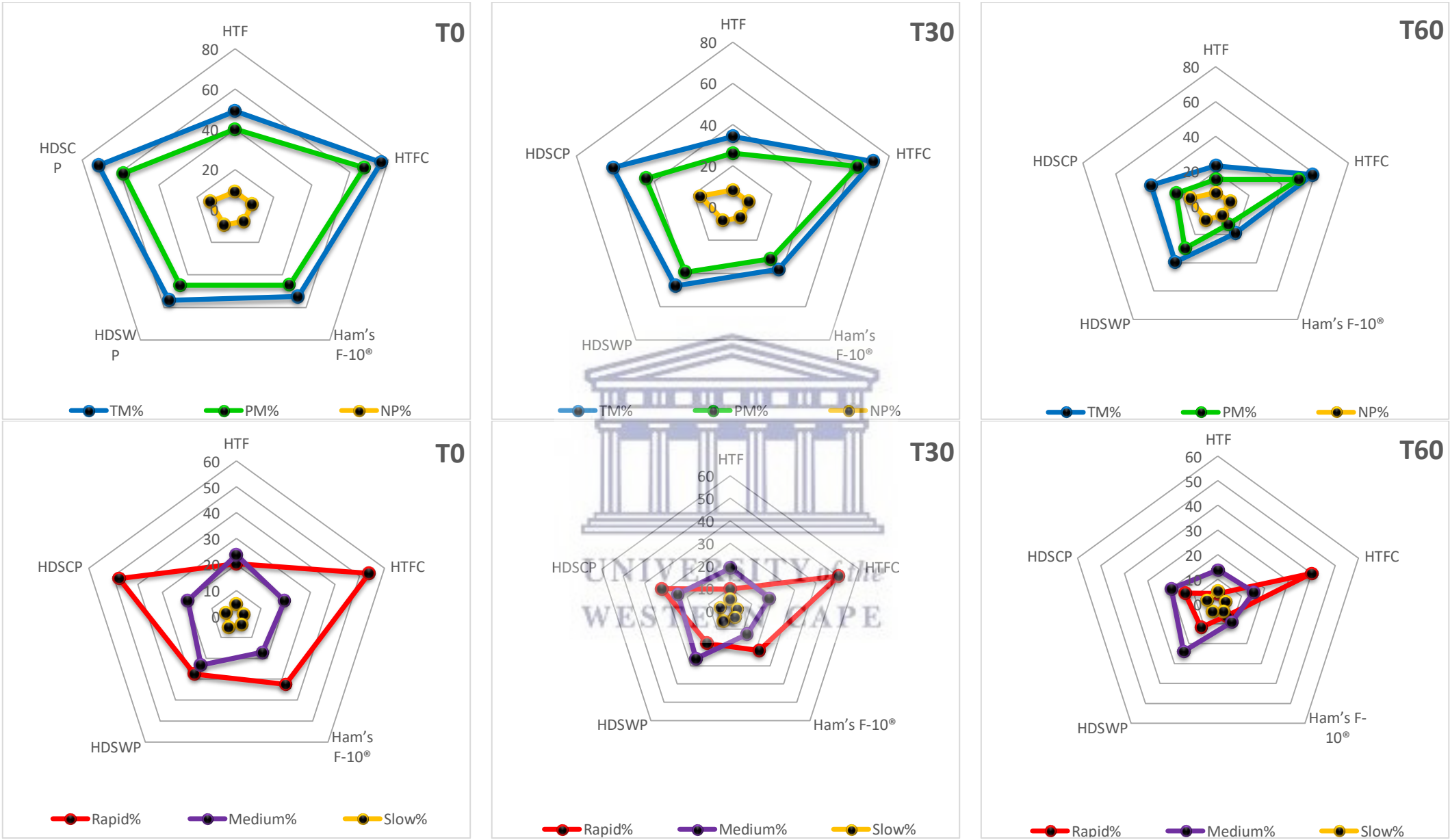


Figure 4.1 Effect of different physiological media on vervet motility parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60). TM% = total motility, PM% = progressive motility, NP% = non-progressive motility; HTF = human tubal fluid, HTFC = human tubal fluid with added caffeine, Ham's F-10®, HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitating Plus, (Baseline n=17, T30 n=16, T60 n=16).

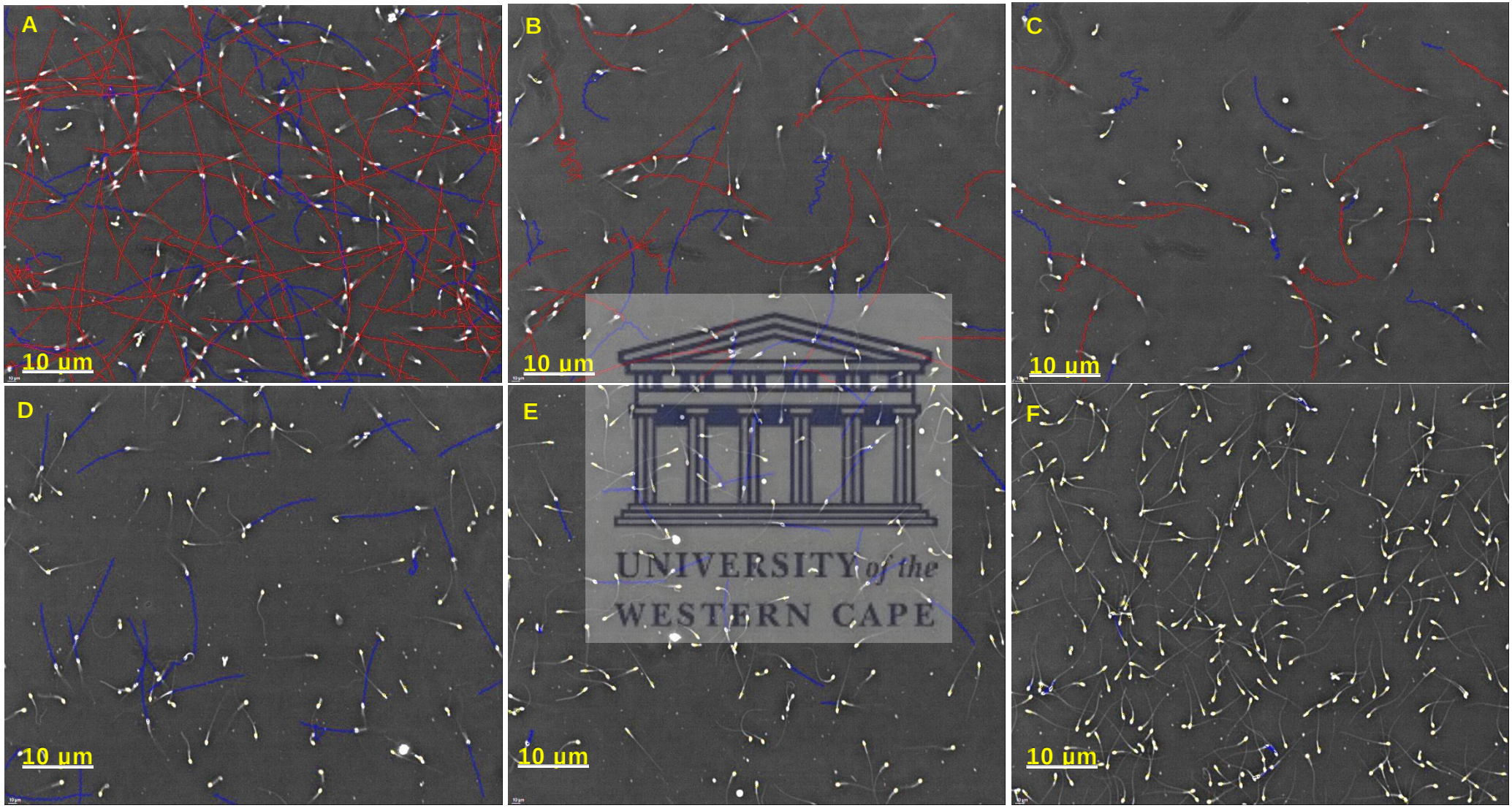


Figure 4. 2 Vervet monkey sperm motility tracks in different physiological media analysed by CASA and SCA® (A) HTFC after 5 minutes, (B) HTFC after 30 minutes, (C) HTFC after 60 minutes, (D) HTF after 5 minutes, (E) HTF after 30 minutes, and, (F) HTF after 60 minutes. Red tracks indicate rapid progressive swimming sperm. Blue tracks indicate medium progressive swimming sperm.

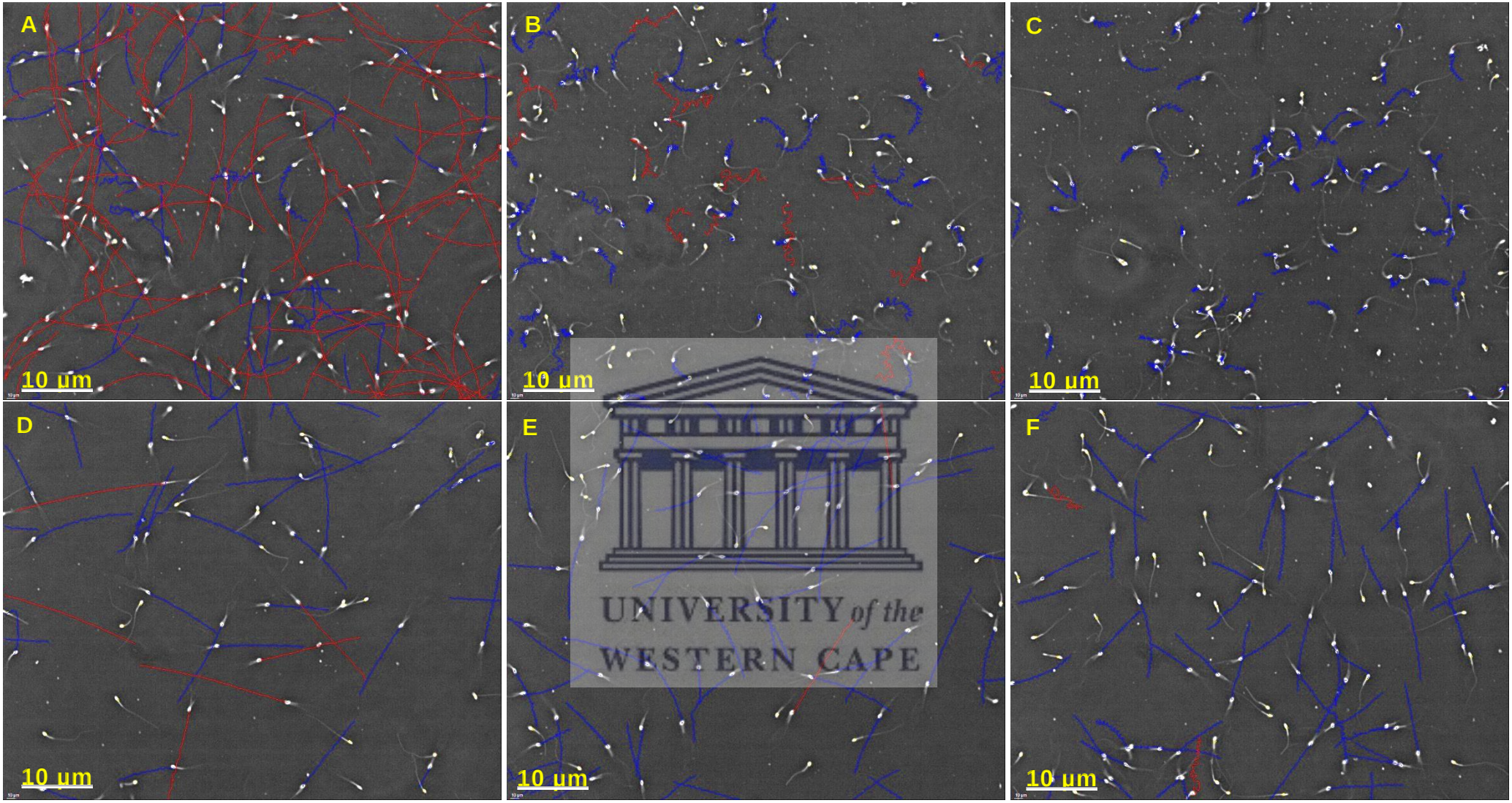


Figure 4.3 Vervet sperm motility tracks in different physiological media analysed by CASA and SCA® , (A) HDSCP after 5 minutes, (B) HDSCP after 30 minutes, (C) HDSCP after 60 minutes, (D) HDSWP after 5 minutes, (E) HDSWP after 30 minutes, and, (F) HDSWP after 60 minutes. Red tracks indicate rapid progressive swimming sperm. Blue tracks indicate medium progressive swimming sperm.

When comparing the effects of different physiological media on percentage total sperm significant differences were observed at baseline, after 30 minutes and 60 minutes incubation. At all three time points, it was noted that HTFC presented with significantly higher values compared with HTF for percentage total motility (76.4 ± 15.1% vs 49.0 ± 23.9% (T0), 71.6 ± 15.9% vs 34.2 ± 21.2% (T30), 58.5 ± 25.5% vs 23.2 ± 24.5% (T60)), progressive motility (67.5 ± 16.2% vs 40.0± 23.2% (T0), 63.6 ± 15.6% vs 26.0 ± 19.2% (T30), 49.8 ± 24.6% vs 15.5 ± 18.1% (T60)) and rapid swimming sperm (53.9 ± 20.5% vs 20.4 ± 21.4% (T0), 50.4 ± 20.3% vs 9.8 ± 14.8% (T30), 40.0 ± 26.6% vs 4.2 ± 7.4% (T60)). Furthermore, HTFC also showed significantly higher values for percentage rapid swimming sperm compared with HDSWP (27.4 ± 21.7% (T0), 17.6 ± 18.5% (T30), 11.8 ± 13.1% (T60)) at all the time points, as well as Ham's F-10® at 30 minutes (21.6 ± 23.5% vs 50.4 ± 20.3%) and 60 minutes (6.1 ± 10.2% vs 40.0 ± 26.6%) time intervals. No significant differences between media were observed for medium and slow swimming sperm over the 60 minute time period. For percentage non-progressive motility, HDSCP had shown significantly higher values compared to Ham's F-10® at baseline (12.9 ± 5.4% vs 7.2 ± 6.1%), and compared to all media at 30 minutes incubation.

Appendix C (Table 4.3) presents the data as obtained for vervet sperm motility parameters in different physiological media at different time points.

In general total motility, progressive motility, and rapid swimming sperm in all the media had the tendency to decrease from baseline up to 60 minutes incubation. Percentage total motility and progressive motility significantly decreased when comparing baseline with 60 minutes for HTF (TM = P = 0.011; PM = P = 0.005), HTFC (TM = P = 0.031; PM = P = 0.03), Ham's F-10® (TM = P = 0.003; PM = P = 0.001), and HDSCP (TM = P = 0.012; PM = P = 0.002). The percentage of rapid swimming sperm also showed a significant decrease comparing baseline with 60 minutes for HTF (P = 0.019), Ham's F-10® (P = 0.005) and HDSCP (P = 0.001). HDSWP only resulted in a near-significant decrease in rapid swimming sperm from baseline to 60 minutes (P = 0.054).

HTFC indicated higher motility percentages (TM and PM) over the time periods, compared to all the media, with significance as indicated (TM = P = 0.031 and PM = P = 0.03). Ham's F-10® and HTF displayed similar results when comparing the different time points, with both media resulting in lower motility and larger decreases in values over the time periods compared to all the media.

Kinematic parameters (average for entire subpopulation)

Figures 4.4, 4.5 and Appendix D (Table 4.4) presents the effects of different physiological media on sperm kinematic parameters (average of entire subpopulation) at baseline (T0), 30 minute (T30), and 60 minute (T60) time intervals, as obtained from the adjusted CASA cut-off values. At baseline, significant differences were observed among media for VCL ($P < 0.001$), VSL ($P = 0.009$), LIN ($P < 0.001$), ALH ($P < 0.001$) and BCF ($P < 0.001$). Regarding VCL, HTFC indicated significantly higher mean values (186.2 ± 26.4 µm/s) in comparison to HTF (141.6 ± 31.5 µm/s) and HDSWP (149.4 ± 28.7 µm/s) at baseline. The VCL for HTF at baseline was also significantly lower compared with HTFC and HDSCP (177.4 ± 26.7 µm/s). VSL values for HDSCP (93.5 ± 26.5 µm/s) was significantly lower compared to HTFC (131.2 ± 27.0 µm/s), Ham's F-10® (124.3 ± 41.6 µm/s) and HDSWP (115.2 ± 24.3 µm/s) at baseline. Results for LIN and STR percentages at baseline for HDSCP (53.6 ± 14.1%, 72.7 ± 9.6%) was significantly lower to all the other physiological media. No significant differences were observed for WOB at baseline although HDSCP indicated lower values compared to other physiological media. ALH values for both HDSCP (3.8 ± 1.2 µm/s) and HTFC (3.0 ± 0.8 µm/s) were significantly higher compared with HTF (2.1 ± 0.4 µm/s), Ham's F-10® (2.3 ± 0.5 µm/s) and HDSWP (2.2 ± 0.4 µm/s) at baseline. The results for BCF at baseline displayed significantly lower values for HDSCP (19.6 ± 2.6 Hz) compared to HTF (24.2 ± 3.8 Hz), Ham's F-10® (23.4 ± 3.5 Hz) and HDSWP (24.4 ± 3.4 Hz). HTFC (20.7 ± 1.8 Hz) also revealed significantly lower mean values compared with HTF.

After 30 minutes incubation VCL values for HTFC (184.5 ± 25.5 µm/s) were significantly higher compared to all the physiological media, except HDSCP (147.5 ± 41.4 µm/s) which in turn showed a significantly higher mean VCL value compared with HTF (121.6 ± 28.9 µm/s). For VSL, HDSCP (64.6 ± 28.1 µm/s) presented significantly lower values compared with HTFC (129.2 ± 17.5 µm/s), Ham's F-10® (104.8 ± 40.7 µm/s) and HDSWP (101.0 ± 26.6 µm/s) after 30 minutes incubation. After 30 minutes HTFC resulted in a significantly higher mean VSL value compared to HTF (92.3 ± 32.7 µm/s). VAP values for HTFC (150.0 ± 18.8µm/s) were significantly higher compared to the other physiological media (HTF, Ham's F-10®, HDSWP and HDSCP). Ham's F-10® (118.7 ± 44.3 µm/s) demonstrated significantly higher VAP values compared with HDSCP (86.1 ± 34.0 µm/s) after 30 minutes incubation. For LIN, STR and WOB, HDSCP resulted in significantly lower values compared to other physiological media. BCF values after 30 minutes incubation for HDSWP (27.3 ± 3.7 Hz) were significantly higher compared with HTFC (20.7 ± 2.4 Hz), Ham's F-10® (22.1 ± 3.1 Hz) and HDSCP (20.8 ± 3.7 Hz). HTF (26.4 ± 3.1 Hz) also resulted in a significantly higher mean value compared with HTFC, Ham's F-10®, and HDSCP.

After 60 minutes incubation HTFC (175.7 ± 38.7 µm/s) resulted in significantly higher VCL values compared to the other physiological media. Also for VSL, HTFC (115.8 ± 30.3 µm/s) resulted in significantly higher values compared with HTF (59.3 ± 27.0 µm/s), Ham's F-10®

(67.6 ± 39.4 µm/s) and HDSCP (37.2 ± 35.2 µm/s) following 60 minutes incubation. HDSCP, compared with HDSWP, indicated a significantly lower mean value (81.2 ± 30.5 µm/s) for VSL. The results for VAP after 60 minutes revealed that HTFC (139.4 ± 33.7 µm/s) ensued higher values compared to other media and that values for HDSWP (91.6 ± 30.2 µm/s) were significantly lower in comparison with HDSCP (49.5 ± 42.6 µm/s).

As for the other time points HDSCP resulted in significantly lower mean values compared with other physiological media for parameters LIN ($P < 0.001$), STR ($P < 0.001$), and WOB ($P < 0.001$) after 60 minutes. For STR, HDSWP values (82.3 ± 16.7 %) were significantly higher compared with Ham's F-10® (73.1 ± 20.3 %). The results for WOB showed significantly higher values for HTFC (78.5 ± 9.0 %) compared with HTF (65.8 ± 16.2 %), Ham's F-10® (64.5 ± 22.4 %) and HDSCP (36.0 ± 25.8 %) after 60 minutes. No significance was observed for ALH ($P = 0.187$) after 60 minutes whereas for BCF ($P = 0.002$), HDSWP (24.9 ± 6.9Hz) showed significantly higher values compared to other media, except HTF (24.1 ± 6.2 Hz). HTF displayed a significantly higher mean value compared with HTFC (20.8 ± 2.2 Hz) and HDSCP (15.9 ± 10.1) following 60 minutes.

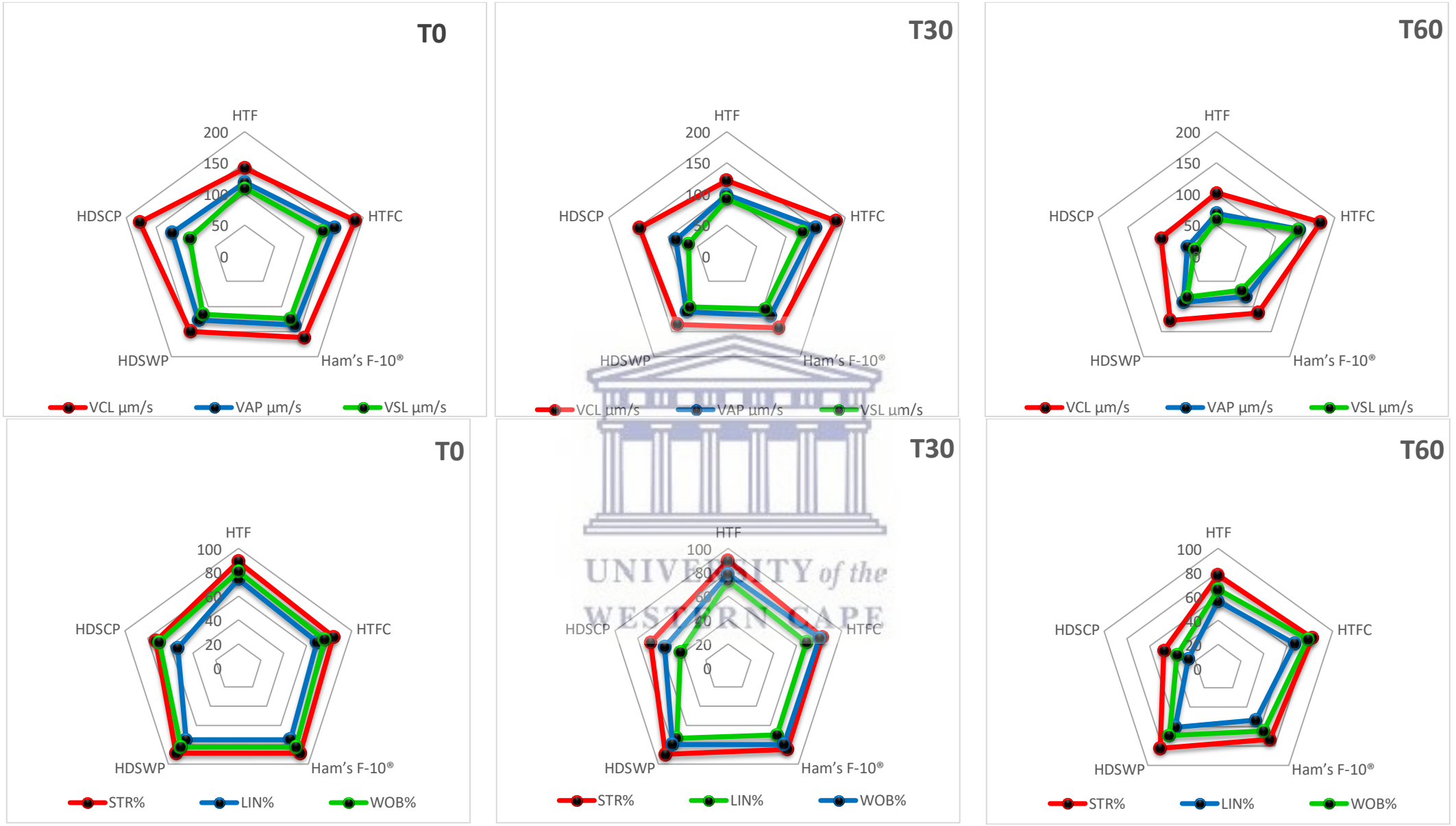


Figure 4.4 Effects of different physiological media on vervet sperm kinematic parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60). VCL µm/s = curvilinear velocity, VAP µm/s = average path velocity, VSL µm/s = straight line velocity, STR %= straightness, LIN % = linearity, WOB % = wobble; HTF = human tubal fluid, HTFC = human tubal fluid with added caffeine, Ham's F-10®, HDSWP = HD Sperm Wash Plus, HDSCP = HD sperm capacitating plus. (Data represented as µm/s for VCL, VAP and VSL and % for STR, LIN and WOB) (Baseline n= 17, T30 n=16, T60 n=16).

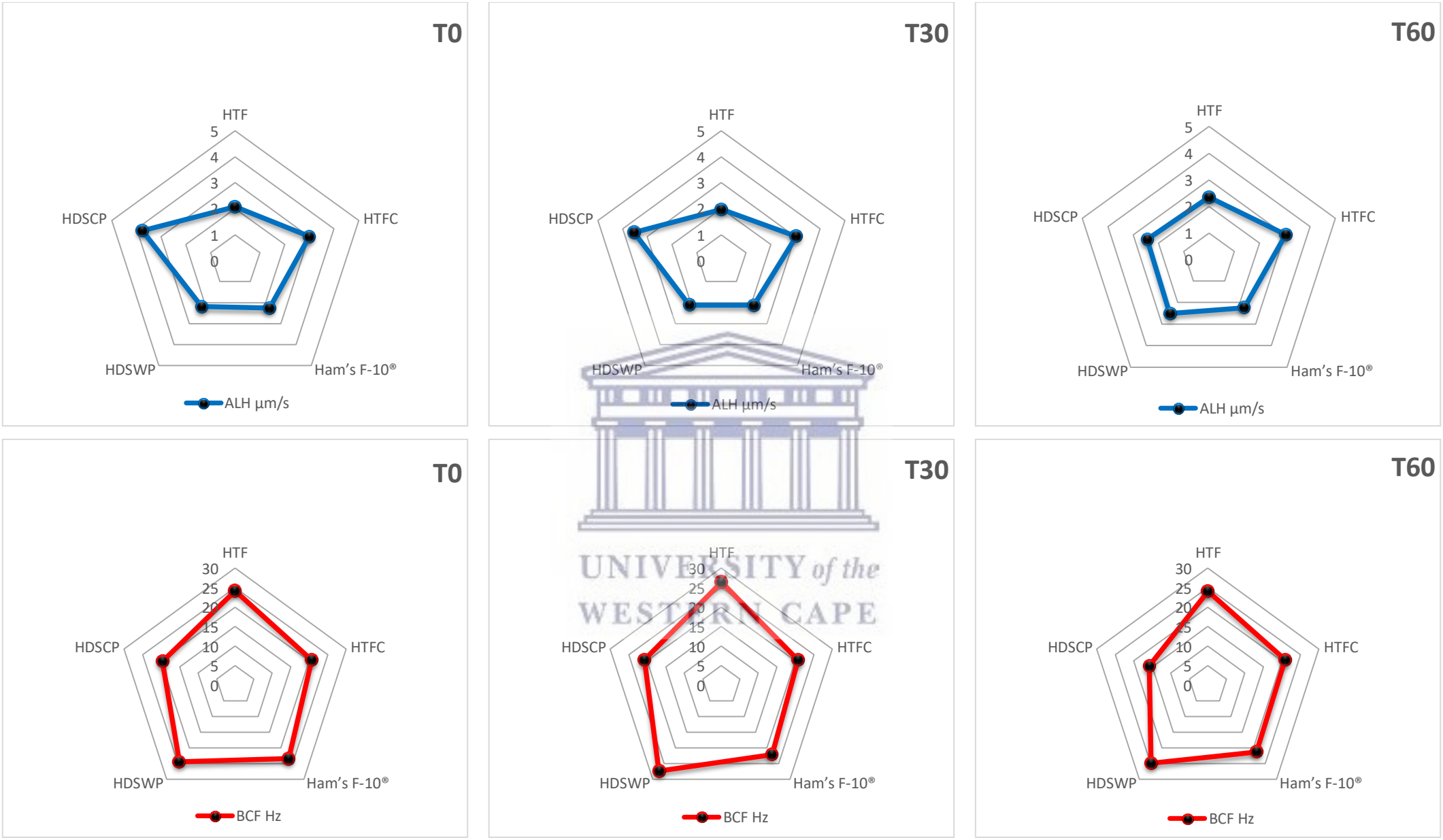


Figure 4.5 Effects of different physiological media on vervet sperm kinematic parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60). ALH µm/s = altitude of lateral head displacement, BCF Hz = beat cross frequency; HTF = human tubal fluid, HTFC = human tubal fluid with added caffeine, Ham's F-10®, HDSWP = HD Sperm Wash Plus, HDSCP = HD sperm capacitating plus (Baseline n= 17, T30 n=16, T60 n=16).

Appendix D (Table 4.5) presents the comparison of the effect of different media on sperm kinematic parameters between the different time points.

For kinematic parameters as measured for the vervet species the majority of values tend to decline over time for all the media. Although significant differences were observed for kinematic parameters over the time period for majority of physiological media, for HTFC, most of the parameters' values remain relatively unchanged over the 1 hour time period. For HTF, a significant decline was observed for VCL ($P < 0.001$), VSL ($P < 0.001$), VAP ($P < 0.001$), LIN ($P = 0.002$), and WOB ($P = 0.002$) when comparing results at baseline with 60 minutes incubation. Additionally, VSL ($P < 0.001$), values were also significantly lower after 30 minutes when compared to baseline. Also for Ham's F-10® significantly lower mean values were observed for VCL ($P = 0.002$), VSL ($P = 0.002$), VAP ($P = 0.002$), LIN ($P = 0.003$), STR ($P = 0.007$), and WOB ($P = 0.006$) when comparing baseline with 60 minutes. The results for HDSWP revealed a significant decrease in mean values for VSL ($P = 0.003$), VAP ($P = 0.004$), LIN ($P = 0.005$), and WOB ($P = 0.002$) when comparing baseline with 60 minutes. HDSCP displayed significant decreased values for kinematic parameters VCL ($P < 0.001$), VSL ($P < 0.001$), VAP ($P < 0.001$) as well as for WOB ($P < 0.001$) comparing baseline with both 30 and 60 minutes. LIN ($P < 0.001$) and ALH ($P = 0.017$) only decreased significantly from baseline to 60 minutes. Overall STR and BCF values did not significantly decrease over the time period for the different media.

A noticeable difference was observed in sperm motility results when comparing the initial CASA and adjusted cut-off values (motility parameters and kinematic parameters). The initial cut-off values presented with lower motility and kinematic values compared to the adjusted cut-offs.

Due to the large standard deviations obtained for the entire subpopulation kinematic results, the individual swimming speed kinematic parameter results were determined to aid in showing the effects of the different physiological media.

Rapid swimming sperm

The kinematic results for rapid swimming sperm are presented in Appendix D (Table 4.6). Results of the kinematic parameters for the different sperm swimming speeds (rapid, medium and slow) showed significant differences similar to obtained kinematic parameters of the entire populations' results. No significant differences were indicated in among the media for VCL at baseline ($P = 0.282$). HDSCP indicated significantly lower mean values for VSL ($P = 0.001$), LIN ($P < 0.001$), STR ($P < 0.001$) and WOB ($P < 0.001$) compared to the other physiological media at baseline and after 30 min. For ALH, HTF resulted in significantly lower mean values compared to all the physiological media ($P < 0.001$) with HDSCP noting the higher mean values at T0 and T30. No significant differences were observed for BCF ($P = 0.253$) at baseline.

For LIN, HDSCP (54.4 ± 10.4 %) resulted in significantly lower mean values compared to HTFC (70.6 ± 10.9 %), Ham's F-10® (67.8 ± 21.1 %) and HDSWP (65.2 ± 12.7 %) with HTF (61.0 ± 17.1 %) being significantly lower to HTFC. HTFC (83.9 ± 7.5 %) resulted in significantly higher STR values compared to HDSCP (69.7 ± 12.9 %). WOB noted significantly higher values when comparing HTFC (80.7 ± 8.3 %) to HDSCP (68.7 ± 7.1 %) and HTF (72.6 ± 11.5 %). Furthermore, Ham's F-10® (75.8 ± 15.4 %) also noted a significantly higher mean value compared to HDSCP after 60 minutes incubation for WOB. No significantly different values were observed for VCL (P = 0.964), VSL (P = 0.159), VAP (P = 0.265), ALH (P = 0.147) or BCF (P = 0.063) at this time interval.

Medium swimming sperm

The kinematic results for medium swimming sperm are presented in Appendix D (Table 4.7). At baseline significant differences were obtained for VSL (P = 0.008), LIN (P = 0.009), STR (P = 0.001), WOB (P = 0.007) and BCF (P < 0.001). Although not significantly, Ham's F-10® resulted in the highest mean values for majority of the parameters (VSL = 90.1 ± 17.3 µm/s, VAP = 99.4 ± 14.1 µm/s, LIN = 73.7 ± 11.0 % and WOB = 81.3 ± 7.5 %). No significant differences were observed in VCL (P = 0.250), VAP (P = 0.106) and ALH (P = 0.127) at baseline. HDSCP indicated significantly lower mean values for VSL (74.6 ± 11.8 µm/s) compared to Ham's F-10® (90.1 ± 17.3 µm/s) and HDSWP (88.5 ± 10.0 µm/s) at baseline. HDSCP also displayed significantly lower mean values for LIN (62.6 ± 9.5 %), STR (78.1 ± 8.3 %), and WOB (73.0 ± 6.8 %) compared to Ham's F-10® (LIN = 73.7 ± 11.0 %; STR = 86.5 ± 8.3 %; WOB = 81.3 ± 7.5 %) initially. For BCF observed HTFC (14.8 ± 2.4 Hz) and HDSCP (15.9 ± 2.9 Hz) revealed significantly lower mean values compared to HTF (20.3 ± 4.1 Hz) and HDSWP (20.8 ± 3.1 Hz).

After 30 minutes incubation significant mean differences were observed in VSL (P < 0.001), VAP (P < 0.001), LIN (P < 0.001), STR (P < 0.001), WOB (P < 0.001), ALH (P < 0.001) and BCF (P < 0.001). HDSCP resulted in significantly lower mean values compared to all other physiological media for VSL (63.1 ± 14.3 µm/s), VAP (77.2 ± 12.4 µm/s), LIN (53.1 ± 10.5 %), STR (75.7 ± 7.5 %) and WOB (64.8 ± 8.8 %). For BCF, HTFC (14.9 ± 3.6 Hz) and HDSCP (15.6 ± 4.8 Hz) noted significantly lower values to HTF (21.3 ± 2.7 Hz) and HDSWP (20.8 ± 4.8 Hz) respectively.

After 60 minutes incubation, HTFC noted significantly higher mean values for majority of slow swimming vervet sperm kinematic parameters, VSL (88.9 ± 22.4 µm/s), VAP (100.4 ± 24.4 µm/s), LIN (69.7 ± 8.4 %), WOB (78.8 ± 5.6 %), and ALH (2.2 ± 0.4 4 µm/s) compared with HDSCP noting the lowest mean values for VSL (60.4 ± 19.9 µm/s), VAP (71.6 ± 18.1 µm/s), LIN (53.1 ± 16.9 %), WOB (64.7 ± 13.8 %) and ALH (3.0 ± 0.7 µm/s). No significant differences were obtained for VCL (P = 0.058) and STR (P = 0.066) at this time point. For ALH Ham's F-10® noted a significantly lower mean value (2.1 ± 0.6 µm/s) compared to HDSCP after 60 minutes, with HDSCP resulting in the highest value (3.0 ± 0.7µm/s).

HDSWP (20.3 ± 5.2 Hz) resulted in a significantly higher BCF value compared to HTFC (15.6 ± 3.8 Hz), Ham's F-10® (15.0 ± 4.9 Hz) and HDSCP (13.1 ± 6.6 Hz).

Slow swimming sperm

The kinematic results for slow swimming sperm are presented in Appendix D (Table 4.8). At baseline all the kinematic parameters, except VCL (P = 0.657), ALH (P = 0.296) and BCF (P = 0.052), resulted in significant differences when comparing the different physiological media. For kinematic parameters VSL (32.3 ± 10.9 µm/s), VAP (41.9 ± 9.5 µm/s;), LIN (47.8 ± 12.2 %); STR (65.5 ± 18.4 %); and WOB (60.4 ± 13.1 %), HDSCP resulted in significantly lower mean values compared to HTF (VSL = 45.1 ± 7.8 µm/s; VAP = 51.1 ± 5.7 µm/s; LIN = 64.9 ± 10.6 %; STR = 83.0 ± 9.3 %; WOB = 73.5 ± 7.3 %) and Ham's F-10® (VSL = 45.0 ± 13.6 µm/s; VAP = 51.4 ± 9.9 µm/s; LIN = 64.7 ± 18.7 %; STR = 80.4 ± 15.6 %; WOB = 74.0 ± 13.4 %).

After 30 minutes incubation, only two of the kinematic parameters, VSL (P = 0.035) and STR (P = 0.023), noted significant differences when comparing the different physiological media. For VSL, HDSCP (32.3 ± 10.9 µm/s) resulted in a significantly lower value compared to HTF (45.1 ± 7.8 µm/s), Ham's F-10® (45.0 ± 13.6 µm/s) and HDSWP (44.5 ± 11.7 µm/s). For STR, HDSCP (71.4 ± 8.7 %) presented a significantly lower value compared with HDSWP (83.1 ± 13.0 %).

The results after 60 minutes incubation noted significant differences. For VSL (P < 0.001), VAP (P < 0.001), LIN (P < 0.001), STR (P = 0.001), WOB (P < 0.001) and ALH (P < 0.001), with no significance obtained for VCL (P = 0.444) and BCF (P = 0.615). Overall HDSCP resulted in significantly lower values for VSL (25.1 ± 10.4 µm/s), VAP (36.9 ± 7.8 µm/s), LIN (36.3 ± 15.2 %), STR (57.7 ± 21.3 %) and WOB (53.6 ± 11.6 %) compared with HTF (VSL = 38.7 ± 7.7 µm/s, VAP = 46.5 ± 5.9 µm/s, LIN = 55.2 ± 11.0 %, STR = 75.3 ± 9.4 %, WOB = 66.3 ± 8.8 %), HTFC (44.2 ± 11.7 µm/s, 51.9 ± 12.1 µm/s, 60.5 ± 11.7 %,78.2 ± 8.6 %,71.1 ± 10.0 %) and HDWSP (39.3 ± 9.7 µm/s, 46.5 ± 7.8 µm/s, 55.6 ± 13.7 %,75.6 ± 12.3 %,66.0 ± 10.8 %). For VAP and WOB, Ham's F-10® (37.6 ± 13.3 µm/s; 53.5 ± 18.8 %) and HDSCP (36.9 ± 7.8 µm/s; 53.6 ± 11.6 %) showed significantly lower means compared to HTF (46.5 ± 5.9 µm/s, 66.3 ± 8.8 %), HTFC (51.9 ± 12.1 µm/s; 71.1 ± 10.0 %) and HDSWP (46.5 ± 7.8 µm/s; 66.0 ± 10.8 %). HDSCP resulted in the highest value for ALH (2.3 ± 0.6 µm/s) compared to HTFC (1.7 ± 0.4 µm/s) and HDSWP (1.8 ± 0.5 µm/s).

Comparing the significance indicated at baseline for the combined subpopulations to the individual swimming sperm kinematics (rapid, medium and slow), the results differ in significance obtained for rapid swimming sperm (VCL = P = 0.282, BCF = P = 0.253), medium swimming sperm (VCL = P = 0.250, VAP = P = 0.106 and ALH = P = 0.127) and slow swimming sperm (VCL = P = 0.657, ALH = P = 0.296 and BCF = P = 0.052) with the combined kinematic results noting significance in all parameters.

After 30 minutes incubation significance differed within rapid swimming sperm (VCL = P = 0.983, VAP = P = 0.08 and BCF = P = 0.018), medium swimming sperm (VCL = P = 0.091) and slow swimming sperm (VCL = P = 0.504, VAP = P = 0.076, LIN = P = 0.055, WOB = P = 0.110, ALH = P = 0.160 and BCF = P = 0.073), compared to the combined results again noting significance in all parameters.

After 60 minutes incubation the combined results noted significant differences in all kinematic parameters except ALH (P= 0.187). The different swimming sperm subpopulations (rapid medium and slow) differed from the combined results with rapid swimming sperm noting no significant differences for VCL (P = 0.964), VSL (P = 0.159) and VAP (P = 0.265) with medium swimming sperm differing within VCL (P = 0.058) and STR (P = 0.066), whereas slow swimming sperm results differed in VCL (P = 0.444), ALH (P = 0.001) and BCF (P = 0.615). The results obtained from the medium and slow swimming sperm for ALH indicate that the averages obtained masked the actual effects of the media seen at the level of the subpopulations by producing differing standard deviations and significance.

Large differences were observed between the swimming speeds for the velocity kinematics, e.g. rapid swimming speed values were close to double that of medium swimming speed values, while slow swimming speed results were much lower compared to medium swimming speed results. This could thus have contributed to the large standard deviations observed within the combined subpopulation kinematic results. However, there are still large standard deviations for rapid swimming speeds' velocity parameters, which could be owed to differences between individual samples.

Hyperactivation

The results obtained for the effects of different physiological media to induce hyperactivation and differences between time intervals are demonstrated in Figures 4.6 and 4.7 (n=20). Due to the high standard deviation obtained for this part of the study Figures 4.8 and 4.9 present different results obtained for selected vervet samples, in order to demonstrate the variation observed in individual samples. Complete results are presented in Appendix E in Tables 4.9 and 4.10. Percentage hyperactivation showed significant differences among different physiological media used after the respective time intervals, i.e. baseline, 15, and 30 minute intervals. At baseline, 15 and 30 minute time intervals HDSCP demonstrated a significantly higher percentage hyperactivation (40.0 ± 25.4 % (T0), 32.6 ± 25.6 % (T15), 24.6 ± 21.8 % (T30)) compared to HTF (8.6 ± 15.2 % (T0), 8.0 ± 10.3 % (T15), 10.1 ± 13.6 % (T30)), Ham's F-10® (10.6 ± 18.1 % (T0), 6.6 ± 13.2 % (T15), 7.2 ± 14.1 % T30) and HDSWP (10.5 ± 16.2 % (T0), 12.2 ± 17.7 % (T15), 9.1 ± 12.5 % (T30)). Although insignificant, HDSCP presented with a slightly higher percentage hyperactivation compared to HTFC at baseline, 15, 30 and 45 minute time intervals. After 45 and 60 minutes HDSCP percentage hyperactivation was still higher compared to the other media, but not significantly so. As for HDSCP, HTFC also presented with significantly higher values compared to other media after 5, 15 and 30 minute

intervals. HDSCP resulted in percentage hyperactivation above 20% for all of the time intervals, while HTFC only maintained more than 20% hyperactivation up to 45 minutes.

Overall, no significant difference between time intervals was noted for all the physiological media tested. For both the capacitating media, HDSCP and HTFC, the percentage hyperactivation tend to decrease from after 30 minutes up to 60 minutes incubation. Although not significantly different from other time intervals, the highest percentage hyperactivation for HDSCP was reached already at baseline (40.0 ± 25.4 %), from where after values decreased, while for HTFC its maximum value was reached following 15 minutes (27.9 ± 22.6 %) of incubation.

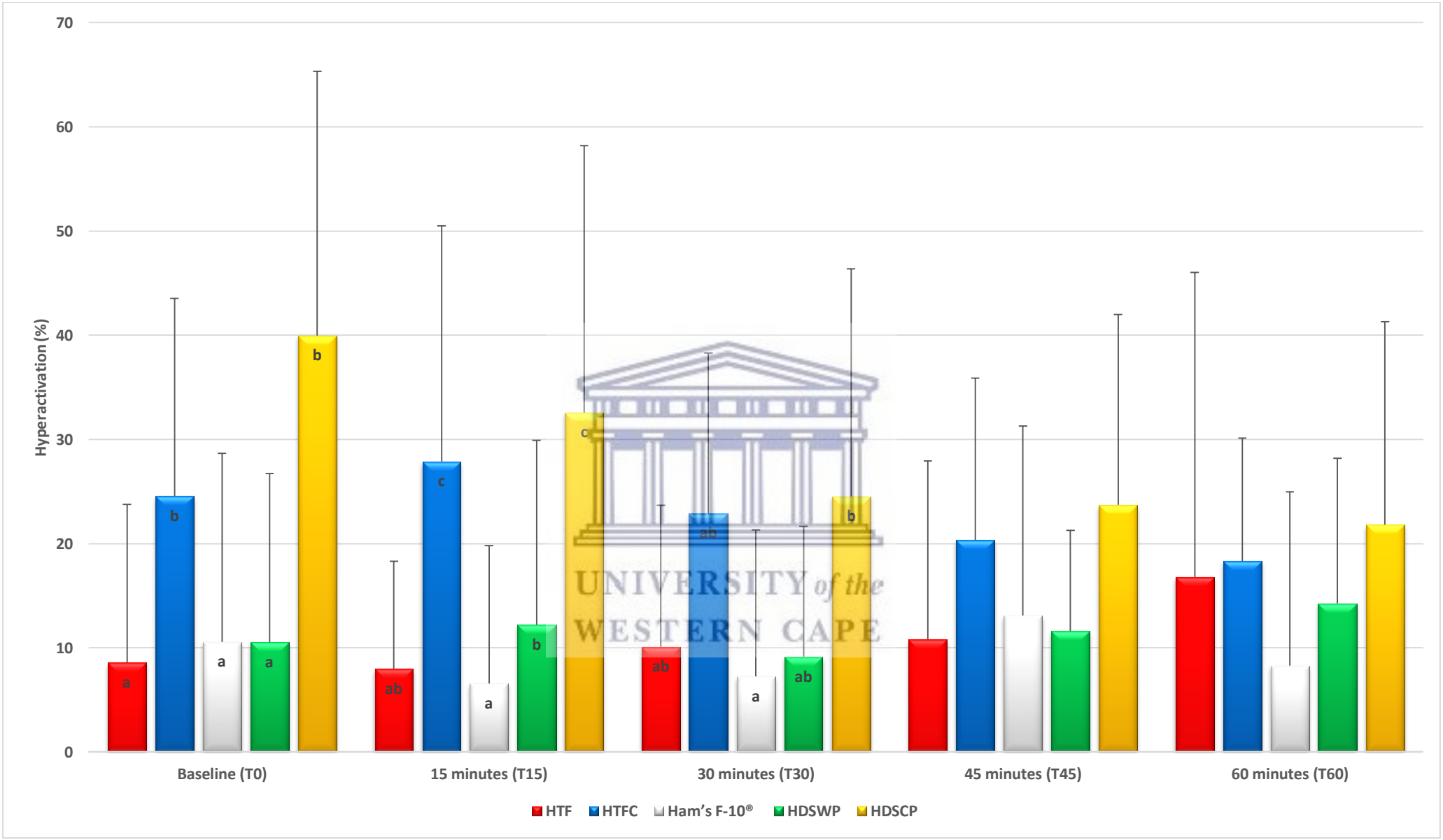


Figure 4.6 Bar graph of percentage hyperactivation of vervet sperm over a period of 60 minutes after incubation with HTF = human tubal fluid, HTFC= human tubal fluid with caffeine, Ham's F-10®, HDSWP/ HD Sperm Wash Plus, and HDSCP/ HD Sperm Capacitation Plus; [abc]Means with different letters differed significantly as obtained from ANOVA; P<0.05= significance (mean ± SD); SD represented as error bars (n=20).

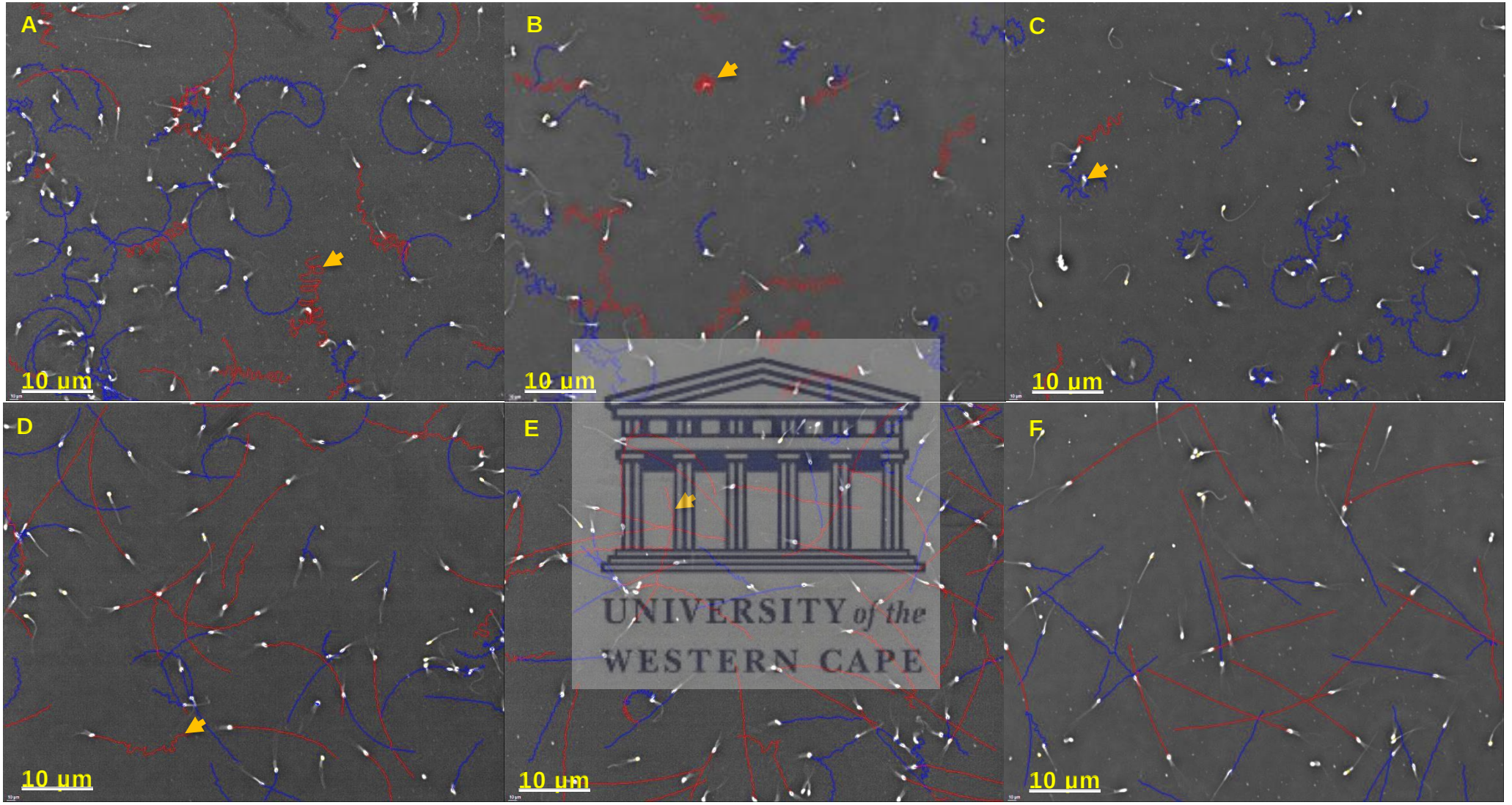
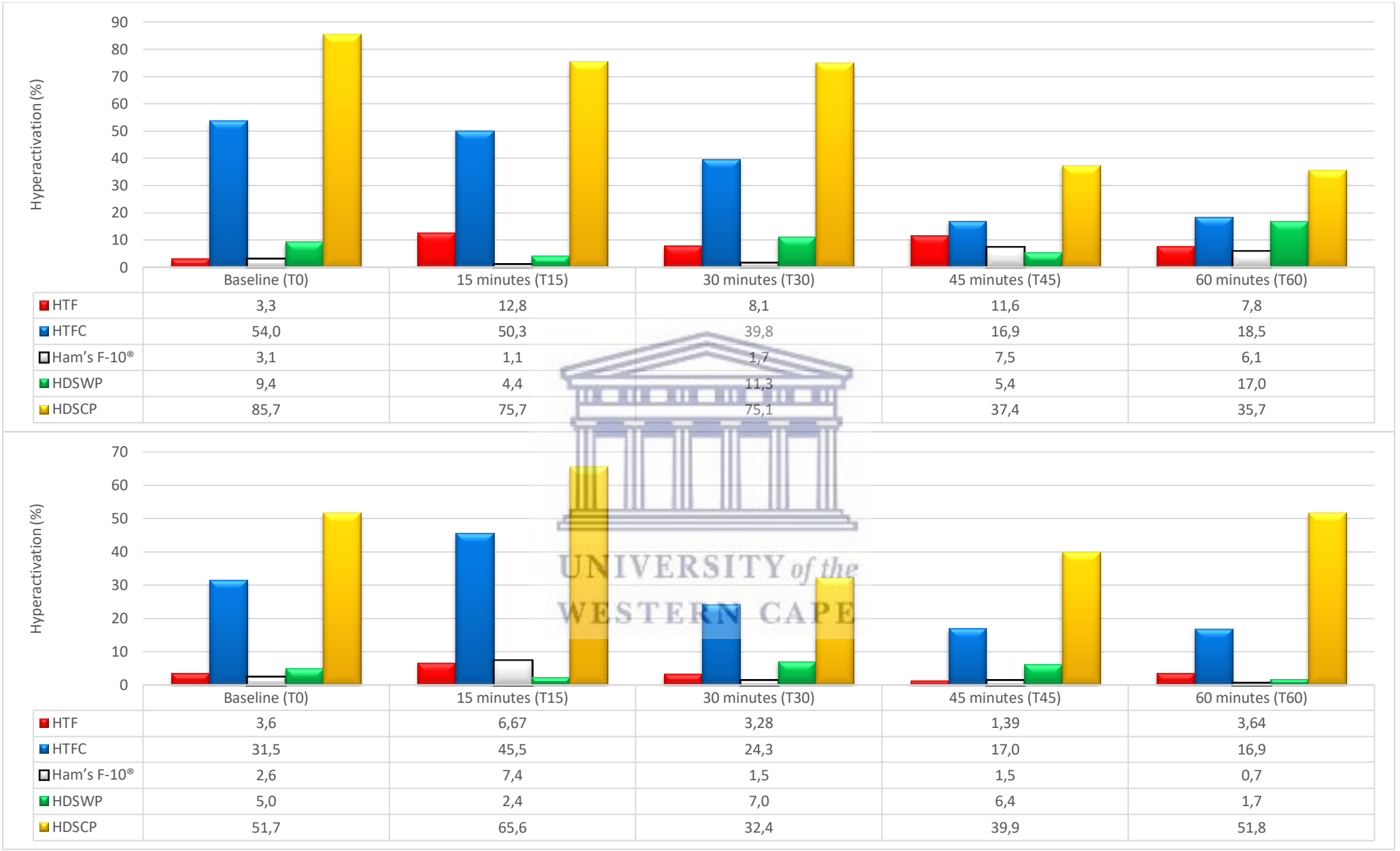


Figure 4.7 Vervet sperm hyperactivation swimming tracks in different physiological media analysed by CASA and SCA® (A) HDSCP after 5 minutes, (B) HDSCP after 15 minutes, (C) HDSCP after 30 minutes, (D) HTFC after 5 minutes, (E) HTFC after 15 minutes, and, (F) HTFC after 30 minutes. Red tracks indicate rapid progressive swimming sperm. Blue tracks indicate medium progressive swimming sperm. Yellow arrows indicate different hyperactivated sperm motility tracks.



	Baseline (T0)	15 minutes (T15)	30 minutes (T30)	45 minutes (T45)	60 minutes (T60)
HTF	3,3	12,8	8,1	11,6	7,8
HTFC	54,0	50,3	39,8	16,9	18,5
Ham's F-10®	3,1	1,1	1,7	7,5	6,1
HDSWP	9,4	4,4	11,3	5,4	17,0
HDSCP	85,7	75,7	75,1	37,4	35,7

	Baseline (T0)	15 minutes (T15)	30 minutes (T30)	45 minutes (T45)	60 minutes (T60)
HTF	3,6	6,67	3,28	1,39	3,64
HTFC	31,5	45,5	24,3	17,0	16,9
Ham's F-10®	2,6	7,4	1,5	1,5	0,7
HDSWP	5,0	2,4	7,0	6,4	1,7
HDSCP	51,7	65,6	32,4	39,9	51,8

Figures 4.8 and 4. 9 Bar graphs of percentage hyperactivation of selected vervet sperm showing varying effects of media over a period of 60 minutes after incubation with HTF = human tubal fluid, HTFC = human tubal fluid with caffeine, Ham's F-10®, HDSWP/ HD Sperm Wash Plus, and HDSCP/ HD Sperm Capacitation Plus (n=1).

Mitochondrial activity

Motility parameters

The cut off values used for sperm exposed to different concentrations of Oligomycin were adjusted from Rapid (µm/s)>280, Slow-Medium (µm/s) = 164, Static (µm/s) <64, to Rapid (µm/s)>150, Slow-Medium (µm/s) = 70, Static (µm/s) <50, as was done for motility analysis in section 4.2.2. The effects of different Oligomycin concentrations on motility parameters between baseline (T0) and 30 (T30) -minute time points are presented in Figure 4.10 (n=17), and complete results are presented in Appendix F (Table 4.11). The effects of time on these parameters are presented in Table 4.12 (Appendix F).

No significant differences were observed for motility parameters when comparing the control with two different Oligomycin concentrations (5 µM and 25 µM) at baseline and 30 minute intervals. Interestingly, at both time points, the 5 µM Oligomycin concentration tends to have higher total motility (T0 = 51.2 ± 29.8 %; T30 = 35.0 ± 31.7 %) compared to the control (T0 = 43.7 ± 35.5 %; T30 = 32.5 ± 33.2 %) and 25 µM Oligomycin concentration (T0 = 43.1 ± 29.5 %; T30 = 28.6 ± 28.4 %), while progressive motility (37.8 ± 35.4%) and rapid swimming sperm percentage (23.9 ± 28.3%) were seemingly higher in the control group compared to the two concentrations. The 25 µM Oligomycin concentration appeared to have an inhibitory effect on total motility and progressive motility, with the latter being lower (TM = 43.1 ± 29.6 % (T0), 28.5 ± 28.3 % (T30); PM = 33.1 ± 28.2 % (T0), 21.2 ± 23.8 % (T30)) compared to the 5 µM Oligomycin concentration (TM = 51.2 ± 29.8 % (T0), 35.0 ± 31.7 % (T30), PM = 36.4 ± 27.8 % (T0), 24.6 ± 22.7 % (T30)) at both time points, but not significantly so. Furthermore, at both time points, the 5 µM Oligomycin tend to have more non-progressive sperm (14.8 ± 12.2 % (T0), 10.4 ± 12.3 % (T30)) compared to the control (5.8 ± 5.0 % (T0), 5.0 ± 4.2 % (T30)) and 25 µM Oligomycin concentration (10.0 ± 9.5 % (T0), 7.3 ± 7.7 % (T30)). Both the Oligomycin concentrations had very similar effects on % rapid swimming sperm at the time points. In the present study both the Oligomycin concentrations resulted in reduced progressive motility, as well as lower values for rapid swimming sperm compared with the control samples. The majority of motility parameters, except the percentage of slow swimming sperm, decreased over the time period in the control and two Oligomycin concentration groups, but no significant differences were noted between the time points.

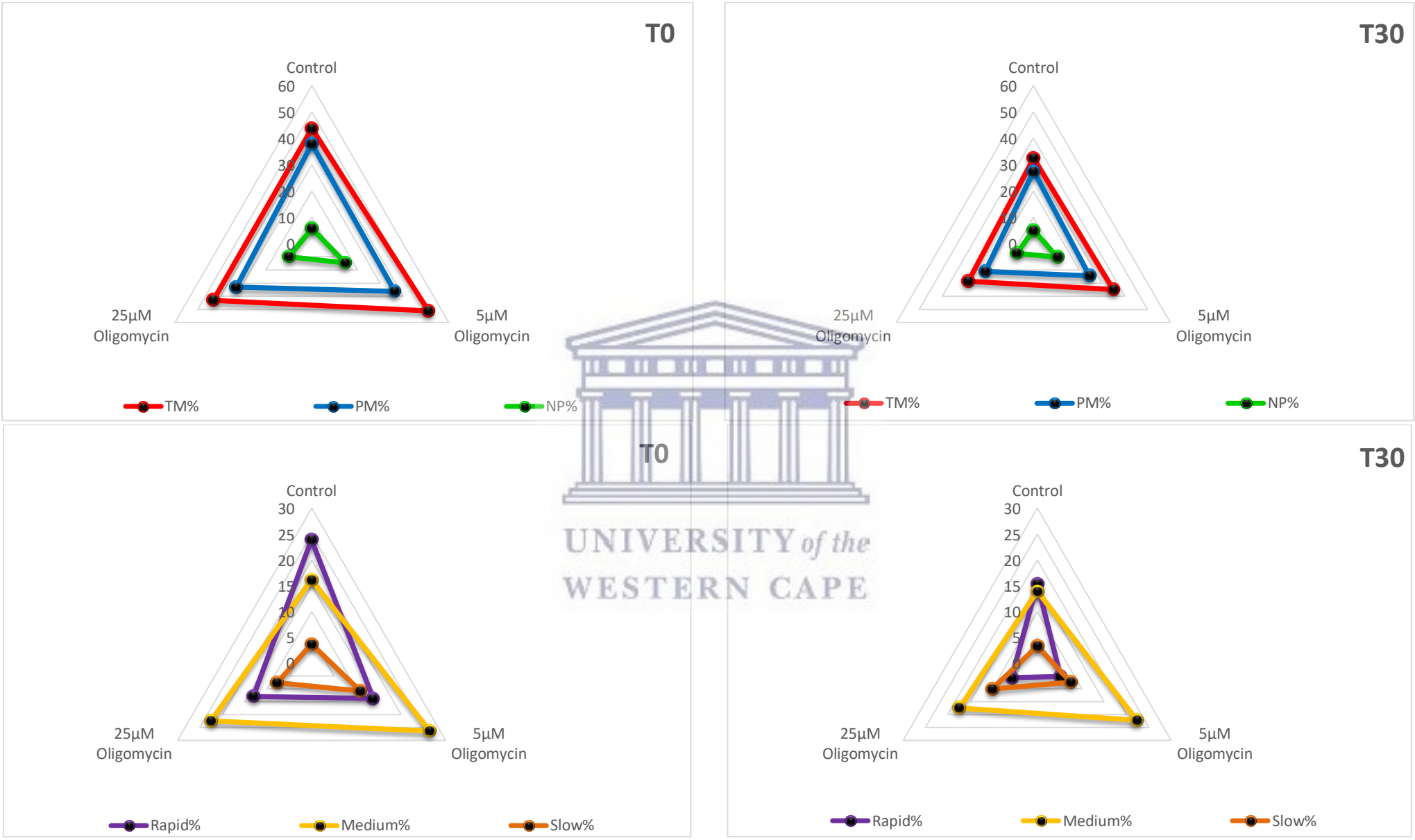


Figure 4.10 Effect of different Oligomycin concentrations on vervet motility parameters at baseline (T0) and 30 minutes (T30). TM% = total motility, PM% = progressive motility, NP% = non-progressive motility; 5µM = Oligomycin, 25µM = Oligomycin, (Control n=6, Oligomycin, 5µM n=17, Oligomycin, 25µM n=17).

Kinematic parameters

Kinematic parameter results obtained for sperm exposed to different concentrations of Oligomycin at different time points are presented in Figures 4.11, 4.12 and 4.13. Complete results are presented in Appendix G (Table 4.13). The CASA cut-off values used were similar to those used above for the motility portion of this study. Comparison of Oligomycin concentration results between different time points is illustrated in Appendix G (Table 4.14).

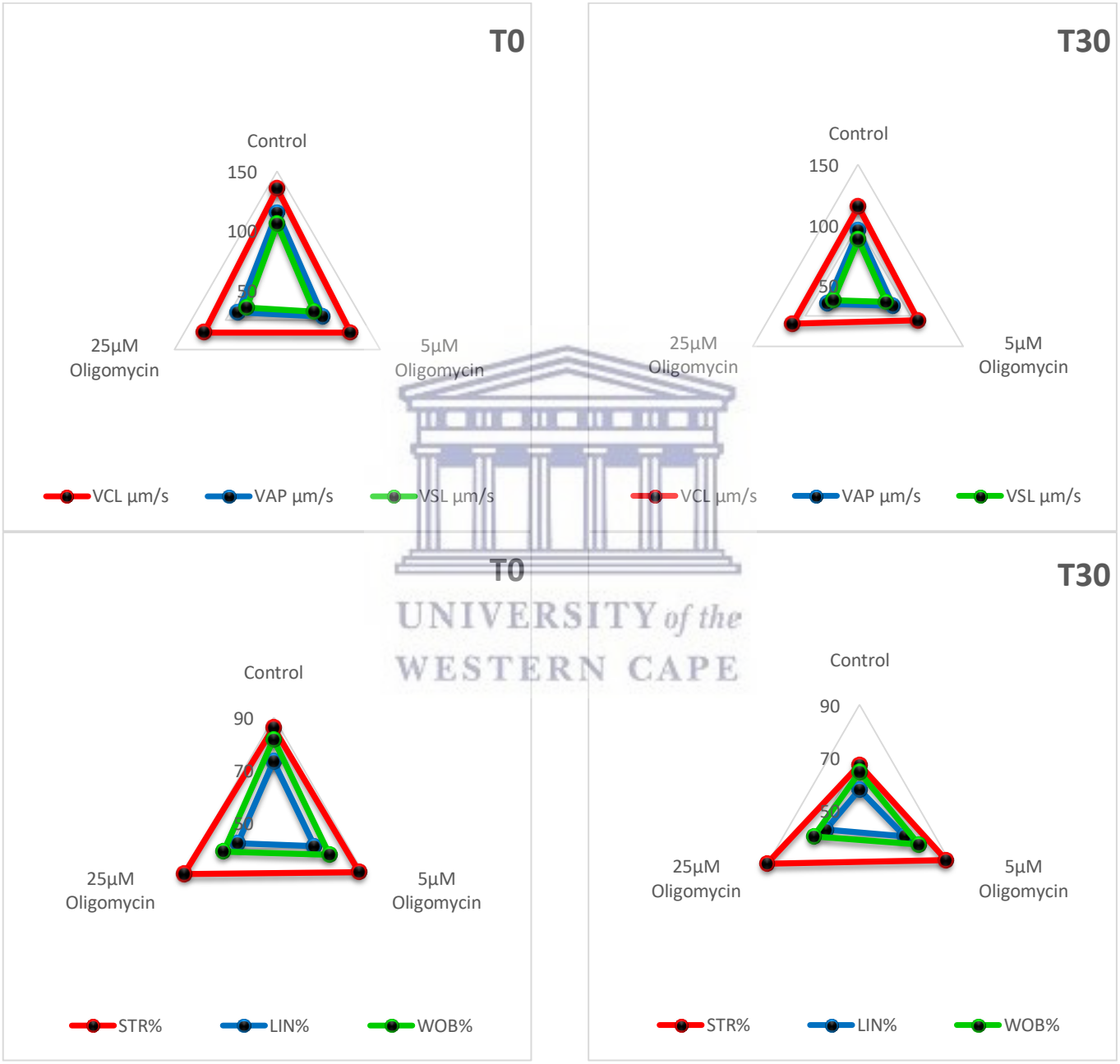


Figure 4.11 Effect of different Oligomycin concentrations on vervet motility parameters at baseline (T0) and 30 minutes (T30). VCL µm/s = curvilinear velocity, VAP µm/s = average path velocity, VSL µm/s = straight line velocity, STR% = straightness, LIN% = linearity, WOB% = wobble, 5µM = Oligomycin, 25µM = Oligomycin (Control n=6, Oligomycin, 5µM n=17, Oligomycin, 25µM n=17).

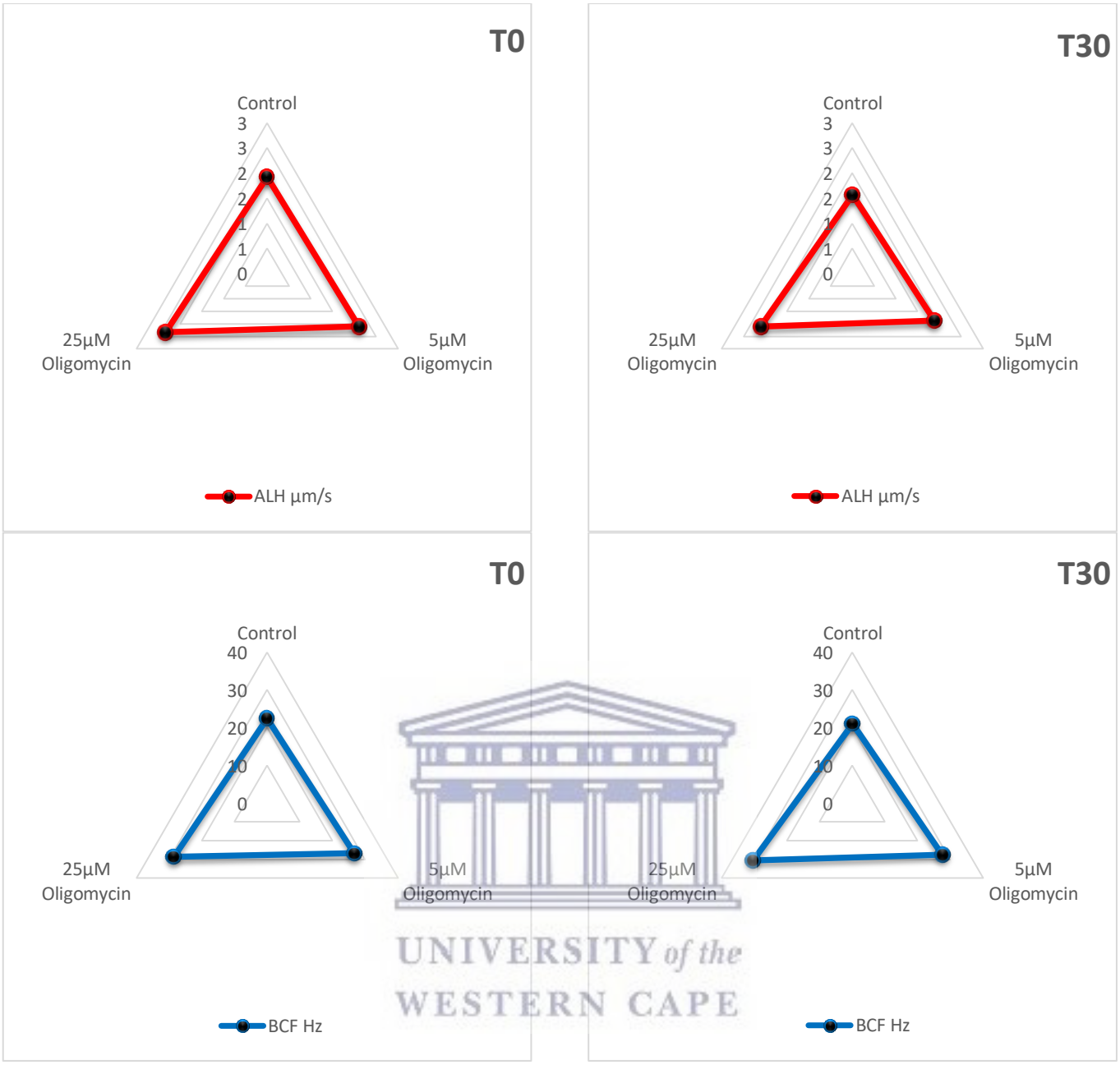


Figure 4.12 Effect of different Oligomycin concentrations on vervet kinematic parameters at baseline (T0) and 30 minutes (T30). ALH µm/s = average lateral head displacement, BCF µm/s = beat cross frequency; 5µM = Oligomycin, 25µM = Oligomycin (Control n=6, Oligomycin, 5µM n=17, Oligomycin, 25µM n=17).

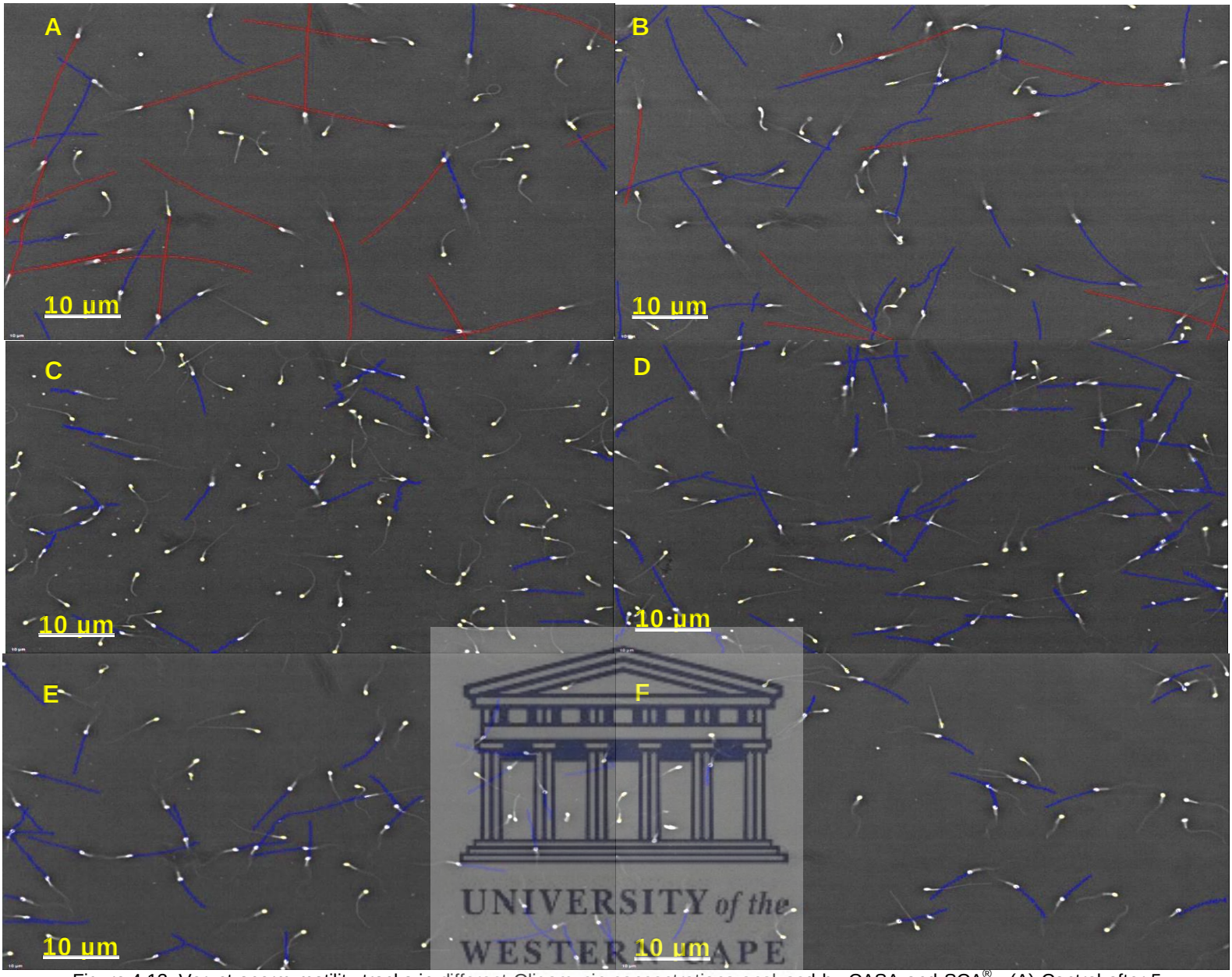


Figure 4.13 Vervet sperm motility tracks in different Oligomycin concentrations analysed by CASA and SCA® , (A) Control after 5 minutes, (B) Control after 30 minutes, (C) 5 µM Oligomycin after 5 minutes, (D) 5 µM Oligomycin after 30 minutes, (E) 25 µM Oligomycin after 15 minutes, and, (F) 25 µM Oligomycin after 30 minutes. Red tracks indicate rapid progressive swimming sperm. Blue tracks indicate medium progressive swimming sperm.

Kinematic parameters (average for entire sperm population)

The results obtained for sperm incubated with different concentrations of Oligomycin when focussing on kinematic parameters are presented in Appendix G (Table 4.13). Table 4.14 (Appendix G) presents the results of sperm subjected to different concentrations of Oligomycin over time. For the kinematic parameters, no significant differences were observed between the control and two Oligomycin concentrations over the time period, except for BCF, for which the 25 µM concentration showed a significantly higher value compared to the control samples at baseline (P = 0.01) and after 30 minutes (P = 0.004). In general, the control held higher mean values for VCL, VSL, and VAP compared to both Oligomycin concentrations, at both the time intervals, but not significantly higher. This result was expected due to the direct relationship between motility parameters and kinematic

parameters. At baseline, the control also indicated the higher mean values for LIN (73.3 ± 21.9 %) and WOB (81.8 ± 16.8 %), whereas STR (89.3 ± 5.3 %), ALH (2.4 ± 0.7 µm/s) and BCF (28.6 ± 2.9 Hz) values for the 25 µM concentration displayed the higher mean values. Regarding the effects of Oligomycin on sperm kinematic parameters, no significantly different results effects over time were noted for any of the groups. Kinematic parameter values for VCL, VSL, and VAP decreased from baseline to 30 minutes in the control group, as well as for the two Oligomycin concentrations, but not significantly so. STR values increased after 30 minutes in the 5 and 25 µM Oligomycin groups. In both the 5 µM and 25 µM concentration Oligomycin groups STR and BCF values slightly increased after 30 minutes.

Rapid swimming sperm

The results obtained for sperm incubated with different concentrations of Oligomycin when focussing on the different swimming speed (rapid, medium and slow) kinematic parameters are presented in Appendix G (Tables 4.15, 4.16 and 4.17). At baseline and after 30 minutes, for rapid swimming sperm, no significant differences were observed for any of the kinematic parameters and the values obtained showed little variation. Although not significantly so, at baseline, values of the velocity parameters (VCL, VAP, and ALH were higher in 25 µM Oligomycin (VCL = 247.8 ± 55.9 µm/s; VAP = 206.6 ± 74.9 µm/s; ALH = 3.3 ± 2.2 µm/s) compared to the other concentrations, control (VCL = 238.8 ± 39.4 µm/s; VAP = 200.9 ± 29.2 µm/s; ALH = 2.6 ± 1.6 µm/s) and 5 µM Oligomycin (VCL = 239.1 ± 43.1 µm/s; VAP = 200.3 ± 36.1 µm/s; ALH = 2.9 ± 1.0 µm/s). For the vervet rapid swimming patterns LIN, STR and WOB, 5 µM Oligomycin (LIN = 78.8 ± 11.0 %; STR = 89.3 ± 8.6 %; WOB = 85.1 ± 8.7%) noted higher mean values as compared to 25 µM (LIN = 70.2 ± 20.9; STR = 83.5 ± 16.8; 78.1 ± 15.4 %) at baseline. As for BCF, the control (16.5 ± 9.2 Hz) presented the higher mean value with the 25 µM Oligomycin (12.2 ± 11.7 Hz) presenting the lowest.

After the 30 minute incubation period 5 µM Oligomycin resulted in the highest mean values for VSL (188.1 ± 70.8 µm/s), VAP (200.9 ± 67.5 µm/s), LIN (74.3 ± 22.0 %), STR (86.0 ± 13.7 %), WOB (79.8 ± 18.6 %) and BCF (9.5 ± 10.1 Hz), but not significantly so.

Medium swimming sperm

The results obtained for the kinematic parameters of medium swimming sperm incubated in different concentrations of Oligomycin (5 µM and 25 µM) at two different time points results are described below. At baseline, and after 30 minutes, no significant differences were observed between the different concentrations Oligomycin. For all the parameters, at baseline, closely similar results were observed for the two Oligomycin concentrations (5 µM and 25 µM). Although not significantly (P = 0.508) so, the 5 µM Oligomycin concentration resulted in a lower VCL (109.9 ± 11.7 µm/s) value compared to the control (116.1 ± 11.0 µm/s) and 25 µM Oligomycin concentration (110.7 ± 11.2 µm/s), while the control noted the

higher mean values for VSL (88.6 ± 22.7 µm/s), VAP (96.6 ± 18.4 µm/s), LIN (77.7 ± 8.1 %), STR (86.7 ± 13.6 %) and WOB (85.1 ± 13.0 %), after 30 minutes.

Slow swimming sperm

Similar to the medium swimming sperm kinematic parameters, at baseline, for majority of the kinematic parameters the control presented with the higher mean values and 25 µM Oligomycin with the lowest for VSL (Control = 50.8 ± 9.3 µm/s; 25 µM = 70.1 ± 2.1 µm/s), VAP (Control = 56.3 ± 6.2 µm/s; 25 µM = 47.6 ± 6.1 µm/s), LIN (Control = 72.8 ± 13.7 %; 25 µM = 60.9 ± 9.7 %), STR (Control = 86.1 ± 10.4 %; 25 µM = 85.1 ± 9.2 %) and WOB (Control = 81.1 ± 9.3 %; 25 µM = 68.0 ± 8.9 %). Different to the entire subpopulation kinematic results, slow swimming sperm results at baseline showed significant differences between the control and 25 µM Oligomycin concentration for VAP (P = 0.024) and WOB (P = 0.014), with significantly higher mean values observed in the control for VAP (56.3 ± 6.2 µm/s) and WOB (81.1 ± 9.3 %) compared to the 25 µM Oligomycin (47.6 ± 6.1µm/s) and (68.0 ± 8.9 %).

After 30 minutes incubation, no significant differences were observed for any of the kinematic parameters. The control noted the higher mean values for the majority of the kinematic parameters excluding ALH (1.1±0.6 µm/s) and BCF (13.4±9.6 Hz) where it presented with the lowest means.

Similar to the motility study kinematic results, the results obtained for Oligomycin kinematic parameters produced high standard deviations. Due to this, the subpopulation (rapid, medium and slow) swimming sperm data was extracted to assist in identifying different results obtained with different concentrations. The different Oligomycin concentration subpopulation kinematic results (rapid, medium and, slow swimming sperm) and time points were compared to the entire population kinematic results.

The results obtained for the different subpopulations (rapid medium and slow) kinematic parameters are presented in Figures 4.14 and 4.15. The rapid swimming sperm kinematic parameters noted high standard deviation similar to the entire sperm population results at baseline. After 30 minutes majority of the rapid swimming sperm kinematic standard deviations noted drastic increases mostly for the 5 µM and 25 µM Oligomycin concentrations. The medium and slow swimming sperm kinematic parameter results noted lower or similar standard deviations as compared to the entire population and rapid swimming sperm kinematic parameter results. When comparing the different subpopulations' VCL results to the entire population results, higher mean values within the rapid swimming sperm were present. Consistent swimming pattern results (LIN, STR, and WOB) was obtained throughout the different time points for the entire and subpopulation results compared to the variation obtained within the velocity parameters (VCL, VSL, and VAP).

Figures 4.14 and 4.15 illustrate the high standard deviation and variation obtained within the different subpopulation kinematic results. The high variation obtained within the entire sperm population is attributed to the variation obtained from the rapid swimming sperm. The high standard deviation in the rapid swimming sperm could be attributed to the varying quality and variation of vervet samples used in this part of the study.

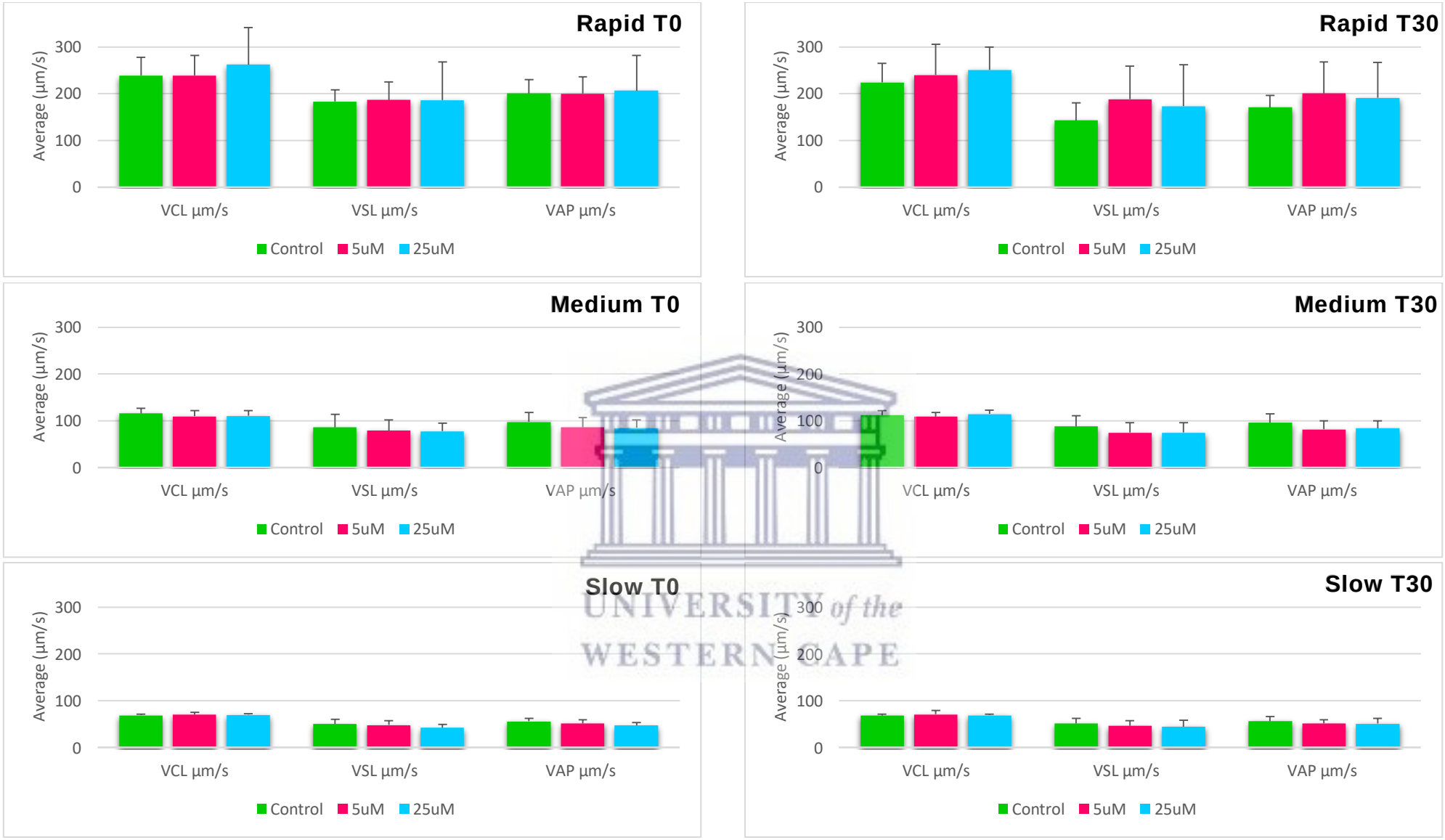


Figure 4.14 Bar graphs of subpopulations (rapid, medium and slow) of vervet sperm velocity parameters compared from 5 minutes (T0/ baseline) to 30 minutes (T30) after incubation with a Control, 5 µM Oligomycin and 25 µM Oligomycin; VCL µm/s = curvilinear velocity, VAP µm/s = average path velocity, VSL µm/s = straight line; SD represented as error bars (Control n=6, Oligomycin, 5µM n=17, Oligomycin, 25µM n=17).

Figure 4.15 Bar graphs of subpopulations (rapid, medium and slow) of vervet sperm swimming patterns compared from 5 minutes (T0/ baseline) to 30 minutes (T30) after incubation with a Control, 5 µM Oligomycin and 25 µM Oligomycin; STR % = straightness, LIN % = linearity, WOB % = wobble; SD represented as error bars (Control n=6, Oligomycin, 5µM n=17, Oligomycin, 25µM n=17).

4.2.3 Sperm structural characteristics

Vitality

Appendix H (Table 4.18) and Figure 4.16 presents the percentage vitality for vervet sperm exposed to different physiological media at baseline, 30 and 60 minute time intervals and Table 4.19 present the effects of time on sperm vitality for each of the media (n=17). The effects of physiological media on vervet sperm vitality showed no significant differences when comparing the values at each time interval (T0 = P = 0.260; T30 = P = 0.405; T60 = P = 0.128). At baseline HTFC (64.0 ± 11.7 %) showed the higher vitality compared to other media (HTF= 64.0 ± 11.7 %; Ham's F-10® = 57.0 ± 13.1 %; HDSWP = 64.2 ± 9.2 %; HDSCP = 63.3 ± 14.6 %). Although insignificant, HDSWP (T30 = P = 0.260, T60 = P = 0.405) showed slightly higher vitality percentage compared to the other media after 30 (61.5 ± 13.0 %), and 60 minutes (53.6 ± 12.0 %). Overall, Ham's F-10® (T0 = 57.0 ± 13.1 %, T30 = 51.0 ± 18.1 %, T60 = 38.4 ± 22.5 %) resulted in lower vitality over time compared to the other media, but not significantly so.

All the media except Ham's F-10® and HDSCP scored above the recommended 58% vitality percentage for normal human sperm as recommended by the WHO manual (2010) at baseline. Vitality results for most media ranged between 50%-66% vitality, except for Ham's F-10® with lower values ranging from 38%-57% vitality.

For all the media sperm vitality tends to decrease from baseline up to 60 minutes, with significantly decreased vitality observed within HTFC (T0 = 65.3 ± 8.4 %, T60 = 50.5 ± 12.5 %), Ham's F-10® (T0= 57.0 ± 13.1 %, T60 = 38.4 ± 22.5 %), and HDSWP (T0 = 64.2 ± 9.37 %, T60 = 53.6 ± 12.0 %) when comparing baseline with 60 minutes incubation time. Additionally, for HTFC, a significant decrease from baseline (65.3 ± 8.4 %) compared to 30 minutes (60.4 ± 8.2 %) was observed.

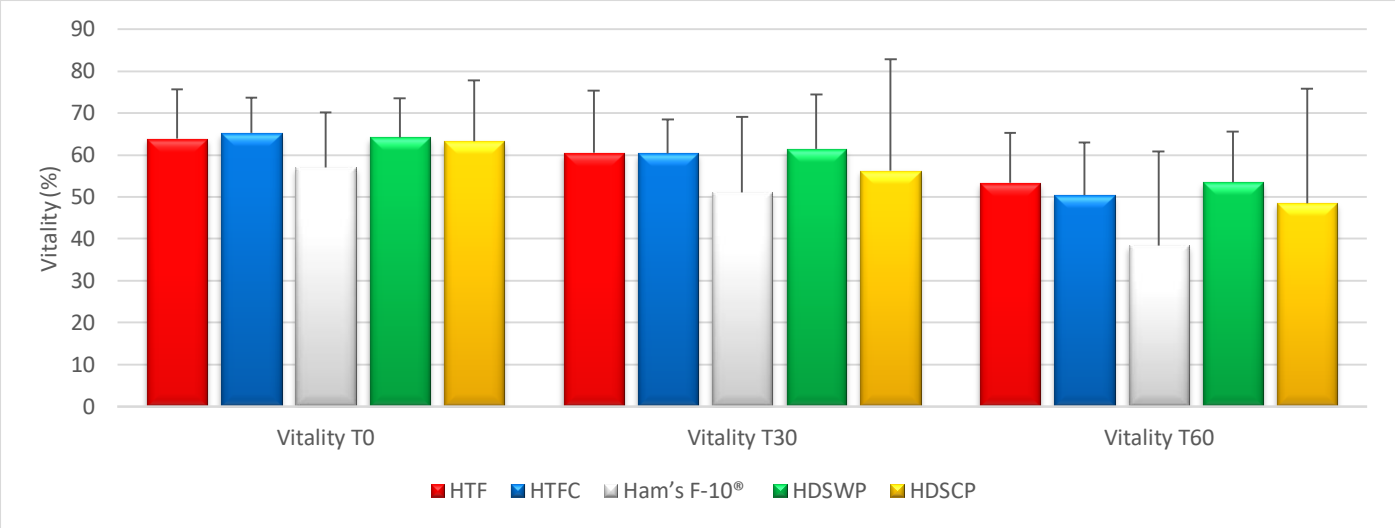


Figure 4.16: Effect of different physiological media on vervet sperm vitality at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD). As obtained from ANOVA; SD represented as error bars. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine, Ham's F-10®, HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus (Baseline n= 17, T30 n=16, T60 n=15).

Acrosome integrity

The acrosome integrity for vervet samples was analysed manually as explained in Chapter 3. Appendix I (Table 4.20) and Figure 4.17 illustrates the effect of different physiological media on vervet sperm acrosome integrity at different time intervals, and Table 4.21 compares the differences in results among these time points (n=11).

No significant differences were noted for acrosome integrity when comparing the different physiological media at three different time points; neither did the media have a significant effect over time. When comparing the different physiological media, HDSWP (T0 = 92.4 ± 3.9 %, T30 = 87.6 ± 6.9 %, T60 = 89.0 ± 3.5 %) noted slightly higher mean percentage intactness at all time points.

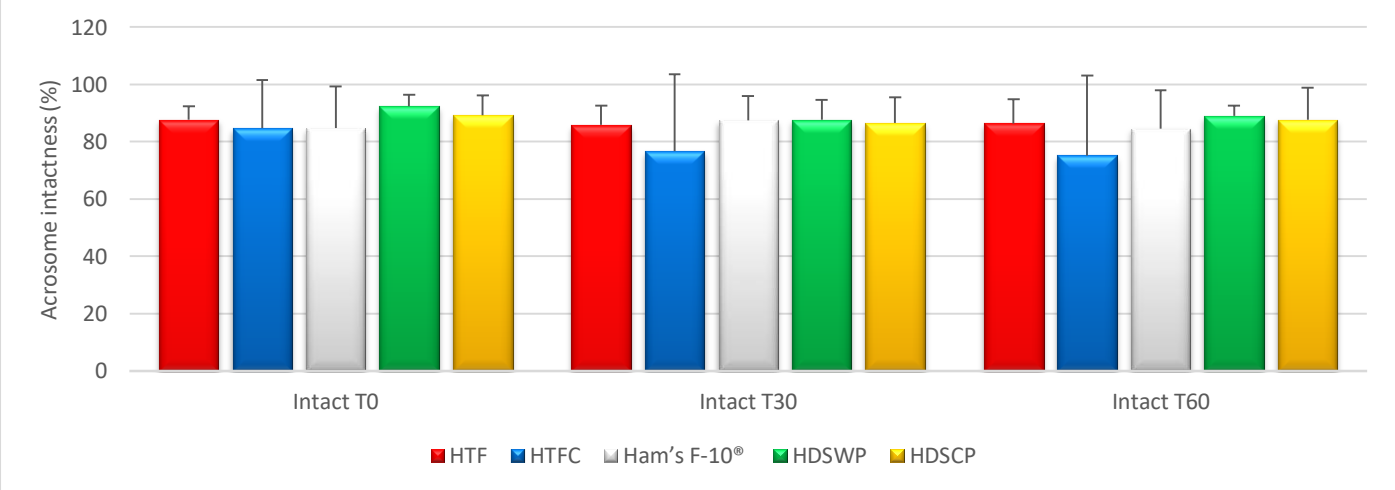


Figure 4.17 Effect of different physiological media on vervet sperm acrosome integrity at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD). As obtained from ANOVA; SD represented as error bars. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine, Ham's F-10®, HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus (HTF n=11, HTFC n=11, Ham's F-10® n=9, HDSWP n= 10, HDSCP n=10)..

4.3 Rhesus monkey

4.3.1 Baseline semen sample characteristics

The baseline semen sample characteristics for rhesus monkeys are presented in Table 4.22 (n=9).

The baseline motility parameters, reflected overall low values for samples, with a mean total motility of 64.3 ± 23.6%, of which the mean progressive motile sperm represented 25.0 ± 27.8%, and non-progressive 39.3 ± 21.8%, respectively. The mean semen volume was 122 ± 78 µl and for sperm concentration, the mean value was 40.5 ± 52.6 mil/ml.

Table 4. 2 Baseline rhesus semen sample characteristics (mean ± SD) n=9

Characteristics	**Range (min - max)**	**Total sample distribution (n= 9)**
Semen volume (µl)	50 - 300	121.8 ± 78.0
Sperm concentration (mil/ml)	0.5 – 52	40.5 ± 52.6
Total motility (PR+ NP, %)	6.7 – 84.2	64.3 ± 23.6
Progressive motility (PR, %)	0 – 26.5	25.0 ± 27.8
Non-progressive (NP, %)	6.7 – 66.7	39.3 ± 21.8
Immotile (%)	15.8 – 93.3	35.7 ± 25.0

4.3.2 Sperm functional characteristics

Motility and kinematic parameters

Motility parameters

Figures 4.18, 4.19 and 4.20 represent the rhesus sperm motility parameter results obtained, exposed to different physiological media at three different time points namely baseline (T0), 30 minutes (T30) and 60 minutes (T60). Appendix J (Table 4.23) presents the effect of the different physiological media and Table 4.24 (Appendix J) presents the effect of time on sperm motility over a 60 minute time period. The cut-off values used for rhesus samples were: Rapid (µm/s) > 240, Slow-Medium (µm/s) ≥ 80 ≤ 240, Static (µm/s) <80.

No significant differences were observed among media for motility parameters at each time interval. Although not significantly so, HTFC maintained the highest total motility percentage throughout each separate time point (T0 = 74.6 ± 22.6 %, T30 = 78.1 ± 17.9 %, T60 = 78.6 ± 22.0 %) compared to the other media. Furthermore, after 30 and 60 minute time intervals, HTFC resulted in the highest progressive motility (T30 = 51.1 ± 17.0 %, T60 = 34.9 ± 6.6 %), as well as the maximum rapid swimming sperm percentages (T30 = 40.3 ± 30.3 %, T60 = 21.6 ± 7.9 %) when compared to the other media, but not significantly so.

Comparing the motility parameters among different time points the majority of media did not indicate any significant differences. The only significant (P = 0.013) change was observed for HDSWP for which progressive motility significantly decreased after 60 minutes when compared to baseline (T0 = 54.9 ± 17.4%, T60 = 11.8 ± 7.3%). Overall, total motility and progressive motility tend to decrease from baseline to 30 minutes incubation in most media. Interestingly, total motility tended to drop from baseline to 30 minutes and then increase from 30 to 60 minutes in HTF, Ham's F-10®, HDSWP, and HDSCP. The percentage of rapid swimming sperm also tends to decrease in most media comparing the different time points. For HTFC, although not significantly so, total motility, progressive motility, and rapid swimming sperm values increased from baseline (TM = 74.6 ± 22.6 %, PM = 45.3 ± 7.4 %, Rapid = 35.8 ± 8.3 %) to 30 minutes (TM = 78.1 ± 17.9 %, PM = 51.1 ± 17.0 %, Rapid = 40.3 ± 30.3 %), but decreased after 60 minutes (TM = 78.6 ± 22.0 %, PM = 34.9 ± 6.6 %, Rapid = 21.6 ± 7.9 %) as observed in all other media.

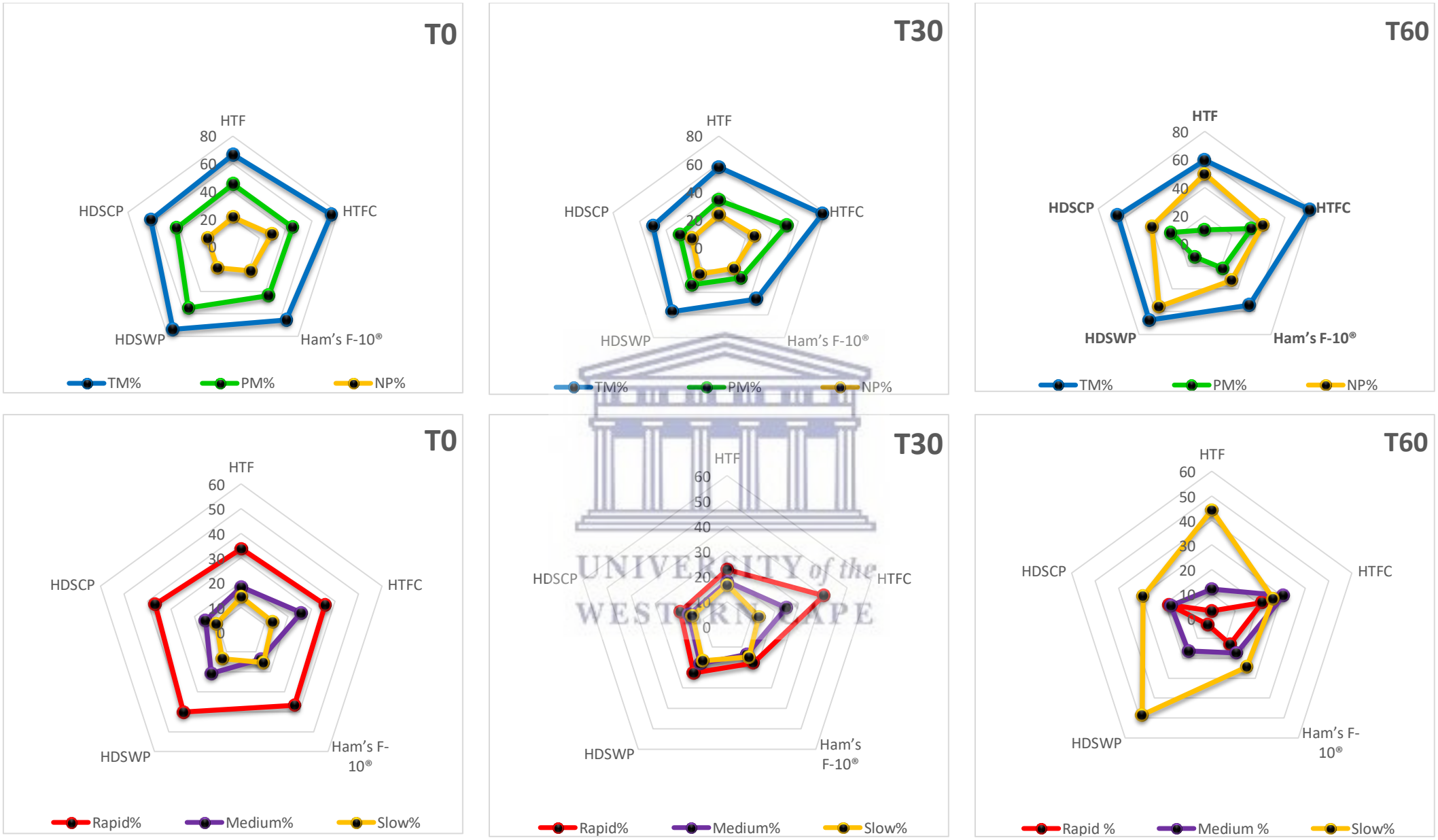


Figure 4.18 Effect of different physiological media on rhesus motility parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60)(data presented in percentage). TM% = total motility, PM% = progressive motility, NP% = non- progressive motility; HTF= human tubal fluid, HTFC= human tubal fluid with added caffeine, Ham's F-10®, HDSWP= HD Sperm Wash Plus, HDSCP= HD Sperm Capacitating Plus (Baseline n=9, T30 n=7, T60 n=2).

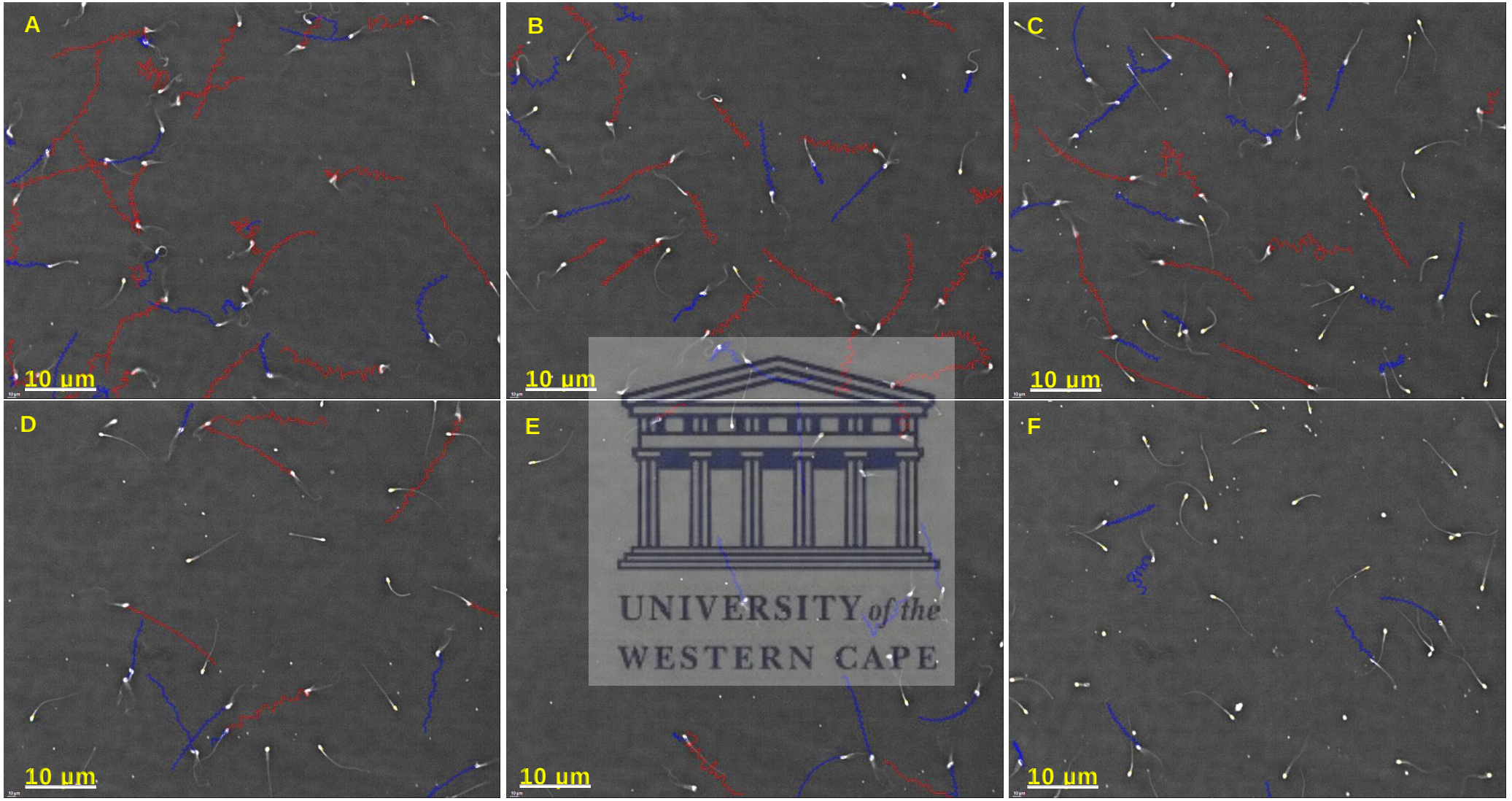


Figure 4.19 Rhesus sperm motility tracks analysed with CASA and SCA® (A) HTFC after 5 minutes, (B) HTFC after 30 minutes, (C) HTFC after 60 minutes, (D) Ham's F-10® after 5 minutes, (E) Ham's F-10® after 30 minutes, and, (F) Ham's F-10® after 60 minutes. Red tracks indicate rapid progressive swimming sperm. Blue tracks indicate medium progressive swimming sperm.

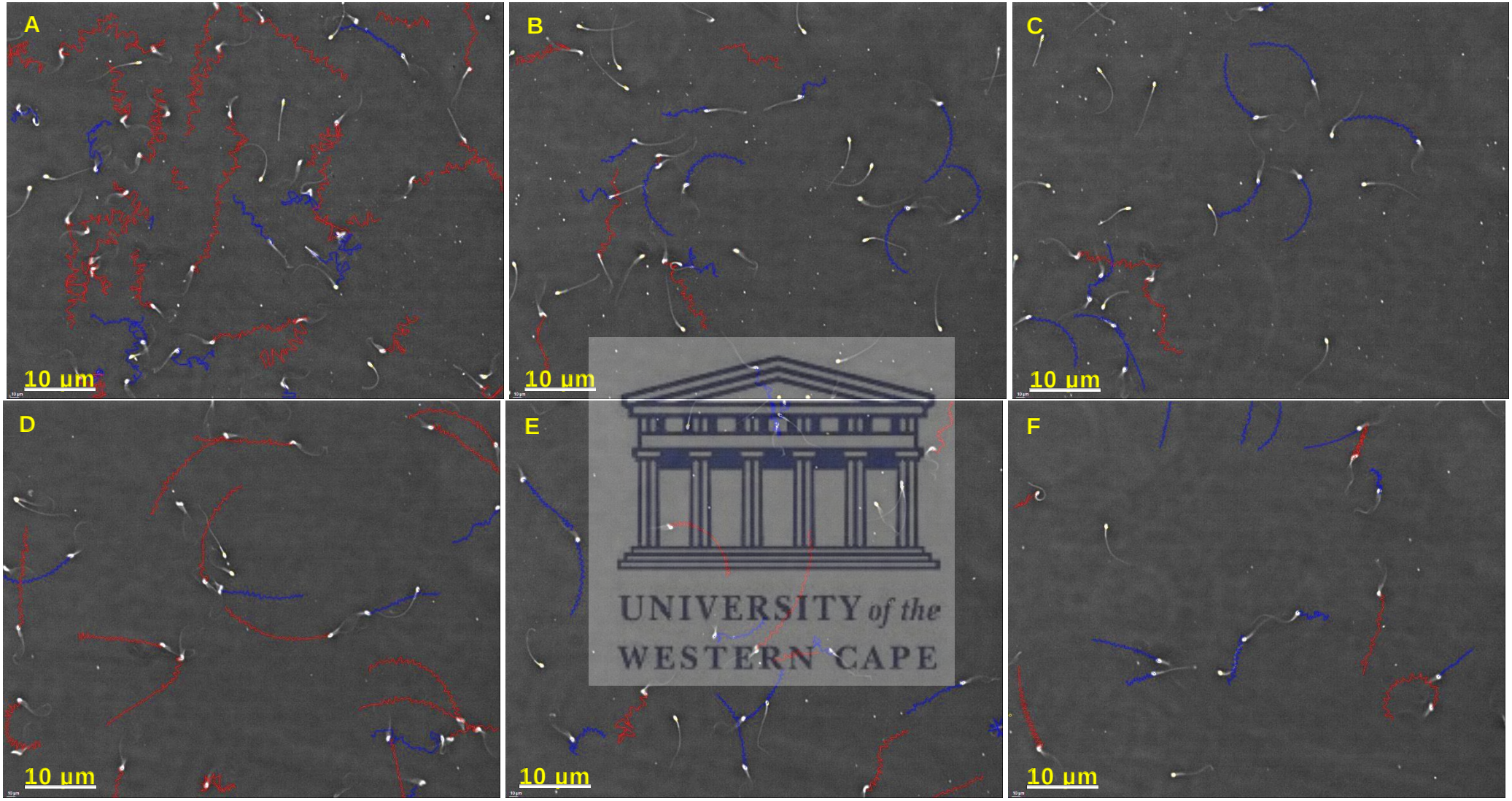


Figure 4.20 Rhesus sperm motility tracks (A) HDSCP after 5 minutes, (B) HDSCP after 30 minutes, (C) HDSCP after 60 minutes, (D) HDSWP after 5 minutes, (E) HDSWP after 30 minutes, and, (F) HDSWP after 60 minutes. Red tracks indicate rapid progressive swimming sperm. Blue tracks indicate medium progressive swimming sperm.

Kinematic parameters (average for entire subpopulation)

The effects of different physiological media on sperm kinematic parameters at different time points are presented in Figures 4.21 and 4.22 (T0, T30 and T60). Complete results are presented in Appendix K (Table 4.25). For most of the kinematic parameters no significant differences were observed among the media at each time point, the only significance was observed at baseline for LIN (P = 0.004), STR (P = 0.017), WOB (P = 0.002), ALH (P = 0.052) and BCF (P = 0.026). LIN was significantly lower in HDSCP (34.5 ± 12.1 %) compared with HTF (52.7 ± 10.3 %) and HDSWP (51.5 ± 7.1 %) at baseline. STR was also significantly lower in HDSCP (82.1 ± 6.9 %) when comparing with HTF (81.2 ± 8.1 %) and HDSWP (82.1 ± 6.9 %) initially. As for HDSCP, HTFC also presented with significantly lower STR values (69.6 ± 9.1 %) compared to HTF (81.2 ± 8.1 %) and HDSWP (82.1 ± 6.9 %). For WOB, baseline values for HDSCP (47.3 ± 11.2%) were significantly lower compared to HTF (62.7 ± 9.2 %), Ham's F-10® (57.9 ± 14.0 %) and HDSWP (60.7 ± 6.7 %), whereas HTFC resulted in significantly lower values for WOB (51.7 ± 3.6 %) compared to HTF and HDSWP.

When observing ALH at baseline, significantly higher values were observed in HTFC (5.7 ± 1.0 µm/s) compared to HTF (3.8 ± 0.8 µm/s), Ham's F-10® (4.0 ± 1.8 µm/s) and HDSWP (4.0 ± 0.7 µm/s). HDSCP (5.0 ± 1.8 µm/s) also noted a significantly higher value compared to HTF. For BCF, HDSWP (27.1 ± 4.1 Hz) was significantly higher to HTFC (21.4 ± 3.2 Hz) and HDSCP (21.7 ± 3.2 Hz) with HTF (25.5 ± 3.8 Hz) also noting a significantly higher mean value compared with HDSCP.

HTFC indicated the highest values for VCL when comparing the different physiological media at each time point, however this was not significant (T0 = P = 0.709; T30 = 0.086; T60 = 0.415). HTFC and HDSCP further revealed higher values for most of the swimming speed parameters and ALH at each of the time intervals, but lower values for LIN, STR, and BCF. HDSCP, overall, revealed the lowest straight swimming percentages throughout all the time points (T0 = 68.4 ± 14.3 %; T30 = 69.0 ± 22.5 %; T60 = 73.1 ± 21.6 %).

A comparison of sperm kinematic parameters between time intervals is presented in Appendix K (Table 4.26). When comparing the effects of media between the different time points, no significant changes were observed. After 60 minutes HTFC, Ham's F-10®, and HDSCP displayed increased effects on VSL, VAP, and LIN compared to baseline, however, this was not significant. In general, HTFC demonstrated insignificant increases from baseline (T0) up to 30 minutes for all the kinematic parameters except for ALH.

Rapid swimming sperm

Appendix K (Table 4.27) presents the results obtained for rapid swimming sperm incubated with different physiological media at different time points. When comparing the effects of the different physiological media on rapid swimming sperm kinematic parameters significant differences were observed for VSL (P = 0.026), LIN (P = 0.003), STR (P = 0.017), WOB (P =

0.013) and ALH (P = 0.002). At baseline, for LIN, STR, and WOB, Ham's F-10® (LIN = 52.9 ± 10.2 %, STR = 83.2 ± 8.6 %, WOB = 61.6 ± 8.7 %) displayed significantly higher mean values compared to HTFC (LIN = 40.5 ± 6.9 %, STR = 70.5 ± 8.0 %, WOB = 54.3 ± 5.6 %) and HDSCP (LIN = 41.0 ± 5.9 %, STR = 75.3 ± 7.4 %, WOB = 52.5 ± 4.4 %). For ALH, a significantly higher mean value was observed in HDSCP (6.8 ± 0.9 µm/s) when compared to HTF (4.7 ± 1.2 µm/s) and HDSWP (4.8 ± 1.1 µm/s) at baseline.

After 30 minutes incubation the only significantly different results obtained were for VSL (P = 0.026), with Ham's F-10® resulting in a significantly higher result compared to all other media. Generally, Ham's F-10® indicated higher results after 30 minutes incubation, for VAP (164.7 ± 37.9 µm/s), LIN (61.0 ± 13.6 %), STR (88.9 ± 10.2 %) and WOB (67.3 ± 11.4 %), however not significantly.

After 60 minutes incubation, no significance was observed between the results for the kinematic parameters of the rapid swimming sperm. Although insignificant, HTF displayed higher values for the velocity parameters (VCL, VSL, and VAP) and WOB, while Ham's F-10® exhibited higher values for the swimming pattern parameters (LIN, STR), and HDSCP resulted in higher values for ALH and BCF compared to other media.

Medium swimming sperm

The kinematic results for medium swimming sperm exposed to different physiological media are presented in Appendix K (Table 4.28). No significant differences were observed between the different physiological media for the sperm kinematic parameters at the different time points. For VSL, LIN and WOB Ham's F-10® (VSL = 85.8 ± 41.4 µm/s, LIN = 59.5 ± 7.1 %, WOB = 67.7 ± 5.9 %) resulted in higher mean values compared to all the physiological media. Though insignificant, HTFC produced the highest VCL value at baseline (T0 = 172.5 ± 69.2 µm/s). Regarding the capacitating media, HDSCP displayed the lowest mean values for VSL (73.6 ± 27.0 µm/s), VAP (91.4 ± 33.6 µm/s), WOB (59.8 ± 11.9 %) and BCF (15.9 ± 6.4 Hz), whereas HTFC was lowest for LIN (48.1 ± 11.9 %) and STR (74.4 ± 11.9 %).

After 30 minutes incubation, Ham's F-10® demonstrated insignificant higher mean values for VSL (90.8 ± 54.6 µm/s), VAP (101.2 ± 58.9 µm/s), LIN (58.3 ± 15.0 %), STR (87.2 ± 9.2 %), WOB (64.7 ± 14.0 %) and BCF (23.0 ± 5.6 Hz) compared to the other physiological media. At baseline (T0), HTFC also resulted in higher values for VCL after 30 and 60 minutes, but lower mean values, as compared to the other physiological media for VSL (71.5 ± 24.9 µm/s), LIN (45.5 ± 12.2 %), STR (73.7 ± 10.8 %) and BCF (17.5 ± 4.7 Hz).

After 60 minutes incubation, Ham's F-10® continued yielding higher values for VSL (118.9 ± 15.6 µm/s), VAP (141.2 ± 13.1 µm/s), WOB (67.0 ± 5.3 %) and BCF (24.2 ± 3.3 Hz) but not significantly so.

Slow swimming sperm

The kinematic results obtained for slow swimming sperm presented no significant differences between the different physiological media at any of the time points (T0, T30, and T60) and are presented in Appendix K (Table 4.29). At baseline, HTFC observed higher mean values for VCL (103.7 ± 46.2 µm/s), VSL (57.2 ± 24.9 µm/s) and VAP (69.7 ± 30.9 µm/s), whereas HDSWP generated insignificant higher mean values for LIN (59.1 ± 13.8 %), STR (78.4 ± 11.5 %), and WOB (69.6 ± 8.7 %). Furthermore, initially, insignificant higher mean values were observed in HDSCP for ALH (2.4 ± 1.8 µm/s) and BCF (15.9 ± 7.8 Hz) respectively whereas the lowest values were detected for VSL (39.0 ± 25.5 µm/s), VAP (48.3 ± 28.6 µm/s), LIN (47.9 ± 22.8 %), STR (72.0 ± 16.3 %) and WOB (58.8 ± 19.9 %) compared to other media.

After 30 minutes incubation, HTFC persevered insignificant higher mean values for VSL (53.7 ± 28.5 µm/s), VAP (62.9 ± 31.4 µm/s), for LIN (56.9 ± 7.1 %), STR (77.8 ± 7.5 %) and WOB (66.8 ± 5.6 %). Similar to baseline (T0), HDSCP persisted in producing the lowest mean values for VSL (39.4 ± 23.6 µm/s), VAP (49.7 ± 28.2 µm/s), LIN (42.6 ± 13.7 %), STR (71.3 ± 10.7 %) and WOB (53.8 ± 13.0 %) compared to other media.

After 60 minutes incubation, similar to baseline HTFC produced higher mean values for VCL (132.8 ± 11.5 µm/s), VSL (75.4 ± 12.1 µm/s) and VAP (88.4 ± 6.6 µm/s) compared to the other physiological media. As for LIN, STR and WOB, Ham's F-10® (LIN = 60.0 ± 11.4 %; STR = 83.3 ± 11.2 %; WOB = 68.9 ± 6.3 %) noted the higher mean values at this time point.

The subpopulation (rapid, medium and slow) swimming sperm data was extracted to assist in showcasing the different results obtained when media and time points were compared using the entire sperm population. At baseline, the results obtained for the entire population, rapid, medium and slow swimming sperm revealed no significant differences. Dissimilarities were observed for medium and slow swimming sperm kinematic results compared to the entire population. Medium and slow swimming sperm results indicated no significance for LIN, STR, WOB, ALH, and BCF as compared to the entire population and rapid swimming sperm kinematic results. After 30 minute incubation, no significance was observed for any of the kinematic parameters for the entire subpopulation results compared to the subpopulations individually except for rapid swimming sperm results noting significant differences in the media for VSL.

High standard deviations were obtained for the different subpopulations compared to the entire population kinematic results in the rhesus samples. Subsequently, observations for the entire population indicate that the velocity parameters (VCL, VSL and VAP) produced high variation compared to the remaining kinematic parameters due to the different effects produced by the physiological media in the rhesus sperm.

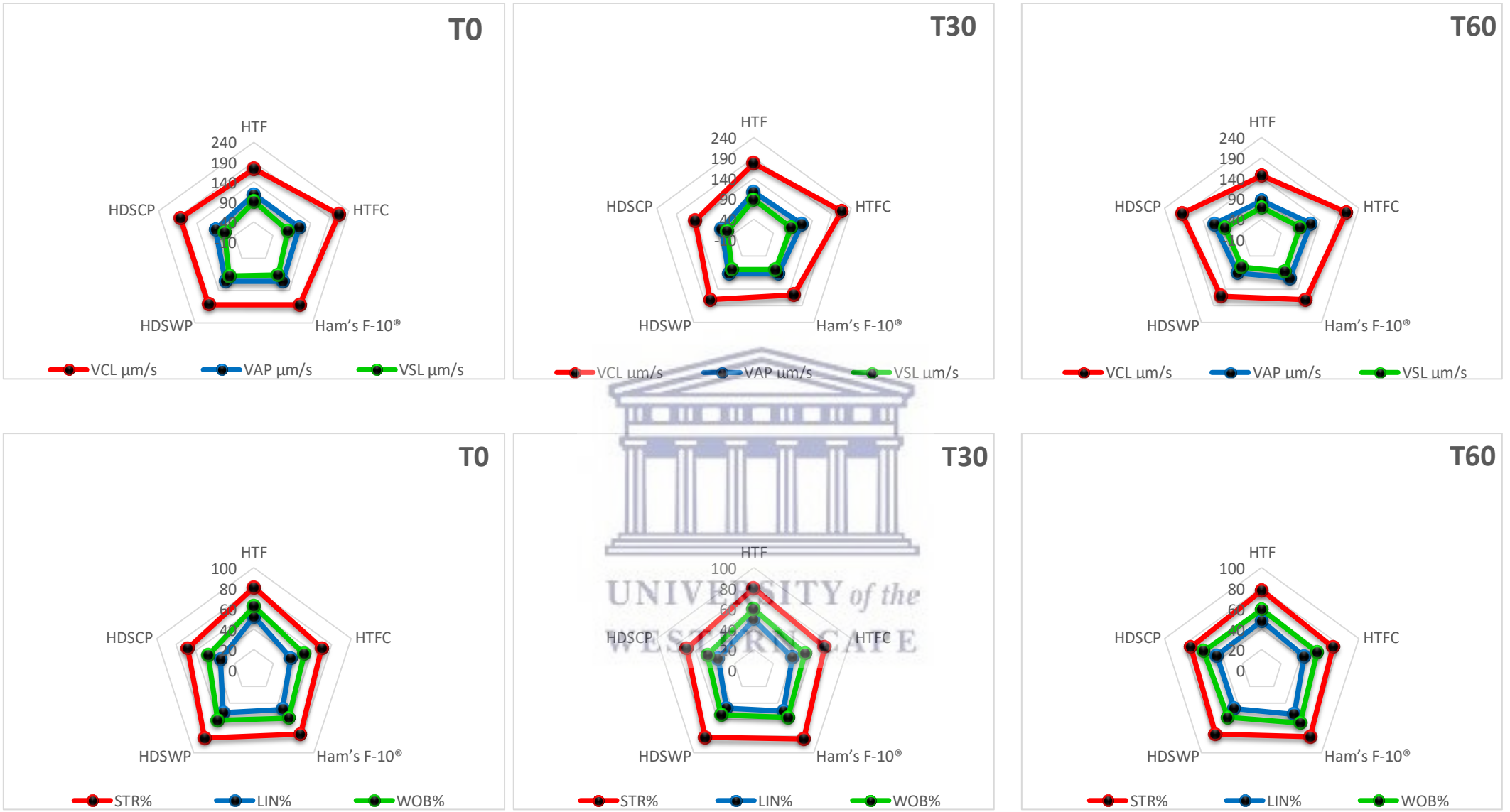


Figure 4.21 Effects of different physiological media on rhesus sperm kinematic parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60). VCL µm/s = curvilinear velocity, VAP µm/s = average path velocity, VSL µm/s = straight line, STR% = straightness, LIN% = linearity, WOB% = wobble; HTF= human tubal fluid, HTFC= human tubal fluid with added caffeine, Ham's F-10®, HDSWP= HD Sperm Wash Plus, HDSCP= HD sperm capacitating plus. (Data represented as µm/s for VCL, VAP and VSL, and % for STR, LIN and WOB (Baseline n=9, T30 n=7, T60 n=2).

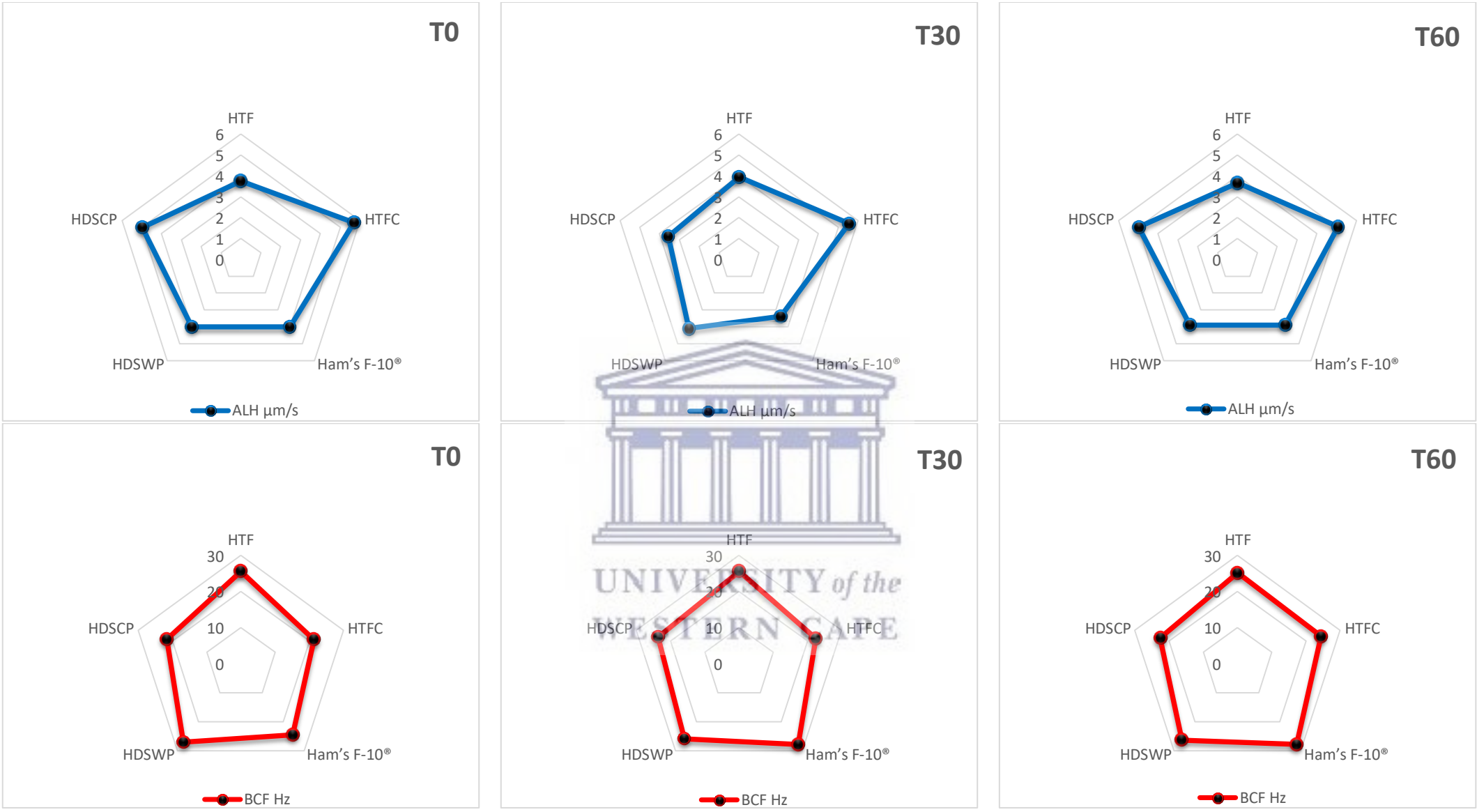


Figure 4.22 Effects of different physiological media on rhesus sperm kinematic parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60). ALH µm/s = average lateral head displacement, BCF Hz = beat cross frequency; HTF = human tubal fluid, HTFC = human tubal fluid with added caffeine, Ham's F-10®, HDSWP = HD Sperm Wash Plus, HDSCP = HD sperm capacitating plus (Baseline n=9, T30 n=7, T60 n=2).

Hyperactivation

The effects of different physiological media on hyperactivation in rhesus sperm at different time intervals are shown in Figure 4.23 and Appendix L (Table 4.30), n=5. As a result of limited and highly agglutinated samples, data for HTF at T45 and T60 could not be obtained as too for HDSCP at T60. No significant differences were observed for any of the physiological media at any time point; neither were there significant differences among time points within the different physiological media.

Although not significant, HTFC resulted in the highest percentage hyperactivation at baseline (54.7 ± 39.4%), 15 minutes (54.0 ± 36.0%) and 30 minutes (43.5 ± 37.8 %) incubation compared to other media. For all the media, hyperactivation tends to decrease after 15 minutes (HTFC, Ham's F-10® HDSWP and HDSCP) or remain in the same range until 30 minutes (HTFC). Following 45 minutes of incubation, hyperactivation results were only obtained for one rhesus sample and are presented in Figure 4.24 below. No data could be obtained for HTF and HDSCP after 60 minutes. Although insignificant, hyperactivation was reduced from 45 minutes up to 60 minutes for HTFC and HDSWP while Ham's F-10® continued to increase within the single sample referred to.

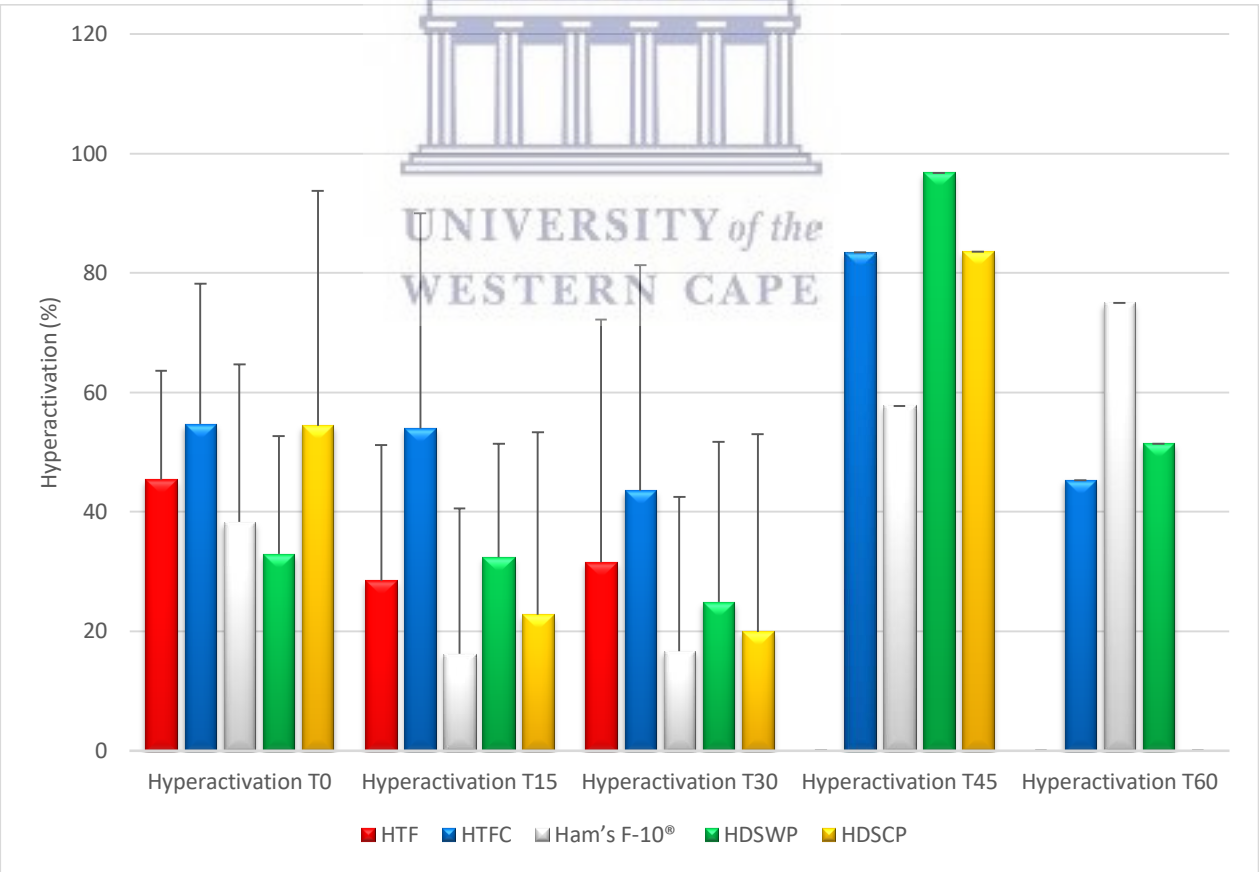


Figure 4. 23 Bar graph of percentage hyperactivation of rhesus sperm over a period of 60 minutes after incubation with HTF/ human tubal fluid, HTFC = human tubal fluid with caffeine, Ham's F-10®, HDSWP = HD sperm was plus, and HDSCP = HD Sperm Capacitation Plus (mean ± SD); SD represented as error bars (T5 n= 5, T15 n=5, T30 n=4, T45 n=1, T60 n=1).

Appendix L (Table 4.31) presents differences in the hyperactivation results between different time points. Limited and highly agglutinated samples resulted in little and/or no data being obtained at certain time points.

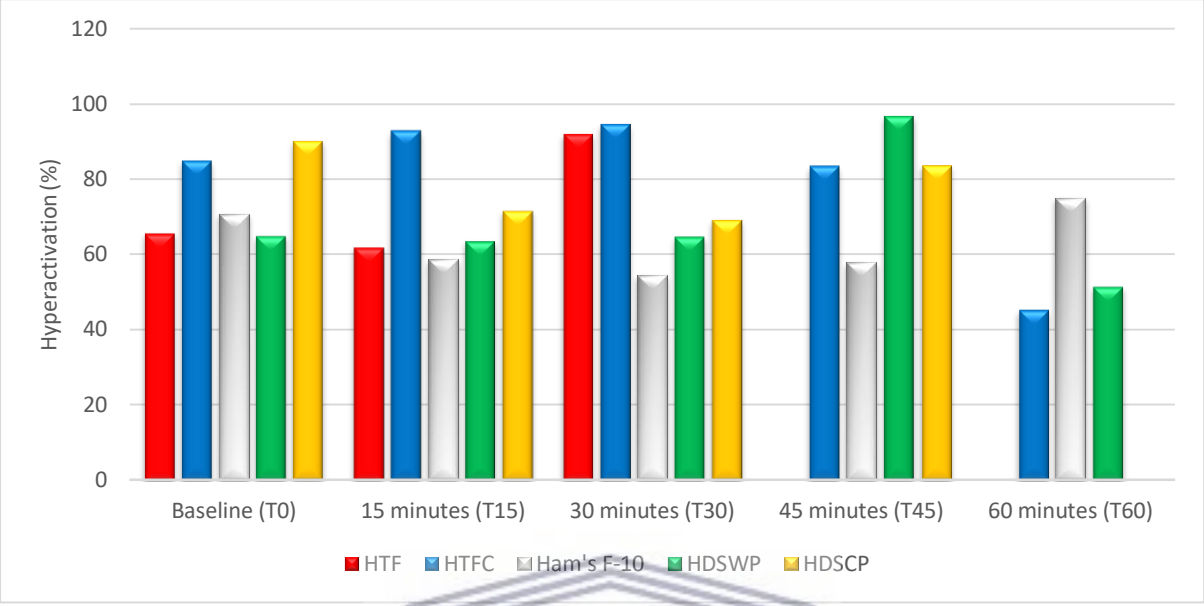


Figure 4. 24 Bar graph of percentage hyperactivation of one individual rhesus sperm sample over a period of 60 minutes after incubation with HTF/ human tubal fluid, HTFC = human tubal fluid with caffeine, Ham's F-10®, HDSWP = HD Sperm Wash Plus, and HDSCP = HD Sperm Capacitation Plus (n=1).

4.3.3 Sperm structural characteristics

Vitality

The results obtained for rhesus sperm vitality exposed to different physiological media, are presented in Appendix M (Table 4.32) and Figure 4.25. Table 4.33 (Appendix M) illustrates the differences in results between the different time points. For vitality, no significant differences between the media at any of the time intervals and when comparing the results between the different time points were observed. At baseline (T0), all the media showed vitality percentages that exceeded the WHO recommendation of 58% vitality, while HTFC (52.3 ± 10.7 %) displayed the lowest percentage vitality compared to the other media, however not significantly. After 30 minutes incubation, vitality percentage for most media increased while vitality for HTFC (49.4 ± 10.0 %) and Ham's F-10® (57.5 ± 2.1 %) decreased. HDSCP resulted in the highest vitality after 30 minutes, compared to the other media, but revealed to be insignificant. No vitality data was obtained for Ham's F-10®, HDSWP, and HDSCP at the 60 minute time point.

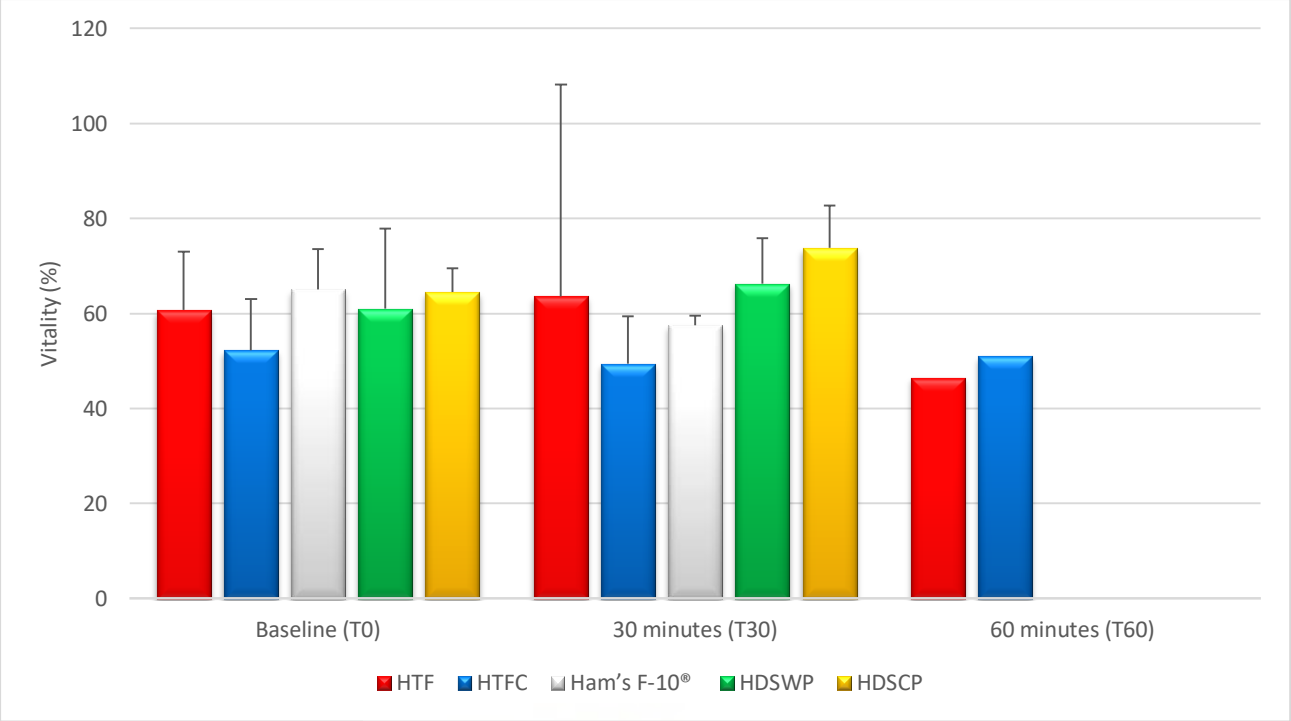


Figure 4.25 Bar graphs of percentage rhesus vitality of different physiological media used at baseline (T0), 30minutes (T30) and 60 minutes (T60) (mean ± SD). As obtained from ANOVA; SD represented as error bars. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine, Ham's F-10®, HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus (Baseline n= 3, T30 n=2, T60 n=1).

Acrosome integrity

Appendix N (Table 4.34) and Figure 4.26 present the effects of different physiological media on acrosome integrity of rhesus sperm at different time intervals. At large no significant differences were observed for acrosome integrity between the different physiological media used. The results indicate that rhesus sperm do undergo the AR when exposed to different physiological media. The findings indicate that at the extended periods at which sperm were exposed to HTF, Ham's F-10®, HDSWP, and HDSCP the more acrosomes reacted when compared to the different time intervals. Acrosome integrity for Ham's F-10® revealed a decrease after 30 minutes (84.5 ± 5.0 %) incubation, but this number increased when exposed for 60 minutes (89.0 ± 8.5 %).

Table 4.35 (Appendix N) presents the comparison of rhesus sperm acrosome integrity between different time points. The acrosome integrity results over time observed high intactness during the different time intervals ranging between 80 - 98% acrosome integrity. Minor increases and decreases were observed within the different media over time with HTF and HDSCP indicating decreases from baseline (T0) up to 60 minutes incubation. HTFC and HDSWP increased from baseline up to 30 minutes with a decrease observed up to the 60 minutes (T60) interval. Concerning Ham's F-10®, an initial decrease was observed from

baseline up to 30 minutes followed an increase recorded after 60 minutes incubation. No significant results were recorded for this part of the study.

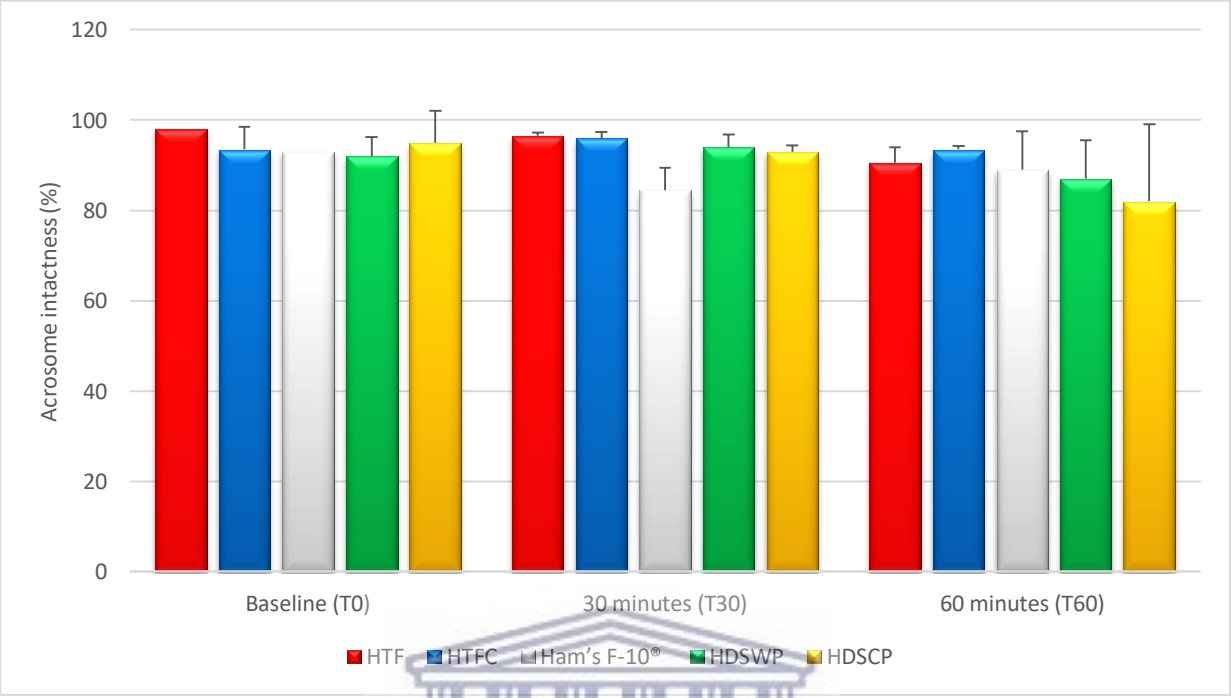


Figure 4.26 Bar graphs of rhesus acrosome integrity of different physiological media used at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD). As obtained from ANOVA; SD represented as error bars. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine, Ham's F-10®, HDSWP = HD Sperm Wash Plus, HDSCP= HD Sperm Capacitation Plus (Baseline n=2, T30 n=2, T60 n=2).

Chapter 5: Discussion

5.1 Introduction

Continual infertility treatment investigations, e.g. IVF, sperm preparation techniques, and especially the use of suitable media for sperm separation from SP are very important (Palmieri *et al.,* 2016). Research results from human studies indicated that the use of physiological media in sperm preparation methods result in higher-quality sperm and sperm function (Kim *et al.,* 2015), and improved ART outcomes have been reported with the use of either sperm selection or dilution methods (WHO, 2010; Kim *et al.,* 2015). The importance of using suitable physiological media in NHP has further been supported by VandeVoort (2004). The findings from this study indicated that rhesus monkey sperm do not capacitate spontaneously *in vitro*, without the addition of capacitating factors in physiological media.

The most commonly physiological media used for human sperm preparation are Ham's F-10®, HTF, AllGrad wash, Percoll, Pure sperm wash, Vitasperm, and physiological media with capacitating substances including caffeine, progesterone, myo-inositol, and procaine to name a few (Vijayakumar *et al.,* 1987; Zollner *et al.,* 2001; Maree, 2011; Khalili *et al.,* 2016; Prag, 2017; Sharma *et al.,* 2019). Although several physiological media are used during sperm preparation, studies investigating the effect of commonly used physiological media on semen parameters are lacking in humans and NHP's.

Therefore, the aim of this study was to evaluate the sperm functionality in NHP's (vervet and rhesus monkeys), focussing on sperm capacitation. In addition, five different physiological media were also compared to determine which media would be more suitable for NHP sperm preparation and capacitation. The study included ordinarily used media, i.e. HTF and Ham's F-10®, as well as capacitation media, HTF with added caffeine (10 mM), and two recently developed physiological media, HD Sperm Wash Plus (HDSWP) and HD Sperm Capacitating Plus (HDSCP). As mentioned in the rhesus monkey experiments highly agglutinated samples as well as limited number of samples hampered the completion or execution of certain functional tests. The agglutination is a characteristic of the rhesus sperm, highlighted by VandeVoort (2004), to have the ability of seemingly high-quality sperm forming clumps appearing as mats of sperm when observed on a microscope causing errors in analysis.

5.2 Sperm functional characteristics in non-human primates

5.2.1 Motility and kinematic parameters

Motility parameters

The evaluation of sperm motility classes (i.e. progressive, rapid, medium, and slow swimming sperm) is of great value to determine fertility potential in a given semen sample (Holt, *et al.,* 1989; Maree and van der Horst, 2013; Prag, 2017). However, these swimming speed subpopulations can be affected by various external factors, including treatments and environmental factors, therefore, the importance of evaluating individual subpopulations aids in highlighting the true effects of these external factors and helps to determine fertility potential of a sample (Maree, 2011; Maree and van der Horst, 2013; Ntanjana, 2014). In our study, we measured sperm motility parameters by means of CASA and applied cut-off values to detect sperm subpopulations in the two NHPs, based on the method described by Maree and van der Horst (2013) using VCL intervals as cut-off points. Values obtained in the current study for vervet monkey percentages total motility, progressive motility, and the three sperm swimming speed subpopulations were very low compared to previous studies evaluating motility parameters in this species. For example, Prag (2017) reported a total motility of 83% and 59% progressive motility for vervet samples using Ham's F-10® in the swim-up method, whilst in the current study Ham's F-10® resulted in 48% total motility, and 22% progressive motility, respectively, at baseline. Furthermore, the percentage rapid swimming sperm measured from our samples was also much lower (0.5%) compared to results obtained by Prag (2017) (56%), and Maree and van der Horst (2013) (75%). In the current study, most of the sperm at baseline were classified as medium and slow swimming sperm. In the case of rhesus samples the motility parameters as well as the swimming speeds, although lower, correlated better with the findings of Prag (2017), and Maree and van der Horst (2013).

Vervet motility parameter results in this study were indicative of too high VCL cut-off values, thus reduced, adjusted VCL intervals were implemented and data were re-analysed using adjusted cut-offs. With the application of lower VCL cut-off points, motility parameters, particularly percentage progressive motility and sperm swimming speeds for media were notably different (data obtained from initial and adjusted cut-offs were not statistically compared to test for significant differences) and more representative of the collected samples. The percentage sperm swimming speed classes after adjustment increased and presented with similar values for medium and slow swimming speeds, with a somewhat lower number of rapid swimming sperm (20.4 – 47.5 % for different physiological media used), compared to Prag (2017), Maree and van der Horst (2013). Contrary to vervet monkey samples, most rhesus monkey sperm were rapid swimming sperm, but the

percentage of rapid swimming sperm was much lower (37%) compared to Prag (66%), and Maree and van der Horst (76%). Furthermore, the percentage of medium swimming sperm in this study was in agreement with findings by Maree and van der Horst (2013) (13.6% versus 13.9%),. whereas slow swimming sperm presented with considerably higher values within our sample population at baseline,15% versus 5% by Maree and van der Horst (2013). Thus, the VCL cut-off values used for rhesus samples did not necessitate any adjustments. These results highlight the importance of evaluation of samples in order to select VCL cut-off values that are more representative of the sample population.

Taken into account that sperm subpopulations are subjective to change upon exposure to several factors (Maree and van der Horst., 2013), the factors that could have possibly contributed to lower motility and swimming class values in this study population include low sperm quality (as indicated with semen analysis), sperm agglutination (particularly in rhesus samples), temperature fluctuations, physiological media being contaminated, extended sample analysis after liquefaction or sperm washing. Also, after sperm activation, sperm agglutination can occur in NHP species (VandeVoort, 2004) with agglutinated sperm appearing in mats of sperm with heads adhering and causing of rapid decrease in sperm motility as observed with CASA in this study for both NHP studies. In this study different media used to prepare samples also have shown to affect sperm swimming speed over time, along with other motility parameters measured, and should therefore also be taken into account when evaluating these parameters.

As discussed in chapter 4, the vervet monkey sperm motility results indicated significant differences among media at certain time points, whereas rhesus monkey sperm lacked sufficient sample numbers to conclude any significant differences among media over time. Rationally, it could be hypothesised that the same media could have different effects on motility parameters when used in other species, though no studies could be found to back this statement. In human sperm, the use of different quality sperm preparation media has been shown to result in different physiological outcomes (Khalili *et al.,* 2016). By understanding the nutrient makeup of sperm preparation media the results can be hypothesized before use. For example, the use of caffeine in physiological media initiates hyperactivation *in vitro* in NHP and human sperm compared to a basic sperm wash medium (VandeVoort, 2004), and functions as an inducer of hyperactivation in physiological media (van der Horst *et al*., 2018).

Sperm function is highly dependent on its ionic environment. One of the functions of physiological media would be to perform as SP by providing similar compounds in order to mimic this ionic environment (Maxwell *et al.,* 2007). Cations present in SP, such as Na^+ and

K^+ exert osmotic balance and are components of many important enzymes (Cevik *et al.,* 2007). Sperm motility is affected by the balance of Na^+ to K^+ in SP (Massa´nyi *et al.,* 2003); the active Na^+ and K^+ exchange in sperm is under control of the Na^+, K^+-ATPase which is present in sperm, aiding in sperm motility (Jimenez *et al.,* 2011).

In accordance with other studies on NHP's, the quality of samples deteriorates over time, as seen in total motility, and progressive motility, along with rapid swimming sperm (Prag, 2017; de Villiers, 2018). In this study, this tendency was noted for all media used over the time period. When comparing the different physiological media, HTF and Ham's F-10® in both vervet and rhesus monkey samples, similar results for motility parameters were observed. In the case of the vervet monkey, however, these two media, if compared to other studies (de Villiers, 2006; Maree, 2011; Kim *et al.,* 2015; Prag, 2017) resulted in much lower values, particularly for total motility, progressive motility and rapid swimming sperm.

One characteristic of Ham's F-10® as a sperm preparation medium is the possibility of ROS production (Quinn, 2000; de Villiers, 2006). The production of ROS components is known to originate from leukocytes and sperm (Henkel *et al.,* 2005). The presence of leukocytes is counteracted by sperm preparation methods including centrifugation. However, in this study it was not performed due to rhesus sperm that are known to be negatively affected during this process. Henkel *et al.* (2005) reported that leukocytes are the main source of ROS in ejaculates resulting in extremely detrimental effects on sperm motility, sperm membrane function, and sperm penetration potential. OWM sperm are capable of superoxide (O_2-) and hydrogen peroxide (H_2O_2) production, aiding in signal transduction pathways controlling sperm capacitation. If however, these oxygen metabolites become elevated, it can result in oxidative stress causing damage to sperm DNA and the plasma membrane (Henkel *et al.,* 2005; de Villiers., 2006) due to the high content of PAFU's present in the plasma membrane. This makes sperm highly susceptible to oxidative stress, which is known to impair sperm function (Henkel *et al.,* 2005).

Compared to the study by de Villiers, (2006) where new Ham's F-10® was made up for every experiment conducted, in this study, Ham's F-10® was used over a longer period of time before using newer/fresher Ham's F-10®, which could have potentiated the results obtained. The newly formulated HDSWP media tested in this study indicated improved motility and swimming speed compared to HTF and Ham's F-10® in vervet monkeys. However, in rhesus monkeys, these three media produced very similar results. Within the vervet samples, HDSWP was noted to be the best medium to sustain motility percentage over the 60 minute time period, noting no significantly lower motility percentages compared to the other physiological media. HDSWP contains myo-inositol which is involved in increasing cytosolic

and inner mitochondrial Ca^{2+} which in turn is important in maintaining sperm motility and increased MMP, supporting motility (Oliva *et al.,* 2016; Palmieri *et al.,* 2016; Condorelli *et al.,* 2017; Korosi *et al.,* 2017). Studies on myo-inositol have identified the positive effects on *in vitro* quality of sperm for reproductive studies especially in patients with OAT reporting increased MMP, an indicator of high cell viability (Condorelli *et al.,* 2017). Thus, because this ingredient seems to improve sperm quality in humans with OAT it is implied that HDSWP has the potential to enhance sperm motility in samples with very low motility percentages as compared to those with good motility percentages, as seen within the vervet samples.

In the vervet monkey, sperm motility declines rapidly when sperm are maintained in semen (Maxwell *et al.,* 2007), thus different culture media and treatments are typically used for sperm recovery in clinical insemination programmes and reproductive research (Nichols and Bavister, 2006; Kim *et al.,* 2015; Khalili *et al.,* 2016; Sharma *et al.,* 2019). Physiological medium functions to extend the motility of sperm and imitate the environment of the female reproductive tract (Khalili *et al.,* 2016, Kim *et al.,* 2015). Ham's F-10® is a widely used medium for the improvement of motility of ejaculated sperm and was developed specifically to support the growth of various cells with specific nutritional requirements (Kim *et al.,* 2015). Compared with other studies using Ham's F-10® in NHP species and humans, reporting improved sperm motility, vitality and acrosome integrity (de Villiers, 2006; Kim *et al.,* 2015; Prag, 2017), Ham's F-10® in this study resulted in contradictive findings with decreased motility, vitality (not significant) and acrosome integrity (not significant), compared to the other physiological media used.

Regarding capacitation, physiological media supplemented with caffeine are known to initiate NHP sperm capacitation and hyperactivation *in vitro* (VandeVoort, 2004; Baumber and Meyers, 2006; Nyacheio *et al.,* 2010) and also increases total motility of the sample. Caffeine-supplemented media have also demonstrated a positive effect on sperm motility by inducing capacitation and increased the intensity of tyrosine phosphorylation in boar sperm (Jin and Yang, 2016). The use of different concentrations of caffeine to initiate sperm hyperactivation is a known practice, with concentrations ranging from 1 mM to 10 mM of caffeine in mammals and NHP (Baumber and Meyer, 2006; Colas *et al.,* 2010; Jin and Yang, 2016). In this study a 10 mM concentration of caffeine was used based on the results reported by Colas *et al.* (2010) on the increases in ram sperm capacitation and tyrosine phosphorylation with a caffeine concentration of 10 mM, and Baumber and Meyer (2006) demonstrating that a concentration of 5 mM caffeine in rhesus samples increased motility.

In agreement with previous studies showing improved sperm motility when using caffeine-supplemented media, our study has shown that caffeine-supplemented media HTFC (HTF

supplemented with 10mM caffeine) resulted in significantly higher values for total motility, progressive motility, as well as rapid swimming sperm, particularly after 30 and 60 minute incubation, compared with the majority of non-capacitating media. Caffeine is a PDE, known to function in preventing the inactivation of intracellular second messengers and the release of internal Ca^{2+} stores (Ntanjana, 2014). The effects of Ca^{2+} were introduced in chapter 2 section 2.4.1, and its effects are presented in our results on both vervet and rhesus sperm samples exposed to physiological media. In boar sperm, the presence of caffeine results in increased intracellular cAMP, stimulating sperm capacitation, spontaneous AR and the increase of sperm motility (Barakat *et al.,* 2015). Similar results were reported by VandeVoort (2004) in rhesus sperm. Caffeine's effect on sperm motility parameters over time, in this study, supports its effectiveness.

Following HTFC, the newly developed capacitating medium, HDSCP, resulted in higher sperm motility percentages in both NHP species. Within the vervet sample results, HTFC displayed significantly higher values for HTF during all the different time intervals, further supporting the positive effect of caffeine in NHP sperm functionality.

In rhesus samples, similar results were observed for HDSCP up to 30 minutes, but sperm motility increased in HDSCP after 60 minutes, which could be attributed to the presence of procaine and progesterone present in HDSCP as too the low number of samples tested after 60 minutes. Research showed that procaine is known to increase the permeability of the sperm plasma membrane to Ca^{2+}, thereby increasing the positive effects produced by increased Ca^{2+} in sperm (Ntanjana, 2014) (described in chapter 2 section 2.4.1). An animal study on stallion sperm has shown that treatment with procaine resulted in significantly decreased sperm motility variables, VSL, STR and LIN, indicative of sperm hyperactivation (McPartlin *et al.,* 2009; Ortgies *et al.,* 2012). Similar to the aforementioned study, both NHP species in this study, resulted in significantly lower values, VSL, STR, and LIN compared to the other physiological media indicative of hyperactivation. As indicated HDSCP contains both procaine and progesterone, which have shown to be beneficial for sperm capacitation in humans guinea pig and cattle (Mújica *et al.,* 1994; Suarez, 2008; McPartlin *et al.,* 2009; Ortgies *et al.,* 2012). Although this study did not investigate the effect of the capacitating media without procaine in NHPs, the results obtained for hyperactivation and kinematics are indicative of the potential beneficial role thereof and will be discussed later in this chapter.

Kinematic parameters (average for entire subpopulation) in NHP

Kinematic parameters in both vervet and rhesus samples were lower for the velocity parameters compared to studies by Maree (2010) and Prag (2017). Similar to the motility parameters, the kinematic parameters improved after applying lower cut-off values. An

increase was observed in the kinematic results when using the adjusted cut-off values for vervet samples and produced values closer to those obtained by the above-mentioned studies. The findings of this study are comparable to Maree (2010) and Prag (2017) whom reported a decrease over time when comparing sperm velocity results. Similar to the vervet motility parameters HTFC resulted in significantly higher values for velocity parameters (VCL, VSL, and VAP), and ALH when compared to the majority of non-capacitating media after 30 and 60 minute time intervals. For HTFC, although values for velocity parameters decreased over time, it was not significantly so, compared to HDSCP, for which these parameters significantly decreased over the time period for the vervet samples. For rhesus samples, similar to Prag (2017), limitations caused the lack of obtaining accurate and complete results. Further, similar to Maree (2010), rhesus samples noted no significant changes over the time periods within sperm velocity parameters over the time periods though no deductions can be made due to low number of samples used. Results from this study indicate that in both NHP samples different physiological media produced different swimming patterns, as per the kinematic results obtained. In vervets, specifically, when focussing on LIN, STR and WOB, it is evident that HDSCP produced less straight and linear swimming sperm, which is suggestive of a star spin movement, an indicator of hyperactivated sperm (van der Horst, 2014). Procaine and progesterone present in HDSCP are known to induce sperm hyperactivation (Calogero, *et al.,* 2000; Colas, 2010; Ntanjana, 2014) and the characteristic star-spin swimming pattern as presented by van der Horst *et al*. (2014) opposed to Calogero *et al*., (2010) presenting no star-spin pattern (Figure 2.7 and 2.11). Furthermore, Calogero *et al.,* (2000) reported that progesterone with concentrations as low as 3 ng/ml increased BCF and VCL while decreasing LIN and STR of motile sperm in humans. HDSCP resulted in significant decreased VCL and LIN values between baseline and 60 minute incubation and could possibly be attributed to the presence of progesterone.

In the vervet samples, the kinematic parameters VCL, VSL, VAP, LIN, STR, WOB, and BCF showed significantly different results when comparing the different physiological media at all-time points (baseline, 30 minutes and 60 minutes). Within the rhesus kinematic parameter results, significant differences were observed for LIN, STR, WOB, ALH, and BCF at baseline. The values obtained for rhesus sample velocity parameters indicated an insignificant variation. The absence of significant differences between media over time in rhesus samples could be due to the large standard deviations observed and a low number of rhesus samples used in the study. As mentioned in chapter 4, large standard deviations were present within both NHP species' results for the majority of the study, thus the results for kinematic parameters were divided into subpopulations of the different swimming speed kinematic parameters to help see a better picture on the effects of the different physiological

media. Maree and van der Horst (2013) stated that the use of sperm subpopulations should rather be evaluated compared to relying on mean values for the entire sperm population only. Subsequently, mean values oversimplify the analysis and decrease the usefulness of data by masking the true effects of drug treatments on sperm. Also, assessment of sperm subpopulations has been linked to fertility potential of ejaculates (Maree and van der Horst, 2013).

The results for vervet rapid swimming sperm kinematics produced higher mean values as compared to the results for the entire vervet subpopulation results. For the medium swimming sperm kinematics lower values were obtained compared to the rapid swimming sperm and so too the slow swimming sperm compared to the medium swimming sperm results. These results show the masking effect of using only the entire populations mean results as compared to individual subpopulations. Also, the subpopulation results show that the high standard deviation obtained for the entire population results were due to the rapid swimming sperm subpopulation noting high variation as compared to the medium and slow swimming sperm showing more uniform averages. Therefore, it can be deducted that different physiological media resulted in higher variation in rapid swimming sperm, within the vervet samples, as compared to the medium and slow swimming sperm even though significant differences were observed in all subpopulations.

Similar to the vervet sample kinematics the findings pertaining to the rhesus sample kinematic parameters observed in the entire population masked high variations of the subpopulation results due to higher standard deviations being observed in the subpopulation results. Similar to the vervet results, the effects of different physiological media displayed higher mean values in the rapid swimming sperm as compared to the medium and slow swimming sperm values obtained, but less significance was observed between the different physiological media used.

In HTF, HTFC and Ham's F-10® media, vervet sperm exhibited linear and straight swimming patterns, compared to rhesus samples with less noticeable variation in LIN and STR. When comparing sperm swimming patterns in the two capacitating media used in our study, HDSCP noted curving/spiraling swimming patterns versus linear, straight swimming patterns observed for HTFC in vervets, especially over time. These swimming patterns observed in HDSCP is indicative of sperm hyperactivation (star-spin), as previously mentioned.

The findings of this study successfully compared the effects of different physiological media at different time points within vervet motility and kinematic parameters.

5.2.2 Hyperactivation

Hyperactivation is an essential function that permits sperm to overcome various obstacles in the female reproductive tract and to reach and further penetrate the oocyte for fertilization. This change of motility pattern is classified by the modification of the sperm flagellar beat, resulting in an increase in the flagellar bend amplitude and production of a vigorous, whiplash swimming pattern termed the star spin pattern (Jones, 2009; Lu *et al.,* 2014; van der Horst, 2014).

As indicated previously, there are no hyperactivation cut-off values for vervets, and therefore established rhesus monkey cut-off values were used in our study in order to determine percentage hyperactivation for both NHP species in this study. These cut-off values were: VCL ≥ 130; LIN ≤ 69%; ALH ≥ 7.5µm (≥ 3.75µm for SCA®). Both NHP species samples were exposed to different physiological media for 5, 15, 30, 45 and 60 minutes as suggested by WHO (2010) for human sperm.

As expected for hyperactivation, the physiological media that noted higher percentages in both NHP species were HDSCP and HTFC, significantly so compared to HTF, Ham's F-10® and HDSWP. In the vervet monkey, HDSCP induced the maximum percentage hyperactivation after 5 minutes as compared to HTFC reaching its maximum effect after 15 minutes. Following their highest percentage hyperactivation, values seemed to decline in both media at further time points. In rhesus sperm samples, HDSCP and HTFC resulted in hyperactivation after 5 minutes, where after HTFC maintained similar percentage hyperactivation up to 30 minutes. On the contrary, percentage hyperactivation rapidly declines in HDSCP following 5 minutes incubation.

The hyperactivation results obtained in HDSCP can be ascribed to the ingredients, progesterone, myo-inositol, and procaine hydrochloride, which are all known initiators of hyperactivation due to their roles in intracellular Ca^{2+} concentration (Calogero, *et al.,* 2000; Colas, 2010; Alasmari *et al.,* 2013).

Calogero *et al.,* (2000), compared the effects of progesterone on sperm function and mechanisms in humans. It was stated that sperm has two-specific sperm binding sites for progesterone that are located on the plasma membrane of the sperm. Once the sperm are exposed to progesterone, a rapid (within 10 min incubation) increase of the intracellular free Ca^{2+} concentration attributes to the initiation of hyperactivation, followed by a sustained rise lasting for several minutes (plateau phase) (Calogero *et al.,* 2000). Myo-inositol is found in high concentrations along the male reproductive system of humans, with high levels present in the seminiferous tubule fluid (Condorelli *et al.,* 2012). Myo-inositol functions as a second messenger causing an increase in Ca^{2+} initiating the increase in sperm motility, capacitation

and the AR (Palmieri *et al.,* 2016). The use of myo-inositol was tested and resulted in significant increases in motile sperm concentration and progressively swimming sperm of humans (Palmieri *et al.,* 2016).

Different studies using a local anaesthetic, procaine, have reported positive effects on sperm hyperactivation (Mujica *et al.,* 1994; McPartlin *et al.,* 2008, 2009; Ortgies *et al.,* 2012; Prag, 2017). An increase in ATP concentration and cAMP levels in procaine treated sperm have been previously reported (Mujica *et al.;* 1994). McPartlin *et al. (*2009) concluded that the addition of procaine induced sperm hyperactivation in horse species. This addition was also shown to induce the star-shaped motility pattern of hyperactivated stallion sperm (Ortgies *et al.,* 2012). This was similar to the results of Calogero *et al.* (2000) who demonstrated that addition of progesterone produced hyperactivation, but without the presence of the star spin pattern. In a recent study by Prag (2017), NHP's sperm was shown to produce better hyperactive swimming sperm after incubation with caffeine as to procaine. The presence of procaine was associated with a negative effect on human sperm motility, as reported by Hong et al. (1981).

The only other medium with values close to HDSCP was HTFC, which noted the highest and most consistent motility results for vervet sperm in this study; this can be attributed to caffeine present in HTFC. The positive effect of caffeine on sperm motility was introduced earlier in this chapter. Several studies support the ability of caffeine, in different concentrations, to induce hyperactivation in animal species. For example a study by Barakat *et al.,* (2015) concluded that caffeine had increased sperm motility and progressive motility as well as initiated hyperactivation in frozen bovine semen samples. This study included the use of different concentrations of caffeine (1 mM, 5 mM, and 10 mM). Colas et al. (2010) noted that caffeine exhibited a significant increase in the proportion of capacitated ram sperm and concluded that it was a potent inducer of hyperactivation. A publication by van der Horst, Maree, and du Plessis, (2018) identified that human semen samples with 20% hyperactivation are related to sperm with higher fertilizing ability. In the present study vervet samples were observed to undergo hyperactivation when incubated with physiological media known to induce hyperactivation (HTFC and HDSCP) while non-capacitating physiological media (HTF, Ham's F-10® and HDSWP) resulted in very low percentage hyperactivation. In rhesus samples, that revealed better motility vs vervet samples, hyperactivation was almost double (>50% vs 22%). Furthermore, the majority of non-capacitating media resulted in hyperactivation >30% after 5 minutes and >28% after 30 minutes in rhesus samples. It should, however, be noted that there were a much smaller number of rhesus samples analysed. For vervets both HDSCP and HTFC seems thus to be suitable physiological media to use to induce hyperactivation over time, while HTFC seems to result in better-

prolonged hyperactivation in rhesus samples. These results proved the stimulatory effect of caffeine, procaine, progesterone, and myo-inositol to induce hyperactivation in vervet and rhesus samples according to VCL, LIN and ALH cut-off values (Maree, 2011).

5.3 Sperm structural characteristics in non-human primates

5.3.1 Vitality

Vitality is measured as a percentage and mainly involves examining the permeability and integrity of the cell membranes (Björndahl *et al.,* 2003; Cooper and Hellenkemper, 2009). The percentage of viable cells must exceed that of motile cells (Cooper and Hellenkemper, 2009; Björndahl *et al.,* 2003; WHO, 2010; van der Horst, 2014). In a clinical andrology laboratory, it is important to verify the fraction of live sperm if the immotile fraction exceeds 40% (WHO, 2010; Maree, 2011; van der Horst, 2014, Prag, 2017). The WHO recommends a lower reference limit for vitality of 58% (WHO, 2010).

The results for vervet samples in majority of the media produced percentages higher or close to the reference limit (58%), with Ham's F-10® noting the lowest percentages over all the time points and when compared to all the media after 60 minutes incubation. In the rhesus samples, the majority of media resulted in vitality values above 58% at baseline and after 30 minutes incubation, while HTFC noted lower than 58% vitality at all the time points.

Vervet sample vitality values in this study, for the most part, exceeded that of motile sperm in some of the physiological media, excluding HTFC at all the time points, and HDSCP at baseline and 30 minutes incubation. The rhesus sample vitality values, at baseline, exhibited higher motility compared to vitality percentages for all the media, except HDSCP, and after 30 minutes all media resulted in higher vitality compared to motility, but not Ham's F-10®. These results could be due to the low number of rhesus samples obtained in this section of the study, thus not accurately depicting the outcomes of incubation with different physiological media.

Overall the differences noted between vitality and motility, for both NHP species, could possibly be attributed to the delay in time (from when motility was analysed up until the BrightVit smears were made), the stain not staining the sperm accurately, and temperature control which could have altered the optimal survival conditions of the NHP sperm samples. Another factor would be the low number of samples used within the rhesus study.

In accordance with other studies (Thomsen, 2013; Maya-Soriano *et al.,* 2015; Prag, 2017) vervet samples displayed a decrease in total motility over time accompanied by a decrease in vitality, but vitality remained higher versus motility for some of the media (Ham's F-10® and HDSWP).

Ham's F-10®, in the vervet, as with total motility, resulted in very low values compared to the other media at all-time points, implying that vervet sperm did not thrive in Ham's F-10® as compared to capacitating media in this study. This view is contradictory to older studies using Ham's F-10® as the culture medium for sperm preparation (Vijayakumar *et al.,* 1987; Oliva *et al.,* 1991; van der Horst *et al.,* 1999). In a study by de Villiers (2008) it was indicated that Ham's F-10® produced high-quality motile live sperm in vervet samples. One possible reason for the poor results obtained using Ham's F-10® in this study could be the production of ROS as alluded to in a study by Gomez and Aitkin (1996) and the fact that the dead sperm present at baseline could potentiate the decreased values obtained further in the time points as dead sperm is known to give rise to free radical production (Davila *et al.,* 2015). When comparing the results of Ham's F-10® on the rhesus vs vervet samples, it seems that the rhesus samples flourish more in this physiological medium compared to the vervet. However, as a result of a small number of rhesus samples used in this study, this finding should be treated with caution.

5.3.2 Acrosome integrity

Sperm with intact acrosomes undergo the AR (after capacitation has taken place), an essential process for penetration of the zona pellucida and fusion with the oolemma (Guraya, 2000; Wolf, 2009; Buffone *et al.,* 2014; Prag, 2017). In this study, we made use of FITC-PNA and Hoechst staining to differentiate between intact acrosomes of two NHP species.

A study by Tollner *et al.* (2003) on rhesus sperm observed that the AR was initiated within 11 seconds of binding to the ZP. In the present study, for rhesus sperm, the percent intact acrosomes changed within 5 minutes of incubation with all the physiological media. At all the time points, these intact percentages stayed consistent, with no significant differences observed when comparing the different media. The decline noted in % integrity could be due to PDEs present in the physiological media used which is known to cause irreversible premature AR but also the enhancement of sperm motility (Glen *et al.,* 2007). In 1992, Tesarik and co-workers suggested that PDEs (specifically caffeine and pentoxifylline) might signal destabilization on the sperm plasma membrane resulting in the amplification of susceptibility of sperm to the AR. Thus, sperm is sensitised for stimulation resulting in activation/ influx of other (Ca^{2+}) components known to induce AR. The data acquired for the rhesus samples, as stated, show high a percentage of intact acrosomes, but the results should be interpreted with caution due to the low number of samples used. The cause for the low number of samples used was due to low-quality sperm produced by the rhesus monkeys. The inability to stain the rhesus sperm may well be attributed to the staining method not executed properly for the rhesus species which is in agreement with Prag,

(2017) also reporting the inability of NHP sperm staining. However, the use of this staining method has been previously proven to work in rhesus and vervet monkey sperm (de Villiers, 2006; Moran *et al.,* 2016). Therefore, premature AR seems to be a more plausible reason for the results obtained.

In the vervet samples the results tend to mimic that of the rhesus, observing high mean values with no significance being observed for acrosome integrity at all the time points. In this study, the percentage intact acrosomes were much higher compared to the reacted acrosomes at all-time points meaning that the physiological media used in this study do not increase the percentage reacted compared to the intact acrosomes within these NHP species incubated over 60 minutes.

Regarding the effect of physiological media, in the vervet samples, Ham's F-10® noted unexpected results for acrosome integrity (also seen in motility and vitality results) as it produced similar results to capacitating medium HTFC containing caffeine (known to induce AR), appearing to have a negative effect on the samples. Evidence on the effects of different physiological media on NHP species acrosome integrity are scarce, making it difficult to compare results. De Villiers, (2006), however, reported higher means with the use of Ham's F-10® on vervet sperm acrosome integrity as compared to this study. Prag (2017) demonstrated more intact acrosomes for both vervet and rhesus samples, but at different time intervals (15 minutes and 45 minutes) using Ham's F-10® compared to this study (15 minutes, 30 minutes and 60 minutes). Due to the lower number of samples used for the rhesus study, further testing is required with a larger sample size to determine the effect of physiological media on acrosome integrity.

5.4 Role of mitochondria in sperm motility

The function of the mitochondrion in all somatic cells is the production and metabolism of chemical energy, and in the sperm this function is no different (Ramalho-Santos *et al.,* 2009; Piomboni *et al.,* 2012). The chemical energy produced and metabolised by the sperm is used to further facilitate continued forward movement (Piomboni *et al.,* 2012). Mitochondrial function in sperm functionality has been investigated in a number of mammalian species, including human (Ramalho-Santos *et al.,* 2009; Piomboni *et al.,* 2012; Amaral *et al.,* 2013) and NHP's (van der Horst *et al.,* 2005; Aitken *et al.,* 2007; Maree, 2011). Understanding the role of mitochondria in sperm functioning is thus of importance as sperm motility represents a major determinant of male fertility (Maree, 2011; Amaral *et al.,* 2013).

Studies focussing on the role of mitochondrial energy production in sperm are focussed on inhibiting specific complexes of the electron transport chain to further help explain the mechanisms by which it functions in energy production. Commonly used inhibitors include:

Rotenone (an inhibitor of complex-I) (Hong, Chiang and Wei, 1983; Plaza Davila *et al.,* 2015), Antimycin-A (an inhibitor of complex-III) (Maree, 2011; Plaza Davila *et al.,* 2015), carbonyl cyanide m-chlorophenylhydrazone (CCP) (lowers mitochondrial membrane potential and protons available for ATP synthase complex-V), and Oligomycin-A (specific inhibitor of the F_0 component of the mitochondrial ATP synthase) (Ramió-Lluch *et al.,* 2014), which was used as inhibitor in this study.

Oligomycin-A is known to specifically inhibit the F_0 component of the mitochondrial ATP synthase and furthermore inhibit sperm to achieve *in vitro* capacitation (Ramió-Lluch *et al.,* 2013). ATP synthase functions to form ATP from ADP and inorganic phosphate. The formation of ATP includes interaction with protons formed by ETC, as explained in Chapter 2. To date, there are no studies focussing on the use of Oligomycin to inhibit the mitochondria of NHP. A study by Hung *et al.* (2008) demonstrated with the use of the OXPHOS inhibitor/ uncoupler, pentachlorophenol (PCP) that energy from OXPHOS was not required for hyperactivated rhesus macaque sperm motility, tyrosine phosphorylation, sperm-zona binding, and AR. Davilla *et al* (2015) noted, in stallion sperm, that Oligomycin-A significantly decreases the inner-MMP when 30 µM and 60 µM concentrations were used compared to a control. Contrasting to the aforementioned study Ramió-Lluch *et al* (2014) noted no significant results with regards to Oligomycin inhibiting ATP synthase observed, when using 2.4µM Oligomycin in boar sperm.

The rationale for the inclusion of Oligomycin-A in this study was due to the lack of studies focussing on NHP sperm and to determine whether NHP sperm metabolism is mainly focussed on glycolysis or OXPHOS. In this study, sperm motility and kinematics was selected as the main parameters to indicate the importance of the metabolic pathway in vervet sperm only. Due to time constraints and the seasonal fertility periods of the rhesus monkeys mitochondrial activity was not investigated in these species. CASA was used for objective analyses of vervet sample parameters.

5.4.1 Motility parameters

As stated previously, two different concentrations of Oligomycin (5µM Oligomycin, and 25µM Oligomycin) were compared to a control (HTF) in this study. Although not significantly so, the control noted a higher percentage of progressive motility compared to the two different concentrations of Oligomycin-A. The control medium could sustain sperm motility at both time points. A study investigating the effects of different concentrations of CCP on ram sperm showed similar results with progressive motility and rapid percent swimming sperm having lower mean values as compared to the control containing no mitochondrial inhibitor (Losano *et al.,* 2017).

The results obtained in this study supports the functioning of Oligomycin-A, by showing the decreased percent progressive motility of vervet samples when incubated with different concentrations of Oligomycin-A and the increased values obtained for slow swimming sperm when compared to the control. The decline observed in motility parameters after 30 minutes incubation were anticipated, due to numerous studies showing the inhibitory effect of Oligomycin on ATPase synthase in the ETC, reducing ATP availability and motility (Ardón *et al.,* 2009; Amaral *et al.,* 2013; Ramió-Lluch *et al.,* 2014; Plaza Davila *et al.,* 2015). The significantly higher rapid and medium swimming sperm values obtained for the control after 30 minutes approves the effect of Oligomycin-A on sperm motility, but also on swimming speed classes, which have not yet been described in the literature for the vervet monkey. The question of whether OXPHOS is the main energy producer in vervet sperm motility is unclear due to the large standard deviations observed within this part of the study.

The results showed that an Oligomycin-A concentration of 5µM inhibited vervet sperm motility more compared the 25 µM Oligomycin concentration noting lower percentages obtained for NP, medium and slow swimming sperm at baseline and after 30 minutes.

Although vervet sperm motility was lowered by both concentrations of Oligomycin, motility was not halted and significant differences were not obtained, indicating that vervet sperm uses an alternative pathway to produce forward moving sperm even when OXPHOS is inhibited. The physiological media used in this part of the study was HTF, which is made up of nutrients that facilitate glycolysis (glucose) and OXPHOS (sodium pyruvate) (Appendix A). Thus it was anticipated that both glycolysis and OXPHOS would be active since initiators of both pathways were present. With the inhibition of OXPHOS by Oligomycin it is apparent that the sperm should make use of alternative means of producing ATP for sperm motility. Due to the fact that glycolysis produces much less net ATP than OXPHOS (Ferramosca and Zara, 2014), our results lean to the previous theory of alternative pathway usage (glycolysis), noting reduced motility (but motility none the less) and kinematic percentages for both Oligomycin concentrations at each time point. Another possible explanation for incomplete inhibition of sperm motility can be a buffering effect of HTF as reported by Nascimento *et al.,* (2008), whom investigated the effects of antimycin-A on sperm motility and observed that HTF had a buffering effect on sperm motility by maintaining motility percentage after incubation with this OXPHOS inhibitor.

The lower values observed in the motility parameters in this study could also indicate that under normal conditions the ATP required for sperm motility is mainly achieved through mitochondrial respiration (Ramió-Lluch *et al.,* 2013; Losano *et al.,* 2017). Furthermore, depending on the environment conditions present, the results are in line with the concept

that the maintenance of ATP production in mammalian sperm is a result of the stability between energy obtained from glycolysis and mitochondrial respiration (Amaral *et al.,* 2013; Lluch *et al.,* 2013; Losano *et al.,* 2017).

5.4.2 Kinematic parameters

Similar to the motility parameters, the kinematic parameters showed a decrease over time, with the control noting the higher values. The 5µM Oligomycin concentration resulted in higher mean values as compared to a 25µM Oligomycin concentration, with significance noted only in BCF. Our results mimic those of a study done on stallion sperm (Plaza Davila *et al.,* 2015) where a complex-I inhibitor (rotenone) noted decreased sperm velocity percentages over time. Even though a different complex was inhibited and different media were used (Breeders Choice INRA-96 extender and Biggers, Whitter and Whittingham medium) in the stallion study, the overall effect was the inhibition of the mitochondria and thus OXPHOS. Yeung and Cooper (2001) demonstrated the effects of quinine (ATPase blocker) on sperm kinematic parameters and noted increased ALH compared to a control (washed with Biggers, Whitter and Whittingham medium), which is in agreement with our results for the 25µM Oligomycin concentration, showing increased ALH mean values compared to the control, at both time points, though not significant. However, different to Yeung and Cooper (2001) that have shown no significant changes in BCF values, in our study BCF at baseline noted a significantly higher value than the control (baseline and 30 minutes). Further, for LIN our results resembled that of Yeung and Cooper (2001) with decreases being noted compared to the control samples. Furthermore in our study STR was higher than the control and in the study by Yeung and Cooper STR values were lower than the control.

5.5 Conclusion

The main aim of this study was to evaluate sperm functionality utilizing the sperm of two NHP species (vervet and rhesus monkey sperm), and further evaluate the effect of physiological media (including commonly used, and newly formulated sperm wash and sperm capacitating media) on NHP sperm functionality. This comparative study evaluated sperm structural and functional characteristics, particularly related to capacitation (including motility, kinematics, hyperactivation, and acrosome integrity), in NHP, and also revealed the possible relationships among different physiological media on NHP sperm functional and structural characteristics. Consequently, this study confirmed the variations in sperm functionality when using five different physiological media, in different NHP species. The evaluation of motility (including three classes of swimming speeds) and sperm kinematic parameters disclosed the sperm swimming characteristics in two different NHP species. The importance of using suitable species-specific cut-offs in CASA for determining motility and

kinematic parameters were further emphasized. The effects of the different physiological media on vervet sperm over time noted significant results contrary to the rhesus sperm results lacking in this regard due to the low number of samples studied. Examining the role of mitochondria in vervet sperm metabolism revealed that these organelles are related to sperm function in terms of sperm motility. Additionally, it was suggested that both glycolysis and OXPHOS are important processes for the preservation of vervet sperm motility.

The development of physiological media for IVF is an evolutionary path. Physiological media designed for a particular cell must come as close as possible to provide the nutritional and physical environment that allows the unique functionality of the specific cell.

Sperm functionality is an important characteristic of sperm to fertilize the female oocyte. The evaluation of sperm functionality by measurement of all motility parameters, including subpopulations of swimming speeds, is an essential criterion of most semen or sperm populations. Values obtained for motility and kinematic parameters, vitality, hyperactivation, and acrosomal integrity tend to decline after 60 minutes incubation for most physiological media used within vervet samples, suggesting immediate use of prepared sperm for ART to avoid deteriorating sperm function, however, not in all physiological media. The study further confirmed that different physiological media used have significantly different effects, not only on sperm functionality but also on sperm hyperactivation in NHP's when compared to each other and over time. The study indicates that the composition of media, varying from simple to more complex, used for semen preparation plays an important role in determining sperm functionality. Based on these findings further investigation of larger sample groups are required to evaluate the role of certain ingredients in the development of more cost-effective media producing satisfactory results in terms of sperm functionality for ART.

Various factors influenced the results of the experiments in this study, as indicated where applicable (notably the sections pertaining to the rhesus sperm experiments), and should be taken into account for any prospective studies on NHP sperm.

As male infertility is increasing, the evaluation of different physiological media aiming to improve on techniques currently used within the field of reproduction would be of great value. The results obtained within this thesis should add to the lack of knowledge currently present within the field and evaluations of sperm functionality for the use in ART.

5.6 Recommendations

The results obtained in this study focused on the effects of different physiological media in NHP functionality focussing on sperm capacitation and are presented in Figure 5.1 (vervet) and Figure 5.2 (rhesus). The results point to the use of HTFC for increased motility for vervet and rhesus sperm owing to the significantly higher values obtained compared to the other

physiological media. With regard to the physiological process of hyperactivation, the results point to the use of the capacitating media (HTFC and HDSCP) due to the significantly higher percentages hyperactivation produced as compared to the non-capacitating media (HTF, Ham's F-10®) in both NHP species. Incubation with HTFC results in hyperactivation within 5 minutes and remains consistent through the 60 minute period. Whereas, HDSCP produces approximately a two-fold effect on hyperactivation initially (within 5 minutes) but displays a decline up to 60 minutes incubation. Thus for HDSCP, it is recommended to assess hyperactivation within 5 minutes as compared to HTFC resulting in consistent levels of hyperactivation up to 60 minutes.

With regard to vitality and acrosome integrity in both species, all the physiological media produced similar results with no significance observed between the media. The results obtained for mitochondrial inhibition pointed to possible interaction between OXPHOS and glycolysis owing to the partial inhibition of vervet sperm motility.

The outcome of this study forms the basis of what could become a much larger comparative study. For example, the experimental protocols might be utilized in prospective studies to evaluate similar sperm functional and structural characteristics in other mammalian species and humans. Additionally, comparing the different physiological media could be compared for future use in reproductive technology. The additional data can be further investigated and compared to the findings reported in the current study. Additionally, it will be possible to test the soundness of the proposed influence of different physiological media on NHP sperm functional and structural characteristics as described in this study.

Studies pertaining to NHP sperm metabolism are required to confirm the present study's results specifically regarding rhesus sperm. An inhibitor for the glycolytic pathway could be introduced to compare the results of this study. This could assist in resolving the contention in the literature on which pathway, glycolysis or OXPHOS is the most important route to maintain sperm motility. Further, this study should be conducted in human sperm to shed light on any similarities between physiological medias effects on human sperm functionality as seen in the present study on NHP species.

The effects of different physiological media on sperm morphology were not tested and could be implemented in future studies, due to the fact that these media are used in sperm preparation techniques for use in ART. The results highlighted the variation of the media to cause different swimming patterns which could be studied more in-depth to aid in the specific use of certain physiological media for specific ART.

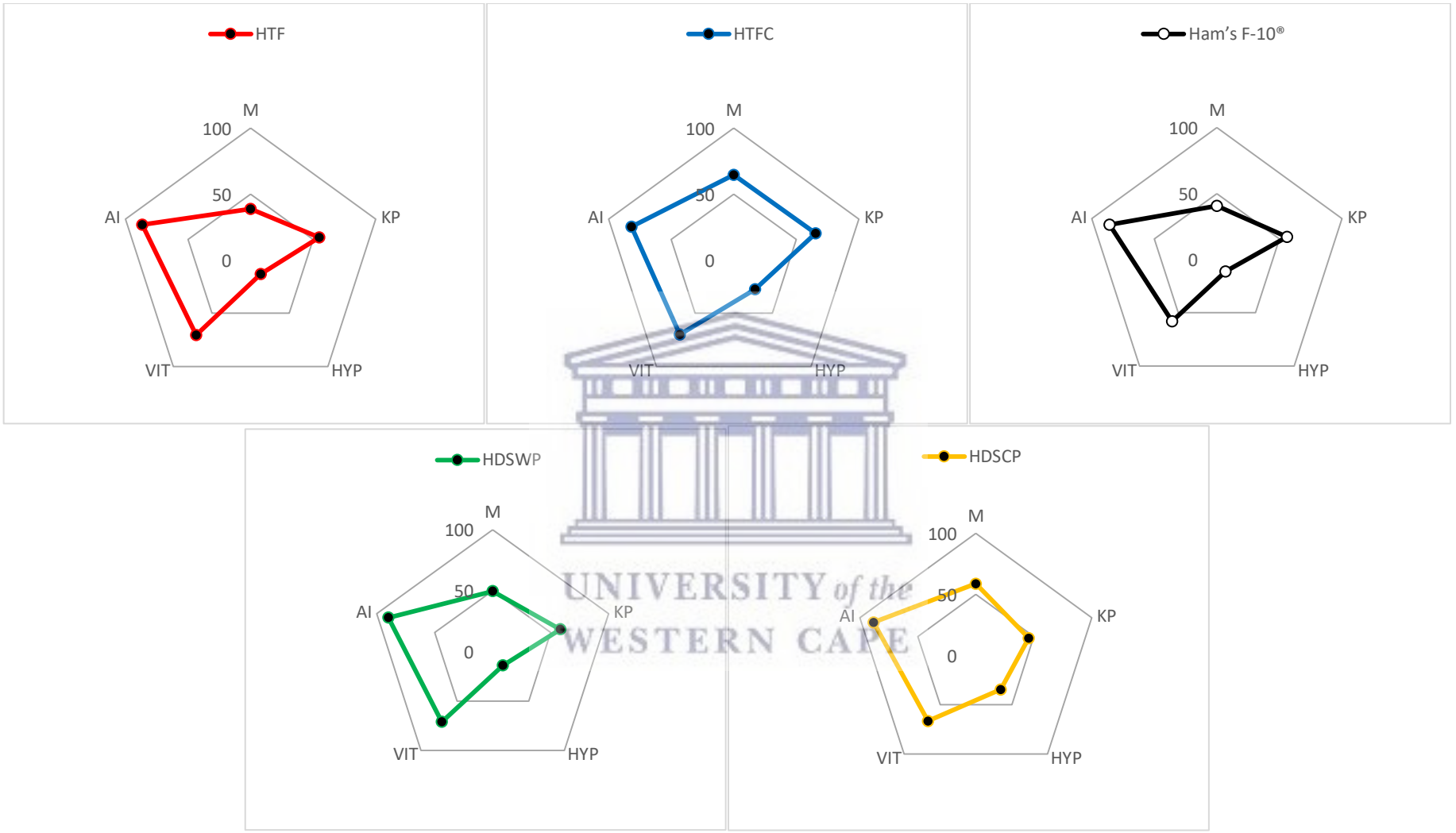


Figure 5. 1 Overall effects of different physiological media on vervet sperm in the present study. TM = total motility, KP = kinematic parameters, HYP = hyperactivation, VIT= vitality, AI = acrosome integrity; HTF= human tubal fluid, HTFC = human tubal fluid with added caffeine, Ham's F-10®, HDSWP = HD sperm wash plus, HDSCP = HD sperm capacitating plus. (Data represented as %).

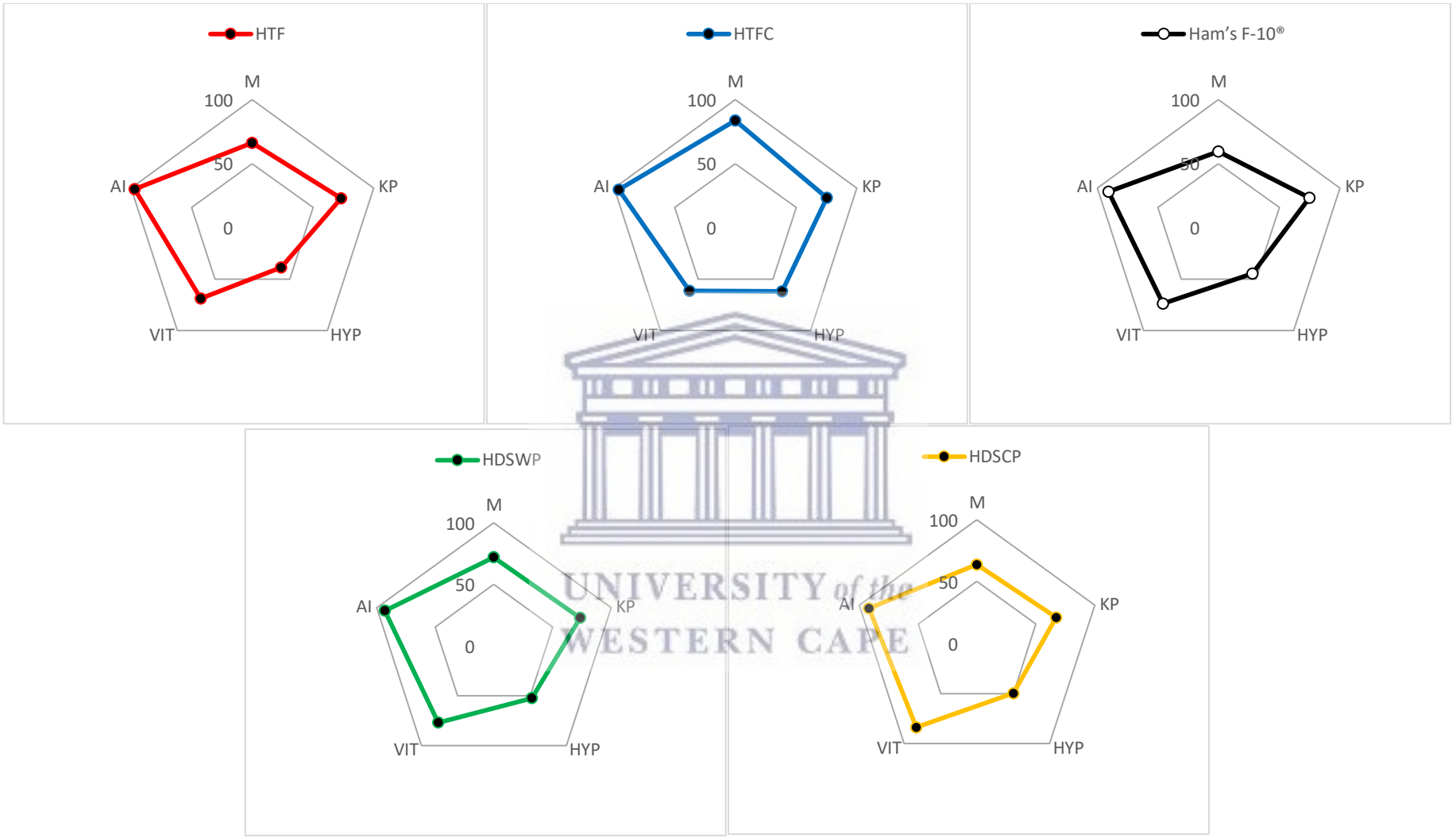


Figure 5.2 Overall effects of different physiological media on rhesus sperm in the present study. TM = total motility, KP = kinematic parameters, HYP = hyperactivation, VIT = vitality, AI = acrosome integrity; HTF = human tubal fluid, HTFC = human tubal fluid with added caffeine, Ham's F-10®, HDSWP = HD sperm wash plus, HDSCP = HD sperm capacitating plus. (Data represented as %).

References

Abou-haila, A., & Tulsiani, D. (2009). Signal transduction pathways that regulate sperm capacitation and the acrsome reaction. *Archives of Biochemistry and Biophysics, 485*(1), 72-81.

Abou-Haila, A., Bendahmane, M., & Tulsiani, D. (2014). Significance of egg's zona pellucida glycoproteins in sperm-egg interaction and fertilization. *Minerva Ginecologica, 66*(4), 409-419.

Abu, D., Franken, D., Hoffman, B., & Henkel, R. (2012). Sequential analysis of sperm functional aspects involved in fertilization: a pilot study. *Andrologia, 44*, 175-181.

Ahmad, K., Bracho, G., Wolf, D., & Tash, J. (1995). Regulation of human sperm motility and hyperactivation components by calcium, calmodulin, and protein phosphotases. *Archives of Andrology, 35*(3), 187-208.

Aitkin, R., Nixon, B., Lin, M., Koppers, A., Lee, Y., & Baker, M. (2007). Proteomic changes in mammalian spermatozoa during epididymis maturation. *Asian Journal of Andrology, 9*(4), 554-564.

Alasmari, W., Barratt, C., Publicover, S., Whalley, K., Forster, E., Kay, V., . . . Oxenham, S. (2013). The clinical significance of calcium-signalling pathways mediating human sperm hyperactivation. *Human Reproduction, 28*(4), 866-876.

Alvarez, J., Sharma, R., Ollero, M., Saleh, R., Lopez, M., Thomas, A., . . . Agarwal, A. (2002). Increased DNA damage in sperm from leukocytospermic semen samples as determined by the sperm chromatin structure assay. *Fertility and Sterility, 78*(2), 319-329.

Amann, R., & Waberski, D. (2014). Computer-assisted sperm analysis (CASA): Capabilities and potential development. *Theriogenology, 81*(1), 5-17.

Amaral, A., Lourenço, B., Marques, M., & Ramalho-Santos, J. (2013). Mitochondria functionality and sperm quality. *Reproduction, 146*(5), 163-174.

Ardón, F., Rodríguez-Miranda, E., Beltrán, C., Hernández-Cruz, A., & Darszon, A. (2009). Mitochondrial inhibitors activate influx of external Ca^{2+} in sea urchin sperm. *Biochimica et Biophysica Acta (BBA), 1787*(1), 15-24.

Ashrafi, I., Kohram, H., & Tayefi-Nasrabadi, H. (2013). Antioxidant effects of bovine serum albumin on kinetics, microscopic and oxidative characters of cryopreserved bull spermatozoa. *Spanish Journal of Agricultural Research, 11*(3), 695.

Austin, C. (1952). The 'capacitation' of the mammalian sperm. *Nature, 170*(4321), 326-326.

Barakat, I., Danfour, M., Galewan, F., & Dkhil, M. (2015). Effect of various concentrations of caffeine, pentoxifyline, and kallikrein on hyperactivation of frozen bovine semen. *BioMed Research International, 2015*, 1-7.

Barros, C., & Berrios, M. (1976). Is the activated spermatozoon really capacitated? *Journal of Experimental Zoology, 201*(1), 65-71.

Baskaran, S., Agarwal, A., Leisegang, K., Pushparaj, P., Panner Selvam, M., & Henkel, R. (2019). An in-depth bibliometric analysis and current perspective on male infertility research. *The World Journal of Men's Health, 37*.

Baumber, J., & Meyer, S. (2006). Hyperactivated motility in rhesus macaque (Macaca mulatta) spermatozoa. *Journal of Andrology, 27*(3), 459-468.

Bavister, B., Boatman, D., Collins, K., Dierschke, D., & Eisele, S. (1984). Birth of rhesus monkey infant after in vitro fertilization and nonsurgical embryo transfer. *Proceedings of the National Academy of Sciences, 81*(7), 2218-2222.

Bedford, J. (2011). Site of the mammalian sperm physiological acrosome reaction. *Proceedings of the National Academy of Sciences, 108*(12), 4703-4704.

Berg, J.M., Tymoczko, J.L., Stryer, L. Biochemistry. 5th edition. New York: W H Freeman; 2002. Available from: https://www.ncbi.nlm.nih.gov/books/NBK21154/ Date of access: 6 May 2019.

Bielert, C., & Vandenbergh, J. (1981). Seasonal influences on births and male sex skin coloration in rhesus monkeys (Macaca mulatta) in the southern hemisphere. *Journal of Reproduction and Fertility, 62*(1), 229-233.

Bjorndahl, L. (2003). Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. *Human Reproduction, 18*(4), 813-816.

Boatman, D., & Robbins, R. (1991). Bicarbonate: carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions1. *Biology of Reproduction, 44*(5), 806-813.

Borland, R., Biggers, J., Lechene, C., & Taymor, M. (1980). Elemental composition of fluid in the human fallopian tube. *Journal of Reproduction and Fertility, 58*(2), 479-482.

Boshoff, N., Lambrechts, H., Maree, L., Cloete, S., & Van der Horst, G. (2018). A novel flush technique to simulate natural dispersal of spermatozoa in the female reproductive tract and expedite motility assessment of fresh ejaculated Merino (*Ovis aries*) sperm. *South African Journal of Animal Sciences, 48*(3), 469-476.

Buffone, M., Hirohashi, N., & Gerton, G. (2014). Unresolved questions concerning mammalian sperm acrosomal exocytosis1. *Biology of Reproduction, 90*(5), 112.

Cai, X., Wang, X., Patel, S., & Clapham, D. (2015). Insights into the early evolution of animal calcium signaling machinery: A unicellular point of view. *Cell Calcium, 57*(3), 166-173.

Calogero, A., Burrello, N., Barone, N., Palermo, I., Grasso, U., & D'Agata, R. (2000). Effects of progesterone on sperm function: mechanisms of action. *Human Reproduction, 15*(Suppl 1), 28-45.

Carlomagno, G., Nordio, M., Chiu, T., & Unfer, V. (2011). Contribution of myo-inositol and melatonin to human reproduction. *European Journal of Obstetrics & Gynecology and Reproductive Biology, 159*(2), 267-272.

Cevik, M., Tuncer, P., Tasdemir, U., & Ozgurtas, T. (2007). Comparison of spermatological characteristics and biochemical seminal plasma parameters of normozoospermic and oligoasthenozoospermic bulls of two breeds. *Turkish Journal of Veterinary and Animal Sciences, 31*(6), 381-387.

Colás, C., Cebrián-Pérez, J., & Muiño-Blanco, T. (2010). Caffeine induces ram sperm hyperactivation independent of cAMP-dependent protein kinase. *International Journal of Andrology, 33*(1), e187-e197.

Condorelli, R., La Vignera, S., Bellanca, S., Vicari, E., & Calogero, A. (2012). Myoinositol: does it improve sperm mitochondrial function and sperm motility? *Urology, 79*(6), 1290-1295.

Condorelli, R., La Vignera, S., Mongioi, L., Vitale, S., Langana, A., Cimino, L., & Calogero, A. (2017). Myo-inositol as a male fertility molecule: Speed them up! *European Review for Medical and Pharmacological Sciences, 21*(2), 30-35.

Cooper, T., & Hellenkemper, B. (2009). Method-related estimates of sperm vitality. *Journal of Andrology, 30*(3), 214-218.

Correa, T., Jakob, S., & Takala, J. (2015). Mitochondrial function in sepsis. *Critical Care Horizons, 1*, 31-41.

Cseh, S., Chan, P., Corselli, J., & LL., B. (2000). Electroejaculated baboon (*Papio anubis*) sperm requires a higher dosage of pentoxifylline to enhance motility. *Journal of Assisted Reproduction & Genetics, 17*, 449-453.

Dancet, E., Spiessens, C., Vangenechten, R., Billiet, J. T., Welkenhuysen, M., & D'Hooghe, T. (2011). Acceptability of preclinical research on nonhuman primates in reproductive medicine. *Reproductive Sciences, 18*(1), 70-78.

de Villiers, C. (2006). The effect of gonadotropin-releasing hormones (GnRH) I & II on sperm motility and acrosome status of the vervet monkey (*Chlorocebus aethiops*) in vitro. *University of the Western Cape, Medical Bioscience Department*, 1-147 (Thesis-PhD).

de Villiers, C. (2018). A comparison between the semen and sperm parameters from the captive-bred vervet monkey (*Chlorocebus aethiops*) and rhesus monkey (*Macaca mulatta*). *Journal of Medical Primatology, 47*(4), 211-216.

Du Plessis, S., Agarwal, A., Halabi, J., & Tvrda, E. (2015). Contemporary evidence on the physiological role of reactive oxygen species in human sperm function. *Journal of Assisted Reproduction and Genetics, 32*(4), 509-520.

Dunson, D., Weinberg, C., Perreault, S., & Chapin, R. (1999). Summarizing the motion of self-propelled cells: applications to sperm motility. *Biometrics, 55*(2), 537-543.

Eddy, E., Toshimori, K., & O'Brien, D. (2003). Fibrous sheath of mammalian spermatozoa. *Microscopy Research and Technique, 61*(1), 103-115.

Fawcett, D. (1962). Sperm tail structure in relation to the mechanism of movement. *Spermatozoon Motility*,72,147-169.

Fawcett, D. (1975). The mammalian spermatozoon. *Developmental Biology, 44*(2), 394-436.

Ferramosca, A., & Zara, V. (2014). Bioenergetics of mammalian sperm capacitation. *BioMed Research International*, 1-8. Available at: https://www.hindawi.com/journals/bmri/2014/902953/cta/. Date of access: 6 May 2019.

França, L., Hess, R., Dufour, J., Hofmann, M., & Griswold, M. (2016). The Sertoli cell: one hundred fifty years of beauty and plasticity. *Andrology, 4*(2), 189-212.

Freitas, M., Vijayaghavan, S., & Fardilha, M. (2017). Signaling mechanisms in mammalian sperm motility. *Biology of Reproduction, 96*(1), 2-12.

Gadella, B. (2008). Sperm membrane physiology and relevance for fertilization. *Animal Reproduction Science, 107*(3-4), 229-236.

Gadella, B., & van Gestel, R. (2004). Bicarbonate and its role in mammalian sperm function. *Animal Reproduction Science, 82-83*, 307-319.

Gervasi, M., & Visconti, P. (2016). Chang's meaning of capacitation: A molecular perspective. *Molecular Reproduction and Development, 83*(10), 860-874.

Glenn, D., McVicar, C., McClure, N., & Lewis, S. (2007). Sildenafil citrate improves sperm motility but causes a premature acrosome reaction in vitro. *Fertility and Sterility, 87*(5), 1064-1070.

Gomez, E., & Aitken, J. (1996). Impact of in vitro fertilization culture media on peroxidative damage to human spermatozoa. *Fertility and Sterility, 65*(4), 880-882.

Goodson, S., White, S., Stevans, A., Bhat, S., Kao, C., Jaworski, S., . . . O'Brien, D. (2017). CASAnova: a multiclass support vector machine model for the classification of human sperm motility patterns. *Biology of Reproduction, 97*(5), 698-708.

Guraya, S. (2000). Cellular and molecular biology of capacitation and acrosome reaction in spermatozoa. *International Review of Cytology, 199*, 1-64.

Harrison, R., & Gadella, B. (2005). Bicarbonate-induced membrane processing in sperm capacitation. *Theriogenology, 63*(2), 342-351.

Henkel, R. (2012). Sperm preparation: state-of-the-art-physiological aspects and application of advanced sperm preparation methods. *Asian Journal of Andrology, 14*(2), 260-269.

Henkel, R., Kierspel, E., Stalf, T., Mehnert, C., Menkveld, R., Tinneberg, H., . . . Kruger, T. (2005). Effect of reactive oxygen species produced by spermatozoa and leukocytes on sperm functions in non-leukocytospermic patients. *Fertility and Sterility, 93*(3), 635.

Hewitson, L., Takahashi, D., Dominko, T., C., S., & Schatten, G. (1998). Fertilization and embryo development to blastocysts after intracytoplasmic sperm injection in the rhesus monkey. *Human Reproduction, 13*, 3449-3455.

Holt, W., Shenfield, F., Leonard, T., Hartman, T., North, R., & Moore, H. (1989). The value of sperm swimming speed measurements in assessing the fertility of human frozen semen. *Human Reproduction, 4*(3), 292-297.

Holt, W., Shenfield, F., Leonard, T., Hartman, T., North, R., & Moore, H. (1989). The value of sperm swimming speed measurements in assessing the fertility of human frozen semen. *Human Reproduction*, 4(3):292-297.

Hong, C., Chiang, B., & Wei, Y. (1983). Mitochondrial respiration inhibitors and human sperm motility: implication in the development of spermicides. *British Journal of Clinical Pharmacology, 16*(5), 487-490.

Hong, C., Chiang, B., Wu, P., Wei, Y., & Fong, J. (1985). Involvement of calcium in the caffeine stimulation of human sperm motility. *British Journal of Clinical Pharmacology, 19*(6), 739-743.

Hong, C., Saintonge, C., & Turner, P. (1981). The inhibitory action of procaine, (+)-propranolol and (+/-) - propranolol on human sperm motility: antagonism by caffeine [letter]. . *British Journal of Clinical Pharmacology, 12*(5), 751-753.

Hung, P., Miller, M., Meyers, S., & VandeVoort, C. (2008). Sperm mitochondrial integrity is not required for hyperactivated motility, zona binding or acrosome reaction in the rhesus macaque. *Biololgy and Reproduction,* 79(2):367-375.

.Ickowicz, D., Finkelstein, M., & Breitbart, H. (2012). Mechanism of sperm capacitation and the acrosome reaction: role of protein kinases. *Asian Journal of Andrology, 14*(6), 816-821.

Jimenez, T., McDermott, J., Sanchez, G., & Blanco, G. (2011). Na,K-ATPase alpha4 isoform is critical for sperm motility and fertility. *Biology of Reproduction, 85*(Suppl 1), 802-805.

Jimenez-Gonzalez, C., Michelangeli, F., Harper, C., Barratt, C., & Publicover, S. (2005). Calcium signalling in human spermatozoa: a specialized 'toolkit' of channels, transporters and stores. *Human Reproduction Update, 12*(3), 253-267.

Jin, S., & Yang, W. (2016). Factors and pathways involved in capacitation: how are they regulated? *Oncotarget,, 8*(2), 3600-3627.

Juyena, N., & Stelletta, C. (2011). Seminal plasma: an essential attribute to spermatozoa. *Journal of Andrology, 33*(4), 536-551.

Khalili, M., Anbari, F., Halvaei, I., Nabi, A., Ghazali, S., & Johansson, L. (2016). The quality of sperm preparation medium affects the motility, viability, and DNA integrity of human spermatozoa. *Journal of Human Reproductive Sciences, 9*(4), 254.

Kim, E., Kim, E., Kim, E., Lee, K., Shin, J., & Kwon, H. (2015). Comparison of the effect of different media on the clinical outcomes of the density-gradient centrifugation/swim-up and swim-up methods. *Clinical and Experimental Reproductive Medicine, 42*(1), 22.

Kizilay, F., & Altay, B. (2017). Sperm function tests in clinical practice. *Türk Üroloji Dergisi/Turkish Journal of Urology, 43*(4), 393-400.

Kocak-toker, N., Aktan, G., & Aykac-toker, G. (2002). The role of Na,K-ATPase in human sperm motility. *International Journal of Andrology, 25*(3), 180-185.

Korosi, T., Barta, C., Rokob, K., & Torok, T. (2017). Physiological Intra-Cytoplasmic Sperm Injection (PICSI) outcomes after oral pretreatment and semen incubation with myo-inositol in oligoasthenoteratozoospermic men: results from a prospective, randomized controlled trial. *European Review of Medical Pharmaceutical Sciences, 21*(Suppl_2), 66-72.

Kraemer, M., Fillion, C., Martin-Pont, B., & Auger, J. (1998). Factors influencing human sperm kinematic measurements by the Celltrak computer-assisted sperm analysis system. *Human Reproduction, 13*(3), 611-619.

Lanzafame, F., La Vignera, S., Vicari, E., & Calogero, A. (2009). Oxidative stress and medical antioxidant treatment in male infertility. *Reproductive BioMedicine Online, 19*(5), 638-659.

Larsen, L., Scheike, T., Jensen, T., Bonde, J., Ernst, E., Hjollund, N., . . . Giwercman, A. (2000). Computer-assisted semen analysis parameters as predictors for fertility of men from the general population. *Human Reproduction, 15*(7), 1562-1567.

Lehti, M., & Sironen, A. (2017). Formation and function of sperm tail structures in association with sperm motility defects†. *Biology of Reproduction, 97*(4), 522-536.

Lindemann, C., & Lesich, K. (2016). Functional anatomy of the mammalian sperm flagellum. *Cytoskeleton, 73*(11), 652-669.

Lippes, J., Enders, R., Pragay, D., & Bartholomew, W. (1972). The collection and analysis of human fallopian tubal fluid. *Contraception, 5*(2), 83-103.

López-Torres, A., & Chirinos, M. (2016). Modulation of human sperm capacitation by progesterone, estradiol, and luteinizing hormone. *Reproductive Sciences, 24*(2), 193-201.

Losano, J., Padín, J., Méndez-López, I., Angrimani, D., García, A., Barnabe, V., & Nichi, M. (2017). The stimulated glycolytic pathway is able to maintain ATP levels and kinetic patterns of bovine epididymal sperm subjected to mitochondrial uncoupling. *Oxidative Medicine and Cellular Longevity*, 1-8. Available at: https://www.hindawi.com/journals/omcl/2017/1682393/. Date of access: 6 May 2019

Lu, J., Huang, Y., & Lü, N. (2014). Computer-aided sperm analysis: past, present and future. *Andrologia, 46*(4), 329–338.

Lybeart, P., Danqury, A., Leleux, F., Meuris, S., & Lebrun, P. (2009). Improved methodology for the detection and quantification of the acrosome reaction in mouse spermatozoa. *Histology and histopathology, 24*(8), 999-1007.

Maegawa, M., Kamada, M., Irahara, M., Yamamoto, S., Yoshikawa, S., Kasai, Y., . . . no, T. (2001). A repertoire of cytokines in human seminal plasma. *Journal of Reproductive Immunology, 54*(1-2), 33-42.

Mann, T., & Lutwak-Mann, C. (1981). Biochemistry of seminal plasma and male accessory fluids; application to andrological problems. *Male Reproductive Function and Semen*, 269-336.Available at: file:///C:/Users/Admin/Documents/Masters/978-1-4471-1300-3_9.pdf. Date of access: 6 May 2019.

Maree, L. (2011). Sperm mitochondria: species specificity and relationships to sperm morphometric features and sperm function in selected mammalian species. *University of the Western Cape, Medical Bioscience Department*. (Thesis-PhD).

Maree, L., & van der Horst, G. (2013). Quantification and identification of sperm subpopulations using computer-aided sperm analysis and species-specific cut-off values for swimming speed. *Biotechnic & Histochemistry, 88*(3-4), 181-193.

Marti, E., Perez-Pe, R., Muino-Blanco, T., & Cebrian-Perez, J. (2006). Comparative study of four different sperm washing methods using apoptotic markers in ram spermatozoa. *Journal of Andrology, 27*(6), 746-753.

Martini, F., & Nath, J. L. (2009). The reproductive system. (*In* Fundamentals of anatomy & physiology. *Eds:* Martini, F., & Nath, J. L. 11th ed. San Francisco: Pearson/Benjamin Cummings, 1101-1148.

Massányi, P., Trandžík, J., Nad, P., Koréneková, B., Skalická, M., Toman, R., . . . Turčan, J. (2003). Concentration of copper, iron, zinc, cadmium, lead and nickel in boar semen and relation to the spermatozoa quality. *Jornal of Environmental Science and Health, 38*(11), 2643-2651.

Massen, J. O.-d., de Vos-Rouweler, A., Spruijt, B., Doxiadis, G., & Sterck, E. (2011). Male mating tactics in captive rhesus macaques (*Macaca mulatta*): the influence of dominance, markets, and relationship quality. *International Journal of Primatology, 33*(1), 73-92.

Matoušek, J. (1985). Biological and immunological roles of proteins in the sperm of domestic animals (review). *Animal Reproduction Science, 8*(1-2), 1-40.

Maxwell, W., de Graaf, S., Ghaoui, R., & Evans, G. (2007). Seminal plasma effects on sperm handling and female fertility. *Reproduction in Domestic Ruminants, 6*(1), 13-38.

Maya-Soriano, M., Abello, M., Fernandez-Bellon, H., Martin, M., Vidal, J. S., & Lopez-Bejar, M. (2015). Reproductive assessment and preliminary evaluation of assisted reproductive technologies in drills (Mandrillus leucophaeus). *Journal of zoo and aquarium research, 3*(4), 116-122.

May-Panloup, P., Chrétien, M., Savagner, F., Vasseur, C., Jean, M., Malthièry, Y., & Reynier, P. (2003). Increased sperm mitochondrial DNA content in male infertility. *Human Reproduction, 18*(3), 550-556.

McPartlin, L., Littell, J., Mark, E., Nelson, J., Travis, A., & Bedford-Guaus, S. (2008). A defined medium supports changes consistent with capacitation in stallion sperm, as evidenced by increases in protein tyrosine phosphorylation and high rates of acrosomal exocytosis. *Theriogenology, 69*(5), 639-650.

McSwiggin, H., & O'Doherty, A. (2018). Epigenetic reprogramming during spermatogenesis and male factor infertility. *Reproduction, 156*(2), 9-21.

Mdhluli, M., Seier, J., & G., v. d. (2004). The male vervet monkey: sperm characteristics and use in reproductive research. *Gynecologic and Obstetric Investigation, 57*(1), 17-18.

Metafora, S., Peluso, G., Persico, P., Ravagnan, G., Esposito, C., & Porta, R. (1989). Immunosuppressive and anti-inflammatory properties of a major protein secreted from the epithelium of the rat seminal vesicles. *Biochemical Pharmacology, 38*(1), 121-131.

Mishra, A., Kumar, A., Swain, D., Yadav, S., & Nigam, R. (2018). Insights into pH regulatory mechanisms in mediating spermatozoa functions. *Veterinary World, 11*(6), 852-858.

Moran, S., Chi, T., Pruncha, M., Aqca, Y., & Chan, A. (2016). Cryotolerance of sperm from transgenic rhesus macaques (*Macaca mulatta*). *Journal of the American Association for Laboratory Animal Science, 55*(5), 520-524.

Mortimer, D., & Mortimer, S. (1990). Kinematics of human spermatozoa incubated under capacitating conditions. *Journal of Andrology, 11*, 195-203.

Mortimer, D., Mortimer, S., & Horst, G. (2015). The future of computer-aided sperm analysis. *Asian Journal of Andrology, 17*(4), 545.

Mújica, A., Neri-Bazan, L., Tash, J., & Uribe, S. (1994). Mechanism for procaine-mediated hyperactivated motility in guinea pig spermatozoa. *Molecular Reproduction and Development, 38*(3), 285-292.

Murase, T., Imaeda, N., Yamada, H., Takasu, M., Taguchi, K., & Katoh, T. (2010). Dilution of boar ejaculates with BTS containing HEPES in place of bicarbonate immediately after ejaculation can reduce the increased inducibility of the acrosome reaction by treatment with calcium and calcium ionophore A23187. *Journal of reproduction and development, 56*(3), 309-314.

Nascimento, J., Shi, L., Tam, J., Chandsawangbhuwana, C., Durrant, B., Botvinick, E., & Berns, M. (2008). Comparison of glycolysis and oxidative phosphorylation as energy sources for mammalian sperm motility, using the combination of fluorescence imaging lazer tweezers, and real-time automated tracking and trapping. *Journal of cellular physiology, 217*(3), 745-751.

Nichols, S., & Bavister, B. (2006). Comparison of protocols for cryopreservation of rhesus monkey spermatozoa by post-thaw motility recovery and hyperactivation. *Reproduction, Fertility and Development, 18*(7), 777-780.

Nieschlag, E., De Geyter, C., De Geyter, M., & Koppers, B. (1998). Diagnostic accuracy of computer-assisted sperm motion analysis. *Human Reproduction, 13*(9), 2512–2520.

Nishigaki, T., González-Cota, A., & Orta, G. (2014). CatSper in male Infertility. *Pathologies of Calcium Channels*, 713-728.Available at: https://link.springer.com/chapter/10.1007/978-3-642-40282-1_34. Date of access: 6 May 2019.

Nyachieo, A., Spiessens, C., Chai, D., Kiulia, N. M., & D'Hooghe, T. (2010). Separate and combined effects of caffeine and dbcAMP on olive baboon (*Papio anubis*) sperm. *Journal of Medical Primatology, 39*(3), 137-142.

Okamura, N., Tajima, Y., Soejima, A., Masuda, H., & Sugita, Y. (1985). Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. *The Journal of Biological Chemistry, 260*(17), 9699-9705.

Oliva, A., Bartolomeo, C., Czille, A., & Porstmann, E. (1991). Sperm motility analysis using multi-exposure photography (MEP) before and after in vitro-treatment of the semen. *Andrologia, 23*(6), 439-442.

Oliva, M., Minutolo, E., Lippa, A., Iaconianni, P., & Vaiarelli, A. (2016). Effect of myoinositol and antioxidants on sperm quality in men with metabolic syndrome. *International Journal of Endocrinology, 2016*, 1-5.

Ortgies, F., Klewitz, J., Görgens, A., Martinsson, G., & Sieme, H. (2012). Effect of procaine, pentoxifylline and trolox on capacitation and hyperactivation of stallion spermatozoa. *Andrologia,* 44 (Suppl 1), 130-138.

Palmieri, M., Papale, P., Della Ragione, A., Quaranta, G., Russo, G., & Russo, S. (2017). Corrigendum to "in vitro antioxidant treatment of semen samples in assisted reproductive technology: effects of myo-Inositol on nemaspermic parameters". *International Journal of Endocrinology,* 1-2. Available at: https://www.hindawi.com/journals/ije/2017/5942741/. Date of access: 6 May 2019.

Pesch, S., & Bergmann, M. (2006). Structure of mammalian spermatozoa in respect to viability, fertility and cryopreservation. *Micron, 37*(7), 597-612.

Piomboni, P., Focarelli, R., Stendardi, A., Ferramosca, A., & Zara, V. (2012). The role of mitochondria in energy production for human sperm motility. *International Journal of Andrology, 35*(2), 109-124.

Plaza Davila, M. M., Tapia, J., Ortega Ferrusola, C., Balao da Silva C, C., & Peña, F. (2015). Inhibition of mitochondrial complex I leads to decreased motility and membrane integrity related to increased hydrogen peroxide and reduced ATP production, while the inhibition of glycolysis has less impact on sperm motility. *PLoS ONE, 10*(9).1-21. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4583303/pdf/pone.0138777.pdf. Date of access: 6 May 2019.

Publicover, S., & Barratt, C. (2011). Reproductive biology: Progesterone's gateway into sperm. *Nature, 471*(7338), 313-314.

Puga Molina, L., Luque, G., Balestrini, P., Marín- Briggiler, C., Romarowski, A., & Buffone, M. (2018). Molecular basis of human sperm capacitation. *Frontiers in Cell and Developmental Biology, 6*(72). 1-23. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6078053/pdf/fcell-06-00072.pdf. Date of access: 6 May 2019.

Quinn, P., Warnes, G., Kerin, J., & Kirby, C. (1985). Culture factors affecting the success rate of in vitro fertilization and embryo transfer. *Annals of the New York Academy of Sciences, 442*(1), 195-204.

Rahman, M., Kwon, W., & Pang, M. (2014). Calcium influx and male fertility in the context of the sperm proteome: an update. *BioMed Research International,* 1-13. Avalaible at: https://www.hindawi.com/journals/bmri/2014/841615/. Date of access: 6 May 2019.

Rahman, M., Kwon, W., & Pang, M. (2017). Prediction of male fertility using capacitation-associated proteins in spermatozoa. *Molecular Reproduction and Development, 84*(9), 749-759.

Ramalho-Santos, J., Amaral, A., Sousa, A., Rodrigues, A., Martins, L., Baptista, M., . . . Gamboa, S. (2007). Probing the structure and function of mammalian sperm using optical and fluorescence microscopy. *Modern Research and Educational Topics in Microscopy, 1,* 394-402. Available at: file:///C:/Users/Admin/Downloads/MicroscopyChapter07.pdf. Date of access: 6 May 2019.

Ramalho-Santos, J., Schatten, G., & Moreno, R. (2002). Control of membrane fusion during spermiogenesis and the acrosome reaction1. *Biology of Reproduction, 67*(4), 1043-1051.

Ramalho-Santos, J., Varum, S., Amaral, S., Mota, P., Sousa, A., & Amaral, A. (2009). Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells. *Human Reproduction Update, 15*(5), 553-572.

Ramió-Lluch, L., Yeste, M. &.N., Laura & Paqui, E. &.P., Muiño-Blanco, I., Concha, I., Ramirez, A., & Rodríguez-Gil, J. (2013). Inhibiting the activity of mitochondrial ATP synthase with Oligomycin-A suppresses motility and 'in vitro' capacitation achievement of boar spermatozoa but does not affect the sperm energy levels. *FEBS Journal, 280*, 266.

Ramió-Lluch, L., Yeste, M., Fernández-Novell, J., Estrada, E., Rocha, L., Cebrián-Pérez, J., . . . Rodríguez-Gil, J. (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), 883-897.

Ramulondi, E. (2018). Vervet monkey - SANBI. Available at: https://www.sanbi.org/animal-of-the-week/vervet-monkey/. Date of access: March 13 2019.

Rovan, E. (2001). Biochemistry of spermatozoa. (B. W. Holzmann A, Ed.) *Veterinary Medicine Andrology, Physiology and Pathology of Reproduction in Male Animals (Schattauer, Stuttgart)*, 23-54.

Ruiz-Pesini, E., Díez-Sánchez, C., López-Pérez, M., & Enríquez, J. (2007). The role of the mitochondrion in sperm function: is there a place for oxidative phosphorylation or is it purely a glycolytic process? *Current Topics in Developmental Biology, 77*, 3-19.

Saeednia, S., Shabani Nashtaei, M., Bahadoran, H., Aleyasin, A., & Amidi, F. (2016). Effect of nerve growth factor on sperm quality in asthenozoosprmic men during cryopreservation. *Reproductive Biology and Endocrinology, 14*(1), 29.

Sangeeta, S., Arangasamy, A., Kulkarni, S., & Selvaraju, S. (2015). Role of amino acids as additives on sperm motility, plasma membrane integrity and lipid peroxidation levels at pre-freeze and post-thawed ram semen. *Animal Reproduction Science, 161*, 82-88.

Scarselli, F., Lobascio, A., Terribile, M., Casciani, V., Greco, P., Franco, G., . . . Greco, E. (2016). Analysis of myo-inositol effect on spermatozoa motility, in hyper viscous ejaculates and in patients with grades II and III varicocele. *Archivio Italiano di Urologia e Andrologia, 88*(4), 279-283.

Seier, J., Fincham, J., Menkveld, R., & Venter, F. (1989). Semen characteristics of vervet monkeys. *Laboratory Animals, 23*(1), 43-47.

Sharma, R., Seifarth, K., Garlak, D., & Agarwal, A. (2019). Comparison of three sperm preparation media. *International Journal of Fertility and Woman's Medicine, 44*(3), 163-167.

Sharpe, R. (2010). Environmental/lifestyle effects on spermatogenesis. *Philosophical Transactions of the Royal Society B: Biological Sciences, 365*(1546), 1697-1712.

Sibley, C., & Ahlquist., J. (1987). DNA hybridization evidence of hominoid phylogeny: results from an expanded data set. *Journal of Molecular Evolution, 26 (1-2)*, 99-121.

Suarez, S. (2008). Control of hyperactivation in sperm. *Human Reproduction Update, 14*(6), 647-657.

Sumigama, S., Mansell, S., Miller, M., Lishko, P., Cherr, G., Meyers, S., & Tollner, T. (2015). Progesterone accelerates the completion of sperm capacitation and activates CatSper channel in spermatozoa from the rhesus macaque1. *Biology of Reproduction, 93*(6), 1-11.

Sun, X., Zhu, Y., Wang, L., Liu, H., Ling, Y., Li, Z., & Sun, L. (2017). The Catsper channel and its roles in male fertility: a systematic review. *Reproductive Biology and Endocrinology, 15*(65), 1-12. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5558725/pdf/12958_2017_Article_281.pdf. Date of access: 6 May 2019.

Tamburrino, L., Marchiani, S., Minetti, F., Forti, G., Muratori, M., & Baldi, E. (2014). The CatSper calcium channel in human sperm: relation with motility and involvement in progesterone-induced acrosome reaction. *Human Reproduction, 29*(3), 418-428.

Tesarik, J., Mendoza, C., & Carreras, A. (1992). Effects of phosphodiesterase inhibitors caffeine and pentoxifylline on spontaneous and stimulus-induced acrosome reactions in human sperm. *Fertility and Sterility, 58*(6), 1185-1190.

Thomsen, R. (2013). Non-invasive collection and analysis of semen in wild macaques. *Primates, 55*(2), 231-237.

Tollner, T., Yudin, A. C., & Overstreet, J. (2003). Real-time observations of individual macaque sperm undergoing tight binding and the acrosome reaction on the zona pellucida1. *Biology of Reproduction, 68*(2), 664-672.

Topfer-Petersen, E., & Waberski, D. (2001). Sperm maturation and fertilization. (B. W. Holzmann A, Ed.) *Veterinary Medicine Andrology, Physiology*, 69-72.

Toshimori, K., & Ito, C. (2015). Sperm function and male infertility. *Journal of Reproductive Immunology, 112*, 143-144.

Tulsiani, D., & Abou-Haila, A. (2012). Biological processes that prepare mammalian spermatozoa to interact with an egg and fertilize It. *Scientifica*, 1-12. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3820447/pdf/SCIENTIFICA2012-607427.pdf.Date of access: 6 May 2019.

van der Horst, G. (2005). Reproduction: male. (e. Wolfe-Coote, Ed.) *In the Laboratory Primate*, 527-536.

van der Horst, G. (2014). Sperm motility subpopulations and fertility. *The NAAB 25th Technical Conference on Artificial Insemination and Reproduction*, 73-77.

van der Horst, G., Maree, L., & du Plessis, S. (2018). Current perspectives of CASA applications in diverse mammalian spermatozoa. *Reproduction, Fertility and Development, 30*(6), 875.

van der Horst, G., Seier, J., Spinks, A., & Hendricks, S. (1999). The maturation of sperm motility in the epididymis and vas deferens of the vervet monkey, Cercopithecus aethiops. *International Fournal of Andrology, 22*(3), 197-207.

VandeVoort, C. A. (2004). High quality sperm for nonhuman primate ART: production and assessment. *Reproductive Biology and Endocrinology, 2*(33), 1-5.

Vijayakumar, R., Simoni, J., Ndubisi, B., Deleon, F., & Heine, W. (1987). Mouse embryo growth in different culture media: selection of a medium for quality control cross-testing of human in vitro fertilization conditions. *Archives of Andrology, 19*(2), 149-158.

Visconti, P. (2009). Understanding the molecular basis of sperm capacitation through kinase design. *Proceedings of the National Academy of Sciences, 106*(3), 667-668.

Visconti, P. (2012). Sperm bioenergetics in a nutshell1. *Biology of Reproduction, 87*(3), 1-4.

Wilson, J., & Hunt, T. (2002). *Molecular biology of the cell* (4th ed.). New York: Garland Science.

Witte, T. S.-S. (2007). Involvement of cholesterol, calcium and progesterone in the induction of capacitation and acrosome reaction of mammalian spermatozoa. *Animal Reproduction Science, 102*(3-4), 181-193.

Wolf, D. (2004). Assisted reproductive technologies in rhesus macaques. *Reproductive Biology and Endocrinology, 2*(37), 1-11.

Wolf, D. (2009). Artificial insemination and the assisted reproductive technologies in non-human primates. *Theriogenology, 71*(1), 123-129.

World Health Organization (WHO). (1999). "WHO laboratory manual for the examination of human semen and semen-cervical mucus interaction." (Cambridge University Press).

World Health Organization (WHO). (2010). WHO laboratory manual for the examination and processing of human semen. Geneva: World Health Organization. Available at: https://apps.who.int/iris/bitstream/handle/10665/44261/9789241547789_eng.pdf?sequence=1&isAllowed=y. Date of access: 6 May 2019.

Yanagimachi, R. (1994). Fertility of mammalian spermatozoa: its development and relativity. *Zygote, 2*(4), 371-372.

Yanagimachi, R., & Noda, Y. (1970). Electron microscope studies of sperm incorporation into the golden hamster egg. *American Journal of Anatomy, 128*(4), 429-461.

Yeung, C., & Cooper, T. (2001). Effects of the ion-channel blocker quinine on human sperm volume, kinematics and mucus penetration, and the involvement of potassium channels. *Molecular Human Reproduction, 7*(9), 819-828.

Zhang, Z., Liu, J., Meriano, J., Ru, C., Xie, S., Luo, J., & Sun, Y. (2016). Human sperm rheotaxis: a passive physical process. *Scientific Reports, 6*(1), 1-4.

Zollner, U., Zollner, K., Dietl, J., & Steck, T. (2001). Semen sample collection in medium enhances the implantation rate following ICSI in patients with severe oligoasthenoteratozoospermia. *Human Reproduction, 16*(6), 1110-1114.

Appendices

Appendix A: Results of vervet sperm motility parameters (initial cut-offs)

Table 4.A: Effects of physiological media on vervet sperm motility parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD)(Initial cut-off values)

Time (minutes)	Media	Number (n)	Total motility (%)	Progressive motility (%)	Non-progressive motility (%)	Rapid (%)	Medium (%)	Slow (%)
Baseline	HTF	17	45.8±24.1^{b}	14.9±15.9^{b}	30.9±15.7^{ab}	0.1±0.4^{a}	17.3±20^{b}	28.4±15.1
	HTFC	17	75.7±14.5^{a}	35.1±17.7^{a}	40.6±13.9^{ab}	1.9±3.6^{b}	45.3±23^{a}	28.5±16.1
	Ham's F-10®	17	47.7±26.4^{b}	22.2±20.7^{ab}	25.5±14.5^{a}	0.5±1.0^{ab}	25.6±23.8^{ab}	21.6±12.4
	HDSWP	17	52.3±25.2^{ab}	16.2±13.9^{b}	36.1±18.6^{ab}	0.2±0.4^{a}	19.6±18.4^{b}	32.6±18.5
	HDSCP	17	64.5±25.2^{ab}	19.1±16.5^{ab}	45.4±18.6^{a}	1.8±3.4^{b}	35.6±26.5^{ab}	27.0±16.7
P-Value			0.001	0.007	0.006	0.047	0.002	0.387
30 min	HTF	16	30.4±21.1^{b}	6.4±12.2^{a}	23.9±16.3^{ab}	0±0^{a}	7.5±13.6^{a}	22.7±15.1^{a}
	HTFC	16	70.1±15.2^{a}	31.3±13.0^{b}	38.8±9.6^{ab}	1.0±1.1^{c}	44.4±21.0^{b}	24.8±8.1^{a}
	Ham's F-10®	16	33.1±28.7^{b}	13.4±15.9^{a}	19.7±15.1^{a}	0.2±0.6^{ab}	16.7±19.5^{a}	16.3±11.7^{b}
	HDSWP	16	45.0±27.5^{ab}	9.6±12.7^{a}	35.4±23.4^{ab}	0.2±0.5^{ab}	11.6±16.5^{a}	33.2±22.6^{a}
	HDSCP	16	54.9±34.4^{ab}	11.2±11.3^{a}	43.8±27.4^{a}	1.4±3.1^{b}	22.8±23.8^{a}	30.8±23.1^{a}
P-Value			<0.001	<0.001	0.003	0.049^{a}	<0.001	0.012
60 min	HTF	16	21.1±22.7^{b}	3.0±5.8^{a}	18.1±19.8^{ab}	0±0^{b}	3.9±6.9^{a}	17.3±18.5^{a}
	HTFC	16	56.±25.4^{a}	26.3±16.3^{b}	30.4±13.9^{c}	1.6±2.8^{a}	36.1±25.3^{b}	19.0±9.5^{a}
	Ham's F-10®	16	15.4±18.8^{b}	3.2±5.8^{a}	12.2±14.6^{a}	0.1±0.4^{a}	4.2±7.8^{a}	11.1±12.7^{a}
	HDSWP	16	37.2±27.8^{ab}	5.0±7.4^{a}	32.1±24.7^{bc}	0±0.1^{a}	6.4±8.9^{a}	30.6±23.4^{b}
	HDSCP	16	35.2±34.7^{ab}	6.4±9.7^{a}	28.8±27.9^{abc}	0±0.1^{a}	11.2±18.7^{a}	23.9±24.2^{a}
P-Value			<0.001	<0.001	0.033	0.001	<0.001	0.05

Parametric data presented as mean and standard deviation (SD). HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, ^{abc}Means with different letters in the same column differed significantly, as obtained from ANOVA.

Appendix B: Results of vervet sperm kinematic parameters (initial cut-offs)

Table 4.B: Effects of physiological media on vervet sperm kinematic parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD)(Initial cut-off values)

Time (minutes)	Media	Number(n)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Baseline	HTF	17	147±29.6^{a}	114.4±34.1^{ab}	123.7±35.5^{b}	75.3±12.6^{a}	89.3±7.2^{a}	81.8±10.8^{a}	2.1±0.5^{a}	24.0±3.9^{a}
	HTFC	17	189.4±25.6^{b}	135.3±25.6^{a}	152.7±28.3^{a}	71.4±10.9^{a}	83.2±8.5^{a}	80.8±8.5^{a}	3.0±0.9^{bc}	20.8±1.9^{ab}
	Ham'sF-10®	17	164.3±34.5^{abc}	128.5±37.4^{a}	138.8±39.3^{ab}	75.9±11.1^{a}	89.1±6.6^{a}	83.9±8.2^{a}	2.3±0.5^{ab}	23.8±3.7^{ab}
	HDSWP	17	155.6±25.7^{ac}	121.1±22.5^{ab}	132.9±25.7^{ab}	76.3±6.6^{a}	89.3±4.3^{a}	83.7±5.3^{a}	2.2±0.5^{ab}	24.3±3.2^{a}
	HDSCP	17	178.8±29.7^{bc}	94±26.7^{b}	122.9±25.7^{b}	54.0±14.1^{b}	73.6±10.1^{b}	69.5±11.6^{b}	3.8±1.3^{c}	20.2±3.5^{b}
P-Value			<0.001	0.002	0.042	<0.001	<0.001	<0.001	<0.001	<0.001
30 min	HTF	16	129.6±26^{a}	100.0±31.7^{a}	106.8±31.1^{bc}	74.9±11.5^{a}	90.9±5.5^{a}	80.3±9.3^{a}	2.0±0.6^{b}	26.2±3.7^{a}
	HTFC	16	188±24.7^{b}	131.1±16.7^{a}	153.3±17.9^{a}	69.7±9.2^{a}	83.6±6.8^{a}	81.1±5.6^{a}	3.1±0.8^{ac}	19.4±5.1^{b}
	Ham'sF-10®	16	150.4±36.7^{a}	109.5±39.2^{a}	124.5±42.5^{ab}	70.1±11.7^{a}	85±7.1^{a}	80.3±10.7^{a}	2.3±0.6^{ab}	21.6±3.6^{b}
	HDSWP	16	140.8±24.0^{a}	106.8±25^{a}	116.1±25.6^{bc}	74.8±10.9^{a}	90.3±5.4^{a}	81.3±8.8^{a}	2.1±0.6^{b}	27.5±3.8^{a}
	HDSCP	16	151±39.1^{a}	65.6±28.8^{b}	87.6±34.1^{c}	42±17.4^{b}	68.5±18.9^{b}	55.9±17.1^{b}	3.7±1.1^{c}	20.4±3.6^{b}
P-Value			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
60 min	HTF	16	114.9±27.2^{bc}	73.8±37.1^{bc}	84.1±35.6^{ab}	62.1±22.5	81.1±18.8^{a}	80.3±9.3^{a}	2.3±0.8	23.6±6.7^{a}
	HTFC	16	180.4±37.9^{a}	122.8±31.2^{a}	143±32.7^{c}	67.8±11.6	83.1±7.3^{a}	81.1±5.6^{a}	3.6±2.3	24±12.4^{a}
	Ham'sF-10®	16	115.2±42.8^{bc}	72.3±43.8^{bc}	85.2±46.4^{ab}	54.2±28.0	70.4±28.1^{a}	80.3±10.7^{a}	2.2±0.8	19.6±6.6^{ab}
	HDSWP	16	131.7±23.7^{ab}	84.6±31.6^{ab}	95.1±31.3^{a}	61.5±18.6	82.2±16.6^{a}	81.3±8.8^{a}	2.6±0.6	25±7^{a}
	HDSCP	16	80.7±76.6^{c}	38.5±38.2^{c}	46.2±47.6^{b}	24.1±23.9	41.9±38.7^{b}	55.9±17.1^{b}	2.0±1.9	12.6±11.7^{b}
P-Value			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.151	0.002

Parametric data presented as mean and standard deviation (SD). HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, VCL = Curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency. ^{abc}Means with different letters in the same column differed significantly, as obtained from ANOVA.

Appendix C: Results of vervet sperm motility parameters (adjusted)

Table 4.2: Effects of physiological media on vervet sperm motility parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD)

Time (minutes)	Media	Number (n)	Total motility (%)	Progressive motility (%)	Non-progressive motility (%)	Rapid (%)	Medium (%)	Slow (%)
Baseline	HTF	17	49.0 ± 23.9^{a}	40.0± 23.2^{a}	9.0 ± 5.0^{ab}	20.4 ± 21.4^{a}	23.7 ± 13.3	4.89 ± 3.58
	HTFC	17	76.4 ± 15.1^{b}	67.5 ± 16.2^{b}	8.9 ± 4.4^{ab}	53.9 ± 20.5^{b}	19.6 ± 9.0	2.94 ± 1.29
	Ham'sF-10®	17	53.3 ± 25.5^{ab}	46.0 ± 25.1^{ab}	7.2 ± 6.1^{a}	32.4 ± 25.9^{abc}	17.1 ± 9.9	3.81 ± 2.68
	HDSWP	17	55.7 ± 24.0^{ab}	46.4 ± 23.4^{ab}	9.3 ± 4.3^{ab}	27.4 ± 21.7^{ac}	23.2 ± 9.3	5.06 ± 3.48
	HDSCP	17	71.3 ± 19.9^{ab}	58.4 ± 22.0^{ab}	12.9 ± 5.4^{b}	47.5 ± 23.6^{bc}	19.7 ± 6.9	4.11 ± 2.56
P-Value			0.001	0.004	0.032	<0.001	0.289	0.199
30 min	HTF	16	34.2 ± 21.2^{b}	26.0 ± 19.2^{b}	8.2 ±6.6^{a}	9.8 ± 14.8^{b}	19.1 ± 13.0	5.3 ± 3.6
	HTFC	16	71.6 ± 15.9^{a}	63.6 ± 15.6^{a}	8.0 ± 3.1^{a}	50.4 ± 20.3^{a}	18.1 ± 5.8	3.2 ± 1.6
	Ham's F-10®	16	37.5 ± 29.2^{b}	31.2 ± 26.9^{b}	6.3 ± 5.1^{a}	21.6 ± 23.5^{b}	12.6 ± 8.4	3.4 ± 2.5
	HDSWP	16	47.5 ± 27.6^{ab}	39.3 ± 24.3^{ab}	8.2 ± 5.8^{a}	17.6 ± 18.5^{b}	26.0 ± 17.8	5.5 ± 7.3
	HDSCP	16	61.3 ± 33.1^{ab}	44.6 ± 29.1^{ab}	16.7 ± 10.9 ^{b}	32.0 ± 27.3^{ab}	24.5 ± 18.7	4.9 ± 2.9
P-Value			<0.001	<0.001	0.001	<0.001	0.0058	0.0342
60 min	HTF	16	23.2 ± 24.5^{b}	15.5 ± 18.1^{b}	7.7 ± 7.2	4.2 ± 7.4^{a}	13.8 ± 15.8	5.24 ± 3.32
	HTFC	16	58.5 ± 25.5^{a}	49.8 ± 24.6^{a}	8.7 ± 5.6	40.0 ± 26.6^{b}	15.3 ± 8.5	3.20 ± 1.63
	Ham's F-10®	16	19.1 ± 20.6^{b}	12.7 ± 16.0^{b}	6.5 ± 6.1	6.1 ± 10.2^{a}	9.3 ± 10.0	3.82 ± 3.25
	HDSWP	16	39.5 ± 28.9^{ab}	29.9 ± 23.5^{ab}	9.6 ± 7.2	11.8 ± 13.1^{a}	23.9 ± 18.7	3.90 ± 2.78
	HDSCP	16	38.9 ± 36.6^{ab}	23.8 ± 26.3^{b}	15.1 ± 17.7	14.1 ± 21.7^{a}	20.0 ± 20.8	4.82 ± 5.70
P-Value			0.002	<0.001	0.141	<0.001	0.098	0.0520

Parametric data presented as mean and standard deviation (SD). HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, ^{abc}Means with different letters in the same column differed significantly, as obtained from ANOVA.

Table 4.3: Comparison of vervet sperm motility parameters between time intervals (mean ± SD)				
Time interval	Baseline	30 min	60 min	P-Value
Number (n)	17	16	16	
HTF				
Total motility (%)	49.0 ± 23.9[a]	34.2 ± 21.2[ab]	23.2 ± 24.5[b]	0.011
Progressive motility (%)	40.0 ± 23.1[a]	26.0 ± 19.2[ab]	15.5 ± 18.1[b]	0.005
Non-progressive motility (%)	9.0 ± 5.0	8.2 ± 6.6	7.7 ± 7.2	0.847
Rapid%	20.4 ± 21.4[a]	9.8 ± 14.8[ab]	4.2 ± 7.4[b]	0.019
Medium%	23.7 ± 13.3	19.1 ± 13.0	13.8 ± 15.8	0.144
Slow%	4.9 ±3.6	5.3 ± 3.6	5.2 ± 3.3	0.934
HTFC				
Total motility (%)	76.4 ± 15.1[a]	71.6 ± 15.9[ab]	58.5 ± 25.5[b]	0.031
Progressive motility (%)	67.5 ± 16.2[a]	63.6 ± 15.6[ab]	49.8 ± 24.8[b]	0.03
Non-progressive motility (%)	8.9 ± 4.4	8.0 ± 3.1	8.7 ± 5.6	0.853
Rapid%	53.9 ± 20.5	50.4 ± 20.3	40.0 ± 26.6	0.199
Medium%	19.6 ± 9.0	18.1 ± 5.8	15.3 ± 8.5	0.305
Slow%	2.9 ± 1.3	3.2 ± 1.6	3.2 ± 1.6	0.872
Ham's F-10®				
Total motility (%)	53.3 ± 25.5[a]	37.5 ± 29.2[ab]	19.1 ± 20.6[b]	0.003
Progressive motility (%)	46.0 ± 25.1[a]	31.2 ± 26.9[ab]	12.7 ± 16.0[b]	0.001
Non-progressive motility (%)	7.2 ± 6.1	6.3 ± 5.1	6.5 ± 6.1	0.894
Rapid%	32.4 ± 25.9[a]	21.6 ± 23.5[ab]	6.1 ± 10.2[b]	0.005
Medium%	17.1 ± 9.9	12.6 ± 8.4	9.3 ± 10.0	0.088
Slow%	3.8 ± 2.7	3.4 ± 2.2	3.8 ± 3.3	0.882
HDSWP				
Total motility (%)	55.7 ± 24.0	47.5 ± 27.6	39.5 ± 28.9	0.236
Progressive motility (%)	46.40 ± 23.43	39.30 ± 24.34	29.89 ± 23.54	0.147
Non-progressive motility (%)	9.3 ± 4.3	8.2 ± 5.8	9.6 ± 7.2	0.764
Rapid%	27.4 ± 21.7	17.6 ± 18.5	11.8 ± 13.1	0.054
Medium%	23.2 ± 9.3	26.0 ± 17.8	23.9 ± 18.7	0.866
Slow%	5.1 ± 3.5	5.5 ± 7.3	4.0 ± 2.8	0.625
HDSCP				
Total motility (%)	71.3 ± 19.9[a]	61.3 ± 33.1[ab]	38.9 ± 36.6[b]	0.012
Progressive motility (%)	58.4 ± 22.0[a]	44.6 ± 29.1[ab]	23.8 ± 26.3[b]	0.002
Non-progressive motility (%)	12.9 ± 5.4	16.7 ± 10.9	15.1 ± 17.7	0.682
Rapid%	47.5 ± 23.6[a]	32.0 ± 27.3[ab]	14.1 ± 21.7[b]	0.001
Medium%	19.7 ± 6.9	24.5 ± 18.7	20.0 ± 20.8	0.662
Slow%	4.1 ± 2.6	4.9 ± 2.9	4.8 ± 5.7	0.834

Parametric data presented as mean and standard deviation (SD). HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, [ab]Means with different letters in the same row differed significantly, as obtained from ANOVA.

Appendix D: Results of vervet sperm kinematic parameters (adjusted cut-offs)

Table 4.4: Effects of physiological media on vervet sperm kinematic parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD)

Time (minutes)	Media	Number(n)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Baseline	HTF	17	141.6 ± 31.5^{a}	109.6±34.2^{ab}	118.6± 36.1^{b}	74.3 ± 11.9^{a}	89.0 ± 6.7^{a}	80.9 ± 10.2^{a}	2.1 ± 0.4^{a}	24.2 ± 3.8^{b}
	HTFC	17	186.2 ± 26.4^{b}	131.2± 27.0^{a}	150.8± 27.5^{a}	69.3 ± 11.0^{a}	83.7 ± 6.4^{a}	75.9 ± 19.5^{a}	3.0 ± 0.8^{b}	20.7 ± 1.8^{ac}
	Ham's F-10®	17	161.8±38.3^{abc}	124.3±41.6^{ab}	136.7± 42.5^{ab}	74.1 ± 11.3^{a}	88.3 ± 6.3^{a}	82.1 ± 9.0^{a}	2.3 ± 0.5^{a}	23.4± 3.5^{ab}
	HDSWP	17	149.4± 28.7^{ac}	115.2±24.3^{ab}	126.7± 28.0^{b}	74.7 ± 6.4^{a}	88.7 ± 4.7^{a}	82.3 ± 5.1^{a}	2.2 ± 0.4^{a}	24.4 ± 3.4^{b}
	HDSCP	17	177.4± 26.7^{bc}	93.5± 26.5^{b}	123.1± 24.2^{b}	53.6 ± 14.1^{b}	72.7 ± 9.6^{b}	69.7 ± 11.7^{b}	3.8 ± 1.2^{b}	19.6 ± 2.6^{c}
P-Value			<0.001	0.009	0.050613	<0.001	<0.001	0.014	<0.001	<0.001
30 min	HTF	16	121.6± 28.9^{b}	92.3± 32.7^{ac}	98.9± 32.7^{ab}	72.8 ± 11.1^{a}	89.9 ± 5.5^{a}	78.5 ± 9.0^{a}	2.0 ± 0.5^{a}	26.4 ± 3.1^{a}
	HTFC	16	184.5± 25.5^{a}	129.2± 17.5^{b}	150.0± 18.8	69.0 ± 9.2^{a}	83.2 ± 6.8^{a}	80.5 ± 5.6^{a}	3.0 ± 0.8^{b}	20.7 ± 2.4^{b}
	Ham's F-10®	16	142.3± 38.4^{b}	104.8±40.7^{ab}	118.7± 44.3^{a}	69.7 ± 11.8^{a}	84.6 ± 7.5^{a}	79.7 ± 10.9^{a}	2.1 ± 0.3^{a}	22.1 ± 3.1^{b}
	HDSWP	16	135.1± 27.4^{b}	101.0±26.6^{ab}	110.1±28.0^{ab}	73.2 ± 10.4^{a}	89.7 ± 5.6^{a}	79.7 ± 8.5^{a}	2.1 ± 0.6^{a}	27.3 ±3.7^{a}
	HDSCP	16	147.5±41.4^{ab}	64.6± 28.1^{c}	86.1± 34.0^{b}	42.0 ± 17.3^{b}	68.4 ± 19.4^{b}	55.9 ± 16.9^{b}	3.5± 1.1^{b}	20.8± 3.7^{b}
P-Value			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
60 min	HTF	16	101.8± 22.4^{a}	59.3 ±27.0^{b}	69.0 ±25.3^{ab}	55.9 ± 20.4^{a}	77.8±18.8^{ab}	65.8 ± 16.2^{b}	2.3 ± 0.6	24.1± 6.2^{ab}
	HTFC	16	175.7± 38.7^{b}	115.8±30.3^{a}	139.4± 33.7^{c}	66.9 ± 12.0^{a}	82.5 ± 7.4^{ab}	78.5 ± 9.0^{a}	3.0 ± 1.0	20.8 ± 2.2^{c}
	Ham's F-10®	16	112.8± 29.8^{a}	67.6± 39.4^{b}	80.1 ±40.8^{ab}	53.3 ± 23.8^{a}	73.1 ±20.3^{a}	64.5 ± 22.4^{b}	2.2 ± 0.4	21.2 ± 3.7^{ac}
	HDSWP	16	127.5± 23.6^{a}	81.2± 30.5^{ab}	91.6 ± 30.2^{a}	60.8 ±18.4^{a}	82.3 ±16.7^{b}	69.1± 15.0^{ab}	2.5 ± 0.6	24.9± 6.9^{b}
	HDSCP	16	93.9 ± 64.7^{a}	37.2± 35.2^{c}	49.5 ± 42.6^{b}	26.0 ± 22.2^{b}	47.6 ±35.0^{c}	36.0 ± 25.8^{c}	2.5 ± 1.6	15.9 ± 10.1^{c}
P-Value			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.187	0.002

Parametric data presented as mean and standard deviation (SD). HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, VCL = Curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency. ^{abc}Means with different letters in the same column differed significantly, as obtained from ANOVA.

Table 4.5: Comparison of vervet sperm kinematic parameters between time intervals (mean ± SD)				
Time interval	Baseline	30 min	60 min	P-Value
Number (n)	17	16	16	
HTF				
VCL (µm/s)	141.6 ± 31.5^{a}	121.6 ± 28.9^{ab}	101.8 ± 22.4^{b}	0.001
VSL (µm/s)	109.6 ± 34.2^{a}	92.3 ± 32.7^{a}	59.3 ± 27.0^{b}	<0.001
VAP (µm/s)	118.6 ± 36.1^{a}	98.9 ± 32.7^{a}	69.0 ± 25.3^{b}	<0.001
LIN (%)	74.3 ± 11.9^{a}	72.8 ± 11.1^{a}	55.9 ± 20.4^{b}	0.002
STR (%)	89.0 ± 6.7	89.9 ± 5.5	77.8 ± 18.8	0.068
WOB (%)	80.9 ± 10.2^{a}	78.5 ± 9.1^{a}	65.8 ± 16.2^{b}	0.002
ALH (µm/s)	2.1 ± 0.4	2.0 ± 0.5	2.3 ± 0.6	0.122
BCF (Hz)	24.2 ± 3.8	26.4 ± 3.1	24.1 ± 6.2	0.258
HTFC				
VCL (µm/s)	186.2 ± 26.4	184.5 ± 25.6	175.7 ± 38.7	0.583
VSL (µm/s)	131.2 ± 27.0	128.2 ± 17.5	137.8 ± 92.5	0.886
VAP (µm/s)	150.8 ± 27.5	150.0 ± 18.8	139.4 ± 33.7	0.421
LIN (%)	69.3 ± 11.0	69.0 ± 9.2	66.9 ± 12.0	0.789
STR (%)	83.7 ± 6.4	83.2 ± 6.8	82.5 ± 7.4	0.876
WOB (%)	75.9 ± 19.5	80.5 ± 5.6	78.5 ± 9.0	0.602
ALH (µm/s)	3.0 ± 0.8	3.0 ± 0.8	3.0 ± 1.0	0.998
BCF (Hz)	20.7 ± 1.8	20.7 ± 2.4	20.8 ± 2.2	0.979
Ham's F-10®				
VCL (µm/s)	161.8 ± 38.3^{a}	142.3 ± 38.4^{ab}	112.8 ± 29.8^{b}	0.002
VSL (µm/s)	124.3 ± 41.6^{a}	104.8 ± 40.7^{ab}	67.6 ± 39.4^{b}	0.002
VAP (µm/s)	136.7 ± 42.5^{a}	118.7 ± 44.3^{ab}	80.1 ± 40.8^{b}	0.002
LIN (%)	74.1 ± 11.3^{a}	69.7 ± 11.8^{a}	53.3 ± 23.8^{b}	0.003
STR (%)	88.3 ± 6.3^{a}	84.6 ± 7.5^{a}	73.1 ± 20.3^{b}	0.007
WOB (%)	82.1 ± 9.0^{a}	79.7 ± 10.9^{a}	64.5 ± 22.4^{b}	0.006
ALH (µm/s)	2.3 ± 0.5	2.1 ± 0.3	2.2 ± 0.4	0.651
BCF (Hz)	23.4 ± 3.5	22.1 ± 3.1	21.2 ± 3.7	0.237
HDSWP				
VCL (µm/s)	149.4 ± 28.7	135.1 ± 27.4	127.5 ± 23.6	0.066
VSL (µm/s)	115.2 ± 24.3^{a}	101.0 ± 26.6^{ab}	81.2 ± 30.5^{b}	0.003
VAP (µm/s)	126.7 ± 28.0^{a}	110.1 ± 28.0^{ab}	91.6 ± 30.2^{b}	0.004
LIN (%)	74.7 ± 6.4^{a}	73.2 ± 10.4^{a}	60.8 ± 18.4^{b}	0.005
STR (%)	88.7 ± 4.7	89.7 ± 5.6	82.3 ± 16.7	0.102
WOB (%)	82.3 ± 5.1^{a}	79.7 ± 8.5^{a}	69.1 ± 15.0^{b}	0.002
ALH (µm/s)	2.2 ± 0.4	2.1 ± 0.6	2.5 ± 0.6	0.088
BCF (Hz)	24.4 ± 3.4	27.3 ± 3.7	24.9 ± 6.9	0.195
HDSCP				
VCL (µm/s)	177.4 ± 26.7^{a}	147.5 ± 41.4^{b}	93.9 ± 64.7^{c}	<0.001
VSL (µm/s)	93.5 ± 26.5	64.6 ± 28.1^{a}	37.2 ± 35.2^{a}	<0.001
VAP (µm/s)	123.1 ± 24.2^{a}	86.1 ± 34.0^{b}	49.5 ± 42.6^{c}	<0.001
LIN (%)	53.6 ± 14.1^{a}	42.0 ± 17.3^{ab}	26.0 ± 22.2^{b}	<0.001
STR (%)	72.7 ± 93.6	68.4 ± 19.4	47.6 ± 35.0	0.1343
WOB (%)	69.7 ± 11.7^{a}	55.9 ± 16.9^{b}	36.0 ± 25.8^{c}	<0.001
ALH (µm/s)	3.8 ± 1.2^{a}	3.5 ± 1.1^{ab}	2.5 ± 1.6^{b}	0.017
BCF (Hz)	19.6 ± 2.6	20.8 ± 3.7	15.9 ± 10.1	0.087

Parametric data presented as mean and standard deviation (SD). HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, VCL = Curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency. ^{abc}Means with different letters in the same row differed significantly, as obtained from ANOVA.

Table 4.6: Effects of physiological media on rapid swimming vervet sperm kinematic parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD)

Time (minutes)	Media	Number(n)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Baseline	HTF	17	221.6±28.6	171.1±31.9^{a}	186.7±24.5^{a}	79.3±13.1^{a}	88.6±10.7^{a}	85.8±9.7^{a}	2.5±1.3^{a}	17.3±4.8
	HTFC	16	216.8±18.1	156.3±26.2^{a}	175.5±24.5^{ab}	72.5±10.3^{a}	85.6±5.6^{a}	82.3±8.1^{a}	3.2±0.9^{a}	19.5±1.7
	Ham's F-10®	16	224.5±18.8	169±26.6^{a}	186.5±24.3^{a}	775.4±11.4^{a}	87±8.3^{a}	83.3±9.1^{a}	2.9±0.9^{a}	18.5±7.6
	HDSWP	16	209.8±14.4	158.7±18.5^{a}	175.8±17.4^{ab}	77.6±6.9^{a}	87.6±4.8^{a}	85.3±6.1^{a}	2.7±0.6^{a}	19.9±4.2
	HDSCP	17	220.3±16.6	121.2±26.4^{b}	155.4±21.3^{b}	58.1±12.3^{b}	72.7±12.9^{b}	71.9±11.4^{b}	4.8±2.7^{b}	16.6±4.2
P-Value			0.282	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	0.253
30 min	HTF	16	226.1±36.4	159.9±40.8^{a}	177.4±35.5	71.7±16.1^{a}	83.0±14.4^{a}	79.4±11.3^{a}	3.0±1.3^{a}	15.3±7.3
	HTFC	16	219.5±9.7	157.4±15.3^{a}	180.5±10.1	72.6±9.0^{a}	85.2±6.1^{a}	82.8±6.1^{a}	3.1±0.7^{a}	18.6±3.0
	Ham's F-10®	16	221.8±38.6	147.1±47.2^{a}	171.0±35.8	69.1±21.2^{a}	81.0±18.8^{a}	79.5±17.2^{a}	3.0±1.2^{a}	16.3±4.6
	HDSWP	16	222.1±41.5	162.2±40.2^{a}	179.4±38.7	73.8±11.3^{a}	85.7±6.8^{a}	81.7±9.1^{a}	2.9±0.8^{a}	17.7±6.0
	HDSCP	16	225.3±38.8	116.9±54.7^{b}	148.4±49.2	51.7±16.5^{b}	72.3±15.4^{b}	65.8±13.0^{b}	4.5±0.8^{b}	16.2±6.0
P-Value			0.983	0.018	0.08	0.001	0.038	0.001	<0.001	0.468
60 min	HTF	16	242.2±53.7	144.6±35.3	172.1±27.1	61.0±17.1^{ac}	74.7±15.8^{ab}	72.6±11.5^{ac}	3.7±1.3	10.8±6.4
	HTFC	16	233.7±30.8	162.8±37.8	186.7±32.8	70.6±10.9^{b}	83.9±7.5^{a}	80.7±8.3^{b}	3.3±1.0	16.5±6.1
	Ham's F-10®	16	243.6±50	159.1±51.5	184.2±44.0	67.8±21.1^{ab}	78.0±13.9^{ab}	75.8±15.4^{ab}	3.4±1.6	11.3±6.4
	HDSWP	16	235.0±59.1	148.0±28	170.2±30.3	65.2±12.7^{ab}	81.6±10.4^{ab}	74.1±10.5^{abc}	3.5±1.0	14.8±7.2
	HDSCP	16	236.0±27.8	130.6±35.5	163.5±30.0	54.4±10.4^{c}	69.7±12.9^{b}	68.7±7.1^{c}	4.3±1.0	11.3±6.8
P-Value			0.964	0.159	0.265	0.036	0.018	0.045	0.147	0.063

Parametric data presented as mean and standard deviation (SD). HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, VCL = Curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency, ^{abc}Means with different letters in the same column differed significantly, as obtained from ANOVA.

Table 4.7: Effects of physiological media on medium swimming vervet sperm kinematic parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD)

Time (minutes)	Media	Number(n)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Baseline	HTF	17	117.6±6.1	84.5±10.9^{ab}	93.6±8.8	70.8±9.0^{ab}	86.0±7.1^{a}	78.8±6.2^{ab}	2.1±0.7	20.3±4.1^{ab}
	HTFC	17	122.2±3.9	84.7±12.7^{ab}	94.6±14.7	68.7±9.2^{ab}	83.5±5.5^{ab}	78.4±7.5^{ab}	2.1±0.4	14.8±2.4^{c}
	Ham's F-10®	17	120.8±7.7	90.1±17.3^{a}	99.4±14.1	73.7±11.0^{a}	86.5±8.3^{a}	81.3±7.5^{a}	1.9±0.5	16.8±4.3^{ac}
	HDSWP	17	121.5±6.3	88.5±10.0^{a}	97.5±9.2	72.0±6.1^{ab}	87.6±4.6^{a}	79.5±4.7^{ab}	2.1±0.3	20.8±3.1^{b}
	HDSCP	17	121.1±6.4	74.6±11.8^{b}	89.1±9.8	62.6±9.5^{b}	78.1±8.3^{b}	73.0±6.8^{b}	2.4±0.4	15.9±2.9^{c}
P-Value			0.250	0.008	0.106	0.009	0.001	0.007	0.127	<0.001
30 min	HTF	16	113.6±7.1	83.8±14.1^{a}	91.0±12.3^{a}	73.3±9.3^{a}	88.8±5.1^{a}	79.6±7.3^{a}	2.0±0.6^{a}	21.3±2.7^{ab}
	HTFC	16	120.2±2.9	86.2±7.8^{a}	96.7±5.8^{a}	71.1±6.1^{a}	85.7±4.5^{a}	79.8±4.1^{a}	2.0±0.3^{a}	14.9±3.6^{c}
	Ham's F-10®	16	116.7±9.4	83.2±13.7^{a}	94.2±13.0^{a}	70.6±8.6^{a}	83.5±8.6^{a}	80.1±6.8^{a}	2.0±0.4^{a}	17.1±4.0^{ac}
	HDSWP	16	117.9±6.7	86.9±11.7^{a}	95.4±10.7^{a}	73.1±8.1^{a}	88.0±5.0^{a}	80.4±7.2^{a}	1.9±0.3^{a}	20.8±4.8^{b}
	HDSCP	16	117.7±5.2	63.1±14.3^{b}	77.2±12.4^{b}	53.1±10.5^{b}	75.7±7.5^{b}	64.8±8.8^{b}	2.7±0.5^{b}	15.6±4.8^{c}
P-Value			0.091	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
60 min	HTF	16	116.3±9.9	75.6±20.6^{ab}	86.6±16.4^{ab}	65.0±17.3^{a}	82.0±13.5	74.2±12.3^{ab}	2.6±0.9^{ab}	18.1±6.1^{ab}
	HTFC	16	126.3±25.4	88.9±22.4^{a}	100.4±24.4^{a}	69.7±8.4^{a}	85.0±6.1	78.8±5.6^{a}	2.2±0.4^{a}	15.6±3.8^{ac}
	Ham's F-10®	16	112.8±7.7	72.7±20.3^{ab}	84.5±17.1^{ab}	65.9±19.1^{a}	80.2±15.7	73.6±13.7^{ab}	2.1±0.6^{a}	15.0±4.9^{ac}
	HDSWP	16	117.7±6.4	77.8±11.2^{ab}	88.3±9.3^{ab}	65.8±9.2^{a}	83.4±7.8	74.5±6.9^{ab}	2.3±0.7^{a}	20.3±5.2^{b}
	HDSCP	16	113.4±10.6	60.4±19.9^{b}	71.6±18.1^{b}	53.1±16.9^{b}	73.4±14	64.7±13.8^{b}	3.0±0.7^{b}	13.1±6.6^{c}
P-Value			0.058	0.002	0.001	0.028	0.066	0.011	0.002	0.004

Parametric data presented as mean and standard deviation (SD). HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, VCL = Curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency, ^{abc}Means with different letters in the same column differed significantly, as obtained from ANOVA.

Table 4.8: Effects of physiological media on slow swimming vervet sperm kinematic parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD)

Time (minutes)	Media	Number(n)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Baseline	HTF	17	69.7±1.2	45.1±7.8^{a}	51.1±5.7^{a}	64.9±10.6^{a}	83.0±9.3^{a}	73.5±7.3^{a}	1.5±0.5	15.4±5.6
	HTFC	17	68.8±2.3	37.3±9.0^{ab}	44.7±10.3^{ab}	54.1±13.1^{ab}	72.5±10.8^{ab}	67.5±9.2^{ab}	1.6±0.4	13.3±3.6
	Ham's F-10®	17	69.2±2.0	45.0±13.6^{a}	51.4±9.9^{a}	64.7±18.7^{a}	80.4±15.6^{a}	74.0±13.4^{a}	1.5±0.6	14.3±5.0
	HDSWP	17	70.7±1.8	42.5±8.3^{ab}	49.7±5.7^{ab}	59.9±12.3^{ab}	78.8±10.7^{ab}	70.0±8.3^{ab}	1.6±0.4	17.5±6.1
	HDSCP	17	69.0±2.2	32.3±10.9^{b}	41.9±9.5^{b}	47.8±12.2^{b}	65.5±18.4^{b}	60.4±13.1^{b}	1.8±0.5	12.9±3.3
P-Value			0.657	0.001	0.003	0.001	0.002	0.002	0.296	0.052
30 min	HTF	16	69.6±1.1	41.2±9.3^{a}	48.9±7.4	59.4±14.0	78.0±9.3^{ab}	70.4±11.1	1.7±0.6	16.9±5.8
	HTFC	16	70.0±1.0	40.1±8.7^{ab}	48.6±7.3	57.2±12.6	75.7±8.6^{ab}	69.5±10.4	1.6±0.4	13.3±4.2
	Ham's F-10®	16	69.4±2.6	35.9±9.4^{a}	44.6±7.7	51.9±14.2	73.4±11.7^{ab}	64.4±12.0	1.9±0.5	15.1±5.5
	HDSWP	16	70.4±2.0	44.5±11.7^{a}	49.9±8.6	62.8±16.4	83.1±13.0^{a}	70.6±11.7	1.5±0.3	18.0±6.1
	HDSCP	16	69.7±1.6	34.8±8.5^{b}	43.4±8.2	49.7±11.4	71.4±8.7^{b}	62.0±11.0	1.8±0.5	13.9±4.9
P-Value			0.504	0.035	0.076	0.055	0.023	0.110	0.160	0.073
60 min	HTF	16	70.3±1.5	38.7±7.7^{ab}	46.5±5.9^{a}	55.2±11.0^{a}	75.3±9.4^{a}	66.3±8.8^{a}	2.0±0.4^{ab}	16.1±5.5
	HTFC	16	72.6±12.0	44.2±11.7^{a}	51.9±12.1^{a}	60.5±11.7^{a}	78.2±8.6^{a}	71.1±10.0^{a}	1.7±0.4^{a}	14.3±3.2
	Ham's F-10®	16	69.4±2.4	29.1±13.8^{bc}	37.6±13.3^{b}	43.4±21.3^{ab}	64.7±18.01^{ab}	53.5±18.8^{b}	1.8±0.4^{ab}	16.1±4.4
	HDSWP	16	70.2±2.0	39.3±9.7^{ab}	46.5±7.8^{a}	55.6±13.7^{a}	75.6±12.3^{a}	66.0±10.8^{a}	1.8±0.5^{a}	15.0±6.6
	HDSCP	16	69.0±1.9	25.1±10.4^{c}	36.9±7.8^{b}	36.3±15.2^{b}	57.7±21.3^{b}	53.6±11.6^{b}	2.3±0.6^{b}	13.7±5.5
P-Value			0.444	<0.001	<0.001	<0.001	0.001	<0.001	0.001	0.615

Parametric data presented as mean and standard deviation (SD). HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, VCL = Curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency, ^{abc}Means with different letters in the same column differed significantly, as obtained from ANOVA.

Appendix E: Results of vervet sperm percentage hyperactivation

Table 4.9: Effects of physiological media on vervet sperm percentage hyperactivation (mean ± SD)

	Hyperactivation %				
Time	5 min	15 min	30 min	45 min	60 min
HTF	8.6 ± 15.2[a] (n=20)	8.0 ± 10.3[ab] (n=20)	10.1 ± 13.6[a] (n=19)	10.8 ± 17.2 (n=19)	16.8 ± 29.2 (n=18)
HTFC	24.6 ± 18.6[b] (n=20)	27.9 ± 22.6[c] (n=20)	22.9 ± 15.4[b] (n=19)	20.3 ± 15.6 (n=19)	18.2 ± 12.2 (n=18)
Ham's F-10®	10.6 ± 18.1[a] (n=19)	6.6 ± 13.2[a] (n=19)	7.2 ± 14.1[a] (n=19)	13.1 ± 18.2 (n=17)	8.3 ± 16.7 (n=17)
HDSWP	10.5 ± 16.2[a] (n=20)	12.2 ± 17.7[b] (n=20)	9.1 ± 12.5[a] (n=19)	11.6 ± 9.7 (n=19)	14.3 ± 13.9 (n=18)
HDSCP	40.0 ± 25.4[b] (n=20)	32.6 ± 25.6[c] (n=20)	24.6 ± 21.8[b] (n=20)	23.7 ± 18.2 (n=19)	22.7 ± 19.9 (n=18)
P-Value	<0.001	<0.001	0.001	0.065	0.324

Parametric data presented as mean and standard deviation (SD). HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, [abc]Means with the different letters in the same column differed significantly, as obtained from ANOVA.

Table 4.10 Comparison of vervet sperm percent hyperactivation between time intervals (mean ± SD)

	Hyperactivation %					
Time interval	5 min	15 min	30 min	45 min	60 min	P=Value
HTF	n=20	n=20	n=18	n=19	n=14	0.641
	8.6 ± 15.2	8.0 ± 10.3	10.1 ± 13.6	10.8 ± 17.2	16.8± 29.2	
HTFC	n=20	n=20	n=19	n=19	n=18	0.485
	24.6 ± 19.0	27.9 ± 22.6	22.9 ± 15.4	20.3 ± 15.6	18.3 ± 11.8	
Ham's F-10®	n=19	n=19	n=19	n=17	n=16	0.744
	10.6 ± 18.1	6.6 ± 13.2	7.3 ± 14.1	13.1 ± 18.2	8.3 ± 16.7	
HDSWP	n=20	n=20	n=18	n=18	n=17	0.869
	10.5 ± 16.2	12.2 ± 17.7	9.1 ± 12.5	11.6 ± 9.7	14.3 ± 13.9	
HDSCP	n=20	n=20	n=20	n=17	n=16	0.082
	40.0 ± 25.4	32.6 ± 25.6	24.6 ± 21.8	23.7 ± 18.3	21.8 ± 19.5	

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus.

Appendix F: Results of vervet Oligomycin sperm motility parameters

Table 4.11: Effects of Oligomycin concentrations on vervet sperm motility parameters at baseline (T0) and 30 minutes (T30) (mean ± SD)

Time (minutes)	Media	Number (n)	Total motility (%)	Progressive motility (%)	Non-progressive %	Rapid%	Medium%	Slow%
Baseline	Control	6	43.7 ± 35.5	37.8 ± 35.4	5.8 ± 5.1	23.9 ± 28.3	16.1 ± 12.2	3.6 ± 3.8
	5 µM Oligomycin	17	51.2 ± 29.8	36.4 ± 27.8	14.8 ± 12.2	13.8 ± 21.1	26.5± 22.3	10.9 ± 10.1
	25 µM Oligomycin	17	43.1 ± 29.5	33.1 ± 28.2	10.1 ± 9.4	13.0 ± 22.5	22.5 ± 16.3	7.7 ± 9.0
P-Value			0.724	0.477	0.138	0.464	0.055	0.111
Time (minutes)	Media	Number (n)	Total motility (%)	Progressive motility (%)	Non-progressive %	Rapid%	Medium%	Slow%
30 min	Control	6	32.5 ± 33.2	27.5 ± 31.8	5.0 ± 4.2	15.4 ± 19.6	13.9 ± 13.5	3.2 ± 2.6
	5µM Oligomycin	17	35.0 ± 31.7	24.6 ± 22.7	10.4 ± 12.3	5.2 ± 9.6	22.3 ± 22.8	7.5 ± 9.5
	25µM Oligomycin	17	28.6 ± 28.4	21.2 ± 23.8	7.3 ± 7.7	5.8 ± 14.4	17.4 ± 18.0	10.1 ± 20.2
P-Value			0.767	0.429	0.087	0.113	0.269	0.242

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA.

Table 4.12:Comparison of vervet sperm motility parameters for different Oligomycin concentrations between time intervals (mean ± SD)			
Time interval	Baseline	30 min	P-Value
Control (n=6)			
Total motility (%)	43.6 ± 35.5	32.5 ± 33.2	0.585
Progressive motility (%)	37.8 ± 35.4	27.5 ± 31.7	0.604
Non-progressive motility (%)	5.8 ± 5.0	5.0 ± 4.2	0.771
Rapid%	23.9 ± 28.3	15.3 ± 19.6	0.556
Medium%	16.1 ± 12.2	13.9 ± 13.5	0.768
Slow%	3.6 ± 3.8	3.2 ± 2.6	0.841
5 µM Oligomycin (n=17)			
Total motility (%)	51.2 ± 29.8	35.0 ± 31.7	0.134
Progressive motility (%)	36.4 ± 27.8	24.6 ± 22.7	0.184
Non-progressive motility (%)	14.8 ± 12.2	10.4 ± 12.3	0.305
Rapid%	13.8 ± 21.1	5.2 ± 9.6	0.136
Medium%	26.5 ± 22.3	22.3 ± 22.8	0.593
Slow%	10.9 ± 10.1	7.5 ± 9.5	0.315
25 µM Oligomycin (n=17)			
Total motility (%)	43.1 ± 29.6	28.5 ± 28.3	0.164
Progressive motility (%)	33.1 ± 28.2	21.2 ± 23.8	0.206
Non-progressive motility (%)	10.0 ± 9.5	7.3 ± 7.7	0.378
Rapid%	13.0 ± 22.5	5.8 ± 14.4	0.281
Medium%	22.5 ± 16.3	17.4 ± 17.9	0.414
Slow%	7.7 ± 9.0	10.1 ± 20.2	0.668
Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA.			

Appendix G: Results of vervet sperm Oligomycin kinematic parameters

Table 4.13: Effects of Oligomycin concentrations on vervet sperm kinematic parameters at baseline(T0) and 30 minutes (T30) (mean ± SD)

Time (minutes)	Media	Number (n)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Baseline	Control	6	135.5 ± 49.9	105.7 ± 58.2	115.3 ± 56.9	73.3 ± 21.9	86.2 ± 13.5	81.8 ± 16.8	1.9 ± 0.7	22.5 ± 4.2[a]
	5 µM Oligomycin	17	121.7 ± 43.4	86.4 ± 42.4	94.7 ± 45.8	68.0 ± 13.6	87.7 ± 6.8	74.6 ± 12.8	2.1 ± 0.5	26.6 ± 4.5[ab]
	25 µM Oligomycin	17	120.9 ± 36.2	79.4 ± 30.6	87.8 ± 33.2	65.7 ± 14.3	89.3 ± 5.3	72.1 ± 13.0	2.4 ± 0.7	28.6 ± 2.9[b]
P-Value			0.427	0.189	0.311	0.567	0.170	0.747	0.337	0.010
30 min	Control	6	115.3 ± 63.5	88.0 ± 56.9	95.6 ± 59.3	57.8 ± 32.8	67.1 ± 36.3	64.5 ± 34.8	1.6 ± 0.9	21.1 ± 10.6[a]
	5 µM Oligomycin	17	107.1 ± 24.3	76.8 ± 25.0	83.1 ± 25.5	69.8 ± 14.1	87.9 ± 10.3	76.0 ± 12.6	1.9 ± 0.4	27.5 ± 4.3[ab]
	25 µM Oligomycin	17	112.6 ± 24.6	73.8 ± 29.5	79.8 ± 30.0	64.7 ± 17.0	90.6 ± 5.0	70.0 ± 16.1	2.1 ± 0.6	30.5 ± 4.0[b]
P-Value			0.30797	0.085	0.093	0.150	0.316752	0.063	0.253	0.004

Parametric data presented as mean and standard deviation (SD). VCL = Curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency. [abc]Means with different letters in the same column differed significantly, as obtained from ANOVA.

Table 4.14:Comparison of vervet sperm kinematic parameters for two oligomycin concentrations between time intervals (mean ± SD)			
Time interval	Baseline	30 min	P-Value
Control (n=6)			
VCL (µm/s)	135.5 ± 49.9	115.3 ± 63.5	0.553
VSL (µm/s)	105.7 ± 58.1	88.0 ± 58.9	0.605
VAP (µm/s)	115.3 ± 56.9	95.6 ± 59.3	0.571
LIN (%)	73.3 ± 21.9	57.8 ± 32.8	0.358
STR (%)	86.1 ± 13.5	67.1 ± 36.3	0.257
WOB (%)	81.7 ± 16.8	64.4 ± 34.8	0.299
ALH (µm/s)	1.9 ± 0.7	1.6 ± 0.9	0.439
BCF (Hz)	23.7 ± 3.2	21.0 ± 10.6	0.601
5 µM Oligomycin (n=17)			
VCL (µm/s)	121.7 ± 43.4	107.1 ± 24.3	0.233
VSL (µm/s)	86.4 ± 42.4	76.8 ± 25.0	0.423
VAP (µm/s)	94.7 ± 45.8	83.1 ± 25.5	0.370
LIN (%)	68.0 ± 13.6	69.8 ± 14.1	0.708
STR (%)	87.7 ± 6.8	87.9 ± 10.3	0.953
WOB (%)	74.6 ± 12.8	76.0 ± 12.6	0.759
ALH (µm/s)	2.1 ± 0.5	1.9 ± 0.4	0.128
BCF (Hz)	26.3 ± 4.4	27.4 ± 4.3	0.454
25 µM Oligomycin (n=17)			
VCL (µm/s)	120.9 ± 36.2	112.6 ± 24.6	0.452
VSL (µm/s)	79.4 ± 30.6	73.8 ± 29.5	0.600
VAP (µm/s)	87.8 ± 33.2	79.8 ± 30.0	0.480
LIN (%)	65.7 ± 14.3	64.7 ± 17.0	0.865
STR (%)	89.3 ± 5.3	90.5 ± 5.0	0.501
WOB (%)	72.1 ± 13.0	70.0 ± 16.1	0.698
ALH (µm/s)	2.3 ± 0.7	2.1 ± 0.6	0.318
BCF (Hz)	28.5 ± 3.0	30.5 ± 4.1	0.151

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. VCL = Curvilinear velocity, VSL =straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

Table 4.15: Effects of Oligomycin concentrations on rapid swimming vervet sperm kinematic parameters at baseline(T0) and 30 minutes (T30) (mean ± SD)										
Time (minutes)	Media	Number (n)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Baseline	Control	6	238.8±39.4	183.2±25.2	200.9±29.2	78.9±14.4	85.9±11.2	85.6±11.7	2.6±1.6	16.5±9.2
	5 µM Oligomycin	17	239.1±43.1	186.9±38.0	200.3±36.1	78.8±11.0	89.3±8.6	85.1±8.7	2.9±1.0	14.7±8.6
	25 µM Oligomycin	17	247.8±55.9	186.5±81.3	206.6±74.9	70.2±20.9	83.5±16.8	78.1±15.4	3.3±2.2	12.2±11.7
P-Value			0.868	0.992	0.947	0.295	0.472	0.240	0.714	0.646
30 min	Control	6	224.2±41.1	143.2±37.1	170.7±26.2	67.4±23.8	76.4±21.6	79.2±16.6	3.0±1.3	15.1±9.4
	5 µM Oligomycin	17	239.9±65.6	188.1±70.8	200.9±67.5	74.3±22.0	86.0±13.7	79.8±18.6	4.3±6.0	9.5±10.1
	25 µM Oligomycin	17	250.9±48.8	173.4±88.3	191.2±76.2	66.3±24.5	81.7±23.4	73.9±18.2	3.7±1.7	12.5±9.5
P-Value			0.602	0.458	0.643	0.600	0.563	0.626	0.807	0.431
Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. VCL = Curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.										

Table 4.16: Effects of Oligomycin concentrations on medium swimming vervet sperm kinematic parameters at baseline(T0) and 30 minutes (T30) (mean ± SD)										
Time (minutes)	Media	Number (n)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Baseline	Control	6	116.1±11.0	86.52±27.5	98.0±20.3	73.2±19.0	83.9±15.6	83.4±11.9	2.0±1.1	18.4±5.1
	5 µM Oligomycin	17	109.9±11.7	79.8±22.2	86.5±20.5	72.0±12.9	87.2±12.3	79.4±10.2	1.9±0.5	21.4±4.9
	25 µM Oligomycin	17	110.7±11.2	77.9±17.7	85.1±17.2	69.8±12.2	88.6±7.6	76.2±10.2	1.9±0.7	21.7±5.1
P-Value			0.508	0.701	0.361	0.835	0.681	0.350	0.898	0.377
30 min	Control	6	112.3.1±10.0	88.6±22.7	96.6±18.4	77.7±18.1	86.7±13.6	85.1±13.0	1.6±0.7	17.1±5.6
	5 µM Oligomycin	17	109.5±8.9	75.1±21.3	82.1±18.2	68.4±18.1	85.9±13.4	74.8±15.0	3.0±4.2	18.5±8.3
	25 µM Oligomycin	17	114.9±8.2	75.0±21.7	84.4±16.1	64.8±19.5	81.7±19.2	73.3±13.5	2.2±0.8	20.0±7.9
P-Value			0.222	0.382	0.223	0.360	0.701	0.218	0.508	0.730
Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. VCL = Curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.										

Table 4.17: Effects of Oligomycin concentrations on slow swimming vervet sperm kinematic parameters at baseline(T0) and 30 minutes (T30) (mean ± SD)										
Time (minutes)	Media	Number (n)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Baseline	Control	6	69.2±1.8	50.8±9.3	56.3±6.2[a]	72.8±13.7	86.1±10.4	81.1±9.3[a]	1.4±0.5	16.4±4.6
	5 µM Oligomycin	17	70.7±5.1	48.4±9.2	52.0±7.4[ab]	68.6±12.4	85.3±11.7	75.4±10.4[ab]	1.4±0.4	18.3±5.9
	25 µM Oligomycin	17	70.1±2.1	42.7±6.7	47.6±6.1[b]	60.9±9.7	85.1±9.2	68.0±8.9[b]	1.4±0.4	18.5±8.2
P-Value			0.719	0.067	0.024	0.061	0.981	0.014	0.968	0.808
30 min	Control	6	68.8±2.5	51.7±11.0	56.9±9.4	75.0±16.6	85.9±9.6	82.5±13.8	1.1±0.6	13.4±9.6
	5 µM Oligomycin	17	71.2±8.5	47.0±10.5	51.9±7.9	67.3±17.6	85.5±13.0	73.9±14.8	2.6±4.9	16.3±7.6
	25 µM Oligomycin	17	67.0±2.4	45.1±13.3	51.2±10.9	65.2±18.4	83.3±16.5	73.9±15.9	1.3±0.4	19.7±9.0
P-Value			0.494	0.519	0.452	0.520	0.884	0.447	0.456	0.268
Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. VCL = Curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency, [ab]Means with different letters in the same column differed significantly, as obtained from ANOVA.										

Appendix H: Results of vervet sperm vitality

Table 4.18: Effects of physiological media on vervet sperm vitality at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD)

Parameter	Physiological media					
	HTF	HTFC	Ham's F-10®	HDSWP	HDSCP	P-Value
	Baseline					
Vitality (%)	n=17	n=17	n=17	n=17	n=17	
	64.0 ± 11.7	65.3 ± 8.4	57.0 ± 13.1	64.2 ± 9.4	63.3 ± 14.6	0.260
	30 min					
	n=16	(n=16)	(n=16)	(n=16)	n=16	
	60.5 ± 14.9	60.4 ± 8.2	51.0 ± 18.1	61.5 ± 13.0	56.3 ± 26.6	0.405
	60 min					
	n=15	n=15	n=15	n=15	n=15	
	53.4 ± 11.8	50.5 ± 12.5	38.4 ± 22.5	53.6 ± 12.0	48.6 ± 27.3	0.128

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus. T0 = baseline, T30 = 30 minutes, T60 = 60 minutes.

Table 4.19: Comparison of vervet sperm vitality between time intervals (mean ± SD)

Time interval	Baseline	30 min	60 min	P-Value
		Vitality (%)		
HTF	64.0 ± 11.7 (n=17)	60.5 ± 14.9 (n=16)	53.4 ± 11.8 (n=15)	0.067
HTFC	65.3 ± 8.4^{a} (n=17)	60.4 ± 8.2^{b} (n=16)	50.5 ± 12.5^{c} (n=15)	<0.001
Ham's F-10®	57.0 ± 13.1^{a} (n=17)	51.0 ± 18.1^{ab} (n=16)	38.4 ± 22.5^{b} (n=15)	0.019
HDSWP	64.2 ± 9.37^{a} (n=17)	61.5 ± 12.9^{ab} (n=16)	53.6 ± 12.0^{b} (n=15)	0.035
HDSCP	63.3 ± 14.6 (n=17)	56.3 ± 26.6 (n=16)	48.6 ± 27.3 (n=15)	0.243

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus. ^{abc}Means with different letters in the same column differed significantly, as obtained from ANOVA.

Appendix I: Results of vervet sperm acrosome parameters

Table 4.20: Effects of physiological media on vervet sperm acrosome parameters at baseline (T0), 30 minutes (T30), and 60 minutes (T60) (mean ± SD)

Parameter	Physiological media					
	HTF (n=11)	HTFC (n=11)	Ham's F-10® (n=9)	HDSWP (n=10)	HDSCP (n=10)	P-Value
Baseline						
Intact (%)	87.7 ± 4.8	84.8 ± 16.8	84.8 ± 14.6	92.4 ± 3.9	89.3 ± 6.9	0.430
30 min						
Intact (%)	86.0 ± 6.8	84.6 ± 14.0	87.4 ± 8.4	87.6 ± 6.9	86.6 ± 8.9	0.360
60 min						
Intact (%)	86.4 ± 8.5	84.9 ± 11.5	84.6 ± 13.5	89.0 ± 3.5	87.7 ± 11.1	0.311

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus. T0 = baseline, T30 = 30 minutes, T60 = 60 minutes.

Table 4.21: Comparison of vervet sperm acrosome integrity between time intervals (mean ± SD)

Time interval	Baseline	30 min	60 min	P-Value
Acrosome intactness (%)				
HTF (n=11)	87.7 ± 4.8	86.0 ± 6.8	86.4 ± 8.5	0.820
HTFC (n=11)	84.8 ± 16.8	84.6 ± 14.0	75.3 ± 27.9	0.622
Ham's F-10® (n=9)	84.8 ± 14.6	87.4 ± 8.4	84.6 ± 13.5	0.851
HDSWP (n=10)	92.4 ± 3.9	87.6 ± 6.9	89.0 ± 3.5	0.097
HDSCP (n=10)	89.3 ± 6.9	86.6 ± 8.9	87.7 ± 11.1	0.795

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus.

Appendix J: Results of rhesus sperm motility parameters

Table 4.23: Effects of physiological media on rhesus sperm motility parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD)

Time (minutes)	Media	Number (n)	Total motility (%)	Progressive motility (%)	Non-progressive %	Rapid%	Medium%	Slow%
Baseline	HTF	9	66.4 ± 28.1	45.1 ± 24.0	21.3 ± 22.7	33.8 ± 25.8	18.3 ± 9.7	14.3± 19.5
	HTFC	5	74.6 ± 22.6	45.3 ± 7.4	29.3 ± 21.6	35.8 ± 8.3	25.6 ± 18.0	13.3 ± 9.1
	Ham's F-10®	9	65.5 ± 30.3	43.7 ± 31.7	21.8 ± 21.2	36.7 ± 32.8	13.6 ± 10.5	15.3 ± 15.8
	HDSWP	9	74.0 ± 14.0	54.9 ± 17.4	19.1 ± 20.0	40.1 ± 22.2	20.7 ± 11.7	13.3 ± 14.9
	HDSCP	9	62.6 ± 28.8	43.1 ± 25.8	19.5 ± 17.0	36.7 ± 26.8	15.3 ± 11.5	10.6 ± 9.5
P-Value			0.853	0.834	0.916	0.991	0.394	0.972
30 min	HTF	7	57.4 ± 29.0	33.9 ± 19.5	23.5 ± 26.3	22.8 ± 22.1	18.0 ± 12.8	16.7 ± 21.6
	HTFC	5	78.1 ± 17.9	51.1 ± 17.0	27.0 ± 19.7	40.3 ± 30.3	24.6 ± 14.7	13.2 ± 10.1
	Ham's F-10®	7	45.8 ± 31.5	27.1 ± 21.9	18.6 ± 16.3	17.6 ± 21.2	13.4 ± 9.2	14.7 ± 12.2
	HDSWP	7	57.1 ± 25.8	33.2 ± 20.1	23.8 ± 25.1	22.5 ± 20.4	18.0 ± 10.7	16.6 ± 21.8
	HDSCP	7	49.8 ± 35.4	29.4 ± 26.1	20.4 ± 19.3	19.5 ± 20.0	15.9 ± 13.3	14.5 ± 11.8
P-Value			0.413	0.393	0.968	0.412	0.617	0.995
60 min	HTF	2	59.6 ± 30.4	9.9 ± 9.8	49.7 ± 20.6	3.2 ± 4.5	12.1 ± 12.4	44.3 ± 13.5
	HTFC	2	78.6 ± 22.0	34.9 ± 6.6	43.7 ± 15.4	21.6 ± 7.9	30.7 ± 13.2	26.3 ± 0.9
	Ham's F-10®	2	54.1 ± 45.0	21.8 ± 28.1	32.4 ± 16.9	12.7 ± 18.0	17.2 ± 19.8	24.2 ± 7.2
	HDSWP	2	67.4 ± 11.1	11.8 ± 7.3	55.6 ± 18.4	2.9 ± 0.4	16.2 ± 5.7	48.3 ± 17.2
	HDSCP	2	65.2 ± 31.8	25.6 ± 16.2	39.7 ± 15.8	18.3 ± 18.8	17.5 ± 11.2	29.5 ± 1.9
P-Value			0.936	0.551	0.724	0.501	0.7	0.196

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus.

Table 4.24 Comparison of Rhesus sperm motility parameters in five physiological media (mean ± SD) between time intervals				
Time interval	Baseline	30 min	60 min	P-Value
HTF (baseline n=9, 30 min n=7 , 60 min n=2)				
Total motility (%)	66.4 ± 28.1	57.4 ± 29.0	59.6 ± 30.4	0.818
Progressive motility (%)	45.1 ± 24.0	33.9 ± 19.5	9.9 ± 9.8	0.136
Non-progressive motility (%)	21.3 ± 22.7	23.5 ± 26.3	49.7 ± 20.6	0.337
Rapid%	33.8 ± 25.8	22.8 ± 22.1	3.2 ± 4.5	0.253
Medium%	18.3 ± 9.7	18.0 ± 12.8	12.1 ± 12.4	0.777
Slow%	14.3 ± 19.5	16.7 ± 21.6	44.3 ± 13.5	0.186
HTFC (baseline n=5, 30 min n=5 , 60 min n=2)				
Total motility (%)	74.6 ± 22.6	78.1 ± 17.9	78.6 ± 22.0	0.956
Progressive motility (%)	45.3 ± 7.4	51.1 ± 20.0	34.9 ± 6.6	0.343
Non-progressive motility (%)	29.3 ± 21.6	27.0 ± 19.7	43.7 ± 15.4	0.617
Rapid%	35.8 ± 8.3	40.3 ± 20.3	21.6 ± 7.9	0.360
Medium%	25.6 ± 18.0	24.6 ± 14.7	30.7 ± 13.2	0.901
Slow%	13.3 ± 9.1	13.2 ± 10.1	26.3 ± 0.9	0.233
Ham's F-10® (baseline n=9, 30 min n=7 , 60 min n=2)				
Total motility (%)	65.5 ± 30.3	45.8 ± 31.5	54.1 ± 45.0	0.487
Progressive motility (%)	43.7 ± 31.7	27.1 ± 21.9	21.8 ± 28.1	0.412
Non-progressive motility (%)	21.8 ± 21.5	18.6 ± 16.3	32.4 ± 16.9	0.676
Rapid%	36.7 ± 32.8	17.6 ± 21.2	12.7 ± 18.0	0.328
Medium%	13.6 ± 10.5	13.4 ± 9.2	17.2 ± 19.8	0.903
Slow%	15.3 ± 15.8	14.7 ± 12.2	24.2 ± 7.2	0.689
HDSWP (baseline n=9, 30 min n=7 , 60 min n=2)				
Total motility (%)	74.0 ± 14.0	57.1 ± 25.8	67.4 ± 11.1	0.256
Progressive motility (%)	54.9 ± 17.4[a]	33.2 ± 20.1[ab]	11.8 ± 7.3[b]	0.013
Non-progressive motility (%)	19.1 ± 20.0	23.8 ± 25.1	55.6 ± 18.4	0.140
Rapid%	40.1 ± 22.4	22.5 ± 20.4	2.9 ± 0.4	0.069
Medium%	20.7 ± 11.7	18.0 ± 10.7	16.2 ± 5.7	0.824
Slow%	13.3 ± 14.9	16.6 ± 21.8	48.3 ± 17.2	0.073
HDSCP (baseline n=9, 30 min n=7 , 60 min n=2)				
Total motility (%)	62.6 ± 28.8	49.8 ± 35.4	65.2 ± 31.8	0.693
Progressive motility (%)	43.1 ± 25.8	29.4 ± 26.1	25.6 ± 16.2	0.488
Non-progressive motility (%)	19.5 ± 17.0	20.4 ± 19.3	39.7 ± 15.6	0.361
Rapid%	36.7 ± 28.8	19.5 ± 20.0	18.3 ± 18.8	0.323
Medium%	15.3 ± 11.5	15.9 ± 13.3	17.5 ± 11.2	0.974
Slow%	10.6 ± 9.5	14.5 ± 11.8	29.5 ± 1.9	0.093

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, [ab]Means with the same letter differed significantly, as obtained from ANOVA.

Appendix K: Results of rhesus sperm kinematic parameters

Table 4.25: Effects of physiological media on rhesus sperm kinematic parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD)

Time (minutes)	Media	Number (n)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Baseline	HTF	9	173.3 ± 29.2	91.9 ± 23.8	109.0 ± 26.1	52.7 ± 10.3^{a}	81.2 ± 8.1^{a}	62.7 ± 9.2^{b}	3.8 ± 0.8^{a}	25.5 ± 3.8^{ab}
	HTFC	5	213.5 ± 24.7	79.4 ± 18.3	109.1 ± 15.3	37.8 ± 7.5^{ab}	69.6 ± 9.1^{b}	51.7 ± 3.6^{ac}	5.7 ± 1.0^{b}	21.4 ± 3.2^{ac}
	Ham's F-10®	9	184.5 ± 70.6	92.3 ± 45.1	111.9 ± 51.3	47.5 ± 14.4^{ab}	77.4 ± 8.3^{ab}	57.9 ±14.0^{ab}	4.0 ± 1.8^{ac}	24.5 ± 3.6^{abc}
	HDSWP	9	182.8 ± 24.4	95.3 ± 18.2	111.5 ± 17.8	51.5 ± 7.1^{a}	82.1 ± 6.9^{a}	60.7 ± 6.7^{b}	4.0 ± 0.7^{ac}	27.1 ± 4.1^{b}
	HDSCP	9	183.3 ± 66.8	68.1 ± 32.6	91.1 ± 38.9	34.4 ± 12.1^{b}	68.4 ± 14.3^{b}	47.3 ± 11.2^{c}	5.0 ± 1.8^{bc}	21.7 ± 3.2^{c}
P-Value			0.709	0.314	0.681	0.004	0.017	0.0017	0.05178	0.025951
30 min	HTF	7	176.5 ± 35.7	88.3 ± 16.3	106.1 ± 20.2	49.7 ± 7.2	80.2 ± 5.5	59.9 ± 7.9	3.9 ± 0.8	25.5 ± 2.3
	HTFC	5	216.1 ± 11.4	85.7 ± 12.0	113.4 ± 7.7	39.7 ± 6.7	72.9 ± 8.9	52.5 ± 4.5	5.5 ± 0.8	22.4 ± 2.8
	Ham's F-10®	7	157.1 ± 51.4	80.4 ± 27.5	92.7 ± 32.0	49.6 ± 14.8	83.2 ± 10.7	57.1 ± 13.1	3.4 ± 1.5	27.9 ± 4.2
	HDSWP	7	171.4 ± 29.8	79.8 ± 23.5	93.7 ± 23.7	45.6± 111.4	81.3 ± 9.0	54.2 ± 10.1	4.1 ± 0.7	25.9 ± 3.7
	HDSCP	5	140.4 ± 66.6	57.8 ± 33.2	73.0 ± 39.5	36.5 ± 17.9	69.0 ± 22.5	47.6 ± 14.7	3.6 ± 1.9	23.8 ± 5.9
P-Value			0.086	0.185	0.131	0.239	0.235	0.310	0.068	0.188
60 min	HTF	2	146.9± 26.8	68.6 ± 1.5	85.6 ± 9.9	48.3 ± 6.8	78.1 ± 7.4	59.4 ± 3.4	3.7 ± 0.9	25.1 ± 1.3
	HTFC	2	207.3 ± 10.0	87.82±20.1	116.5 ±7.0	43.4 ±12.8	73.1 ± 13.9	56.8 ± 6.8	5.1 ± 1.1	24.4 ± 5.5
	Ham's F-10®	2	171.6 ± 55.9	85.9 ± 5.6	106.4 ± 23.6	53.6 ± 15.0	80.5 ± 15.0	64.0 ± 7.6	3.9 ± 2.0	27.8 ± 8.3
	HDSWP	2	160.8 ± 16.1	73.3 ± 27.3	91.0 ± 21.7	46.3 ± 13.1	77.6 ± 11.0	57.1 ± 8.4	3.9 ± 0.6	26.3 ± 7.4
	HDSCP	2	195.5 ± 30.4	85.2 ± 32.1	112.8 ± 12.0	46.4 ± 23.7	73.1 ± 21.6	59.9 ± 15.9	5.0 ± 2.3	22.6 ± 7.5
P-Value			0.415	0.842	0.363	0.969	0.978	0.932	0.806	0.938

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, VCL = Curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency, ^{abc}Means with the same letters in the same column differed significantly, as obtained from ANOVA.

Table 4.26: Comparison of Rhesus sperm kinematic parameters between time intervals (mean ± SD)				
Time interval	5 min	30 min	60 min	P-Value
HTF (baseline n=9, 30 min n=7 , 60 min n=2)				
VCL (µm/s)	173.3 ± 29.2	176.5 ± 35.7	146.9 ± 26.8	0.511
VSL (µm/s)	91.9 ± 23.8	88.3 ± 16.3	68.6 ± 1.5	0.359
VAP (µm/s)	109.0 ± 26.1	106.1 ± 20.2	85.6 ± 9.9	0.448
LIN (%)	52.7 ± 10.3	49.7 ± 7.2	48.3 ± 6.8	0.734
STR (%)	81.2 ± 8.1	80.2 ± 5.5	78.1 ± 7.4	0.851
WOB (%)	62.7 ± 9.2	59.9 ± 7.9	59.4 ± 3.4	0.763
ALH (µm/s)	3.8 ± 0.8	3.9 ± 0.8	3.7 ± 0.9	0.863
BCF (Hz)	25.5 ± 3.8	25.5 ± 2.3	25.1 ± 1.3	0.984
HTFC (baseline n=5, 30 min n=5 , 60 min n=2)				
VCL (µm/s)	213.5 ± 24.7	216.1 ± 11.4	207.3 ± 10.0	0.851
VSL (µm/s)	79.4 ± 18.3	85.7 ± 12.0	87.8 ± 20.1	0.76
VAP (µm/s)	109.1 ± 15.3	113.4 ± 7.7	116.1 ± 7.0	0.739
LIN (%)	37.8 ± 7.5	39.7 ± 6.7	43.4 ± 12.8	0.709
STR (%)	69.6 ± 9.1	72.9 ± 8.9	73.1 ± 13.9	0.840
WOB (%)	51.7 ± 3.6	52.5 ± 4.5	56.8 ± 6.8	0.413
ALH (µm/s)	5.7 ± 1.0	5.5 ± 0.8	5.1 ± 1.1	0.715
BCF (Hz)	21.4 ± 3.2	22.4 ± 2.8	24.4 ± 5.5	0.586
Ham's F-10® (baseline n=9, 30 min n=7 , 60 min n=2)				
VCL (µm/s)	184.5 ± 70.6	157.1 ± 51.4	171.6 ± 55.9	0.692
VSL (µm/s)	92.3 ± 45.1	80.4 ± 27.5	85.9 ± 5.6	0.820
VAP (µm/s)	111.9 ± 51.3	92.7 ± 32.0	106.4 ± 23.6	0.679
LIN (%)	47.5 ± 14.4	49.6 ± 14.8	53.6 ± 15.0	0.864
STR (%)	77.4 ± 8.3	83.2 ± 10.7	80.5 ± 15.0	0.518
WOB (%)	57.9 ± 14.0	57.12 ± 13.0	64.0 ± 7.6	0.807
ALH (µm/s)	4.0 ± 1.8	3.4 ± 1.5	3.9 ± 2.0	0.768
BCF (Hz)	24.5 ± 3.6	27.9 ± 4.2	27.8 ± 8.3	0.289
HDSWP (baseline n=9, 30 min n=7 , 60 min n=2)				
VCL (µm/s)	182.8 ± 24.4	171.4 ± 29.8	160.8 ± 16.1	0.491
VSL (µm/s)	95.3 ± 18.2	79.8 ± 23.5	73.3 ± 27.3	0.252
VAP (µm/s)	111.5 ± 17.8	93.7 ± 23.7	91.0 ± 21.7	0.196
LIN (%)	51.5 ± 7.1	45.7 ± 11.4	46.3 ± 13.1	0.474
STR (%)	82.1 ± 6.9	81.3 ± 9.0	77.6 ± 11.0	0.775
WOB (%)	60.7 ± 6.7	54.2 ± 10.1	57.1 ± 8.4	0.326
ALH (µm/s)	4.0 ± 0.7	4.1 ± 0.7	3.9 ± 0.6	0.932
BCF (Hz)	27.1 ± 4.1	25.9 ± 3.7	26.3 ± 7.4	0.866
HDSCP (baseline n=9, 30 min n=5 , 60 min n=2)				
VCL (µm/s)	183.3 ± 66.8	140.4 ± 66.6	195.5 ± 30.4	0.371
VSL (µm/s)	68.1 ± 32.6	57.8 ± 33.2	85.2 ± 32.1	0.574
VAP (µm/s)	91.1 ± 38.8	73.0 ± 39.5	112.8 ± 12.0	0.396
LIN (%)	34.4 ± 12.1	36.5 ± 17.9	46.4 ± 23.7	0.622
STR (%)	68.4 ± 14.3	69.0 ± 22.5	73.1 ± 21.6	0.946
WOB (%)	47.3 ± 11.2	47.6 ± 14.7	59.9 ± 15.9	0.459
ALH (µm/s)	5.0 ± 1.8	3.6 ± 1.9	5.0 ± 2.3	0.325
BCF (Hz)	21.7 ± 3.2	23.8 ± 5.9	22.6 ± 7.5	0.695

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus. VCL = Curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

Table 4.27: Effects of physiological media on rapid swimming rhesus sperm kinematic parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD)

Time (minutes)	Media	Number(n)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Baseline	HTF	9	267.0±48.9	124.7±24.2	147.5±31.3	52.9±8.4[a]	82.4±7.8[ac]	62.2±7.5[a]	4.7±1.2[a]	21.5±3.7
	HTFC	6	265.8±37.6	109.2±24.5	145.8±29.0	40.5±6.9[b]	70.5±8.0[b]	54.3±5.6[b]	6.5±1.2[ab]	17.9±4.1
	Ham's F-10®	8	262.6±45.9	140.3±39.0	164.0±39.6	52.9±10.2[a]	83.2±8.6[a]	61.6±8.7[a]	5.1±1.2[ab]	22.1±6.5
	HDSWP	8	239.3±53.6	124.1±29.0	147.9±37.7	52.1±7.8[a]	81.7±7.0[ac]	61.7±6.3[a]	4.8±1.1[a]	22.9±6.4
	HDSCP	8	260.0±34.6	105.5±17.7	135.8±22.7	41.0±5.9[b]	75.3±7.4[bc]	52.5±4.4[b]	6.8±0.9[b]	18.2±2.9
P-Value			0.586	0.132	0.559	0.003	0.017	0.013	0.002	0.194
30 min	HTF	9	251.9±50.9	117.1±15.0[a]	148.9±23.8	47.9±9.9	76.1±11.3	59.9±6.9	5.5±1.4	22.8±6.0
	HTFC	5	260.7±26.8	109.1±8.7[a]	141.1±12.2	42.8±8.0	74.7±8.6	54.8±6.3	6.2±1.2	19.3±2.5
	Ham's F-10®	8	248.4±56.3	147.7±31.2[b]	164.7±37.9	61.0±13.6	88.9±10.2	67.3±11.4	4.6±1.5	21.5±5.1
	HDSWP	8	245.1±53.7	111.1±30.0[a]	135.1±36.5	45.3±10.1	78.0±12.5	55.0±8.1	5.4±1.1	22.1±6.2
	HDSCP	6	245.7±54.6	116.3±9.3[a]	142.8±17.4	49.3±12.8	78.8±9.7	59.7±10.2	5.6±2.2	19.6±7.4
P-Value			0.985	0.026	0.339	0.073	0.118	0.070	0.420	0.759
60 min	HTF	3	327.1±44.1	166.0±28.7	209.7±34.6	52.5±8.1	72.4±1.9	65.3±6.1	5.0±1.2	12.9±7.9
	HTFC	3	304.5±23.3	125.1±13.4	167.9±1.2	41.1±6.3	67.9±13.3	55.1±3.0	5.8±1.1	16.5±3.8
	Ham's F-10®	3	305.3±12.7	165.5±34.2	196.6±39.0	54.8±11.3	77.0±11.7	64.7±11.3	4.8±2.8	14.6±13.7
	HDSWP	3	308.1±34.9	159.3±26.4	192.9±36.6	49.2±6.5	73.7±8.2	60.5±5.8	5.8±1.0	14.1±6.1
	HDSCP	3	293.0±15.2	133.3±29.3	164.5±20.5	45.0±10.1	76.8±14.1	55.7±5.4	6.7±0.8	17.1±3.9
P-Value			0.700	0.276	0.346	0.364	0.830	0.284	0.610	0.963

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, VCL = Curvilinear velocity, VSL= straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency, [abc]Means with different letters in the same column differed significantly, as obtained from ANOVA.

Table 4.28: Effects of physiological media on medium swimming rhesus sperm kinematic parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD)

Time (minutes)	Media	Number(n)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Baseline	HTF	9	148.9±60.5	82.3±36.2	98.5±42.6	54.8±5.9	80.2±7.0	65.8±5.9	3.0±1.2	20.3±3.9
	HTFC	6	172.5±69.2	77.0±27.8	102.7±41.4	48.1±11.9	74.4±11.9	61.1±8.1	4.2±2.1	17.7±3.7
	Ham's F-10®	9	146.1±66.2	85.8±41.4	98.2±45.7	59.5±7.1	81.8±8.7	67.7±5.9	2.6±1.4	17.6±4.6
	HDSWP	8	143.0±58.9	81.4±34.7	95.2±40.8	57.4±6.2	82.7±6.5	66.8±4.4	2.9±1.2	20.1±5.1
	HDSCP	8	157.3±67.7	73.6±27.0	91.4±33.6	49.0±15.4	77.1±10.1	59.8±11.9	5.2±3.9	15.9±6.4
P-Value			0.920	0.958	0.990	0.110	0.381	0.172	0.113	0.337
30 min	HTF	9	158.0±63.2	83.0±32.5	100.2±40.6	53.5±9.3	81.0±6.8	63.7±8.8	3.3±1.4	21.5±5.7
	HTFC	5	168.6±70.3	71.5±24.9	95.2±35.6	45.5±12.2	73.7±10.8	58.1±8.5	4.1±2.2	17.5±4.7
	Ham's F-10®	8	150.9±67.2	90.8±54.6	101.2±58.9	58.3±15.0	87.2±9.2	64.7±14.0	2.7±1.3	23.0±5.6
	HDSWP	8	150.8±62.7	74.5±39.7	88.5±42.8	47.7±12.5	79.5±10.5	57.1±10.8	3.4±1.2	22.6±4.3
	HDSCP	7	162.5±65.0	74.8±21.3	91.4±31.9	48.5±10.2	80.8±10.8	57.9±9.1	3.8±1.8	19.3±3.5
P-Value			0.985	0.867	0.972	0.284	0.190	0.485	0.539	0.218
60 min	HTF	3	207.0±6.0	118.9±22.9	138.4±18.9	57.7±10.6	82.1±7.7	67.0±8.1	4.1±0.9	19.4±1.6
	HTFC	3	214.0±2.1	116.1±30.8	139.5±20.5	54.5±14.4	79.6±12.0	65.5±9.6	4.3±1.0	22.9±3.6
	Ham's F-10®	3	211.7±4.2	118.9±15.6	141.2±13.1	56.5±7.1	80.8±6.2	67.0±5.3	4.1±0.6	24.2±3.3
	HDSWP	3	206.1±1.6	107.3±19.0	128.0±8.9	52.0±9.7	80.0±11.3	62.0±5.0	4.2±1.0	23.2±7.8
	HDSCP	3	213.7±1.1	105.8±26.2	129.8±16.5	49.5±12.5	78.2±13.6	60.8±8.0	4.8±0.8	19.8±5.1
P-Value			0.054	0.920	0.789	0.894	0.993	0.771	0.842	0.659

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, VCL = Curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

Table 4.29: Effects of physiological media on slow swimming rhesus sperm kinematic parameters at baseline (T0), 30 minutes (T30) and 60 minutes (T60) (mean ± SD)

Time (minutes)	Media	Number(n)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Baseline	HTF	9	82.5±43.7	45.4±29.9	54.3±32.8	51.7±12.6	73.1±14.3	63.2±8.5	1.7±1.0	14.7±7.4
	HTFC	6	103.7±46.2	57.2±24.9	69.7±30.9	56.4±4.7	75.5±5.3	67.4±3.1	2.2±0.9	12.9±4.1
	Ham's F-10®	9	83.1±43.4	45.1±29.0	52.4±32.6	50.2±9.7	75.0±9.4	59.2±9.8	1.8±0.8	15.7±7.2
	HDSWP	8	78.7±42.3	46.9±27.8	55.2±30.9	59.1±13.8	78.4±11.5	69.6±8.7	1.6±0.9	14.0±6.5
	HDSCP	9	83.3±42.7	39.0±25.5	48.3±28.6	47.9±22.8	72.0±16.3	58.8±19.9	2.4±1.8	15.9±7.8
P-Value			0.856	0.809	0.770	0.537	0.854	0.279	0.584	0.914
30 min	HTF	8	98.6±45.2	50.8±26.5	62.8±30.4	49.5±7.4	73.2±7.2	62.6±5.4	2.2±0.9	17.5±4.0
	HTFC	5	95.4±47.2	53.7±28.5	62.9±31.4	56.9±7.1	77.8±7.5	66.8±5.6	1.8±0.9	13.3±4.6
	Ham's F-10®	8	86.8±43.2	45.8±29.8	55.7±32.0	49.2±11.1	73.8±11.2	62.1±12.3	1.9±0.9	18.9±5.7
	HDSWP	8	87.3±44.9	47.9±29.2	56.0±30.9	54.1±24.5	76.9±18.7	64.1±19.1	1.7±1.0	18.1±10.3
	HDSCP	7	89.8±40.1	39.4±23.6	49.7±28.2	42.6±13.7	71.3±10.7	53.8±13.0	2.0±0.9	19.8±6.6
P-Value			0.980	0.909	0.924	0.493	0.866	0.436	0.964	0.549
60 min	HTF	3	126.8±6.7	68.4±8.3	82.8±6.6	54.2±7.0	78.1±4.2	65.4±5.6	2.8±0.5	20.2±3.3
	HTFC	3	132.8±11.5	75.4±12.1	88.4±6.6	57.2±11.0	80.4±10.5	66.9±6.7	2.6±0.6	18.7±3.1
	Ham's F-10®	3	124.3±8.0	74.7±9.6	85.9±2.7	60.0±11.4	83.3±11.2	68.9±6.3	2.4±0.8	20.7±6.6
	HDSWP	3	132.0±4.6	72.7±17.7	84.8±11.7	55.5±12.3	81.5±10.1	64.6±7.8	2.8±0.9	21.6±5.2
	HDSCP	3	131.3±5.5	68.9±19.4	84.6±14.8	53.4±13.8	76.5±12.0	65.1±9.6	2.8±0.9	18.1±5.0
P-Value			0.606	0.954	0.961	0.952	0.922	0.947	0.945	0.893

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus, VCL = Curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

Appendix L: Results of rhesus sperm percent hyperactivation

Table 4.30: Effects of physiological media on Rhesus sperm percent hyperactivation (mean ± SD)					
Hyperactivation %					
Time interval	5 min (n=5)	15 min (n=5)	30 min (n=4)	45 min (n=1)	60 min (n=1)
HTF	45.4 ± 18.2	28.5 ± 22.7	31.6 ± 40.7	No data	No data
HTFC	54.7 ± 23.5	54.0 ± 36.0	43.5 ± 37.8	83.5	45.3
Ham's F-10®	38.3 ± 26.4	16.2 ± 24.4	16.7 ± 25.8	57.8	75
HDSWP	32.9 ± 19.8	32.4 ± 19.0	24.9 ± 26.9	96.8	51.4
HDSCP	54.4 ± 39.4	22.8 ± 30.5	19.9 ± 33.1	83.6	No data
P-Value	0.624	0.272	0.799	No Data	No Data

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus.

Table 4.31: Comparison of Rhesus sperm percent hyperactivation between time intervals (mean ± SD)						
Hyperactivation %						
Time interval	5 min	15 min	30 min	45 min	60 min	P-Value
HTF	n=5	n=5	n=4	n=0	n=0	0.605
	45.4 ± 18.2	28.5 ± 22.7	31.7 ± 40.7	No Data	No Data	
HTFC	n=5	n=5	n=4	n=1	n=1	0.750
	54.7 ± 23.5	54.0 ± 36.0	43.5 ± 37.8	83.5	45.3	
Ham's F-10®	n=5	n=5	n=4	n=1	n=1	0.316
	38.3 ± 26.4	16.2 ± 24.4	16.7 ± 25.8	57.8	75	
HDSWP	n=5	n=5	n=4	n=1	n=1	0.075
	32.9 ± 19.7	32.4 ± 19.0	24.9 ± 26.9	96.8	51.4	
HDSCP	n=5	n=5	n=4	n=1	n=0	0.239
	54.4 ± 39.4	22.8 ± 30.5	19.9 ± 33.1	83.6	No Data	

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus.

Appendix M: Results of rhesus sperm vitality

Table 4.32: Effects of physiological media on rhesus sperm vitality at baseline (0 minutes), 30 minutes (T30), and 60 minutes (T60) (mean ± SD)

Parameter	Physiological media				
	HTF	HTFC	Ham's F-10®	HDSWP	HDSCP
Vitality (%)	Baseline				
	n=3	n=3	n=2	n=2	n=2
	60.7 ± 12.3	52.3 ± 10.7	65.0 ± 8.5	61.0 ± 16.9	64.0 ± 5.0
	30 min				
	n=2	n=2	n=2	n=2	n=2
	63.6 ± 44.6	49.4 ± 10.0	57.5 ± 2.1	66.3 ± 9.5	73.8 ± 8.9
	60 min				
	n=1	n=1	n=0	n=0	n=0
	46.5	51.0	No Data	No Data	No Data

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus. T0 = baseline, T30 = 30 minutes.

Table 4.33: Comparison of rhesus sperm vitality between time intervals (mean ± SD)

	Vitality %		
Time interval	Baseline	30 min	60 min
HTF	n=3	n=2	n=1
	60.7 ± 12.3	63.6 ± 44.6	46.5
HTFC	n=3	n=2	n=1
	52.3 ± 10.7	49.4 ± 10.0	51.0
Ham's F-10®	n=2	n=2	n=0
	65.0 ± 8.5	57.5 ± 2.1	No Data
HDSWP	n=2	n=2	n=0
	61.0 ± 16.9	66.3 ± 9.5	No Data
HDSCP	n=2	n=2	n=0
	64.0 ± 5.0	73.8 ± 8.9	No Data

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus.

Appendix N: Results of rhesus sperm acrosome parameters

Table 4.34: Effects of physiological media on rhesus sperm acrosome parameters at baseline (T0), 30 minutes (T30), and 60 minutes (T60) (mean ± SD)

Parameter	Physiological media				
	HTF	HTFC	Ham's F-10®	HDSWP	HDSCP
Baseline					
Intact (%)	n=1	n=2	n=1	n=2	n=2
	98	93.5 ± 5.0	93	92 ± 4.2	95 ± 7.1
30 min					
Intact (%)	n=2	n=2	n=2	n=2	n=2
	96.5 ± 0.7	96 ± 1.4	84.5 ± 5.0	94 ± 2.8	93 ± 1.4
60 min					
Intact (%)	n=2	n=2	n=2	n=2	n=2
	90.5 ± 3.5	93.5 ± 0.7	89 ± 8.5	87 ± 8.5	82 ± 17.0

Parametric data presented as mean and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP= HD Sperm Capacitation Plus. T0 = baseline, T30 = 30 minutes, T60 = 60 minutes.

Table 4.35: Comparison of rhesus sperm acrosome integrity between time intervals (mean ± SD)

	Acrosome intactness %		
Time interval	Baseline	30 min	60 min
Number (n)	n=1	n=2	n=2
HTF	98	96.5 ± 0.7	90.5 ± 3.5
Number (n)	n=2	n=2	n=2
HTFC	93.5 ± 5.0	96 ± 1.4	93.5 ± 0.7
Number (n)	n=1	n=2	n=2
Ham's F-10®	93	84.5 ± 5.0	89 ± 8.5
Number (n)	n=2	n=2	n=2
HDSWP	92 ± 4.2	94 ± 2.8	87 ± 8.5
Number (n)	n=2	n=2	n=2
HDSCP	95 ± 7.1	93 ± 1.4	82 ± 17.0

Parametric data presented as mean (95% confidence interval) and standard deviation (SD). As obtained from ANOVA. HTF = Human tubal fluid, HTFC = Human tubal fluid with caffeine; HDSWP = HD Sperm Wash Plus, HDSCP = HD Sperm Capacitation Plus.