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SPERM FUNCTION IN DIFFERENT HUMAN SPERM SUBPOPULATIONS

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Medical Bioscience, University of the Western Cape

By Shannen Keyser

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Sperm function in different human sperm subpopulations

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KEYWORDS

Semen heterogeneity

Differential density centrifugation

Computer-aided sperm/semen analysis

Acrosome reaction

Hyperactivation

Mitochondrial membrane potential

Reactive oxygen species

Sperm motility

Sperm kinematics

Progesterone

Myo-inositol

Prolactin

Dopamine

HD-C

FAST

ABSTRACT

Sperm function in different human sperm subpopulations

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Human semen contains a heterogenous population of spermatozoa of which only a certain fraction is physiologically suitable for fertilization. Functional characteristics of these subpopulations and sperm separation techniques are not well understood. Furthermore, the questionable reliability of basic semen analyses has led to more emphasis being placed upon the assessment of detailed sperm functional parameters in determining male factor infertility as well as media supplements that could possibly enhance male fertility. Our study was designed to investigate and compare the functional characteristics of two sperm motility subpopulations and to determine which group of semen parameters provide predictive value into the presence of a high motility sperm subpopulation. In addition, the study aimed to investigate the effects of biological components present in follicular fluid on sperm functionality of different sperm motility subpopulations. Furthermore, it was aimed to determine whether the use of a new flagellar analysis and sperm tracking program (FAST) may provide more information on sperm motility functions as compared to computer-aided sperm analysis (CASA).

Semen was separated into a high motile (HM) and low motile (LM) sperm subpopulation via double density gradient centrifugation. Subpopulations in human tubal fluid (HTF) were assessed for functional characteristics namely sperm viability, motility and kinematics, hyperactivation, acrosome reaction, reactive oxygen species and mitochondrial membrane potential, chromatin intactness and maturity. In the second phase, subpopulations were exposed to various media namely, HTF, capacitating medium (CAP), HD capacitating medium (HD-C), progesterone, myo-inositol, dopamine and prolactin, and subsequently subjected to the same functional tests as mentioned above. In the final phase of the study, subpopulations were exposed to selected concentrations of the media and individual spermatozoa further analysed with CASA and FAST for comparison of kinematic and flagellar characteristics.

Two distinct sperm motility subpopulations were observed, with HM sperm subpopulations displaying significantly improved functionality compared to LM sperm

subpopulations. Semen morphology abnormalities correlated with grouped motility parameters of the LM subpopulation. On the other hand, a combined group of semen total motility, progressive motility, viscosity and mucus penetration correlated with HM subpopulations' grouped motility parameters. Media did improve sperm functionality, however progesterone and myo-inositol had the greatest effects on HM subpopulations, whereas dopamine and prolactin were more favourable to LM subpopulations. Furthermore, variable effects were observed for media on functional parameters.

Collectively, total and progressive motility, viscosity and mucus penetration present a reliable group of semen characteristics for predicting the quality of a HM sperm subpopulation. Separating the same donor semen samples into two significantly varying motility sperm subpopulations can be a new approach to mimic the qualities of fertile and subfertile males' sperm populations. Although motility subpopulations respond differently to various media, the study concludes that treatment of subfertile semen samples with biological substances present in follicular fluid could assist to develop new strategies for IVF treatment. Finally, FAST and SCA present with similar kinematic parameters, however flagellar parameters provide predictive value into rapid and medium progressive speed groups of individual spermatozoa.

January 2022

DECLARATION

Hereby I, the undersigned, declare that the thesis *"Sperm function in different human sperm subpopulations"* is my own work, that it has not been submitted previously 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.

Shannen Keyser

Signed:  **Date:** 13/04/2022

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ABBREVIATIONS

%	Percentage
˚C	degrees Celsius
µL	microliters
µM	micro molar
µm	micrometres
µm/s	micrometres per second
10^6/ejaculate	million per ejaculate
10^6/mL	million per millilitre
ADP	adenosine diphosphate
ALH	amplitude of lateral head displacement
AMH	anti-mullerian hormone
ANOVA	analysis of variance
AR	acrosome reaction
ART	artificial reproductive techniques
ATP	adenosine triphosphate
AVI	audio video interleave file
BCF	beat cross frequency
BSA	basic semen analysis
Ca^{2+}	calcium ions
CaCl	calcium chloride
Caf	caffeine
cAMP	cyclic adenosine monophosphate
CAP	capacitating medium
CASA	computer-aided sperm analysis
CatSper	cation channel of sperm
CI	confidence interval
CIAR	calcium ionophore induced acrosome reaction
CICR	calcium induced calcium release
CO_2	carbon dioxide
COC	cumulus oophorus complex
cP	centipoise

DAG	diacylglycerol
DAT	dopamine transporter5
DGC	density gradient centrifugation
dH_2O	distilled water
DHE	dihydroethidium
DI	deformity index
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOPA	dopamine
DRD	dopamine-like receptors
E/N	eosin nigrosin
ETC	electron transfer chain
f/s	frames per second
FACS	fluorescence activated cell sorting
FADH	flavin adenine dinucleotide
FAST	flagellar analysis and sperm tracking
fAWL	flagellar arc-wavelength
fAWS	flagellar arc-wave speed
fBF	flagellar beat frequency
FF	follicular fluid
FITC-PNA	fluorescein isothiocyanate-labelled peanut agglutinin assay
FL	flagellar length
FS	fibrous sheath
FSH	follicle stimulating hormone
g	grams
Gd	glycodelin
GH	growth hormone
GTP	guanosine triphosphate
HA	hyperactivation
hCG	human chorionic gonadotropin
HCl	hydrogen chloride
HCO^{3-}	bicarbonate
HD-C	HD capacitating medium
HM	high motile

HSA	human serum albumin
HTF	human tubal fluid
Hz	hertz
ICSI	intracytoplasmic sperm injection
IMM	inner mitochondrial membrane
IP3	inositol 1,4,5-trisphosphate
IUI	intrauterine insemination
IVF	*in-vitro* fertilization
Jak2	janus kinase
KCl	potassium chloride
kDa	kilodaltons
KH_2PO_4	monopotassium phosphate
L-DOPA	levodopa
LH	luteinizing hormone
LIN	linearity
LM	low motile
MAD	mean angular displacement
MAI	multiple anomalies index
MAPK	mitogen-activated protein kinase
Max	maximum
Med	medium
$MgSO_4$	magnesium sulphate
Min	minimum
min	minutes
mL	millilitres
mM	millimolar
MMP	mitochondrial membrane potential
MP	medium progressive
mROS	mitochondria-produced ROS
mtDNA	mitochondrial DNA
MYO	myo-inositol
Na lactate	sodium lactate
Na pyruvate	sodium pyruvate
NaCl	sodium chloride

NADH	nicotinamide adenine dinucleotide
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
ng/mL	nanogram per millilitre
nM	nanomolar
nm	nanometres
NOXs	NADPH oxidases
NP	non-progressive
ODF	outer dense fibers
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
PA	particle area
PAF	platelet-activating factor
PBS	phosphate buffered saline
PI	phosphatidylinositol
PI3K	phosphoinositide 3-kinase
PIP	polyphospho-inositides
PKA	protein kinase-A
PKC	protein kinase-C
PLC	phospholipase-C
Pos	positive
PRG	progesterone
PRL	prolactin
Pro	procaine
Prog	progressive
PSA	prostate-specific antigen
PTK	protein tyrosine kinase
PUFA	poly unsaturated fatty acids
Rap	rapid
ROS	reactive oxygen species
RP	rapid progressive
RT	room temperature
SACY	soluble adenylyl cyclase
SCA	Sperm Class Analyser

SD	standard deviation
sDF	sperm DNA fragmentation
SOCs	store operated channels
SP *p*	significant differences between subpopulations
STATs	signal transducer and activator of transcription
STR	straightness
TCS	track centroid speed
Total Mot	total motility
TP *p*	significant differences between time points
TZI	tetrazoospermic index
UJI	utero-tubal junction
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight-line velocity
WHO	World Health Organisation
WOB	wobble
ZP	zona pellucida

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CHAPTER ONE: Outline

1.1 | Introduction

Infertility is an increasing global condition affecting 70 million couples, with about 50% of cases being due to a male factor (Jungwirth *et al*., 2012; Gatica *et al*., 2013; Punab *et al*., 2017; Shah *et al*., 2021). A few large-scale epidemiological studies have addressed this topic (Pierik *et al.,* 2000; Tüttelmann *et al.*, 2011); however, it remains essential that more research is focussed on the diverse causes providing explanation in male infertility and that more personalized treatments for the condition be explored. The common path followed for investigating the conditions that effect male fertility usually comprise of patient history, physical examination, and basic semen analysis (Esteves, 2016). However, in approximately 15% of cases, conventional semen analysis results do not reveal obvious abnormalities, thereby conveying the importance of sperm heterogeneity and alternative approaches such as functional and structural sperm tests (Evgeni *et al*., 2014; Esteves *et al*., 2017).

Human semen consists of a heterogeneous cell population with different degrees of maturation, varying in functional quality and fertilizing ability (Alvarez *et al*., 2003; Sousa *et al*., 2011; García-Peiró *et al*., 2012; Santolaria *et al*., 2016). Knowledge of semen heterogeneity has led to many attempts to characterize and improve the sorting and selection of distinct subpopulations of spermatozoa for assisted reproductive technology (ART) purposes (Varum *et al*, 2007). A spermatozoon's capability to reach and fertilize an ovum depends on its competence of undergoing various functional processes, therefore separation of subpopulations for ART should ultimately result in a highly functional subpopulation of recovered spermatozoa.

Assisted reproductive technology represents an important advance in human reproductive medicine and has become a standard intervention for couples with infertility problems (Grafodatskaya *et al*., 2013). Considering the decline in male fertility and the increasing delay of pregnancy by couples, it is likely that ART will be even more widely used in the future (Bellieni, 2016). Despite the rise in ART and total number of pregnancies, ART may be associated with potential risks to the mother and foetus (Grafodatskaya *et al.*, 2013). Moreover, success of these techniques is largely dependent upon lifestyle, and failure can inflict heavy financial and psychological burdens on patients (Zeinab *et al*., 2015). In addition, with the advent of procedures such as intracytoplasmic sperm injection (ICSI), appropriate

investigations including diagnosis and treatment of male factor infertility remain a challenge. As such, men with medical pathologies such as primary infertility are often neglected, as informative andrological analysis is not explored before opting for ART (Punab *et al*., 2017).

Furthermore, despite increased use of ART, 68.5% of *in-vitro* fertilization (IVF) cycles do not result in a live birth (Miner *et al*., 2018) and overall, ART success rates have remained at around 25% for the past 30 years (Drevet, 2016). Thus, a major challenge remains to better characterize and isolate particularly good sperm subpopulations or to even improve subpopulations' functionality. ICSI has become the main selected ART option in more than half of the assisted reproductive cycles conducted each year, with many programs applying 100% ICSI cycles (Leisinger *et al*., 2021). The selection of normal, highly progressive sperm for ICSI is suggested to result in improved fertilization and lower miscarriage rates (Leisinger *et al*., 2021). Therefore, exploring alternative techniques which may improve aspects such as progressive motility or fertilizing capacity can have a significant effect on the outcome of a cycle for patients seeking fertility treatment (Leisinger *et al*., 2021). In addition, such improvements may even broaden the option for patients to select a less invasive and cost effect procedure such as intrauterine insemination (IUI), which can assist several fertility disorders such as idiopathic infertility (Tonguc *et al*., 2010; Ombelet, 2017).

1.2 | Aims

The main aim of this study was to investigate and compare a comprehensive set of 96 functional characteristics of two motility sperm subpopulations in human semen. This study attempted to elucidate if specific basic semen parameters relate to individual motility subpopulations, and whether a grouping of semen parameters could provide a possible predictive outcome towards the HM subpopulation. This study also investigated the unique effects of various components of follicular fluid on individual subpopulations and whether a medium containing progesterone and myo-inositol would be more effective in enhancing subpopulations than an individual component alone. A concomitant aim of the study was to establish whether selected concentrations of media alter the flagellar beat characteristics across sperm motility subpopulations and whether flagellar beat characteristics would be more predictive of sperm quality than normal CASA parameters.

1.3 | Objectives

The objectives of this study were to:

- Perform a basic semen analysis on each semen sample, including assessment of volume (mL), pH, viscosity (cP), total sperm motility (%), progressivity (%), mucus penetration (10^6/ejaculate), concentration (both in 10^6/ejaculate and 10^6/mL measured) and morphology (%).
- Separate semen seminal plasma from spermatozoa and further divide spermatozoa into two subpopulations, namely highly motile (HM) spermatozoa and less motile (LM) fractions.
- Evaluate and compare the two subpopulations for various sperm functional parameters such as vitality (%), sperm motility and kinematics parameters, hyperactivation (%), reactive oxygen species (%, ROS), mitochondrial membrane potential (%, MMP), acrosome reaction (%, AR), sperm maturity and chromatin integrity (%).
- Relate individual subpopulations' functional parameters back to neat semen samples basic semen analysis (BSA) and to determine whether grouped semen parameters have a predictive power on HM subpopulations.
- Expose both sperm subpopulations to human tubal fluid (HTF), capacitating medium (CAP), HD Capacitating medium (HD-C), progesterone (1.98 µM, 3.96 µM and 19.8 µM), myo-inositol (11 mM), dopamine (20 nM, 100 nM and 1 µM) and prolactin (50 ng/mL, 100 ng/mL, 250 ng/mL, and 500 ng/mL)

- Evaluate the effect of each medium on subpopulations functional characteristic namely, vitality (%), motility and kinematics parameters, hyperactivation (%), ROS (%), MMP (%), and AR (%).
- Expose subpopulations to selected concentrations of progesterone, myo-inositol, dopamine, and prolactin and to analyse both CASA and FAST motility and kinematics parameters after treatment.
- Compare the various motility and kinematic parameters of CASA and FAST.
- Determine if selected FAST parameters provide a more reliable predictive model than CASA parameters for motility and kinematic parameters.

1.4 | Overview of Thesis Chapters

This thesis is divided into five main chapters.

Chapter 1: *General introduction*

This chapter includes a general introduction including the aims and objectives of the undertaken study. Furthermore, at the end of the thesis references and various appendices related to the investigation are included.

Chapter 2: *Literature overview*

An overview of the literature relating to semen and sperm heterogeneity, barriers of the female reproductive tract, components of follicular fluid and their effects in sperm functionality of sperm subpopulations.

Chapter 3: *Methodology*

Chapter three includes a description of the methods and materials used in the three different phases of the study. Phase one of the study included the separation of semen into motility subpopulations and the functional testing of the subpopulations in HTF medium. Phase two included the separation of semen into subpopulations and treatment of the subpopulations with HTF, CAP, HD-C, progesterone, myo-inositol, dopamine, and prolactin. After incubation in the treatments using different concentrations, subpopulations were tested for the various functional parameters. Phase three of the study involved the separation of semen into subpopulations, after which subpopulations were treated with selected concentrations of the media used in phase two. After incubation the subpopulations motility and kinematic parameters were analysed with both SCA® and FAST software.

Chapter 4: *Results*

Chapter four includes the results produced from the initial, second and third phase of the current study. The results for each phase of the study are describe in different subheadings as indicated in Chapter four. This chapter contains various tables, graphs, and multivariate visualisations as part of the results.

Chapter 5: *Discussion*

This chapter involves a discussion of the results and relates the findings to previous investigations and literature.

Chapter 6: *Conclusion*

Chapter six critically extracts the major findings of this investigation.

1.5 | Research Output

1.5.1 | Publications

Publish abstract - Keyser, S., van der Horst, G. and Maree, L., 2021. Improved Sperm Function in Human Sperm Subpopulations: A Model for Studying Subfertility. In "*XIIIth International Symposium on Spermatology*" (pp. 367-368). Springer, Cham (Appendix 1).

Published Article - Keyser, S.; van der Horst, G.; Maree, L. Progesterone, Myo-Inositol, Dopamine and Prolactin Present in Follicular Fluid Have Differential Effects on Sperm Motility Subpopulations. *Life* 2021, 11, 1250. https://doi.org/10.3390/ life11111250 (Appendix 2).

Published Article - Keyser, S.; van der Horst, G.; Maree, L. New approaches to define the functional competency of human sperm subpopulations and its relationship to semen quality. *IJFS,* accepted and in press (Appendix 3).

1.5.2 | Conference proceedings

Oral presentation - Symposium of Scientific Training of the Polish Society of Andrology – 19th day of Andrology Kraków, 17–18.11.2017- *"A model to study poor and good sperm functionality of the same patient using CASA".*

Poster presentation - XIIIth International Symposium on Spermatology Stockholm, May 9-13, 2018 – "*Improved sperm function in human sperm subpopulations: a model for studying subfertility"* (Appendix 4).

Poster presentation – Reproductive Medicine Conference, Johannesburg, November 23-25, 2018 – "Progesterone and Myo-inositol improve sperm functionality in subfertile human sperm subpopulations". (Appendix 5).

Oral online presentation - SASREG SIG Embryology Meeting, Tuesday, 24th August 2021, 4:00 PM Cape Town, Cairo, Harare, Johannesburg, Pretoria GMT +2
"Title: *Does media matter? Evidence from two sperm motility subpopulations".*

CHAPTER TWO: Literature Review

2.1 | Basic Semen Analysis and Semen Heterogeneity

At least 15% of couples engaged in unprotected intercourse experience infertility (Agarwal *et al*.,2015). In 50% of involuntarily childless couples, male infertility is associated with abnormal semen parameters (Pásztor *et al*., 2021), as identified using reference values that have been published and modified by the World Health Organization (WHO) since 1987 (Milachich and Dyulgerova-Nikolova, 2020). However, the usefulness and significance of basic semen analysis parameters are a point of contention (Björndahl, 2010; Patel *et al*., 2017), with some deliberations that the results of basic semen analysis are poor indicators of true male fertility (Tomlinson, 2016). Furthermore, there is also confusion about different purposes of semen analysis since a basic semen analysis is also used to generate markers for male fertility in population-based epidemiological and toxicological studies (Tomlinson, 2016).

The wide variation in reported normal values for semen analysis contributes to confusion and scepticism, with even the most objective parameter (concentration) varying amongst laboratories (van der Horst and du Plessis, 2017). In addition, other parameters, such as morphology and motility, are difficult to interpret since research and clinical laboratories differ greatly in their uniformity of analysis and methods (Chong *et al*.1983). Nevertheless, total motile sperm count is suggested to have a greater predictive value of high-quality embryo production, pregnancy outcomes and the odds of miscarriage, as compared to any other WHO 2010 cut-off value (Leisinger *et al*. 2021). Recent publications that have better defined populations and better awareness of the need for quality control have demonstrated that traditional semen parameters can provide some degree of prognostic and diagnostic information to infertile couples (Tomlinson *et al*., 2013). Such information includes a prediction of the general quality of a semen sample and possible indication of the general reproductive health of the donor/patient (van der Horst and du Plessis, 2017). Even with rigorous quality control procedures, these parameters are most useful at the lower end of the spectrum-even then, they should only be used as guidance for couples, not as absolute fertility measures (van der Horst and du Plessis, 2017). Furthermore, the presence of various functional abnormalities is typically associated with an inability to conceive naturally in addition to fertilization failure or low rates of fertilization during *in-vitro* fertilization (IVF) therapy (Oehninger and Franken, 2006). As such, basic semen analysis is not a reliable diagnostic tool for male infertility, but rather an initial evaluation.

Human semen is known to contain a distinct heterogeneous cell population with varying maturation, functionality, and fertilizing ability (Alvarez *et al*., 2003; Sousa *et al*., 2011; García-Peiró *et al*., 2012; Ramone *et al*., 2014; Santolaria *et al*., 2016). These subpopulations serve a physiological purpose, and although the exact role remains ambiguous, correlations have been observed between sperm quality and fertility as well as the ability to withstand cryopreservation damage (Henkel and Schill, 2003; Quintero-Moreno *et al*., 2003; Buffone *et al*., 2004; Dorado *et al*., 2011; Jayaraman *et al*., 2012; Ramone *et al*., 2014; Peña *et al*., 2012; Santolaria *et al*., 2016; Ibănescu *et al*., 2018). Subpopulations are often ignored in basic semen analysis, which could impede an accurate assessment of sperm quality in general, with overlaps previously observed between the characteristics of both fertile and infertile men (Lewis, 2007; Hamada *et al*., 2012; Bompart *et al*., 2018; Oehninger and Ombelet, 2019). In practice, most routine semen analysis is based on small amounts of spermatozoa from a larger heterogeneous group present in the single ejaculate, meaning that results will inherently have a large variability (Hossain *et al*., 2011). Therefore, semen parameter values do not necessarily reflect the functional integrity of all the spermatozoa in the different subpopulations. As a result, identifying the properties of the various individual subpopulations would also provide insight into the development of relevant tests to assess semen quality (Holt and van Look, 2004; Dorado *et al*., 2011; Caroppo, 2013).

While it is possible to separate the various sperm subpopulations through utilizing distinct techniques, characterizing and isolating superior sperm subpopulations remains a challenge in modern andrology (Sousa *et al*., 2011; Gloria *et al*., 2016). There has been an increasing number of sperm selection techniques developed based on differentiation methods for sperm density, membrane surface charge, morphology, motility, and membrane integrity (Vaughn and Sakkas, 2019). An example of this is colloid centrifugation, which separates a subpopulation of motile spermatozoa with good functional and structural integrity (Morrell, 2016). Nonetheless, discontinuous density gradient centrifugation (DGC) has been observed to result in high levels of DNA damage and reactive oxygen species (ROS) production (Muratori *et al*., 2019). Nonetheless, reports have indicated higher percentages of motile and morphologically normal spermatozoa present in the pellet of density gradient separated semen, in comparison with lower density fractions (Buffone *et al*., 2004; Johannisson *et al*., 2009; Morrell *et al*., 2010; Martínez-Pastor *et al*., 2011; Gosálvez *et al*., 2014; Gamboa *et al*., 2017). In addition, the recovered higher motile sperm subpopulations contained more spermatozoa with functional mitochondria, lower levels of ROS and few spermatozoa with apoptotic and necrotic markers (Sousa *et al*., 2011). More comprehensive and in-depth sperm assessment methodologies may reveal unknown factors affecting male fertility particularly in cases of

idiopathic male infertility, thereby assisting in more individualized infertility treatments (Punab *et al*., 2017). As such, advancements in understanding and evaluating the biochemical and molecular mechanisms regulating human sperm functionality and subpopulations can assist the clinician in selecting the best ART treatment and in characterizing and isolating superior sperm subpopulations (Oehninger *et al*., 2014; Talwar and Hayatnagarkar, 2015).

2.2 | Sperm Structure and Functional Tests

Several structural and functional abnormalities have been detected in the spermatozoa of subfertile men's semen (Oehninger and Franken, 2006). Such abnormalities predominantly include failure to achieve capacitation, inability to bind to the zona pellucida (ZP) and undergo acrosomal exocytosis, as well as the presence of nuclear/chromatin defects such as deoxyribonucleic acid (DNA) fragmentation and aberrations of nuclear proteins (Oehninger and Franken, 2006; Björndahl, 2010). Despite being commonly associated with subfertile semen, these abnormalities can, however, be observed when basic semen parameters are normal, subnormal, or abnormal (Oehninger and Franken, 2006; Björndahl, 2010). Consequently, 25% of infertile men are diagnosed as normozoospermic. However, despite their normal appearing sperm, such patients often fail to achieve parenthood (Puga Molina *et al*., 2020). Thus, standard semen assessment needs to be expanded to incorporate alternative diagnostic approaches (Jakubik-Uljasz *et al*., 2020), especially when borderline semen analysis results are identified (Talwar and Hayatnagarkar, 2015).

The advancements in IVF technology, such as intracytoplasmic sperm injection (ICSI), have led many to feel that sperm functional tests are irrelevant (Talwar and Hayatnagarkar, 2015). Nevertheless, semen analysis has limited diagnostic value for assessing sperm fertilization competency due to the existence of multiple types of sperm defects that cannot be identified by conventional semen analysis (Jakubik-Uljasz *et al*., 2020). It is desirable that sperm functional tests diagnose particular sperm aberrations, predict fertility and pregnancy rates, as well as indicate an appropriate treatment for the disorder (Kizilay and Altay, 2017). For example, by determining whether spermatozoa are able to undergo acrosome reaction (AR), hyperactivation, or if DNA fragmentation is present, the fertilizing potential of samples can be estimated with more certainty (Ansari *et al*., 2018).

In the following section, we discuss and describe several sperm structural and functional tests performed during this study. Tests selected included both the basic semen analysis (motility, vitality, and morphology) as well as more in-depth parameters, including motility speeds and kinematics, hyperactivation, acrosome reaction, reactive oxygen species,

mitochondrial membrane potential, and DNA fragmentation. In addition to their informative nature, functional and structural tests were selected to assess the fertilization potential of spermatozoa.

2.2.1 | Morphology

Human spermatozoa have a head, partially hidden by an acrosome, a neck, and a flagellum consisting of a connecting piece, mid-piece, principal piece and end piece (Rahman *et al*., 2013) (Figure 1.1). Formation of various accessory structures during its development provide spermatozoa with motility and protection from harsh environments in the female reproductive tract (Sutovsky and Manandhar, 2006). Sperm morphological assessments related to structural normality of sperm head, midpiece, flagellum, and flagellar components, are thus proposed as useful diagnostic tools in the treatment of male infertility (Gu *et al*., 2019).

As the acrosome and nuclei vary in size, a range of sperm heads differing in width and length can be observed in a human semen sample (Gu *et al*., 2019). During spermatogenesis, sperm-specific DNA-binding proteins, known as protamines, replace histones in the nucleus, enabling higher chromatin packaging and reducing cell size which improves aerodynamic properties and facilitates fertilization (Bloom, 1975; Sutovsky and Manandhar, 2006; Henkel, 2012). The acrosome contains hydrolytic enzymes that are released during the acrosome reaction (AR) and function in sperm penetration of the glycoprotein-based ZP surrounding the oocyte (Tosti and Menezo, 2016). Adjacent to the sperm head lies the connecting piece containing atypical centrioles. The centrioles lack structural characteristics and protein compositions compared with centrioles in somatic cells (Gu *et al*., 2019).

At the anterior portion of the tail lies the midpiece that is composed of cytoskeletal structures and surrounded by packed mitochondria (Lamirande and Gagnon, 1993; Ramalho-Santos *et al*., 2009; Zabeo *et al*., 2018, Gu *et al*., 2019). The mitochondria produce adenosine triphosphate (ATP) and transport energy to the axoneme in the principal piece, thereby producing the sperm's driving force. Sperm swimming velocity may therefore be determined by the length of the midpiece and the principal piece. The mitochondrial sheath further produces optimal levels of ROS and ATP while preventing internal structures from buckling out of the flagellum during swimming (Gu *et al*., 2019).

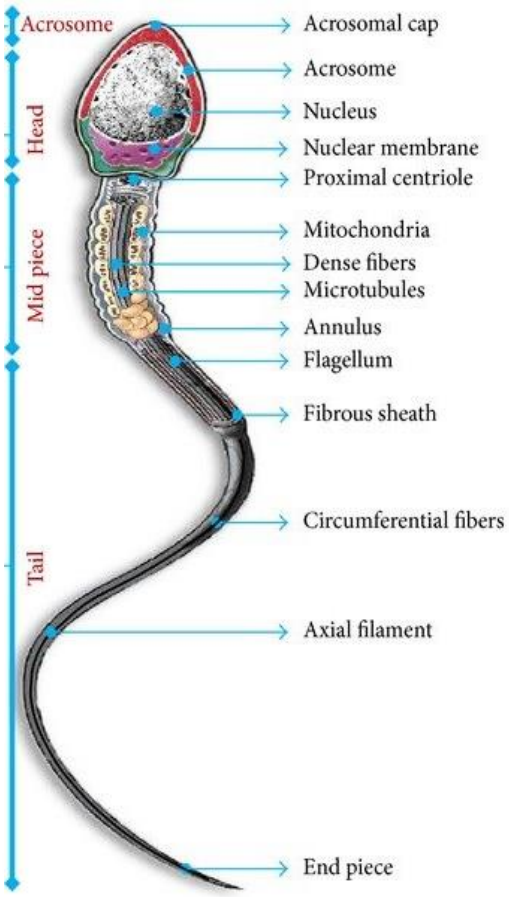

Figure 1.1 Diagram of a mature human spermatozoon and accessory structures. ***Note:*** proteins contained in the acrosome, head, midpiece, and tail are primarily involved in capacitation and acrosome reaction, zona pellucida binding, energy production and metabolism, and motility and metabolism, respectively (Rahman *et al*., 2013).

Encasing the cytoskeleton is the fibrous sheath (FS) of the principal piece, whereas the end piece without any accessory structure, can be described as a continuation of the principal piece (Figure 1.2). Studies suggest that the FS supports glycolysis, cyclic-AMP (cAMP) mediated signalling transduction and mechanical support (Miki *et al*., 2004; Eddy, 2007; Gu *et al*., 2019). The sperm tail consists of a 9+2 microtubular axoneme with nine doublet pairs of peripheral microtubules arranged in a circle around the central pair (Alvarez, 2017). The outer dense fibers (ODF) and FS of the axoneme are thought to protect the tail during epididymal transport and ejaculation as well as increasing flagellum stiffness and stabilization (Baltz *et al*., 1990; Zhao *et al*., 2018; Gu *et al*. 2019) (Figure 1.2).

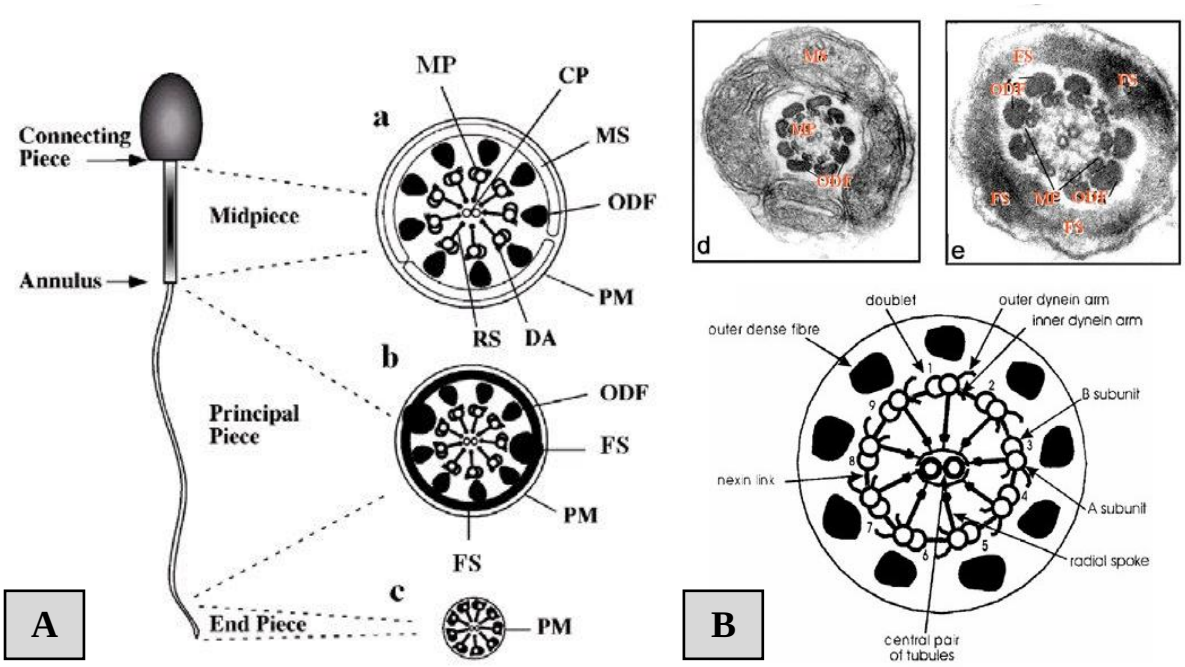

Figure 1.2 Schematic illustration of a human spermatozoa. **(A)**. Longitudinal section illustrating the sperm head, midpiece, principal piece and end piece. **(B)** Organization of the axoneme. ***Note:*** **(A)** The insets on the right indicate the cytoskeletal organization of the sperm tail in transverse sections at different levels; middle (a), principal (b) and end piece (c). Electron microscopy of the transverse sections of the sperm tail at the level of the middle (d) and the principal piece (e) is reported in the lower part of the figure. **Abbreviations:** CP, central pair; DA, dynein arms; FS, fibrous sheath; MP; microtubule pairs MS: mitochondria; ODF: outer dense fibers; PM: plasma membrane; RS, radial spoke (Luconi *et al*., 2006).

The use of sperm morphology as a fertility indicator and its correlation with success rates in ART has been subject to substantial debate (Danis and Samplaski, 2019; Lüpold and Pitnik, 2021). Older reports suggest sperm morphology as a potential predictor for fertilizing ability, which is said to correlate with IVF rates (Shibahara *et al*., 2002). Growth *et al*. (1994) proposed sperm morphology as an excellent predictor of sperm fertility, independent of motility and concentration, and that the presence of <4% normal sperm might indicate a poor likelihood of conceiving. However, according to more recent data, even couples with abnormal sperm morphology (0%) can achieve pregnancy through natural conception, IUI, or IVF (Danis and Samplaski, 2019).

Despite subfertile men having lower proportions of normal forms compared to men with proven fertility, the question of "Does form impact function?" remains (Danis and Samplaski, 2019). For instance, spermatozoa in cervical mucus cannot be proven to be more functional than those with the same appearance and form in seminal plasma (Kovac *et al.,* 2017). Furthermore, spermatozoa's genetic makeup and fertilization capabilities cannot be solely determined by morphology. As such, strict morphology should not be used to predict fertilization, pregnancy, or live birth potential, and in cases of men with 0% normal forms, alternative reproductive tests should be considered before immediate IVF (Danis and Samplaski, 2019).

2.2.2 | Vitality

Apart from a high percentage of abnormal spermatozoa, male infertility can result from an impairment in the structural and functional competence of the sperm membranes (Eskandari and Momeni, 2016). Although sperm membranes play an important role in multiple cellular processes, their molecular mechanisms are poorly understood (Ushiyama *et al*., 2017). Rich in polyunsaturated fatty acids (PUFA) and differing from the membranes of somatic cells, spermatozoa are said to cease lipid and protein synthesis in the plasma membrane after leaving the testis (Flesch and Gadella, 2000). However, once sperm cells are activated by capacitation factors, reorientation and modification the plasma membrane molecules are evident, thus linking the plasma membrane integrity to sperm capacitation (Flesch and Gadella, 2000; Zhou *et al*., 2010). Damage to the plasma membrane impairs the ability for sperm to function properly, which leads to reduced fertility (Aurich, 2005). Additionally, intact and functioning plasma membranes are essential to protect against the harsh environment of the vagina and oxidative stress (Talwar and Hayatnagarkar, 2015).

In modern basic semen analysis, robust, reliable and easy-to-use assessment methods are fundamental, including sperm vitality conducted promptly at controlled temperatures (37℃). Vitality analysis is fundamental in differentiating between immotile dead and immotile live sperm (Björndahl *et al*., 2003). Vitality is therefore known to correlate with motility and is essential to assess in the presence of very low motility (<40% progressive motility) (World Health Organization, 2010). Vitality provides a guarantee of the motility assessments' accuracy, as the percentage of live spermatozoa should exceed the percentage of motile spermatozoa (Talwar and Hayatnagarkar, 2015). Measured by either dye exclusion or osmo-regulatory capability under hypo-osmotic conditions, sperm vitality represents the proportion of live, membrane-intact spermatozoa (Munuce *et al*., 2000; Moskovtsev and Librach, 2013). As part of ART procedures like ICSI, tests determining sperm vitality are often used to identify and select only spermatozoa capable of sustaining life (Kovac *et al*., 2017).

2.2.3 | Motility

The acquisition, maintenance, and modulation of sperm motility is essential in male fertility (Freitas *et al*., 2017; Nowicka-Bauer *et al*., 2021). Characterized by significantly reduced sperm motility (<40% total motility and <32% progressive motility), asthenozoospermia is a common cause of male infertility (Zhao *et al*., 2018). Three typical movement patterns can be observed in sperm samples, namely progressive motility (a linear movement of spermatozoa in the field of view), non-progressive motility (spermatozoa that has

no progression) and immotility (WHO, 2010; Moscatelli *et al.*, 2019). Primary/activated motility is acquired in the epididymis and drives spermatozoa in a straight line in seminal plasma (Freitas *et al.*, 2017). Once in the fallopian tubes, sperm must acquire hyperactivated motility which is characterized by high amplitude and asymmetric flagellar bending (Freitas *et al.*, 2017).

According to the WHO manual (2021), spermatozoa can be classified according to various progressive speed groups. Swimming progressive speed classifications include rapid progressive (≥25 µm/s), slowly progressive (>5 µm/s and <25 µm/s) and non-progressive (<5 µm/s) (WHO, 2021). Kinematics are 'time-varying geometric aspects of motion that are distinct from calculations of mass and force' (Mortimer, 1997). Generally, three velocity values are used as a description of sperm centroid movement (Mortimer, 1997; Dunson *et al.*, 1999; Lu *et al.*, 2014), including curvilinear velocity (VCL), straight-line velocity (VSL) and average-path velocity (VAP) (Figure 1.3). Computer-assisted sperm analysis (CASA) allows for accurate and precise assessment of various motility parameters and illustrating the pattern of sperm kinematic movements (van der Horst *et al.*, 2018). Kinematic parameters have been shown to be a useful diagnostic and prognostic tool for fertility (Kraemer *et al.*, 1998). Findeklee *et al.* (2020) demonstrated a correlation between sperm motility of male patients and successful clinical pregnancy outcomes in IUI cases. Ola *et al.* (2003) found a significant relationship between cervical mucus penetration and aspects of sperm movement, namely highly progressive and rapid motility with significant lateral head movement (Mortimer, 1997; Larsen *et al.*, 2000; Björndahl, 2010). VCL, VSL, rapid progressivity, amplitude of lateral head displacement (ALH), linearity (LIN) and VAP all demonstrate some predictive power on the success of IVF (Larsen *et al.*, 2000; Lewis, 2007; Lu *et al.*, 2014).

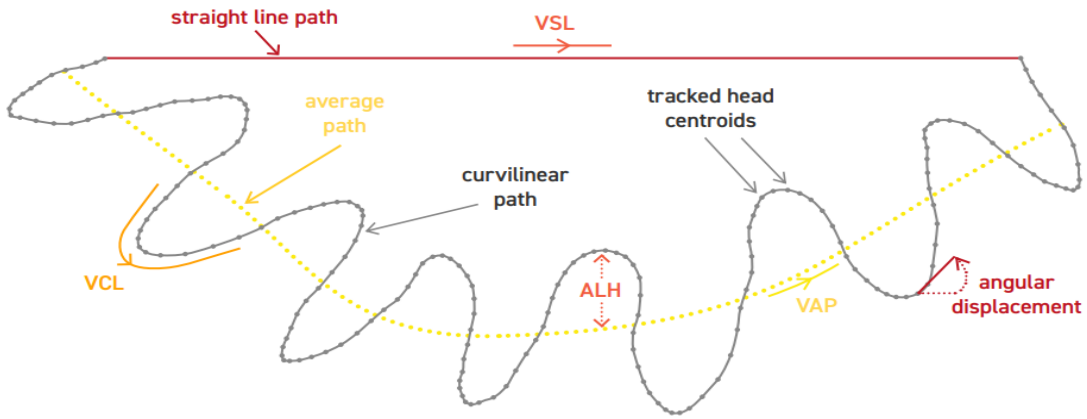

Figure 1.3 Schematic illustration of the various sperm kinematic motility parameters. ***Note:*** Calculated kinematic parameters include STR (VSL/VAP x 100), LIN (VSL/VCL x 100) and WOB (VAP/VCL x 100). **Abbreviations:** ALH, amplitude of later head displacement; LIN, linearity; MAD, mean angular displacement; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble (World Health Organization, 2021).

To acquire and maintain motility, spermatozoa require a morphologically intact flagellum, energy production capabilities, and functional signalling pathways (Freitas *et al*., 2017). Since male infertility is frequently associated with impaired motility, understanding how the sperm tail forms and functions can be crucial to resolving male infertility issues (Lehti and Sironen, 2017).

The axoneme is known as the propulsive engine, and if containing defects can result in male infertility (Freitas *et al*., 2017; Zhao *et al*., 2018). The microtubule doublets are connected via nexin links which connect to the central pair by radial spokes that position the doublets in a perfect circle around the central microtubule pair (Freitas *et al*., 2017; Figure 1.2). Dynamin-ATPase catalyzes the hydrolysis of ATP permitting axonemal components to move asymmetrically along microtubules (Lamirande and Gagnon, 1993; Mortimer, 1997). Each doublet pair and its associated dynein arms (projecting from the microtubule doublets) should therefore produce torque which bends the flagellum in a different direction (Freitas *et al*., 2017; Lindemann and Lesich, 2021). To obtain waveform movement and motility, the process must occur on one side of the axoneme whilst being inactive on the opposite side, thereby forming an "on-and-off" flagellar beat pattern (Freitas *et al*., 2017) (Figure 1.4).

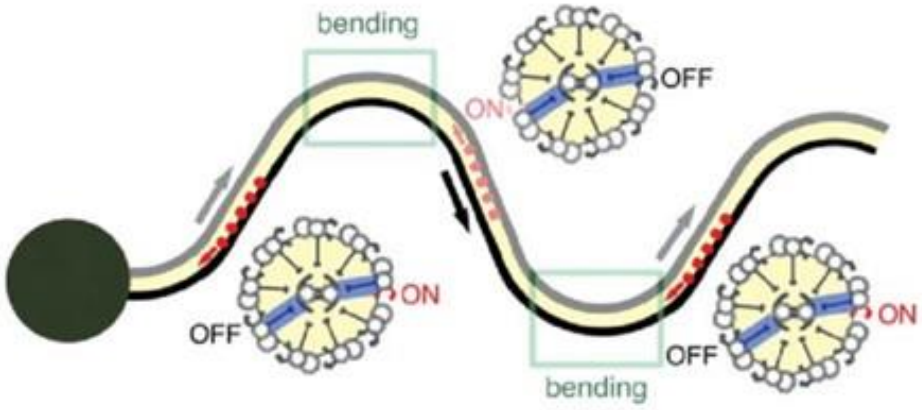

Figure 1.4 Schematic diagram illustrating the oscillatory mechanism of flagellar bending. ***Note:*** Dyneins on two sides on the plane of the CP apparatus (red and pink) are regulated by signals of the RS/CP apparatus (blue), resulting in the formation of a planar wave. The axonemes are fixed at the basal body near the sperm head. The base or tip of the flagellum points toward the minus or plus end of doublet microtubules, respectively. Dyneins are minus-ended motors (red and pink arrows), sliding adjacent microtubules to the plus end (black and grey arrows). The bending provides feedback and switches active dyneins, resulting in the sliding of opposite microtubules across the bend. **Abbreviations:** CP, central pair; RS, radial spoke (Inaba, 2011).

Abnormalities in morphology can occur as a single defect or in association with other defects resulting in reduced sperm functionality (Pereira *et al*., 2017). Many immotile sperm exhibit nonspecific flagellar abnormalities accompanied by a disruption of normal axoneme patterns and other components of the sperm tail (Pereira *et al*., 2017). Correct preassembly of

structural components may therefore play a significant role in sperm motility, in addition to how proteins are modified and assembled prior to transport to the developing tail (Lehti and Sironen, 2017). Abnormalities in these genes can thus lead to defective or even absent flagellar components essential for motility (Lehti and Sironen, 2017). In asthenozoospermic samples, several proteins important for flagellar formation and function were found to be differentially expressed, suggesting they may reduce sperm motility (Shen *et al*., 2013). Despite motility results primarily being focused on likelihood of successful ART, this functional parameter can ultimately be used more directly to address problems affecting the male and his reproductive organs (Björndahl, 2010).

Activation and maintenance of sperm motility is dependent on membrane potential, intracellular pH, and proper balancing of intracellular ions (Nowicka-Bauer *et al*., 2021). Thus, both extrinsic and intrinsic factors influence sperm motility, making it a complex physiological component of sperm function (Dcunha *et al*., 2020). Mediated through calcium ions (Ca^{2+}) and bicarbonate ions (HCO_3^-), cAMP/protein kinase A (PKA) and phosphoinositide 3-kinase (PI3Ks) signalling contribute to sperm motility (Schlingmann *et al*., 2007; Dey *et al*., 2019; Dcunha *et al*., 2020). Bicarbonate activation of adenylate cyclase during epididymal maturation and ejaculation results in the production of cAMP (Lamirande and Gagnon, 1993; Mortimer, 1997; Nowicka-Bauer *et al*., 2021). Thus, cAMP activates protein kinases, causing phosphorylation of proteins participating in the conversion of axonemal sliding to flagellar bending (Lamirande and Gagnon, 1993; Mortimer, 1997; Nowicka-Bauer *et al*., 2021). Low intracellular Ca^{2+} concentrations trigger symmetrical beating of the flagellum, while higher concentrations induce hyperactivation, and very high concentrations suppress motility (Nowicka-Bauer *et al*., 2021). Ca^{2+} regulation therefore plays an essential role in regulating secondary messengers involved in activation of signalling pathways controlling motility.

2.2.4 | Capacitation

Mammalian spermatozoa require contact with molecules present in the female reproductive tract to acquire fertilizing capacity, which makes the understanding of capacitation crucial for ART (Visconti *et al*., 2011; Henkel, 2012). Although the exact site of capacitation varies among species, studies suggest capacitation occurs when spermatozoa pass through the cervix, uterus and oviduct (Jin and Yang, 2017). During their residence in the female reproductive tract, spermatozoa undergo various alterations leading to capacitation (Puga Molina *et al*., 2018; Luque *et al*., 2020; Zigo *et al*., 2020). When encountering capacitation triggers, the sperm plasma membrane should undergo several molecular and

biochemical changes (Harrison, 1996; Puga Molina *et al*., 2018; Leemans *et al*., 2019) (Figure 1.5), including the following:

- Increases in membrane fluidity.
- Lateral movement of cholesterol to the apical region of the sperm head.
- Cholesterol efflux from the sperm plasma membrane.
- Cytoplasmic changes such as protein tyrosine phosphorylation and elevated pH, cAMP and Ca^{2+} concentrations.

Capacitation is thus not referring to one functional change, but rather all the events enabling the spermatozoon's transition from progressive to hyperactivated motility and induction of AR (Leemans *et al*., 2019). As hyperactivation takes place prior to AR, each process will mark the end of the early and late phases of capacitation (Ferramosca and Zara, 2014). Furthermore, these processes will facilitate releasing of spermatozoa from the isthmic reservoir, responding to thermotaxis and chemotaxis, penetration of the cumulus layers, binding to the oocyte, and AR for oocyte penetration (Harrison *et al*., 1996; Armon and Eisenbach, 2011; Zigo *et al*., 2020). Only a few spermatozoa can undergo capacitation, which is a short-lived process (1–4 hrs in humans *in vitro*) (Coy *et al*., 2012).

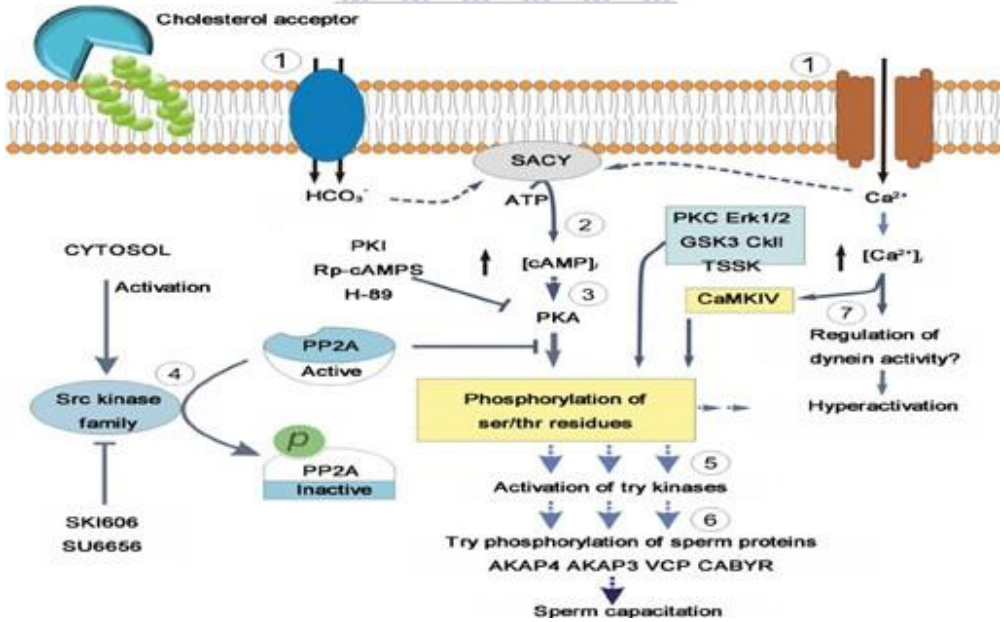

Figure 1.5 Model of the regulation of phosphorylation pathways during mammalian sperm capacitation. ***Note:*** The overall pathway is modulated by cholesterol efflux of sperm plasma membrane. **(1)** Influx of HCO_{3-} and Ca^{2+} stimulates SACY. **(2)** Increasing intracellular cAMP concentrations. **(3)** High cAMP levels activate PKA. This activation can be blocked *in-vitro* by addition of inhibitors. **(4)** *In-vivo*, phosphorylation of PKA substrates is subjected to regulation by a ser/thr phosphatase. This phosphatase is in turn down regulated by a member of the Src kinase family. Activation of Src kinase family members can be blocked by *in-vitro* addition of the inhibitors. The onset of PKA substrates phosphorylation is followed by activation of tyrosine kinases **(5)** and the promotion of tyrosine phosphorylation of sperm proteins **(6)** (e.g., AKAP4, AKAP3, VCP and CABYR). **(7)** Intracellular Ca^{2+} increase might affect the axoneme directly, through selective regulation of dyneins. In addition, Ca^{2+} up regulates CaMKIV, leading to hyperactivation. **Abbreviations:** cAMP, cyclic AMP; PKA, protein kinase A; SACY, soluble adenylyl cyclase; ser/thr, serine/threonine (Visconti *et al*., 2011).

The cholesterol efflux from the sperm membrane will ultimately cause an increase in bicarbonate ions, soluble adenylyl cyclase (SACY) activities and Ca^{2+} levels in spermatozoa (Castillo *et al*., 2019; Zigo *et al*., 2020). These changes will further initiate critical signal transduction cascades and remodelling of the sperm surface via phospholipid scramblase activity (Castillo *et al*., 2019; Zigo *et al*., 2020). Consequently, the activation of the previously mentioned pathways will lead to several intracellular biochemical processes including oxidation of energy substrates, phosphorylation of signal transduction proteins, and axoneme energy conversion (Ferramosca and Zara, 2014). As spermatozoa are essentially transcriptionally, and translationally silent, functional alterations will be accomplished via post-translational modifications during epididymal maturation and capacitation (Rahman *et al*., 2017). Presence of peptide residues potentially harbouring post-translational modification sites was found in spermatozoa during capacitation. Thereby suggesting that protein modification may be a key part of sperm maturation as previously mentioned. Changes or deletions in such genes or proteins may therefore prevent spermatozoa from undergoing this essential process (Castillo *et al*., 2019). Thus, the proportion of spermatozoa that can undergo capacitation can serve as a proxy for the ability of the sperm to fertilize an egg (Rahman *et al*., 2017). In fact, the addition of decapacitating factors have been demonstrated to partially reverse capacitation and therefore render spermatozoa incapable of recognizing and fertilizing an oocyte (Zigo *et al*., 2020).

2.2.5 | Hyperactivation

Sperm motility is acquired through the gradual transition from phosphatase to kinase activities as well as protein phosphorylation in the epididymis (Nowicka-Bauer *et al*., 2021). While spermatozoa exhibit linear patterns and small bend amplitudes in active motility, hyperactivated motility involves large amplitudes and asymmetric beat patterns (Figure 1.6) (Suarez, 2008; Badcock *et al*., 2014; Ferramosca and Zara, 2014; Fujinoki *et al*., 2016; Nowicka-Bauer *et al*., 2021). It has been proposed that hyperactivated motility provides spermatozoa with the ability to detach from the isthmus, migrate through the viscous oviduct environment, and penetrate the cumulus cells and ZP surrounding the oocyte (Breznik *et al*., 2013; Wang *et al*., 2012). As sperm are hyperactivated by tyrosine phosphorylation, any impairment in this process will prevent hyperactivation, such as in the case of asthenozoospermia (Wang *et al*., 2021).

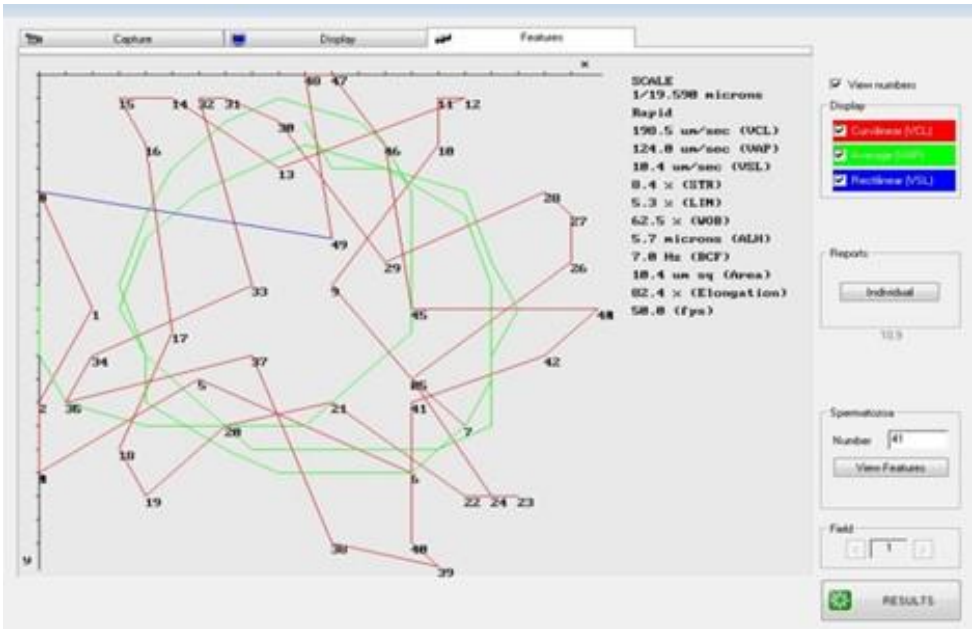

Figure 1.6 Starspin hyperactivation pattern of human spermatozoa. ***Note:*** the spermatozoa depicted displays a VCL of 190 um/s, LIN 5.3% and an ALH of 5.7 um, which is equivalent to 11.4 um in CASA systems using 2x ALH. Hyperactivation is typically characterized by VCL >150 µm/s; LIN <50 %; ALH >3.5/7 (van der Horst, 2018).

While several physiological factors, such as Ca^{2+}, cAMP, HCO^{3-} and metabolic substrates, are essential for the initiation and maintenance of hyperactivated motility *in vitro* (Ho and Suarez, 2001; López-Úbeda and Matás, 2015), Ca^{2+} is the primary second messenger that triggers hyperactivated motility (Figure 1.7) (Suarez, 2008). Hyperactivation is initiated by extracellular Ca^{2+} influx through sperm Ca^{2+} (CatSper) channels; however, intracellular Ca^{2+} stores may also play a role (Loux *et al*., 2013). Increased intracellular cAMP levels were additionally observed during *in-vitro* hyperactivation (White and Aitken, 1989). Thus, cAMP dependent phosphorylation of proteins localized in the region of the FS regulate sperm motility (Kinukawa *et al*., 2003). Together with various physiological stimuli, protein-protein interactions will therefore modify the Ca^{2+} influx mechanisms in spermatozoa (Rahman., *et al* 2014). CatSper proteins are polymodal, chemosensory channels encoded by multiple testis-specific genes and localized in the plasma membrane of the principal piece of mature spermatozoa (Luque *et al*., 2020; Rahban and Nef, 2020).

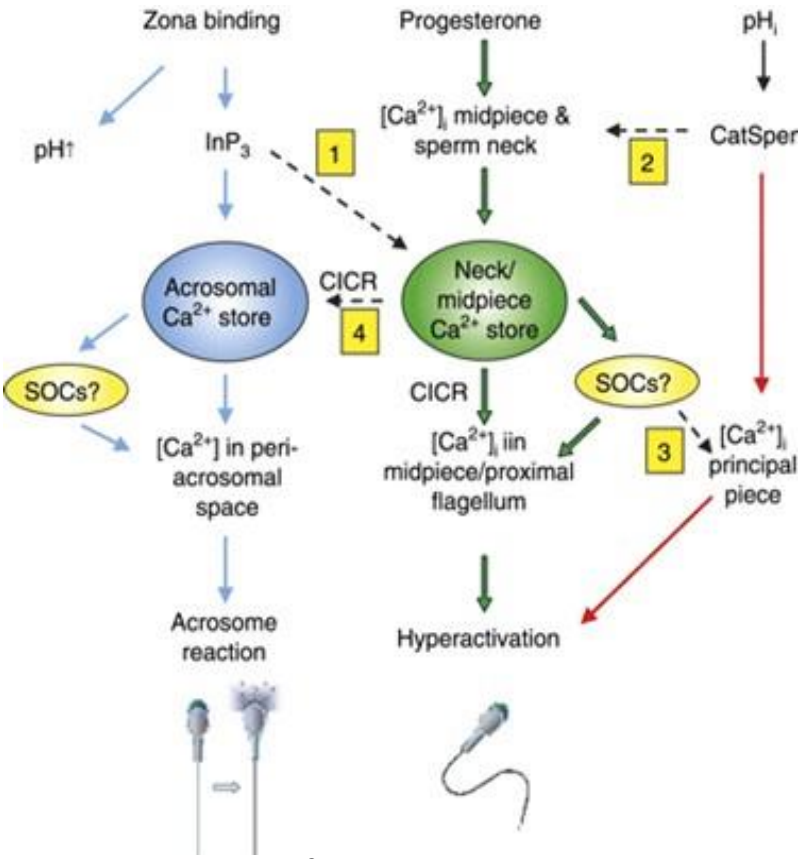

Figure 1.7 Model for roles and interactions of Ca^{2+} stores in mammalian sperm hyperactivation and acrosome reaction. ***Note:*** Zona binding (pathway shown in blue) induces generation of IP3, mobilisation of the acrosomal store and activation of store operated Ca^{2+} influx, leading to elevated intracellular Ca^{2+} concentrations in the peri-acrosomal space and acrosome reaction. SOCs may be recruited during this process. Progesterone (shown in green) mobilise Ca^{2+} stored at the sperm neck/midpiece by CICR and activation of store operated Ca^{2+} influx, causing elevation of Ca^{2+} in the midpiece/proximal flagellum leading to regulation of flagellar activity and hyperactivation. CatSper channels in the principal piece of the flagellum are activated by increased pH, causing influx of Ca^{2+}, elevation of intracellular Ca^{2+} concentrations and hyperactivation (shown in red). Dashed arrows (numbered) show potential crosstalk between these pathways. **(1)** IP3 generated downstream of zona binding may activate IP3Rs at the sperm neck, leading to Ca^{2+} mobilisation and hyperactivation. **(2)** Ca^{2+} influx through CatSper in the principal piece may affect intracellular Ca^{2+} concentrations at the sperm neck/midpiece, mobilising stores Ca^{2+} by CICR. **(3)** SOCs in the principal piece may be activated downstream of store mobilisation. **(4)** Ca^{2+} mobilisation in the neck/midpiece may spread forward into the head, potentially mobilising acrosomal Ca^{2+} by CICR. **Abbreviations:** CICR, calcium induced calcium release; IP3, inositol triphosphate; SOC, store operated channels (Costello *et al*., 2009).

The CatSper channel forms four linear columns of Ca^{2+} signalling domains along the principal piece of flagellum, which organize the spatiotemporal pattern of tyrosine phosphorylation of flagellar proteins (Yang *et al.*, 2021). This complex channel is known to contain seven proteins: four that form a pore through which Ca^{2+} can enter, and three accessory proteins whose roles in hyperactivated motility are less clear. The formation of the CatSper channel groups (known as complexes) with several other proteins is therefore essential for hyperactivated motility (Figure 1.8). Any aberrations in the genes that encode these proteins or the inability to form the complex can therefore lead to infertility in males (Chung *et al*., 2017).

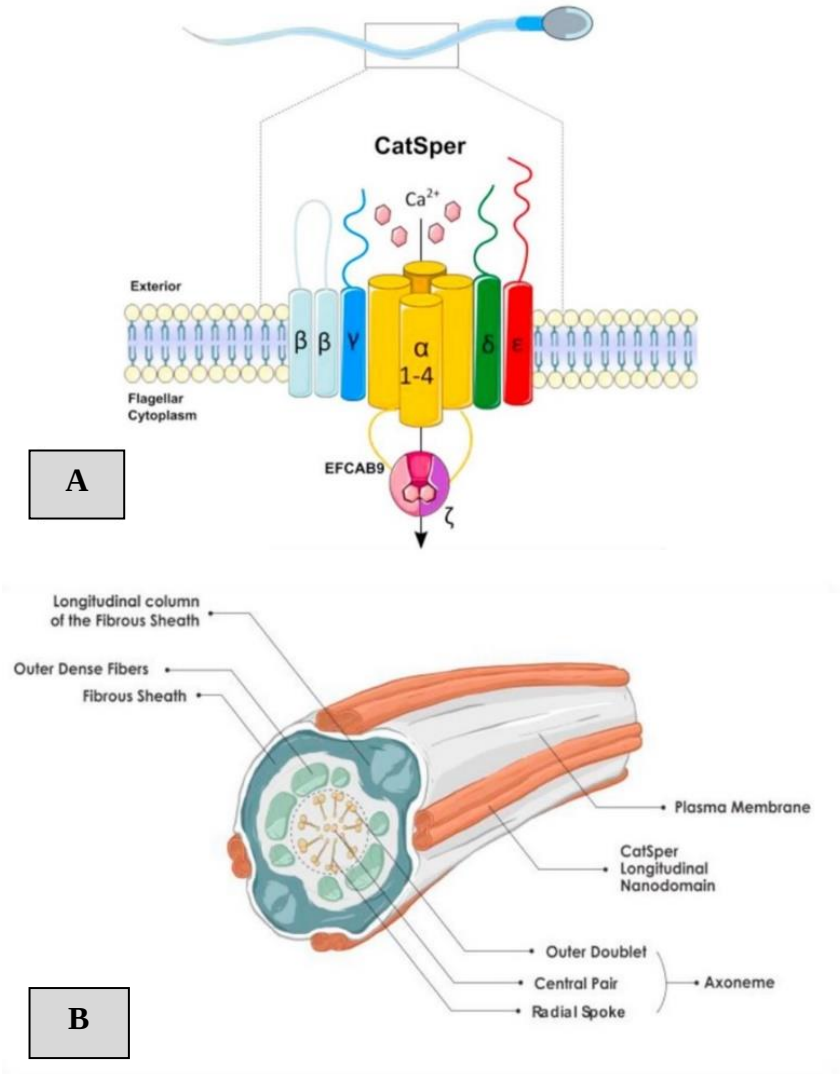

Figure 1.8 Illustration of the CatSper channel in mammalian spermatozoa. ***Note:*** **(A)** Located in the principal piece of the flagellum is the heteromeric voltage-dependant, pH sensitive CatSper channel composed of ten subunits. The channel consists of four pore-forming α-subunits (CatSper 1-4), in addition to five auxiliary subunits: CatSper β (beta), CatSper γ (gamma), Catsper δ (delta), CatSper ζ (zeta) and CatSper ε (elipson). A new member of the complex recently identified as EF-hand calcium binding domain containing protein 9 (EFCAB9) is a calmodulin like protein which binds Ca^{2+} and acts as a dual calcium and pH sensor for CatSper. **(B)** The channel forms functional domains within the principal piece to permit the rapid propagation of Ca^{2+} signals along the flagellum. The channel complex is organised in a quadrilateral longitudinal domain that forms a unique pattern of four linear threads running down the principal piece (Rahban and Nef, 2020).

When raising the intracellular pH, CatSper channels are activated, which increase soluble adenyl cyclase (SAC), thereby stimulating protein tyrosine kinases (PTKs) and phosphorylating flagellar proteins related to sperm motility (Rahban and Nef, 2020; Nowicka-Bauer and Szymczak-Cendlak, 2021). The alkaline environment is further required by dyneins forming peripheral doublets for axonemes and exhibiting ATPase activity (Nowicka-Bauer and Szymczak-Cendlak, 2021). Hyperactivation can therefore be mimicked *in vitro* after performing density gradient centrifugation and by incubating sperm cells in a medium containing HCO_3^-, Ca^{2+} and albumin (Leemans *et al.*, 2019). Based on clinical research,

hyperactivated spermatozoa correlate with fertilization rates *in-vitro* (Breznik *et al*., 2013). Accordingly, significant differences in the proportion of hyperactivated cells were observed between men with normal semen parameters and sub-fertile patients (Alasmari *et al*., 2013).

2.2.6 | Acrosome Reaction

The sperm head contains a large secretory granule called the acrosome, which should be well defined and comprise about 40-70% of the head area (Figure 1.9) (Mayorga *et al*., 2007; Oberheide *et al*., 2017; WHO, 2021). There should be no larger vacuoles in the acrosomal region, and not more than two small vacuoles, which should occupy less than a fifth of the sperm head. Ideally, there should be no vacuoles in the post-acrosomal region (WHO, 2021). Membranes covering the nucleus and underlying the plasma membrane are called 'inner' and 'outer' acrosomal membranes, respectively (Mayorga *et al*., 2007).

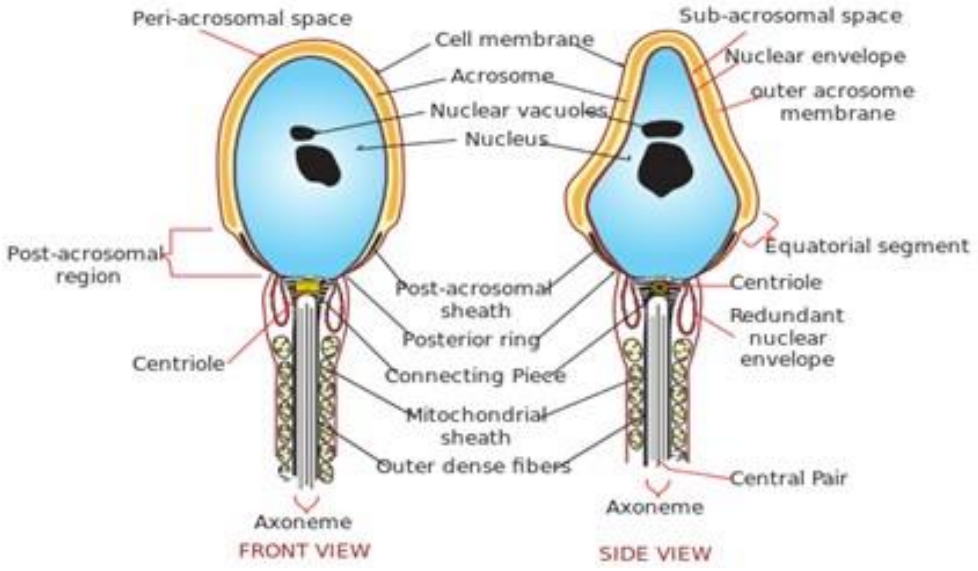

Figure 1.9 Diagram of spermatozoa and the acrosome (Wikipedia, 2006).

The acrosome reaction (AR), a crucial step for successful fertilization, is triggered when mammalian spermatozoa encounter the ZP (Khawar *et al*., 2019) or may even occur before encountering the ZP as when mediated by progesterone (Simons and Fauci, 2018). The carbohydrate-dependent event of sperm-egg fusion will occur through interactions between glycan-binding molecules (receptors) present on the sperm plasma membrane, with their corresponding glycans (ligands) localized to the ZP (oocyte) (Abou-haila and Tulsiani, 2009; Khawar *et al*., 2019). Irreversible binding of the opposite gametes initiates a Ca^{2+}-dependent signal transduction pathway (Bendahmane *et al*., 2002). Ca^{2+} modulation is known to play a role in capacitation and AR, as Ca^{2+}-adenosine 5'-triphosphatase (ATPase) function is

inhibited during capacitation, leading to increased levels of acrosomal Ca^{2+} triggering exocytosis (Santos, 2018). AR is thus characterized by a biphasic Ca^{2+} response: an initial sharp rise in Ca^{2+} concentrations in the sperm head followed by a relaxation phase where the Ca^{2+} concentration tends toward a steady, elevated level (Simons and Fauci, 2018).

Glycohydrolases and proteases released at the sperm binding site, as well as the enhanced thrust generated by the hyperactive beat patterns of bound spermatozoa, are important factors that regulate the ZP penetration (Tulsiani *et al*., 1998). The reaction can be induced physiologically by follicular fluid serum albumin, glycosaminoglycans, or glycoproteins from the ZP (Henkel *et al*., 1993; Abou-haila and Tulsiani, 2009; Okabe, 2018). The substantial efflux of cholesterol from the sperm plasma membrane increases permeability allowing the lateral movements of integral proteins and rendering the membrane fusogenic (Ikowictz *et al*., 2012). Thus, making it more responsive to ZP glycoproteins and the influx of Ca^{2+} and HCO_{3-} ions that activate intracellular secondary messengers (Abou-haila and Tulsiani, 2009, Reid *et al*., 2011).

Spermatozoa bind to the ZP, phosphorylating both the principal and mid-piece regions (Figure 1.7) (Abou-haila and Tulsiani, 2009; Hirohashi and Yanagimachi, 2018). The temporary increase of Ca^{2+} and other secondary messengers triggers a cascade of signalling events that raise internal sperm pH and trigger the fusion of the sperm plasma membrane and outer acrosomal membrane (Hirohashi and Yanagimachi, 2018). Hybrid vesicles containing acrosomal contents begin to form and are released via exocytosis in a time dependent manner (Hirohashi and Yanagimachi, 2018). Finally, there is a disappearance of the acrosomal contents and vesicles which are held together by the acrosomal matrix (Henkel *et al*., 1993; Mayorga *et al*, 2007; Abou-haila and Tulsiani, 2009). The powerful action of hydrolytic enzymes released at the surface of the ZP makes it possible for the hyperactivated spermatozoon to penetrate the oocyte and to fertilize it (Figure 1.10) (Henkel *et al*., 1993; Mayorga *et al*, 2007; Abou-haila and Tulsiani, 2009).

Acrosome structural or functional abnormalities can impair sperm fusion, resulting in infertility - therefore its occurrence is predictive of *in vitro* fertilization (Menkveld *et al*., 2003; Mayorga *et al*., 2007; Costa *et al*., 2010; Khawar *et al*., 2019). As such, only acrosome-reacted spermatozoa can penetrate the ZP (Henkel *et al*., 1993). In fact, investigations have indicated that intra-cytoplasmic insemination of spermatozoa containing such abnormalities, did not lead to successful fertilization, even in the absence of fertilization barriers (Khawar *et al*., 2019).

A homozygous deletion of the DPY19L2 gene has been shown to affect sperm head elongation and acrosome formation in globozoospermia patients (Harbuz *et al*., 2011). Furthermore, $Na^{+/}H^{+}$ exchanger NHE8, has been demonstrated to play a role in acrosome biogenesis and its loss of function results in similar deformities as seen in globozoospermia (Oberheide *et al*., 2017). Infertile sperm show substantial morphological changes in the sperm head and acrosome membrane as compared to fertile sperm (Yu *et al*., 2014). Acrosome membranes with several morphological aberrations/alterations might be unable to respond to certain signals triggering capacitation, suggesting that abnormal acrosome ultrastructure may contribute to male infertility (Yu *et al*., 2014).

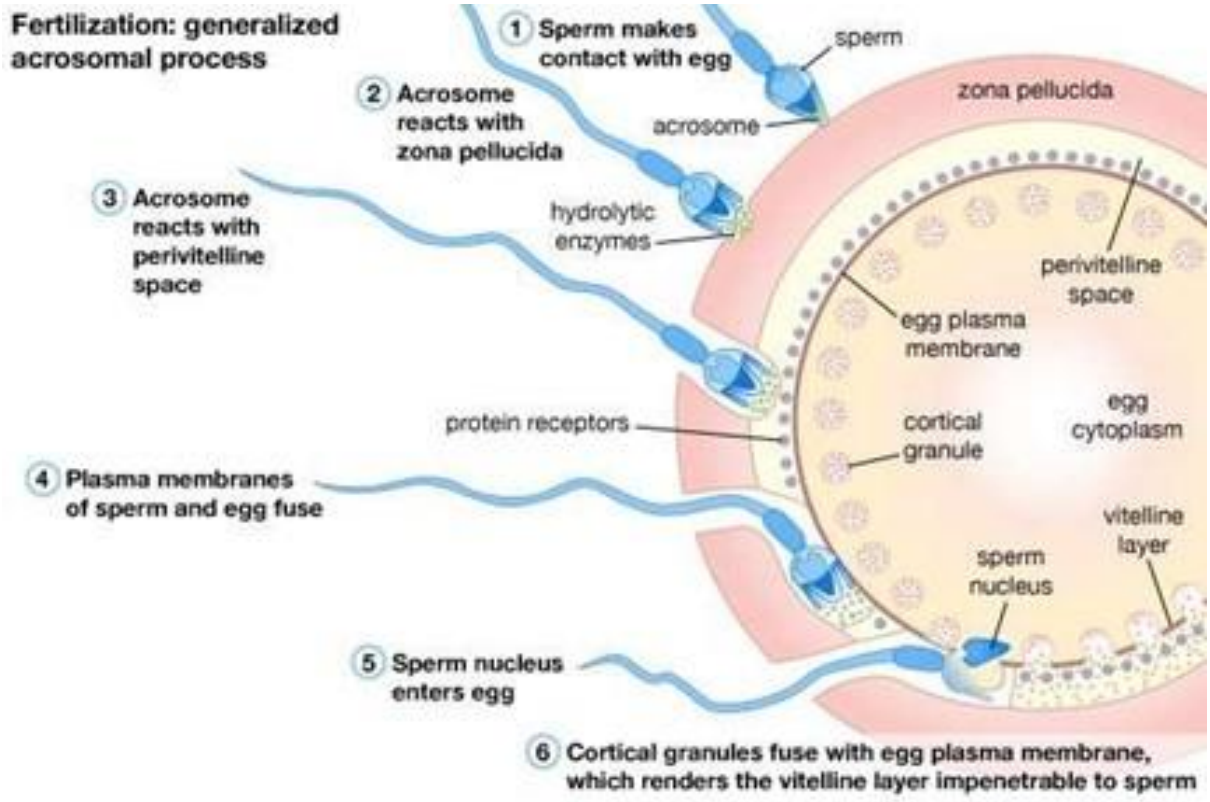

Figure 1.10 Diagram of human sperm acrosome reaction. (Monroy, 2018).

2.2.7 | Mitochondrial Membrane Potential

Sperm mitochondria have peculiar characteristics distinguishing them from normal somatic cells (Moraes *et al*., 2018). In spermatozoa, mitochondria are tightly packed in the midpiece and rearranged in tubular structures helically anchored around the anterior portion of the ODF of the axoneme, as well as covering the striated columns of the connecting piece and proximal centriole (Figure 1.12) (Sutovsky *et al*., 1997; Piomboni *et al*., 2012; Amaral *et al*., 2013; Barbagallo *et al*., 2020). Furthermore, it has been well-established that sperm

mitochondria possess specific isoforms of many proteins and isoenzymes (Piomboni *et al*., 2012; Strivastava *et al*., 2016). The preservation of mitochondria in spermatozoa suggests an important role in fertilization (Sousa *et al*., 2011; Amaral *et al*., 2013).

Mitochondria play a key role in the production of ATP via oxidative phosphorylation (OXPHOS), which is preceded by the generation of reduced electron carriers, both in the cytoplasm and in the mitochondrial matrix (Piomboni *et al*., 2012; Amaral *et al*., 2013; Cheng *et al*., 2017). In the IMM, several complexes make up the electron transfer chain (ETC), which transports electrons obtained from oxidation of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH) (Figure 1.11) (Guo *et al*., 2018). The chemiosmotic theory suggests the proton gradient formed across the IMM drives oxidative phosphorylation, further coupling respiratory oxygen to adenosine diphosphate (ADP) phosphorylation/ATP synthesis (Cheng *et al*., 2017). The gradient consists of two components, namely a minor chemical (pH) component and a major electric component, which is usually translated into the mitochondrial membrane potential (MMP) (Piomboni *et al*., 2012; Amaral *et al*., 2013; Kuhlbrandt, 2015).

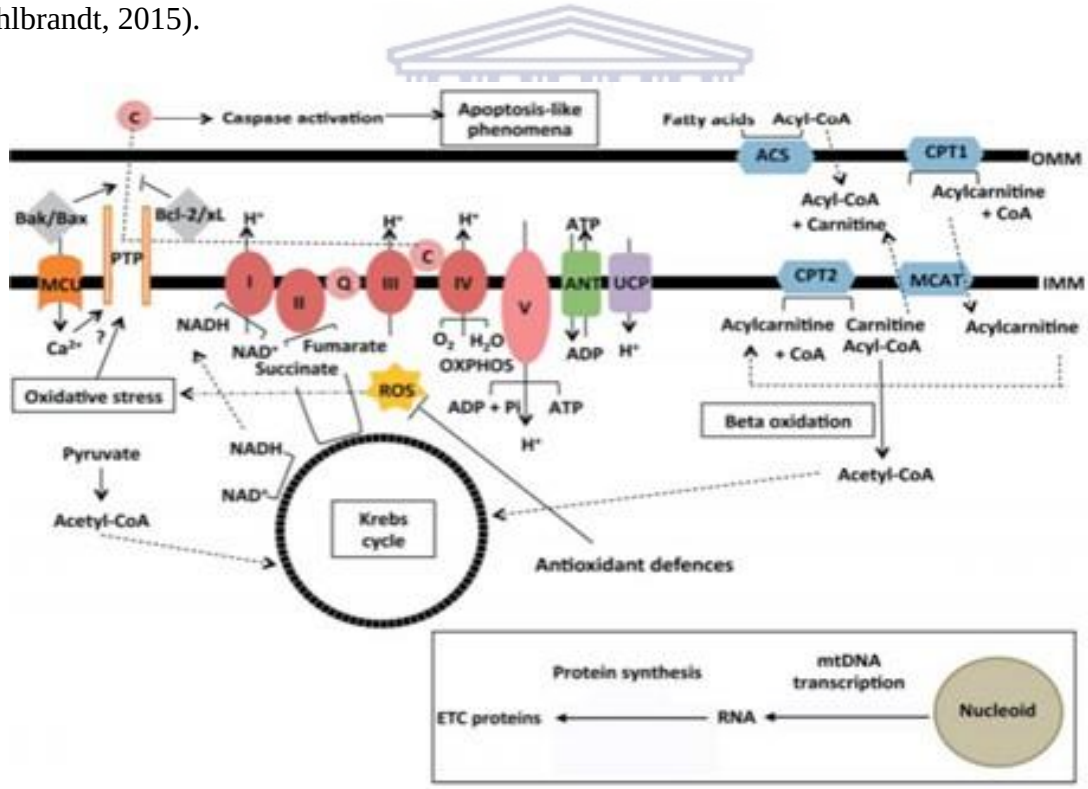

Figure 1.11 Overview of the pathways likely to be active in mammalian sperm mitochondria. (Amaral *et al.*, 2013).

The electric nature of the MMP can sequester Ca^{2+} and therefore participate in Ca^{2+} homeostasis (Piomboni *et al*., 2012; Amaral *et al*., 2013). In addition to ATP production, mitochondrial ETC produces reactive oxygen species (ROS), which can both function in signalling pathways and cause oxidative damage (Amaral *et al*., 2013; Santos *et al*., 2014).

Mitochondria also produce sex steroid hormones, which in turn control mitochondrial function (Ferramosca *et al*., 2021). The interaction between mitochondrial function and steroid hormone signalling can therefore contribute to the energy maintenance of sperm motility (Ferramosca *et al*., 2021).

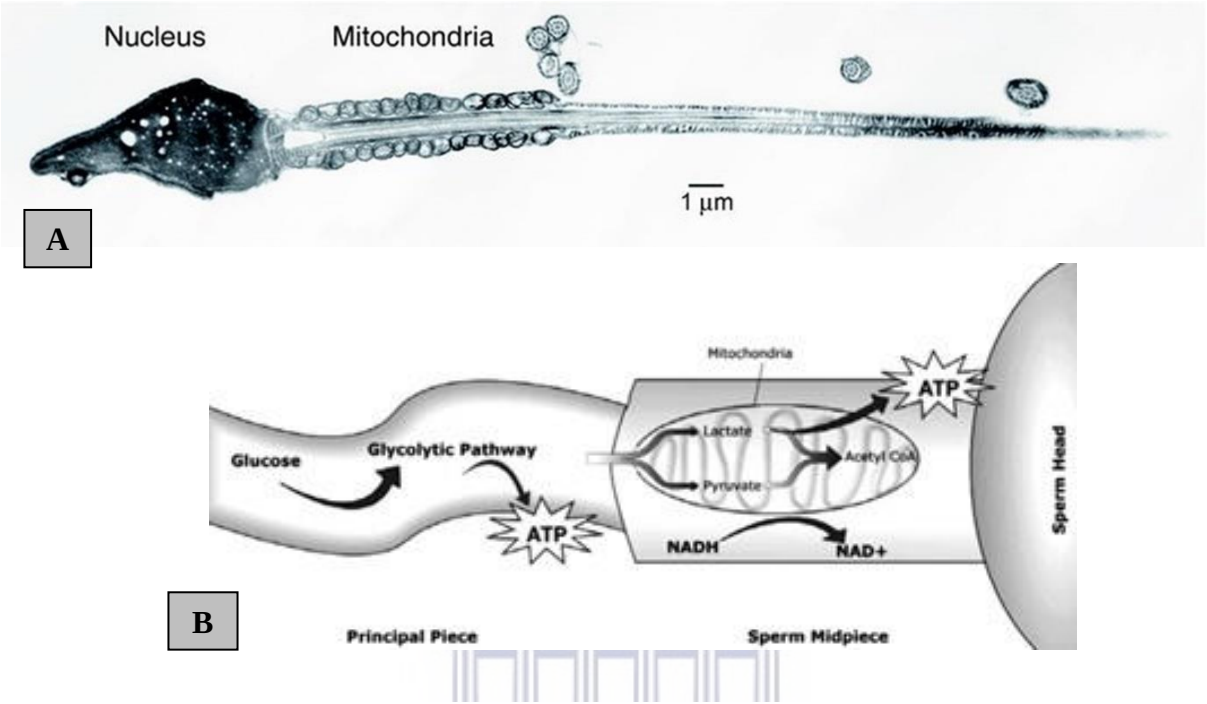

Figure 1.12 Illustration of the **s**permatozoa mitochondria. ***Note:*** **(A)** Montage transmission electron micrograph of a human sperm cell (Barrett *et al.*, 2009). **(B)** Illustration of sperm mitochondria (Vadnais *et al.*, 2007)

In addition to their importance and uniqueness, ongoing research continues to reveal new ways in which mitochondria function in cells (Amaral *et al*., 2013). To meet cellular needs, mitochondria must be fully integrated into the whole cell's regulation and coordination mechanisms (Dong *et al*., 2017). Although mitochondrial DNA codes for only 13 proteins, their expression is crucial to mitochondrial function (Amaral *et al*., 2013). Sperm function may be adversely affected by changes to the mitochondrial genome, transcriptome, proteome, or metabolome, or by other cellular events that compromise mitochondrial function (Cassina *et al*., 2015). Therefore, biomarkers to predict fertility based on differentially expressed mitochondrial proteins should be explored (Samanta *et al*., 2018).

Mitochondria are crucial for motility and capacitation of spermatozoa (Barbagallo *et al*., 2020; Ferramosca *et al*., 2021). Thus, assessment of mitochondrial function, specifically MMP, can distinguish better-quality gametes (Santos *et al.*, 2014). Based on comparative proteomic data, several sperm mitochondrial proteins may be altered in asthenozoospermic patients (Santos *et al*., 2014). It is suggested that mitochondrial enzymes and the ETC complex activity

correlates with sperm parameters, such as concentration, vitality, motility, and fertilizing capacity (Barbagallo *et al*., 2020). Due to the intimate association of mitochondrial functionality with both sperm quality and fertilization ability (Sousa *et al*., 2011; Amaral *et al*., 2013; Zhang *et al*., 2016), it is evident that mitochondrial dysfunction may be responsible for idiopathic asthenozoospermia (Barbagallo *et al*., 2020).

2.2.8 | Reactive Oxygen Species

Oxygen derivatives, known as reactive oxygen species (ROS), are more reactive than molecular oxygen and elevated levels thereof can cause molecular damage (Sies and Jones, 2020). Both enzymatic and non-enzymatic mechanisms can generate ROS in mammalian cells (Villaverde *et al*., 2019). Reduction of oxygen through the addition of electrons leads to the formation of several ROS species, including superoxide, hydrogen peroxide, hydroxyl radical, hydroxyl ion, and nitric oxide (Figure 1.13) (Choudhury *et al*., 2017; Kalyanaraman *et al*., 2017).

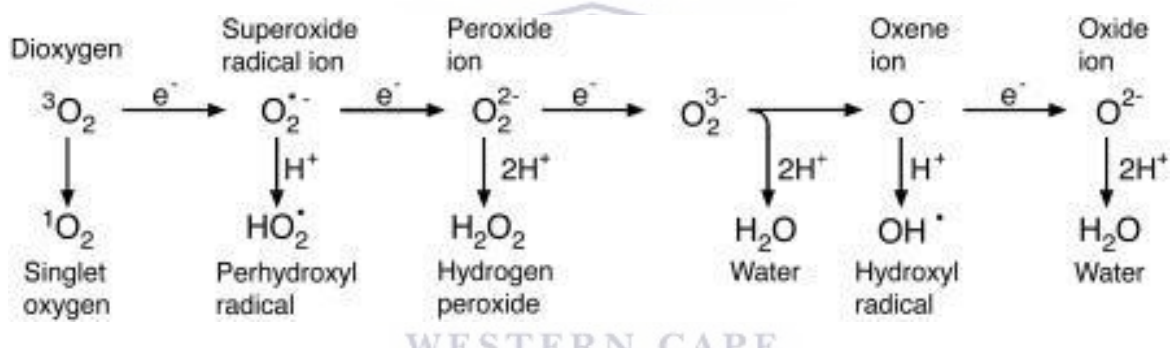

Figure 1.13 Generation of different reactive oxygen species by energy transfer or sequential univalent reduction of ground-state triplet oxygen (Rodriguez and Redman, 2005).

Discrete ROS and mitochondria-produced ROS (mROS) levels play a role in the activation of male gametes (de Lamirande *et al*., 2008; Santos *et al*., 2014; Aitken, 2017). However, oxidative stress associated with their ubiquitous production has been implicated in the pathogenesis of male infertility (Villaverde *et al*., 2019). Cells produce ROS mainly through NADPH oxidates (NOXs), cytochrome P450 dependent oxygenases, xanthine oxidase, and the mitochondrial ETC (Santos *et al*., 2014; Otasevic *et al*., 2020). Until recently, ROS producing enzymatic systems in spermatozoa was unclear. However, the Ca^{2+} dependent NADPH oxidase called NOX5 (primary located in testes and found in the acrosome and mid-piece of the flagellar) has been found to use NADPH as a source of electrons for reduction of oxygen to ROS and is responsible for ROS production both in testes and spermatozoa (Otasevic *et al*., 2020).

The difference between beneficial or detrimental effects of ROS depends on aspects of timing and the amount of ROS production (Amaral *et al*., 2013; Santos *et al*., 2014). Incubation of sperm cells with low concentrations of hydrogen peroxide have been found to stimulate sperm capacitation, hyperactivation, AR and oocyte fusion (Sanocka and Kurpisz, 2004; Otasevic *et al*., 2020). This mechanism by ROS is unclear but may involve tyrosine phosphorylation of sperm proteins (Aziz *et al*., 2004). It's suggested that ROS stimulates the AR adenyl cyclase-mediated activation of downstream targets to start the exocytotic event (Otasevic *et al*., 2020).

Up to 40% of infertile men have high seminal ROS levels (Irvine *et al*., 2000; Aziz *et al*., 2004; Santos *et al*., 2014). Thus, a relationship between poor semen parameters and ROS levels exists (Homa *et al*., 2015; Wagner *et al*., 2017). Excessive amounts of leukocytes and dysfunctional spermatozoa are additionally responsible for contact with exogenous ROS (Irvine *et al*., 2000; Aziz *et al*., 2004; Santos *et al*., 2014; Bui *et al*., 2018). Infertile males who produce high levels of ROS have a five-fold lower chance of initiating a pregnancy than those producing low levels of ROS (Aziz *et al*., 2004; Santos *et al*., 2014). High levels of seminal ROS are associated with poor sperm morphology, decreased viability, motility, and MMP, and especially DNA damage (Figure 1.14) (Irvine *et al*., 2000; Aziz *et al*., 2004; Amaral *et al*., 2013; du Plessis *et al*., 2015; Lobascio *et al*., 2015; Alahmar, 2019). As uncontrolled generation of ROS adversely affects sperm morphology and functions through lipid peroxidation (Dutta *et al*., 2020), ROS levels could ultimately be correlated with male infertility, hereby suggesting a possible diagnostic application (Santos *et al*., 2014).

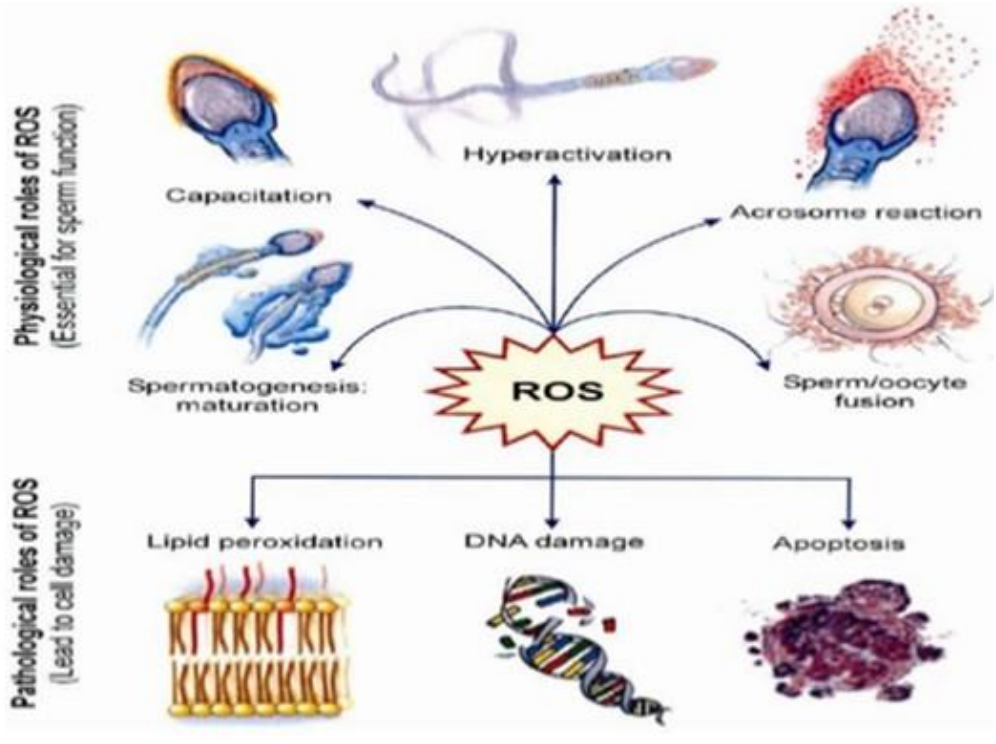

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Figure 1.14 Pathological and physiological roles of reactive oxygen species in spermatozoa (Katiyar, 2016)

2.2.9 | DNA and Chromatin Fragmentation

To transfer genetic and epigenetic information from sperm to embryo, sperm chromatin needs to be highly condensed, protecting the paternal DNA and epigenome from various external factors (Stiavnická *et al*., 2020). Spermatozoa replace histones with protamines through DNA protamination; however, only a small number of histones undergo post-translational modifications (Stiavnická *et al*., 2020). During spermatogenesis, chromatin remodelling is sensitive to environmental conditions, thus ROS exposure can ultimately result in DNA fragmentation and apoptosis (Štiavnická *et al*., 2020). Consequently, sperm quality can be negatively impacted by abnormalities in sperm chromatin structure or histones (Štiavnická *et al*., 2020).

Sperm DNA fragmentation (sDF) consists of both single and double stranded DNA breaks and high levels can be present in semen with normal motility, morphology and count (Muratori and De Geyter, 2019). Therefore, sDF is independent from conventional semen parameters (Muratori and De Geyter, 2019). Moreover, high sDF levels have been reported to reduce fertility and the probability of clinical pregnancy after IVF and ICSI treatments (Muratori and De Geyter, 2019). Sperm DNA defects can be identified in men with ART failure using sperm DNA sequencing and ultrastructural analysis (Xie *et al*., 2015). It is therefore an important goal of reproductive research to identify the parameters that predict ART success (Marchiani *et al*., 2017).

2.2.10 | CASA and FAST Analysis

As the quality of semen continues to decline, infertility has become a serious public health concern, especially since an accurate diagnosis of its causes remains elusive (Gallagher *et al*., 2019; Wan *et al*., 2019). Therefore, additional techniques for assessing sperm functionality and fertilization capacity are needed (van der Horst *et al*., 2017). Over a thousand years of technological development has provided biologists with the ability to examine semen and observe spermatozoa (Holt *et al*., 2018). The spermatozoon's ability to swim led to the oversimplified conclusion that swimming speed reflects potential fertility, ultimately leading to the development of methods for assessing and measuring sperm swimming speed (Holt *et al*., 2018). Manual assessment of sperm motility presents with considerable discrepancies between obtained results, due to inaccuracies in the examination processes (individual subjectivism), as well as lack of internal and external quality control in many laboratories worldwide (van der Horst and du Plessis, 2017; Talarczyk-Desole *et al*., 2017). The

repeatability of measurements and objectivism therefore makes CASA the most popular and intensively improved tool over the years (Talarczyk-Desole *et al*., 2017).

Without integrating CASA into clinical analyses, we are throwing away huge quantities of latent information with a wide-ranging potential diagnostic impact (Gallahger *et al*., 2018). During the past two decades, CASA systems have been revolutionized largely due to advances in both hardware and software providing accuracy and verification (van der Horst, 2020). Amongst the various functional parameters that can be assessed by CASA such as vitality, morphology, concentration, acrosome reaction and DNA integrity – motility analysis is most commonly used (Sikka and Hellstrom, 2016). In CASA, individual sperm head centroids are tracked - thereby producing useful kinematic parameters providing insights into sperm motion characteristics closely related to fertilization rates and time to conception (Gallagher *et al*., 2018; Wan *et al*., 2019; Wei *et al*., 2019). Although highly effective, current techniques fail to analyse the beating flagellum, thus lacking mechanistic insight (Gallagher *et al*., 2019; van der Horst, 2020). Sperm motility is induced by the beating of a single flagellum with the head causing drag and the flagellum acting as a motor (Gallagher *et al*., 2018; Wan *et al*., 2019; Gallagher *et al*., 2019; van der Horst, 2020).

Flagellar and Sperm Tracking (FAST) software, developed by the School of Mathematics and Centre for Human Reproductive Science, Institute of Metabolism and Systems Research, at the University of Birmingham (Gallagher *et al*., 2019, van der Horst, 2020), quantitatively measures several sperm flagellar parameters. Flagellar waves are characterized in terms of the tangent angle (Gallagher *et al*., 2019). The flagellar curvature is then calculated, from which the beat frequency and arc-wavespeed can be derived (Gallagher *et al*., 2019). FAST flagellar analysis can therefore provide additional sperm motion parameters as well as the standard kinematic parameters (van der Horst, 2020). By analysing key features of the flagellar beat, it may be possible to determine the energy expenditure of sperm subpopulations and relate that to sperm quality or even track the change of flagellar beat in different environments, among other benefits (Gallagher *et al*., 2019, van der Horst, 2020). FAST also accurately determines tail length thus having a great potential both in studies on fertility assessment as well as in sperm competition studies in animals (van der Horst, 2020).

2.3 | Sperm Selection by the Female Reproductive Tract

Spermatozoa undergo a highly competitive journey in the female genital tract to reach their target, the oocyte (Ikawa *et al*., 2010; Anifandis *et al*., 2014; Okabe, 2014), during which they are exposed to harsh environments, strict selection criteria, and various thermotactic and

chemotactic signals (Miller, 2018). Through these various factors, spermatozoa are prepared for fertilization by inducing physiological and morphological changes in their structure (Ikawa *et al*., 2010; Okabe, 2013; Okabe, 2014; Tulsiani and Abou-Haila, 2011; Tulsiani and Abou-Haila, 2012; Georgadaki *et al*., 2016). Since around 40–50% of infertility cases are caused by male factors and non-functional spermatozoa, the evaluation of sperm interactions within the female reproductive system is valuable in identifying possible causes of infertility (Zandieh *et al*., 2019).

2.3.1 | Vagina

Following ejaculation, semen becomes viscous due to clotting factors and fibrinolysins such as tissue factor, thromboplastin and CD142 facilitating sperm coagulation (Lavanya *et al*., 2022). After coagulation the gel like consistency will be broken down by the enzyme, Prostate-Specific Antigen (PSA), thereby freeing spermatozoa from being trapped in the seminal plasma (Lilja and Lundwall, 1992). Despite the essential nutrients, protective proteins, and acidic pH neutralizing properties of seminal plasma, spermatozoa need to escape this environment soon after entering the female reproductive tract (Maxwell *et al*., 2007). Vaginal fluid is thought to influence sperm motility characteristics (Henkel, 2012). Sperm are exposed to the first luminal medium after semen deposition in the vagina, and the acidic pH of the vagina makes it inhospitable for spermatozoa, although buffers in semen can neutralize the local pH (Miller, 2018). Nevertheless, spermatozoa spend only a short time in the vagina before entering the cervical canal, avoiding damage caused by the low vaginal pH (Henkel, 2012).

Researchers have hypothesized that components in seminal plasma could either prevent or delay the natural sperm maturation process, leading to cell death. Hence the importance of sperm separation techniques in functional sperm testing and ART should be highlighted (Maxwell *et al*., 2007). However, some laboratories process the ejaculate still containing seminal plasma, while others remove the plasma to avoid loss of motility and to maintain normal metabolic activity (Tvrdá *et al*., 2018). Thus, there is no consensus about the use or disposal of seminal plasma for ART purposes, and no specific recommendations have been made by the WHO regarding the possible positive or negative role of seminal plasma during *ex vivo* semen processing (Tvrdá *et al*., 2018).

2.3.2 | Cervix

After vaginal deposition, human sperm leave the seminal pool and move into the cervical canal (Suarez and Pacey, 2006). Only 10% of spermatozoa will enter the highly hydrated

cervical mucus in the cervical os (Figure 1.15) (Henkel, 2012). The degree of hydration will ultimately depend on the stage of the female menstrual cycle (Brunelli *et al*., 2007; Han *et al*., 2017). Spermatozoa undergo a strict selective process in the cervix (Tollner *et al*., 2008; Ikawa *et al*., 2010). Progressive sperm motility is essential for passage through cervical mucus, thus sperm with poor motility parallel with abnormal morphology are filtered out during this passage (Sakkas *et al*., 2015). A spermatozoon containing cytoplasmic droplets is also suggested to be prohibited due to its association with impaired cellular function (Fetic *et al*., 2006; Henkel, 2012). However, recent observations suggest that cytoplasmic droplets may be the site of osmotic volume regulation, thereby may be linked to good motility and the ability to morphologically adapt to fit through the mucin mesh of the cervical mucus (Xu *et al*., 2013). Cytoplasmic droplets may further assist in maintaining rapid progressive motility essential for evading phagocytosis by leukocytes and for penetrating the cervix (Katz *et al*., 1997; Keating *et al*., 1997; Fischer *et al*., 2003). The physical interaction between spermatozoa and the mucus macrostructure of cervical mucus participates in the initiation and preparation of sperm capacitation and stimulation of hyperactivation (Jokiniemi *et al*., 2019). The cervix further aids in removal of Glycodelin-S (GdS), which suppresses sperm capacitation process such as acrosome reaction (Figure 1.16).

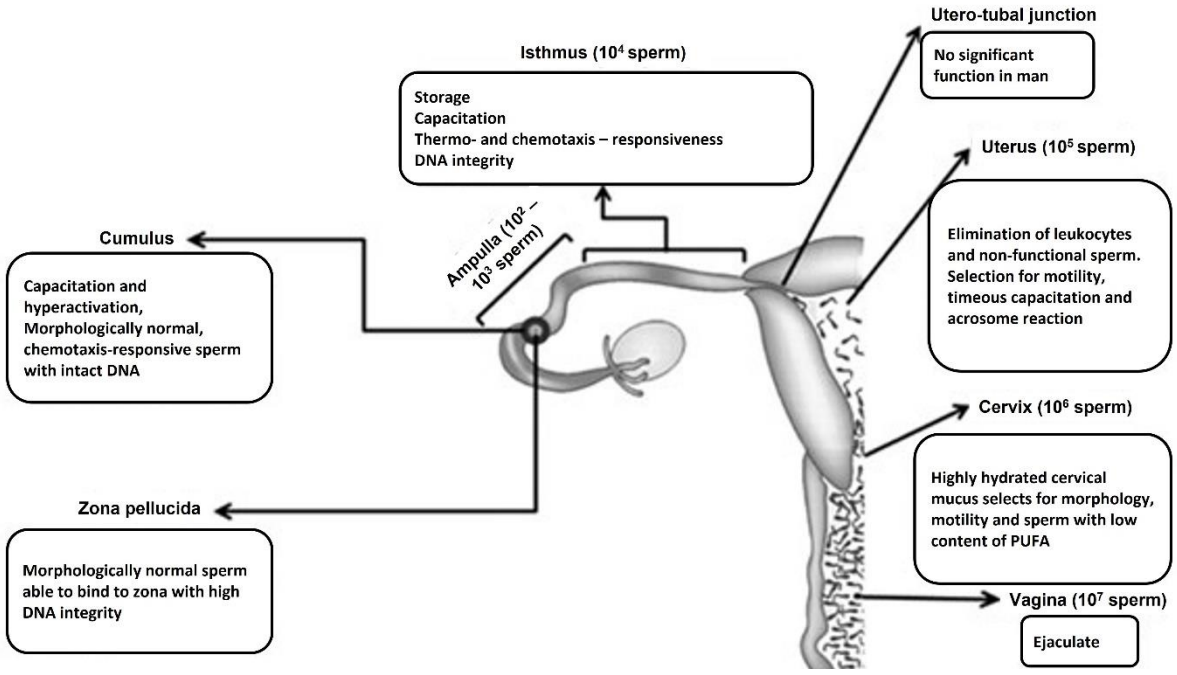

Figure 1.15 Sites of sperm selection in the human female. ***Note:*** Estimated numbers of spermatozoa in the respective sections of the female genital tract are given in brackets. Structural and functional character selection at each barrier is presented in the circles (Henkel, 2012).

2.3.3 | Uterus and Utero-tubal Junction

Approximately, 1% of ejaculated spermatozoa will enter the uterus via vaginal-uterine contractions and progressive motility. However, sperm will only spend a brief period of time in this environment (Yanagimachi, 1994; Eisenbach and Giojalas, 2006; Tulsiani and Abou-Haila, 2012). The uterus contains a hostile environment that functional spermatozoa need to evade to survive (Suarez, 2006). However, during its time spent in the uterus, spermatozoa need to undergo many structural changes which will make them capable of undergoing hyperactivation, AR and ZP binding (Tulsiani and Abou-Haila, 2012).

Leukocytes within the uterus produce ROS that causes increasing membrane permeability to ions essential for chemotaxis and hyperactivated motility (Suarez, 2006; Olaniyan *et al*., 2021). The albumin within the uterus triggers cholesterol efflux from the plasma membrane lipid bilayer, ultimately increasing membrane fluidity and permeability to ions essential for initiation of capacitation (Jin and Yang, 2017). A decrease in the cholesterol to phospholipid ratio results in the alteration of sperm surface glycoproteins, such as the lectin-binding protein, which bind to sugar residues of the ZP (Tulsiani and Abou-Haila, 2012). As a result, only capacitated spermatozoa can bind to the oocyte (Tulsiani and Abou-Haila, 2012).

From the uterus, only about 0.1% of the spermatozoa will reach and enter the utero-tubal junction (UTJ) (Figure 1.15) (Henkel, 2012). Acting as a mucus barrier, the UTJ impedes the progress of spermatozoa as they attempt passing through the narrow, mucus-filled lumen (Suarez, 2006). As such, the greatest obstacle for spermatozoa that enters the uterus, is the passage through the UTJ (Xiong *et al*., 2019).

2.3.4 | Fallopian Tube

The fallopian tubes provide an environment for oocyte maturation, sperm maturation, fertilization, and the transport of gametes and embryos (Zandieh *et al*., 2019). About 10 000 spermatozoa will enter the isthmus of the fallopian tube, designed to preserve and enhance sperm viability and motility, stimulate capacitation and prevent polyspermy by slow release of functionally capable spermatozoa (Figure 1.15) (Suarez, 2016). Spermatozoa will bind to the epithelial cells, prolonging their lifespan and delaying capacitation until ovulation or a maximum of five days in the human female reproductive tract (Maillo *et al*., 2016; Xiong *et al*., 2019). At ovulation, only spermatozoa having undergone capacitation respond to various signals, freeing them from the isthmus and directing them towards the

oocyte (Moody *et al*., 2017). The ability to capacitate spermatozoa *in-vitro* is therefore paramount in successful IVF and other ART procedures (Moody *et al*., 2017). Additionally, toll-like receptors (TLRs) participate in sperm-fallopian tube interactions and contribute to ovulation, sperm activation, fertilization, and pregnancy (Zandieh *et al*., 2019). It has been reported that spermatozoa with high levels of DNA fragmentation activate higher levels of TLRs and pro-inflammatory cytokines which ultimately produce a pathologic environment for capacitation, fertilization, embryo development, and implantation (Zandieh *et al*., 2019).

Fertilization may be aided by follicular fluid in the oviduct. Human follicular fluid participates in the induction of AR, affects sperm motility and hyperactivation, improves sperm-hamster oocyte fusion, enhances sperm acrosin activity and attracts spermatozoa to the oocyte (Brown *et al*., 2017). As such, the supplementation of media with human follicular fluid or pre-treatment of spermatozoa improves results in IVF, gamete intra fallopian transfer and IUI, and increases fertilization rates in sub-zonal insemination (Somfai *et al*., 2012). Furthermore, follicular fluid may modulate the progesterone effect and prevent polyspermia through its physiologically inhibitory effect on ZP binding capacity of spermatozoa (Munuce *et al*., 2004).

Spermatozoa respond to the long-range thermotactic signals from oocyte temperature changes during ovulation (Brown *et al*., 2017). Bicarbonate (HCO_3^-) and carbon dioxide (CO_2) present in the oviductal fluid will act as a chemoattractant, activating adenylyl-cyclase in the sperm tail for hyperactivation (Brown *et al*., 2017). In addition, HCO_3^- and CO_2 act as buffers regulating the pH environment and increasing sperm pH essential for motility (Zhou et al., 2015). Follicular fluid will further participate in binding glycodelin A (GdA) and F (GdF) to spermatozoa (Figure 1.16). The latter, but not the former, inhibits progesterone-induced AR, preventing premature AR of spermatozoa (Chiu *et al*., 2007). Spontaneous acrosome loss in spermatozoa incubated *in-vitro* in synthetic media has been demonstrated in several mammalian species including humans (Li *et al*., 2017). Moreover, an association between the onset of hyperactivated motility and the occurrence of spontaneous AR has been reported (Li *et al*., 2017). Precise timing of the AR is essential for fertilization, and thus must occur on the surface of the ZP (Hirohashi and Yanagimachi, 2018). Accordingly, premature AR leads to the loss of ZP recognition sites from the surface of the spermatozoon, compromising sperm–zona pellucida binding (Hirohashi and Yanagimachi, 2018).

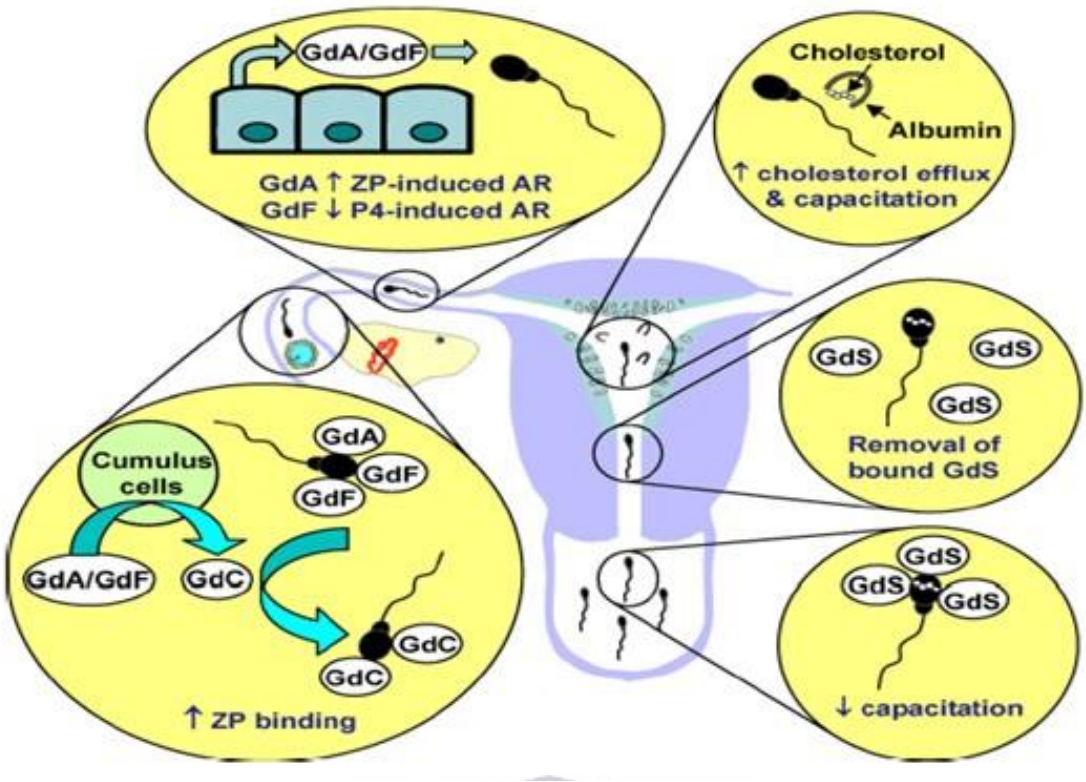

Figure 1.16 Glycodelin glycoforms modulate sperm function in the female reproductive tract. Spermatozoa interact with the glycoforms in succession. GdS in the seminal plasma suppresses albumin-induced cholesterol efflux and maintains the spermatozoa in an uncapacitated state in the vagina. Sperm-bound GdS is removed during the passage of the spermatozoa through the cervical mucus. Albumin in the uterine lumen induces cholesterol efflux from the uterine spermatozoa and initiates capacitation. The oviduct produces GdA and GdF. GdA primes spermatozoa for ZP-induced AR, while GdF suppresses progesterone induced AR. At the fertilization site, GdC in the cumulus matrix displaces sperm-bound GdA and GdF and enhances spermatozoa–ZP binding. The cumulus cells convert GdA and GdF in the FF into GdC (Yeung *et al.*, 2009).

Various ligand-receptor interactions and functionally active signal transduction pathways oversee spermatozoa's race to the oocyte (De Jonge, 2005). Only genomically-mature, viable and acrosome-intact spermatozoa will bind and penetrate the cumulus oophorus complex (COC) (Henkel, 2012). After penetration, spermatozoa reach the last barrier, the ZP, composed of small glycoproteins, of which ZP3 is essential for sperm binding and penetration (Swann *et al*., 2017). The ZP binds spermatozoa with normal morphology, which is associated with many sperm functions including AR and normal DNA integrity (Menkveld *et al*., 1991). According to previous studies, patients with disordered zona-induced acrosome reaction had low or no fertilization after IVF, but good fertilization and pregnancy rates after ICSI, since the latter procedure bypassed all the barriers of selection in the female reproductive tract (Henkel, 2012).

When ZP3 binds to primary receptors on capacitated spermatozoa, it initiates a cascade of intraspermatic events such as biphasic Ca^{2+} influx (Schuffner *et al*., 2002). The AR, an all or nothing event, will ultimately result in the release of the acrosomal contents containing a rich source of enzymes, assisting in the penetration of spermatozoa through the ZP (Hirohashi and Yanagimachi, 2018; Balestrini *et al*., 2020). Studies using zona pellucida-bound

spermatozoa for ICSI found no difference in fertilization rates compared to the normal ICSI protocol (Black *et al*., 2010; Liu *et al*., 2011; Henkel, 2012). However, the use of ZP-bound spermatozoa did result in significantly higher quality of embryos, implantation and pregnancy rates, thus suggesting that ZP-bound spermatozoa are of superior quality (Black *et al*., 2010; Liu *et al*., 2011; Henkel, 2012).

2.4 | Follicular Fluid

As previously mentioned, during pre- and post-ovulatory periods, the ovary, follicular fluid, and female reproductive tract provide biochemically active biomolecules that facilitate oocyte-sperm communication (Olaniyan *et al*., 2021). Spermatozoa are separated from seminal plasma during passage, making them more susceptible to oxidative damage (Opuwari and Henkel, 2016). Despite contributing to the redox status of the sperm environment, spermatozoa interact with the female reproductive fluids and are further able to retain their physiological function (Holt and Fazeli, 2016; Olaniyan *et al*., 2021).

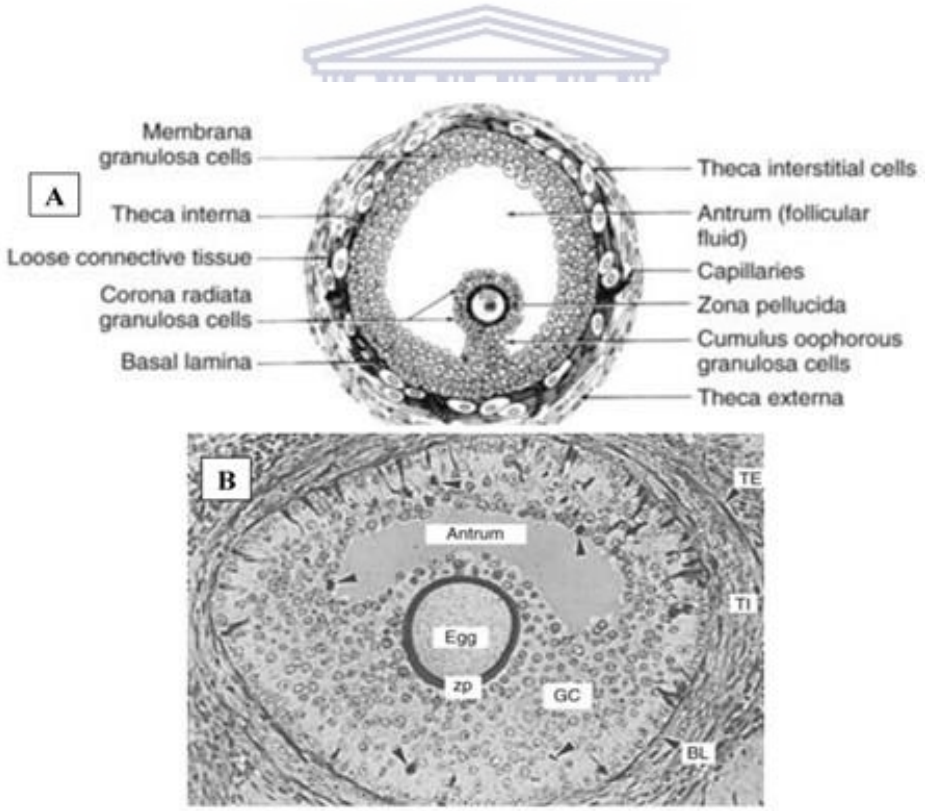

Figure 1.17 The developing oocyte and follicular fluid. ***Note:*** **(A)** Diagram of the cross-sectional appearance of a typical healthy graafian follicle showing the organization of its various cell types (Erickson, 1983). **(B)** Photomicrograph of a tertiary follicle approximately 400 µm in diameter at the time of cavitation or early antrum formation. It comprises a fully growing dictyate oocyte (egg) surrounded by a thick zona pellucida; an antrum containing follicular fluid; multiple layers of granulosa cells organized morphologically into recognizable zones; a basal lamina; a theca interna; and theca externa (Bloom and Fawcett, 1975). **Abbreviations:** BL. Basal lamina; GC, granulosa cells; TE, theca externa; TI, theca interna; ZP, zona pellucida.

An ovarian follicle is the site of a complex network of systemic and local signalling pathways by which the follicle grows, and the oocyte matures in the female reproductive tract (Freitas *et al*., 2017). During folliculogenesis (controlled by FSH and LH), a distinct cellular and liquid environment, consisting mainly of cumulus oophorus cells and follicular fluid (FF), will be established and encircle the evolving oocyte (Figure 1.17) (Revelli *et al*., 2009; Freitas *et al*., 2017). The presence of FF is noticed early in the pre-antral stage of the ovarian follicle, when small, empty spaces appear between granulosa cells (Erickson and Shimasaki, 2001). During the following months the follicles grow, and the dominant follicle stage is attained. The FF (approximately 5 mL) occupies a single compartment that enlarges progressively until it surrounds most of the cumulus oophorus cells and the oocyte (Erickson and Shimasaki, 2001).

The various constituents found in FF are essential for the maturation and modification of both oocyte and spermatozoa in fertilization (Iwasi *et al*., 2013). FF is initially derived from, and is similar in composition to, thecal capillary serum (Rodgers and Irving-Rodgers, 2010; Emori and Drapkin, 2014). Along with the development of the follicle, granulosa cells produce large polysaccharides, hormones, and growth factors, causing an osmotic gradient which further increases follicular fluid volume (Emori and Drapkin, 2014). By the time a mature or Graafian follicle has formed, the FF contains a complex mixture of steroids, metabolites, polysaccharides, proteins and small peptides, ROS and antioxidant enzymes (Kushnir *et al*., 2016; Freitas *et al*., 2017).

The identification of FF peptides and related proteins has been the subject of intense research; however, for ethical reasons and due to methodological difficulties related with retrieving the fluids, obtaining human fluids is not recommended (Robert *et al*., 2008). Many FF proteins have extracellular origins, while the remaining proteins are related to organelles or other cellular features (Shen *et al*., 2017). Biological activities include enzymes, regulatory proteins and forming part of the extracellular matrix or involvment in cell adhesion (Freitas *et al*., 2017). The fact that many of the proteins are enzymes, suggests that the compartment is not a mere reservoir but also supports intense metabolic activity (Chi *et al*., 1998; Freitas *et al*., 2017).

Well-known hormones contained in FF include gonadotropins, such as follicle stimulating hormone (FSH), luteinizing hormone (LH), and human chorionic gonadotropin (hCG), all of which encourage oocyte maturation and are associated with eggs having a high chance of fertilization (Revelli *et al*., 2009). Prolactin (PRL), growth hormone (GH) and anti-

mullerian hormone (AMH) are also present in FF (Klein *et al*., 1996). GH is known to enhance the FSH-dependent Estradiol 2 production of granulosa cells and the formation of FSH and LH receptors in these cells (Lanzone *et al*., 1996). Oestrogens, progesterone and androgens such as testosterone also play a role in FF. For instance, observations have shown that high FF progesterone concentrations are predictive for subsequent implantations and pregnancies (Revelli *et al*., 2009; Carpintero *et al*., 2015).

Glucose is the most important energy substrate in most mammalian cells, and evidence regarding glucose metabolism in the human granulosa cells/oocyte complex is still a matter of investigation (Richardson *et al*., 2009). Remaining under gonadotrophic control, *in vitro* granulosa cells accumulate lactate which is derived from glycolysis (Richardson *et al*., 2009). The insulin-stimulated uptake of glucose in these cells has been shown to partially depend on a transport system involving phosphatidyl-inositol-3 kinase (Józwik *et al*., 2007). Initial investigations observed a five-carbon sugar ribulose-containing compound of peptide nature in human FF, whereas another investigation reported glucose and fructose in pre-ovulatory FF of patients undergoing ovarian stimulation for IVF (Sucha *et al*., 2002). Further research has indicated the presence of myo-inositol, a six-carbon sugar alcohol, in human FF (Chiu *et al*., 2002) and hyaluronan, which is contained in the cumulus extracellular matrix where it is involved in the detachment of the COC that becomes freely floating in FF just before ovulation (Józwik *et al*., 2007; Revelli *et al*., 2009). Neurotransmitters such as norepinephrine and dopamine have also been detected in ovarian homogenates and are suggested to contribute to ROS homeostasis in the follicular microenvironment (Saller *et al*., 2014).

2.5 | Components in Follicular Fluid

A follicle's antrum is filled with FF that is derived both from blood components that cross the blood-follicle barrier as well as from follicle-derived products (Björvang *et al*., 2022). It has been mentioned previously that FF includes several biological components that are essential for fertilization. There is, however, a lack of information on how the biological components affect a comprehensive set of sperm functional parameters, especially for different subpopulations of human spermatozoa. Hence, the purpose of this study was to investigate a comprehensive set of functional parameters related to fertilization of various sperm subpopulations, in addition to how progesterone, myo-inositol, dopamine, and prolactin may alter these parameters.

2.5.1 | Progesterone

Progesterone is a steroid hormone with the chemical formula $C_{21}H_{30}O_2$ and is involved in the regulation of the female reproductive cycle of mammals (Figure 1.18). Progesterone is present around or secreted by cumulus oophorus cells and may also be distributed along a gradient capable of initiating various functional and structural changes *in vitro* in spermatozoa (Meizel, 1990; Calogero *et al*., 2000; Servin-Vences *et al*., 2012; Unates *et al*., 2014; Jung *et al*., 2018; Asavasupreechar *et al*., 2020). It's proposed that only capacitated human sperm can participate in chemotactic swimming using progesterone gradients near the egg (Puga Molina *et al*., 2018).

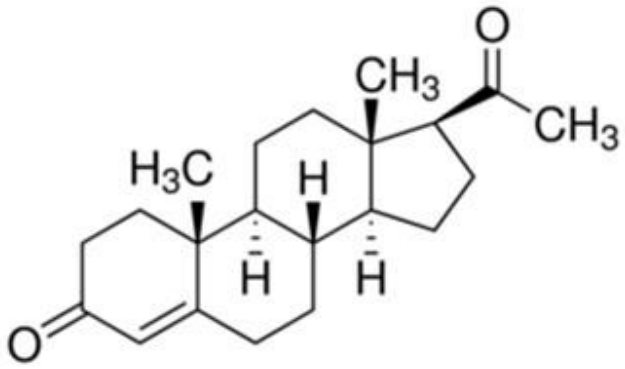

Figure 1.18 Chemical structure of progesterone (Sigma Aldrich, 2018a).

Initially the physiological function of progesterone was attributed to inducing AR, however, it is now known to induce hyperactivation, stimulate AR, increase ZP binding and increase sperm penetration rate into hamster oocytes (Foresta *et al*., 1992; Parinaud *et al*., 1992; Uhler *et al*., 1992; Sueldo *et al*., 1993; Calogero *et al*., 1996; Parinaud and Milhet, 1996; Bronson *et al*., 1999; Jaiswal *et al*., 1999; Harper *et al*., 2004; Kirkman-Brown *et al*., 2004; Calogero *et al*., 2000; Servin-Vences *et al*., 2012). In mammalian spermatozoa, progesterone regulates AR, ZP penetration, and hyperactivation through non-genomic regulation (Servin-Vences *et al*., 2012; Fujinoki *et al*., 2016). Therefore, it is suggested that progesterone, acting alone or in concert with ZP, is another physiological initiator of sperm mechanisms such as AR and hyperactivation (Sanchez-Cardenas *et al*., 2014). Extracellular Ca^{2+} influx is induced by progesterone through CatSper (Figure 1.19), which is a sperm-specific Ca^{2+} channel located in the principal piece of the sperm flagellum (Ren and Xia, 2010; Fujinoki, 2013; Diao *et al*., 2019; Zhang *et al*., 2020). However, in addition to progesterone - CatSper channels and associated proteins have multiple binding sites that can be stimulated by ligands such as neurotransmitters, chemokines, and odorants (Diao *et al*., 2019).

Even though progesterone causes rapid escalation of Ca^{2+} concentrations followed by a brief period of extracellular Ca^{2+} influx, many other mechanisms are also triggered by progesterone (Baldi *et al*., 1995; Calogero *et al*., 2000). These include the stimulation of a trypsin-like activity, biosynthesis of polyamines and platelet-activating factor (PAF), and PTK activity (Baldi *et al*., 1995). Non-genomic regulation of hyperactivation by progesterone is associated with Phospholipase-C (PLC), which activates the production of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from phosphatidylcholine and phosphatidylinositol (PI), respectively (Noguchi *et al*., 2008; Fujinoki, 2013). However, not all these biological effects of progesterone are mediated by the common increase of intracellular Ca^{2+}. Experimental evidence suggests that a chloride ion transmembrane flux is vital for progesterone in promoting AR (Lishko *et al*., 2011). This efflux is activated through the interaction with GABA-like receptors and PTK mediation, as it occurs against a chemical gradient (Lishko *et al*., 2011). This resultant membrane depolarization will ultimately lead to the various biological effects (Campbell *et al*., 2013). In addition, progesterone will increase the intracellular content of cAMP and stimulate the activity of the protein-kinase C (PKC), thereby leading to the mediation of progesterone's biological effects downstream to the Ca^{2+} -increase (Noguchi *et al*., 2008; Lishko *et al*., 2011). Despite progesterone commonly being cited in literature, the effects of progesterone on a collective selection of various sperm functional parameters are lacking for human sperm subpopulations.

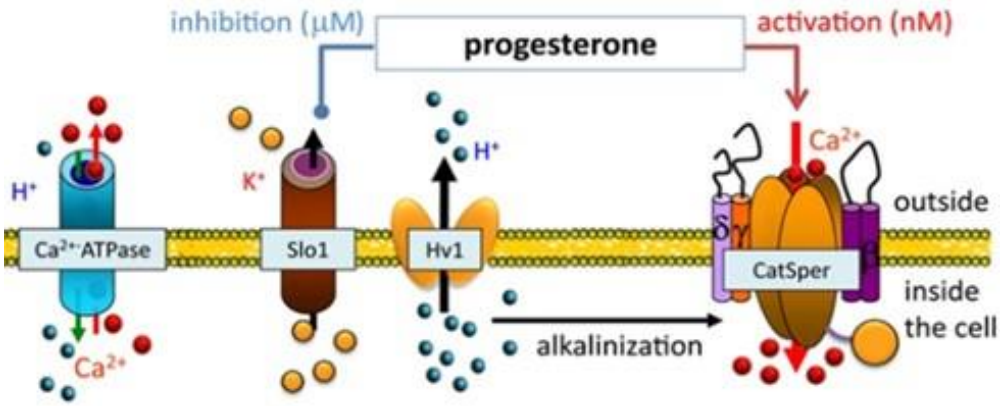

Figure 1.19 Ion channels working together with progesterone. ***Note:*** Spermatozoa contain a variety of ion channels that control the movement of ions and protons into and out of the cell. As a sperm cell moves up the fallopian tube, the CatSper ion channel (right), which controls the movement of Ca^{2+}, is partially activated because of alkalinization inside the cell (caused by protons leaving through the Hv1 ion channel) and low levels of progesterone outside the cell. As the spermatozoa get closer to the egg, the increased levels of progesterone inhibit the Slo1 ion channel, causing K^+ to leave the cell. This hyperpolarizes the cell membrane and leads to full activation of the CatSper ion channel. The resulting influx of large numbers of Ca^{2+} leads to hyperactivation of the spermatozoa—the vigorous tail thrashing motion that is a prerequisite of fertilization. Protons and calcium ions can also move through the Ca^{2+} ATPase transporter (left) (Clapham, 2013).

2.5.2 | Myo-inositol

Myo-inositol is a sugar-like molecule with the chemical compound formula $C_6H_{12}O_6$, a six-fold alcohol (polyol) of cyclohexane with five equatorials and one axial hydroxyl group (Scarselli *et al*., 2016; Condorelli *et al*., 2017). Nine different stereoisomers exist in nature, but myo-inositol is the most common (Figure 1.20) (Condorelli *et al*., 2017). Myo-inositol is found in many plants and in animal tissue. The classification of the inositol as a vitamin-B has been discussed with controversy as inositol is not an essential substance and can be produced in human cells from glucose (Montanino Oliva *et al*., 2016; Regidor and Schindler, 2016).

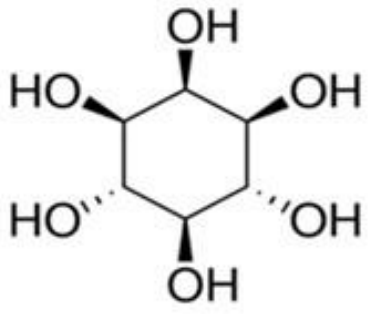

Figure 1.20 Chemical structure of Myo-inositol (Sigma Aldrich, 2018b).

Several studies have proven the direct involvement of inositol with insulin cellular signalling (Montanino Oliva *et al*., 2016; Regidor and Schindler, 2016). Acting as a precursor for secondary messengers such as DAG and IP3, inositol is involved in cellular signal transduction systems and regulation of intracellular Ca^{2+} concentrations (Condorelli *et al*., 2012; Croze and Soulage, 2013). As such, myo-inositol is an important precursor for the PI signalling pathways (Berridge, 2009). Inositol incorporates itself with PI, which will further be successfully converted to the polyphospho-inositides, PIP and PIP2 (Berridge, 2009). Under specific stimuli, PIP2 will be hydrolysed to produce two secondary messengers, DAG and IP3 which respectively modulate specific protein phosphorylation processes and intracellular Ca^{2+} concentrations (Figure 1.21) (Berridge, 2009).

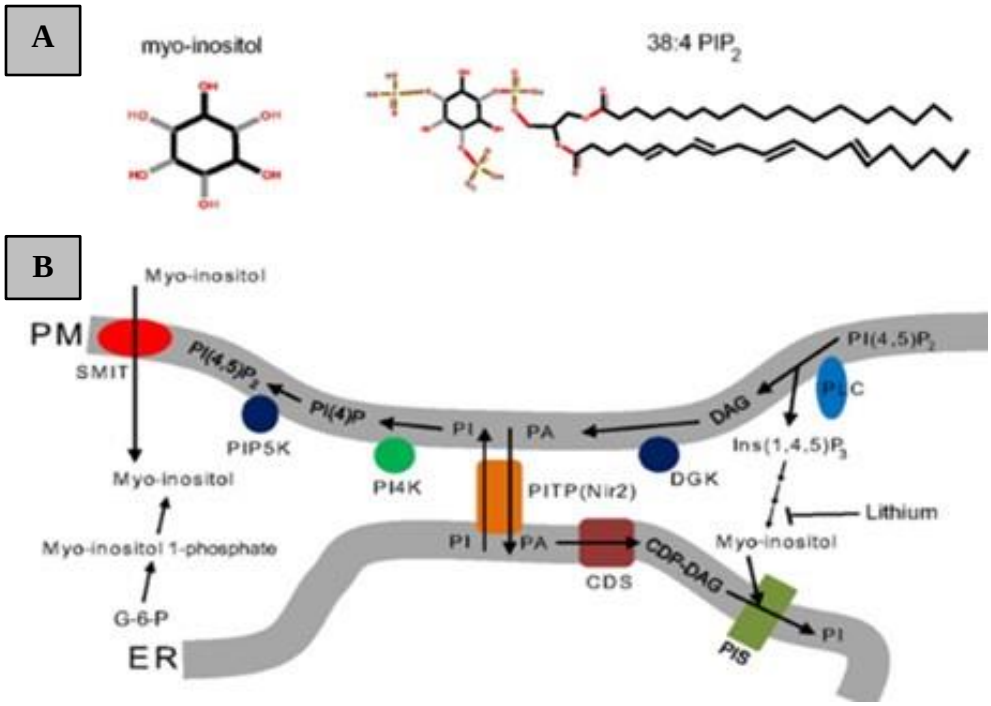

Figure 1.21 Myo-inositol actions on cells. ***Note:*** **(A)** Diagram showing the structure of myo-inositol and PIP2. The black and grey sticks in the myo-inositol structure indicate different geometrical orientations of the chemical bonds connecting the inositol ring and the hydroxyl group. **(B)** Diagram illustrating the metabolism of phosphoinositides. PITP(Nir2) can transfer PI from ER to PM and PA from PM to ER at ER–PM (Dai *et al.*, 2016). **Abbreviations:** CDP-DAG, Cytidine diphosphate DAG; CDS, cytidine diphosphate synthase; DGK, discylglycerol kinase; ER, endoplasmic reticulum; G-6-P, glucose-6-phosphate; PA, phosphatidic acid; PI, phosphatidylinositol; PI4K, phosphatidylinositol-4-kinase; PI(4)P, phosphatidylinositol-(4)-phosphate; PI(4,5)P2, phosphatidylinositol-(4, 5)-biphosphate; PIP5K, phosphatidylinositol-(4)-phosphate 5 kinase; PITP, phosphatidylinositol synthase, phosphatidylinositol transfer protein; PLC, phospholipase C; PM, plasma membrane; SMIT, sodium-coupled myo-inositol transporter

Spermatozoa possess a sophisticated mechanism for regulation of cytoplasmic Ca^{2+} concentrations (Correia *et al*., 2015). The presence of two intracellular Ca^{2+} stores and CatSper in the principal piece has been proposed (Correia *et al*., 2015). One of the Ca^{2+} permeable channels present in the Ca^{2+} storage organelles of spermatozoa is the PIP3-sensitive Ca^{2+} channel, which has been extensively studied in a variety of cell types (Darszon *et al*., 2006). The PIP3 channel binds the second messenger IP3, ultimately leading to elevation of intracellular Ca^{2+} concentrations (Mannowetz *et al*., 2017). Present in FF, myo-inositol is additionally synthesized by two enzymes, namely myo-1-phosphate synthase and myo-monophosphatase, both of which are present in high concentrations in the testes (Condorelli *et al*., 2012, Korosi *et al*., 2017). The human testis synthesizes myo-inositol from glucose-6-phosphate, and further transports the inositol into cells via a sodium/myo-inositol co-transporter protein (Condorelli *et al*., 2012, Korosi *et al*., 2017). Both myo-1-phosphate synthase and myo-monophosphatase-1 are expressed in germinal and Sertoli cells, and only the latter express the sodium/myo-inositol co-transport proteins (Parthasarathy *et al*., 1993).

Myo-inositol is involved in numerous biological processes, including protein binding to the cell surface, cell signalling and vesicle trafficking, membrane excitability, regulation of ion channel opening, intracellular Ca^{2+} signalling, cytoskeleton and chromatin dynamics and remodelling, gene expression, and epigenome regulation (Vazquez-Levin and Veron, 2020). Myo-inositol is suggested to play a role in maturation and migration of spermatozoa from the epididymis, as concentrations are higher in the seminiferous tubules than in serum and increases during the sperm movement through the epididymis and the deferent duct (Cooper, 1986). Inositol has further been suggested to play a role in chemotaxis and thermotaxis through activation of PLC, resulting in IP3 binding which opens Ca^{2+} channels, thereby increasing intracellular Ca^{2+} concentrations in the flagellum (Correia *et al*., 2015). By acting on the sperm plasma membrane, mitochondria, acrosome, and neck region, inositol regulates the intracellular Ca^{2+} concentrations (Condorelli *et al*., 2012; Korosi *et al*., 2017).

Studies show that myo-inositol enhances sperm mitochondrial function, consequently improving sperm motility in patients with altered sperm parameters (Nordio, 2017). In addition, myo-inositol increases the total number of spermatozoa after swim-up in normozoospermic men and, more importantly, in patients with abnormal sperm parameters (Condorelli *et al*., 2012). As such, myo-inositol in the reproductive tract of both female and male play vital roles in improving sperm quality, including motility, viability and cholesterol efflux required for sperm capacitation (Santoro *et al*., 2020). However, few studies have collectively investigated and compared the effects of myo-inositol on a comprehensive set of functional parameters of human sperm subpopulations to different biological components additionally found in FF.

2.5.3 | Dopamine

Monoamines such as the catecholamines, are derived from the amino acid tyrosine and include the three essential neurotransmitters namely norepinephrine, epinephrine, and dopamine (Klein *et al*., 2019). Dopamine participates in many biological processes in mammals, including those related to cognition, emotions, and control of motor activity (Urra *et al*., 2014; Rangel-Barajas *et al*., 2015) (Figure 1.22). Dopamine can be synthesized directly from tyrosine, but also indirectly by the conversion of L-phenylalanine to tyrosine by phenylalanine hydroxylase (Klein *et al*., 2019). Tyrosine hydroxylase converts tyrosine to levodopa (L-DOPA), a precursor of dopamine, using tetrahydrobiopterin, oxygen, and iron as cofactors. Consequently, L-DOPA can then be converted to dopamine by aromatic L-amino acid decarboxylase (DOPA decarboxylase), having pyridoxal phosphate as a cofactor (Klein *et al*., 2019) (Figure 1.23).

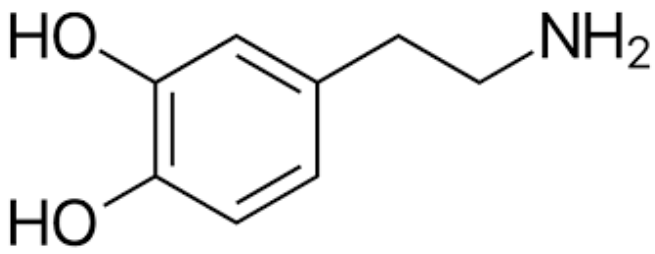

Figure 1.22 Chemical structure of Dopamine (Mills, 2019).

High concentrations of catecholamines have been observed in human semen and at variable concentrations in the oviduct (Bahr *et al*., 1986; Fernandez-Pardal *et al*., 1986; Ramirez *et al*., 2009; Urra *et al*., 2014). Expression of tyrosine hydroxylase, a rate-limiting enzyme in catecholamine synthesis, has been observed in Leydig cells of humans, thus raising the question of whether catecholamines are synthesized from a source other than the testis and oviduct (Rodríguez-Gil, 2019). By implication this means that sperm would be in contact with catecholamines, or at least with L-DOPA, from a very early stage (Rodríguez-Gil, 2019).

Invertebrate and mammalian sperm express receptors for many neurotransmitters and neuromodulators including dopamine (Ramírez-Reveco *et al*., 2017). Western blot analysis of human and rat seminal vesicle tissues has demonstrated peripheral dopamine D1 and D2 receptor proteins and dopamine transporter (DAT) on sperm membranes (Hyu *et al*., 2002; Ferguson, 2021), indicating that dopamine may play a role in sperm functionality. Dopamine receptors are transmembrane trimeric guanosine triphosphate (GTP)-binding protein (G protein)-coupled receptors (Ramirez *et al*., 2009; Harlev *et al*., 2020). Dopamine type 2 receptors (DRD2) have been detected in testis, spermatogenic cells, and sperm of human, rats, bull, and mouse (Ramirez *et al*., 2009; Harlev *et al*., 2020). Classified according to pharmacological characteristics and sequence homology, dopamine type 1-like receptors (DRD1 and DRD4) activate adenylyl cyclase by coupling to stimulatory GTP-binding regulatory protein (Gs protein), thereby increasing cAMP accumulation and activating cAMP-dependent PKA (Ramirez *et al*., 2009). In contrast, D2-like receptors (DRD2, DRD3, and DRD4) inhibits adenylyl cyclase by coupling to inhibitory GTP-binding regulatory protein (Gi/o) and decreasing PKA activity (Ramirez *et al*., 2009). As such, when ligands bind, the G proteins will either activate or inhibit adenylyl cyclase, thereby affecting the levels of secondary messengers, such as cAMP, DAG and IP_3 (Figure 2.24).

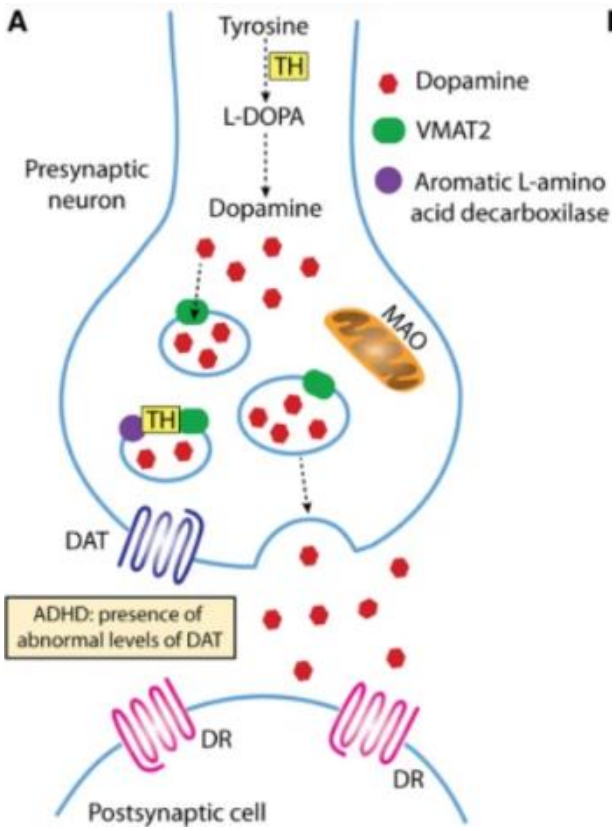

Figure 1.23 An example of Dopamine synthesis in a typica; presynaptic neuron. ***Note:*** Primary metabolic route involving a two-step synthesis. First tyrosine hydroxylase converts tyrosine to L-DOPA which can then be converted to dopamine. Dopamine is transported from the cytosol by a vesicular monoamine transporter into synaptic vesicles where it is stored until release into synaptic cleft. Dopamine degradation pathways with Monoamine oxidase present in outer mitochondrial membrane. Dopamine receptors are present in both post and presynaptic neurons (including dopamine transporter, DAT) (Klein *et al*., 2019). **Abbreviations:** DAT, dopamine transporter; MAO, Monoamine oxidase; TH, tyrosine hydroxylase; VMAT2, vesicular monoamine transporter.

During sperm transit through the female genital tract, several diverse and simultaneous systems regulate sperm functional processes (Rodrguez-Gil, 2019). Signals like neurotransmitters are related to not only sperm reaching the oviduct, but also achieving capacitation (Rodríguez-Gil, 2019). Labelled a “neuron with a tail” (Meizel, 2004), spermatozoa share similar membrane characteristics and features to neurons, but lack synaptic mechanisms (Cortés-Rodriguez *et al*., 2018). Dopamine appears to act as a physiological modulator and studies on animals have demonstrated low concentrations to elicit a positive effect on sperm capacitation, viability and progressive motility (Otth *et al.*, 2007; Ramirez *et al*., 2009; Urra *et al*., 2014; Cariati *et al*., 2016). In contrast, high concentrations were shown to decrease tyrosine phosphorylation and thus negatively affect various sperm functional parameters such as hyperactivation and motility (Otth et al., 2007; Ramirez et al., 2009; Urra et al., 2014; Cariati et al., 2016). Investigation of dopamine on sperm functional characteristics such as ROS, MMP and various sperm kinematics are still lacking.

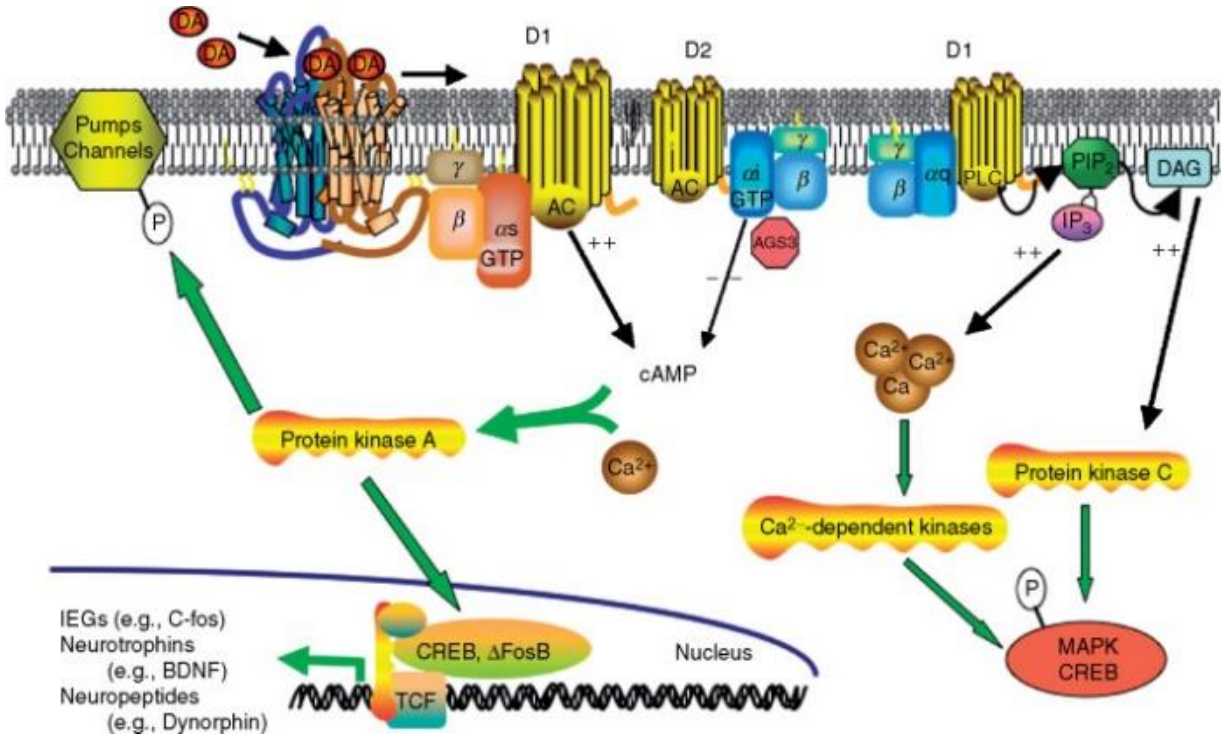

Figure 1.24 Dopamine receptor signalling cascades. ***Note:*** Dopamine acts at G-protein-coupled receptors to produce activation (via D1/D5 receptors) or inhibitory effects (via the D2 family (D2–D4)) overall. Dopamine acting at D1/D5 receptors predominantly activates the stimulatory G-protein-coupled receptors (Gs), which upregulate adenylyl cyclase activity, leading to an increase in cAMP. Higher levels of cAMP activate protein kinase A, which phosphorylates many proteins, including transcriptional activators, and membrane-bound ion pumps and channels. Phosphorylation of ion pumps/channels produce short-term alterations in the membrane potential, causing the cell to become more or less likely to fire. In contrast, longer-term effects are seen through the phosphorylation of transcription factors such as CREB and ΔFosB. These transcription factors ultimately increase the production of proteins, including c-fos, neurotrophins, and neuropeptides. Dopamine at D2–D4 receptors activates the inhibitory G-protein-coupled receptors (Gi), which decreases adenylyl cyclase activity. Dopamine can also act through a secondary D1/D5 pathway, via Gq receptors and the phosphatidylinositol pathway. In this pathway, dopamine binding increases phospholipase C activity, which produces inositol triphosphate and diacylglycerol from phosphatidylinositol. Increased levels of diacylglycerol activate protein kinase C, which phosphorylates other kinases such as mitogen-activated protein kinase, and transcription factors such as CREB. Higher levels of inositol triphosphate increase cytoplasmic Ca^{2+} levels, thereby activating calcium-dependent kinases, which further continue the process of protein phosphorylation and activation (Andersen and Leussis, 2009). **Abbreviations:** AC, adenylyl cyclase; AGS3, activator of G-protein signaling 3; BDNF, brain-derived neurotrophic factor; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; DA, dopamine; DAG, diacylglycerol; ΔFosB, delta-Fos subunit B; GTP, guanosine triphosphate; IEGs, immediate early genes; IP_3, inositol triphosphate; MAPK, mitogen-activated protein kinase; PIP_2, phosphatidylinositol; PLC, phospholipase C; TCF, transcription factor.

2.5.4 | Prolactin

Prolactin is a polypeptide hormone, primarily secreted by lactotrophic cells of the pituitary gland (Raut *et al*., 2019) (Figure 1.25). However, it is also synthesized at extra-pituitary sites such as the mammary epithelium, placenta, uterus, brain and immune system (Harris *et al*., 2004). Prolactin is known to have more actions than all the other pituitary hormones combined (Raut *et al*., 2019). It plays a role in complex activities such as osmoregulation, growth and development, endocrine functions, metabolism, neurobiological functions, immunomodulation as well as reproduction (Raut *et al*., 2019). Plasma prolactin concentrations are said to increase for >1 hour in humans following orgasm. This increase is

suggested to initiate the increased oviductal fluid flow to the uterus (Miki and Clapham, 2013). Besides its important role in female reproduction, prolactin also modulates testicular function (Keyser *et al*., 2021). A functional role of prolactin has been observed in the control of gonadal activity and sexual behaviour, and receptors are widely distributed in a number of cells and tissues, including spermatozoa and testes (Martnez-Fresneda *et al*., 2020).

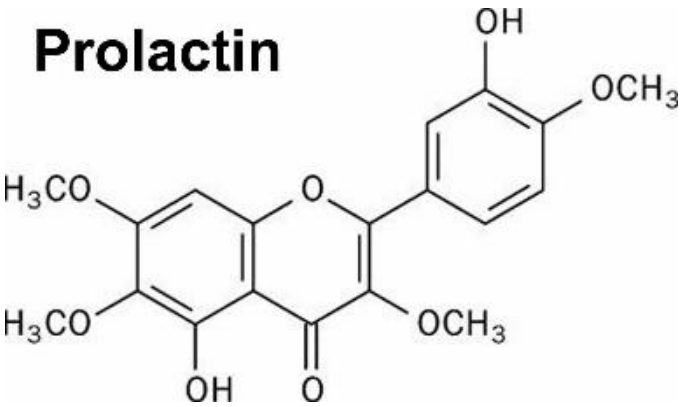

Figure 1.25 Chemical structure of Prolactin (Health by Jade, 2019).

The prolactin receptor has been demonstrated to be localised in the postacrosomal region of the sperm head, neck, and midpiece together with a punctate labelling pattern along the principal piece of the sperm tail (Pujianto *et al*., 2010). Prolactin acts via a transmembrane receptor, PRL-R which belongs to class 1 cytokine receptor superfamily (Harris *et al*., 2004; Raut *et al*., 2019). Prolactin receptor has two distinct domains - the extracellular domain has a ligand binding site to which prolactin binds and brings about dimerization of the receptor (Harris *et al*., 2004; Raut *et al*., 2019). The Intracellular domain is responsible for initiating the signalling cascade for the prolactin receptor, which occurs via transphosphorylation of Janus kinase (Jak2) (Harris *et al*., 2004; Raut *et al*., 2019). Jak2 initiates phosphorylation of other tyrosine residues which act as binding sites for transducer molecules like signal transducer and activator of transcription (STATs) which binds via SH2 domain (Harris *et al*., 2004; Raut *et al*., 2019). The prolactin receptor also activates Ras/Raf/MAPK (mitogen-activated protein kinase) pathways by stimulating a wide variety of transcription factors or early genes by phosphorylation leading to various genomic effects which is involved in various biological functions (Raut *et al*., 2019) (Figure 1.26).

Prolactin promotes both steroidogenesis and spermatogenesis in the testis, as well as the metabolism, motility, and fertilizing potential of spermatozoa (Martínez-Fresneda *et al*., 2020). Prolactin may have pro-survival effects on spermatozoa, but it is unclear whether it stimulates, inhibits, or has no effect on sperm capacitation (Keyser *et al*., 2021). By modulating the FSH

and LH receptors on Sertoli and Leydig cells, prolactin regulates various testicular functions (Raut *et al*., 2019). For example, studies have observed that the restoration of normal prolactin levels in subfertile, hypoprolactinaemic men caused an increase in sperm density and quality, and restored fertility (Hair *et al*., 2002). In contrast, acute hyperprolactinemia is known to suppress testosterone synthesis and male fertility through prolactin-induced hypersecretion of adrenal corticoids or by inhibiting the secretion of GnRH through prolactin receptors on hypothalamic dopaminergic neurons (Gil-Sharma, 2009).

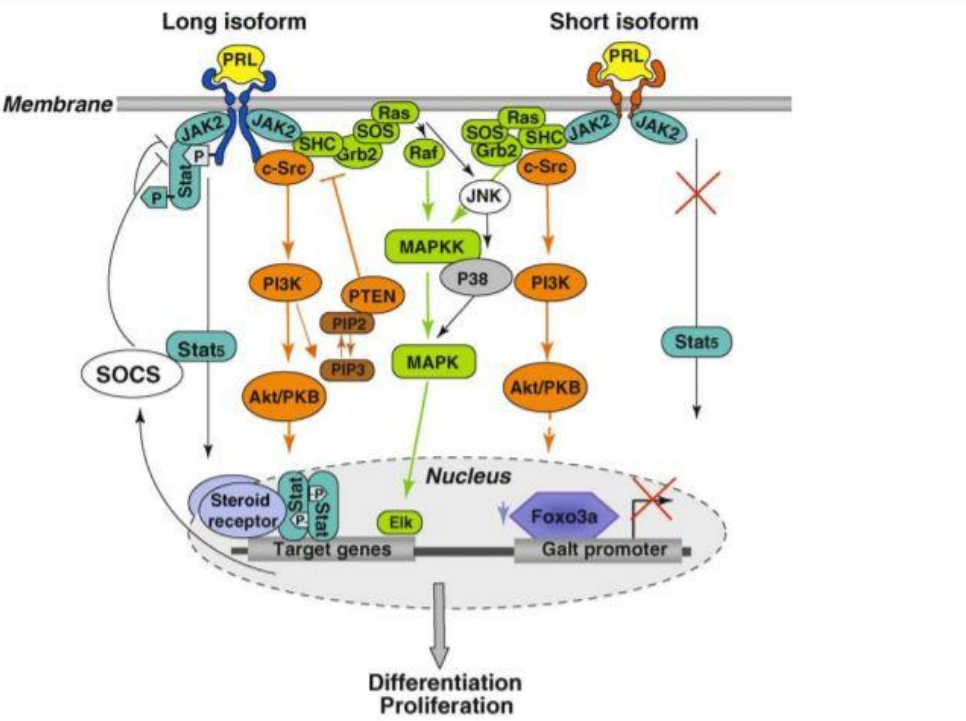

Figure 1.26 Major signalling cascades triggered by long and short prolactin receptor isoforms. ***Note:*** Ligand-induced activation of the PRLR triggers several signalling cascades (Binart *et al*., 2010). **Abbreviations:** Jak2, Janus kinase; MAPK, mitogen-activated protein kinase; PI3K, Phosphoinositide 3-kinase; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PIP2, phosphatidylinositol (3,4,5)-tdisphosphate; PRLR, prolactin receptor; STAT, signal transducer and activator of transcription.

2.6 | Conclusion

While several techniques have been developed over the years to isolate highly functional sperm subpopulations, characterizing such subpopulations remain problematic. A basic semen analysis alone cannot determine the exact fertilizing potential of a sample and only allude as to what the high motile sperm subpopulation's quality may be. Several studies investigated the functional characteristics of high motile sperm subpopulations; however, this was mainly in comparison to the original semen samples' quality. From the few studies comparing low motile and high motile sperm subpopulations, only a select few functional characteristics were examined. Thus, a comprehensive comparison of a collective set of in-depth functional characteristics that best characterise each subpopulation and how it relates to fertility is lacking.

By correlating subpopulation characteristics to the semen parameters, useful information as to which parameters represent each individual sperm subpopulation or its functional parameters can be determined. Such information could also assist in determining the semen parameters that provide the most reliable predictor of fertility. Furthermore, subpopulation characteristics can be correlated and compared to those of fertile and sub-fertile semen samples, thereby providing a possible future research model for investigating different fertility status or effects of various chemicals on such fertile and sub-fertile samples.

Since around 40–50% of infertility cases are caused by male factors and non-functional spermatozoa, the evaluation of sperm interactions within the female reproductive system is valuable in identifying possible causes of infertility. Despite few studies having individually investigated the effects of progesterone, myo-inositol, dopamine and prolactin on spermatozoa, these investigations were only preformed on high motile sperm subpopulations or semen samples. In addition, only a select few functional characteristics were examined, and various biological components of FF (e.g., progesterone, myo-inositol, dopamine and prolactin) were not compared to one another. The current study aimed to expose sperm subpopulations to a selection of biological components in FF (progesterone, myo-inositol, HD-C, dopamine and prolactin) and investigate their effects on a comprehensive set of functional sperm characteristics. Through utilizing the previously mentioned research model, the study can elude as to how spermatozoa of various fertilizing potentials respond the different components of FF. Thus, the knowledge of the different effects of the media on different sperm subpopulations could provide possible alternative methods to improve sperm subpopulations of low quality to be similar to that of subpopulations of a higher quality. As such, sub-fertile samples could be improved prior to ART to increase the chances of successful fertilization.

During the past two decades, CASA systems have been revolutionized largely due to advances in both hardware and software, providing increased accuracy and verification. However, due to only tracking the centroid of the sperm head for motility analysis, the question remains whether valuable information from flagellar movement could be lost. By comparing both a standard CASA system and a new flagellar analysis program, the study aims to determine whether flagellar analysis may provide additional information on the potential fertility of a sample as compared to CASA.

CHAPTER THREE: Methods and Materials

3.1 | Introduction to the Different Phases of the Study

This section provides an overview of the methodology and materials used in the different phases of this study, followed by the detailed protocols employed throughout. Figure 3.1, 3.2 and 3.3 illustrates the basic outline of the experimental procedures included in the first, second and third phases of this study.

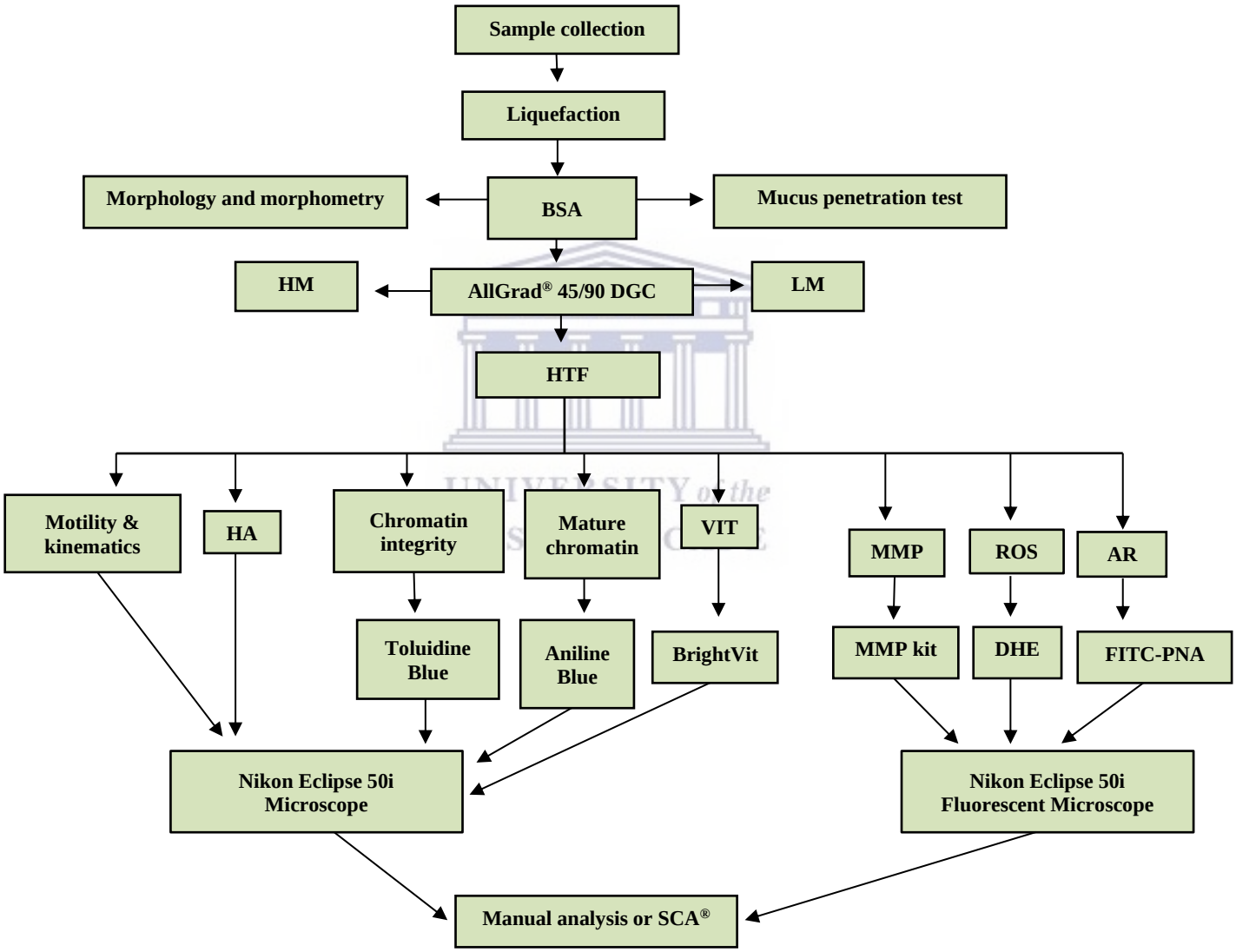

Figure 3.1 Flow diagram illustrating the simplified procedural outline of the first phase of the study involving the separation of donor semen samples into two motility sperm subpopulations (high motile, HM and low motile, LM) and investigating their various functional characteristics in HTF. **Abbreviations:** AR, acrosome reaction; BSA, basic semen analysis; DGC, double density gradient centrifugation; DHE, dihydroethidium; FITC-PNA, Fluorescein isothiocyanate-labelled Peanut Agglutinin assay; HA, hyperactivation; HM, high motile; HTF, non-capacitating human tubal fluid; LM, low motile; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; SCA®, Sperm Class Analyser®; VIT, vitality.

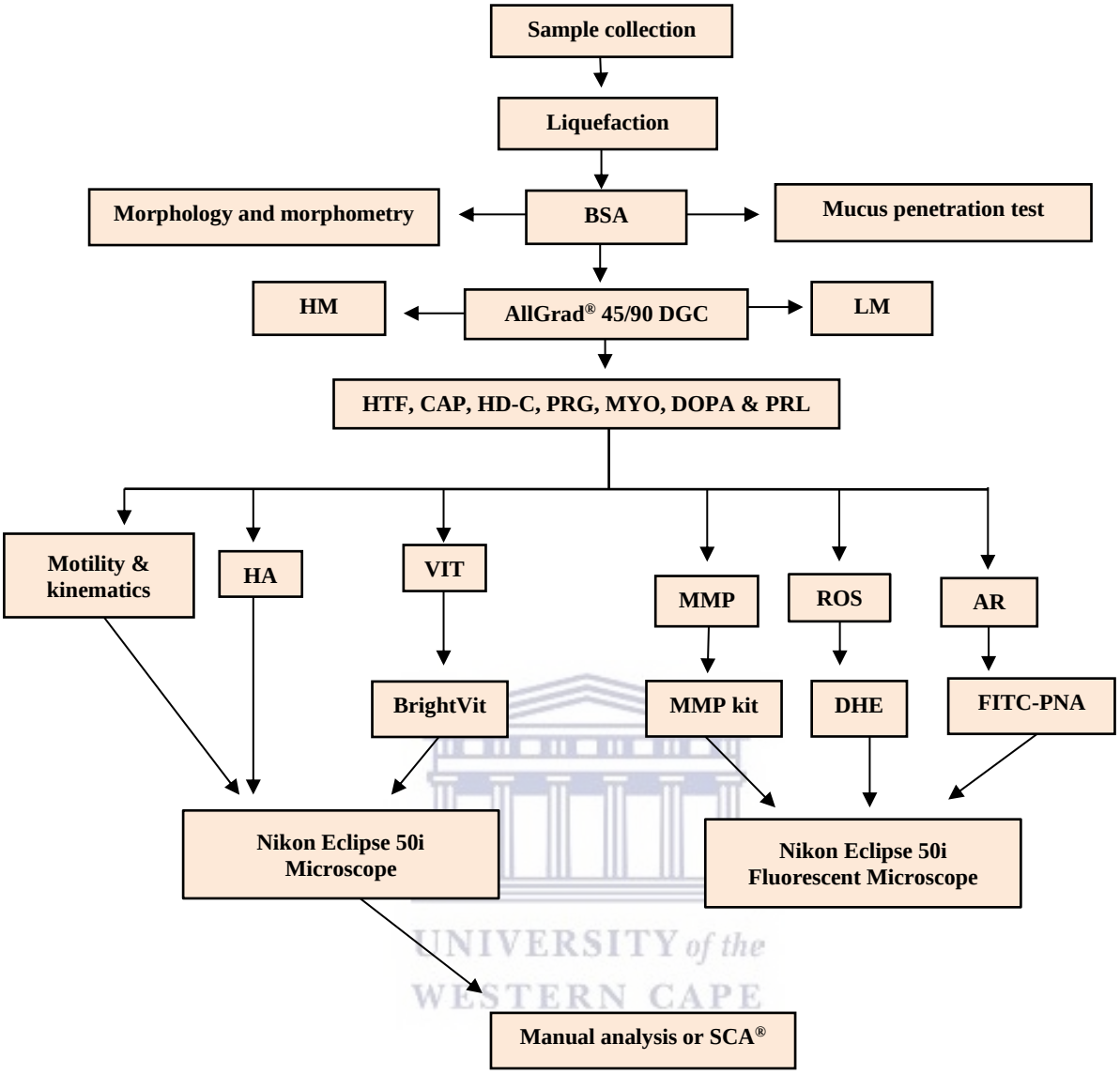

Figure 3.2 Flow diagram illustrating the simplified procedural outline of the second phase of the study involving the separation of donor semen samples into two motility sperm subpopulations (high motile, HM and low motile, LM) and investigating their various functional characteristics after exposure to different media. **Abbreviations:** AR, acrosome reaction; BSA, basic semen analysis; CAP, capacitating HTF medium; DGC, double density gradient centrifugation; DHE, dihydroethidium; DOPA, dopamine; FITC-PNA, Fluorescein isothiocyanate-labelled Peanut Agglutinin assay; HA, hyperactivation; HD-C, HD capacitating medium; HM, high motile; HTF, non-capacitating human tubal fluid; LM, low motile; MMP, mitochondrial membrane potential; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; ROS, reactive oxygen species; SCA®, Sperm Class Analyser®; VIT, vitality.

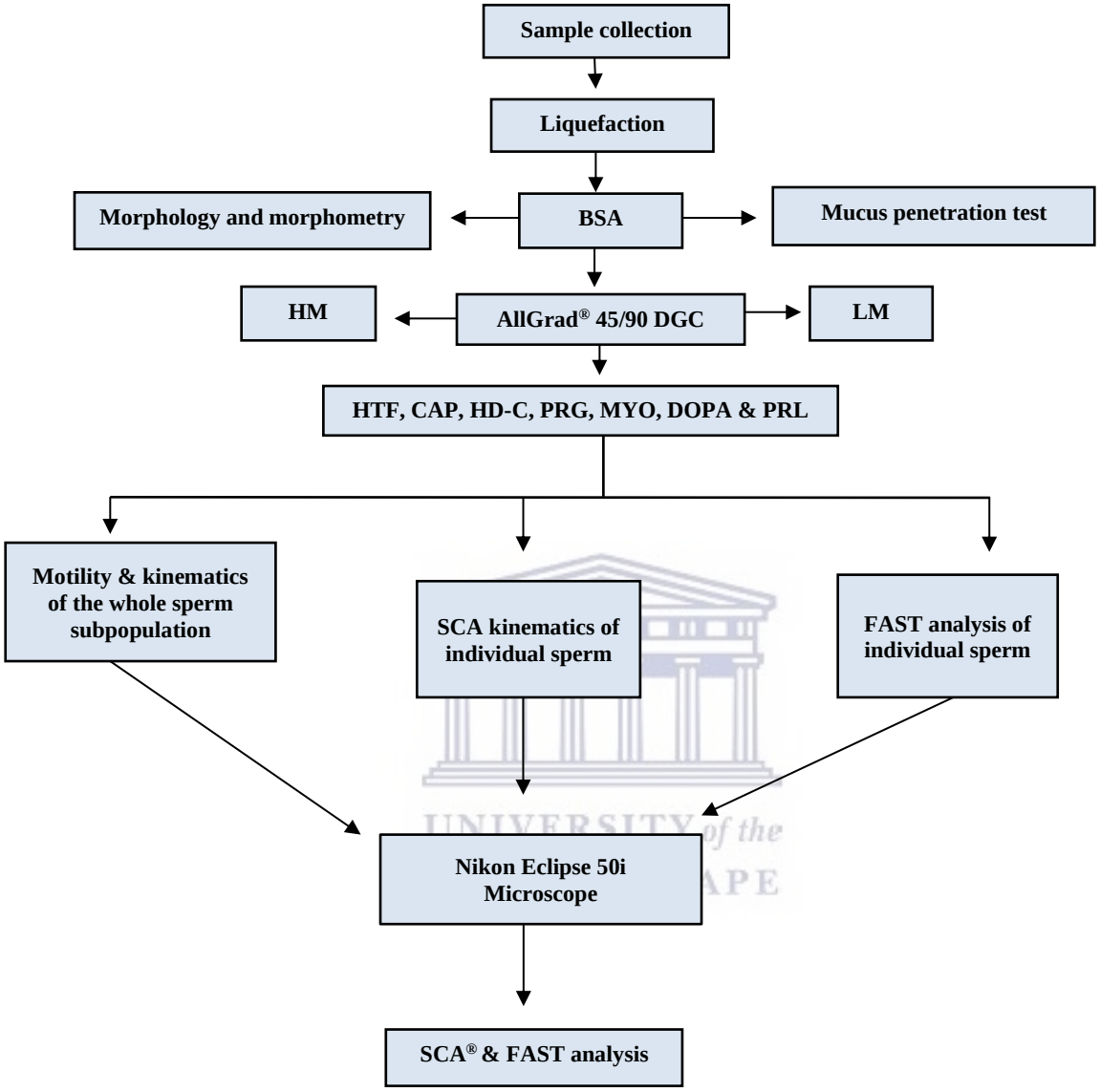

Figure 3.3 Flow diagram illustrating the simplified procedural outline of the second phase of the study involving the separation of donor semen samples into two motility sperm subpopulations (high motile, HM and low motile, LM) and investigating their kinematic parameters by SCA® and FAST software after exposure to different media. **Abbreviations:** BSA, basic semen analysis; CAP, capacitating HTF medium; DGC, double density gradient centrifugation; DOPA, dopamine; FAST, flagellar analysis and sperm tracking; HD-C, HD capacitating medium HM, high motile; HTF, non-capacitating human tubal fluid; LM, low motile; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; SCA®, Sperm Class Analyser®.

3.2 | Ethical Clearance

This study was approved by the ethical boards of the University of the Western Cape (code 13/10/90 and code BM20/9/14), and Stellenbosch University (code N14/06/074). All procedures were in accordance with the ethical guidelines of the Comparative Spermatology Laboratory, Department of Medical Bioscience, University of the Western Cape and the Reproductive Research Laboratory, Division of Medical Physiology, Department of Biomedical Sciences, Stellenbosch University. The Helsinki Declaration (Christie, 2000) governing research on humans has been adhered to and each human donor gave written consent (see Appendix 6).

3.3 | Sample Collection and Basic Semen Analysis

A total of 260 semen samples were obtained via masturbation as part of a donor program (Division of Medical Physiology, Department of Biomedical Sciences, Stellenbosch University and Comparative Spermatology Laboratory, Department of Medical Bioscience, University of the Western Cape) after two to three days of sexual abstinence. Semen samples were split into different phases of the study namely:

- **Phase one (n = 60)** - investigation of the high motile (HM) and low motile (LM) sperm subpopulations' functional parameters.
- **Phase two (n = 160)** - investigation of the HM and LM sperm subpopulations' functional parameters at different time points and after treatment with various media and concentrations of biological substances.
- **Phase three (n = 40)** - investigation of the HM and LM sperm subpopulation's Sperm Class Analyser® (SCA®) and Flagellar Analysis Sperm Tracking (FAST) parameters after exposure to selected media and concentrations of biological substances.

After collection, donor samples were incubated permitting liquefaction (30-60 min at 37 ºC in a 5% CO_2 regulated incubator) and subsequently processed for standard semen analysis as recommended by the World Health Organization (WHO, 2021). Semen volume, pH and viscosity were assessed as well as several sperm parameters, including total motility, progressive motility, sperm concentration, total number of spermatozoa, mucus penetration, vitality and morphology. A minimal cut-off value for percentage total sperm motility at 25% was used for phase one of the study, and although lower than the WHO lower reference limit of 40% (WHO, 2021), was a non-biased reflection of the donors used, who otherwise had good semen parameters. A minimal cut-off value for percentage total sperm motility of 15% was

used for phases two and three as the main purpose of the study was to determine how various concentrations of media can improve different qualities of spermatozoa.

3.4 | Computer-Aided Sperm Analysis

Various parameters were analysed with the use of the different modules of the SCA® (Microptic S.L., Barcelona, Spain) computer-aided sperm analysis (CASA) system, version 6.1 (phases one) and version 6.2 (phases two and three). A Basler A312fc (phases one) or Basler acA1300-200uc digital camera (phases two and three) (Microptic S.L., Barcelona, Spain), attached to a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa), equipped with a heated stage (37 ºC), was used to capture spermatozoa (Figure 3.4).

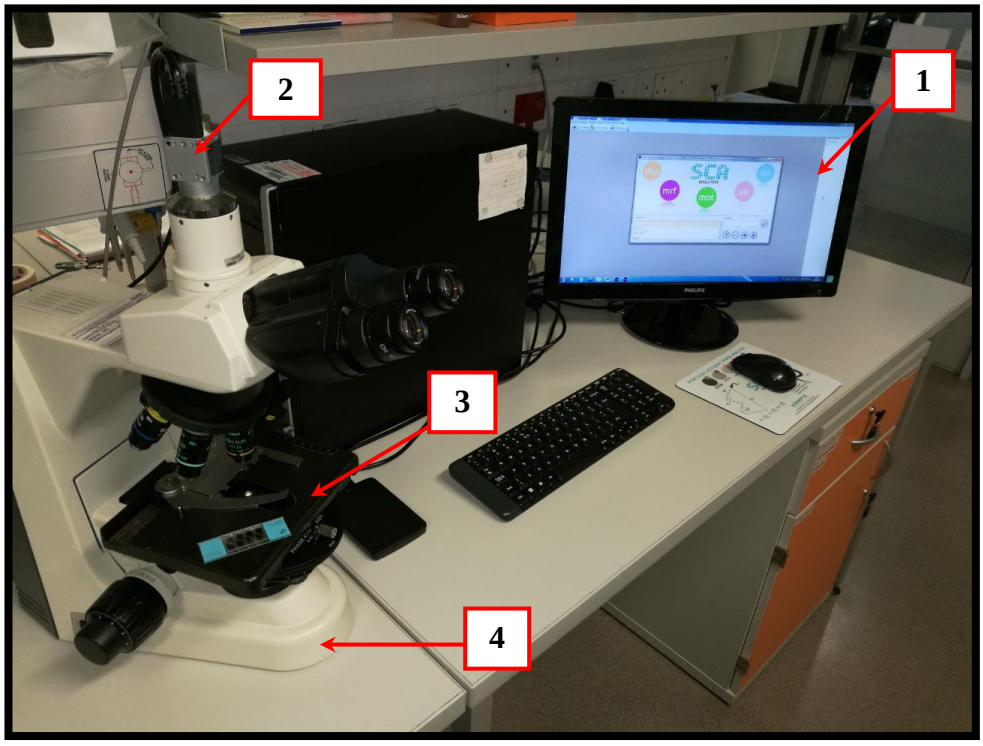

Figure 3.4 Equipment used for assessment of semen/sperm parameters on the SCA® CASA system. ***Note:*** **(1)** Computer with SCA® software for CASA analysis. **(2)** Basler A312fc or acA1300-200uc digital camera. **(3)** Heated stage (37 ºC). **(4)** Nikon Eclipse 50i microscope. **Abbreviations:** CASA, computer aided sperm analysis; SCA®, Sperm Class Analyser®.

3.5 | Volume

Semen volume was determined after liquefied semen samples were decanted into a preheated (37 ºC), graded plastic test tube. An Adam PW184 scale was balanced with the empty graded test tubes before decanting of samples (Figure 3.5). After the scale was balanced, samples were weighed in grams (g). The sample weight was further converted into millilitres (mL) to determine the exact volume of each sample. After the volume of the semen samples were determined, samples were placed into a AccuBlock™ digital dry bath set to 37 ºC (Figure 3.6).

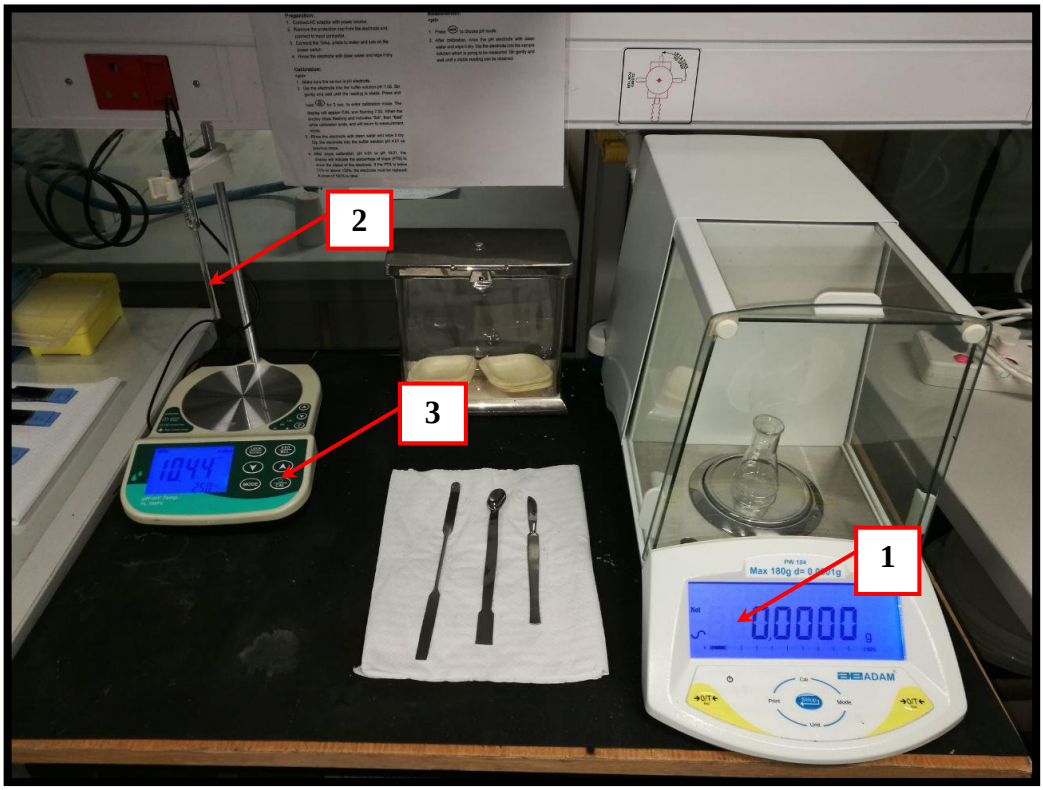

Figure 3.5 Equipment used for measurement of semen volume and pH. ***Note:*** **(1)** Adam PW184 scale. **(2)** PL-700PV pH meter. **(3)** Accsen pH probe.

3.6 | pH

After decanting the liquefied semen samples, pH was measured with the use of a PL-700PV pH meter (Lasec, Cape Town, South Africa), equipped with an Accsen pH probe (Figure 3.5). The probe was immersed into the semen sample and left for approximately 30-60 seconds, until a stable reading was reached and recorded.

3.7 | Viscosity

Using the viscosity evaluation technique described by Rijnders *et al*. (2007), 3 µL semen aliquots were loaded into preheated (37 ºC) four-chamber, 20 µm-depth Leja slides (Leja Products B.V., Nieuw Vennep, The Netherlands) and the filling time was recorded in seconds. Viscosity in centipoise (cP) was subsequently determined by using the following equation:

$$y = 0.34x + 1.34$$

where y = viscosity in cP and x = filling time in seconds.

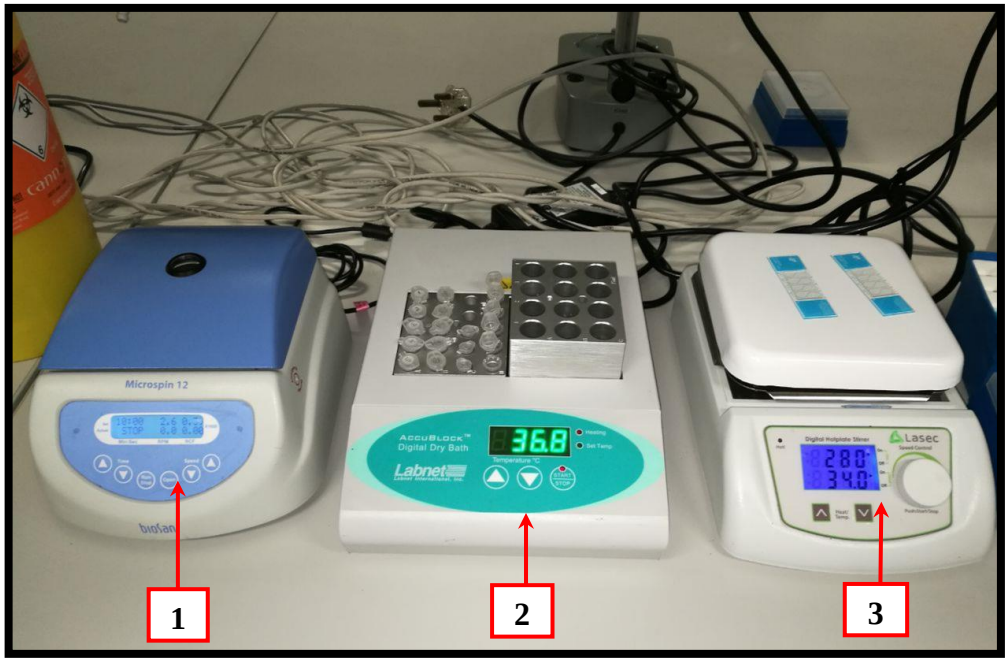

Figure 3.6 Equipment used for incubation and sperm preparation. ***Note:*** **(1)** BioSan Microspin 12 centrifuge. **(2)** Labnet AccuBlock™ digital dry bath set at 37 ºC. **(3)** Lasec digital hot plate set at 37 ºC with preheated eight chamber Leja slides.

3.8 | Sperm Motility, Mucus Penetration and Concentration

Sperm motility, mucus penetration (kinematic cut-off values: average path velocity (VAP) >25 µm/s, straightness (STR) >80 % and 7.5 µm< amplitude of lateral head displacement (ALH) >2.5 µm) and concentration were all analysed with the use of the Motility module of SCA®. A preheated (37 ºC) four chamber, 20 µm-depth Leja slide was loaded with 3 µL semen and placed on a heated stage (37 ºC). At least two fields were captured at 50 frames per second (f/s), and a minimum of 200 motile sperm per sample were analysed using negative phase contrast optics and a 10x objective together with the microscope setup described in Section 3.4. Total sperm motility (%), progressive motility (%), sperm mucus penetration ($x10^6$/sample) and sperm concentration ($x10^6$/mL and $x10^6$/ejaculate) were recorded after each captured field had been verified and any analysis errors were removed. Table 3.1 displays the SCA® Motility module configuration settings used for analyses of human samples.

Table 3.1 Capturing and analysis properties for human spermatozoa in the Motility/Concentration module of SCA®.

Parameter	Setting
Particle area (µm²)	2< PA <60
Area (µm²) (min)	1
Area (µm²) (max)	100
Drifting (µm/s)	10
Static (µm/s)	<25
Slow (µm/s)	25< Slow <60
Medium (µm/s)	60< Medium <89

Rapid (µm/s)	Rapid >89
Progressivity (% of STR)	>80
Circular (% of LIN)	<50
Connectivity	12
VAP points (pixels)	7
ALH (µm)	3.5< ALH >30

Abbreviations: ALH, amplitude of lateral head displacement; LIN, linearity; max, maximum; min, minimum; PA, particle area; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity.

3.9 | Morphology and Morphometry

Semen aliquots (300 µL) were centrifuged in AllGrad Wash® (Delfran, Johannesburg, South Africa) at room temperature (RT) for 20 minutes (min) at 500 *g* (Figure 3.6) and subsequent pellets were re-suspended in non-capacitating human tubal fluid (HTF). Morphology smears were prepared (15 µL) on clean Starfrost slides (Lasec, Cape Town, South Africa) and dried slides were stained with SpermBlue® fixative and stain mixture (Microptic S.L., Barcelona, Spain) as described by van der Horst and Maree (2010) and modified by Microptic (2020). Coverslips were mounted with DPX mounting medium (Sigma Aldrich, Cape Town, South Africa) and at least 100 spermatozoa were analysed with the Morphology module of the SCA® using brightfield optics, a Basler acA1300-200uc digital camera (Microptic S.L., Barcelona, Spain), a blue filter and 60x objective on a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa) (Figure 3.7). Table 3.2 displays the configuration settings used for the SCA® Morphology module for analyses of human samples.

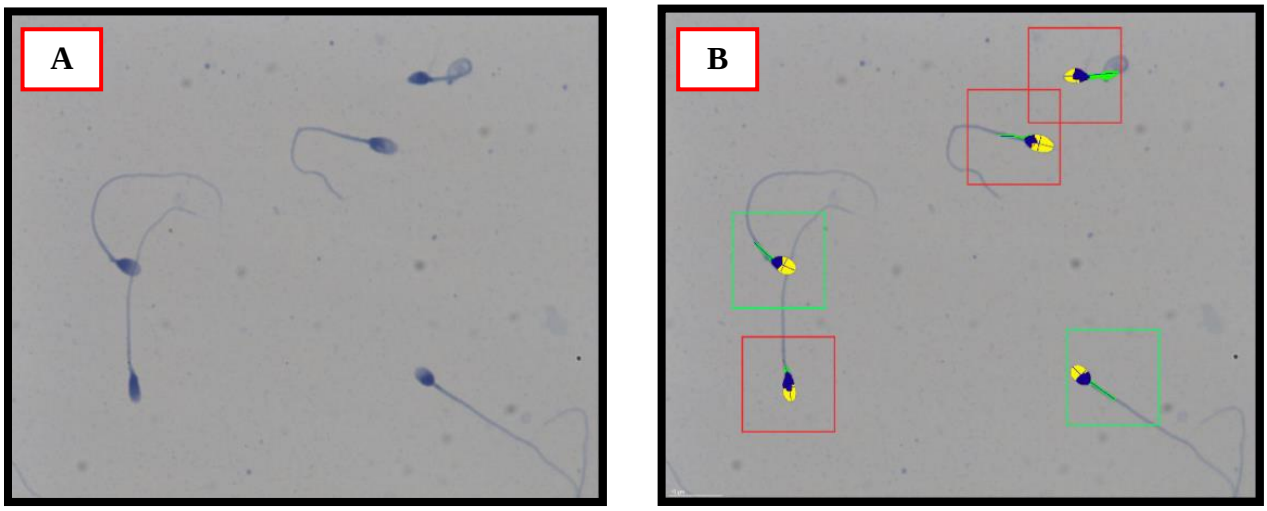

Figure 3.7 SCA® analysis of human spermatozoa stained with SpermBlue® at 1000x magnification. ***Note:*** Five pairs of spermatozoa are shown above. **(A)** Images of the spermatozoa as detected by the Basler acA1300-200uc digital camera. **(B)** Analyses of the acrosome (yellow), head (blue) and mid-piece (green) of the same spermatozoa and the classification of normal (green box) or abnormal (red box) morphology as analysed by the Morphology module of SCA®. **Abbreviations:** SCA®, Sperm Class Analyser®

Table 3.2 Capturing and analysis properties for human spermatozoa in the Morphology module of SCA®.

Sperm component	Parameter	Formula	Minimum	Maximum
Head	Length (µm)	L	4.0	5.0
	Width (µm)	W	2.5	3.5
	Area (µm^2)	A	9.0	15.5
	Perimeter (µm)	P	11.5	15.5
	Ellipticity	L/W	1.3	1.8
	Elongation	(L-W)/(L+W)	0.12	0.3
	Roughness	4π (A/P^2)	0.8	1.32
	Regularity	π (LxW/4xA)	0.8	1.15
	Acrosome (%)		40.0	70.0
	Vacuoles (%)		0.0	20.0
Mid-piece	Width (µm)	W	0.0	1.4
	Distance (µm)		0.0	0.6
	Angle (˚)		0.0	60.0
Tail	Length (µm)	L	12.0	100.0

Abbreviations: A, area; L, length; P, perimeter; W, width; π, pi = 3.14159265359

3.10 | Sperm Subpopulation Preparation

Density gradient centrifugation (DGC) media contain different concentrations of Silane-coated silica particles in an aqueous solution thereby forming different density layers of the medium (Malvezzi *et al*., 2014). This preferred technique in ART for processing of semen removes the spermatozoa from the seminal plasma and cellular debris and further separates the most motile sperm subpopulation from the less motile sperm subpopulation (Malvezzi *et al*., 2014). Highly motile and morphologically normal spermatozoa have a different weight compared to the lower motile, DNA damaged or morphological abnormal spermatozoa. Thus, centrifuging semen using various layered densities enables one to separate the spermatozoa according to quality and functionality (Figure 3.8) (Malvezzi *et al*., 2014).

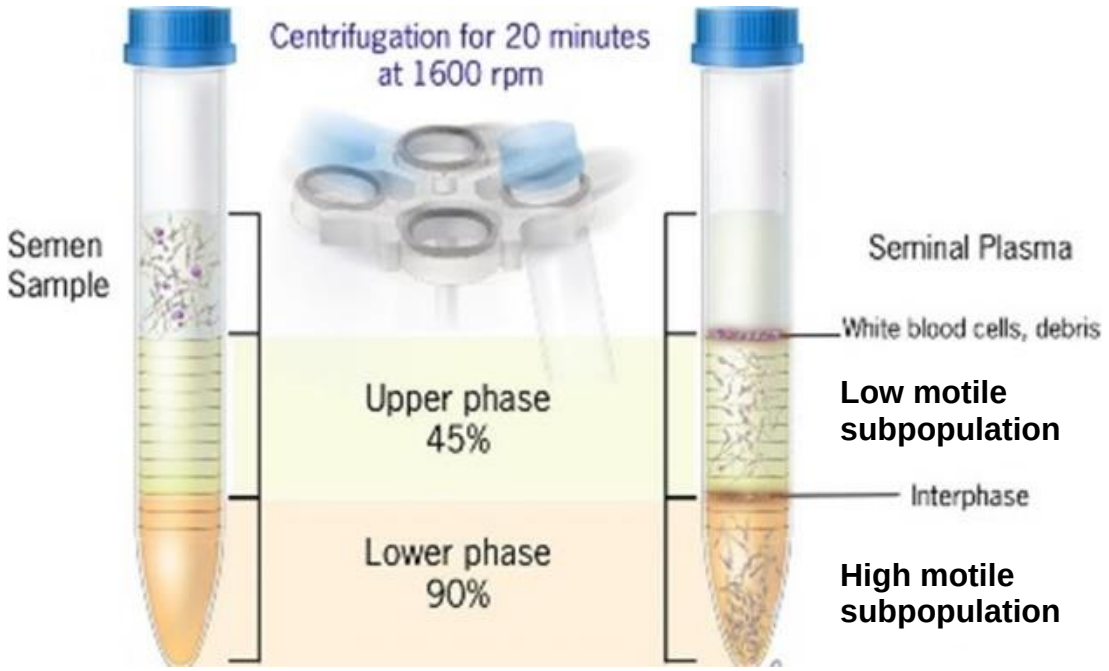

Figure 3.8 Schematic diagram of differential density gradient centrifugation technique. ***Note:*** Indicated on the left is the semen sample layered on equal volumes of the 45% and 90% density media. Indicated on the right is the resultant product formed after centrifugation. Viable motile spermatozoa will form a concentrated pellet at the bottom of the tube, while the less motile and abnormal spermatozoa will remain in the intermediate middle layer. The debris and seminal plasma form a cloud on the surface of the medium (modified from Malvezzi *et al*., 2014).

Semen samples were separated using an AllGrad® 45/90 (Delfran, Johannesburg, South Africa) double density gradient centrifugation technique. Equal volumes (300 µL) of preheated (37 ˚C) AllGrad 90%, AllGrad 45% and semen were pipetted into 2 mL Eppendorf tubes, respectively. Eppendorf tubes were centrifuged at 500 *g* for 20 minutes and samples were subsequently separated into top (abnormal, non-motile spermatozoa) and bottom (viable, motile spermatozoa) factions after removal of seminal plasma and debris (Figure 3.8). Separated subpopulations were re-suspended in 300 µL AllGrad Wash® and centrifuged at 500 *g* for 10 min. Washed pellets were re-suspended in HTF and sperm concentrations were adjusted to 15-25x10^6/mL before further analysis for phase one of the study. For phases two and three, the subpopulations were suspended in the various media and sperm concentrations were adjusted to 15-25x10^6/mL before further analysis.

3.11 | Media Preparation

In the first phase of the study, subpopulations were only exposed to human tubal fluid (HTF) and their functional characteristics tested after being suspended in the medium for 5-10 minutes. In the second phase of the study, subpopulations were treated with HD Capacitating medium (HD-C), progesterone, myo-inositol, dopamine and prolactin, while HTF and

capacitating HTF (CAP) served as the negative and positive controls, respectively. Subpopulations were incubated in the various media for 30 minutes where after functional characteristics were analysed. For phase three, subpopulations were incubated for 15 minutes in selected concentrations of the media used in phase two, where after subpopulations were analysed with SCA® and flagellar analysis and sperm tracking (FAST) software. Four of the five different media (containing progesterone, myo-inositol, dopamine and prolactin) were prepared at various concentrations in CAP. All media were void of 1% human serum albumin (HSA) supplementation as samples were not exposed to extensive incubation hours and the study aimed to determine the individual effects of the media alone on sperm function.

3.11.1 | Controls

Non-capacitating human tubal fluid (HTF) mimics the environment created by the epithelial cells in the fallopian tube (Tay *et al.,* 1997). HTF made up as non-capacitating and capacitating medium was used as a negative and a positive control, respectively. HTF medium consisted of 1.4845 g NaCl, 0.0875 g KCl, 0.024 g $MgSO_4$, 0.0125 g KH_2PO_4, 0.525 g $NaHCO_3$, 0.009 g Na pyruvate, 0.12525 g glucose, 0.0075 g phenol red and 0.784 g Na lactate. Capacitating HTF (CAP) comprised of non-capacitating HTF with the addition of 0.105 g $NaHCO_3$, 1.1915 g HEPES and 0.6 ml NaOH (Mortimer, 1994).

3.11.2 | HD-C Medium

HD Sperm Capacitation™ medium (HD-C, HDSC - 0005; Delfran, Johannesburg, South Africa South African) comprised of a mixture of NaCl, KCl, $MgSO_4.7H_2O$, KH_2PO_4, Na_2HPO_4, $NaHCO_3$, $CaCl_2$, glucose, progesterone and myo-inositol for inducing hyperactivation in spermatozoa.

3.11.3 | Progesterone

Progesterone stock in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Cape Town, South Africa) was prepared in CAP to three working solutions of 1.98 µM, 3.96 µM and 19.8 µM (volume/volume). These specific concentrations were based on previous investigations and the physiological concentrations of progesterone reported in the female reproductive tract (Thomas and Meizel, 1989; Costa *et al*., 2004; Wen *et al*., 2010). For the third phase of the study, 3.96 µM progesterone was selected based on the data collected from phase two of the study and was used to treat subpopulations before analysis.

3.11.4 | Myo-inositol

Myo-inositol (MW = 180.16 g/mol) was weighed out and dissolved in CAP (mass/volume), yielding a working solution of 11 mM which was used for both the second and third phase of the study. The specifc concentration was based on previous investigations and the physiological concentration of myo-inositol reported in the female reproductive tract (Thomas and Meizel, 1989; Costa *et al*., 2004; Wen *et al*., 2010).

3.11.5 | Dopamine

Dopamine hydrochloride (H8502, Sigma-Aldrich, Cape Town, South Africa) was weighed out and dissolved in CAP (mass/volume) to yield a stock solution of 1 mM dopamine, which was further diluted to three working solutions of 20 nM, 100 nM and 1 µM (volume/volume). These specific concentrations were adjusted from previous investigations and based on physiological concentrations of dopamine found within the female reproductive tract (Helm *et al*., 1982; Fait *et al*., 2001; Ramírez *et al*., 2009; Urra *et al*., 2014). For the third phase of the study, 1 µM dopamine was selected based on the data collected from phase two of the study and was used to treat subpopulations before analysis.

3.11.6 | Prolactin

Hydrolyzed Prolactin (L7009, Sigma Aldrich, Cape Town, South Africa) prepared in 4 mM HCl (mass/volume) to a stock solution of 10 µg/mL was diluted in CAP to four working solutions of 50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL. These specific concentrations were adjusted from previous investigations of the effect of prolactin on spermatozoa (Shah *et al.,* 1976; Burkman, 1984; Fukuda *et al*., 1989; Pujianto *et al.,* 2010). For the third phase of the study, both 250 ng/mL and 500 ng/mL prolactin were selected based on the data collected from phase two of the study and were used to treat subpopulations before analysis.

3.12 | Vitality

All reagents and equipment were heated to 37 ºC prior to use. In phase one of the study sperm subpopulations were incubated for 5 – 10 minutes in HTF, whereafter subpopulations were stained accordingly for vitality. Sperm subpopulations for phase two of the study were first incubated in the various media and vitality smears were made at 5- and 30-minutes' incubation, in addition to 60 minutes for prolactin. Semen samples and sperm subpopulations were stained in suspension in an amber Eppendorf tube at a ratio of 1:4 (10 µL semen with 40 µL stain) for 10-15 min at 37 ºC with BrightVit (Microptic S.L., Barcelona, Spain), an eosin-

nigrosin (E/N) based stain. Vitality smears (20 µL) were prepared according to the technique proposed by Microptic (2020) and left to air dry before being mounted with a coverslip using DPX mounting medium (Sigma Aldrich, Cape Town, South Africa). Stained smears were viewed using brightfield optics and a 40x objective on a Nikon Eclipse 50i microscope. Images were captured using the Vitality module of SCA® and a Basler A312fc (phases one) or acA1300-200uc digital camera (phase two) (Microptic S.L., Barcelona, Spain). Percentage live (white, unstained) spermatozoa and dead (pink-stained) spermatozoa were calculated after at least 200 spermatozoa per slide were manually assessed (Figure 3.9).

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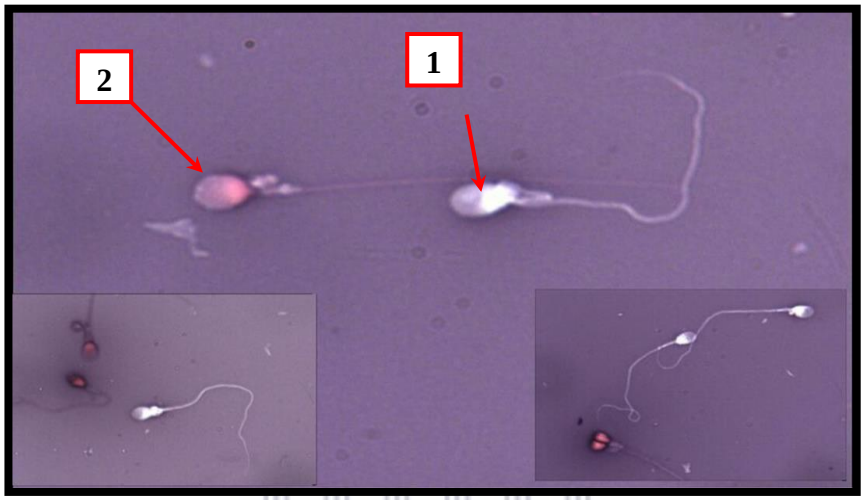


Figure 3.9 Eosin-nigrosin vitality stain of the HM sperm subpopulation in HTF, captured with the Vitality module of SCA®, at 1000xmagnification using brightfield optics. ***Note:*** **(1)** live spermatozoon and **(2)** dead spermatozoon. **Abbreviations:** HM, high motile subpopulation; HTF, non-capacitating human tubal fluid; SCA®, Sperm Class Analyser®.

3.13 | Sperm Motility and Kinematics

For phase one of the study, subpopulations were incubated in HTF for 5-10 minutes before analysis. In the second phase, subpopulations were incubated in the various media and motility and kinematics were analysed at 5- and 30-minutes' incubation, with addition of 60 minutes for prolactin. In the third phase, subpopulations were incubated for 15 minutes in selected concentrations of the biological substances as mentioned in Section 3.11. In addition to the kinematic and motility parameters of the whole HM and LM sperm subpopulations (as was analysed for phases one and two), kinematic parameters for individual spermatozoa were recorded for the third phase of the study.

Motility and kinematics for the average motile, various speed (rapid, medium and slow) and progressivity groups (rapid progressive, medium progressive and non-progressive) were subsequently assessed with the use of the Motility module of SCA®, version 6.5.0.91.

Preheated (37 ºC) eight chamber, 20 µm-depth Leja slides (Leja Products B.V., Nieuw Vennep, The Netherlands) were loaded with 2 µL of incubated (phase one) or treated (phase two) sperm subpopulations and at least two SCA motility fields with a minimum of 200 motile spermatozoa were analysed at 50 frames per second (f/s) using positive phase contrast optics, a green filter and a 10x objective (see Section 3.4 for software, microscope and camera setup). Listed in Table 3.3 are the motility and kinematic parameters analysed for each subpopulation and for the various speed and progressivity groups. ALH was measured as half the width of the Curvilinear (VCL) track and not as the full VCL wave or doubling of riser values (risers' method) as described by Mortimer (1994, 1997).

In the third phase of the study, subpopulations were flushed with the selected media, and kinematics for individual spermatozoa and the whole subpopulation were analysed after 15 minutes. Applying the flush technique described by van der Horst *et al*. (2010), each chamber of a preheated (37 ºC) 10 µm-depth, four chamber Leja slide (Leja Products B.V., Nieuw Vennep, The Netherlands) was loaded with 0.5 µL sperm preparation (HM or LM sperm subpopulations suspended in HTF) and flushed with 1.5 µL of preheated medium as mentioned in Section 3.11. At least two fields with a minimum of 100 motile spermatozoa were analysed at 169 frames per second (f/s) for 120 images, pixel size of 4 µm by 4 µm, using negative phase optics and a 10x objective (see Section 3.4 for microscope and camera setup). Selected frame rates and SCA® configuration settings (see Table 3.4) were derived from previous preliminary investigations. Results for the individual spermatozoa kinematics, in addition to the whole sperm subpopulations were extracted from Excel reports. Captured fields were further exported and saved as an Audio Video Interleave (AVI) file for exporting into the FAST programme. AVI files were saved without any track changes.

Table 3.3 Sperm motility and kinematic parameters analysed with SCA® for the average motile, speed and progressiveness groups.

Parameter	Description
Total Motile (%)	Tailing beating seen (types a+b+c)
Progressive (%)	Space gain of ≥5 µm/s but <25 µm/s (type a+b)
Rapid Progressive (%)	Spermatozoa moving actively, either linearly or in a large circle with a speed of >89 µm/s
Medium Progressive (%)	Spermatozoa moving actively, either linearly or in a large circle with a speed between >60 µm/s and <89 µm/s
Non-Progressive (%)	Tail beating seen but no net space gain (movement <5 µm/s)
Rapid (%)	>89 µm/s
Medium (%)	>60 µm/s and <89 µm/s

Slow (%)	>25 µm/s and <60 µm/s
VCL (µm/s)	Time-averaged velocity of a sperm head along its actual curvilinear path
VSL (µm/s)	Time-averaged velocity of a sperm head along the straight line between its first detected position and its last
VAP (µm/s)	Time-averaged velocity of a sperm head along its average path
LIN (%)	Linearity of the curvilinear path (VSL/VCL)
STR (%)	Linearity of the average path (VSL/VAP)
WOB (%)	Measure of oscillation of the actual path about the average path (VAP/VCL)
ALH (µm)	Magnitude of lateral displacement of a sperm head about its average path
BCF (Hz)	Average rate at which the curvilinear path crosses the average path

Note: Kinematic parameters were analysed for average motile, rapid, medium and slow speeds, in addition to rapid-progressive, medium progressive and non-progressive speeds. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; Hz, hertz; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

Table 3.4 Capturing and analysis properties for human spermatozoa in the Motility/Concentration module of SCA® for the FAST analysis.

Parameter	Setting
Area (µm^2) (min)	2
Area (µm^2) (max)	60
Drifting (µm/s)	10
Static (µm/s)	<15
VCL Slow (µm/s)	15< Slow <72
VCL Medium (µm/s)	72< Medium <127
VCL Rapid (µm/s)	Rapid >127
Progressivity (% of STR)	>58
Circular (% of LIN)	<50
Connectivity (pixels)	10
VAP points (pixels)	13

Abbreviations: LIN, linearity; max, maximum; min, minimum; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity.

3.14 | Hyperactivation

For the first phase of the study, hyperactivation was evaluated with the use of 2 mM procaine hydrochloride in HTF and 5 mM caffeine in HTF, as a previous study observed that these concentrations yielded adequate hyperactivation in human sperm samples (Ntanjana, 2014). For the second phase of the study, HD-C, progesterone, myo-inositol, dopamine and prolactin were all prepared as mentioned in Section 3.11, and further used to investigate its ability to induce hyperactivation in the sperm subpopulations. For both phase one and two,

HTF prepared as capacitating and non-capacitating media were used as positive and negative controls respectively.

Applying the flush technique described by van der Horst *et al*. (2010) - each chamber of a preheated (37 ºC) 20 µm-depth, eight chamber Leja slide was loaded with 0.5 µL sperm preparation (HM or LM sperm subpopulations suspended in HTF) and flushed with 1.5 µL of preheated treatment as mentioned above (Figure 3.10). Percentage hyperactivation (using cut-off values: VCL >150 µm/s; LIN <50%; ALH >7 µm (3.5 for SCA®); Mortimer, 1997) of at least 200 motile spermatozoa was assessed after 5-, 15-, 30-, 45- and 60-minutes exposure to each treatment, for each sperm subpopulation using the Motility module of SCA® and equipment describe in the previous Section 3.4 and 3.13.

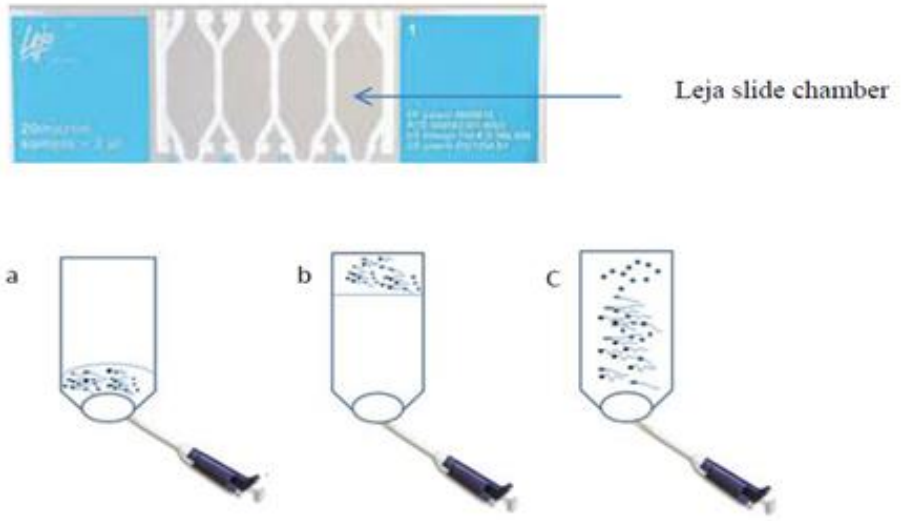


Figure 3.10 Illustration demonstrating the flush technique as describe by van der Horst *et al* (2010) for analyses of hyperactivation of spermatozoa. ***Note:*** **(a)** The Leja slide is loaded with 0.5 µL sperm preparation and **(b)** flushed with 1.5 µL of preheated treatment. **(c)** Spermatozoa swim down into the medium, inducing hyperactivation (Ntanjana, 2014).

3.15 | Reactive Oxygen Species

In phase one of the study, subpopulations were incubated in HTF for 5-10 minutes - whereas in phase two subpopulations were incubated for 30 minutes in media as mentioned in Section 3.11. After incubation subpopulations were stained in suspension with 20 µM Dihydroethidium (DHE, excitation = 518 nm and emission = 605 nm; Molecular Probes, Eugene, OR, USA) to detect spermatozoa positive for reactive oxygen species (ROS). Treated subpopulations were stained in the dark for 15 minutes in suspension (180 µL) with 20 µL

DHE at 37 ºC. Following incubation, 5-10 µL suspension was placed on a clean slide with a coverslip, and immediately analysed using a 100x oil immersion objective and triband filter (MXU440) (excitation wavelengths: 457 nm = blue, 530 nm = green and 628 nm = red) on a Nikon Eclipse 50i fluorescence microscope (IMP, Cape Town, South Africa). The percentage of spermatozoa positive for ROS was calculated after manual assessment of at least 100 spermatozoa. Images of the stained specimens were later captured with a Nikon Eclipse 50i fluorescence microscope and NIS Elements BR. 3. 10 (Build 578) Image Analysis Software (IMP, Cape Town, South Africa) for demonstrative purposes (Figure 3.11).

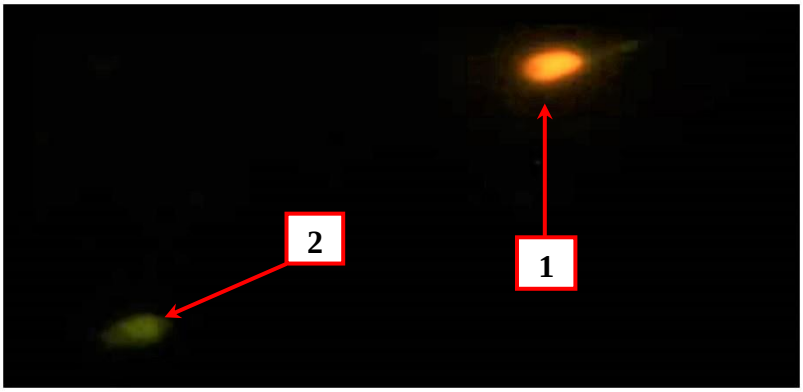


Figure 3.11 High motile **(**HM) sperm subpopulation in HTF, stained with DHE and captured at 1000x magnification using a Triband filter for detection of ROS positive spermatozoa. ***Note***: **(1)** ROS positive spermatozoon **(2)** ROS negative spermatozoon. **Abbreviations:** DHE, dihydroethidium; HM, high motile; HTF, human tubal fluid; ROS, reactive oxygen species.

3.16 | Mitochondrial Membrane Potential (ΔΨm)

Prior to the start of this investigation, the Sigma Aldrich mitochondrial membrane potential (MMP) (CS0390, Sigma Aldrich, Cape Town, South Africa) protocol was optimized accordingly to suit this specific study. In phase one of the study, subpopulations were incubated in HTF for 5-10 minutes, whereas in phase two, subpopulations were incubated for 30 minutes in media as mentioned in Section 3.11. Following incubation, subpopulations were stained at a ratio of 1:1 in the dark at 37 ºC for 20 minutes in suspension (200 µL) with the MMP staining solution (160 µL dH_2O, 40 µL JC-5 buffer and 1 µL frozen MMP 200x stock solution). After incubation, suspensions were centrifuged at 500 *g* for 5 minutes at 5-7 ºC and washed pellets re-suspended in 200 µL JC-1 buffer (80 µL JC-5 buffer and 320 µL dH_2O), prepared and cooled on ice before use. Suspensions were centrifuged for a second time as described above and subsequent pellets re-suspended in the remaining 200 µL JC-1 buffer. A single drop of 5-10 µL suspension was placed on a clean slide and covered with a coverslip. Slide preparations were immediately analysed using the same equipment as described in Section 3.15, and the percentage of spermatozoa with intact MMP calculated after manual assessment of at least 100

spermatozoa. Images of the stained specimens were later captured with a Nikon Eclipse 50i fluorescence microscope and NIS Elements BR. 3. 10 (Build 578) Image Analysis Software for demonstrative purposes (Figure 3.12).

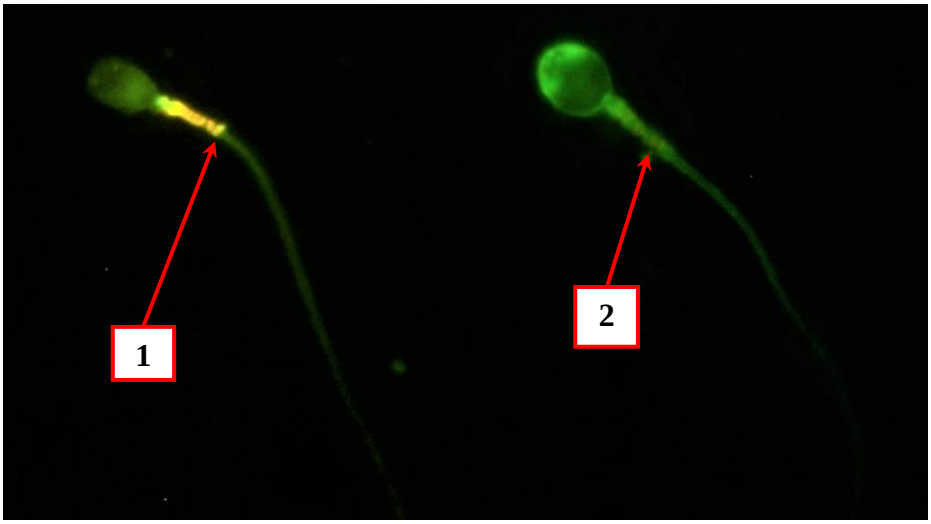


Figure 3.12 Mitochondrial membrane potential (MMP) of the high motile (HM) sperm subpopulation in HTF captured at 1000x magnification using a Triband filter. ***Note:*** **(1)** Mitochondrial membrane intact spermatozoon **(2)** Mitochondrial membrane disrupted spermatozoon**. Abbreviations:** HM, high motile subpopulation; HTF, human tubal fluid.

3.17 | Acrosome Reaction and Capacitation

Acrosome reaction was determined with the use of the FluAcro protocol described by Microptic (Microptic, 2020). Subpopulations were incubated at 37 ºC for 3 hours in preheated (37 ºC) capacitating medium (1 mL HAMS-F10 and 0.03 g of HSA; Sigma-Aldrich, Cape Town, South Africa). After incubation, subpopulations were treated with 10 µL of each of the following media: 1 mM Ca-ionophore made up in DMSO (yielding a final concentration of 0.01 mM); capacitating and non-capacitating HTF (phases one and two); HD-C; progesterone; myo-inositol; dopamine and prolactin (phase two). Samples were incubated in the media for 15 minutes after which reactions were terminated with 100 µL 70% ethanol. Two 5 µL drops of each suspension were placed on a clean slide and left to air dry before fixation in 95% ethanol (United Scientific, Cape Town, South Africa) at 4 ºC for 30 minutes. Fixed slides were stained in a dark room for 30-40 minutes with 80 µL of fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA; Sigma-Aldrich, Cape Town, South Africa) on each drop and dipped twice in dH_2O to remove excess stain. Slides were subsequently counterstained for 7 min with 5 µL Hoechst (H33258, Sigma-Aldrich, Cape Town, South Africa) on the area of the slide previously stained with PNA, followed by destaining in dH_2O and were left to air dry before viewing. The acrosome status of at least 100 spermatozoa per sample was manually assessed by using a 40x objective and a triband fluorescence filter (MXU440) on a Nikon Eclipse 50i

fluorescence microscope. Images were captured with the use of the "no analyse" function of the Acrosome module of SCA® and the Nikon Eclipse 50i florescence microscope (Figure 3.13).

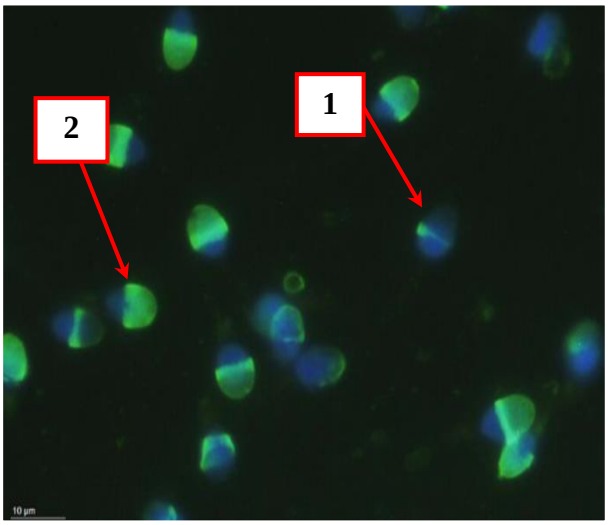


Figure 3.13 Human spermatozoa stained with Hoechst FITC-PNA and viewed at 1000x magnification. for acrosome reaction detection. ***Note:*** **(1)** Acrosome reacted spermatozoon **(2)** Acrosome intact spermatozoon**. Abbreviations:** FITC-PNA; fluorescein isothiocyanate-labelled peanut agglutinin.

3.18 | Chromatin Maturity and Fragmentation

Following 5-10 minutes incubation in HTF, subpopulations of the first phase of the study were stained for detection of chromatin maturity and fragmentation of spermatozoa. Chromatin maturity and fragmentation were determined following the aniline and toluidine blue protocols proposed by Erenpreisa *et al*. (2003) and Erenpreiss *et al.* (2004). Smears made from the two sperm subpopulations in HTF (15 µL) were left to air dry. For assessment of chromatin maturity, dried smears were fixed at room temperature for 30 minutes in 4% formalin and rinsed in dH_2O. Fixed smears were stained for 5 minutes in 5% aniline blue (prepared in 4% acetic acid solution, pH 3.5), and excess stain rinsed off in dH_2O. Smears were subsequently stained in 0.5% eosin for 1 minute, then rinsed in dH_2O and left to air dry.

For assessment of chromatin fragmentation, dried slides were fixed in 96% ethanol-acetone (1:1) at 4 °C for 1 hour, then hydrolyzed in 0.1 N HCl at 4 °C for 5 minutes, and finally rinsed in dH_2O. Smears were subsequently stained for 5 minutes in 0.05% toluidine blue (prepared in 50% McIlvaine's citrate phosphate buffer, pH 3.5) at room temperature, briefly rinsed in dH_2O and left to dry. Stained smears were mounted with DPX mounting medium and viewed using brightfield optics and a 60x objective on a Nikon Eclipse 50i microscope. The percentages of spermatozoa with respectively immature chromatin and fragmented chromatin were calculated after manual assessment of at least 100 spermatozoa per subpopulation. For aniline blue stain, spermatozoa with dark blue heads were scored as having immature

chromatin, whereas spermatozoa with pink heads had mature chromatin. For toluidine blue, the light sperm heads were scored as possessing normal chromatin integrity, and those with dark heads were scored as having damaged chromatin integrity. Images were captured with the use of the Count module of SCA® and a Nikon Eclipse 50i microscope (Figure 3.14 and 3.15).

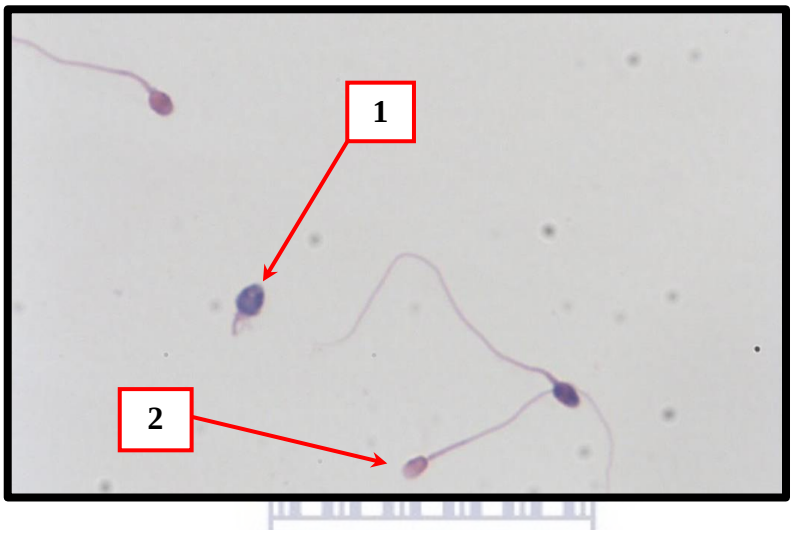


Figure 3.14 Illustration of the high motile (HM) sperm subpopulation stained with aniline blue for detection of chromatin maturity in spermatozoa. ***Note:*** **(1)** Spermatozoon with immature chromatin. **(2)** Spermatozoon with mature chromatin. **Abbreviations:** HM, high motile.

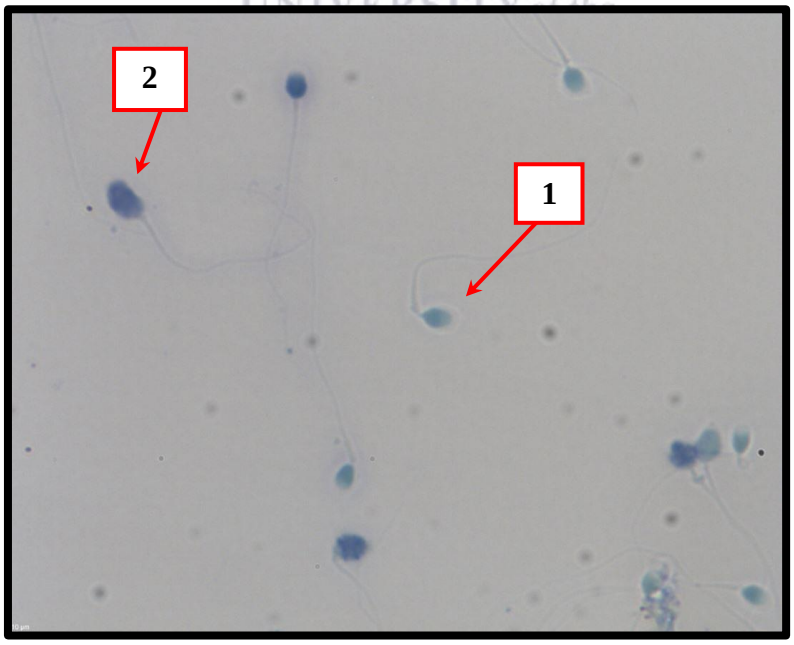


Figure 3.15 Illustration of the high motile (HM) sperm subpopulation stained with toluidine blue for detection of chromatin integrity in spermatozoa. ***Note:*** **(1)** Spermatozoon with normal chromatin integrity. **(2)** Spermatozoon with abnormal chromatin integrity. **Abbreviations:** HM, high motile.

3.19 | Flagellar Analysis and Sperm Tracking (FAST)

For the third and final phase of the investigation, subpopulations were flushed with selected concentrations of biological substances in media used in phase two (see Section 3.11). After 10-15 minutes, 100 individual spermatozoa were analysed with the motility module of SCA® and the fields further saved with no track changes as AVI files (see Section 3.13). The AVI files were further exported as batch files into FAST software (School of Mathematics and Centre of Reproductive Science, Institute for Metabolism and Systems Research, University of Birmingham, United Kingdom) (Gallagher *et al*., 2019), version 1.1.2, and analysed for individual spermatozoa. Table 3.5 displays the configuration settings used for the FAST analysis. Once analysed, individual spermatozoa with a full tangent angle with distinct lines (as illustrated in Figure 3.16) were selected for further evaluation. Flagellar waves are characterized in terms of the tangent angle θ (s, t) (Gallagher *et al*., 2019). A function of both the arc-length along the flagellum (s) and time (t) (Gallagher *et al.*, 2019). The flagellar curvature as κ (s, t) = $\partial\theta/\partial s$ is then calculated, from which the beat frequency (f) and arc-wavespeed (c) can be derived (Gallagher *et al*., 2019). Analysis regions are restricted to sections of the flagellum with 10 µm< s <30 µm, ensuring a wave developed (Gallagher *et al*., 2019). Thus, large enough to contain all the relevant information and small enough to allow for fair comparisons between all cells (Gallagher *et al*., 2019). Data of selected spermatozoa were recorded and was further reduced by selecting spermatozoa with the following criteria:

- 35 µm< Analysed flagellum length >50 µm
- Maximum analysed flagellum <60 µm
- Frames captured >60 frames

Table 3.5 Capturing and analysis properties for human spermatozoa in the FAST program.

Parameter	Setting
Min cell length (µm)	0.5
Max cell length (µm)	57.0
Max swimming speed (µm/s)	1000
Max tail length (µm)	55.2
Max tail width (µm)	1.0
Fluid viscosity (Ps)	0.001
Image frame rate (f/s)	169.2
Initial frame	1.0
Magnification	10x
Image pixel size (µm)	4x4
Image border (µm)	5.0
Head threshold	100.0

Abbreviations: FAST, flagellar analysis sperm tracking; f/s, frames per second.

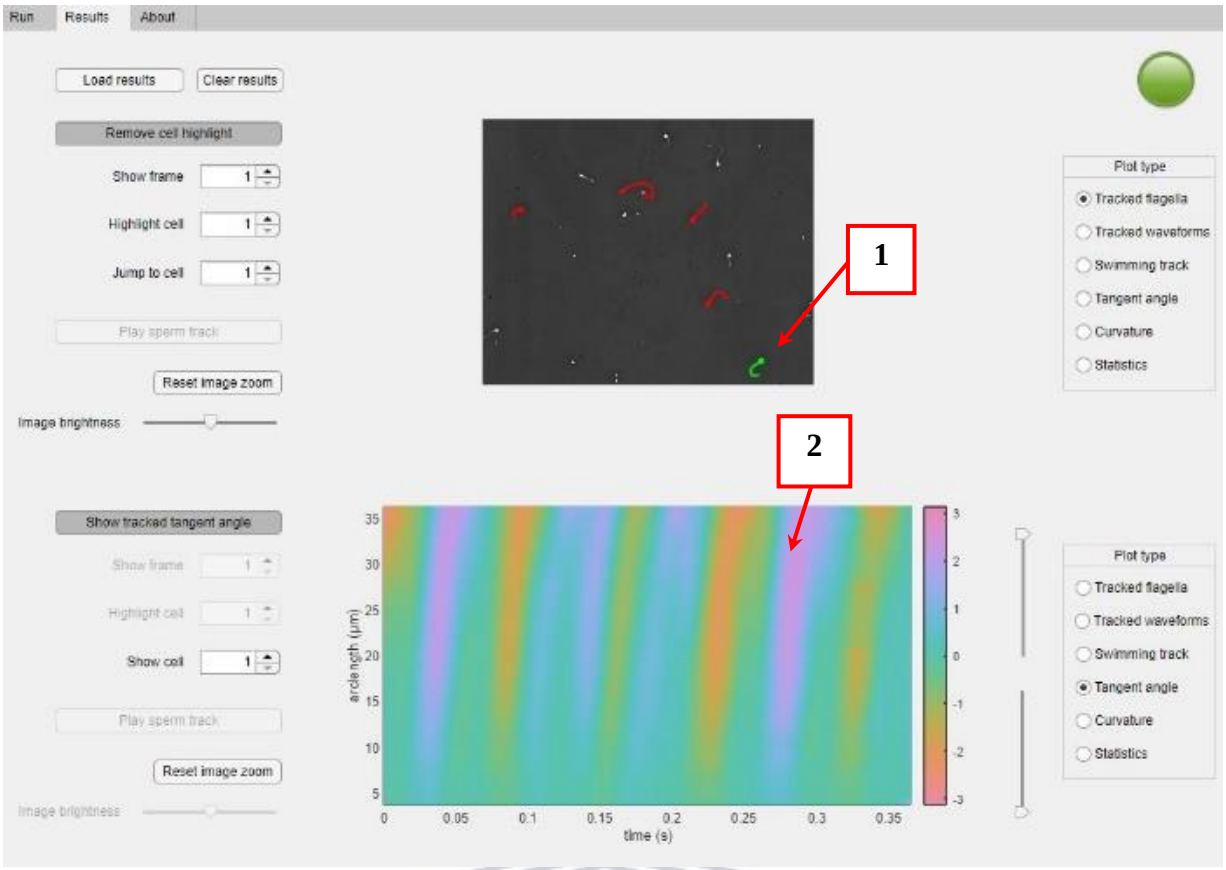


Figure 3.16 Illustration of the high motile (HM) sperm subpopulation in CAP medium analysed with FAST. ***Note:*** **(1)** The individual spermatozoa analysed and the **(2)** tangent angle of the flagellar wave pattern. Spermatozoa selected for further evaluation required a tangent angle as illustrated by label (2). Tangent angles were required to display a distinct separation between colours with clear consistent lines. **Abbreviations:** CAP, capacitating HTF; FAST, flagellar analysis and sperm tracking; HM, high motile.

3.20 | Statistical Analysis

MedCalc statistical software version 14.8.1 (Mariakerke, Gent, Belgium) was used to calculate basic summary statistics, and results were expressed as mean ± standard deviation in all the tables. The Shapiro-Wilk test was used to evaluate the distribution of the data, where after the Student's *t*-test or the Mann-Whitney test (when normal distribution was void) was used to compare subpopulations and time points with one another. Correlations, heat maps and scatterplots were calculated with the Pearson correlation test or Spearman correlation test when normal distribution was not applicable. Where applicable, the one-way analysis of variance (ANOVA) for parametric distributions or the Kruskal-Wallis test for non-parametric distributions were used to compare various subpopulations, time points and treatments. Significance was determined at a level of $p < 0.05$. Tables and radar plots were constructed with the use of Microsoft Office Excel™ 2016 (Microsoft Corporation, Redmond, Washington, United States).

Additional analyses such as correlation coefficients, multifactorial ANOVA and creation of multivariable charts, and multivariate visualisations were performed with Statgraphics® Centurion XVII (Statgraphics Technologies, Inc.) to create Star glyphs and Andrews plots. Star glyphs are useful in identifying differences and similarities amongst observed cases when the number of dimensions is too large to use in a standard scatterplot. A glyph is a geometric object which represents the values of each quantitative variable, where the size of the polygon in each direction is scaled according to the value of each variable for the selected individual semen sample (semen samples with similar characteristics will have a similar size and shape). values. Similarly, the Andrews Plot is a multivariate visualization technique that can be very useful in identifying differences and similarities amongst observed cases when the number of dimensions is too large to use a standard scatterplot. Andrews plots can distinguish small but not necessarily statistically significant differences and thus emphasize potential differences between cases.

Multiple regression analysis and principal component analysis was further executed with the use of STATISTICA, version 10 (StatSoft Inc.). Subpopulations, media and time were additionally used as fixed factors in a mixed model repeated measures ANOVA and samples as the random factors. The Fisher LSD was used for the post hoc test and reports based on third order effects (interactions amongst subpopulations, media and time). If third order effects were void, reports were based on interactions between fixed factors (subpopulations and media, subpopulations and time or media and time). If none of the fixed factors (subpopulations, media or time) were included in interactions, only the main effects were both reported.

CHAPTER FOUR: Results of Separated Motility Subpopulations

The two sperm subpopulations separated from each semen sample are referred to as high motile (HM) and low motile (LM) sperm subpopulations in this chapter. The HM subpopulation represents the sperm subpopulation with good initial motility and the LM subpopulations represents the sperm subpopulation with poor initial motility.

4.1 | Results of Phase One of the Study

4.1.1 | Basic Semen Analysis

Displayed in Table 4.1 are the basic semen analysis results of the selected 55 donor semen samples. The mean values of all the semen parameters were above the lower reference values according to the WHO laboratory manual except for the percentage progressive motility which fell below the reference values (WHO, 2021). In this respect a large range (3.6 – 84.8%) of progressive motility values was obtained.

Table 4.1 Basic semen parameters of selected semen samples used in this investigation (mean ± SD) (n=55).

	Mean	±	SD	95% C.I		
Progressive (%)	27.1	±	16.9	22.6	-	31.7
Total motility (%)	52.2	±	15.5	48.0	-	56.4
Mucus penetration test (x10^6/ejaculate)	23.6	±	28.5	16.0	-	31.3
Sperm concentration (x10^6/mL)	55.0	±	38.3	44.7	-	65.4
Total sperm number (x10^6/ejaculate)	154.4	±	137.3	117.3	-	191.5
pH**	7.7	±	0.5	7.5	-	7.8
Volume (mL)	2.8	±	1.3	2.4	-	3.1
Viscosity (cP)	9.0	±	7.7	6.9	-	11.1
Vitality (live spermatozoa, %) *	57.2	±	14.0	50.7	-	63.8
Immature spermatozoa (%) *	23.3	±	10.8	18.2	-	28.3
Mature spermatozoa (%) *	77.7	±	8.8	73.6	-	81.8
Normal chromatin (%) *	74.8	±	11.1	69.6	-	79.9
Abnormal Chromatin (%) *	25.2	±	11.0	20.0	-	30.4
Normal (%)	11,0	±	7.8	8.8	-	13.1
Head defects (%)	78.5	±	12.2	75.2	-	81.8
Midpiece defects (%)	35.7	±	10.6	32.9	-	38.6
Tail defects (%)	20.5	±	18.8	15.4	-	25.6
Normal Acrosome (%)	62.5	±	16.5	58.1	-	67.0
Cytoplasmic droplets (%)	12.9	±	13.3	9.3	-	16.5
Macro (%) **	44.2	±	21.4	36.9	-	51.5
Micro (%) **	23.1	±	19.3	16.4	-	29.7
TZI	1.6	±	0.3	1.6	-	1.7
MAI	2.4	±	0.3	2.3	-	2.5
DI	2.2	±	0.4	2.0	-	2.3

Note: The mean semen parameters fall above the lower reference limits as recommended by WHO (2021), however mean progressive motility fell between the 2.5th and 5th percentile. **Abbreviations:** C.I, confidence interval; cP, centipoise; DI, deformity index; MAI, multiple abnormalities index; SD, standard deviation; TZI, teratozoospermic index. Values labelled with an asterisk had different number of samples (*n=20, **n=35).

4.1.2 | Motility and Kinematics

Sperm motility subpopulations were separated from whole semen samples via double density gradient centrifugation (DGC) and further exposed to non-capacitating human tubal fluid (HTF) after which motility parameters were analysed with the use of computer aided sperm analysis (CASA). In Table 4.2, the HM subpopulations consistently presented with significantly (p <0.001) higher percentages as compared to the LM subpopulations, for all the motility and speed group parameters when exposed in HTF.

Moreover, values recorded for HM subpopulations were on average four to six times greater compared to the LM subpopulations for percentage total motility (HM 58.6 ± 12.4%; LM 14.7 ± 7.9%, p <0.001), progressive motility (HM 35.5 ± 17.1%; LM 6.3 ± 6.1%, p <0.001), rapid progressive motility (HM 15.7 ± 13.3%; LM 2.5 ± 3.2%, p <0.001), medium progressive motility (HM 21.6 ± 7.9%; LM 3.9 ± 3.9%, p <0.001), rapid- (HM 26.2 ± 17.7%; LM 4.1 ± 5.2%, p <0.001), medium- (HM 16.5 ± 7.6%; LM 4.2 ± 3.6%, p <0.001) and slow-swimming (HM 16.0 ± 8.0%, LM 6.5 ± 4.0%, p <0.001) spermatozoa (Figure 4.1).

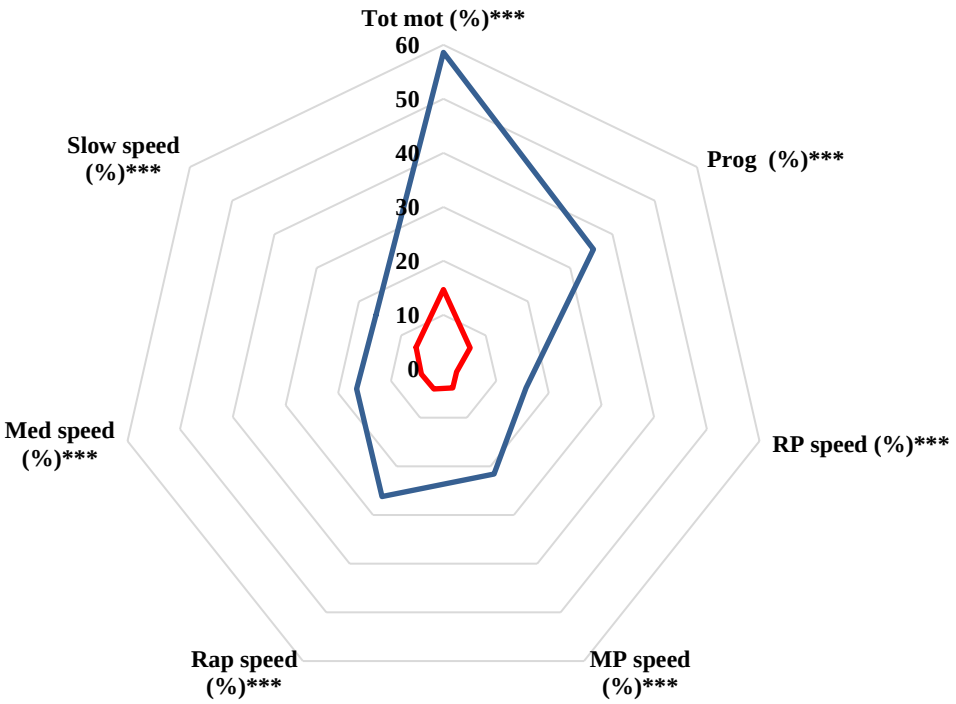


Figure 4.1 Radar plot of the mean sperm motility parameter measurements for comparison of low motility (LM, red line) and high motility (HM, blue line) sperm subpopulations (n=35). ***Note*:** HM sperm subpopulations consistently presented with higher mean percentages for the various sperm motility parameters as compared to the LM sperm subpopulations. **Abbreviations:** HM, high motile; LM, low motile; Med, medium speed; MP, medium progressive; Prog, progressive motility; Rap, rapid speed; RP, rapid progressive and Tot mot, total motility. Values labelled with an asterisk were significantly (p <0.001) different between the two sperm subpopulations for individual parameters.

Table 4.2 Comparison of the motility parameters for both high motile (HM) and low motile (LM) sperm subpopulations in human tubal fluid (HTF) (mean ± SD) (n=35).

		Mean	±	SD	95% C.I			SP *p*
Tot. Mot (%)								
	HM	58.6	±	12.4***	54.4	-	62.9	**<0.001**
	LM	14.7	±	7.9***	12.0	-	17.5	
Prog (%)								
	HM	35.5	±	17.1***	29.7	-	41.4	**<0.001**
	LM	6.3	±	6.1***	4.2	-	8.4	
RP (%)								
	HM	15.7	±	13.3***	11.2	-	20.3	**<0.001**
	LM	2.5	±	3.2***	1.4	-	3.6	
MP (%)								
	HM	21.6	±	7.9***	18.9	-	24.3	**<0.001**
	LM	3.9	±	3.9***	2.6	-	5.2	
Rapid (%)								
	HM	26.2	±	17.7***	20.1	-	32.2	**<0.001**
	LM	4.1	±	5.2***	2.3	-	5.9	
Medium (%)								
	HM	16.5	±	7.6***	13.9	-	19.1	**<0.001**
	LM	4.2	±	3.6***	2.9	-	5.4	
Slow (%)								
	HM	16.0	±	7.8***	13.3	-	18.8	**<0.001**
	LM	6.5	±	4.0***	5.1	-	7.9	

Note: The HM subpopulation yielded significantly (p <0.001) higher percentages for total motility, progressive, rapid- and medium-progressive speeds and for rapid, medium and slow swimming speeds in comparison to the LM subpopulations. **Abbreviations:** C.I, confidence interval; HM, high motile subpopulation; HTF, human tubal fluid; LM, low motile subpopulation; MP, medium progressive; Prog, progressive motility; RP, rapid progressive; SD, standard deviation, SP *p*, t-test between subpopulations; Tot. Mot, total motility. Values in the same column labelled in bold with an asterisk were significantly (p <0.001) different between the two sperm subpopulations.

The kinematic parameters for the average/total motile and different speed and progressivity groups of both HM and LM sperm subpopulations are given in Table 4.3. Although HM sperm subpopulations showed higher values for average kinematic parameters in contrast to the LM subpopulations, significant differences were observed between subpopulations for curvilinear velocity (VCL, HM 105.2 ± 33.3 μm/s; LM 80.9 ± 29.6 μm/s, p <0.001), average path velocity (VAP, HM 57.5 ± 15.9 μm/s; LM 45.5 ± 16.1 μm/s, p <0.001), straight-line velocity (VSL, HM 43.6 ± 14.1 μm/s; LM 34.6 ± 14.4 μm/s, p = 0.010) and amplitude of lateral head displacement (ALH, HM 2.9 ± 0.9 μm/s; LM 2.4 ± 0.8 μm, p <0.001). In addition to average kinematics, HM sperm subpopulations also revealed significantly higher values for non-progressive and medium speed group kinematic parameters (Table 4.3). Significant differences were seen for VCL (HM 126.1 ± 41.2 μm/s; LM 103.6 ± 31.0 μm/s, p = 0.001), VAP (HM 64.8 ± 13.5 μm/s; LM 55.7 ± 11.9 μm/s, p = 0.002) and ALH (HM 3.6 ± 1.3 μm; LM 2.9 ± 1.1 μm, p = 0.010) of the medium speed group and for VAP (HM 32.4 ± 6.7 μm/s; LM 28.6 ± 5.9 μm/s, p = 0.014), VSL (HM 23.8 ± 7.4 μm/s; LM 20.2 ± 6.9 μm/s, p =

0.037), linearity (LIN, HM 44.8 ± 9.9%; LM 39.0 ± 12.1%, $p = 0.033$) and wobble (WOB, HM 59.5 ± 7.8%; LM 56.2 ± 10.2%, $p = 0.039$) of the non-progressivity group. In contrast, the LM subpopulation obtained significantly higher values for medium speed straightness (STR, HM 71.0 ± 9.4%; LM 78.3 ± 13.0%, $p = 0.011$) compared to the HM subpopulation.

Table 4.3 Comparison of the different speed and progressive speed group kinematics of the high motile (HM) and low motile (LM) sperm subpopulations in human tubal fluid (HTF) (mean ± SD) (n=35).

		Mean	±	SD	95% C.I			SP *p*
VCL (µm/s):								
Average								
	HM	**105.2**	**±**	**33.3*****	93.8	-	116.7	**<0.001**
	LM	**80.9**	**±**	**29.6*****	70.7	-	91.0	
Rapid								
	HM	134.4	±	31.0	123.8	-	145.1	0.218
	LM	128.3	±	30.9	114.9	-	141.7	
Medium								
	HM	**126.1**	**±**	**41.2****	111.9	-	140.2	**0.001**
	LM	**103.6**	**±**	**31.0****	92.4	-	114.8	
Slow								
	HM	62.4	±	9.4	59.2	-	65.7	0.221
	LM	59.4	±	8.4	56.5	-	62.2	
RP								
	HM	145.6	±	32.0	134.6	-	156.5	0.580
	LM	135.4	±	29.7	123.9	-	146.9	
MP								
	HM	83.9	±	9.9	80.5	-	87.3	0.773
	LM	82.6	±	9.2	79.4	-	85.8	
NP								
	HM	52.4	±	7.4	49.9	-	55.0	0.057
	LM	49.1	±	6.1	47.0	-	51.2	
VAP (µm/s):								
Average								
	HM	**57.7**	**±**	**15.9*****	52.2	-	63.2	**<0.001**
	LM	**45.5**	**±**	**16.1*****	39.9	-	51.0	
Rapid								
	HM	78.6	±	17.0	72.8	-	84.4	0.249
	LM	76,0	±	19.1	67.7	-	84.3	
Medium								
	HM	**64.8**	**±**	**13.5****	60.1	-	69.4	**0.002**
	LM	**55.7**	**±**	**11.9****	51.4	-	60.0	
Slow								
	HM	33.9	±	7.6	31.3	-	36.5	0.659
	LM	32.5	±	6.8	30.2	-	34.8	
RP								
	HM	81.0	±	18.4	74.7	-	87.3	0.124
	LM	76.6	±	27.2	66.0	-	87.1	
MP								
	HM	53.6	±	7.1	51.2	-	56.1	0.151
	LM	50.7	±	9.2	47.5	-	54.0	

NP								
	HM	**32.4**	**±**	**6.7***	30.1	-	34.7	**0.014**
	LM	**28.6**	**±**	**5.9***	26.6	-	30.6	
VSL (µm/s):								
Average								
	HM	**43.6**	**±**	**14.1***	38.8	-	48.5	**0.010**
	LM	**34.6**	**±**	**14.4***	29.6	-	39.5	
Rapid								
	HM	71.4	±	15.7	66.0	-	76.8	0.571
	LM	68.8	±	18.6	60.8	-	76.9	
Medium								
	HM	45.5	±	7.7	42.8	-	48.1	0.319
	LM	43.3	±	9.6	39.9	-	46.8	
Slow								
	HM	21.5	±	7.1	19.1	-	24.0	0.424
	LM	21.2	±	7.4	18.7	-	23.8	
RP								
	HM	61.9	±	16.8	56.1	-	67.6	0.580
	LM	59.7	±	24.8	50.1	-	69.3	
MP								
	HM	43.8	±	8.8	40.7	-	46.8	0.187
	LM	40.5	±	11.4	36.6	-	44.5	
NP								
	HM	**23.8**	**±**	**7.4***	21.3	-	26.4	**0.037**
	LM	**20.2**	**±**	**6.9***	17.8	-	22.5	
STR (%):								
Average								
	HM	71.2	±	7.4	68.7	-	73.8	0.493
	LM	69.8	±	9.2	66.7	-	73.0	
Rapid								
	HM	90.4	±	2.1	89.7	-	91.1	0.531
	LM	89.9	±	3.1	88.6	-	91.3	
Medium								
	HM	**71.0**	**±**	**9.5***	67.8	-	74.3	**0.011**
	LM	**78.3**	**±**	**13.0***	73.6	-	83.0	
Slow								
	HM	60.3	±	9.6	57.0	-	63.6	0.762
	LM	61.1	±	11.6	57.1	-	65.0	
RP								
	HM	71.2	±	9.7	67.8	-	74.5	0.414
	LM	70.8	±	17.4	64.1	-	77.6	
MP								
	HM	77.1	±	8.6	74.2	-	80.1	0.354
	LM	74.8	±	11.7	70.8	-	78.9	
NP								
	HM	64.4	±	13.0	59.9	-	68.9	0.431
	LM	62.0	±	12.6	57.6	-	66.3	
LIN (%):								
Average								
	HM	42.5	±	7.6	39.9	-	45.1	0.640
	LM	41.5	±	9.4	38.3	-	44.8	
Rapid								

	HM	54.6	±	6.5	52.4	-	56.8	0.917
	LM	54.4	±	8.8	50.6	-	58.2	
Medium								
	HM	42.3	±	10.3	38.8	-	45.8	0.129
	LM	46.9	±	14.0	41.8	-	51.9	
Slow								
	HM	34.5	±	8.9	31.5	-	37.6	0.879
	LM	35.7	±	11.7	31.7	-	39.7	
RP								
	HM	44.2	±	8.7	41.2	-	47.2	0.978
	LM	42.5	±	13.4	37.3	-	47.7	
MP								
	HM	52.1	±	9.5	48.9	-	55.4	0.186
	LM	48.6	±	12.3	44.3	-	52.9	
NP								
	HM	**44.8**	**±**	**9.9***	41.4	-	48.2	**0.033**
	LM	**39.0**	**±**	**12.1***	34.9	-	43.2	
WOB (%):								
Average								
	HM	56.8	±	5.6	54.9	-	58.7	0.931
	LM	56.7	±	6.9	54.3	-	59.0	
Rapid								
	HM	59.7	±	6.2	57.6	-	61.9	0.826
	LM	60.2	±	8.4	56.5	-	63.8	
Medium								
	HM	55.9	±	7.4	53.4	-	58.5	0.563
	LM	57.2	±	10.6	53.4	-	61.1	
Slow								
	HM	54.1	±	6.8	51.8	-	56.4	0.977
	LM	54.9	±	8.7	51.9	-	57.9	
RP								
	HM	56.3	±	5.6	54.4	-	58.2	0.681
	LM	55.3	±	12.0	50.6	-	60.0	
MP								
	HM	63.9	±	6.9	61.6	-	66.3	0.152
	LM	61.1	±	9.2	57.9	-	64.3	
NP								
	HM	**59.5**	**±**	**7.8***	56.8	-	62.1	**0.039**
	LM	**56.2**	**±**	**10.2***	52.7	-	59.7	
ALH (µm):								
Average								
	HM	**2.9**	**±**	**0.9*****	2.6	-	3.2	**<0.001**
	LM	**2.4**	**±**	**0.8*****	2.1	-	2.6	
Rapid								
	HM	3.4	±	0.9	3.1	-	3.7	0.924
	LM	3.3	±	0.9	2.9	-	3.7	
Medium								
	HM	**3.6**	**±**	**1.3***	3.1	-	4.0	**0.010**
	LM	**2.9**	**±**	**1.1***	2.5	-	3.3	
RP								
	HM	3.6	±	0.8	3.3	-	3.9	0.287
	LM	3.3	±	0.9	2.9	-	3.6	
MP								

		HM	2.1	±	0.4	2.0	-	2.2	0.349
		LM	2.2	±	0.5	2.0	-	2.3	
	NP								
		HM	1.5	±	0.2	1.4	-	1.5	0.890
		LM	1.4	±	0.3	1.3	-	1.5	
BCF (Hz):									
	Average								
		HM	20.3	±	2.8	19.4	-	21.3	0.059
		LM	19.0	±	3.1	17.9	-	20.0	
	Rapid								
		HM	25.0	±	6.6	22.7	-	27.3	0.070
		LM	22.2	±	3.6	20.7	-	23.8	
	Medium								
		HM	20.3	±	3.4	19.2	-	21.5	0.459
		LM	21.6	±	5.0	19.8	-	23.4	
	RP								
		HM	17.8	±	4.2	16.4	-	19.3	0.257
		LM	16.2	±	5.5	14.1	-	18.4	
	MP								
		HM	19.2	±	4.0	17.8	-	20.6	0.212
		LM	18.0	±	6.6	15.7	-	20.3	
	NP								
		HM	14.9	±	4.0	13.5	-	16.2	0.488
		LM	13.9	±	4.8	12.3	-	15.6	

Note: The HM sperm subpopulation had significantly higher values for the average VCL, VAP, VSL and ALH; medium speed VCL, VAP and ALH; NP speed VAP, VSL, LIN and WOB kinematic parameters, as compared to LM subpopulations. The LM subpopulation had significantly higher values for medium speed STR, as compared to the HM subpopulations. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; C.I, confidence interval; HM, high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MP, medium progressive; NP, non-progressive; RP, rapid progressive; SD, standard deviation; SP *p*, t-test between subpopulations; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, wobble. Values in the same column labelled in bold with an asterisk were significantly different between the two sperm subpopulations (**p* <0.05, ***p* <0.01 and ****p* <0.001).

Figures 4.2 and 4.3 show the various speed and progressive group kinematic parameters of the HM and LM subpopulations. The radar plots confirm that, HM subpopulations were higher for the various parameters, however not all were significantly different to the LM subpopulation. Majority of the significant differences could be observed between subpopulations for the average, medium speed and non-progressive speed groups, whereas the rapid, slow, rapid-progressive and medium-progressive speed groups were in close range of values between the two subpopulations.

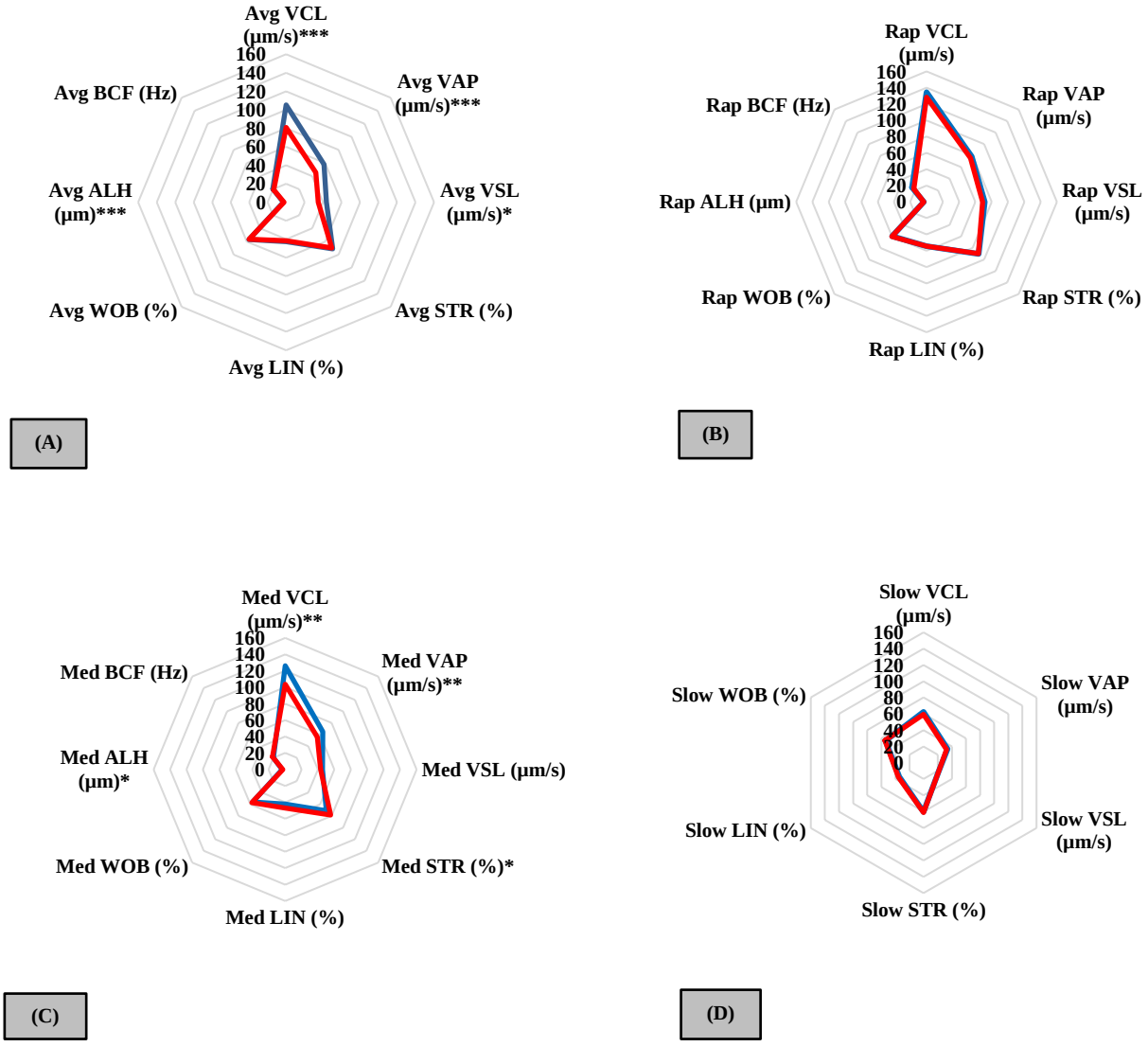
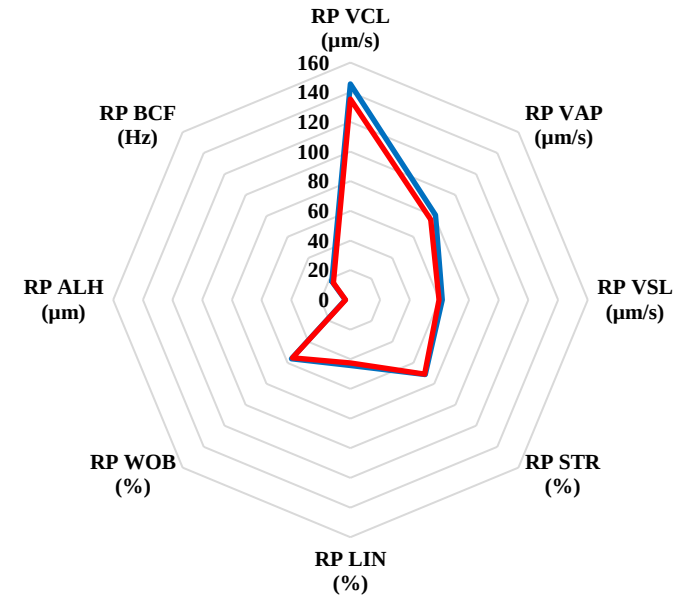
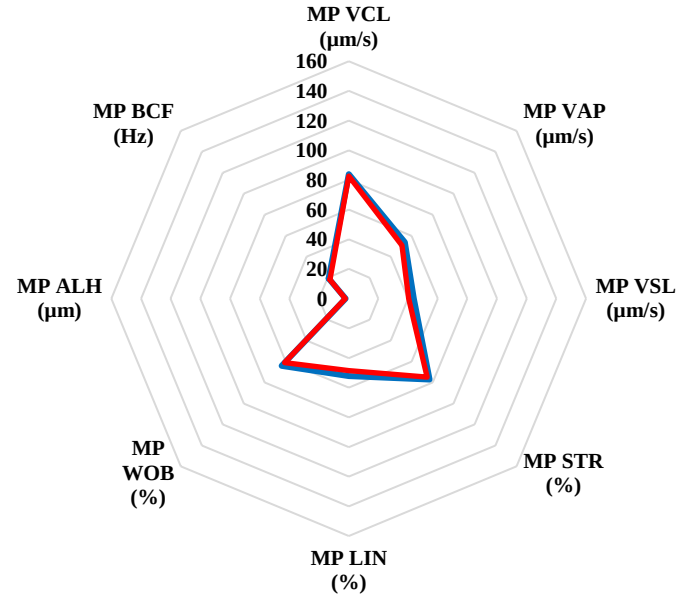


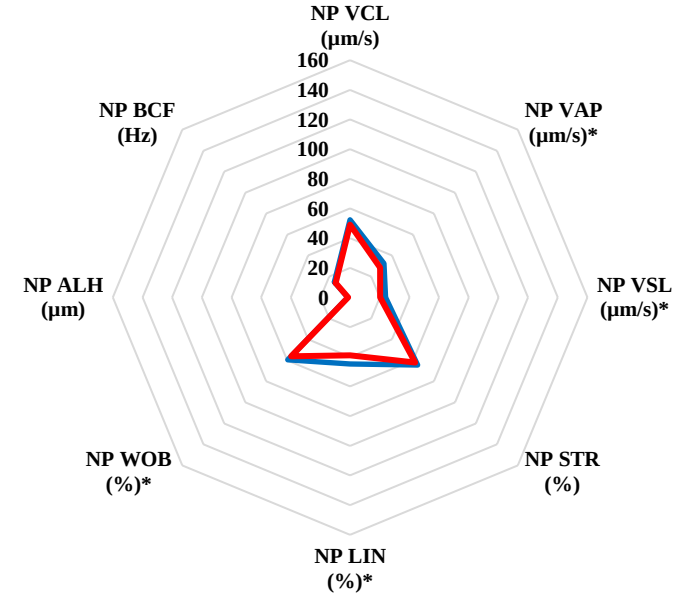
Figure 4.2 Radar plot of the mean sperm motility kinematic parameter measurements for comparison of low motility (LM, red line) and high motility (HM, blue line) sperm subpopulations (n=35). **(A)** Comparison of HM and LM sperm subpopulations' average motile sperm motility kinematic parameters. **(B)** Comparison of HM and LM sperm subpopulations' rapid speed group motility kinematic parameters. **(C)** Comparison of HM and LM sperm subpopulations' medium speed group motility kinematic parameters. **(D)** Comparison of HM and LM sperm subpopulations' slow speed group motility kinematic parameters**.** ***Note:*** the HM sperm subpopulation had significantly higher values for the average VCL, VAP, VSL and ALH, and medium speed VCL, VAP and ALH. The LM subpopulation had significantly higher values for medium speed STR, as compared to the HM subpopulations. **Abbreviations:** ALH, amplitude of lateral head displacement; Avg, average; BCF, beat cross frequency; HM, high motile; LIN, linearity; LM, low motile; Med, medium speed group; Rap, rapid speed group; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble. Values labelled with an asterisk were significantly different between the two sperm motility subpopulations for individual parameters (*p <0.05; **p <0.01; ***p <0.001).



(A)



(B)



(C)

Figure 4.3 Radar plot of the mean sperm motility kinematic parameter measurements for comparison of low motility (LM, red line) and high motility (HM, blue line) sperm subpopulations (n=35). **(A)** Comparison of HM and LM sperm subpopulations' RP sperm motility kinematic parameters. **(B)** Comparison of HM and LM sperm subpopulations' MP motility kinematic parameters. **(C)** Comparison of HM and LM sperm subpopulations NP motility kinematic parameters**.** ***Note:*** the HM sperm subpopulation had significantly higher values for the NP speed VAP, VSL, LIN and WOB kinematic parameters, as compared to LM subpopulations. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; HM, high motile; LIN, linearity; LM, low motile; MP, medium progressive speed group; NP, non-progressive speed group; RP, rapid progressive speed group; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble. Values labelled with an asterisk were significantly (p <0.05) different between the two sperm motility subpopulations for individual parameters.

Figure 4.4 illustrates star-glyphs of the two separated sperm subpopulations from individual semen samples. Star glyphs are a multivariate visualization technique that can be very useful in identifying differences and similarities amongst observed cases when the number of dimensions is too large to use in a standard scatterplot. The glyph is a geometric object which represents the values of each quantitative variable, where the size of the polygon in each direction is scaled according to the value of each variable for the selected individual semen sample (i.e., semen samples with similar characteristics will have a similar size and shape glyphs). Each individual star-glyph was constructed using 12 kinematic parameters as indicated in the graph key below. Whilst distinct differences between sperm subpopulations remain clear, the star-glyph plots assisted in visualizing similarities/differences between subpopulations of individual semen samples and within a single subpopulations group. HM sperm subpopulations illustrate similarities in star-glyph patterns amongst individual semen samples, whereas the LM sperm subpopulation displayed a more heterogeneous pattern (see Figure 4.4), indicating a greater variability in kinematic parameter values within LM sperm subpopulations as compared to HM sperm subpopulations. Furthermore, Figure 4.4 displays the variability between subpopulations prepared from individual ejaculates. For example, substantial differences in the kinematic parameter values can be observed for the two subpopulations of semen samples 11 (S11), 14 (S14) and 24 (S24). In contrast, some semen samples illustrated kinematic parameter values that were largely similar between the two sperm subpopulations, e.g., sample 3 (S3) where both had low values or sample 16 (S16) where both had high values.

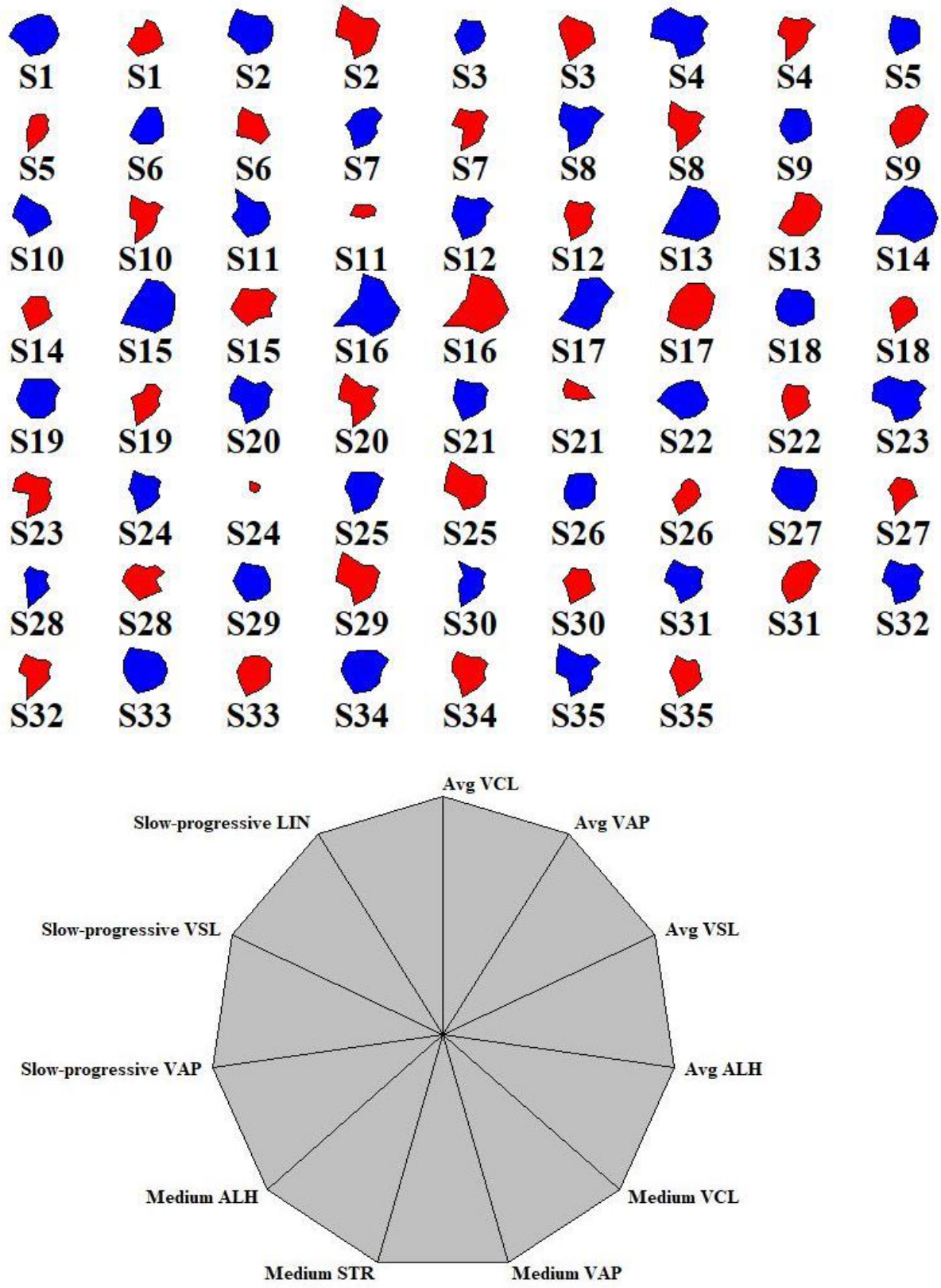


Figure 4.4 Star-glyph plots for comparison of the sperm kinematic characteristics that were significantly different between the two sperm motility subpopulations. Each glyph represents the HM and LM subpopulation separated from individual donor semen samples (n=35) for 12 input parameters (see key above). Data for each parameter was scaled by subtracting its minimum value amongst all the cases and dividing by the range. ***Note:*** HM sperm subpopulations (blue glyphs) had proportionately higher values for most of the selected parameters when compared to LM sperm subpopulations (red glyphs). HM subpopulations additionally presented with greater homogeneity in patterns (size and shape of star-glyphs) when compared to one another, while LM subpopulations had more heterogeneous patterns (variability in input parameters). **Abbreviations:** ALH, amplitude of lateral head displacement; Avg, average; HM, high motile; LIN, linearity; LM, low motile; S#, individual semen sample; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.

4.1.3 | Hyperactivation

Table 4.4 show the hyperactivation percentages of both the HM and LM subpopulations at various time points after exposure to HTF, capacitating-HTF (CAP), 5 mM caffeine, 2 mM procaine. After exposure to the various hyperactivating media, HM subpopulations yielded significantly (p <0.05, p <0.01 and p <0.001) higher mean percentages for sperm hyperactivation as compared to the LM sperm subpopulations. No significant difference was apparent among the media at individual time points; however, significant differences in percentage sperm hyperactivation were observed when comparing different time intervals for individual media.

Both subpopulations generally exhibited a significant reduction in percentage hyperactivation at 60 minutes compared to 5, 15 and 30 minutes for all the hyperactivation inducing media (5 mM caffeine, 2 mM procaine and capacitating HTF). However, no significant differences were observed for HTF medium between the different time points for either subpopulation. A significant reduction was also observed at 45 minutes compared to the 15 minutes time interval for 2 mM procaine in the LM subpopulation (p = 0.003) and 5 mM caffeine in both subpopulations (LM: p = 0.001, HM: p = 0.013). In contrast, sperm hyperactivation was significantly higher at 45 min compared to 60 min for capacitating HTF in the LM subpopulation (p = 0.002) (Figure 4.5).

Table 4.4. Percentage hyperactivation (%) of the high motile (HM) and low (LM) sperm subpopulations at different time intervals and induced with different hyperactivating media (mean ± SD) (n=20).

Media	Time intervals (min)										ANOVA
	5 minutes	SP *p*	15 minutes	SP *p*	30 minutes	SP *p*	45 minutes	SP *p*	60 minutes	SP *p*	
Pro											
HM	19.5 ± 19.2$^{ab\ **}$	**0.008**	21.2 ± 15.8$^{a\ **}$	**0.008**	16.4 ± 11.7$^{ab\ ***}$	**< 0.001**	12.9 ± 11.8$^{bc\ ***}$	**< 0.001**	7.5 ± 7.2$^{c\ ***}$	**< 0.001**	**0.012**
LM	5.6 ± 9.3$^{ab\ **}$		7.1 ± 8.4$^{a\ **}$		7.3 ± 10.2$^{abc\ ***}$		3.1 ± 5.6$^{bc\ ***}$		0.6 ± 1.3$^{c\ ***}$		**0.003**
Caf											
HM	17.9 ± 15.5$^{a\ **}$	**0.004**	21.8 ± 11.9$^{a\ **}$	**0.001**	17.0 ± 10.3$^{a\ *}$	**0.012**	13.6 ± 10.7$^{ab\ **}$	**0.001**	9.7 ± 6.4$^{b\ ***}$	**< 0.001**	**0.013**
LM	7.3 ± 9.1$^{ab\ **}$		11.7 ± 10.9$^{a\ **}$		5.9 ± 7.1$^{ab\ *}$		4.0 ± 7.4$^{bc\ **}$		2.7 ± 4.4$^{c\ ***}$		**0.001**
CAP											
HM	20.6 ± 16.1$^{a\ **}$	**0.004**	22.1 ± 16.6$^{a\ ***}$	**< 0.001**	17.1 ± 13.4$^{a\ ***}$	**< 0.001**	14.3 ± 12.1$^{ab\ ***}$	**< 0.001**	8.2 ± 7.3^{b***}	**< 0.001**	**0.027**
LM	7.7 ± 9.9$^{a\ **}$		7.0 ± 9.7$^{a\ ***}$		5.7 ± 9.9$^{a\ ***}$		3.4 ± 4.0$^{a\ ***}$		0.4 ± 1.3 $^{b\ ***}$		**0.002**
HTF											
HM	13.6 ± 15.0*	**0.025**	15.1 ± 13.1***	**< 0.001**	15.9 ± 14.0***	**< 0.001**	12.0 ± 10.5**	**0.001**	6.7 ± 9.4**	**0.003**	0.163
LM	5.4 ± 8.8*		3.7 ± 6.1***		4.4 ± 6.9***		4.1 ± 6.0**		1.6 ± 4.5**		0.493

***Note*:** HM subpopulations had significantly higher percentages for hyperactivation at all time intervals and for all media. There were no differences between the media for individual subpopulations and time intervals, however differences in percentage hyperactivation between time intervals for individual media were observed. Significantly lower hyperactivation percentages for procaine were observed at the 45- and 60- minute interval in the HM subpopulation, as compared to the 15-minute interval. Both caffeine and CAP for the HM subpopulation had significantly lower percentages at the 60-minute interval as compared to the 5-, 15- and 30- minute intervals. LM subpopulations had significantly lower hyperactivation percentages at 60- and 45- minute intervals for both procaine and caffeine, as compared to the 15-minute interval. At the 60- minutes interval, LM subpopulation hyperactivation percentages were significantly lower for CAP as compared to the 5-, 15-, 30- and 45- minute intervals. **Abbreviations:** ANOVA, analysis of variance between time points for individual media and subpopulations; Caf, caffeine; CAP, capacitating-HTF; HTF, human tubal fluid; HM, high motile; LM, low motile; min, minutes; Pro, procaine; SD, standard deviation; SP *p*, t-test between subpopulations. $^{a, b, c}$ Values labelled with different superscript letters in the same row for each subpopulation were significantly different between the time point for each medium. Values labelled with an asterisk were significantly different between subpopulations for the same time interval and medium (*p <0.05, **p <0.01 and ***p <0.001).

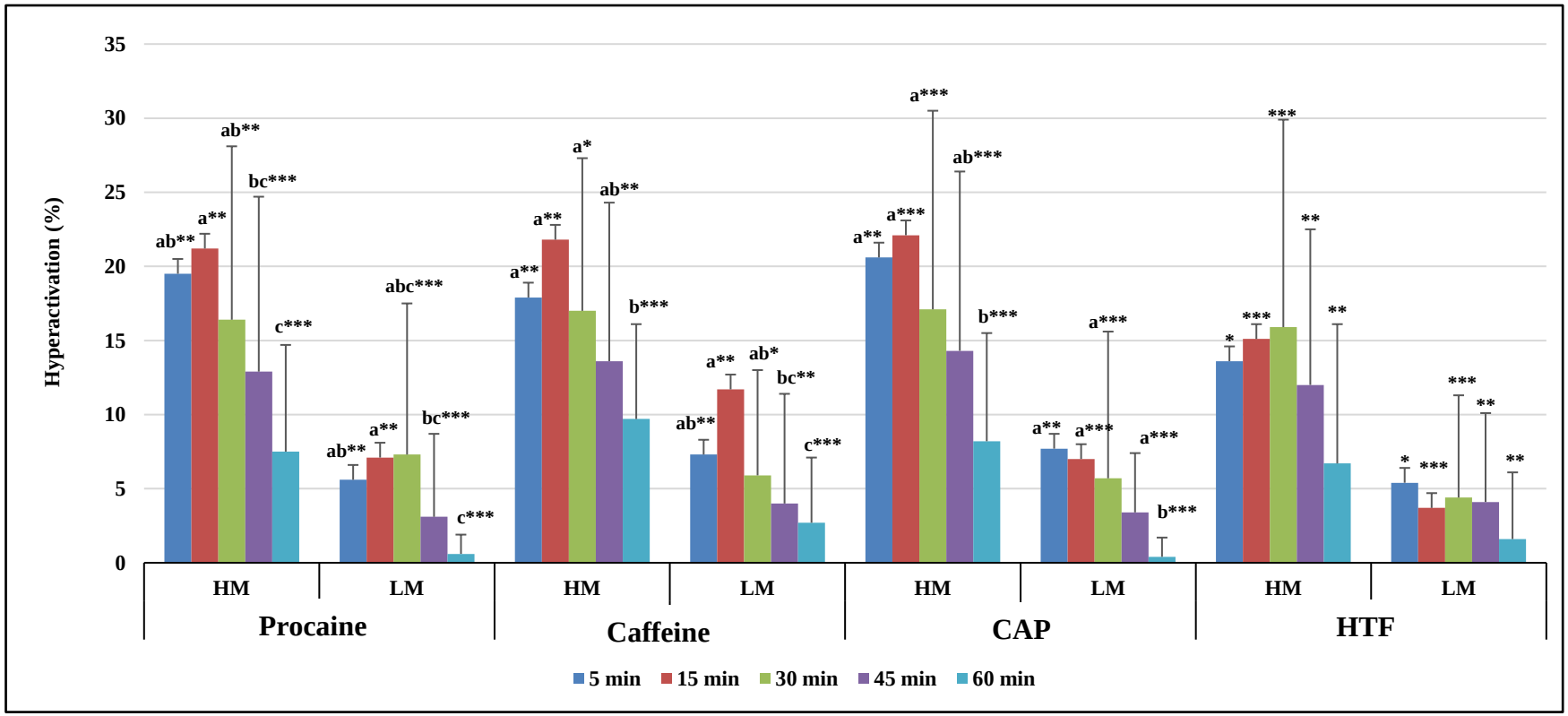


Figure 4.5. Comparison of percentage hyperactivation (%) between the low motile (LM) and high motile (HM) sperm subpopulations at different time intervals and induced with different hyperactivating media (n=20). ***Note:*** Differences between induced hyperactivation percentages between time points for individual media were seen in both subpopulations for CAP, procaine and caffeine. For all four media used, a significantly higher percentage hyperactivation could be induced in HM subpopulations as compared to the LM subpopulations. Time intervals labelled with different superscript letters (a, b and c) were significantly different (p <0.01) in individual subpopulations and media. Corresponding bars labelled with an asterisk were significantly different between HM and LM subpopulations (*p <0.05; **p <0.01; ***p <0.001). **Abbreviations:** CAP, capacitating-HTF; HM, high motile; HTF, human tubal fluid; LM, low motile and min, minutes.

Data from all four media was pooled and the effect of time on percentage hyperactivation for each subpopulation is illustrated in Figure 4.6. Both subpopulations displayed a non-significant increase in percentage hyperactivation between the first two time points followed by a steady decrease up to the 60-minute interval. However, for the HM sperm subpopulation, the decrease in percentage hyperactivation was significant for each time interval from 15 minutes to 60 minutes, whereas for the LM sperm subpopulation the decrease was more gradual. From these results, one can deduce that peak hyperactivation seemed to commonly appear at the 15-minute time interval and decreased thereafter. Thus, it is evident that sperm hyperactivation should be measured after 15 minutes of exposure to the media.

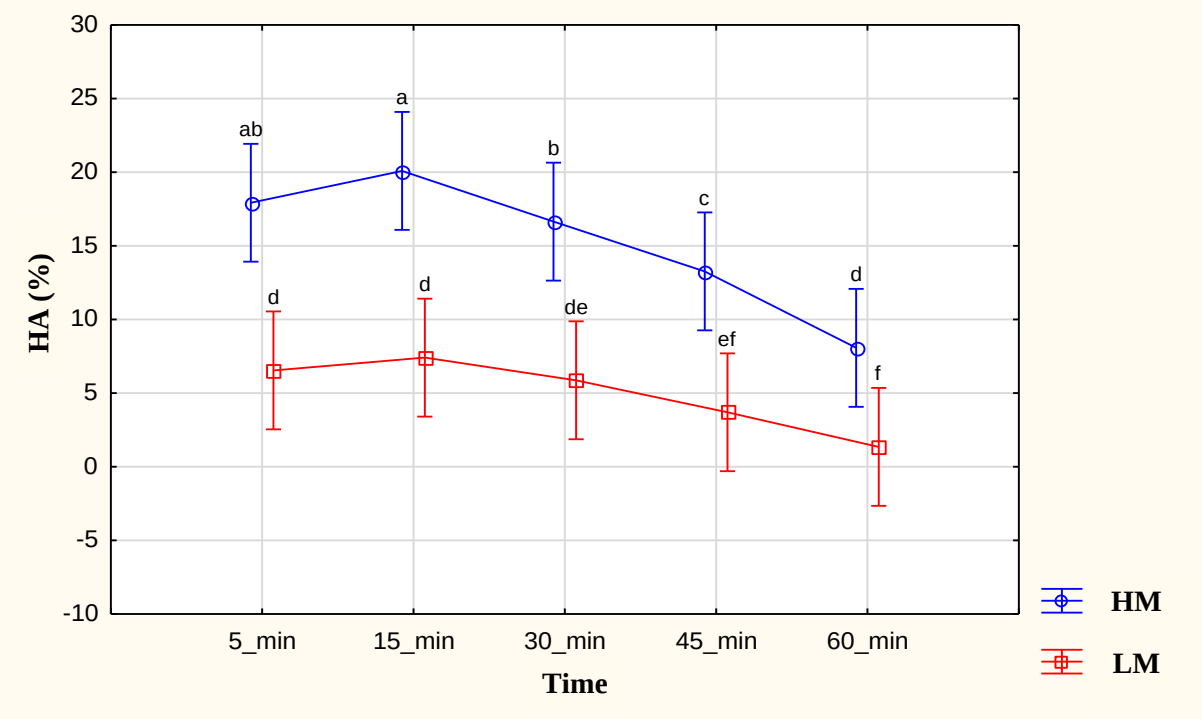


Figure 4.6 Mixed model repeated measures ANOVA illustrating the significant (p <0.01) interaction between subpopulations and time as a combined fixed factor on percentage sperm hyperactivation (n=20) (mean ± SD). ***Note:*** A significant interaction is displayed between subpopulations and time as fixed factors on sperm hyperactivation percentages. HM subpopulations displayed an increase in hyperactivation from 5 to 15 min, followed by a steep decline from 15 to 60 min. LM subpopulations did not appear to have great increased hyperactivation percentages, but rather gradually decreased in percentages. Vertical bars denote 0.95 confidence intervals and bars labelled with different alphabetic letters (a, b, c, d, e, f) were significantly (p <0.01) different between time intervals for individual subpopulations. **Abbreviations:** HA, hyperactivation; HM, high motile; LM, low motile; min, minutes.

4.1.4 | Additional Structural and Functional Characteristics

Shown in Table 4.5 are the percentages acrosome reaction (AR), mitochondrial membrane potential (MMP), reactive oxygen species (ROS), vitality, chromatin integrity and chromatin maturity for both the HM and LM subpopulations exposed to HTF. The HM subpopulations contained significantly higher percentages of mature (p <0.001), viable (p <0.001) and chromatin intact spermatozoa (p <0.001) which also had higher percentages of intact MMP (p <0.001), and lower levels of ROS positive spermatozoa (p <0.001) as compared to the LM subpopulations. In addition, Ca-ionophore induced significantly higher percentages of AR (p <0.001) in HM subpopulations as compared to the LM subpopulations. In contrast, the LM subpopulations had significantly higher percentages of ROS positive spermatozoa and spontaneous acrosome reaction (AR-DMSO) (p <0.001). As such, the LM subpopulation thereby resulted in significantly lower acrosome reaction to Ca-ionophore challenge (ARIC) percentages as compared to HM sperm subpopulations (p <0.001), albeit remaining above the abnormal value (ARIC <15%) as recommended by the WHO (WHO, 2010).

Table 4.5 Comparison of percentage (mean ± SD) vitality (n=35), spontaneous acrosome reaction (AR DMSO, n=35), mature spermatozoa (n=20), intact chromatin (n=20), calcium-ionophore induced acrosome reaction (AR Ca–ionophore, n=35), acrosome reaction after calcium-ionophore challenge (ARIC, n=35), mitochondrial membrane potential (MMP, n=20) and reactive oxygen species (ROS, n=20) for both high motile (HM) and low motile (LM) sperm subpopulations.

	Mean ± SD			95% C.I			SP *p*
AR (Ca-ionophore, %)							
HM	77.5	±	12.2***	73.3	-	81.7	**<0.001**
LM	61.3	±	17.1***	55.4	-	67.2	
AR (DMSO, %)							
HM	21.9	±	11.9***	17.8	-	25.9	**<0.001**
LM	35.8	±	16.5***	30.1	-	41.5	
ARIC (%)							
HM	55.6	±	14.3***	50.7	-	60.5	**<0.001**
LM	25.0	±	16.5***	19.3	-	30.7	
Vitality (live spermatozoa, %)							
HM	70.4	±	9.7***	67.1	-	73.8	**<0.001**
LM	47.9	±	10.3***	44.3	-	51.4	
Mature spermatozoa (%)							
HM	83.4	±	10.0***	78.7	-	88.0	**<0.001**
LM	64.6	±	8.2***	60.8	-	68.4	
Intact chromatin (%)							
HM	80.5	±	8.1***	76.7	-	84.3	**<0.001**
LM	71.3	±	8.0***	67.5	-	75.0	
ROS pos. (%)							
HM	33.9	±	10.5***	28.9	-	38.8	**<0.001**

MMP Intact (%)	**LM**	52.0	±	10.2***	47.3	-	56.8		
	HM	67.2	±	10.4***	62.4	-	72.1		**<0.001**
	LM	44.7	±	15.0***	37.7	-	51.7		

Note: The HM sperm subpopulation yielded significantly (p <0.001) higher mean values for mature and viable spermatozoa with intact mitochondria and chromatin networks. LM sperm subpopulations had significantly (p <0.001) higher percentages of positive reactive oxygen species (ROS) and spontaneous acrosome reaction. **Abbreviations:** AR, acrosome reaction; ARIC, acrosome reaction to Ca-ionophore challenge; C.I, confidence interval; DMSO, dimethyl sulfoxide; HM, high motile; LM, low motile; MMP, mitochondrial membrane potential; ROS pos., reactive oxygen species positive; SD, standard deviation and SP p, t-test between subpopulations. Values in the same column labelled in bold with an asterisk were significantly different between the two sperm subpopulations (*p <0.05, **p <0.01 and ***p <0.001).

Illustrated in Figure 4.7 is an Andrews plot constucted from data of the two subpopulations of individual semen samples. The Andrews plot is designed to distinguish cases that have similar values. Each indiviudal line represents a sepecific subpopulation from a single sample, constructed from the data pertaining to the percentage vitality, ARIC, normal chromatin structure, mature spermatozoa, positive ROS and intact MMP (Table 4.6) . Distinct differences in patterns between the HM (blue lines) and LM sperm subpopulations (red lines) for each semen sample are evident in Figure 4.7 by clear separations of the blue and red lines (label A), thereby illustating the presence of the two distinct groups (subpopulations). In other areas of the plot (label B), the red and blue lines are not dispersed from one another and lacking uniformity. This is an indication of how individual data for certain parameters varied among sample subpopulations; however, the LM sperm subpopulation (red lines) displays larger variation in results compared to the HM sperm subpopulation (blue lines). However, the Andrews plots further show the importance of demostrating differences in subpopulations when a large number of parameters are used in a single test.

Table 4.6 Multivariate visualisation - Andrews plot data input key for individual semen samples (n=40) including bot high and low motile sperm subpopulation values. Data variables included in the Andrews plot are the percentage vitality (%), acrosome reaction after calcium-ionophore challenge scores (%), positive reactive oxygen species (%), intact mitochondrial membrane potential (%), intact chromatin (%) and mature spermatozoa (%). The plot has been standardised according to the minimum and maximum values.

Variables	Mean	±	SD
Vitality (%)	55.3	±	15.1
ARIC (%)	42.9	±	21.4
ROS pos (%)	43.0	±	13.7
MMP intact (%)	56.0	±	17.1
Intact chromatin (%)	75.9	±	9.2
Mature spermatozoa (%)	74.0	±	13.1

Abbreviations: ARIC, acrosome reaction after calcium-ionophore challenge scores; MMP, mitochondrial membrane potential; ROS pos., positive reactive oxygen species; SD, standard deviation.

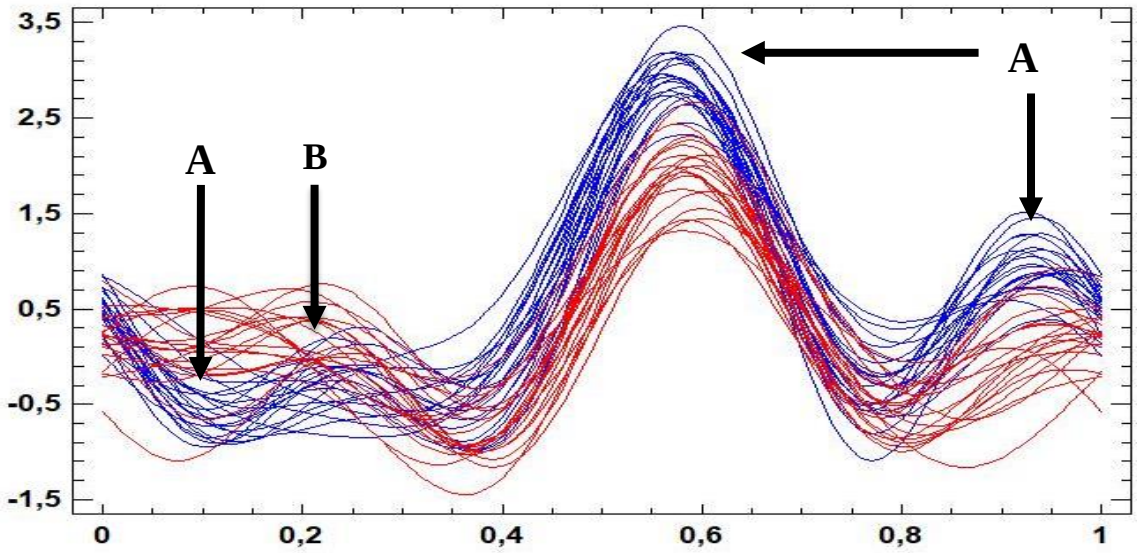


Figure 4.7 Andrews plot for comparison of sperm structural and functional characteristics of two subpopulations separated from individual donor semen samples and constructed using six input parameters (see Table 4.6): percentage vitality (n=35), percentage acrosome reaction (n=35) mature spermatozoa (n=20), normal chromatin (n=20), positive ROS (n=20) and intact MMP (n=20). ***Note:*** Data for each parameter was scaled by subtracting its minimum value amongst all the cases and dividing by the range. **(A)** Clear separation is seen for HM subpopulations (blue lines) and LM subpopulations (red lines) at various peaks and valleys. **(B)** While there is overlap between the two subpopulations in some areas of the plot, the LM subpopulations seem to have larger variation among individual subpopulations for specific parameters. **Abbreviations:** HM, high motile; LM, low motile.

4.1.5 | Correlation and Multiple Regression Analysis of Semen Characteristics with Sperm Motility Subpopulations

Functional and structural parameters of the two sperm motility subpopulations were correlated with the corresponding initial semen parameters as shown in Table 4.7. Subpopulation parameters were divided into two main groups based on the sperm motility and structure. Sperm motility was arranged into different subgroups which comprised of the motility and kinematics for different speed groups (average, rapid, medium and slow) in addition to hyperactivation. The sperm structure group comprised of percentage intact MMP, positive ROS, ARIC and immature spermatozoa. The kinematic subgroup parameters were further arranged and categorized as velocity (VCL, VAP and VSL), linear (LIN and STR) and vigour (WOB, DANCE, BCF and ALH) kinematics. Hyperactivation included both control (non-capacitating HTF) and induced (5 mM caffeine, 2 mM procaine and capacitating HTF) percentages. It should be noted that correlations were calculated for 20 semen parameters and 96 sperm subpopulation parameters, but that data presented in Table 4.7 only indicate significant (p <0.05) correlations where $r \geq 0.40$. Furthermore, the highest correlation between individual parameters was selected to represent each grouped parameter.

Table 4.7 Significant (p <0.05) correlations (r ≥0.40) between conventional standard semen parameters of whole semen and sperm structural and functional parameters of density-gradient centrifugation separated sperm subpopulations (high motile and low motile).

		Motility grouped parameters															Structure grouped parameters			
		Motility sub-group					Kinematics sub-group									HA sub-group				
							Avg			Rapid		Medium			Slow					
Semen characteristics and subpopulations		Total	Progressive	Non-progressive	Rapid	Medium	Velocity	Linear	Vigour	Velocity	Vigour	Velocity	Linear	Vigour	Velocity	HTF	Induced	MMP	ROS	ARIC	Immature
Volume																					
	HM																				
	LM					-0.46															
pH																					
	HM																			-0.49	
	LM																				
Conc.																					
	HM												0.41				-0.49				
	LM																				
MPT																					
	HM																-0.49				
	LM				-0.43																
Visc																					
	HM	0.47	0.69	-0.43	0.69		0.61		0.54	0.51	0.52	0.58	-0.45	0.55		0.53	0.75				
	LM				0.43					0.62	0.57						0.48	0.61		-0.47	
Tot. mot																					
	HM															0.53	0.50				-0.60
	LM																			-0.42	
Prog mot																					
	HM																				
	LM										0.55										
Normal morph																					
	HM																				
	LM			-0.40			0.46	0.51							0.40			-0.46			

Normal acro																					
	HM															0.45					
	LM																0.50				
Head defects																					
	HM																0.49				
	LM														-0.46			0.52			
Midpiece defects																					
	HM												0.41								
	LM				-0.48		-0.47									-0.50					
Tail defects																					
	HM					0.44										-0.53					
	LM										-0.64						-0.75			0.44	
Cytopl droplets																					
	HM												0.41		-0.40						
	LM	-0.45			-0.40				-0.40								-0.52		0.52		
TZI																					
	HM																				
	LM	-0.43	-0.45		-0.40		-0.42		-0.40							-0.63	-0.51				0.49
DI																					
	HM																				
	LM							-0.40							-0.42						
MAI																					
	HM																-0.48				
	LM							-0.41													

Note: Subpopulation parameters are divided into motility and structural groups. Motility groups include the different motility parameters, kinematics for different speed groups (average, rapid, medium and slow) and hyperactivation as HTF (non-capacitating HTF) and induced (5 mM caffeine, 2 mM procaine and capacitating HTF). Kinematics are further categorized into velocity (VCL, VAP and VSL), linear (LIN and STR) and vigour (WOB, DANCE, BCF and ALH) groups and the structural group into the percentage intact MMP, positive ROS, ARIC and immature spermatozoa. Semen viscosity presented with numerous correlations to the motility group of the HM subpopulation, whereas semen morphology parameters presented with numerous correlations to both the LM subpopulation's structural and motility group. Overall semen parameters seemed to equally correlate more with the LM subpopulation's structural and motility group parameters (40 correlations in total), compared to the HM subpopulation where less correlations were observed between semen parameters and was further isolated to the motility group (28 correlations in total). Taking all correlations for both subpopulations into account, total sperm motility and viscosity of semen had positive relationships with fractionated sperm with high motility and swimming speeds in addition to hyperactivation capabilities, whereas sperm morphology defects were indicative of sperm subpopulations with less motile and immature sperm, less potential for hyperactivation and acrosome reaction, lower MMP and more ROS. **Abbreviations:** ALH, amplitude of lateral head displacement; ARIC, acrosome reaction to Ca-ionophore challenge; Avg, average; BCF, beat cross frequency; Conc, concentration; Cytopl droplets, cytoplasmic droplets; DI, deformity index; HA, hyperactivation; HM, high motile; HTF, human tubal fluid; LIN, linearity; LM, low motile; MAI, multiple abnormalities index; MMP, intact mitochondrial membrane potential; MPT, mucous penetration test; Normal acro, normal acrosome; Normal morph, normal morphology; Prog mot, progressive motility; ROS, reactive oxygen species; STR, straightness; Tot. mot, total motility; TZI, tetrazoospermic index; VAP, average path velocity; VCL, curvilinear velocity; Visc, viscosity; VSL, straight-line velocity; WOB, wobble.

Semen parameters appeared to correlate more with the HM sperm subpopulations' grouped motility parameters, whereas correlations between the semen and LM sperm subpopulation parameters appeared to be equally dispersed in both motile and structural grouped parameters. Furthermore, majority of the sperm morphology parameters in semen seemed to largely correlate (mostly negative) with the LM subpopulations parameters, whereas semen viscosity appeared to correlate (majority positive) with majority of the HM subpopulations parameters. Taking all correlations into account, it seems that a positive relationship exists among total sperm motility, viscosity of semen and fractionated sperm with high motility and swimming speeds in addition to hyperactivation capabilities. On the other hand, sperm morphology defects in semen samples were indicative of sperm subpopulations with less motile and immature sperm, less potential for hyperactivation and acrosome reaction, lower MMP and more ROS.

Illustrated in Table 4.8 is the R-squared (R2) and beta coefficients (b*) from the multiple regression analysis used to determine the relationship between four groups of semen parameters (20 predictor variables) and the HM sperm subpopulations motility and structural parameters (96 dependent variables). Group I comprised of semen concentration and volume, group II of total motility, semen viscosity, mucus penetration and progressive motility, group III of semen vitality, normal chromatin integrity and mature spermatozoa, and group IV of semen morphology parameters which were further split into two sub-groups due to multicollinearity (inter-correlations amongst the independent variables which could result in a disturbance of the data) between parameters. Motility and structural parameters of the HM subpopulations were further grouped and categorised as previously mentioned in Table 4.7. Data from semen groups with significant (p <0.05) predictor variables and beta coefficients with b* ≥0.40 were presented in Table 4.8. Due to incomplete cases in results of the LM subpopulations, multiple regression analysis was only preformed on the HM sperm subpopulations.

Table 4.8 Significant (p <0.05 and *p* <0.01) beta coefficients (b*) ≤0.40 and R-squared (R2) values from the multiple linear regression analysis used to determine the relationship between four groups of semen parameter parameters (20 predictor variables) and the HM sperm subpopulation's motility and structural grouped parameters (96 dependent variables).

		Motility grouped parameters																						Structure grouped parameters		
		Motility sub-group							Kinematics sub-group															HA sub-group				
		Avg		Rapid			Medium			Slow			Rap prog			Med prog	Slow prog										
BASIC SEMEN PARAMETER GROUPS		Total	Prog	Non-prog	Rapid	Medium	Slow	Rap Prog	Velocity	Vigour	Velocity	Linear	Vigour	Velocity	Linear	Vigour	Velocity	Linear	Vigour	Velocity	Linear	Vigour	Vigour	Linear	HTF	Induced	Immature	Mature	MMP
GROUP I																													
R																									0.53			
R2																									0.28			
b*	**Conc.**																								-0.47			
	Vol																											
GROUP II																													
R		0.60	0.65	0.51	0.74	0.60		0.62	0.84	0.80	0.78	0.49	0.68	0.81	0.64	0.80	0.45	0.45	0.57	0.80		0.73		0.44	0.68	0.83	0.71	0.75	
R2		**0.36****	**0.43****	0.26	**0.55****	**0.36****		**0.39****	**0.70****	**0.65****	**0.62****	0.24	**0.46****	**0.66****	**0.41****	**0.65****	0.20	0.20	**0.33***	**0.63****		**0.53****		0.19	**0.46***	**0.69****	**0.5***	**0.57****	
b*	**Tot mot**											-0.45															-0.64	0.69	
	Visc	0.48	0.67	-0.52	0.76	-0.57		0.60	0.87	0.83	0.84		0.68	0.84	-0.51	0.80	-0.44	-0.42	-0.50	0.82		0.72		-0.43	0.98	1.14			
	MP																												
	Prog																								-0.62	-0.69			
GROUP IV (A)																													
R		0.62				0.68				0.44		0.55				0.57					0.51					0.85			0.74
R2		0.38				**0.46***				0.19		0.30				0.32					0.26					**0.72***			0.54
b*	**Head def**					0.42																				0.97			
	Midpiece def					0.62										0.53													
	Tail def	-0.42				0.44				-0.48																			
	Cytopl droplets																												
	MAI					-0.57															0.78								
	Micro																												
	Macro																												1.08
	Norm. acro	-0.56										0.48														0.78			
GROUP IV (B)																													
R			0.54		0.53	0.67	0.58			0.66		0.61	0.55											0.57		0.82			
R2			0.30		0.28	**0.45***	0.33			**0.43***		0.37	0.30											0.33		**0.68***			
b*	**Norm. morph**					-0.76				-0.60														-0.70					
	Tail def						-0.52																						
	Cytopl droplets																									0.47			
	TZI		0.73		-0.54	0.51	0.69																			-0.99			
	DI		-0.82			-0.69																							
	Micro											-0.64																	
	Macro											-0.74																	
	Norm. acro									0.45		0.54	0.48																

Note: Semen parameter groups comprised of: (Group I) - concentration and volume; (Group II) - total motility, semen viscosity, mucus penetration and progressive motility and (Group IV) - semen morphology parameters, which were further split into two subgroups (Groups IV (A) and (B)) due to multicollinearity between parameters. HM sperm subpopulations structural and functional sperm parameters are grouped and categorised into motility and structural groups. Motility groups include the different motility parameters, kinematics for different speed and progressivity groups (average, rapid, medium, slow and rapid -, medium - and slow-progressive) and hyperactivation as HTF (non-capacitating HTF) and induced (5 mM caffeine, 2 mM procaine and capacitating HTF). Kinematics are further categorized into velocity (VCL, VAP and VSL), linear (LIN and STR) and vigour (WOB, DANCE, BCF and ALH) groups and the structural group into the percentage intact MMP, vitality, immature and mature spermatozoa. Group (II) of the semen parameter groups had the best predictive value towards the HM sperm subpopulation structural and functional parameters, with semen viscosity contributing the most that model. **Abbreviations:** ALH, amplitude of lateral head displacement; Avg, average; BCF, beat cross frequency; Conc, concentration; Cytopl droplets, cytoplasmic droplets; DI, deformity index; HA, hyperactivation; Head def: head defect; HM, high motile; HTF, human tubal fluid; LIN, linearity; LM, low motile; MAI, multiple abnormalities index; Med prog, medium progressive; Midpiece def: midpiece defect; MMP, intact mitochondrial membrane potential; MP, mucous penetration; Non-prog, non-progressive motility; Norm. acro, normal acrosome; Norm. morph, normal morphology; Prog, progressive motility; Rap prog, rapid progressive; ROS, reactive oxygen species; Slow prog, slow progressive; STR, straightness; Tail def: tail defect; Tot. mot, total motility; TZI, tetrazoospermic index; VAP, average path velocity; VCL, curvilinear velocity; Visc, viscosity; Vol, volume; VSL, straight-line velocity; WOB, wobble; R2, R-squared; b*, beta coefficients. Significant values indicated in bold as **p* <0.05 and ***p* <0.01.

Compared to semen groups I and IV (A) and (B), semen group II (total motility, semen viscosity, mucus penetration and progressive motility) had majority R-squared values range above 0.50 for both structural and motility parameters of the HM subpopulation. However, significance was only seen for percentage rapid swimming spermatozoa, velocity and vigour kinematic parameters of average rapid, medium, and rapid progressive speeds, induced hyperactivation and immature spermatozoa. Consequently, the semen total motility, viscosity, mucus penetration and progressive motility could therefore, together, account for more than 50% of the variation seen in the above-mentioned parameters. Looking at the beta coefficients, semen viscosity had the most impact (positive) on the previously mentioned HM subpopulation parameters, whereas total motility had the most impact on immature spermatozoa in the HM subpopulation.

In groups IV (A) and IV (B), R-squared values for morphology parameters ranged above 0.5 for induced hyperactivation and intact MMP; however, significance was only seen for induced hyperactivation. Together, semen morphology parameters can therefore account for more than 50% of the variability seen in induced hyperactivation results of the HM subpopulation. Beta coefficients of the sperm head defects and normal acrosome in group IV (A), both appeared to have the most impact on induced hyperactivation, whereas in group IV (B), TZI and cytoplasmic droplets had the most impact.

4.2 | Results of Phase Two of the Study

4.2.1 | Basic Semen Analysis

Illustrated in Table 4.9 are the average basic semen analysis results of the selected semen samples used for the second phase of the study. The mean values of all the semen parameters were above the lower reference values according to the WHO laboratory manual (WHO, 2021), however with the exclusion of the percentage progressive motility which fell below the reference values. As the main aim of the second phase of the study was to determine the effects of the various media on different functional quality of spermatozoa, selected semen samples had a large variation in quality of semen parameters.

Table 4.9 Basic semen parameters of selected semen samples used in this investigation (mean ± SD) (n=160).

	Mean	±	SD	95% C.I		
Progressive (%)	27.3	±	16.6	24.8	-	29.8
Total motility (%)	54.5	±	18.5	51.7	-	57.3
Mucus penetration test (x10^6/ejaculate)	28.3	±	27.0	24.2	-	32.4
Sperm concentration (x10^6/mL)	58.6	±	42.8	52.2	-	65.1
Total sperm number (x10^6/ejaculate)	161.3	±	143.5	139.6	-	183.0
pH	7.4	±	0.2	7.4	-	7.5
Volume (mL)	3.2	±	2.9	2.8	-	3.7
Viscosity (cP)	10.8	±	11.1	9.1	-	12.5
Vitality (live spermatozoa, %)	69.9	±	10.7	68.3	-	71.6
Normal (%)	5.8	±	4.4	5.2	-	6.5
Head defects (%)	80.7	±	11.5	79.0	-	82.5
Midpiece defects (%)	51.2	±	14.1	49.0	-	53.3
Tail defects (%)	48.6	±	19.4	45.6	-	51.5
Normal Shape (%)	79.5	±	12.5	77.3	-	81.7
Normal Size (%)	29.0	±	15.3	26.6	-	31.3
Cytoplasmic droplets (%)	17.7	±	13.3	15.7	-	19.7
Macro (%)	17.1	±	10.6	15.4	-	18.7
Micro (%)	9.4	±	9.7	7.9	-	10.9
TZI	2.1	±	0.2	2.1	-	2.1
MAI	2.8	±	2.5	2.4	-	3.2
DI	2.7	±	1.7	2.4	-	3.0

Note: The mean semen parameters fall above the lower reference limits as recommended by WHO (2021), however mean progressive motility fell between the 2.5th and 5th percentile. **Abbreviations:** C.I, confidence interval; cP, centipoise; DI, deformity index; MAI, multiple abnormalities index; SD, standard deviation; TZI, teratozoospermic index.

4.2.2 | Bubble Diagrams

To indicate the comprehensive effects of the media on individual sperm subpopulations, and collectively with media as the main factor, the results were summarized and displayed as bubble diagrams. Overall effects of the different concentrations of the media were additionally summarised as one, and further illustrated with the use of arrows to indicate whether an increase or decrease was observed on the different functional parameters. Figure 4.8 displays the overall effects of the various media on functional parameters, including vitality, MMP, positive reactive ROS, hyperactivation (HA) and AR. Illustrated in Figure 4.9 are the effects of each medium on motility percentages and illustrated in Figures 4.10 are the effects of each medium on average/motile kinematic parameters. Figures 4.11, 4.12 and 4.13 display the effects of the media on the kinematic parameters of the different progressivity groups namely, rapid progressive, medium progressive and non-progressive respectively. Individual functional parameters are considered below in more detail under the subsequent subheadings; however, bubble diagrams will be referred to as a summarised illustration.

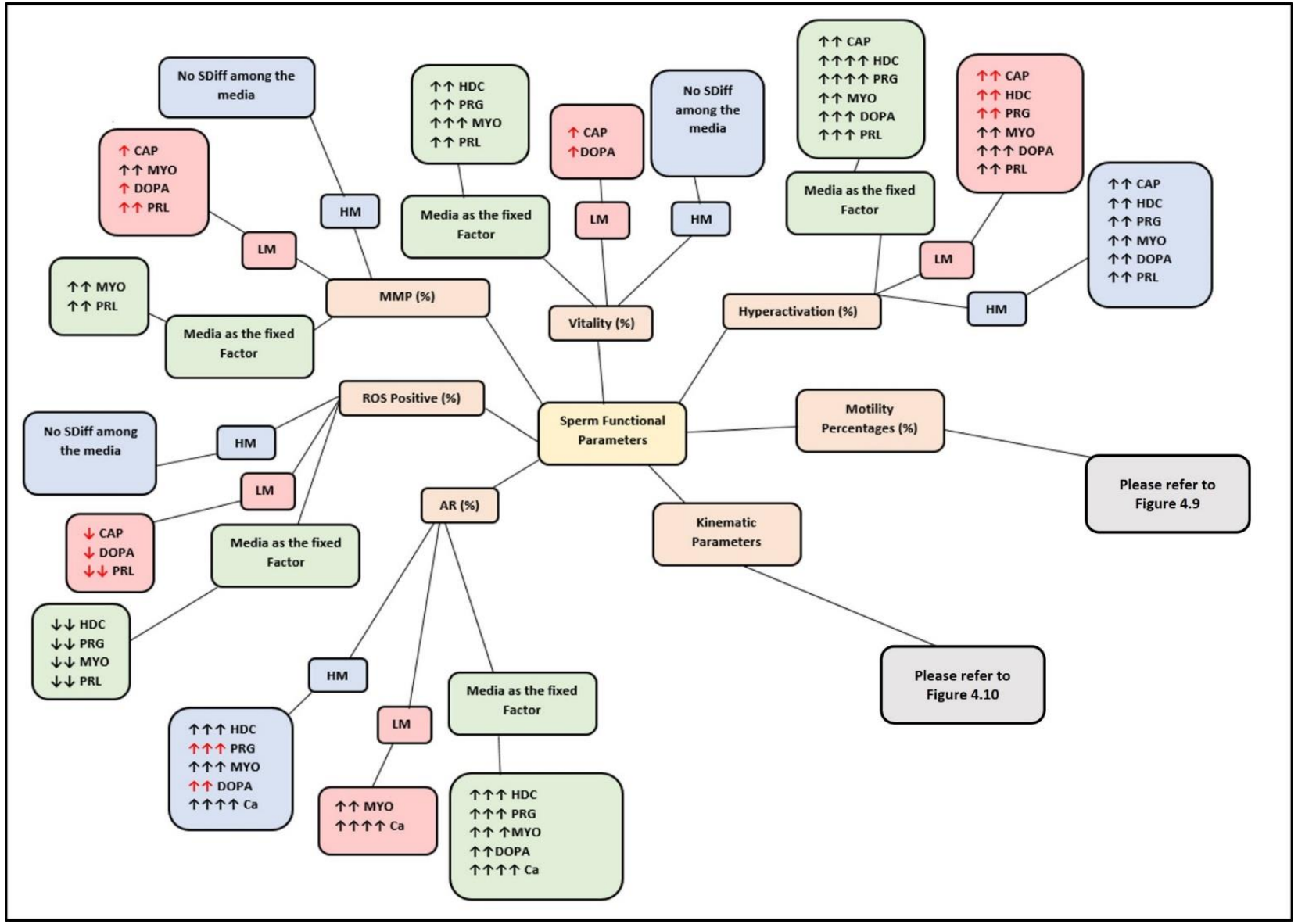


Figure 4.8 Bubble diagram displaying the overall effect of media on various functional parameters of spermatozoa for both subpopulations, and collectively with media as the main factor. ***Note:*** Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values until significant differences between subpopulations were no longer observed. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. Student's *t*-test or the Mann-Whitney test when normal distribution was void in addition to one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions; $p < 0.05$. **Abbreviations:** AR, acrosome reaction; CAP, capacitating-HTF; Ca-ionophore, calcium-ionophore; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LM, low motile subpopulation; MMP, mitochondrial membrane potential; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; ROS, positive reactive oxygen species; SDiff; significant difference.

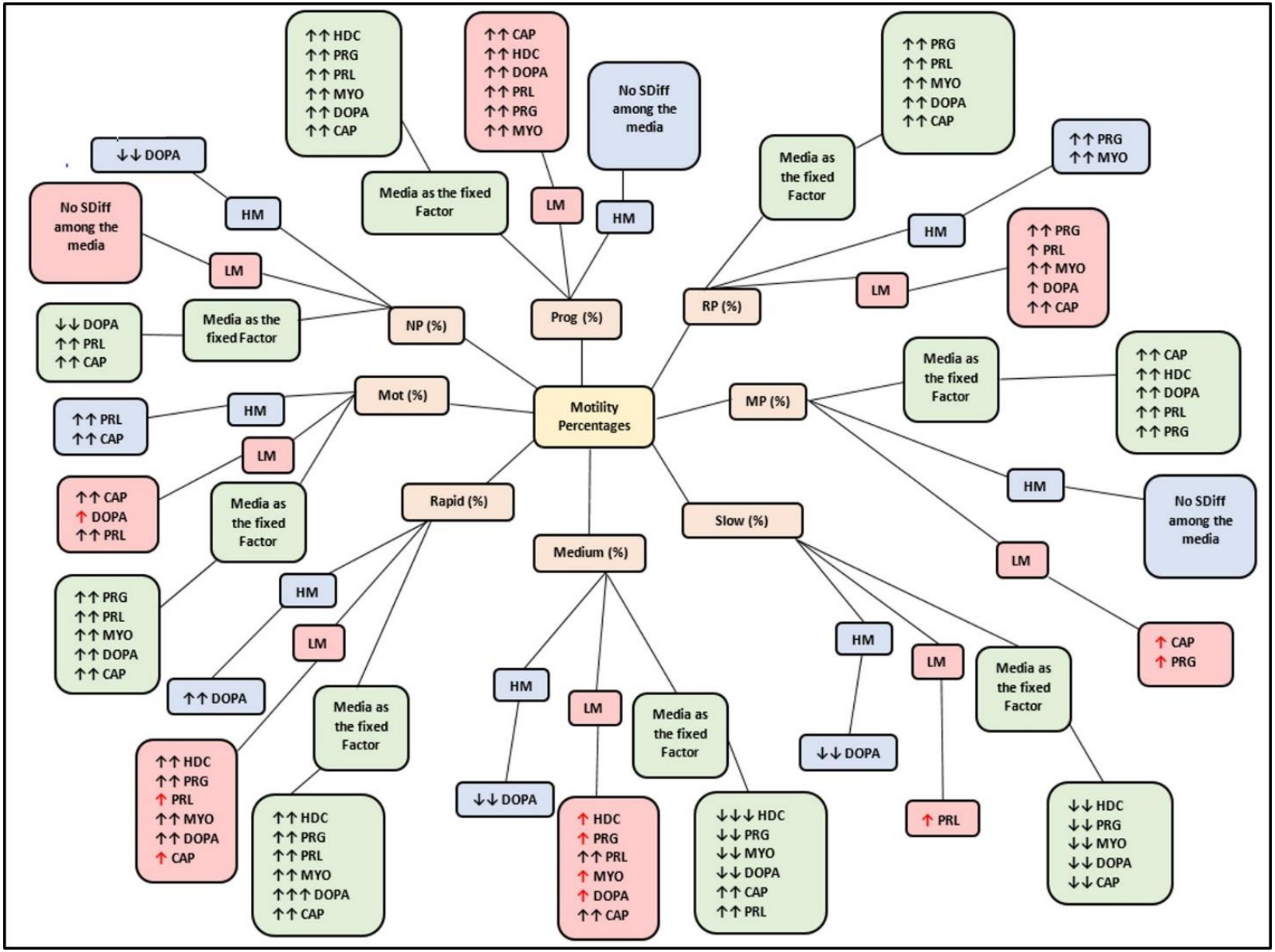


Figure 4.9 Bubble diagram displaying the overall effect of media on various motility parameters of spermatozoa for both subpopulations, and collectively with media as the main factor. ***Note:*** Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values until significant differences between subpopulations were no longer observed. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. Student's *t*-test or the Mann-Whitney test when normal distribution was void in addition to one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions; $p < 0.05$. **Abbreviations:** CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LM, low motile subpopulation; Mot, total motility; MP, medium progressive; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; PRL, prolactin; Prog, progressive motility; RP, rapid progressive; SDiff; significant difference.

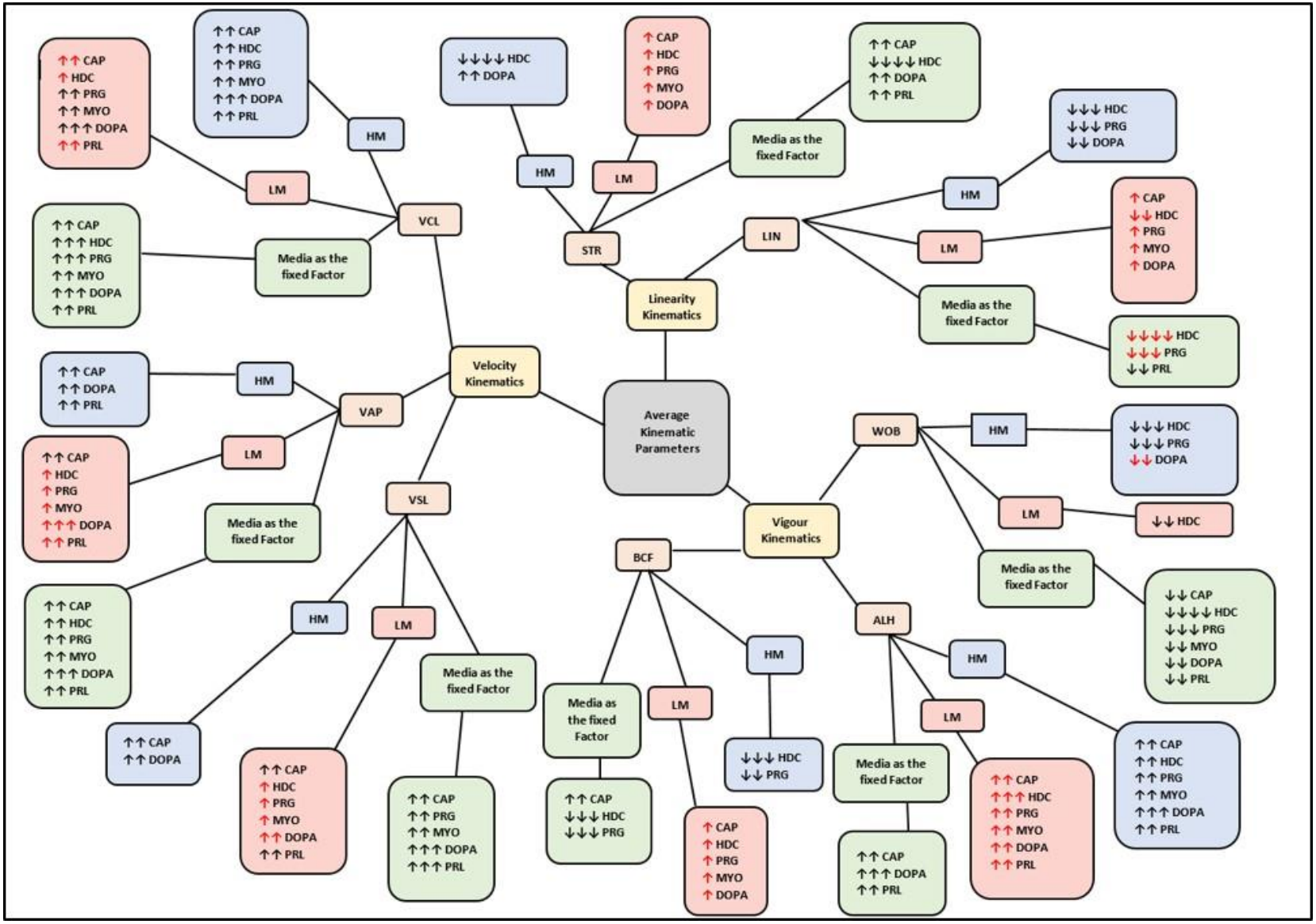


Figure 4.10 Bubble diagram displaying the overall effect of media on various motile/average kinematic parameters of spermatozoa for both subpopulations, and collectively with media as the main factor. ***Note:*** Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values until significant differences between subpopulations were no longer observed. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. Student's *t*-test or the Mann-Whitney test when normal distribution was void in addition to one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions; $p < 0.05$. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; sig, significant; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

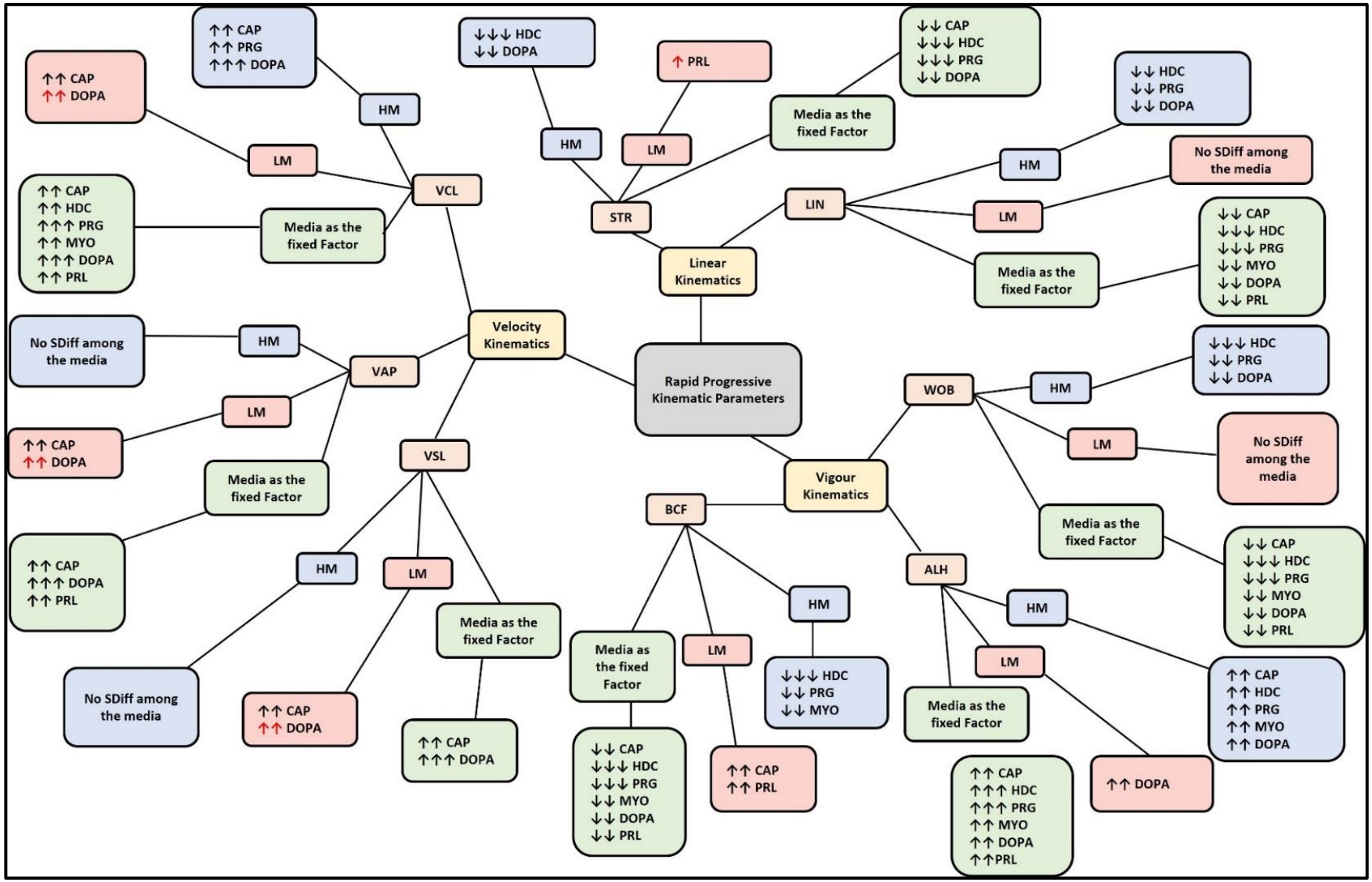


Figure 4.11 Bubble diagram displaying the overall effect of media on various rapid progressive kinematic parameters of spermatozoa for both subpopulations, and collectively with media as a fixed factor. ***Note:*** Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values until significant differences between subpopulations were no longer observed. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. Student's *t*-test or the Mann-Whitney test when normal distribution was void in addition to one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions; $p < 0.05$. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; sig, significant; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

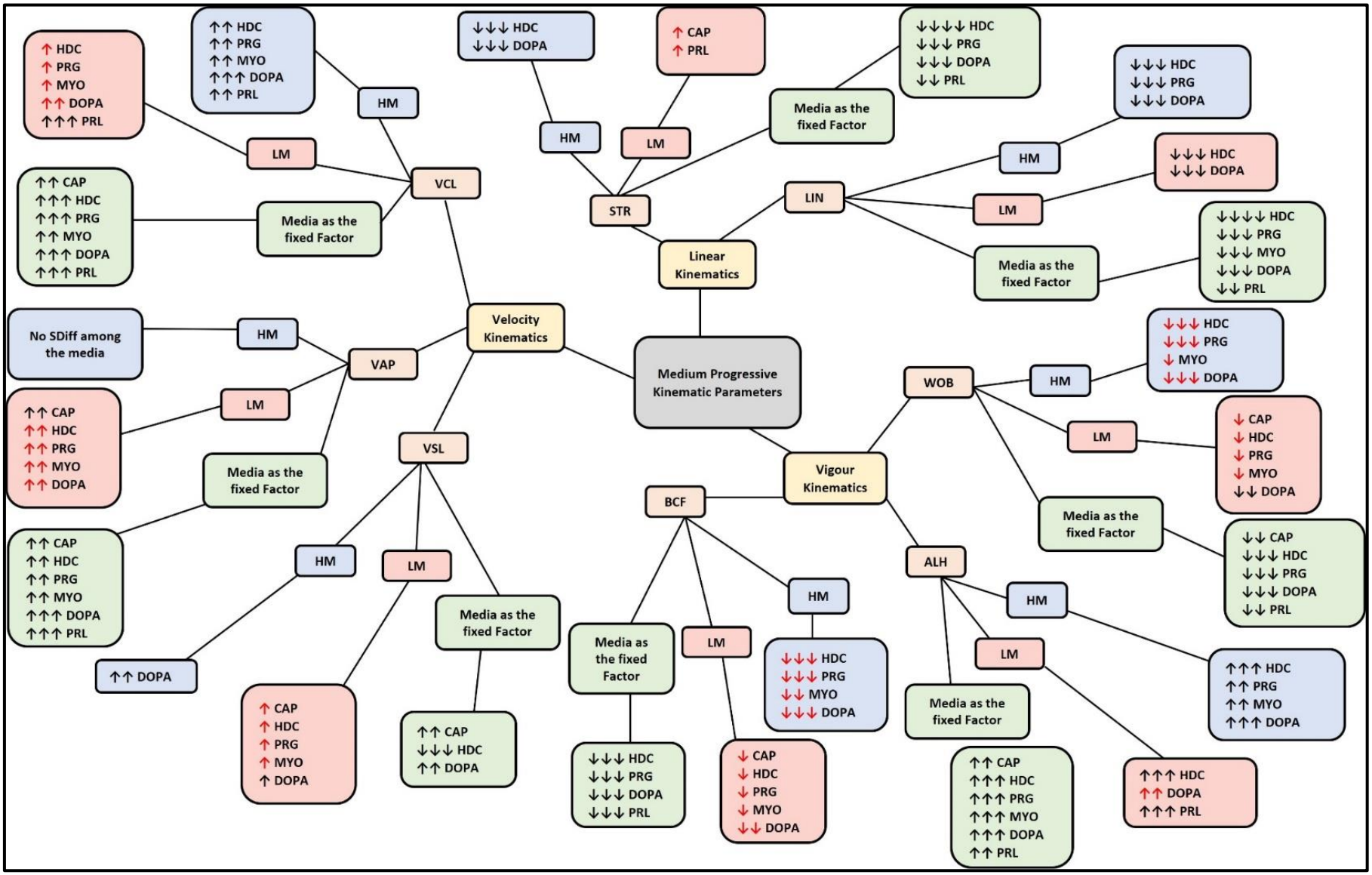


Figure 4.12 Bubble diagram displaying the overall effect of media on various medium progressive kinematic parameters of spermatozoa for both subpopulations, and collectively with media as fixed factor. ***Note:*** Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values until significant differences between subpopulations were no longer observed. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. Student's *t*-test or the Mann-Whitney test when normal distribution was void in addition to one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions; $p < 0.05$. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; sig, significant; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

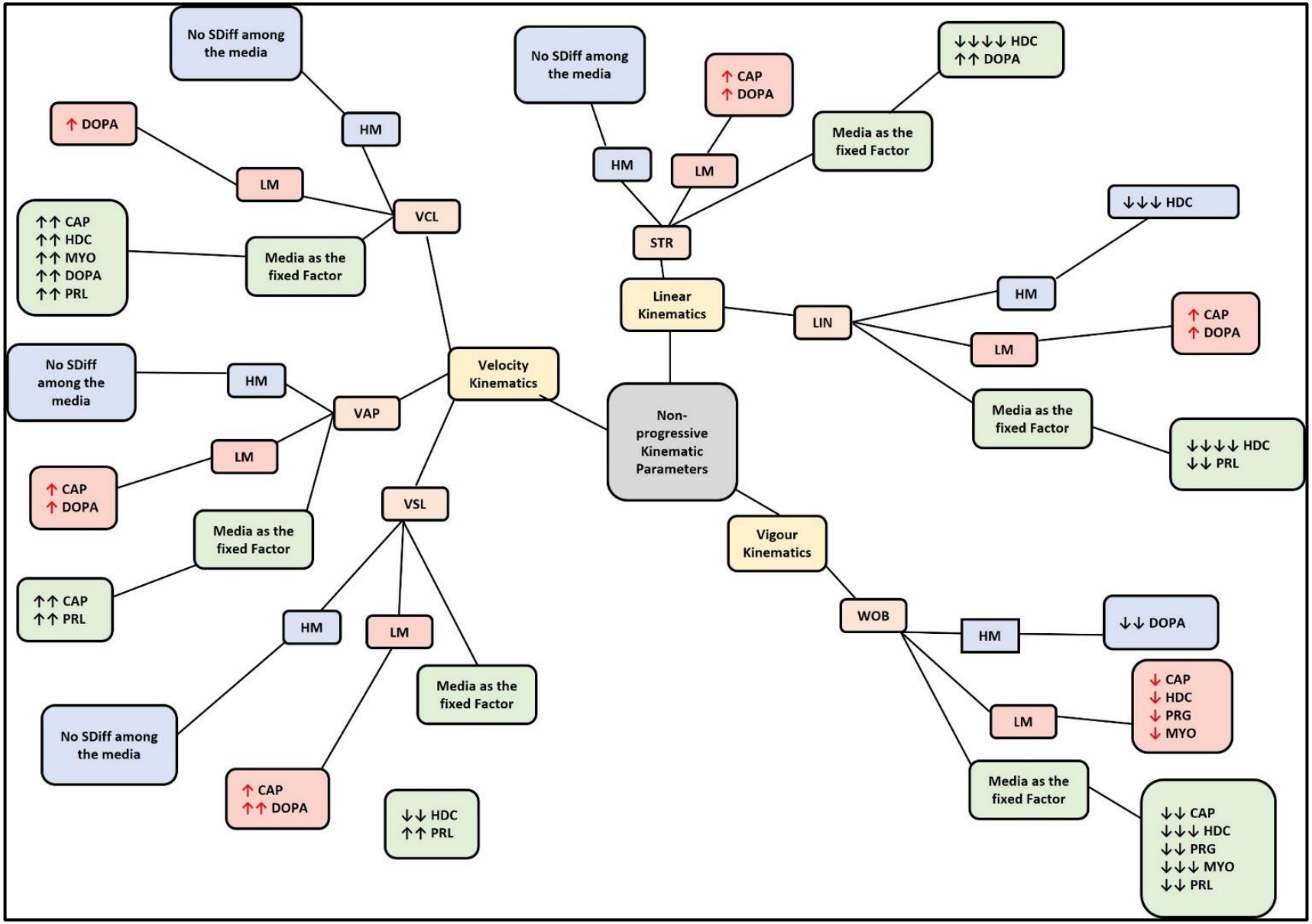
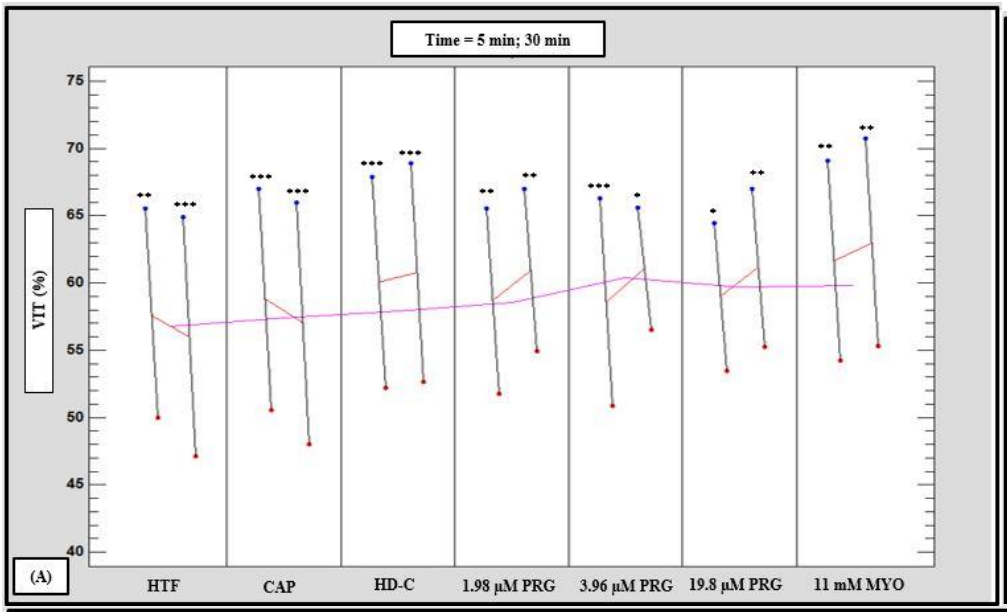


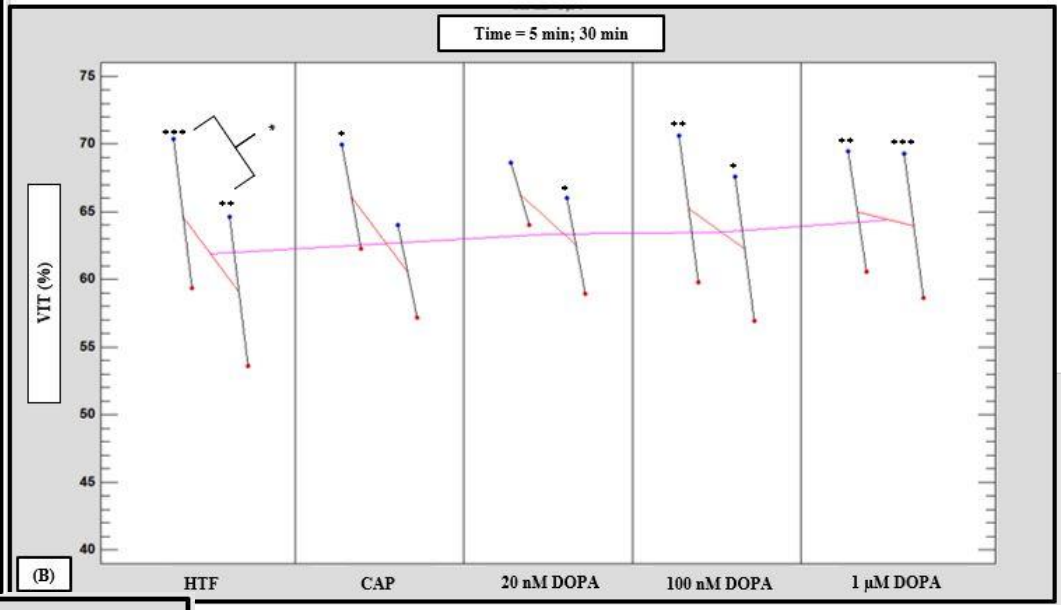
Figure 4.13 Bubble diagram displaying the overall effect of media on various non-progressive kinematic parameters of spermatozoa for both subpopulations, and collectively with media as fixed factor. ***Note:*** Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values until significant differences between subpopulations were no longer observed. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. Student's *t*-test or the Mann-Whitney test when normal distribution was void in addition to one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions; $p < 0.05$. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; sig, significant; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

4.2.3 | Vitality

Illustrated in Supplementary Table 1 and Figure 4.14 are the vitality percentages of both the high motile (HM) and low motile (LM) subpopulations after exposure to HTF, CAP, HD-C, progesterone, myo-inositol, dopamine, and prolactin. After exposure to the various media, the HM subpopulation yielded significantly (p <0.05, p <0.01 and p <0.001) higher vitality percentages as compared to the LM subpopulations. These results were observed for all media and time points; however, at the 5-minute interval 20 nM dopamine improved the percentage viable spermatozoa of the LM subpopulation until significant differences were no longer observed between the two subpopulations (HM 68.7 ± 10.8%; LM 64.2 ± 9.3 %, p = 0.165) (Figure 4.8 & Figure 4.14 B). This trend was further observed at 30 minutes for capacitating-HTF (HM 64.0 ± 10.1%; LM 57.2 ± 13.1%, p = 0.071) (Figure 4.8 & Figure 4.14 B). Vitality percentages were significantly lower at 30 minutes incubation as compared to 5 minutes incubation for the HM subpopulation in HTF (5 minutes 70.7 ± 7.9%; 30 minutes 64.6 ± 6.3%, p = 0.01) (Figure 4.14 B). No other significant differences were observed between vitality percentages at the different time points for individual media and subpopulations.

To determine whether combined factors (subpopulations, time and media) had an interaction on vitality percentages, data was analysed in a mixed model ANOVA. A significant effect (p <0.01) was only observed for media as a fixed factor for HTF, CAP, progesterone, myo-inositol, and prolactin (Figure 4.15 A & C). For the individual effects of media, HD-C (p <0.01), progesterone (1.98 µM, p = 0.03; 3.96 µM, p = 0.03 and 19.8 µM, p = 0.02) (Figure 4.15 A), dopamine (20 nM, p = 0.04 and 1 µM, p = 0.03) (Figure 4.15 B) and prolactin (50 ng/mL, 100 ng/mL and 250 ng/mL, p <0.01) (Figure 4.15 C) significantly improved percentages of viable spermatozoa as compared to HTF. In addition, myo-inositol (p <0.01) significantly improved vitality as compared to both HTF and CAP. Prolactin appeared to display a possible dose-dependent trend as 100 ng/mL resulted in significantly (p <0.01) higher vitality percentages as compared to 500 ng/mL (Figure 4.15 C).





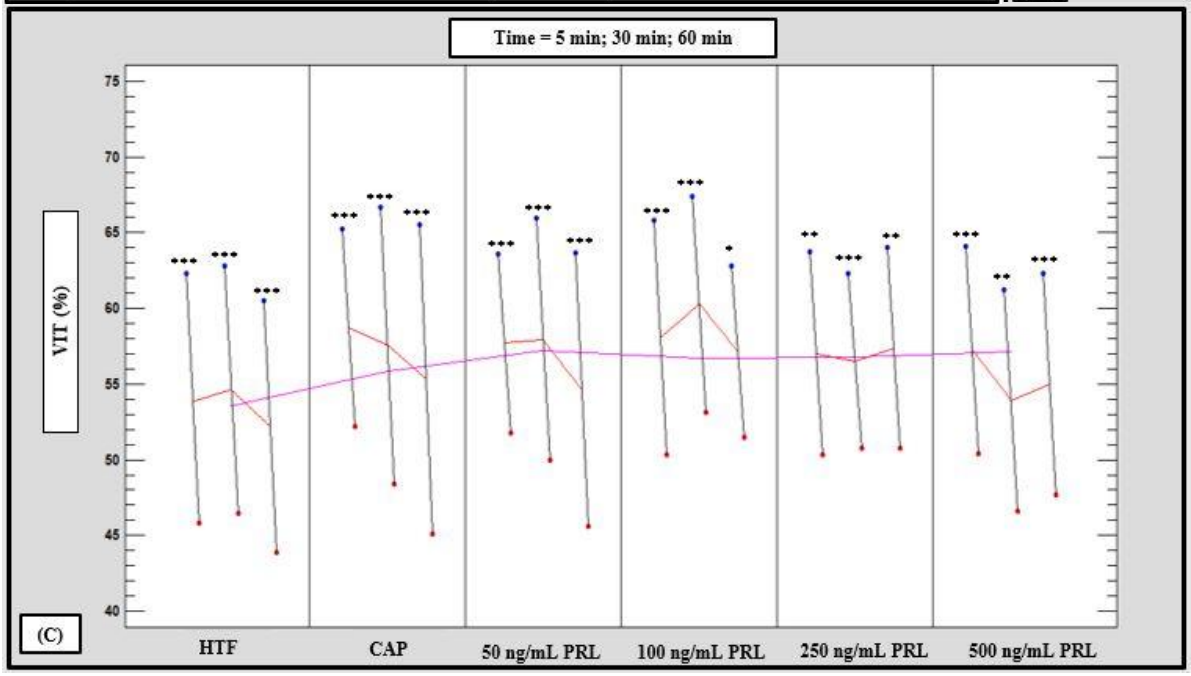
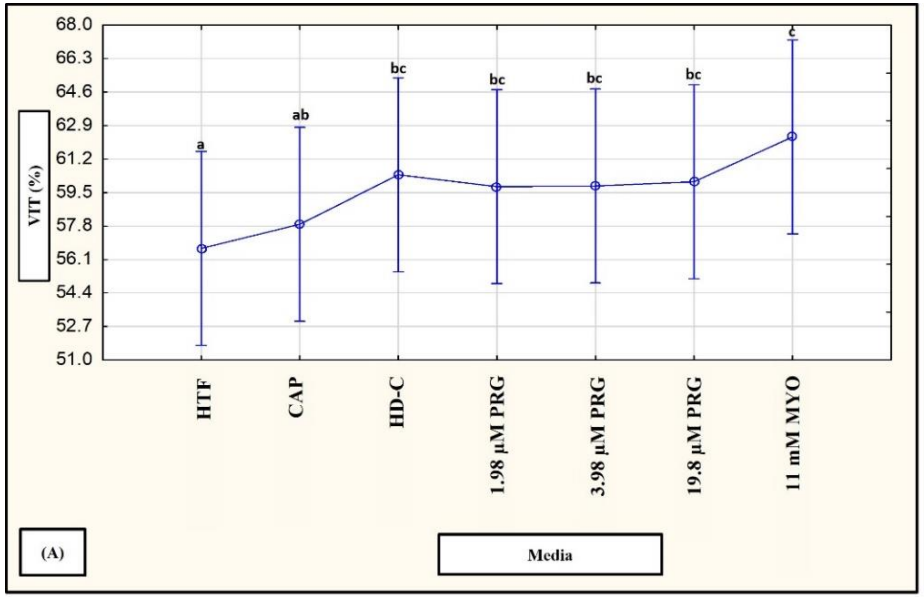
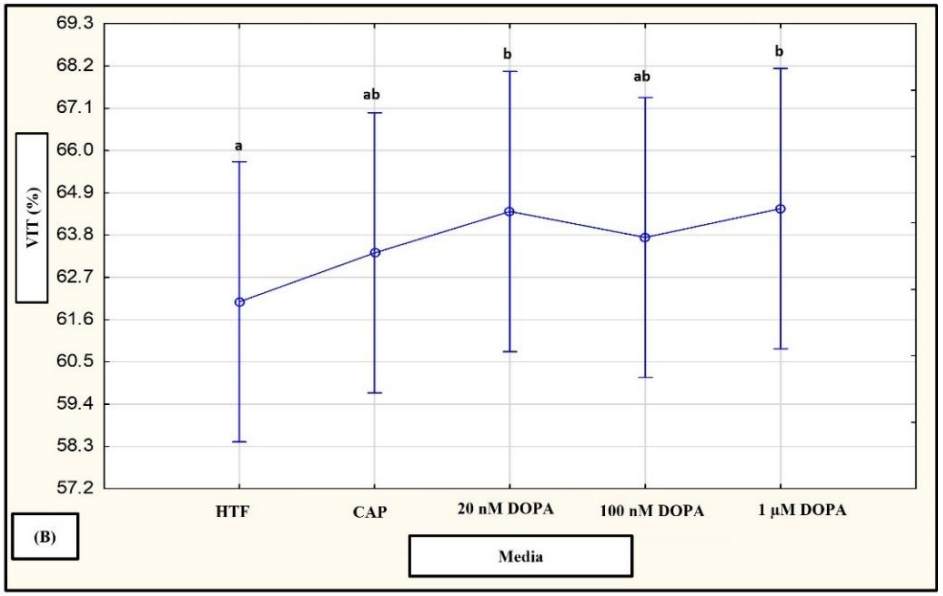


Figure 4.14 Multivariable chart comparing the mean percentage viable (%) spermatozoa of high motile (HM, blue dots) and low motile (LM, red dots) sperm subpopulations at different time points after treatment with various media. **(A)** Comparison of HM and LM subpopulations at 5- and 30-min incubation with HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM). **(B)** Comparison of HM and LM subpopulations at 5 and 30 min after incubation with HTF, CAP and DOPA (20 nM, 100 nM and 1 µM). **(C)** Comparison of HM and LM subpopulations at 5, 30 and 60 min after incubation with HTF, CAP and PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL). ***Note:*** 20 nM dopamine and CAP medium improved the LM subpopulation vitality percentages until significant differences between subpopulations could no longer be observed. Significant differences between subpopulations for individual media and time points are indicated as an asterisk (**p* <0.05, ***p* <0.01 and ****p* <0.001). Student's *t*-test or the Mann-Whitney test when normal distribution was void. **Abbreviations:** CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin and VIT, vitality.





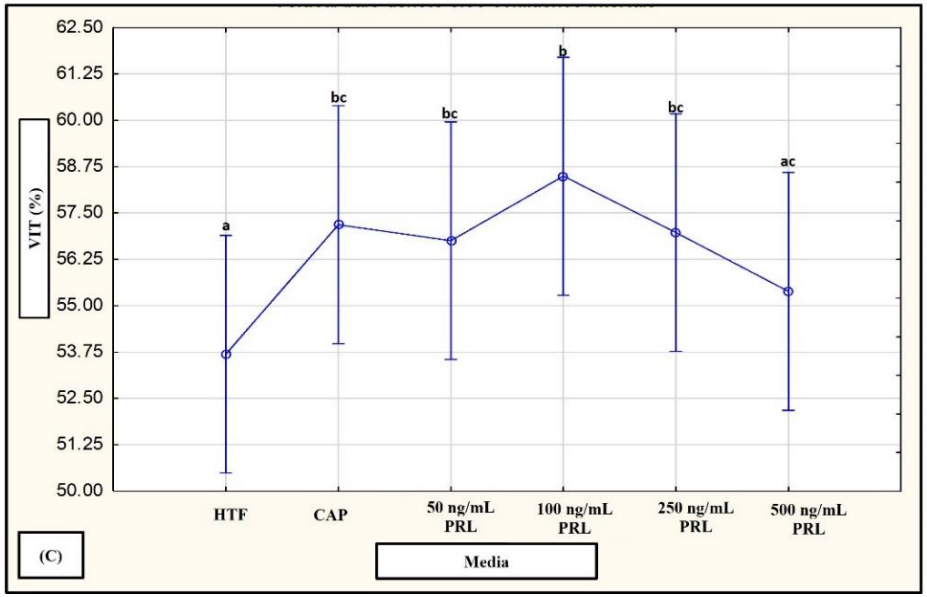


Figure 4. 15 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on percentage viable (%) spermatozoa. **(A)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on percentage viable (%) spermatozoa (F (6,540) = 3.29, p <0.01). **(B)** Effect of HTF, CAP and DOPA (20 nM, 100 nM and 1 µM) on percentage viable (%) spermatozoa (F (4,361) = 1.52, p = 0.20) **(C)** Effect of HTF, CAP and PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on percentage viable (%) spermatozoa (F (5,630) = 4.26, p <0.01). ***Note:*** Only HTF, CAP, HD-C, PRG, MYO and PRL had a significant interaction on vitality percentages as a fixed factor. HD-C, PRG, MYO, PRL (50-250 ng/mL) and DOPA (20 nM and 1 µM) significantly improved viable spermatozoa as compared to HTF. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HTF, human tubal fluid; MYO, myo-inositol; PRL, prolactin; PRG, progesterone and VIT, vitality.

4.2.4 | Motility Parameters

In Supplementary Tables 2 - 4 are the motility and kinematic parameters of the HM and LM subpopulations after exposure to various media for specific time intervals. Compared to the LM subpopulations, HM subpopulations had significantly higher percentages for various motility parameters at 5-, 30- and 60-minutes incubation in HTF medium (see Supplementary Tables 2 - 4). After incubation in media, motility parameters could be improved in the LM subpopulation until significant difference between subpopulations could no longer be observed (See Figure 4.9). This trend was noted for the various media, however, predominantly for slow and medium speed groups of CAP, HD-C and progesterone. In contrast, myo-inositol and higher concentrations of dopamine (100 nM and 1 µM) and prolactin (100 ng/mL and 500 ng/mL) appeared to improve all velocity and progressivity speed groups (Supplementary Table 2 - 4).

CAP, HD-C, progesterone and myo-inositol significantly increased (p <0.05) progressive motility, rapid and RP speed groups of LM subpopulations in comparison to HTF. However, for the HM subpopulation only 3.96 µM progesterone and myo-inositol significantly improved (p = 0.039) the RP speed group (Supplementary Table 2 & Figure 4.9). Likewise, dopamine (p <0.05 and p <0.01) could increase rapid, progressive and MP speeds in the LM subpopulations, however, varied effects were observed between dopamine concentrations. In contrast, dopamine decreased the HM subpopulation (p <0.05, p <0.01 and p <0.001) non-progressive motility, medium and slow speed groups, while subsequently increasing (p <0.05) rapid and RP speed groups (Supplementary Table 3 & Figure 4.9). Prolactin was observed to increase (p <0.05 and p <0.01) the progressive motility, total motility, medium and MP speeds of the LM subpopulations, however, different responses were observed between concentrations at different time points. In contrast to the LM subpopulation, only total motility of the HM subpopulation was significantly increased by prolactin (p = 0.024) (Supplementary Table 4 & Figure 4.9).

To determine the interaction of media as the main factor on motility parameters, data for subpopulations and time points were pooled and analysed in a mixed model ANOVA. Media had a significant (p <0.01) interaction on various motility parameters (See Figures 4.16 – 4.16). For individual effects of the media, CAP, HD-C, progesterone and myo-inositol significantly increased progressive motility (p <0.01) and rapid speed groups (p <0.01) while decreasing slow (p <0.01 and p <0.05) and medium speed groups (p <0.01 and p <0.05)

(Figure 4.16 & 4.17). However, HD-C and 3.96 µM progesterone had the greatest decrease on medium speeds (p <0.01 and p <0.05). Furthermore, CAP, progesterone (3.96 µM and 19.8 µM), and myo-inositol increased total motility (p <0.01 and p <0.05) and RP speed groups (p <0.01) (Figure 4.16 & 4.18), whereas CAP, HD-C and progesterone (3.96 µM and 19.8 µM) increased MP speed groups (p <0.01 and p <0.05). In addition, HD-C further increased percentages as compared to progesterone (1.98 µM and 19.8 µM) and myo-inositol (p <0.01 and p <0.05) (Figure 4.18).

Dopamine and CAP both increased the progressive motility and total motility as compared to HTF controls (p <0.01) (Figure 4.19). Furthermore, while decreasing (p <0.01) NP, slow and medium speeds, dopamine was observed to increase (p <0.01) rapid speeds, RP and MP speed groups (Figure 4.20 & 4.21). However, effects for the motility parameters varied amongst dopamine concentrations. In comparison to HTF, both CAP and prolactin increased (p <0.01) progressive motility, total motility, medium and rapid speeds, NP, MP and RP speed groups (Figure 4.22, 4.23 & 4.24). Despite increasing NP speed groups, prolactin contrastingly decreased (p <0.05) slow speeds. Furthermore, prolactin may display a dose dependent effect as higher concentrations displayed decreased values as compared to the lower concentrations of prolactin for various motility parameters.

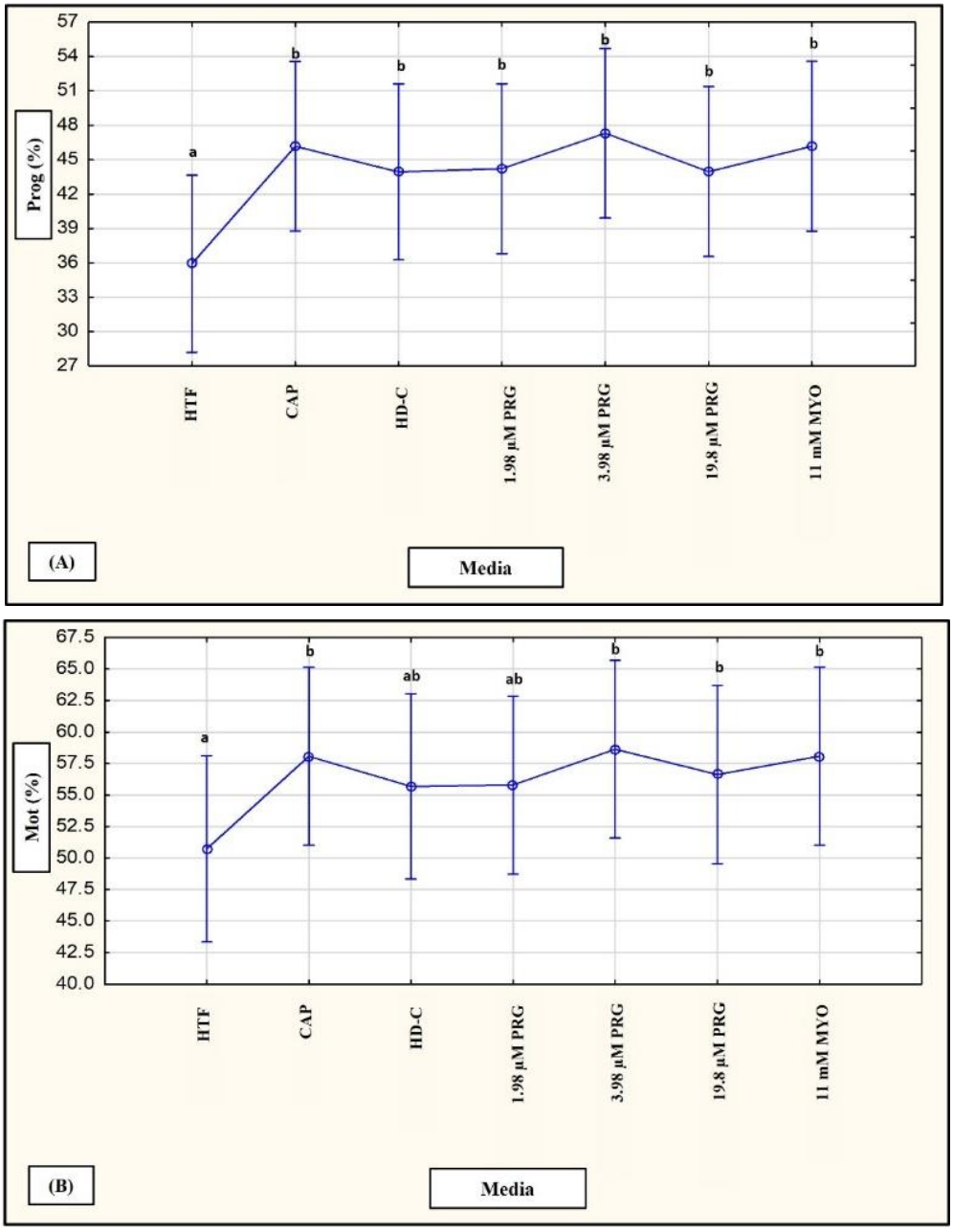
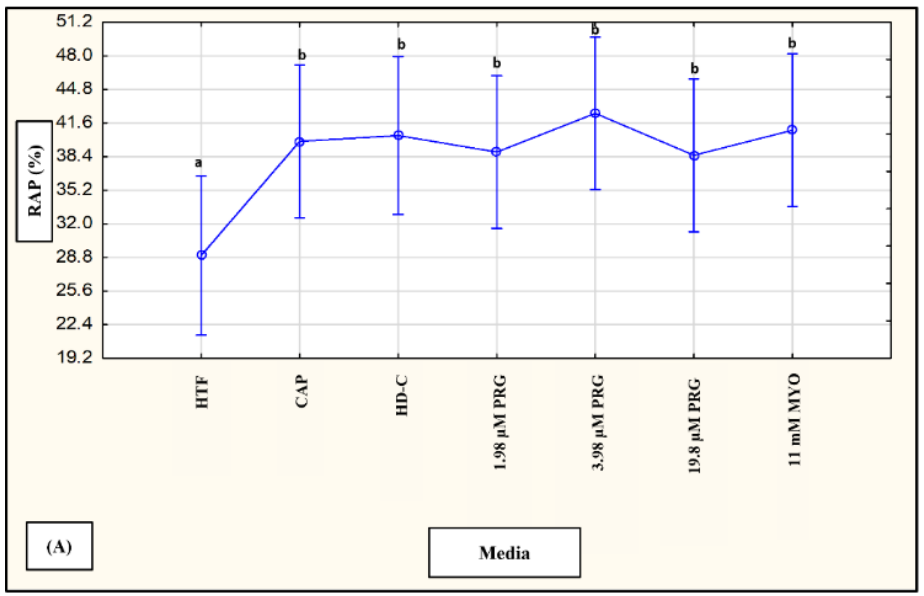
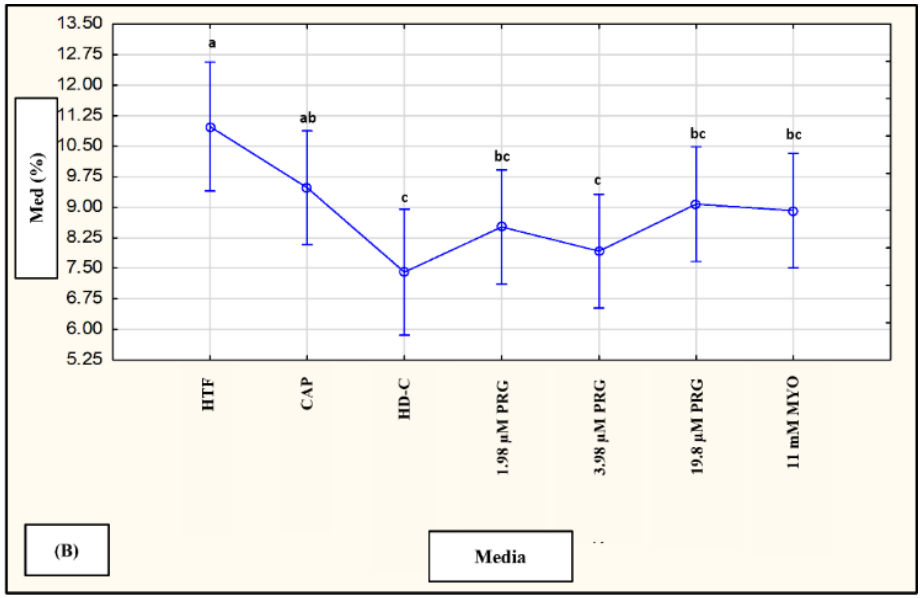


Figure 4.16 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on percentage progressive motility and total motility**. (A)** Effects of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on percentage progressivity (%) (F (6,527) = 3.54, p <0.01). **(B)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on total motility (%) (F (6,528) = 1.87, p = 0,08). ***Note:*** Media as a fixed factor only had a significant interaction of progressive motility. For individual effects media, CAP, HD-C, PRG and MYO all significantly improved progressive motility as compared to HTF. Only CAP, PRG and MYO increased total motility as compared to HTF. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; Mot, total motility; MYO, myo-inositol; PRG, progesterone; PROG, progressive.





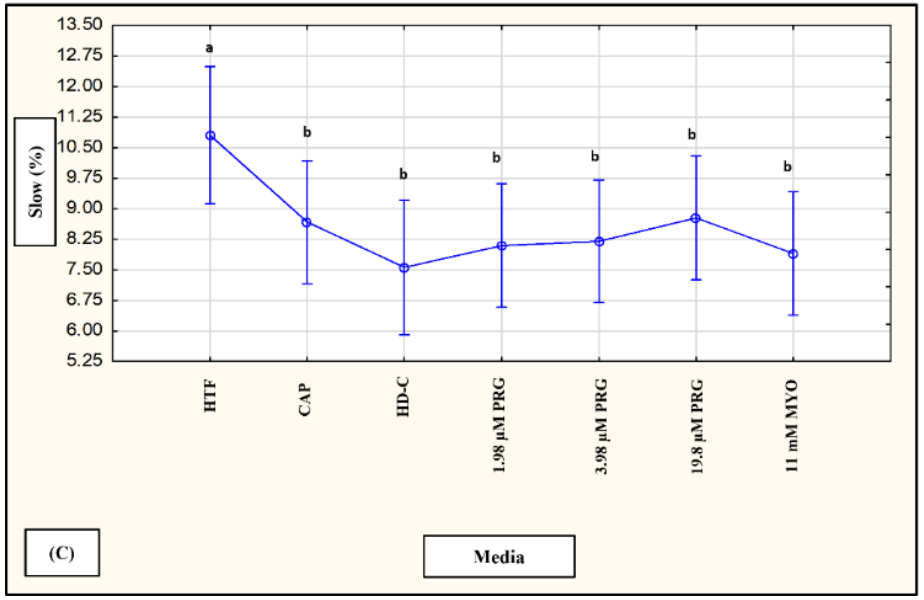
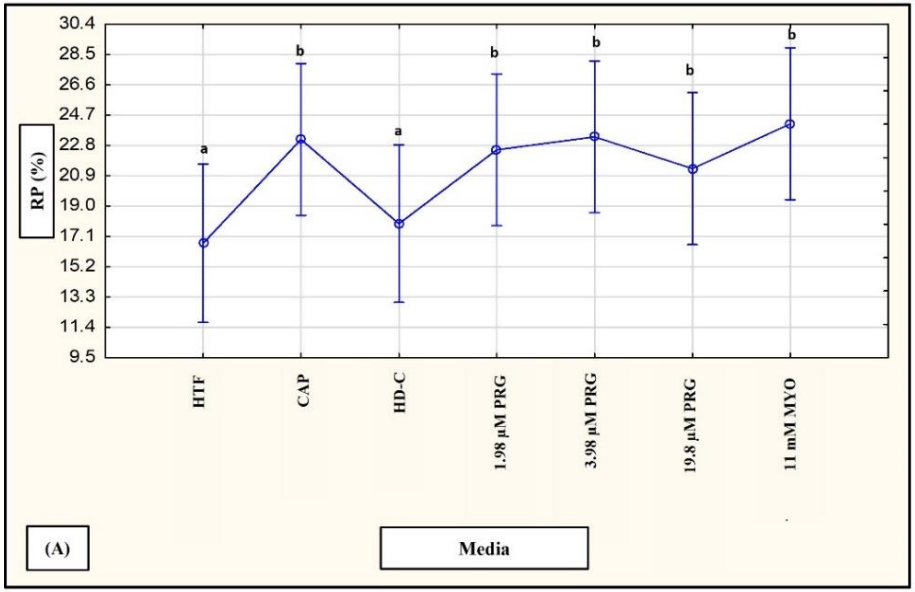
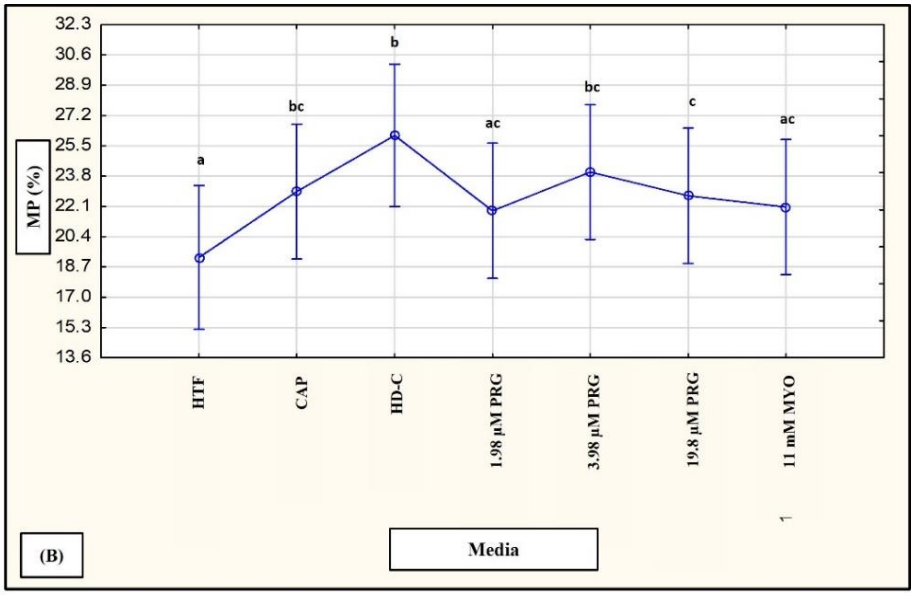


Figure 4.17 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on percentage rapid, medium, and slow speed group. **(A)** Effects of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on percentage rapid speed (%) (F (6,527) = 3.59, p <0.01). **(B)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on percentage medium speed (%) (F (6,529) = 3.35, p <0.01). **(C)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on percentage slow speed (%) (F (6,527) = 2.61, p = 0.02). ***Note:*** Media as a fixed factor had a significant interaction of rapid, medium, and slow speed groups. For individual effects media, CAP, HD-C, PRG and MYO all significantly improved rapid speed groups and decreased slow speed groups as compared to HTF. HD-C, PRG and MYO decreased medium speed groups as compared to HTF. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations**: CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; Med, medium; MYO, myo-inositol; PRG, progesterone; Rap, rapid.





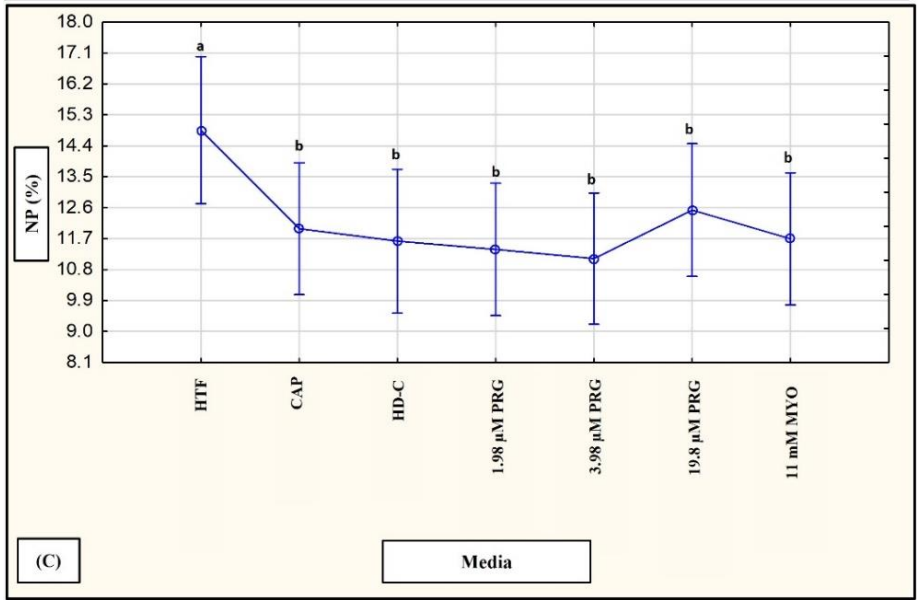


Figure 4.18 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on percentage RP, MP and NP speed group. **(A)** Effects of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on percentage RP speed (%) (F (6,527) = 5.40, p <0.01). **(B)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on percentage MP speed (%) (F (6,528) = 2.92, p <0.01). **(C)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on percentage NP speed (%) (F (6,528) = 2.37, p = 0.03). ***Note:*** Media as a fixed factor had a significant interaction of RP, MP and NP speed groups. For individual effects media, CAP, PRG and MYO all significantly improved RP speed groups, whereas all the media decreased NP speed groups as compared to HTF. CAP, HD-C and PRG increased MP speed groups as compared to HTF. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; MP, medium progressive; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; RP, rapid progressive.

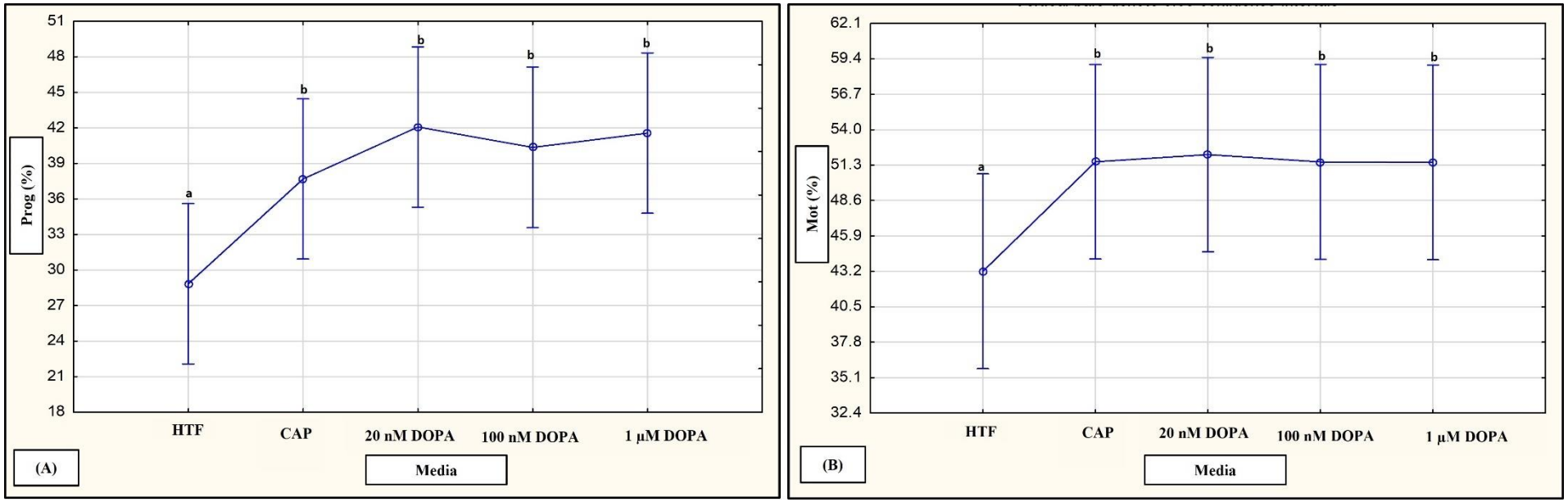
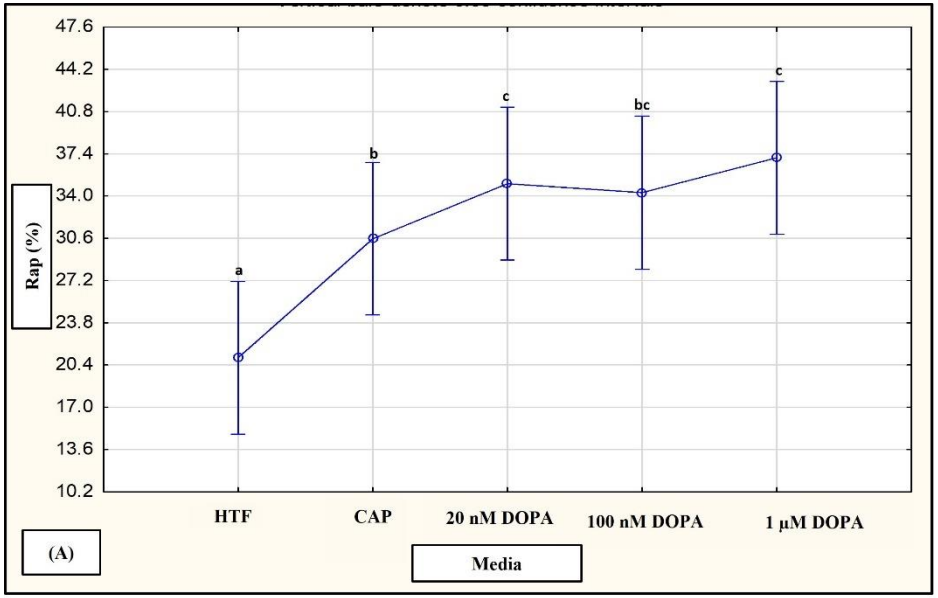
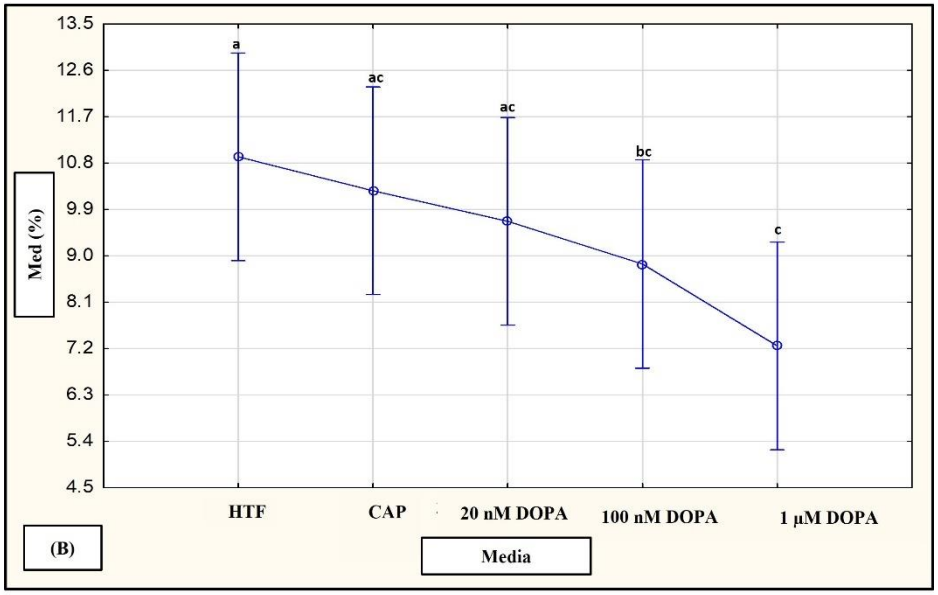


Figure 4.19 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on percentage progressive motility and total motility. **(A)** Effects of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on percentage progressivity (%) (F (4,452) = 11.41, p <0.01). **(B)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on total motility (%) (F (4,452) = 5.04, p <0.01). ***Note:*** Media as a fixed factor had a significant interaction on both progressive and total motility. For individual effects media, CAP and DOPA significantly improved progressive and total motility as compared to HTF. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** CAP, capacitating-HTF; DOPA, dopamine; HTF, human tubal fluid; Mot, total motility; PROG, progressive.





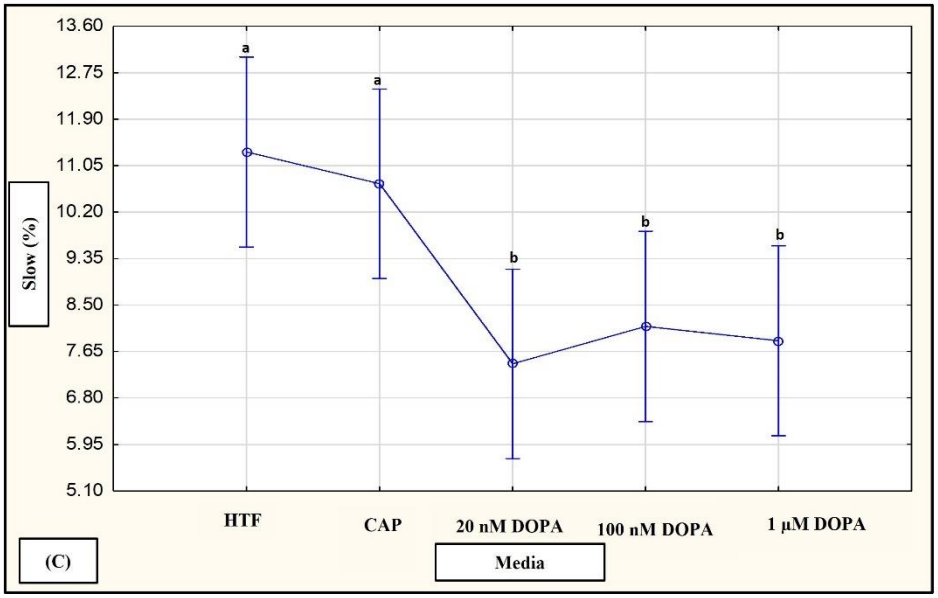


Figure 4.20 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on percentage rapid, medium, and slow speed group. **(A)** Effects of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on percentage rapid speed (%) (F (4,452) = 16.98, p <0.01). **(B)** Effects of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on percentage medium speed (%) (F (4,452) = 4.76, p <0.01). **(C)** Effects of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on percentage slow speed (%) (F (4,452) = 8.74, p <0.01). ***Note:*** Media as a fixed factor had a significant interaction of rapid, medium, and slow speed groups. For individual effects media, CAP and DOPA increased rapid speed groups, whereas DOPA decreased both medium and slow speed groups. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** CAP, capacitating-HTF; DOPA, dopamine; HTF, human tubal fluid; Med, medium; Rap, rapid.

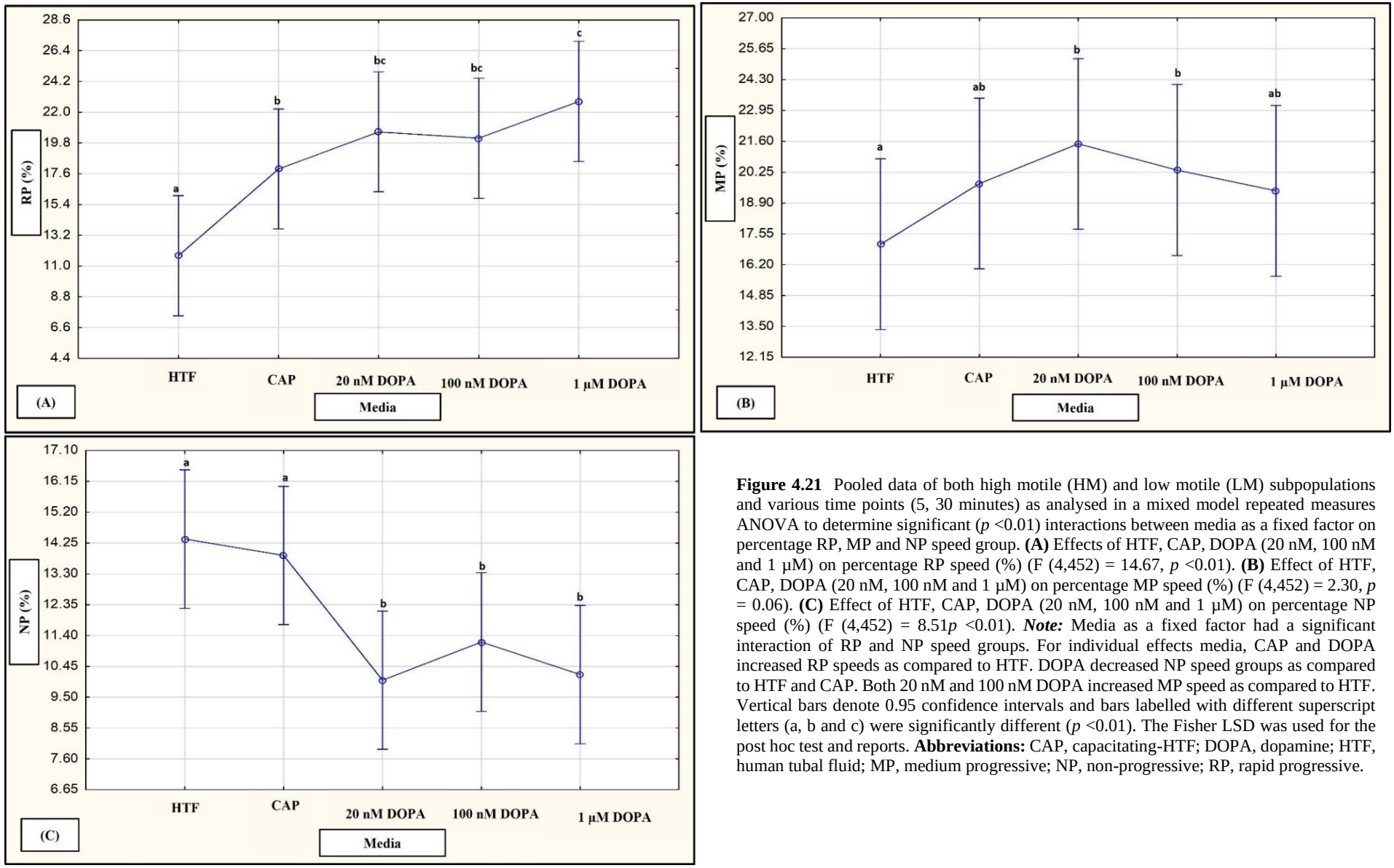


Figure 4.21 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on percentage RP, MP and NP speed group. **(A)** Effects of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on percentage RP speed (%) (F (4,452) = 14.67, p <0.01). **(B)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on percentage MP speed (%) (F (4,452) = 2.30, p = 0.06). **(C)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on percentage NP speed (%) (F (4,452) = 8.51p <0.01). ***Note:*** Media as a fixed factor had a significant interaction of RP and NP speed groups. For individual effects media, CAP and DOPA increased RP speeds as compared to HTF. DOPA decreased NP speed groups as compared to HTF and CAP. Both 20 nM and 100 nM DOPA increased MP speed as compared to HTF. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** CAP, capacitating-HTF; DOPA, dopamine; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; RP, rapid progressive.

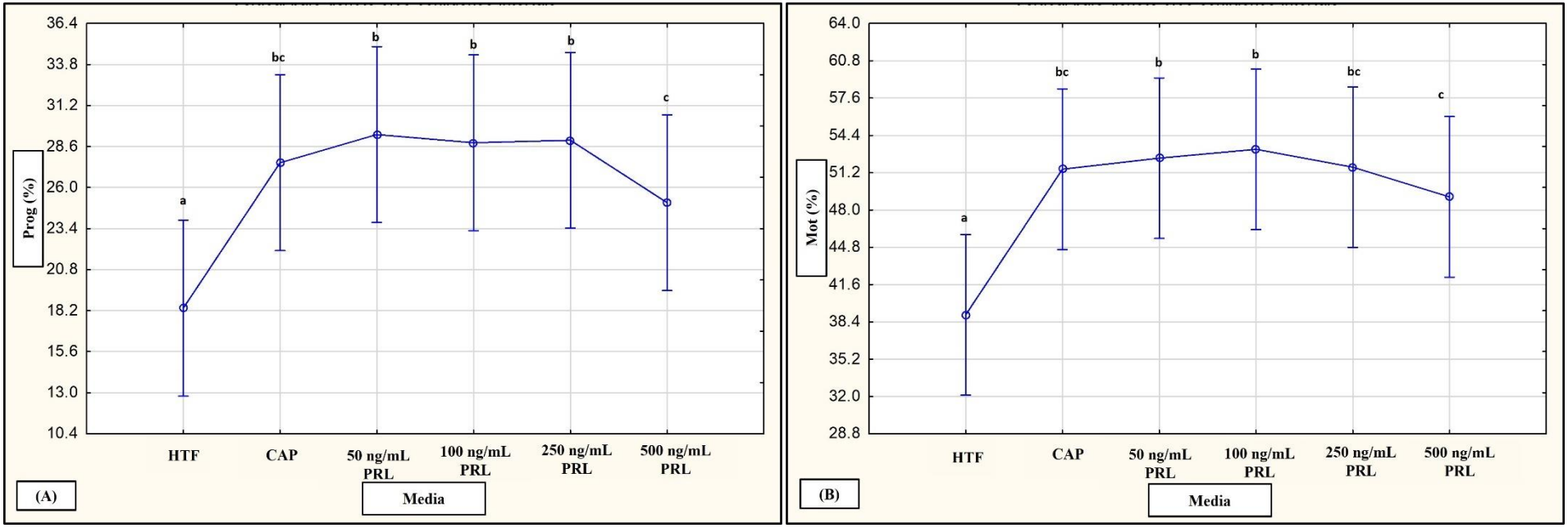


Figure 4.22 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30, 60 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on percentage progressive motility and total motility. **(A)** Effects of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on percentage progressivity (%) (F (5, 741) = 16.28, p <0.01). **(B)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on total motility (%) (F (5,741) = 21.51, p <0.01). ***Note:*** Media as a fixed factor had a significant interaction on both progressive and total motility. For individual effects media, CAP and PRL significantly improved progressive and total motility as compared to HTF. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports**. Abbreviations:** CAP, capacitating-HTF; HTF, human tubal fluid; Mot, total motility; PRL prolactin; PROG, progressive.

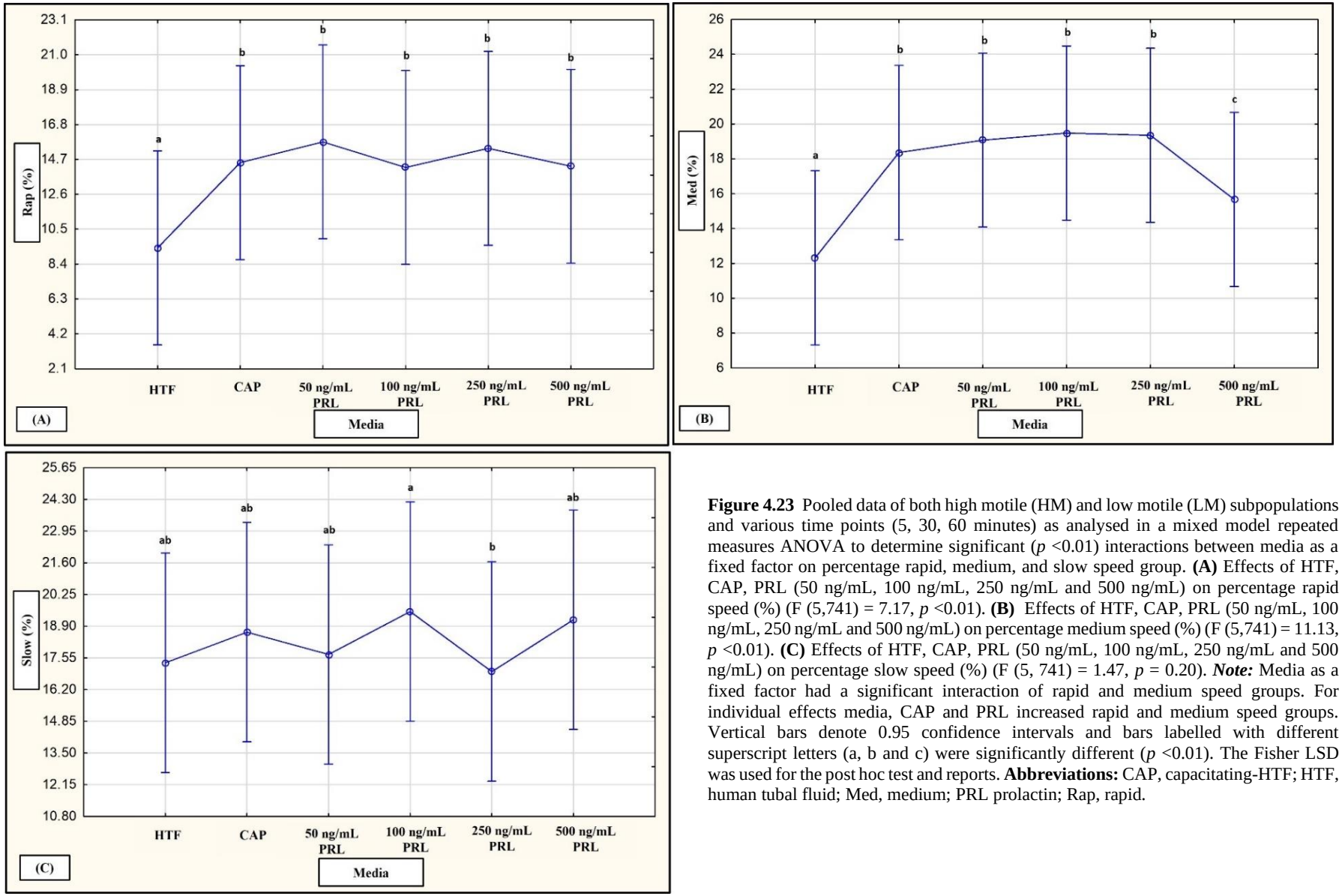
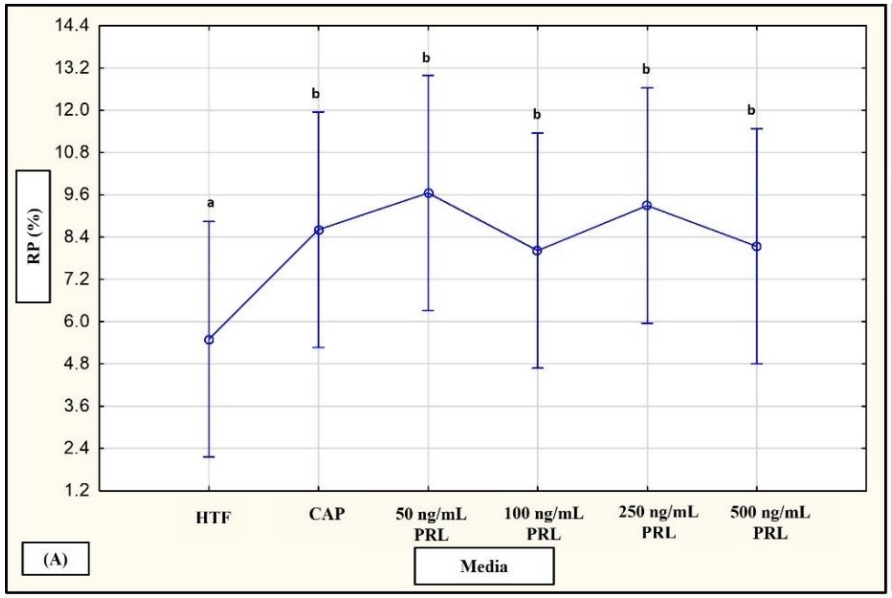
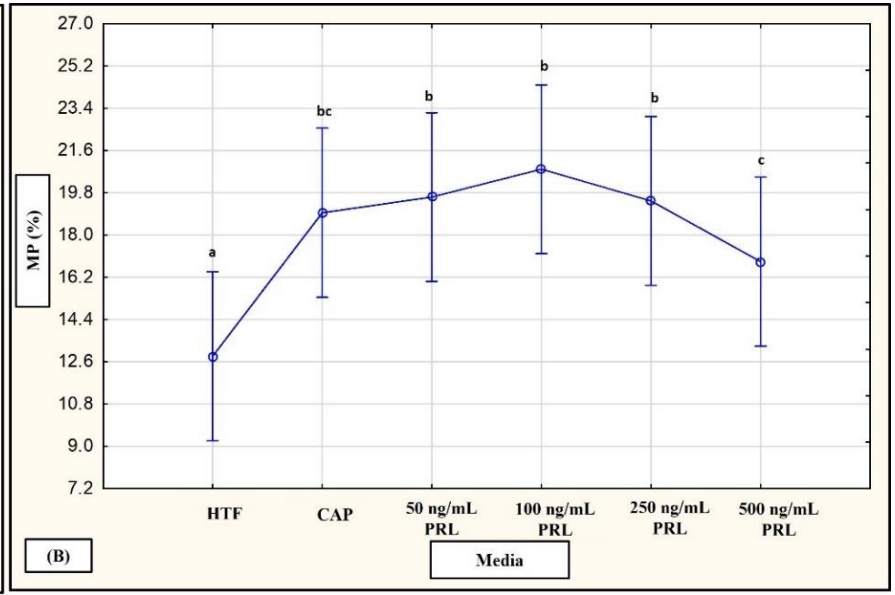


Figure 4.23 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30, 60 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on percentage rapid, medium, and slow speed group. **(A)** Effects of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on percentage rapid speed (%) (F (5,741) = 7.17, p <0.01). **(B)** Effects of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on percentage medium speed (%) (F (5,741) = 11.13, p <0.01). **(C)** Effects of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on percentage slow speed (%) (F (5, 741) = 1.47, p = 0.20). ***Note:*** Media as a fixed factor had a significant interaction of rapid and medium speed groups. For individual effects media, CAP and PRL increased rapid and medium speed groups. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** CAP, capacitating-HTF; HTF, human tubal fluid; Med, medium; PRL prolactin; Rap, rapid.





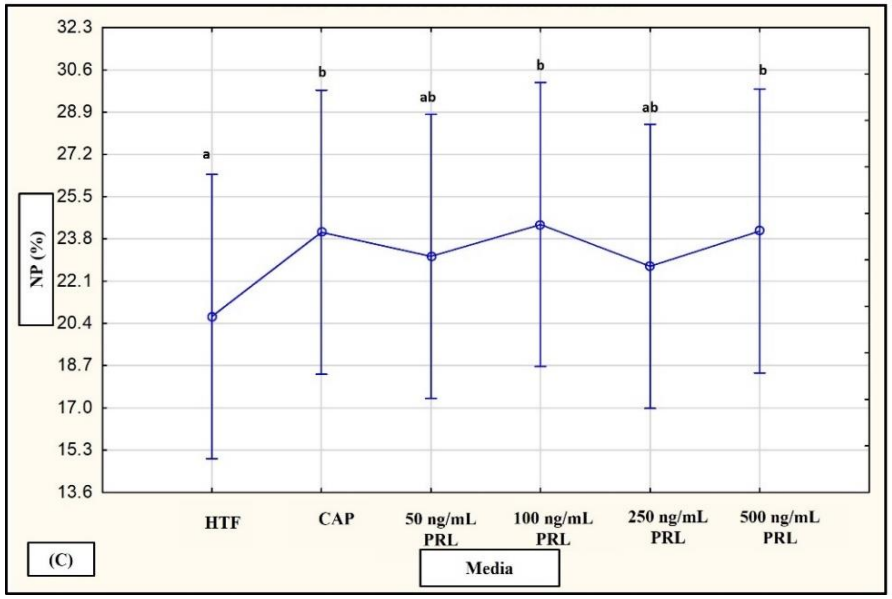


Figure 4.24 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30, 60 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on percentage RP, MP and NP speed group. **(A)** Effects of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on percentage RP speed (%) (F (5,741) = 6.16, p <0.01). **(B)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on percentage MP speed (%) (F (5,741) = 2.30, p <0.01). **(C)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on percentage NP speed (%) (F (5,741) = 2.1, p = 0.05). ***Note:*** Media as a fixed factor had a significant interaction of RP and MP speed groups. For individual effects media, CAP and PRL increased RP and MP speeds as compared to HTF. CAP, 100 ng/mL and 500 ng/mL PRL increased NP speeds as compared to HTF. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** CAP, capacitating-HTF; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; PRL, prolactin; RP, rapid progressive.

4.2.5 | Kinematic Parameters

From here on forward for illustrative and discussion purposes of kinematic parameters for both subpopulations – curvilinear velocity (VCL), average path velocity (VAP) and straight-line velocity (VSL) will be grouped as "velocity" kinematics; linearity (LIN) and straightness (STR) as "linear"; and amplitude of lateral had displacement (ALH), wobble (WOB) and beat cross frequency (BCF) as "vigour" kinematics.

4.2.5.1 | Velocity

HM subpopulations displayed significantly higher values for various velocity kinematics (Supplementary Table 2 - 4). However, media could increase LM subpopulation values until significant differences were no longer observed. All media, except progesterone increased the LM subpopulation motile velocity kinematics (Figure 4.10). Dopamine could increase both MP and NP velocity kinematics, whereas progesterone only increased the former (Figure 4.12 and 4.13). Interestingly, low prolactin concentrations (50 ng/mL) significantly increased HM subpopulation MP velocity kinematics as compared to LM subpopulations, whereas high concentrations (500 ng/mL) increased LM subpopulations as compared to HM subpopulations (Figure 4.12). Furthermore, progesterone, myo-inositol, dopamine and prolactin all increased HM subpopulation RP velocity kinematics resulting in significant differences between subpopulations (Figure 4.11).

CAP, HD-C, progesterone and myo-inositol all increased the average/motile (p = 0.018), and MP (p <0.01) velocity kinematic parameters of the HM subpopulations. However, only 3.96 µM and 19.8 µM progesterone increased (p = 0.041) the RP velocity kinematics as compared to HTF. In the LM subpopulation CAP, HD-C, progesterone and myo-inositol all increased average/motile (p <0.05) and MP (p <0.05 and p <0.01) velocity kinematics (Supplementary Table 2). Both CAP and dopamine increased (p <0.05 and p <0.001) the average/motile velocity kinematics of both subpopulations, however varying effects were observed between dopamine concentrations in the LM subpopulation. In addition, only 100 nM dopamine increased (p = 0.03) the NP velocity kinematics of LM subpopulations, whereas no effects were seen in the HM subpopulations. Dopamine further increased (p <0.05 and p <0.001) both subpopulations MP velocity kinematics, however different effects were observed between concentrations. That is, higher concentrations improved LM subpopulations whereas lower concentrations could improve the HM subpopulations. Furthermore, dopamine improved (p <0.05 and p <0.01) both subpopulations RP velocity

kinematics, however significantly increased values were only observed for higher concentrations in the HM subpopulation (Supplementary Table 3). Compared to HTF, both CAP and prolactin increased (p <0.05, p <0.01 and p <0.001) motile velocity kinematics of both subpopulations. However, varying concentrations of prolactin displayed an increase amongst different velocity kinematic parameters. Compared to HTF, lower concentrations of prolactin displayed an increase (p = 0.02) in MP velocity kinematics of the HM subpopulations, whereas in LM subpopulations higher concentrations resulted in an increase (p = 0.03) in values (Supplementary Table 4).

To determine the effect of media as the main factor on velocity kinematic parameters, data was pooled and analysed in a mixed model ANOVA. Media had a significant interaction on various velocity kinematic parameters (See Figure 4.25 - 4.27). For individual effects of the media - CAP, HD-C, progesterone and myo-inositol increased (p <0.01 and p <0.05) VCL of the various speed groups as compared to HTF. However, HD-C and 3.96 µM progesterone had a greater stimulatory effect on motile VCL (p <0.01 and p <0.05) and MP VCL (p <0.01), whereas 3.96 µM progesterone had a greater stimulatory effect (p <0.01) on RP VCL. In contrast, both HD-C and myo-inositol increased NP VCL (p <0.05) as compared to HTF (Figure 4.25). All dopamine concentrations significantly (p <0.01) increased motile, and MP VCL as compared to HTF and CAP. However, varying effects were observed amongst dopamine concentrations for MP VCL. Furthermore, only higher dopamine concentrations (100 nM and 1 µM) increased (p <0.01 and p <0.05) VCL of the RP and NP speed groups (Figure 4.26). Both CAP and prolactin increased (p <0.01 and p <0.05) the VCL of the various progressive speed groups as compared to HTF. However, varying effects were seen between the prolactin concentrations, thereby displaying a possible dose dependent effect (Figure 2.27).

Compared to HTF – CAP, HD-C, progesterone, and myo-inositol all increased motile VAP (p <0.01) and MP VAP (p <0.01 and p <0.05). In contrast, only CAP, 3.96 µM progesterone and myo-inositol increased (p <0.01 and p <0.05) RP VAP as compared to HD-C (Figure 4.28). Dopamine and CAP increased (p <0.01 and p <0.05) VAP values of the various progressive speed groups as compared to HTF. However, dopamine additionally increased (p <0.01) motile VAP as compared to CAP. Likewise, higher dopamine concentrations (1 µM) increased (p <0.01 and p <0.05) RP VAP and MP VAP as compared to HTF and CAP - but varying effects were observed between concentrations for the RP speed group (Figure 4.29). Prolactin and CAP increased (p <0.01) motile VAP as compared to HTF,

however prolactin concentrations varied in their effects. Only 100 ng/mL increased (p <0.05) RP VAP as compared to HTF and CAP - whereas all prolactin concentrations increased (p <0.01 and p <0.05) MP VAP and varied effects between concentrations were observed for NP VAP (Figure 4.30).

CAP, 3.96 µM progesterone and myo-inositol increased (p <0.01 and <0.05) motile VSL as compared to HTF and HD-C. Interestingly, HD-C decreased (p <0.01) RP VSL, MP VSL and NP VSL (Figure 4.31). Dopamine increased the various speed group VSL values as compared to HTF, however the different concentrations displayed varying effects between the VSL of the different speed groups. Motile VSL could be increased (p <0.01 and p <0.05) by both the lowest and highest dopamine concentrations, but RP VSL was increased (p <0.01 and p <0.05) by the higher concentrations. In contrast, MP VSL was increased (p <0.05) by the lower concentrations (Figure 4.32). CAP and prolactin increased (p <0.01 and p <0.05) motile VSL as compared to HTF, however effects between concentrations varied. Only 100 ng/mL increased (p <0.05) the RP VSL as compared to HTF, whereas MP VSL values varied between concentrations. Furthermore, both 50 ng/mL and 250 ng/mL prolactin increased (p <0.01 and p <0.05) NP VSL as compared to HTF, 100 ng/mL and 500 ng/mL prolactin (Figure 4.33).

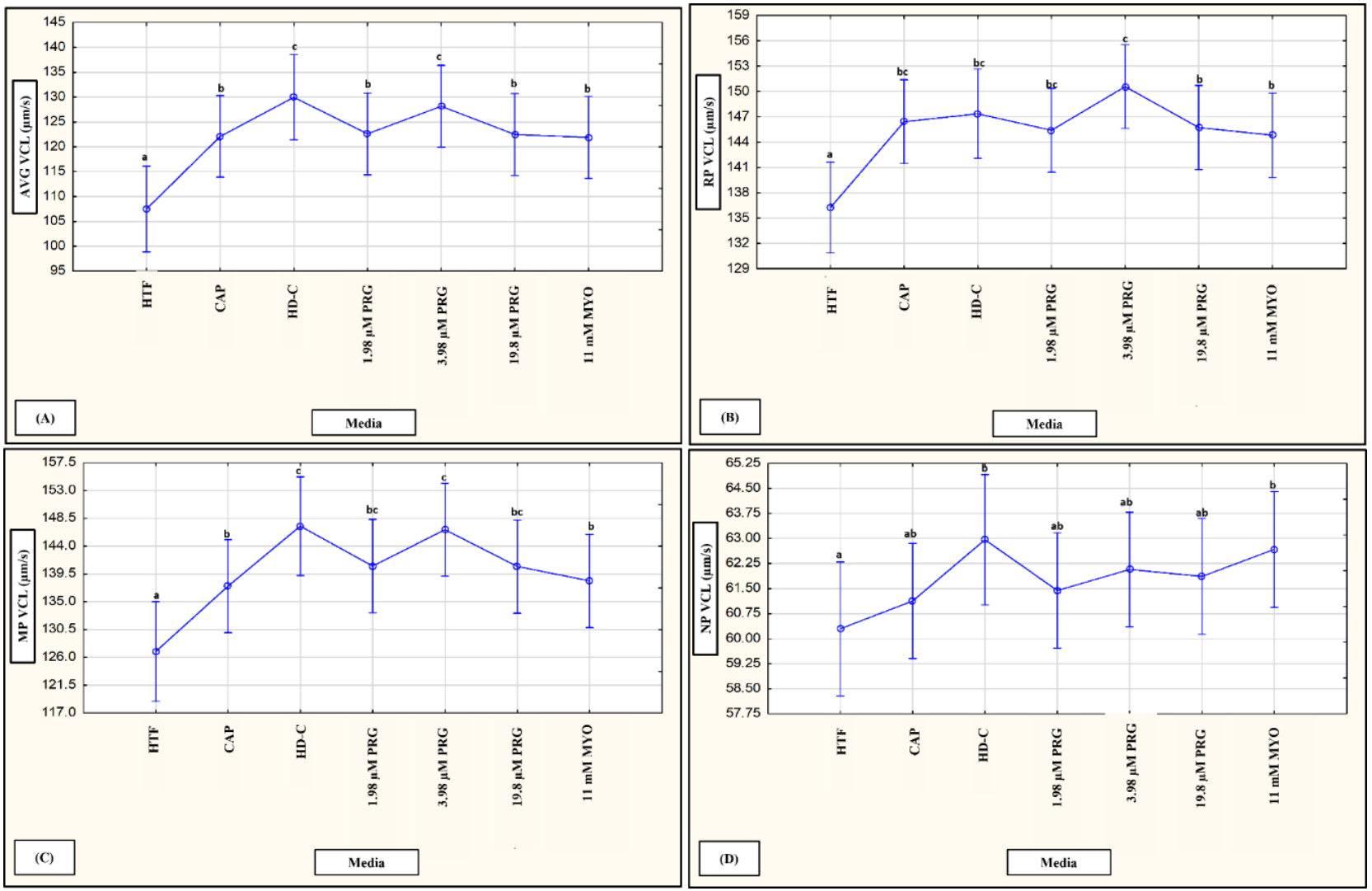


Figure 4.25 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on VCL of various progressive speed groups. **(A)** Effects of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on motile VCL (F (6,526) = 9.80, p <0.01). **(B)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on rapid progressive (RP) speed groups VCL (F (6,520) = 6.16, p <0.01). **(C)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on medium progressive (MP) speed groups VCL (F (6,524) = 7.15, p <0.01). **(D)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on non-progressive (NP) speed group VCL (F (6,529) = 1.13, p = 0.34). ***Note:*** Media as a fixed factor had a significant effect on motile, RP and MP speed group VCL. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports**. Abbreviations:** AVG, average; CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; MP, medium progressive; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; RP, rapid progressive; VCL, curvilinear velocity.

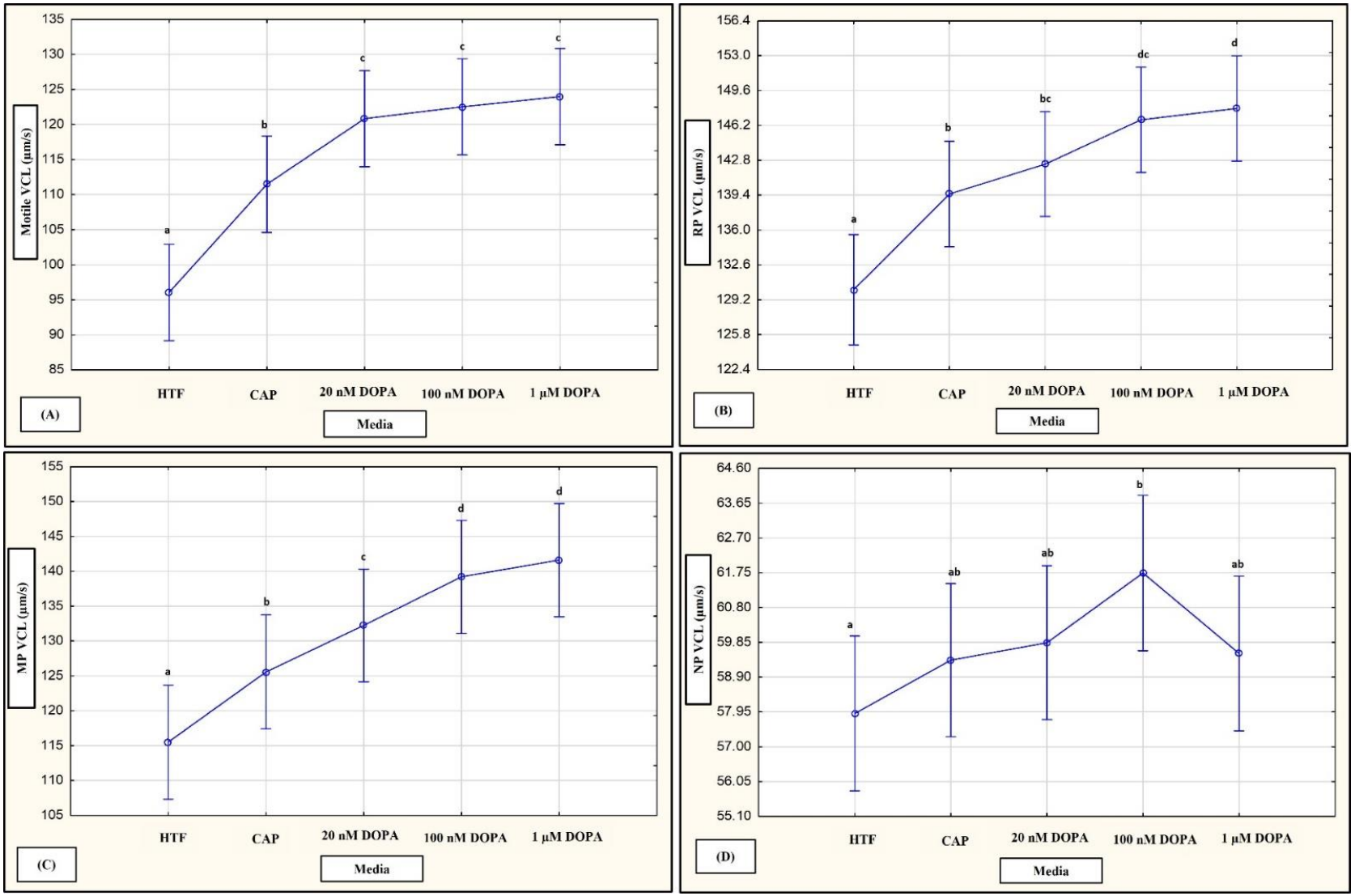


Figure 4.26 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on VCL of various progressive speed groups. **(A)** Effects of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on motile VCL (F (4,451) = 39.70, p <0.01). **(B)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on rapid progressive (RP) speed groups VCL (F (4,421) = 15.24, p <0.01). **(C)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on medium progressive (MP) speed groups VCL (F (4,438) = 23.82, p <0.01). **(D)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on non-progressive (NP) speed group VCL (F (4,438) = 2.37, p = 0.05). ***Note:*** Media as a fixed factor had a significant effect on motile, RP and MP speed group VCL. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; DOPA, dopamine; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; RP, rapid progressive; VCL, curvilinear velocity.

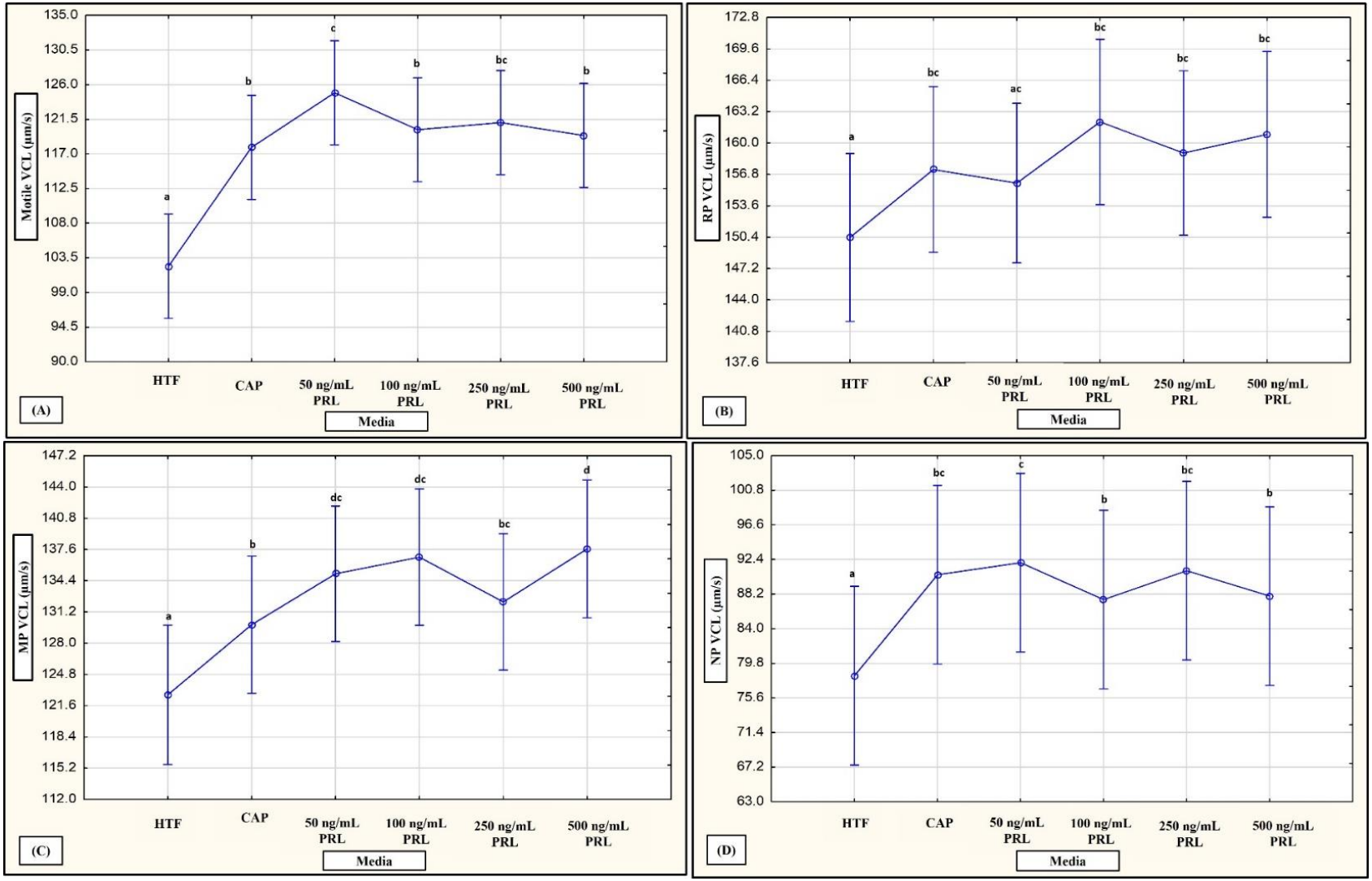


Figure 4.27 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 and 60 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on VCL of various progressive speed groups. (A) Effects of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on motile VCL (F (5,739) = 24.71, p <0.01). **(B)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on rapid progressive (RP) speed groups VCL (F (5,425) = 3.11, p <0.01). (C) Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on medium progressive (MP) speed groups VCL (F (5,661) = 7.90, p <0.01). **(D)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on non-progressive (NP) speed group VCL (F (5,730) = 12.61, p <0.01). ***Note:*** Media as a fixed factor had a significant effect on motile, RP, MP and NP speed group VCL. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; PRL, prolactin; RP, rapid progressive; VCL, curvilinear velocity.

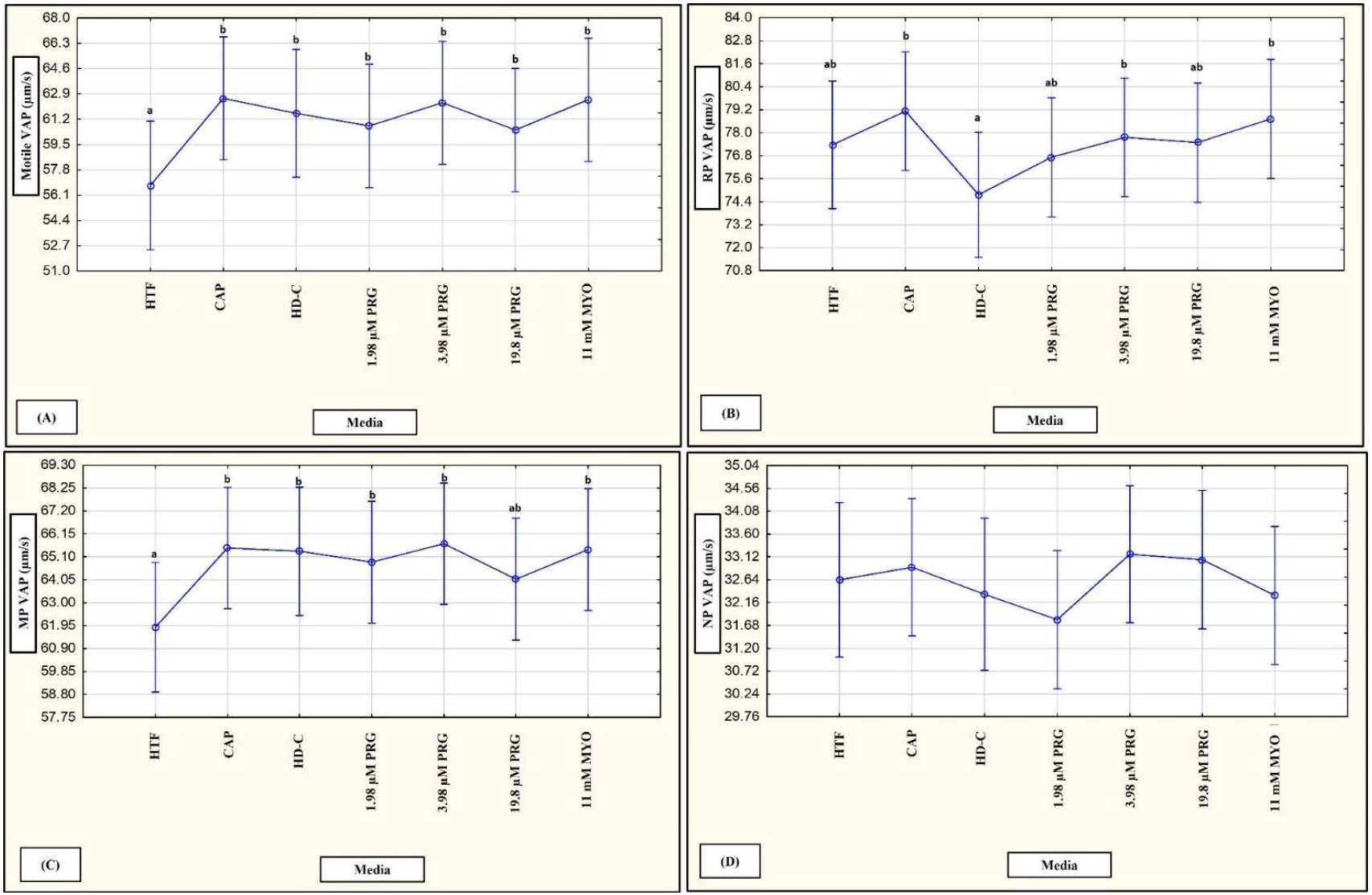


Figure 4.28 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on VAP of various progressive speed groups. **(A)** Effects of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on motile VAP (F (6,526) = 3.46, p <0.01). **(B)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on rapid progressive (RP) speed groups VAP (F (6,520) = 4.18, p = 0.04). **(C)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on medium progressive (MP) speed groups VAP (F (6,525) = 2.19, p = 0.04). (**D)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on non-progressive (NP) speed group VAP (F (6,529) = 0.76, p = 0.61). *Note***:** Media as a fixed factor had a significant effect on motile, RP and MP speed group VAP. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; MP, medium progressive; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; PROG, progressive; RP, rapid progressive; VAP, average path velocity.

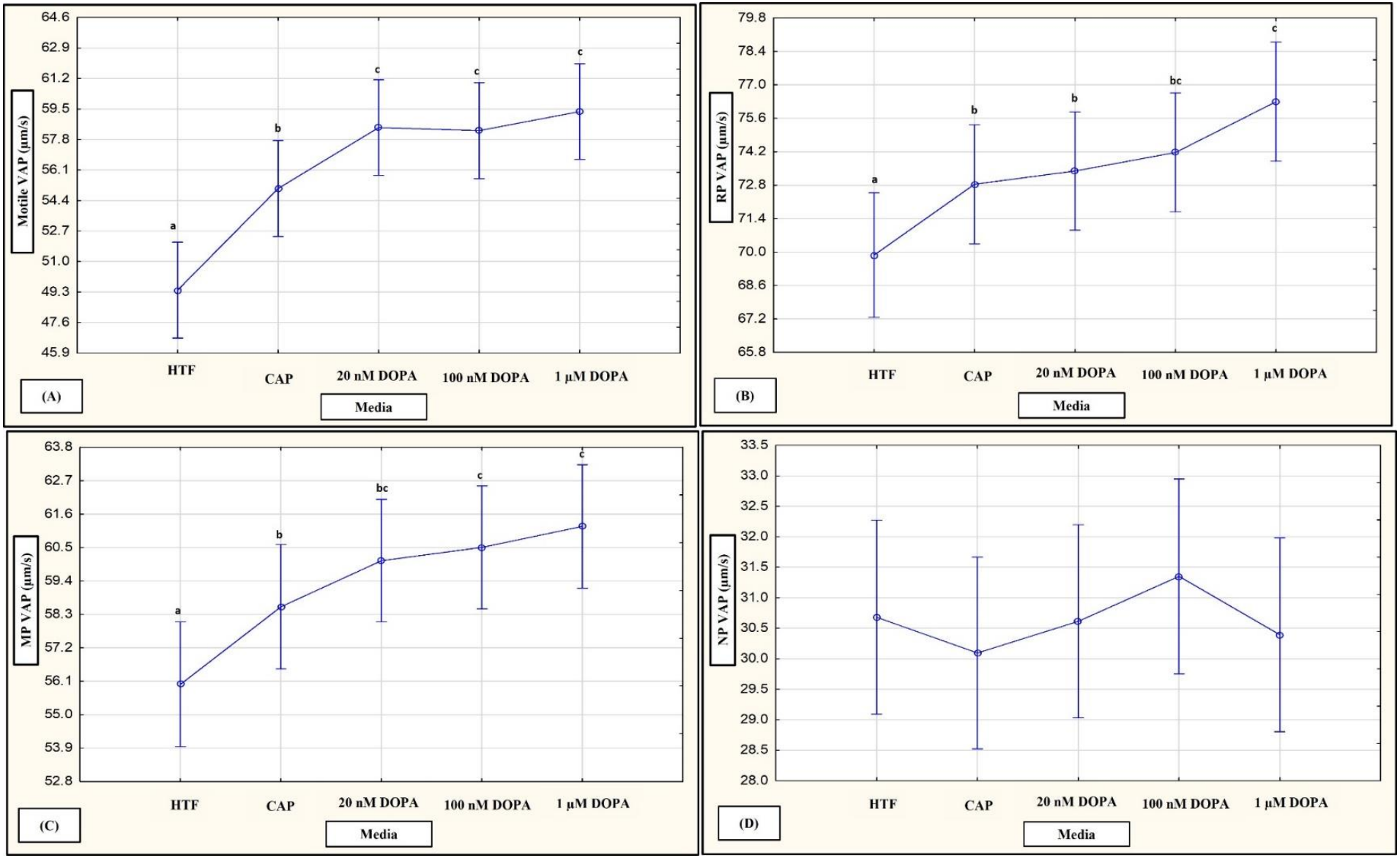


Figure 4.29 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on VAP of various progressive speed groups. **(A)** Effects of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on motile VAP ($F(4,451) = 23.07$, p <0.01). **(B)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on rapid progressive (RP) speed groups VAP ($F(4,421) = 7.22$, p <0.01). **(C)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on medium progressive (MP) speed groups VAP ($F(4,438) = 9.08$, p <0.01). **(D)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on non-progressive (NP) speed group VAP ($F(4,439) = 0.49$, $p = 0.74$**).** ***Note:*** Media as a fixed factor had a significant effect on motile, RP and MP speed group VAP. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; DOPA, dopamine; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; RP, rapid progressive; VAP, average path velocity.

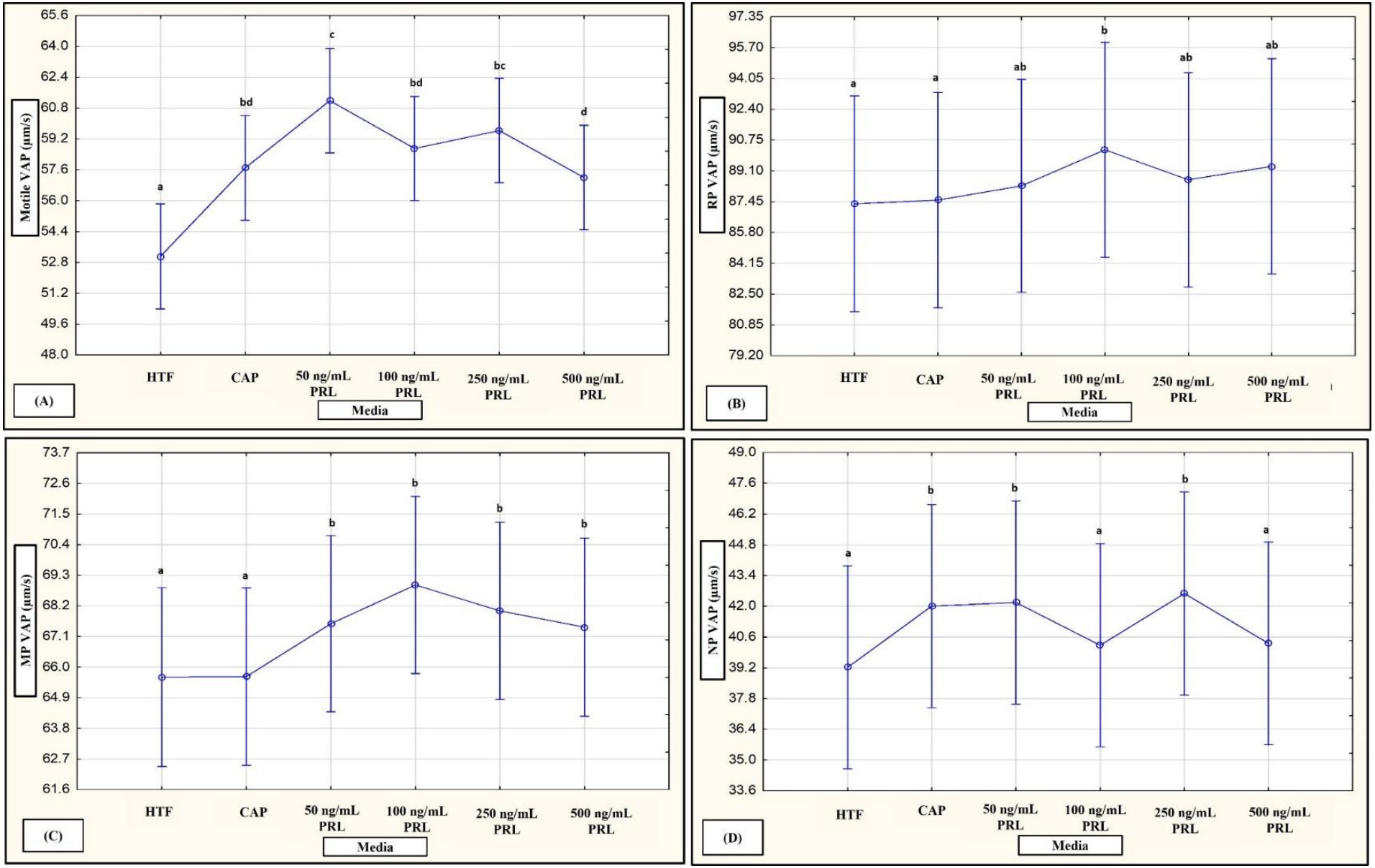


Figure 4.30 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 and 60 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on VAP of various progressive speed groups. **(A)** Effects of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on motile VAP (F (5,739) = 13.93, p <0.01). **(B)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on rapid progressive (RP) speed groups VAP (F (5,425) = 1.58, p = 0.16). **(C)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on medium progressive (MP) speed groups VAP (F (5,661) = 4.45, p <0.01). **(D)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on non-progressive (NP) speed group VAP (F (5,730) = 7.54, p <0.01). ***Note:*** Media as a fixed factor had a significant effect on motile, RP, MP and NP speed group VAP. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; PRL, prolactin; RP, rapid progressive; VAP, average path velocity.

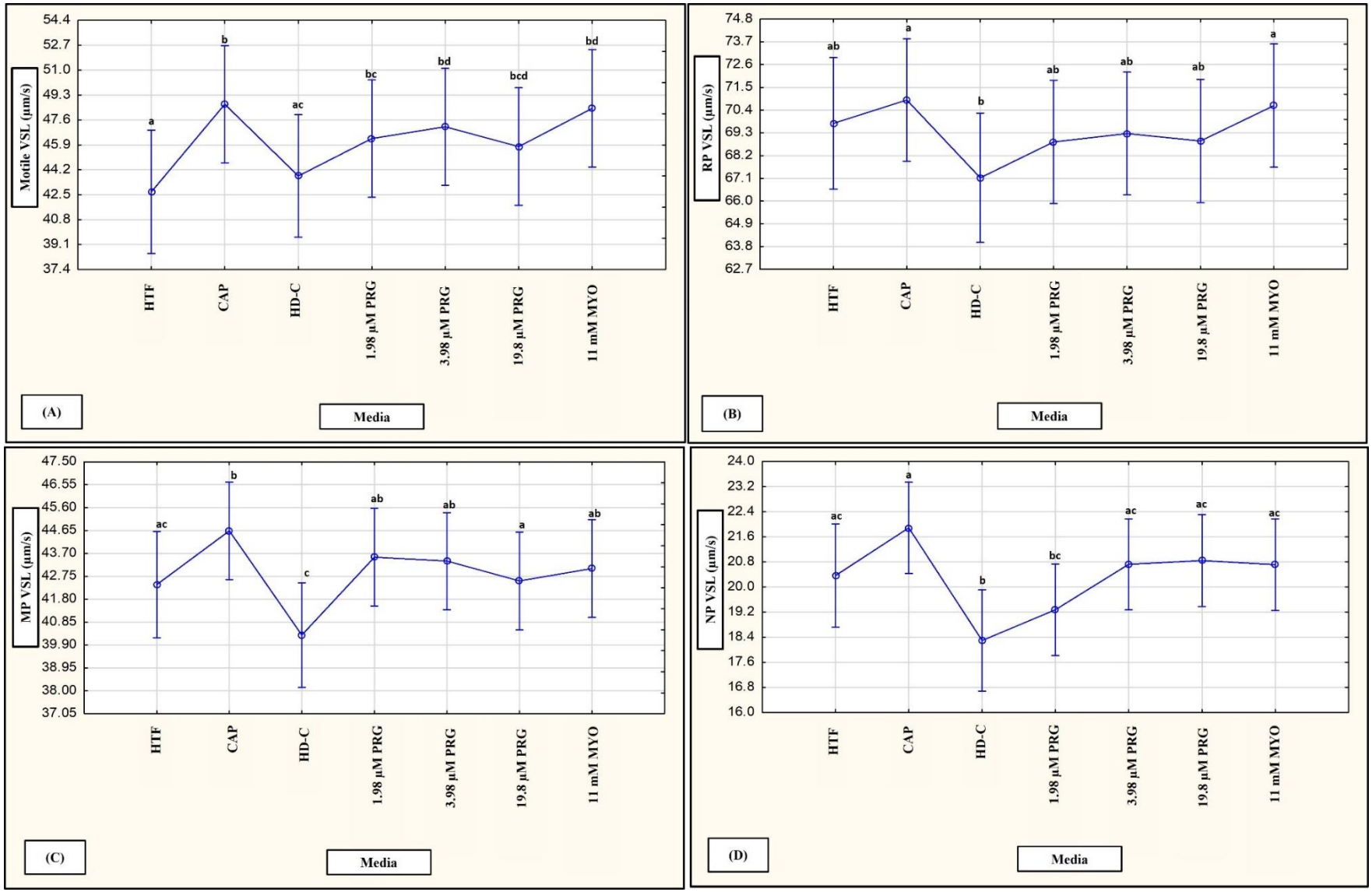


Figure 4.31 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on VAP of various progressive speed groups. **(A)** Effects of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on motile VSL (F (6,528) = 3.98, p <0.01). **(B)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on rapid progressive (RP) speed groups VSL (F (6,520) = 1.98, p = 0.07). **(C)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on medium progressive (MP) speed groups VSL (F (6,527) = 3.33, p <0.01). **(D)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on non-progressive (NP) speed group VSL (F (6,529) = 3.55, p <0.01). ***Note*:** Media as a fixed factor had a significant effect on motile, MP and NP speed group VSL. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; MP, medium progressive; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; PROG, progressive; RP, rapid progressive; VSL, straight-line velocity.

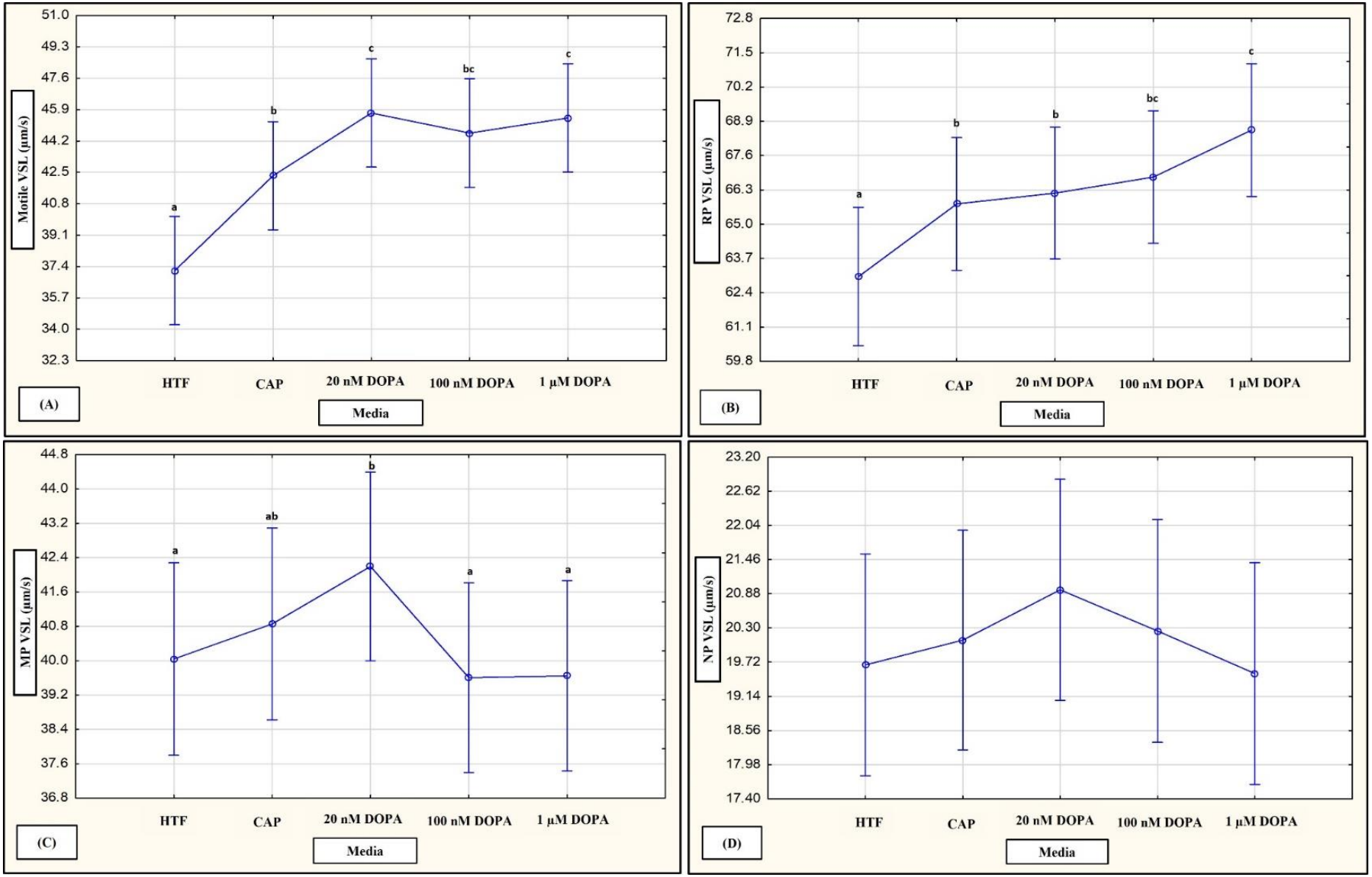


Figure 4.32 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on VSL of various progressive speed groups. **(A)** Effects of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on motile VSL (F (4,451) = 14.89, p <0.01). **(B)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on rapid progressive (RP) speed groups VSL (F (4,421) = 5.71, p <0.01). **(C)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on medium progressive (MP) speed groups VSL (F (4,438) = 1.97, p = 0.10). **(D)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on non-progressive (NP) speed group VSL (F (4,439) = 0.60, p = 0.67). ***Note:*** Media as a fixed factor had a significant effect on motile and RP VSL. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; DOPA, dopamine; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; RP, rapid progressive; VSL, straight-line velocity.

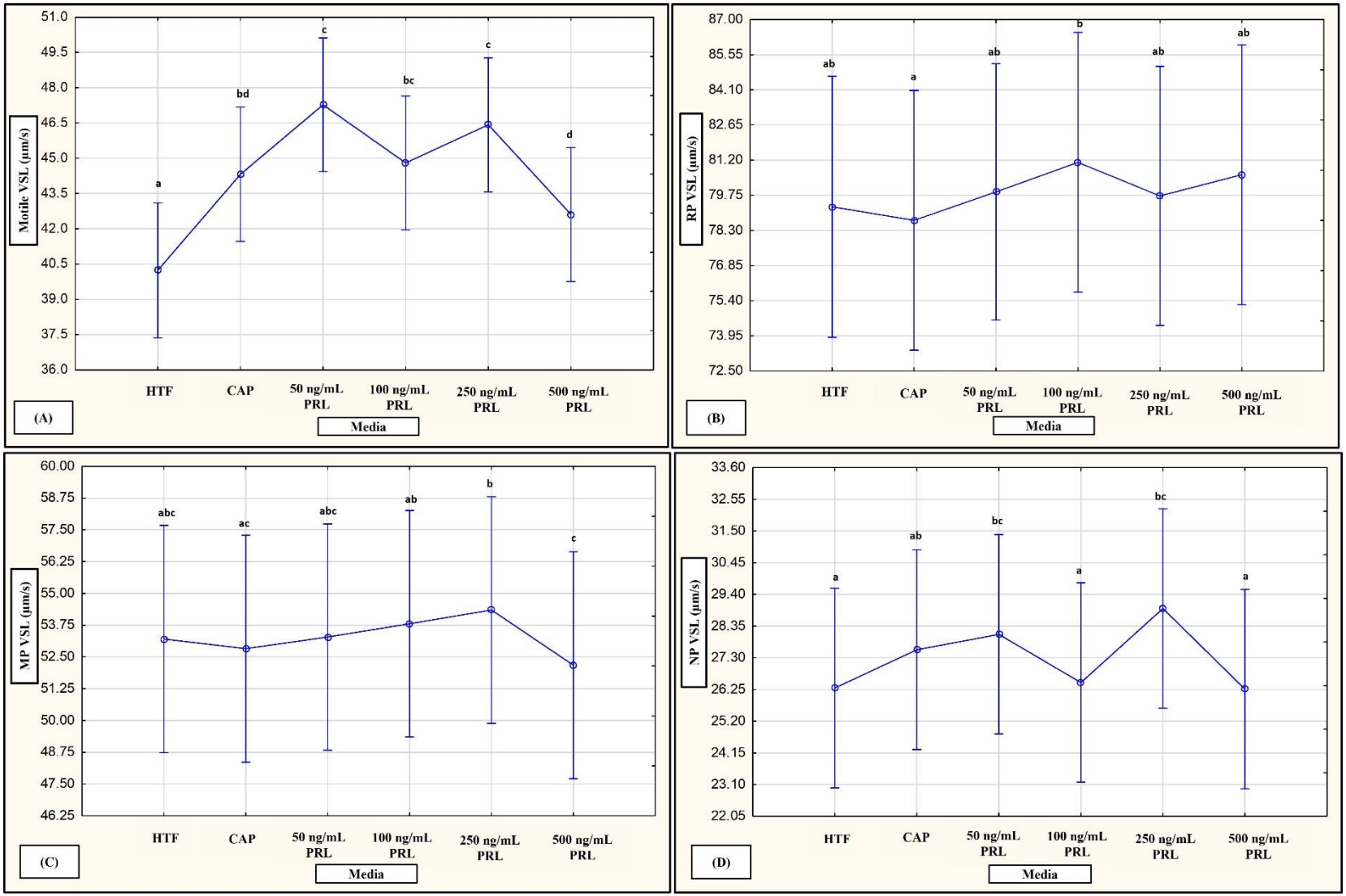


Figure 4.33 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 and 60 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on VSL of various progressive speed groups. **(A)** Effects of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on motile VSL (F (5,739) = 11.28, p <0.01). **(B)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on rapid progressive (RP) speed groups VSL (F (5,425) = 0.97, p = 0.43). **(C)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on medium progressive (MP) speed groups VSL (F (5,661) = 1.92, p = 0.09). **(D)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on non-progressive (NP) speed group VSL (F (5,730) = 5.18, p <0.01). ***Note:*** Media as a fixed factor had a significant effect on motile and NP speed group VSL. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; PRL, prolactin; RP, rapid progressive; VSL, straight-line velocity.

4.2.5.2 | Linear

As seen in Supplementary Table 2 - 4, HTF of HM subpopulations displayed significantly higher values for various linear kinematic parameters. After incubation in CAP, HD-C, 1.98 µM progesterone, myo-inositol and dopamine (20 nM and 1 µM) – LM subpopulation linear kinematics were increased until differences between subpopulations were no longer observed (Figure 4.10). This was further noted in CAP and prolactin improving MP and RP linear kinematics (Figure 4.11 & 4.12). In contrast, NP linear kinematics were increased by 100 nM dopamine and 50 ng/mL prolactin until percentages were significantly higher than the HM subpopulations (Figure 4.13). Moreover, 3.96 µM progesterone significantly increased HM subpopulation RP linear kinematics as compared to LM subpopulations (Figure 4.11), whereas 20 nM dopamine also significantly increased LM subpopulation MP linear kinematics as compared to HM subpopulations (Figure 4.12)

HD-C displayed a continuous decrease (p <0.05, p <0.01 and p <0.001) in linear kinematics of both subpopulations as compared to other media. HD-C additionally decreased (p = 0.023) the HM subpopulation NP linear kinematics as compared to other media; however, MP linear kinematics were decreased (p <0.05 and p <0.01) by both HD-C and 1.98 µM progesterone, but HD-C still had the greatest decrease (p <0.01) in both HM and LM subpopulations MP speed groups. Likewise, HD-C and progesterone decreased (p <0.001) HM subpopulation RP linear kinematics, however HD-C again had the greatest decrease (p <0.05 and p <0.01) in both subpopulations as compared to other media (Supplementary Table 2). Lower dopamine concentrations decreased (p <0.05) motile linear kinematics of the HM subpopulations. Whereas all dopamine concentrations decreased (p <0.001) HM subpopulation MP and RP linear kinematics, but higher concentrations decreased (p = 0.046) the LM subpopulation (Supplementary Table 3). Prolactin had no effect on individual subpopulation linear kinematics as compared to other media (Supplementary Table 4).

To determine the effect of media as the main factor on linear kinematic parameters, data was pooled and analysed in a mixed model ANOVA. Media had a significant (p <0.01) interaction on various linear kinematic parameters of the different progressive speed groups. Compared to all the media, HD-C significantly (p <0.05 and p <0.01) decreased the different progressive speed group STR values. Progesterone additionally decreased RP STR, MP STR and NP STR, however effects varied amongst the concentrations. Moreover, HD-C still displayed the greatest decrease in values (Figure 4.34). Motile STR values were increased (p

<0.05 and p <0.01) by the lower concentrations of dopamine, however values varied between the concentrations. Contrastingly, the higher dopamine concentrations decreased both RP (p = 0.04) and MP STR (p <0.01) values whereas NP STR was increased (p <0.05) by 20 nM dopamine (Figure 4.35). Both CAP and prolactin increased (p <0.01) motile STR, but a dose dependent effect was observed for prolactin as values varied between the concentrations. Likewise, prolactin NP, MP and RP STR values were also different between concentrations with concentrations varying amongst one another in effects (Figure 4.36).

HD-C and progesterone decreased (p <0.05 and p <0.01) motile LIN, however progesterone had variations between concentrations and HD-C displayed the greatest decrease as compared to all media. Despite CAP, HD-C, progesterone and myo-inositol decreasing (p <0.01) RP LIN as compared to HTF - both HD-C and 3.96 µM progesterone had the greatest impact (p <0.05 and p <0.01). Furthermore HD-C, progesterone and myo-inositol also decreased (p <0.05 and p <0.01) MP LIN and NP LIN, but HD-C again had the greatest decrease in LIN values (Figure 4.37). Dopamine decreased (p <0.05 and p <0.01) RP LIN and MP LIN, with higher concentrations displaying a greater decrease in LIN as compared to other media (Figure 4.38). Higher prolactin concentrations (p <0.01) decreased motile LIN values, but fluctuating results were noted between the concentrations. Similar observations of varying values between concentrations were noted for the NP, RP and MP LIN values, however prolactin still decreased LIN (Figure 4.39).

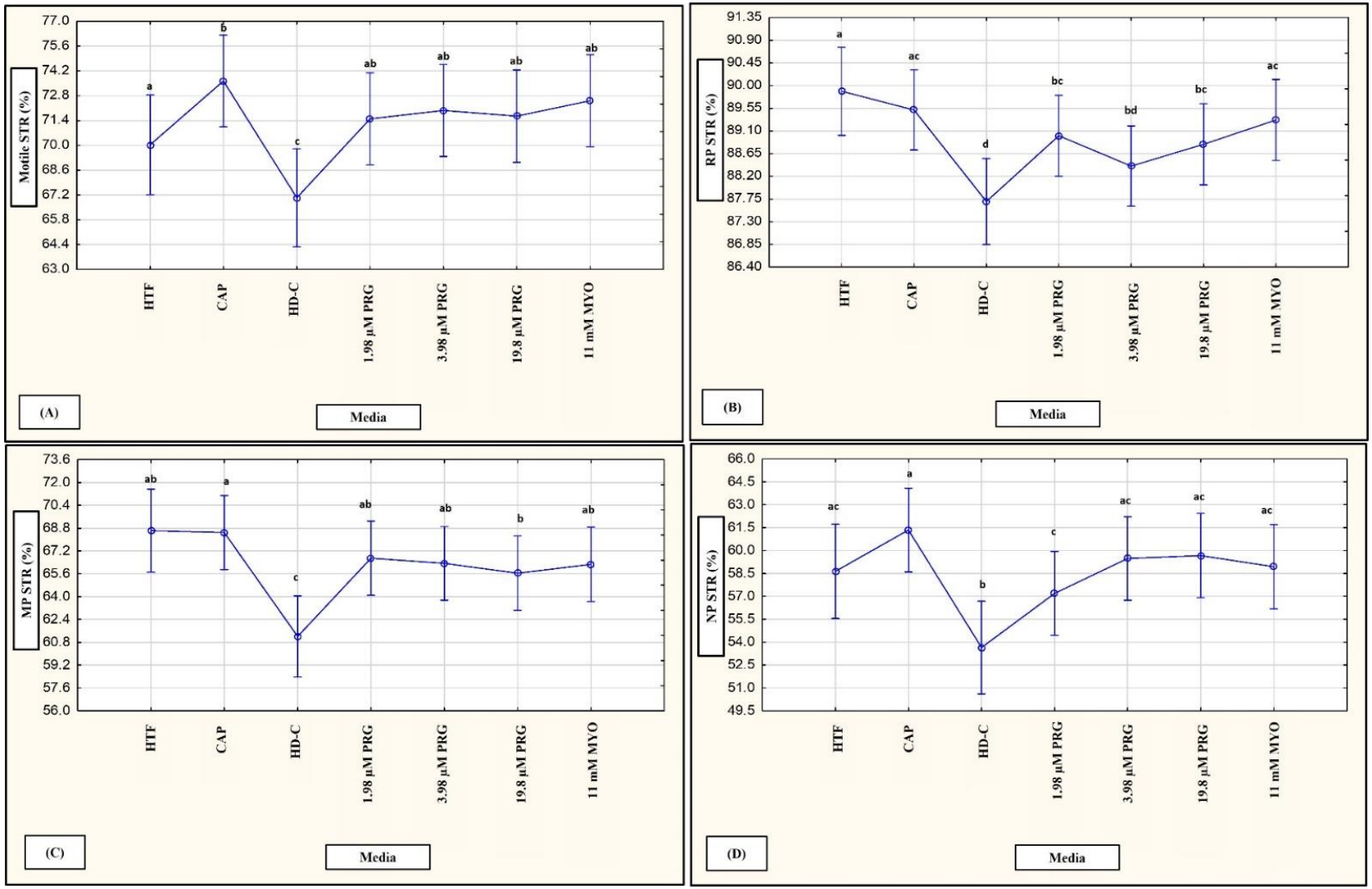


Figure 4.34 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on STR of various progressive speed groups. **(A)** Effects of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on motile STR (F (6,528) = 5.39, p <0.01). **(B)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on rapid progressive (RP) speed groups STR (F (6,520) = 6.49, p <0.01). **(C)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on medium progressive (MP) speed groups STR (F (6,525) = 4.83, p <0.01). **(D)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on non-progressive (NP) speed group STR (F (6,528) = 4.36, p <0.01). ***Note*:** Media as a fixed factor had a significant effect on motile, RP, MP, and NP speed group STR. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; MP, medium progressive; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; PROG, progressive; RP, rapid progressive; STR, straightness.

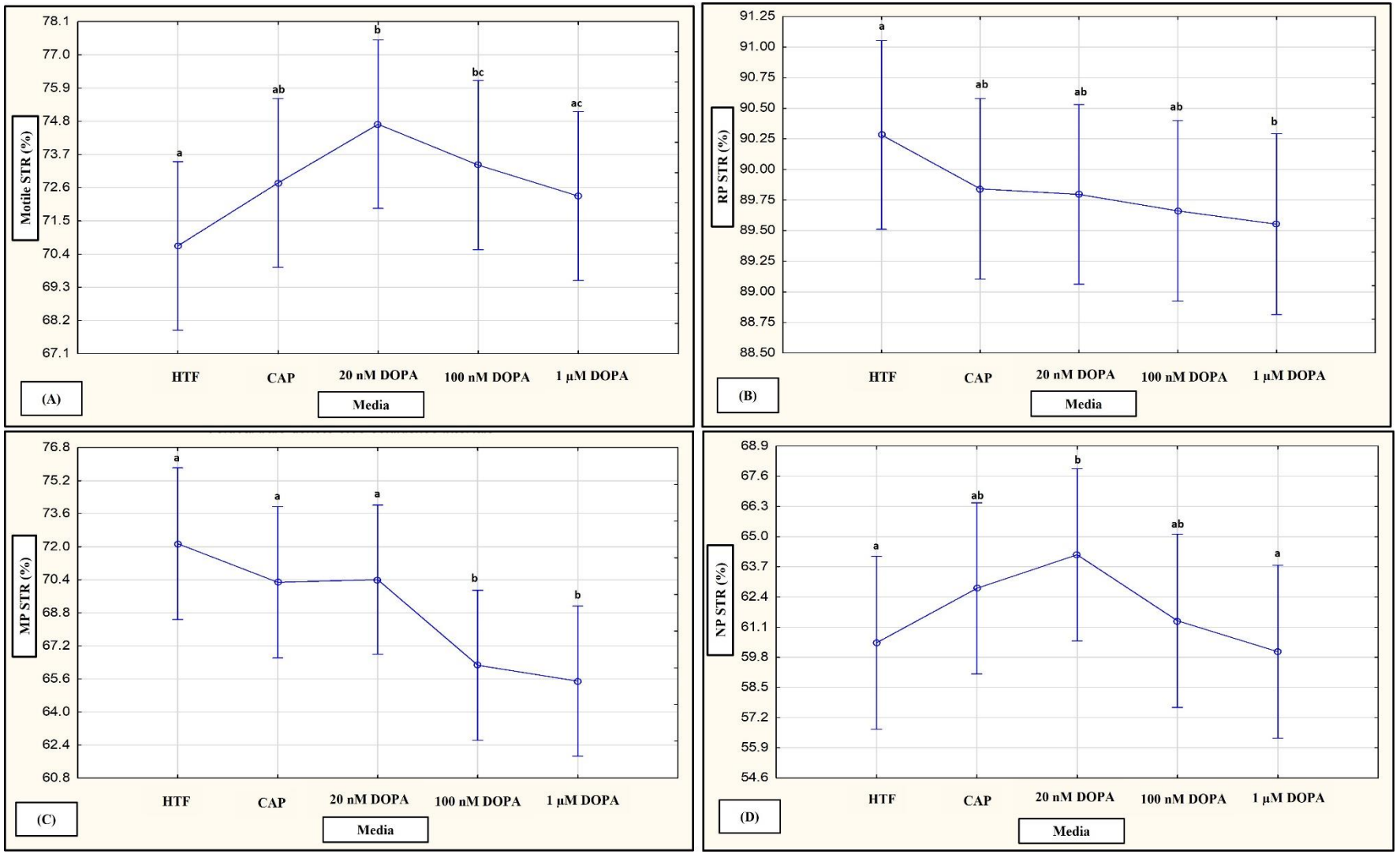


Figure 4.35 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on STR of various progressive speed groups. **(A)** Effects of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on motile STR (F (4,451) = 2.82, p = 0.02). **(B)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on rapid progressive (RP) speed groups STR (F (4,421) = 1.22, p = 0.30). **(C)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on medium progressive (MP) speed groups STR (F (4,438) = 6.51, p <0.01). **(D)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on non-progressive (NP) speed group STR (F (4,439) = 1.51, p = 0.18). ***Note:*** Media as a fixed factor had a significant effect on motile and MP STR. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; DOPA, dopamine; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; RP, rapid progressive; STR, straightness.

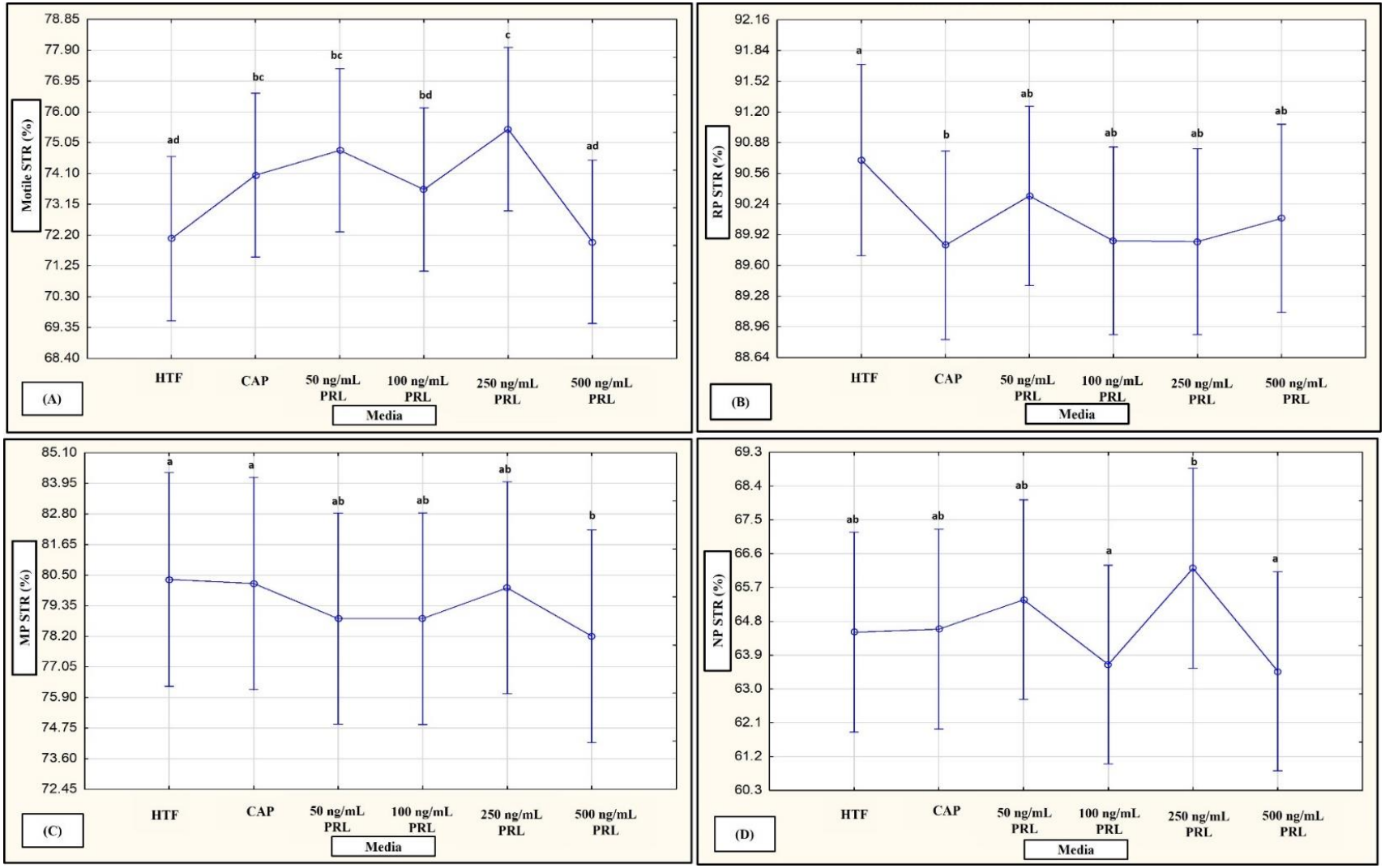


Figure 4.36 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 and 60 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on STR of various progressive speed groups. **(A)** Effects of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on motile STR (F (5,739) = 4.91, p <0.01). **(B)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on rapid progressive (RP) speed groups STR (F (5,425) = 1.17, p = 0.32). **(C)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on medium progressive (MP) speed groups STR (F (5,661) = 1.53, p = 0.18). **(D)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on non-progressive (NP) speed group STR (F (5,730) = 1.49, p = 0.19). ***Note:*** Media as a fixed factor had a significant effect on motile STR. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; PRL, prolactin; RP, rapid progressive; STR, straightness.

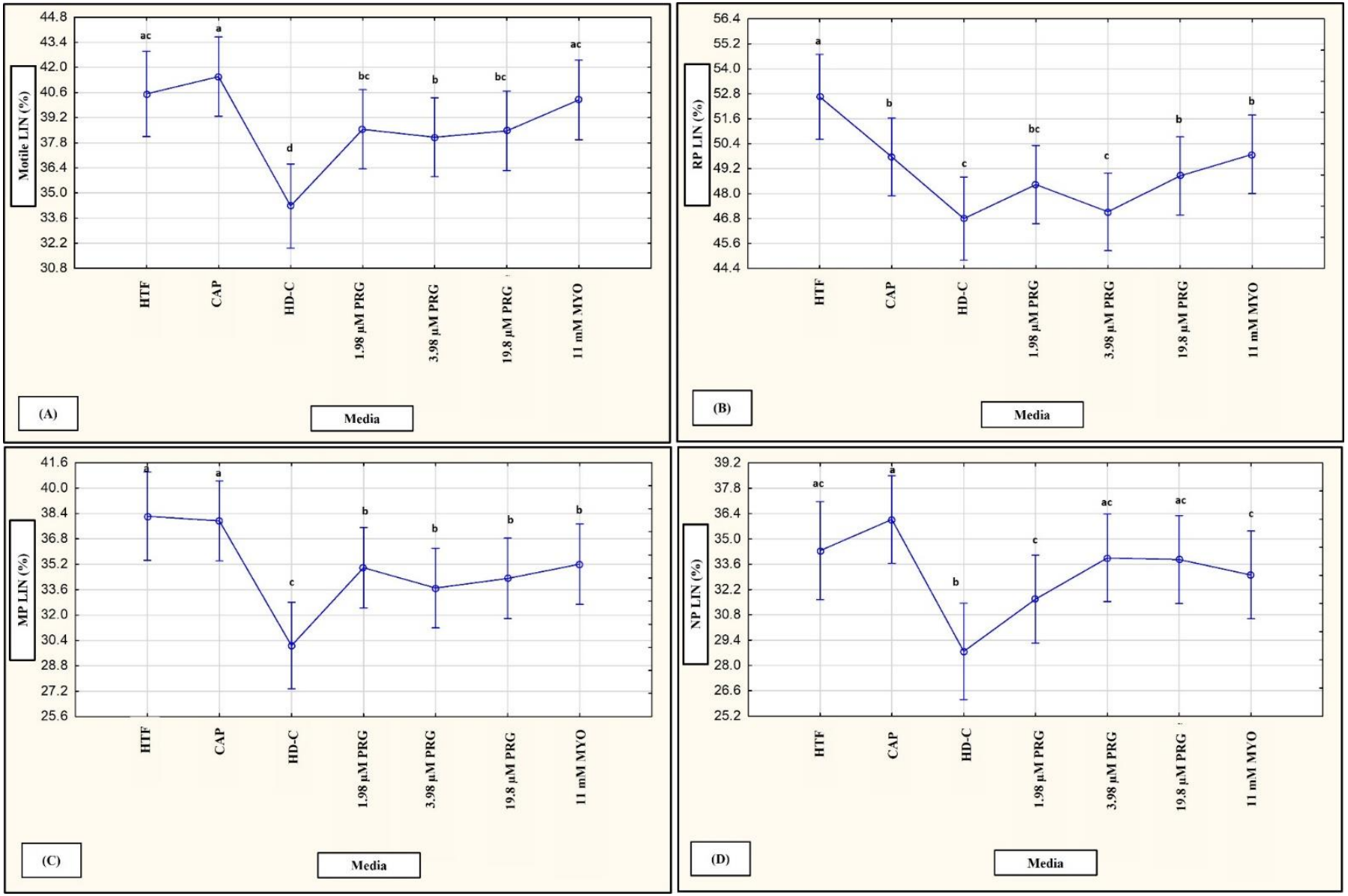


Figure 4.37 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on LIN of various progressive speed groups. **(A)** Effects of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on motile LIN (F (6,528) = 10.05, p <0.01). **(B)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on rapid progressive (RP) speed groups LIN (F (6,521) = 8.26, p <0.01). **(C)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on medium progressive (MP) speed groups LIN (F (6,525) = 7.94, p <0.01). **(D)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on non-progressive (NP) speed group LIN (F (6,528) = 5.56, p <0.01). ***Note*:** Media as a fixed factor had a significant effect on motile, RP, MP and NP speed group LIN. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; LIN, linearity; MP, medium progressive; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; PROG, progressive; RP, rapid progressive.

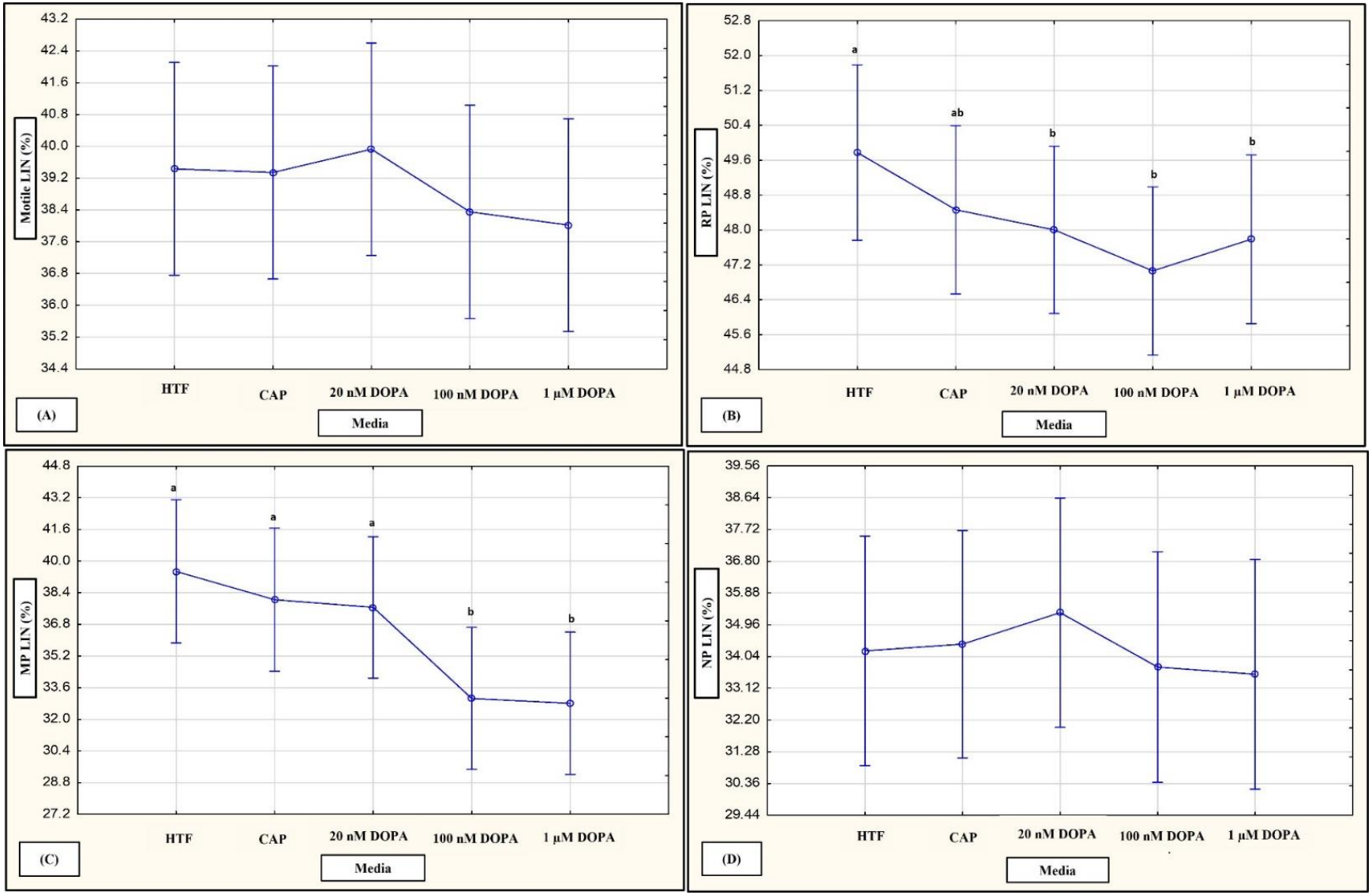


Figure 4.38 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on LIN of various progressive speed groups. **(A)** Effects of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on motile LIN (F (4,451) = 1.12, p = 0.35). **(B)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on rapid progressive (RP) speed groups LIN (F (4,421) = 2.52, p = 0.04). **(C)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on medium progressive (MP) speed groups LIN (F (4,438) = 8.59, p <0.01). **(D)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on non-progressive (NP) speed group LIN (F (4,439) = 0.31, p = 0.87). ***Note:*** Media as a fixed factor had a significant effect on RP and MP LIN. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; DOPA, dopamine; HTF, human tubal fluid; LIN, linearity; MP, medium progressive; NP, non-progressive; RP, rapid progressive.

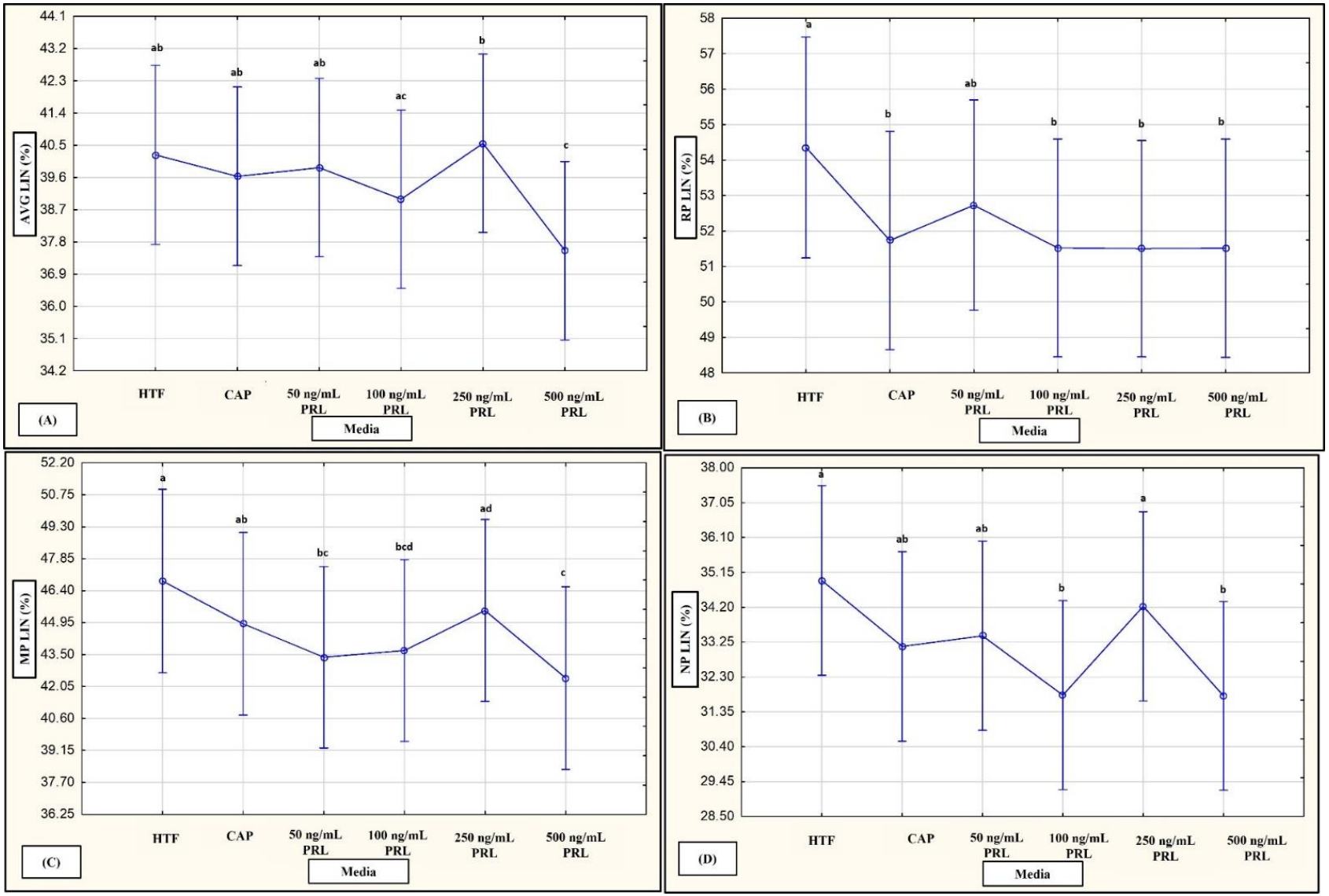


Figure 4.39 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 and 60 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on LIN of various progressive speed groups. **(A)** Effects of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on motile LIN (F (5,739) = 3.72, p <0.01). **(B)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on rapid progressive (RP) speed groups LIN (F (5,425) = 1.71, p = 0.13). **(C)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on medium progressive (MP) speed groups LIN (F (5,661) = 4.43, p <0.01). **(D)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on non-progressive (NP) speed group LIN (F (5,730) = 3.37, p <0.01). ***Note:*** Media as a fixed factor had a significant effect on motile, MP and NP LIN. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; HTF, human tubal fluid; LIN, linearity; MP, medium progressive; NP, non-progressive; PRL, prolactin; RP, rapid progressive.

4.2.5.3 | Vigour

As seen in Supplementary Table 2 - 4, HM subpopulations had significantly higher vigour kinematic parameters as compared to LM subpopulations when exposed to various media. Albeit select media and concentrations could improve the LM subpopulations until significant differences between subpopulations were no longer apparent. This trend was observed for CAP, HD-C, 1.98 µM progesterone and 1 µM dopamine which improved the motile vigour kinematics of LM subpopulations (Figure 4.10). The study further noted this trend of improvement for MP vigour kinematics by CAP, HD-C, progesterone, dopamine (100 nM and 1 µM) and 100 ng/mL prolactin (Figure 4.12). In contrast, 500 ng/mL prolactin increased HM subpopulation MP vigour kinematics resulting in a significant difference between subpopulations. Furthermore, 3.96 µM progesterone and myo-inositol also increased HM subpopulation RP vigour kinematics resulting in significant differences between subpopulations – whereas 100 nM dopamine, 50 ng/mL, 100 ng/mL and 500 ng/mL prolactin improved the LM subpopulation RP vigour kinematics until differences between subpopulations were no longer seen (Figure 4.11). In addition, CAP, 50 ng/mL and 250 ng/mL prolactin increased LM subpopulation NP vigour kinematics as compared to the HM subpopulations (Figure 4.13).

HD-C and progesterone decreased (p <0.05, p <0.01 and p <0.001) motile vigour kinematics of HM subpopulations, but only HD-C decreased (p <0.05) the LM subpopulation. Nevertheless, all media did however increase ALH in both subpopulations. Both HD-C and 1.98 µM progesterone decreased (p <0.01 and p <0.001) MP vigour kinematics in HM subpopulations, where again only HD-C decreased (p <0.01) the LM subpopulations. In contrast, HD-C, progesterone and myo-inositol decreased (p <0.05 and p <0.001) HM subpopulation RP vigour kinematics, but no significant differences between media were seen in the LM subpopulation (Supplementary Table 2). Motile vigour kinematics were decreased (p = 0.001) in HM subpopulations by dopamine, but only CAP and dopamine increased (p <0.001) the LM subpopulation motile ALH values. Dopamine could further decrease (p = 0.001) the HM subpopulation MP vigour kinematics, however only the higher dopamine concentrations displayed a decrease (p <0.05) in the LM subpopulation. The same observations were seen for the RP vigour kinematic values, while only 1 µM dopamine decreased (p = 0.023) NP vigour kinematics of HM subpopulations, as compared to HTF. (Supplementary Table 3). Prolactin increased (p <0.01) motile ALH of both subpopulations as compared to HTF. Furthermore, only higher prolactin concentrations increased (p = 0.001)

MP ALH of LM subpopulations. However, both CAP and prolactin increased ($p = 0.02$) RP vigour kinematics of the LM subpopulations as compared to HTF (Supplementary Table 4).

To determine the effect of media as the main factor on vigour kinematic parameters, data was pooled and analysed in a mixed model ANOVA. Media had a significant ($p < 0.01$) interaction on various vigour kinematic parameters of the different progressive speed groups. For individual effects of the media – HD-C, progesterone and myo-inositol all decreased ($p < 0.01$) motile and RP WOB as compared to HTF. However, both HD-C and 3.96 µM progesterone displayed the greatest effects and additionally decreased ($p < 0.05$ and $p < 0.01$) MP WOB. In contrast, for NP WOB, HD-C and myo-inositol decreased ($p < 0.05$ and $p < 0.01$) values as compared to HTF, CAP and 3.96 µM progesterone (Figure 4.40). Both CAP and dopamine decreased ($p < 0.05$ and $p < 0.01$) motile WOB as compared to HTF - however only dopamine decreased RP speed groups and varying effects were observed between concentrations for MP WOB (Figure 4.41). CAP and prolactin decreased ($p < 0.01$) WOB of the various progressive speed groups, however different effects were observed between the prolactin concentrations for RP and MP speed groups. Higher concentrations of prolactin appeared to decrease WOB as compared to lower prolactin concentrations (Figure 4.42).

CAP, HD-C, progesterone and myo-inositol significantly increased ($p < 0.01$) RP ALH as compared to HTF. However, HD-C and 3.96 µM progesterone had the greatest effect as compared to other media. Likewise, HD-C and progesterone also displayed the greatest effect for increasing MP ALH (Figure 4.43). Both dopamine and CAP increased ALH of the progressive speed groups as compared to HTF, however dopamine displayed the greatest effect ($p < 0.01$). In addition, higher concentrations of dopamine appeared to increase values greater than that of lower dopamine concentrations (Figure 4.44). Both CAP and prolactin increased ($p < 0.01$) motile ALH as compared to HTF, but 50 ng/mL prolactin additionally increased ($p < 0.01$) ALH as compared to CAP. In contrast, 50 ng/mL prolactin was not statistically different to HTF for RP ALH, whereas CAP and other prolactin concentrations significantly increased ($p < 0.05$) ALH as compared to HTF. Furthermore, only 500 ng/mL prolactin increased ($p < 0.05$) MP ALH as compared to HTF and CAP (Figure 4.45).

Progesterone and HD-C decreased ($p < 0.05$ and $p < 0.01$) motile and MP BCF as compared to HTF, CAP and myo-inositol - however HD-C had a greater reduction ($p < 0.01$) in BCF as compared progesterone. This was further observed for RP BCF, however myo-inositol also decreased ($p < 0.01$) BCF as compared to HTF (Figure 4.46). Compared to CAP, 100 nM dopamine decreased ($p < 0.01$) motile BCF – whereas compared to HTF, 100 nM

dopamine decreased (p <0.05) RP BCF. Furthermore, higher dopamine concentrations decreased (p <0.05 and p <0.01) MP BCF as compared to HTF, CAP and 20 nM dopamine (Figure 4.47). Compared to HTF, both CAP and 250 mg/mL prolactin increased (p <0.05) motile BCF, whereas compared to CAP, 50 ng/mL and 250 ng/mL – 500 ng/mL prolactin decreased (p <0.01) motile BCF. Both CAP and prolactin decreased (p <0.05 and p <0.01) RP BCF, whereas 100 ng/mL and 500 ng/mL prolactin decreased (p <0.01) MP BCF (Figure 4.48).

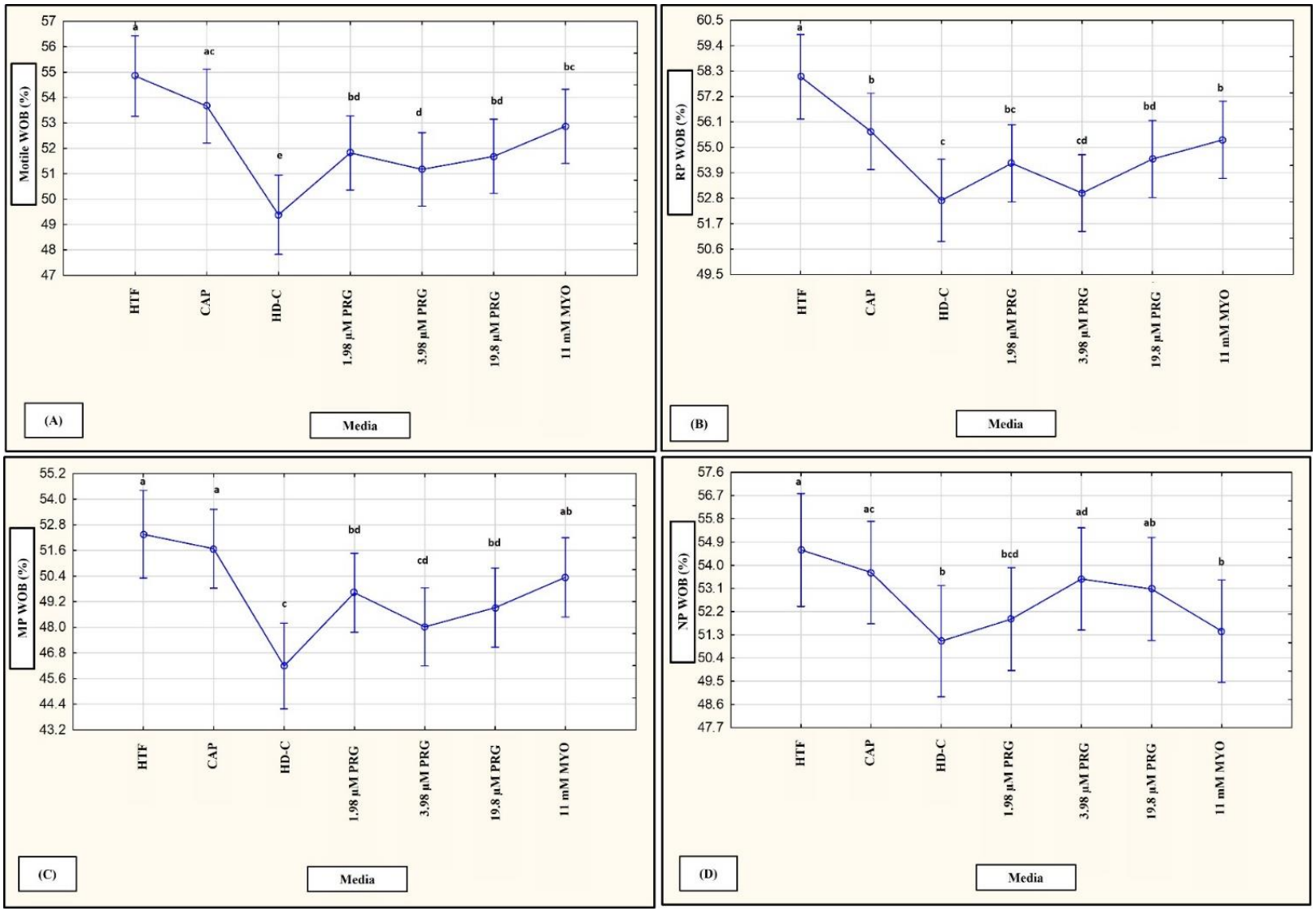


Figure 4.40 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on WOB of various progressive speed groups**. (A)** Effects of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on motile WOB (F (6,529) = 11.26, p <0.01). **(B)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on rapid progressive (RP) speed groups WOB (F (6,521) = 8.35, p <0.01). **(C)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on medium progressive (MP) speed groups WOB (F (6,524) = 7.98, p <0.01). **(D)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on non-progressive (NP) speed group WOB (F (6,528) = 2.91, p <0.01). ***Note*:** Media as a fixed factor had a significant effect on motile, RP, MP and NP speed group WOB. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations**: AVG, average; CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; LIN, linearity; MP, medium progressive; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; PROG, progressive; RP, rapid progressive; WOB, wobble.

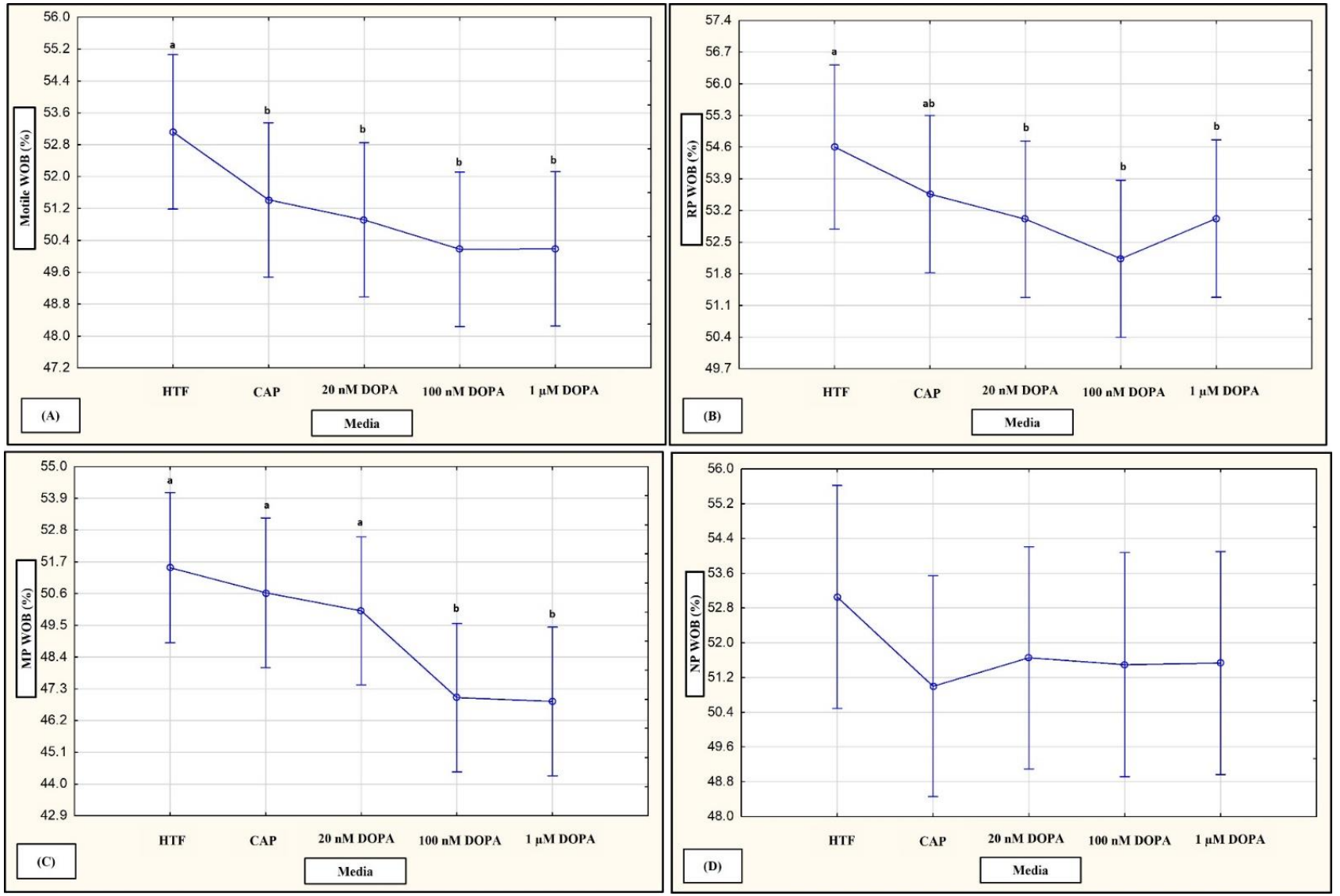


Figure 4.41 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on WOB of various progressive speed groups. **(A)** Effects of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on motile WOB (F (4,451) = 5.12, p <0.01). **(B)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on rapid progressive (RP) speed groups WOB (F (4,421) = 2.44, p = 0.05). **(C)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on medium progressive (MP) speed groups WOB (F (4,438) = 7.64, p <0.01). **(D)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on non-progressive (NP) speed group WOB (F (4,439) = 0.65, p = 0.63). ***Note:*** Media as a fixed factor had a significant effect on motile and MP WOB. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; CAP, capacitating-HTF; DOPA, dopamine; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; RP, rapid progressive, WOB wobble.

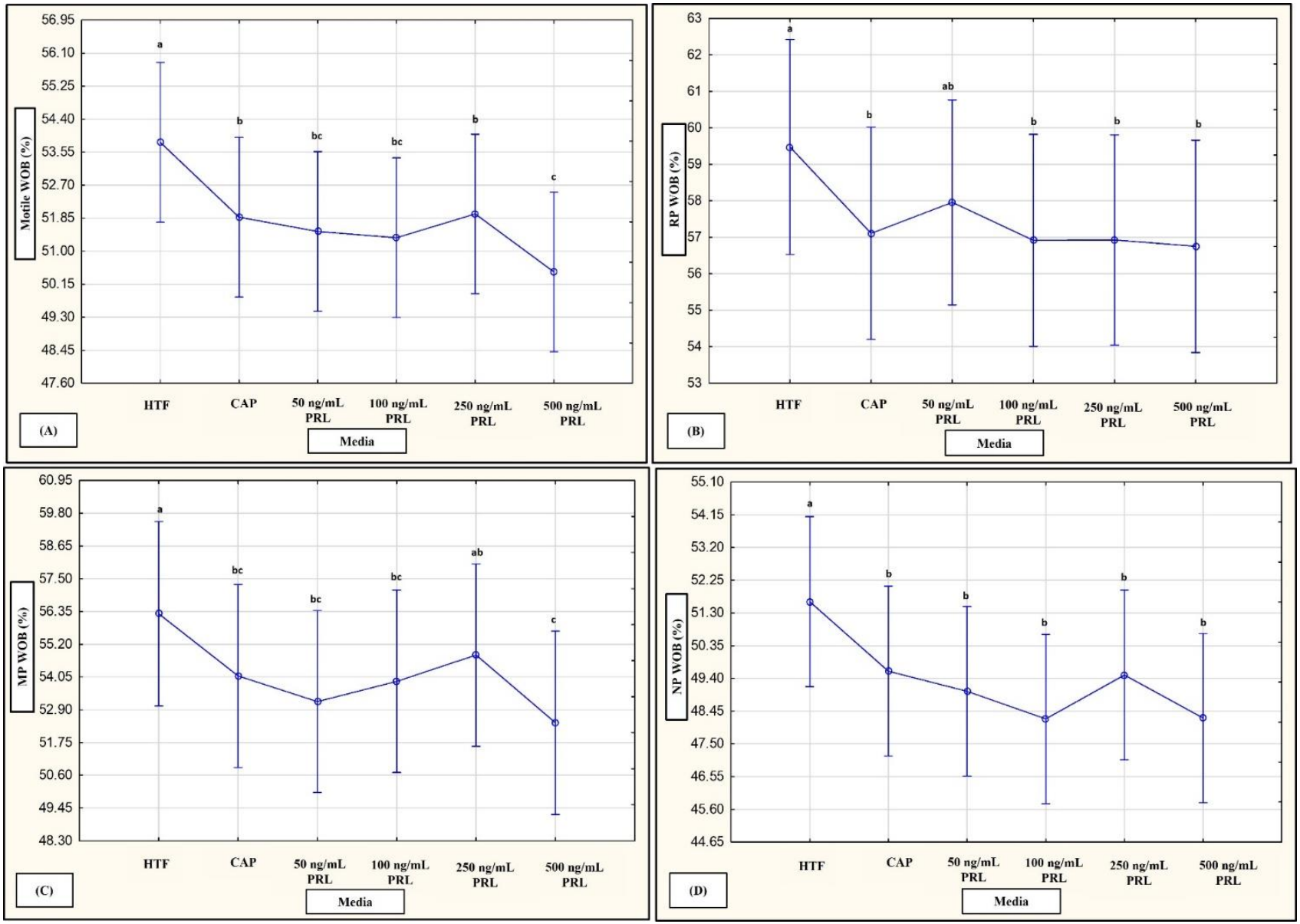


Figure 4.42 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 and 60 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on WOB of various progressive speed groups. **(A)** Effects of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on motile WOB (F (5,739) = 6.57, p <0.01). **(B)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on rapid progressive (RP) speed groups WOB (F (5,425) = 1.68, p = 0.14). **(C)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on medium progressive (MP) speed groups WOB (F (5,661) = 4.14, p <0.01). **(D)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on non-progressive (NP) speed group WOB (F (5,730) = 4.48, p <0.01). ***Note:*** Media as a fixed factor had a significant effect on motile STR. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations**: AVG, average; CAP, capacitating-HTF; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; PRL, prolactin; RP, rapid progressive; WOB, wobble.

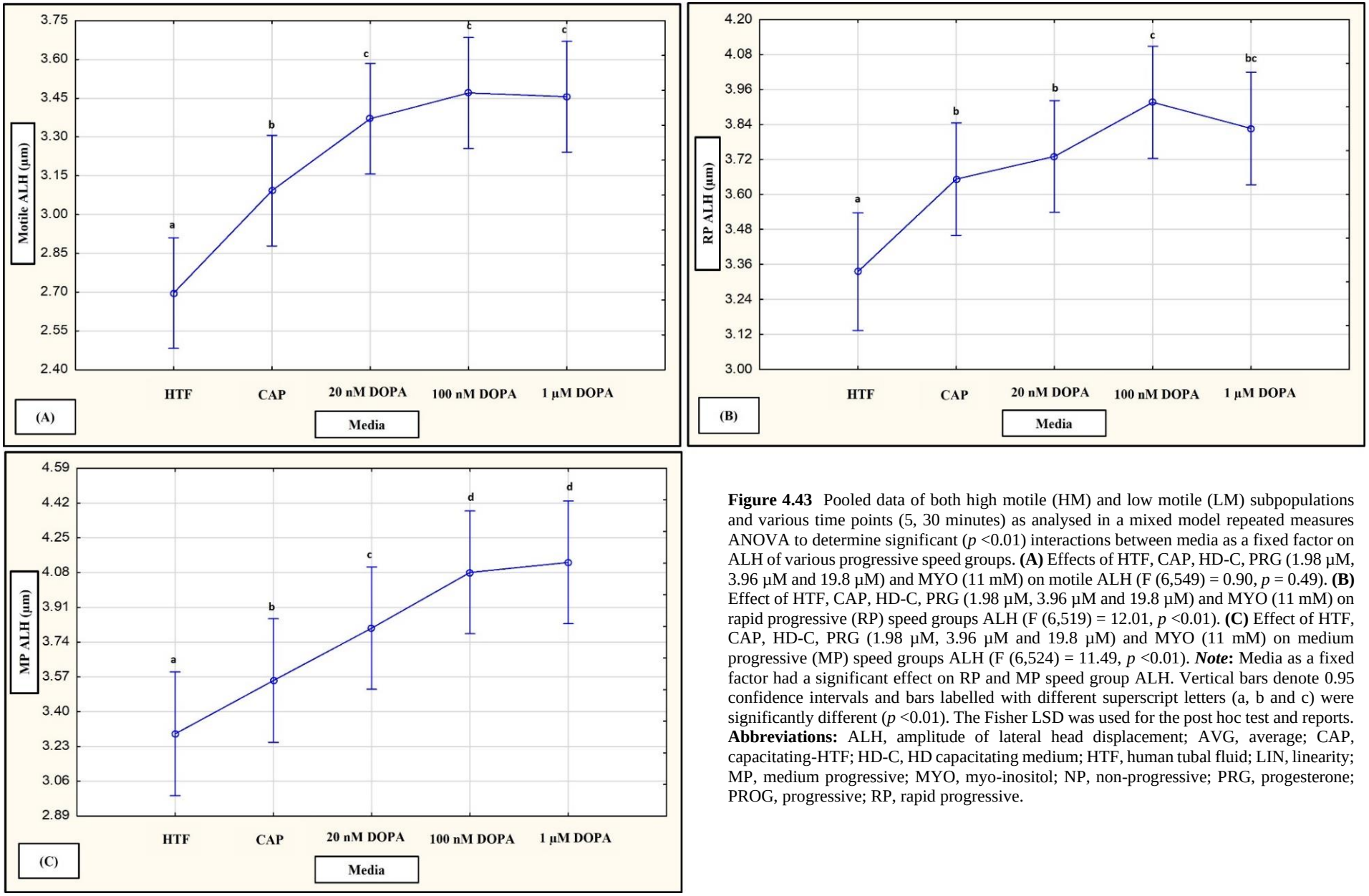
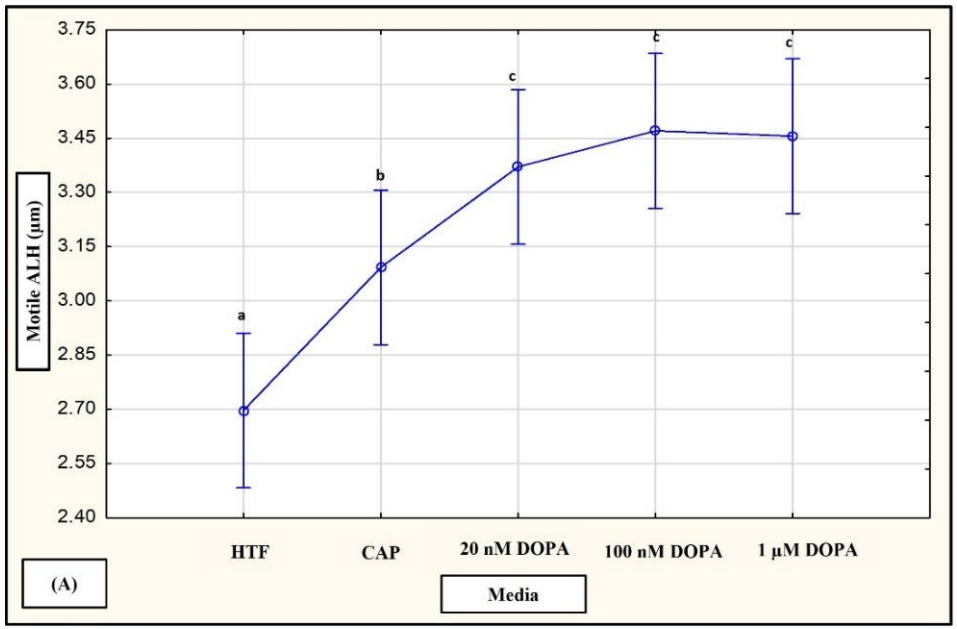
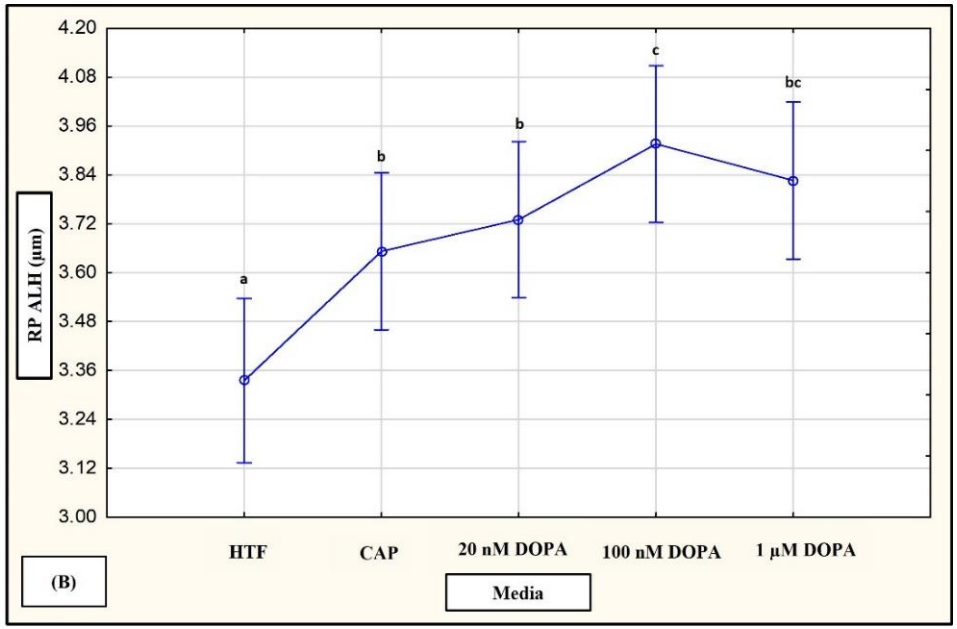


Figure 4.43 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on ALH of various progressive speed groups. **(A)** Effects of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on motile ALH (F (6,549) = 0.90, p = 0.49). **(B)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on rapid progressive (RP) speed groups ALH (F (6,519) = 12.01, p <0.01). **(C)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on medium progressive (MP) speed groups ALH (F (6,524) = 11.49, p <0.01). ***Note*:** Media as a fixed factor had a significant effect on RP and MP speed group ALH. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** ALH, amplitude of lateral head displacement; AVG, average; CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; LIN, linearity; MP, medium progressive; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; PROG, progressive; RP, rapid progressive.





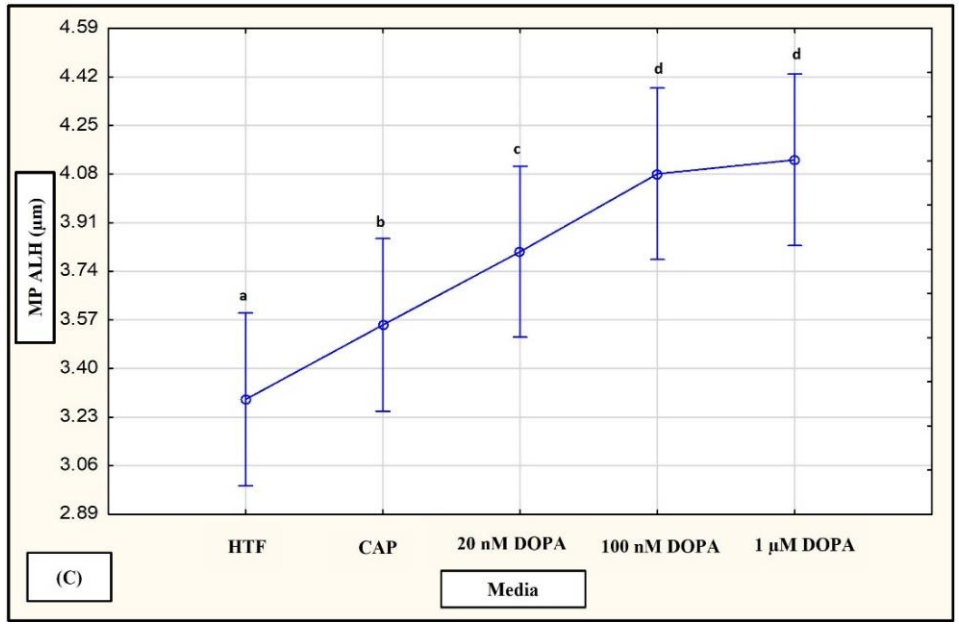


Figure 4.44 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on ALH of various progressive speed groups**. (A)** Effects of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on motile ALH (F (4,451) = 33.92, p <0.01). **(B)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on rapid progressive (RP) speed groups ALH (F (4,421) = 10.57, p <0.01). **(C)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on medium progressive (MP) speed groups ALH (F (4,438) = 18.96, p <0.01). ***Note:*** Media as a fixed factor had a significant effect on motile, RP and MP ALH. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** ALH, amplitude of lateral head displacement; AVG, average; CAP, capacitating-HTF; DOPA, dopamine; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; RP, rapid progressive.

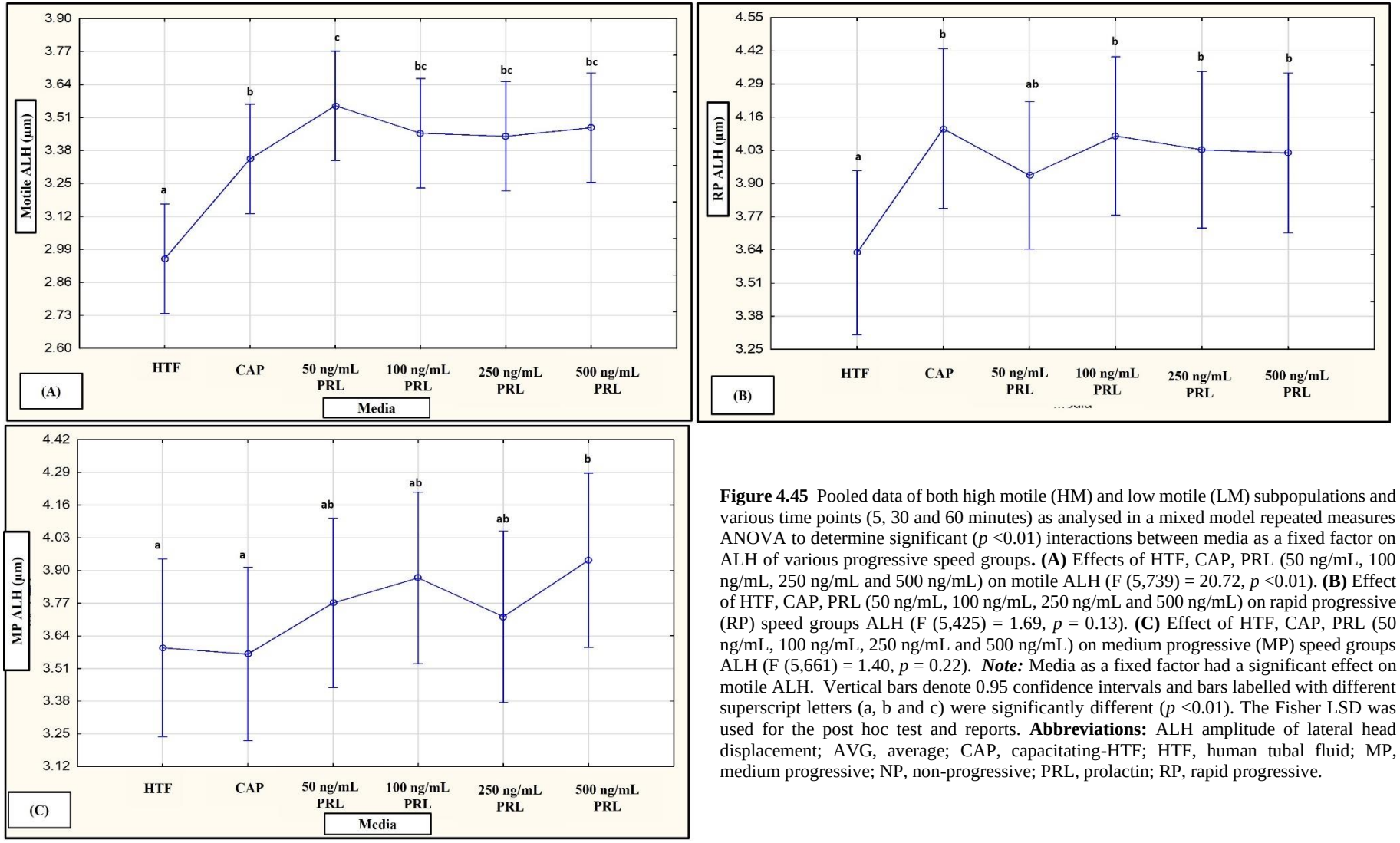


Figure 4.45 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 and 60 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on ALH of various progressive speed groups**. (A)** Effects of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on motile ALH (F (5,739) = 20.72, p <0.01). **(B)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on rapid progressive (RP) speed groups ALH (F (5,425) = 1.69, p = 0.13). **(C)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on medium progressive (MP) speed groups ALH (F (5,661) = 1.40, p = 0.22). ***Note:*** Media as a fixed factor had a significant effect on motile ALH. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** ALH amplitude of lateral head displacement; AVG, average; CAP, capacitating-HTF; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; PRL, prolactin; RP, rapid progressive.

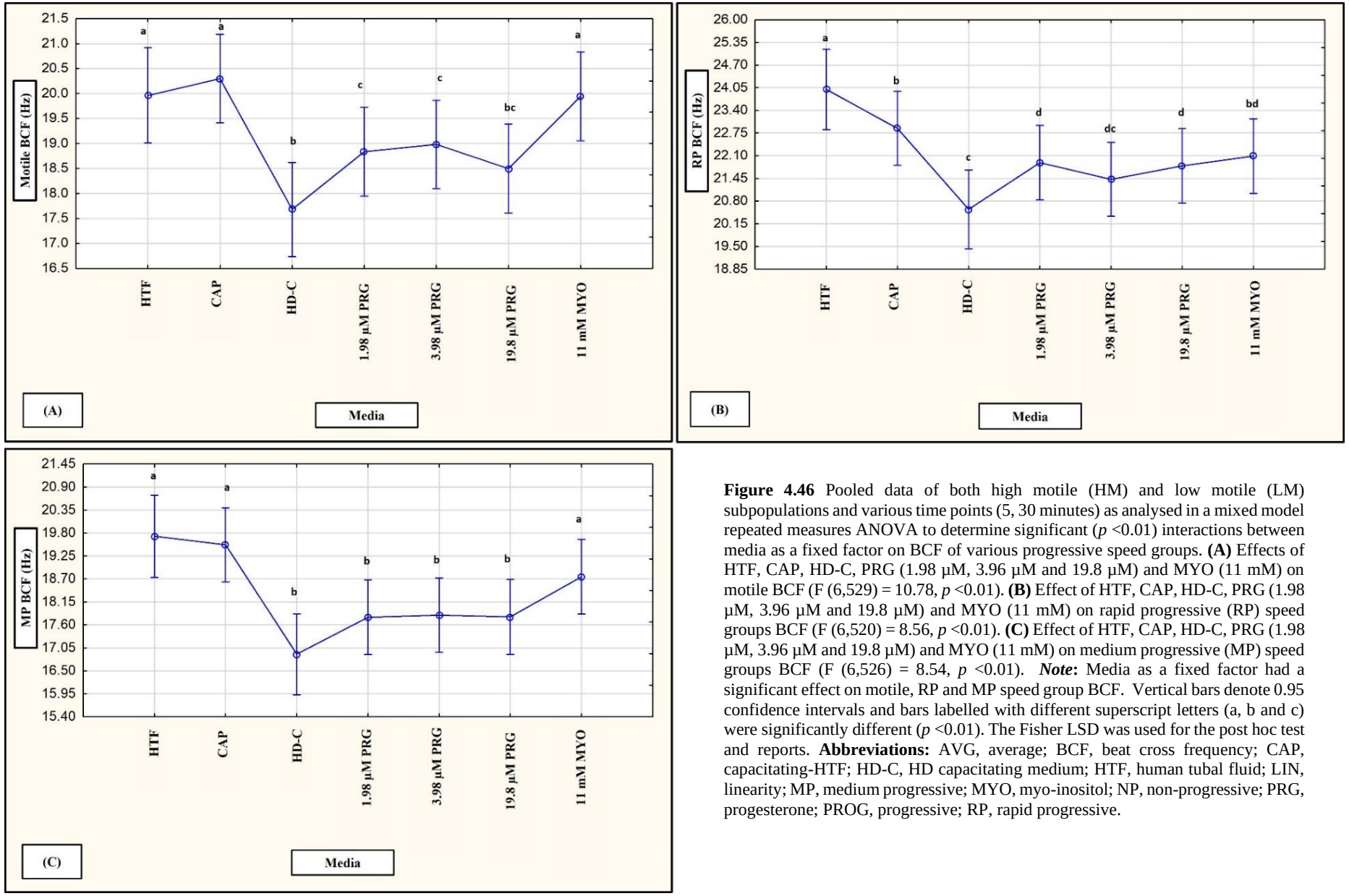


Figure 4.46 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on BCF of various progressive speed groups. **(A)** Effects of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on motile BCF (F (6,529) = 10.78, p <0.01). **(B)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on rapid progressive (RP) speed groups BCF (F (6,520) = 8.56, p <0.01). **(C)** Effect of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on medium progressive (MP) speed groups BCF (F (6,526) = 8.54, p <0.01). ***Note*:** Media as a fixed factor had a significant effect on motile, RP and MP speed group BCF. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; BCF, beat cross frequency; CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; LIN, linearity; MP, medium progressive; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; PROG, progressive; RP, rapid progressive.

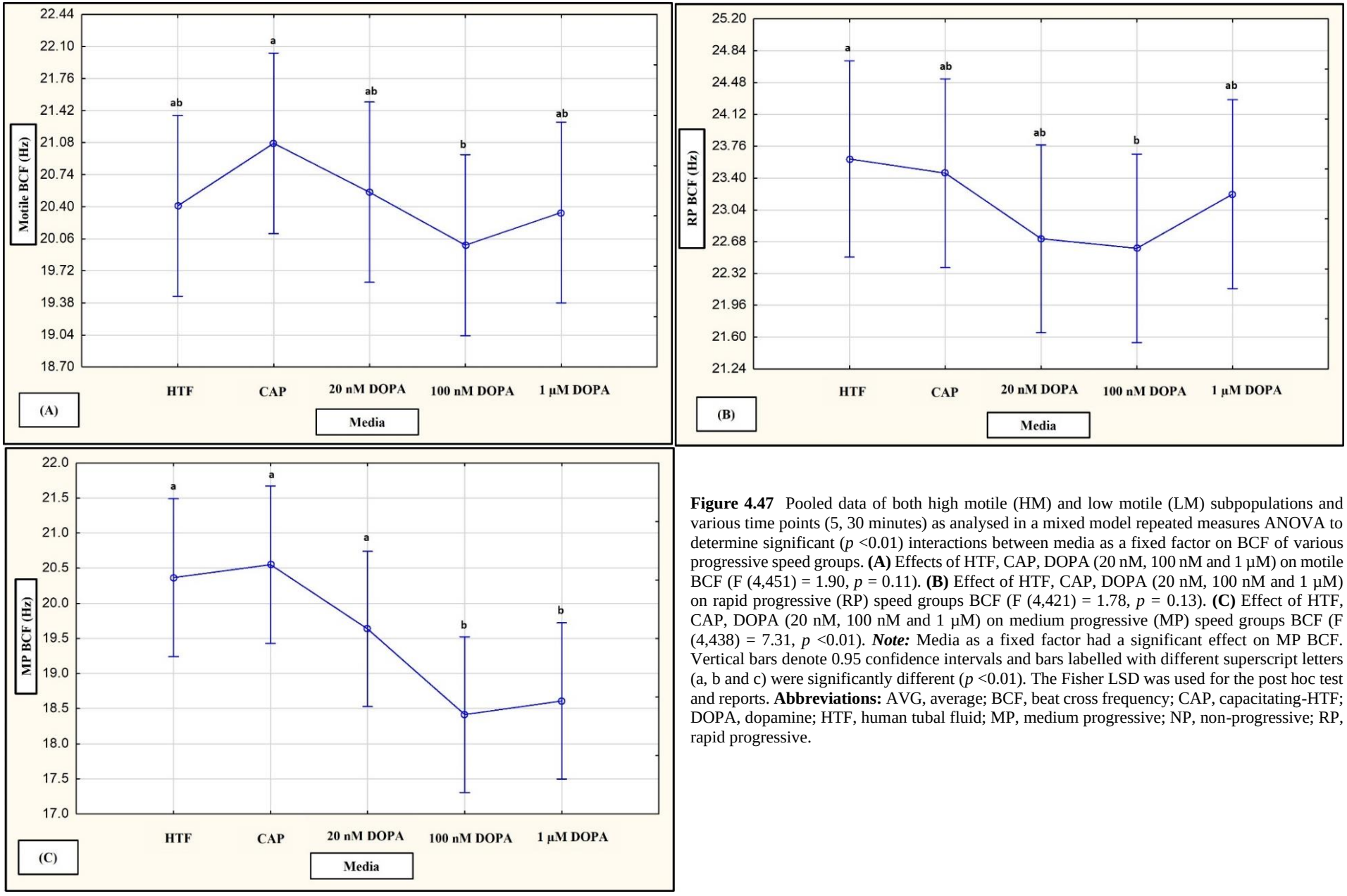
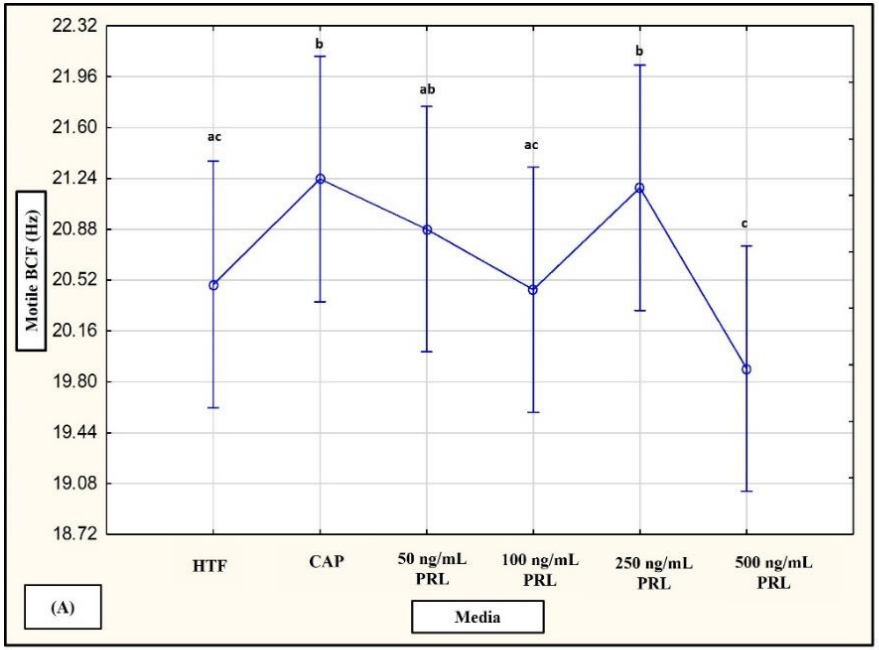
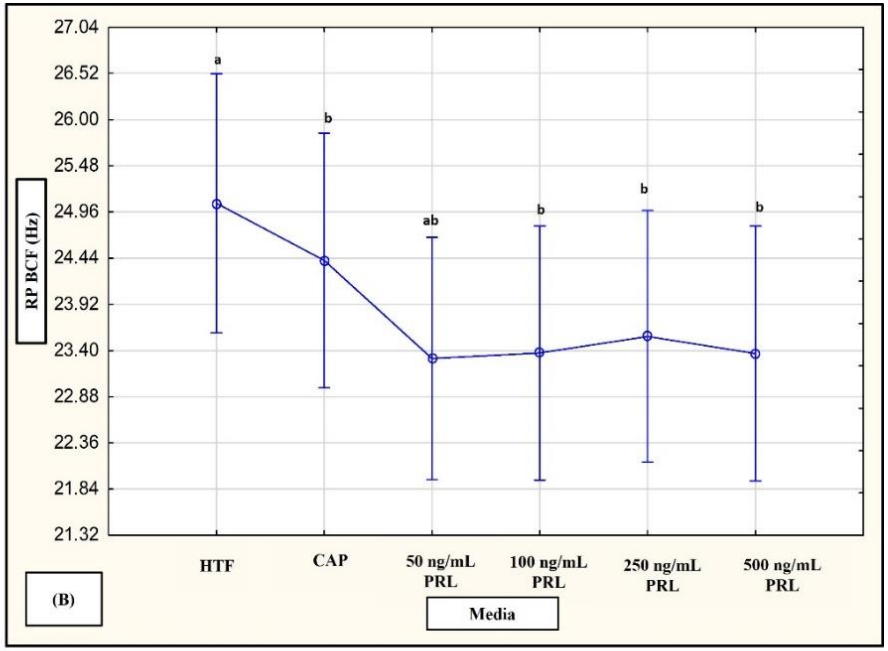


Figure 4.47 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on BCF of various progressive speed groups. **(A)** Effects of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on motile BCF (F (4,451) = 1.90, p = 0.11). **(B)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on rapid progressive (RP) speed groups BCF (F (4,421) = 1.78, p = 0.13). **(C)** Effect of HTF, CAP, DOPA (20 nM, 100 nM and 1 µM) on medium progressive (MP) speed groups BCF (F (4,438) = 7.31, p <0.01). ***Note:*** Media as a fixed factor had a significant effect on MP BCF. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; RP, rapid progressive.





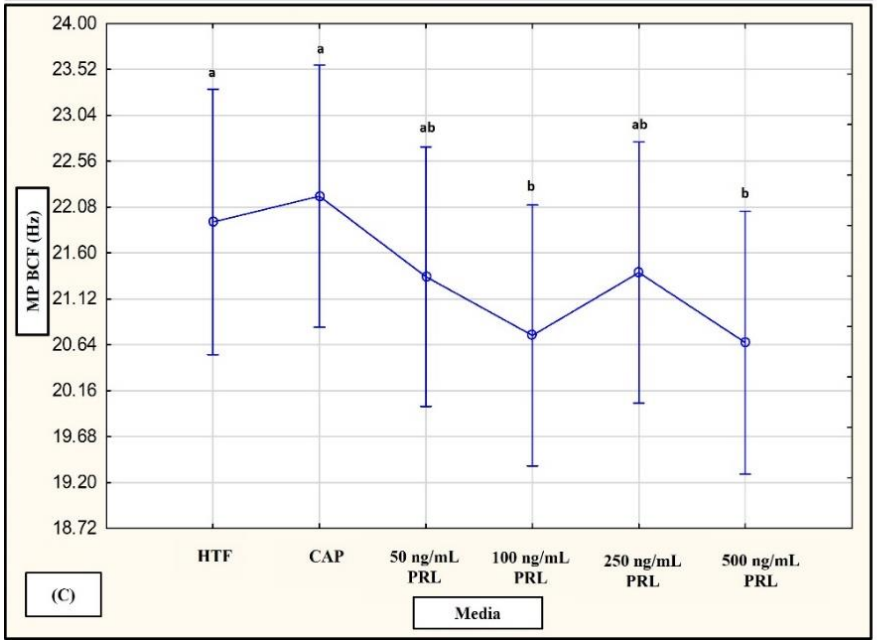


Figure 4.48 Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 and 60 minutes) as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on BCF of various progressive speed groups. **(A)** Effects of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on motile BCF (F (5,739) = 5.20, p <0.01). **(B)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on rapid progressive (RP) speed groups BCF (F (5,425) = 2.39, p = 0.04). **(C)** Effect of HTF, CAP, PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) on medium progressive (MP) speed groups BCF (F (5,661) = 3.19, p <0.01). ***Note:*** Media as a fixed factor had a significant effect on motile, RP and MP BCF. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** AVG, average; BCF, beat cross frequency; CAP, capacitating-HTF; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; PRL, prolactin; RP, rapid progressive.

4.2.6 | Hyperactivation

Seen in Table 4.10 - 4.12 are the percentage hyperactivation results of both the HM and LM subpopulations after exposure to the various media for different time intervals. Compared to the LM subpopulations, the HM subpopulation displayed significantly higher values for percentage hyperactivation at various time points for HTF. However, after exposure to the various media, LM subpopulations were often improved to the point where no significant differences between subpopulations could be observed (Figure 4.8). This trend was observed at various time intervals for CAP, HD-C, progesterone and myo-inositol (Table 4.10). In contrast 3.96 µM progesterone, myo-inositol, dopamine and prolactin all displayed significant differences between subpopulations at various time intervals (Table 4.10 - 4.12).

Compared to HTF, HD-C, progesterone (3.96 µM and 19.8 µM) and myo-inositol had an early stimulatory ($p = 0.005$) effect on HM subpopulation hyperactivation percentages at the 5-minute interval. In contrast, only HD-C and 3.96 µM progesterone increased ($p = 0.009$) hyperactivation in LM subpopulations at 5 minutes. Interestingly, interactions between the LM subpopulations and the media were more notable as compared to the HM subpopulations. For example, at 15- ($p = 0.003$), 45- ($p = 0.008$) and 60-minutes ($p = 0.024$) CAP, HD-C, progesterone and myo-inositol, all significantly increased hyperactivation in LM subpopulations as compared to HTF (Table 4.10 & Figure 4.8).

Dopamine significantly increased hyperactivation in HM subpopulations as compared to HTF, however differences between concentrations were only observed at later time intervals of 45- and 60 minutes. At the 45-minute interval only 100 nM and 1 µM dopamine significantly increased ($p = 0.003$) hyperactivation as compared to HTF, whereas at 60 minutes only 20 nM and 1 µM dopamine significantly improved ($p = 0.041$) hyperactivation. In contrast, differences between concentrations were observed at earlier time intervals for the LM subpopulations. At 5 minutes only 100 nM and 1 µM dopamine significantly improved ($p < 0.001$) hyperactivation in the LM subpopulation as compared to HTF and CAP. Although dopamine increased hyperactivation at later time intervals in the LM subpopulations, 1 µM dopamine displayed the greatest effect as compared to HTF and CAP (Table 4.11 & Figure 4.8).

Prolactin and CAP increased hyperactivation in HM subpopulations as compared to HTF, however differences between concentrations were observed at various time intervals. Compared to HTF of HM subpopulations - 50 ng/mL, 100 ng/mL and 500 ng/mL prolactin

stimulated (p = 0.026) hyperactivation at 30 minutes, whereas at 60 minutes 250 ng/mL increased (p = 0.049) hyperactivation. Prolactin further displayed an increase in hyperactivation of the LM subpopulations as compared to HTF, however at 30 minutes 250 ng/mL prolactin had the greatest increase (p <0.001) as compared to both HTF and CAP (Table 4.12 & Figure 4.8)

Table 4.10 Percentage hyperactivation in both low motile and high motile sperm subpopulations after treatment with HTF, CAP, HD-C, 1.98 µM progesterone, 3.96 µM progesterone, 19.8 µM progesterone and 11 mM myo-inositol at 5, 15, 30, 45 and 60 minutes (mean ± SD) (n=20).

	HTF		CAP		HD-C		1.98 µM PRG		3.96 µM PRG		19.8 µM PRG		11 mM MYO		
	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	ANOVA
5 minutes															
HM	18.0 ± 11.5^{a}	0.081	29.8 ± 17.1^{ab}	0.133	42.4 ± 20.1^{b}	0.163	29.8 ± 17.8^{ab}	0.185	37.9 ± 18.8^{b}	0.210	35.67 ± 17.8^{b}	0.095	36.1 ± 17.0$^{*\,b}$	**0.022**	**0.005**
LM	10.8 ± 9.5^{a}		22.6 ± 14.7^{ab}		33.5 ± 19.3^{b}		23.2 ± 16.9^{ab}		30.2 ± 19.5^{b}		26.0 ± 18.0^{ab}		23.9 ± 16.1$^{*\,ab}$		**0.009**
15 minutes															
HM	25.0 ± 13.3***	**<0.001**	35.4 ± 17.2	0.085	43.1 ± 16.1	0.217	34.5 ± 16.7	0.178	41.0 ± 16.7*	**0.022**	37.1 ± 16.6	0.060	35.6 ± 16.7	0.149	0.067
LM	8.8 ± 5.8$^{***\,a}$		26.2 ± 15.8^{b}		35.7 ± 21.1^{b}		26.2 ± 21.4^{b}		27.7 ± 18.7$^{*\,b}$		27.0 ± 21.7^{b}		27.8 ± 16.9^{b}		**0.003**
30 minutes															
HM	24.1 ± 11.4***	**<0.001**	35.2 ± 17.9	0.082	35.9 ± 16.1	0.230	29.4 ± 16.0	0.404	34.7 ± 16.8	0.091	30.8 ± 17.0	0.151	27.6 ± 13.7	0.414	0.245
LM	8.64 ± 8.5***		25.6 ± 16.3		28.5 ± 21.7		24.5 ± 20.5		25.1 ± 18.2		23.5 ± 20.0		23.3 ± 18.9		0.100
45 minutes															
HM	17.2 ± 9.9***	**<0.001**	27.9 ± 12.5	0.079	27.1 ± 14.9	0.605	25.1 ± 16.7	0.122	30.4 ± 15.2	0.085	23.9 ± 12.2	0.176	23.8 ± 11.9	0.599	0.172
LM	4.0 ± 6.6$^{***\,a}$		20.2 ± 14.6^{b}		24.4 ± 17.7^{b}		17.7 ± 16.9^{b}		21.7 ± 16.2^{b}		19.4 ± 18.8^{b}		21.2 ± 18.1^{b}		**0.008**
60 minutes															
HM	13.8 ± 9.8**	**0.005**	21.0 ± 14.8	0.212	21.7 ± 12.7	0.568	21.1 ± 12.3	0.098	25.5 ± 14.7*	**0.021**	20.8 ± 12.1	0.050	20.1 ± 10.2	0.133	0.304
LM	3.0 ± 7.0$^{**\,a}$		16.0 ± 15.1^{b}		19.4 ± 13.3^{b}		15.46 ± 17.5^{b}		14.8 ± 13.9$^{*\,b}$		12.7 ± 12.9^{b}		14.7 ± 12.0^{b}		**0.024**

Note: Compared to the low motile (LM) subpopulation, high motile (HM) subpopulations had significantly higher percentages of hyperactivated spermatozoa at 15, 30, 45 and 60 min for HTF, in addition to 15 and 60 min for 3.96 µM PRG. In the HM subpopulation, HTF had significantly lower percentages of hyperactivation at 5 min (compared to HD-C, 3.96 µM PRG, 19.8 µM PRG and 11 mM MYO). In the LM subpopulation, HTF had significantly lower hyperactivation percentages at 5 min (compared to HD-C and 3.96 µM PRG); 15 min, 45 min and 60 min (compared to all the other media used). **Abbreviations:** ANOVA, analysis of variance between media for individual time intervals and subpopulations; CAP, capacitating-HTF; HD-C, HD capacitating medium; HM, high motile; HTF, human tubal fluid; LM, low motile; min, minutes; MYO, myo-inositol; PRG, progesterone; SD, standard deviation and SP *p*, t test between subpopulations. $^{a, b,c,d}$ Values labelled with different superscript letters in the same row were significantly different between the various media for individual subpopulations and time points. One-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions was used. Values labelled with an asterisk in the same column were significantly different between the HM and LM subpopulations for individual media and time points (**p* <0.05, ***p* <0.01 and ****p* <0.001). Student's t-test was used or the Mann-Whitney test when normal distribution was void.

Table 4.11 Percentage hyperactivation in both low motile and high motile sperm subpopulations after treatment with HTF, CAP, 20 nM, 100 nM and 1 µM dopamine (mean ± SD) (n=20).

	HTF		CAP		20 nM DOPA		100 nM DOPA		1 µM DOPA		
	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	ANOVA
5 minutes											
HM	11.0 ± 15.0^{a}	0.544	19.7 ± 18.0^{a}	0.054	35.7 ± 21.9$^{** b}$	**0.004**	31.3 ± 15.4^{b}	0.158	35.2 ± 21.0$^{* b}$	**0.028**	**< 0.001**
LM	6.8 ± 7.4^{a}		11.9 ± 12.2^{ac}		19.0 ± 15.0$^{** bc}$		24.0 ± 16.9^{b}		22.1 ± 15.1$^{* b}$		**< 0.001**
15 minutes											
HM	17.8 ± 13.7$^{** a}$	**0.003**	29.4 ± 17.3$^{** b}$	**0.006**	35.1 ± 20.4$^{** b}$	**0.009**	31.3 ± 12.5$^{* b}$	**0.028**	39.5 ± 18.2$^{* b}$	**0.015**	**0.001**
LM	6.9 ± 6.8$^{** a}$		15.7 ± 11.9$^{** b}$		18.9 ± 12.6$^{** bc}$		21.6 ± 14.2$^{* bc}$		26.7 ± 13.4$^{* c}$		**< 0.001**
30 minutes											
HM	23.1 ± 14.0***	**<0.001**	33.6 ± 19.4***	**<0.001**	33.6 ± 18.4*	**0.010**	33.6 ± 15.6*	**0.011**	37.9 ± 19.2	0.060	0.105
LM	6.6 ± 9.3$^{*** a}$		13.7 ± 12.0$^{*** ab}$		20.5 ± 11.7$^{* bc}$		21.4 ± 13.3$^{* bc}$		24.5 ± 15.3^{c}		**< 0.001**
45 minutes											
HM	12.0 ± 13.1^{a}	0.146	24.8 ± 15.6$^{** ab}$	**0.002**	20.8 ± 16.0^{ab}	0.329	24.2 ± 16.8^{b}	0.119	31.8 ± 16.3^{b}	0.056	**0.003**
LM	5.9 ± 8.0^{a}		10.3 ± 10.9$^{** ab}$		16.7 ± 12.1^{bc}		18.2 ± 15.8^{bc}		21.7 ± 16.1^{c}		**0.001**
60 minutes											
HM	9.7 ± 12.1^{a}	0.078	22.2 ± 12.1$^{** ab}$	**0.001**	21.2 ± 16.0^{b}	0.078	18.0 ± 17.1^{ab}	0.430	23.4 ± 18.0^{b}	0.067	**0.041**
LM	4.7 ± 8.9		9.6 ± 10.7**		14.3 ± 12.0		14.4 ± 14.9		13.0 ± 12.5		0.056

Note: Compared to the low motile (LM) subpopulation, the high motile (HM) subpopulation had significantly higher percentages of hyperactivated spermatozoa at 5 min (for 20 nM and 1 µM DOPA); 15 min (for all media); 30 min (for all media except 1 µM DOPA); 45 min and 60 min (for CAP). For the HM subpopulation HTF had significantly lower percentages of hyperactivation at 5 min (compared to all concentrations of DOPA); 15 min (compared to all media); 45 min (compared to 100 nM and 1µM DOPA) and 60 min (compared to 20 nM and 1 µM DOPA). At 5 min CAP also had significantly lower percentages of hyperactivation (compared to all concentrations of DOPA). In the LM subpopulation, HTF had significantly lower percentages of hyperactivation at 5, 30 and 45 min (compared to all DOPA concentrations); 15 min (compared to all the media). At 5 min, 100 nM and 1µM DOPA had significantly higher percentages of hyperactivation (compared to CAP and HTF) and 100 nM DOPA had significantly higher percentages hyperactivation at 15, 30 and 45 min (compared to HTF and CAP). **Abbreviations**: ANOVA, analysis of variance between media for individual time intervals and subpopulations; CAP, capacitating-HTF; DOPA, dopamine; HM, high motile; HTF, human tubal fluid; LM, low motile; min, minutes; SD, standard deviation and SP *p*, t test between subpopulations. $^{a, b,c,d}$ Values labelled with different superscript letters in the same row were significantly different between the various media for individual subpopulations and time points. One-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions was used. Values labelled with an asterisk in the same column were significantly different between the HM and LM subpopulations for individual media and time points (**p* <0.05, ***p* <0.01 and ****p* <0.001). Student's t-test was used or the Mann-Whitney test when normal distribution was void.

Table 4.12 Percentage hyperactivation in both low motile and high motile sperm subpopulations after treatment with HTF, CAP, 50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL prolactin at 5, 15, 30, 45 and 60 minutes (mean ± SD) (n=20).

	HTF		CAP		50 ng/mL PRL		100 ng/mL PRL		250 ng/mL PRL		500 ng/mL PRL		
	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	ANOVA
5 minutes													
HM	18.9 ± 13.7***	**<0.001**	29.6 ± 17.4**	**0.003**	26.4 ± 18.1	0.451	29.7 ± 18.3	0.354	28.0 ± 19.1	0.132	**34.0 ± 18.6***	**0.040**	0.091
LM	7.9 ± 9.5$^{***\,a}$		15.0 ± 13.8$^{**\,ab}$		22.4 ± 18.1^{b}		22.0 ± 17.6^{b}		18.9 ± 18.7^{b}		**23.6 ± 15.3**$^{*\,b}$		**0.005**
15 minutes													
HM	22.3 ± 8.2$^{**\,a}$	**0.001**	34.5 ± 14.8$^{**\,b}$	**0.003**	36.8 ± 19.7^{b}	0.155	32.2 ± 18.2^{b}	0.967	33.3 ± 18.9^{b}	0.302	33.9 ± 17.0^{b}	0.318	**0.029**
LM	11.5 ± 11.6$^{**\,a}$		21.1 ± 14.5$^{**\,b}$		28.9 ± 18.0^{b}		32.5 ± 21.0^{b}		26.7 ± 20.1^{b}		29.1 ± 16.4^{b}		**0.001**
30 minutes													
HM	21.3 ± 10.7$^{***\,a}$	**<0.001**	35.3 ± 17.7$^{**\,ab}$	**0.001**	38.2 ± 18.5$^{**\,b}$	**0.009**	33.6 ± 18.2^{b}	0.380	34.4 ± 21.6^{ab}	0.410	32.9 ± 17.5^{b}	0.155	**0.026**
LM	9.5 ± 10.1$^{***\,a}$		19.6 ± 13.8$^{**\,b}$		24.9 ± 15.6$^{**\,bc}$		28.8 ± 19.2^{bc}		29.7 ± 17.6^{c}		25.4 ± 18.3^{bc}		**<0.001**
45 minutes													
HM	16.1 ± 10.7$^{*\,a}$	**0.014**	26.5 ± 15.9$^{*\,b}$	**0.012**	32.5 ± 16.8$^{*\,b}$	**0.017**	29.0 ± 14.4^{b}	0.642	35.1 ± 18.0$^{*\,b}$	**0.017**	29.4 ± 15.3^{b}	0.188	**0.001**
LM	8.7 ± 10.3$^{*\,a}$		16.1 ± 11.8$^{*\,ab}$		21.3 ± 14.8$^{*\,b}$		25.6 ± 17.7^{b}		23.9 ± 12.9$^{*\,b}$		23.6 ± 14.6^{b}		**<0.001**
60 minutes													
HM	17.7 ± 11.9$^{**\,a}$	**0.001**	25.4 ± 15.4$^{*\,ab}$	**0.015**	31.0 ± 16.6$^{*\,ab}$	**0.010**	26.0 ± 12.9^{ab}	0.692	30.7 ± 19.6$^{**\,b}$	**0.008**	28.8 ± 18.0$^{*\,ab}$	**0.016**	**0.049**
LM	7.0 ± 9.5$^{**\,a}$		14.8 ± 10.8$^{*\,b}$		19.8 ± 12.3$^{*\,b}$		24.4 ± 15.0^{b}		17.1 ± 14.6$^{**\,b}$		17.9 ± 11.9$^{*\,b}$		**<0.001**

Note: Compared to the low motile (LM) subpopulation, the high motile (HM) subpopulation had significantly higher percentages of hyperactivated spermatozoa at all time points for HTF and CAP; 30, 45 and 60 min for 50 ng/mL PRL; 45 and 60 min for 250 ng/mL PRL; 5 and 60 min for 500 ng/mL PRL. For the HM subpopulation significantly lower percentages of hyperactivation was seen for HTF at 15 and 45 min (compared to all media); 30 min (compared to 50 ng/mL, 100 ng/mL and 500 ng/mL PRL) and 60 min (compared to 250 ng/mL PRL). In the LM subpopulation significantly lower percentages of hyperactivation was seen for HTF at 5, 30 and 45 min (compared to all the concentrations of PRL); 15 and 60 min (compared to all the media). LM subpopulation 250 ng/mL PRL additionally had significantly higher percentages of hyperactivation compared to CAP and HTF at 30 min. **Abbreviations**: ANOVA, analysis of variance between media for individual time intervals and subpopulations; CAP, capacitating-HTF; HM, high motile; HTF, human tubal fluid; LM, low motile; min, minutes; PRL, prolactin; SD, standard deviation and SP *p*, t test between subpopulations. $^{a, b,c,d}$ Values labelled with different superscript letters in the same row were significantly different between the various media for individual subpopulations and time points. One-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions was used. Values labelled in bold with an asterisk in the same column were significantly different between the HM and LM subpopulations for individual media and time points (*p <0.05, **p <0.01 and ***p <0.001). Student's t-test was used or the Mann-Whitney test when normal distribution was void.

To determine the interaction of collective and/or individual factors (time, subpopulation, or media) on hyperactivation, data for subpopulations were pooled and analysed in a mixed model ANOVA. Both "media" and "time" as an individual main factors had significant (p <0.01) interactions on hyperactivation percentages (Figure 4.49). The media displayed an early stimulatory (p =0.02) effect on hyperactivation at 5 minutes to 15 minutes, where after a significant decline (p <0.01) was observed (Figure 4.49 A). Thus, indicating that maximum hyperactivation was achieved at 15 minutes and that media had a rapid stimulatory effect on the spermatozoa which was sustained up to 15 minutes after which media no longer stimulated hyperactivation. CAP, progesterone and myo-inositol induced (p <0.01) hyperactivation as compared to HTF. However, both HD-C and 3.96 µM progesterone displayed the greatest stimulatory effect (p <0.01) (Figure 4.49 B).

Hyperactivation data for dopamine was pooled in a mixed model ANOVA and significant (p <0.01) interactions determined for collective factors such as "subpopulations and media", "subpopulations and time", and individual main factors of "media" and "time" (Figure 4.50). Dopamine induced (p <0.01) hyperactivation in HM subpopulations as compared to HTF, however 1 µM dopamine had the best stimulatory (p <0.01) effect as compared to all media. In contrast, for the LM subpopulation all dopamine concentrations increased (p <0.01) hyperactivation as compared to HTF and CAP, however 1 µM dopamine had significantly (p = 0.02) better values as compared to 20 nM dopamine (Figure 4.50 A). HM subpopulations displayed a sudden increase (p = 0.01) in hyperactivation from 5- to 15-minutes which was sustained until 30 minutes where after a steep decline (p <0.05 and p <0.01) was observed. The media thus had an early stimulatory effect on hyperactivation where after percentages could be maintained for up to 30 minutes. In contrast, LM subpopulations displayed an insignificant and minor trend of increase in hyperactivation from 5- to 30-minutes, and only experienced a steep significant (p <0.05 and p <0.01) decline from 45 minutes. LM subpopulations may therefore display a reduced response to the media as compared to HM subpopulations. Media and time displayed a significant (p <0.01) interaction on hyperactivation results (Figure 4.50 B). CAP displayed a sudden increase (p <0.01) in hyperactivation from 5- to 15-minutes, followed by a steep decline (p <0.05 and p <0.01) from 30 minutes. In contrast, dopamine maintained hyperactivation values in proximity over time with a gradual decrease from 45- to 60- minutes (Figure 4.50 C).

Hyperactivation data of prolactin was pooled in a mixed model ANOVA and significant (p <0.01) interactions determined for "media", "subpopulations and media", and "time" as

main factors (Figure 4.51). Although prolactin concentrations equally increased (p <0.01) hyperactivation as compared to HTF and CAP with "media" as the main factor (Figure 4.51 A), when looking at interactions of "media and subpopulations" as collective fixed factors, differences between subpopulations become evident (Figure 4.51 B). In HM subpopulations, both CAP and prolactin significantly increased (p <0.01) hyperactivation as compared to HTF. The same was observed in LM subpopulations, however difference between prolactin concentrations were seen. Prolactin significantly increased (p <0.01) hyperactivation as compared to HTF and CAP, however 250 ng/mL prolactin had a lower (p = 0.02) stimulatory effect on hyperactivation when compared to 100 ng/mL prolactin (Figure 4.51 B). Furthermore, a significant steep increase (p <0.01) in hyperactivation was observed from 5- to 15- minutes, where after steep decline (p <0.05 and p <0.01) was observed from 30- to 60- minutes (Figure 4.51 C). The media therefore had a rapid and early stimulatory effect, after which hyperactivation percentages were maintained from 15- to 30- minutes followed by a sudden decrease.

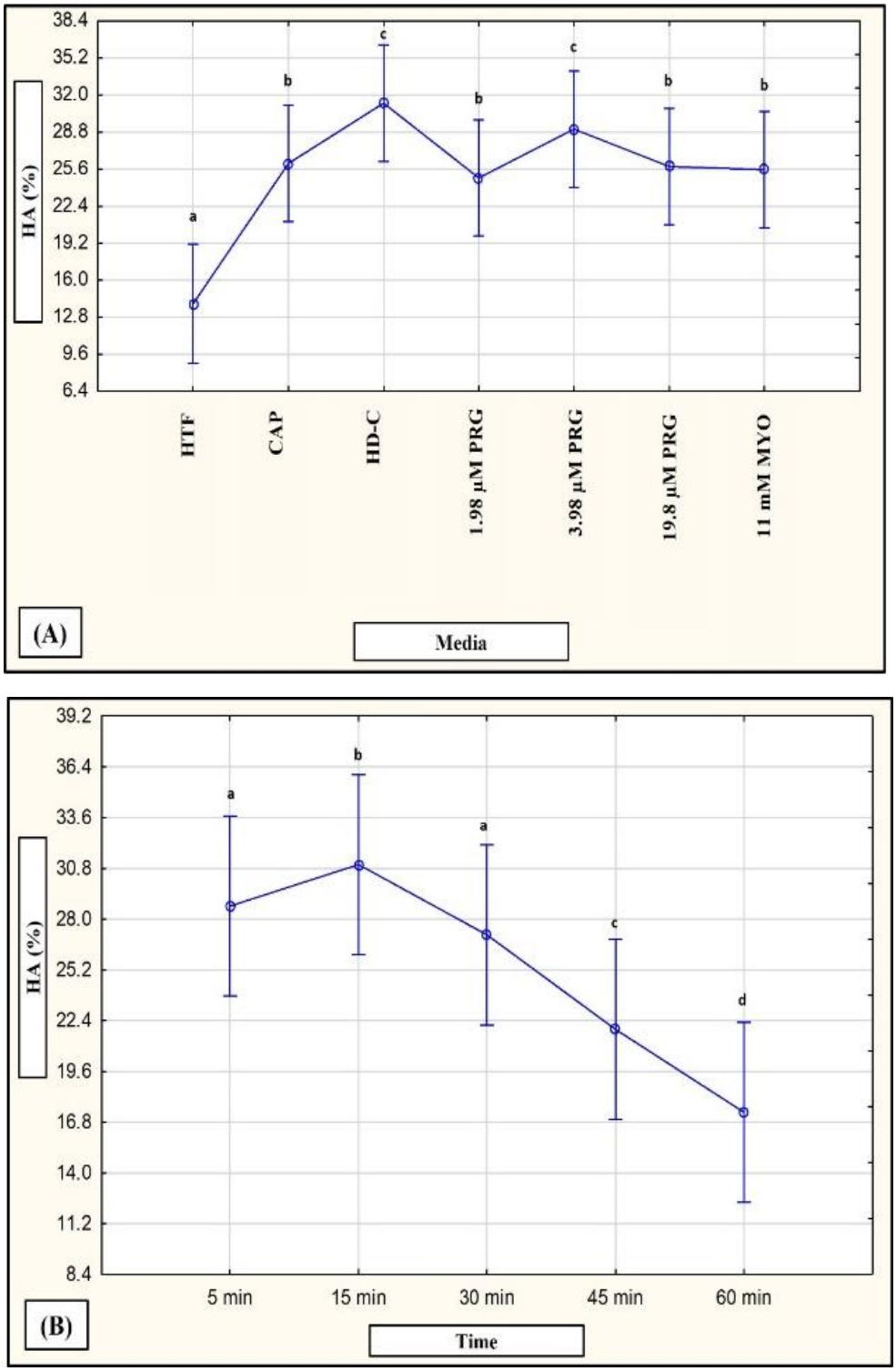


Figure 4.49 Mixed model ANOVA of repeated measures of pooled data from hyperactivation results for HD-C, progesterone, and myo-inositol in the high motile (HM) and low motile (LM) subpopulations (n = 20): **(A)** Mixed model ANOVA illustrating the interaction of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) on percentage sperm hyperactivation (F (6, 1252) = 33.06, p <0.01). **(B)** Mixed model ANOVA illustrating the interaction of time (5, 15, 30, 45 and 60 min) on percentage sperm hyperactivation for pooled data of HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM) (F (4, 1250) = 60.35, p <0.01) of both subpopulations. ***Note:*** (A) HD-C and 3.96 µM PRG significantly increased the percentage hyperactivation compared to all media, whereas CAP, 1.98 µM PRG, 19.8 µM PRG and 11 mM MYO significantly increased hyperactivation compared to the control (HTF). (B) Hyperactivation for pooled data of HTF, CAP, HD-C PRG and MYO significantly increased from 5 min to 15 min, where after a significant decline was seen from 30 min to 60 min. Vertical bars denote 0.95 confidence intervals. Vertical bars labelled with different letters were significantly different (p <0.01) (The Fisher LSD was used for the post hoc test and reports). **Abbreviations:** CAP, capacitating-HTF; HA, hyperactivation; HD-C, HD capacitating medium; HM, high motile; HTF, human tubal fluid; LM, low motile; min, minute; MYO, myo-inositol; PRG, progesterone.

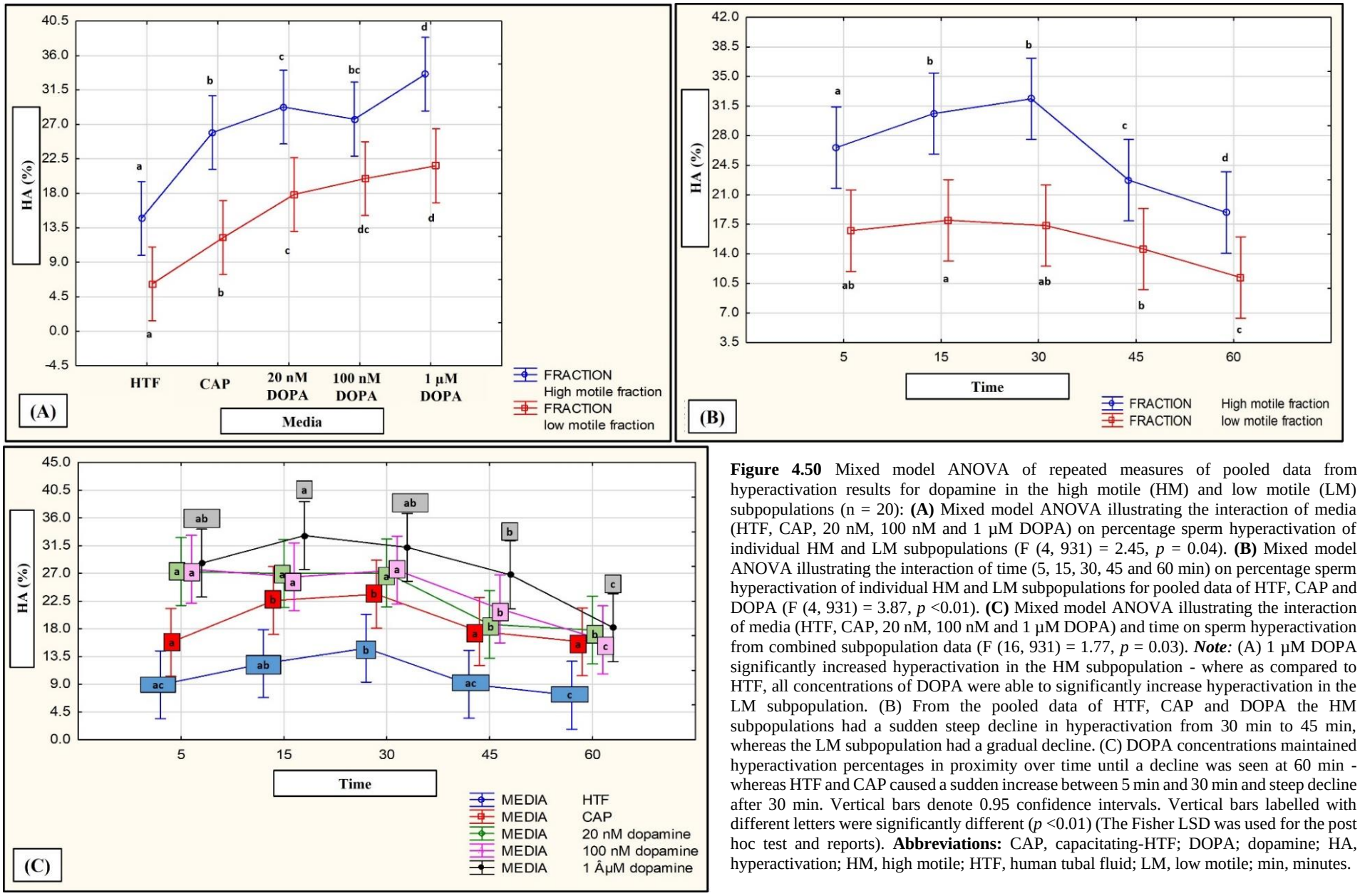
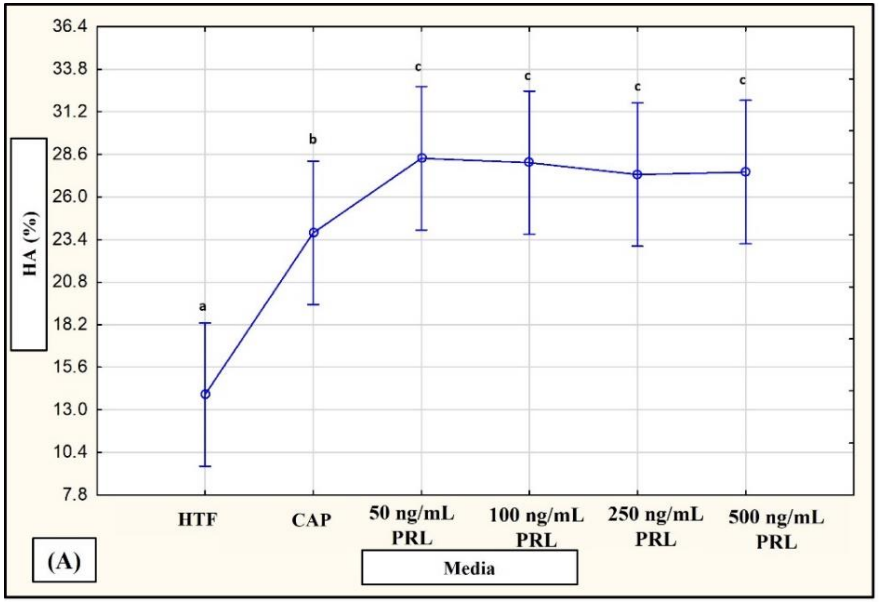
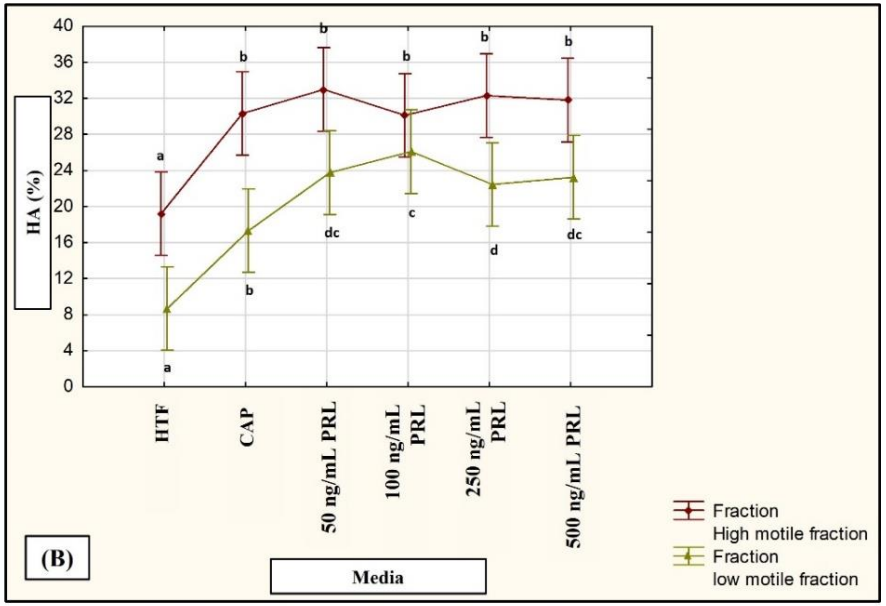


Figure 4.50 Mixed model ANOVA of repeated measures of pooled data from hyperactivation results for dopamine in the high motile (HM) and low motile (LM) subpopulations (n = 20): **(A)** Mixed model ANOVA illustrating the interaction of media (HTF, CAP, 20 nM, 100 nM and 1 µM DOPA) on percentage sperm hyperactivation of individual HM and LM subpopulations (F (4, 931) = 2.45, p = 0.04). **(B)** Mixed model ANOVA illustrating the interaction of time (5, 15, 30, 45 and 60 min) on percentage sperm hyperactivation of individual HM and LM subpopulations for pooled data of HTF, CAP and DOPA (F (4, 931) = 3.87, p <0.01). **(C)** Mixed model ANOVA illustrating the interaction of media (HTF, CAP, 20 nM, 100 nM and 1 µM DOPA) and time on sperm hyperactivation from combined subpopulation data (F (16, 931) = 1.77, p = 0.03). ***Note:*** (A) 1 µM DOPA significantly increased hyperactivation in the HM subpopulation - where as compared to HTF, all concentrations of DOPA were able to significantly increase hyperactivation in the LM subpopulation. (B) From the pooled data of HTF, CAP and DOPA the HM subpopulations had a sudden steep decline in hyperactivation from 30 min to 45 min, whereas the LM subpopulation had a gradual decline. (C) DOPA concentrations maintained hyperactivation percentages in proximity over time until a decline was seen at 60 min - whereas HTF and CAP caused a sudden increase between 5 min and 30 min and steep decline after 30 min. Vertical bars denote 0.95 confidence intervals. Vertical bars labelled with different letters were significantly different (p <0.01) (The Fisher LSD was used for the post hoc test and reports). **Abbreviations:** CAP, capacitating-HTF; DOPA; dopamine; HA, hyperactivation; HM, high motile; HTF, human tubal fluid; LM, low motile; min, minutes.





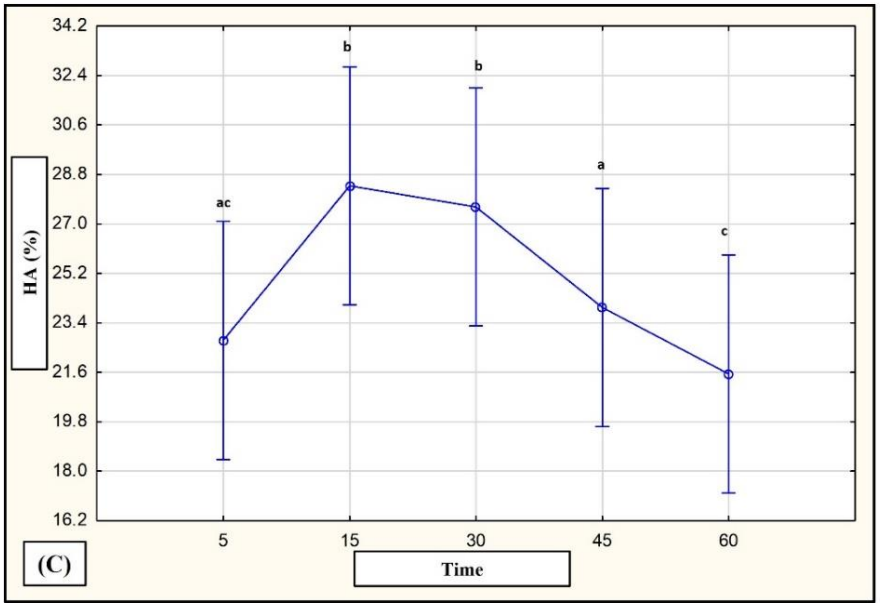


Figure 4.51 Mixed model ANOVA of repeated measures of pooled data from hyperactivation results for prolactin in the high motile (HM) and low motile (LM) subpopulations (n = 20): **(A)** Mixed model ANOVA illustrating the significant interaction of media (HTF, CAP, 50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL PRL) on percentage sperm hyperactivation (F (5, 1357) = 52.15, p <0.01). **(B)** Mixed model ANOVA illustrating the significant interaction of media (HTF, CAP and prolactin) and subpopulations on sperm hyperactivation (F (5, 1357) = 3.66, p <0.01). **(C)** Mixed model ANOVA illustrating the interaction of time (5, 15, 30, 45 and 60 min) on percentage sperm hyperactivation for pooled data of HTF, CAP and PRL (F (4, 1357) = 18.09, p <0.01). ***Note:*** (A) All concentrations of PRL were able to significantly improve hyperactivation compared to HTF and CAP media. (B) All concentrations of PRL equally stimulated the HM subpopulation, whereas 100 ng/mL PRL had a trend of displaying the best stimulatory effects in the LM subpopulation. (C) For pooled data of HTF, CAP and PRL - hyperactivation significantly increased from 5 to 30 min, after which a significant decline occurred at 45- and 60-min. Vertical bars denote 0.95 confidence intervals. Vertical bars labelled with different letters were significantly different (p <0.01) (The Fisher LSD was used for the post hoc test and reports). **Abbreviations:** CAP, capacitating-HTF; HA, hyperactivation; HM, high motile; HTF, human tubal fluid; LM, low motile; min, minutes; PRL, prolactin.

4.2.7 | Reactive Oxygen Species

Observed in Supplementary tables 5 - 7 and Figure 4.52 are the percentages reactive oxygen species (ROS) positive spermatozoa for the HM and LM subpopulations after treatment with HTF, CAP, HD-C, progesterone, myo-inositol, dopamine, and prolactin. The LM subpopulations comprised of significantly (p <0.05 and p <0.01) higher percentages of ROS positive spermatozoa as compared to the HM subpopulations. However, after treatment with specific media, ROS percentages could be decreased in LM subpopulations until significant differences between subpopulations were no longer observed. This trend of improvement was observed for 100 nM and 1 µM dopamine (Figure 4.52 B), 50 ng/mL, 100 ng/mL and 250 ng/mL prolactin (Figure 4.8 & Figure 4.52 C).

Significant differences between the media for individual subpopulations was only observed between prolactin and HTF in the LM subpopulation. Prolactin concentrations had significantly (p = 0.008) decreased ROS levels as compared to HTF (Figure 4.52 C). Furthermore, data for subpopulations was pooled in a multifactorial ANOVA and significant interactions between media, subpopulations and ROS percentages calculated (Figure 4.53). Subpopulations had a significant (p <0.001) interaction on ROS percentages, that is, the HM subpopulations had significantly lower ROS positive spermatozoa as compared to the LM subpopulations (Figure 4.53 A-C). Media had a significant interaction on ROS percentages for HD-C, progesterone, and myo-inositol (p = 0.0059) (Figure 4.53 A), and prolactin (p <0.001) (Figure 4.53 C), but not for dopamine (Figure 4.53 B). Compared to HTF, 1.98 µM and 19.8 µM progesterone, HD-C, and 11 mM myo-inositol significantly (p = 0.0059) decreased ROS in spermatozoa (Figure 4.53 A), whereas all prolactin concentrations decreased (p <0.001) ROS as compared to HTF (Figure 4.53 C). Furthermore, despite being statistically insignificant, it should be noted that 20 nM dopamine had a trend of lower ROS values as compared to HTF, CAP and other dopamine concentrations (Figure 4.53 B).

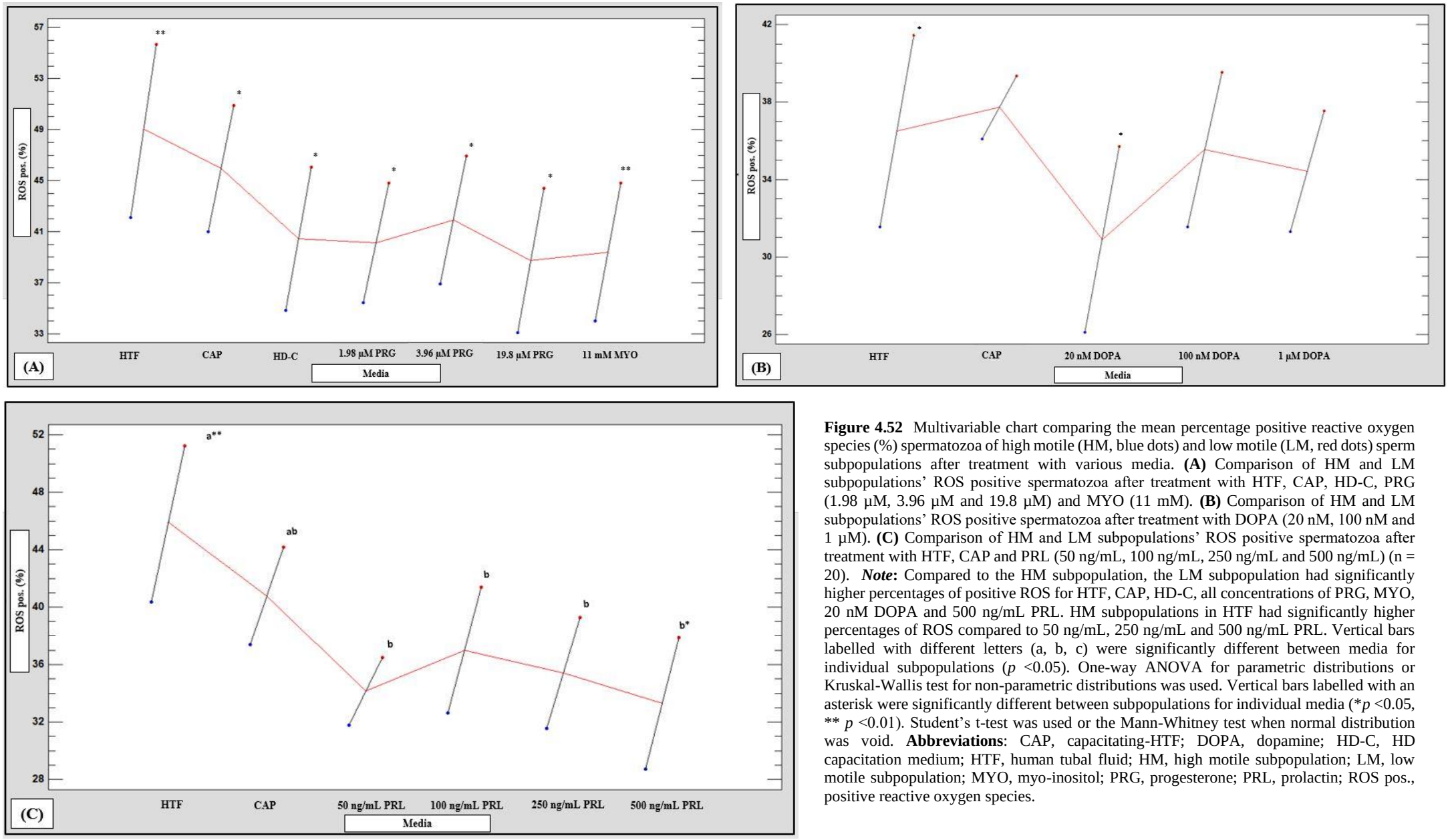


Figure 4.52 Multivariable chart comparing the mean percentage positive reactive oxygen species (%) spermatozoa of high motile (HM, blue dots) and low motile (LM, red dots) sperm subpopulations after treatment with various media. **(A)** Comparison of HM and LM subpopulations' ROS positive spermatozoa after treatment with HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM). **(B)** Comparison of HM and LM subpopulations' ROS positive spermatozoa after treatment with DOPA (20 nM, 100 nM and 1 µM). **(C)** Comparison of HM and LM subpopulations' ROS positive spermatozoa after treatment with HTF, CAP and PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) (n = 20). ***Note*:** Compared to the HM subpopulation, the LM subpopulation had significantly higher percentages of positive ROS for HTF, CAP, HD-C, all concentrations of PRG, MYO, 20 nM DOPA and 500 ng/mL PRL. HM subpopulations in HTF had significantly higher percentages of ROS compared to 50 ng/mL, 250 ng/mL and 500 ng/mL PRL. Vertical bars labelled with different letters (a, b, c) were significantly different between media for individual subpopulations (p <0.05). One-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions was used. Vertical bars labelled with an asterisk were significantly different between subpopulations for individual media (*p <0.05, ** p <0.01). Student's t-test was used or the Mann-Whitney test when normal distribution was void. **Abbreviations**: CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitation medium; HTF, human tubal fluid; HM, high motile subpopulation; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; ROS pos., positive reactive oxygen species.

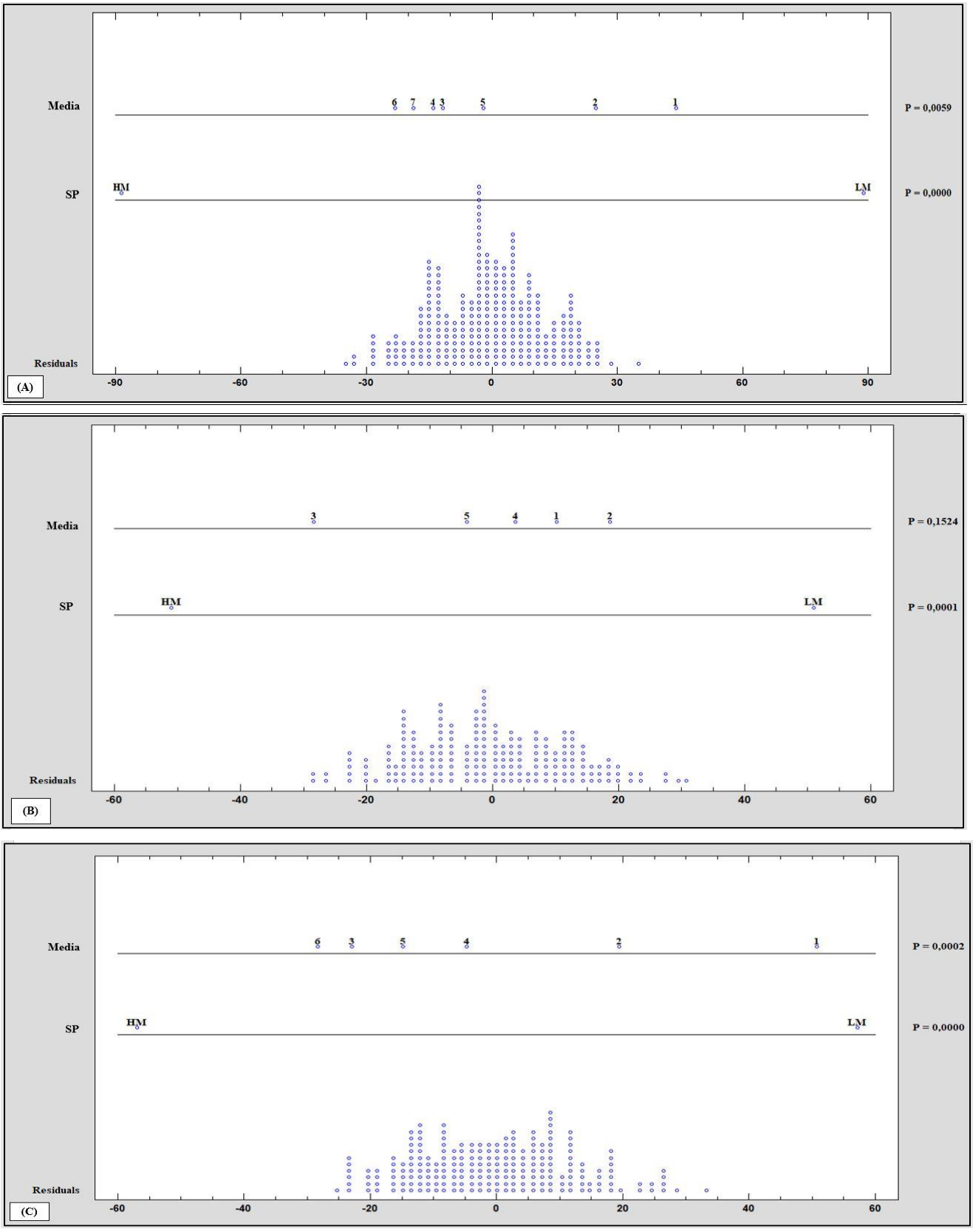


Figure 4.53 Multifactorial graphical ANOVA of the pooled data from the high motile (HM) and low motile (LM) sperm subpopulations percentage reactive oxygen species (ROS) positive spermatozoa. **(A)** Multifactorial graphical ANOVA displaying the significant interactions of subpopulations and media on ROS percentages after treatment with HTF (1), CAP (2), HD-C (3), 1.98 µM PRG (4), 3.96 µM PRG (5), 19.8 µM PRG (6) and 11 mM MYO (7). **(B)** Multifactorial graphical ANOVA displaying the significant interactions of subpopulations and media on ROS percentages after treatment with HTF (1), CAP (2), 20 nM DOPA (3), 100 nM DOPA (4) and 1 µM DOPA (5). **(C)** Multifactorial graphical ANOVA displaying the significant interactions of subpopulations and media on ROS percentages after treatment with HTF (1), CAP (2), 50 ng/mL PRL (3) 100 ng/mL PRL (4), 250 ng/mL PRL (5) and 500 ng/mL PRL (6) ***Note:*** The Graphical ANOVA plot shows the effects of each factor scaled so that they can be compared to the variability of the residuals. For each factor, the deviations of the adjusted level means from the estimated grand mean are displayed. Any factor that shows considerably larger variability than the residuals is likely to be an important factor. Student-Newman-Keuls was used for the post hoc test. **Abbreviations:** ANOVA, analysis of variance; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitation medium; HM, high motile subpopulation; HTF, human tubal fluid; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin.; ROS, positive reactive oxygen species

4.2.8 | Mitochondrial Membrane Potential

Observed in Supplementary tables 5 - 7 and Figure 4.54 are the percentages intact mitochondrial membrane potential (MMP) spermatozoa for the HM and LM subpopulations after treatment with HTF, CAP, HD-C, progesterone, myo-inositol, dopamine, and prolactin. The HM subpopulations had significantly (p <0.05, p <0.01 and p <0.001) higher percentages of spermatozoa with intact MMP as compared to LM subpopulations. Despite comprising of significantly lower percentages of MMP intact spermatozoa, after incubation in dopamine and prolactin LM subpopulation MMP percentages could be improved to the point that significant differences were no longer observed.

This trend was observed for 1 µM dopamine (Figure 4.8 & Figure 4.54 B), and 50 ng/mL, 250 ng/mL and 500 ng/mL prolactin (Figure 4.8 & Figure 4.54 C). No significant differences between the media were seen in HM subpopulations. In contrast, for the LM subpopulations both myo-inositol (p = 0.048) and prolactin (50 ng/mL, 250 ng/mL and 500 ng/mL prolactin; p = 0.012) significantly maintained higher percentages of intact MMP as compared to HTF (Figure 4.54 A & C). Data of media and subpopulations were pooled and analysed in a multifactorial ANOVA to determine significant interactions between media, subpopulations and MMP intactness (Figure 4.55). A significant (p <0.001) interaction was observed between subpopulations and MMP intactness – that is, HM subpopulations had significantly higher percentages of MMP intact spermatozoa as compared to LM subpopulations. Furthermore, a significant interaction was observed between media and percentages of MMP intactness for HD-C, progesterone, myo-inositol (Figure 4.55 A) and prolactin (Figure 4.55 C). Compared to both HTF and CAP, myo-inositol significantly (p = 0.0153) maintained higher MMP intactness (Figure 4.55 A). Whereas, compared to HTF, 500 ng/mL prolactin significantly (p = 0.0037) maintained higher MMP percentages (Figure 4.55 C). Furthermore, despite being statistically insignificant, it should be noted that 20 nM and 100 nM dopamine had a trend of displaying higher MMP intactness percentages as compared to HTF, CAP and 1 µM dopamine (Figure 4.55 B).

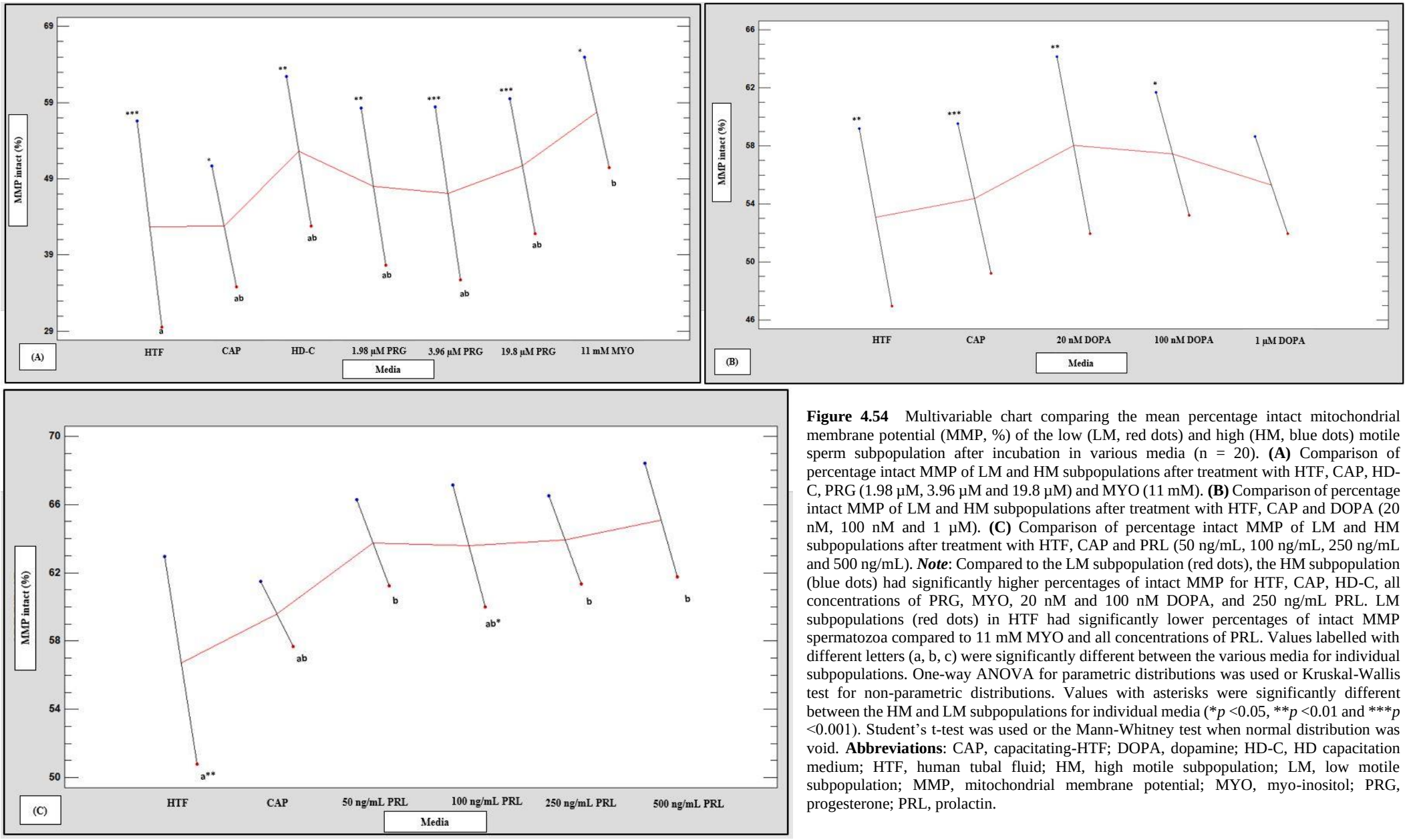


Figure 4.54 Multivariable chart comparing the mean percentage intact mitochondrial membrane potential (MMP, %) of the low (LM, red dots) and high (HM, blue dots) motile sperm subpopulation after incubation in various media (n = 20). **(A)** Comparison of percentage intact MMP of LM and HM subpopulations after treatment with HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM) and MYO (11 mM). **(B)** Comparison of percentage intact MMP of LM and HM subpopulations after treatment with HTF, CAP and DOPA (20 nM, 100 nM and 1 µM). **(C)** Comparison of percentage intact MMP of LM and HM subpopulations after treatment with HTF, CAP and PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL). ***Note***: Compared to the LM subpopulation (red dots), the HM subpopulation (blue dots) had significantly higher percentages of intact MMP for HTF, CAP, HD-C, all concentrations of PRG, MYO, 20 nM and 100 nM DOPA, and 250 ng/mL PRL. LM subpopulations (red dots) in HTF had significantly lower percentages of intact MMP spermatozoa compared to 11 mM MYO and all concentrations of PRL. Values labelled with different letters (a, b, c) were significantly different between the various media for individual subpopulations. One-way ANOVA for parametric distributions was used or Kruskal-Wallis test for non-parametric distributions. Values with asterisks were significantly different between the HM and LM subpopulations for individual media (*p <0.05, **p <0.01 and ***p <0.001). Student's t-test was used or the Mann-Whitney test when normal distribution was void. **Abbreviations**: CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitation medium; HTF, human tubal fluid; HM, high motile subpopulation; LM, low motile subpopulation; MMP, mitochondrial membrane potential; MYO, myo-inositol; PRG, progesterone; PRL, prolactin.

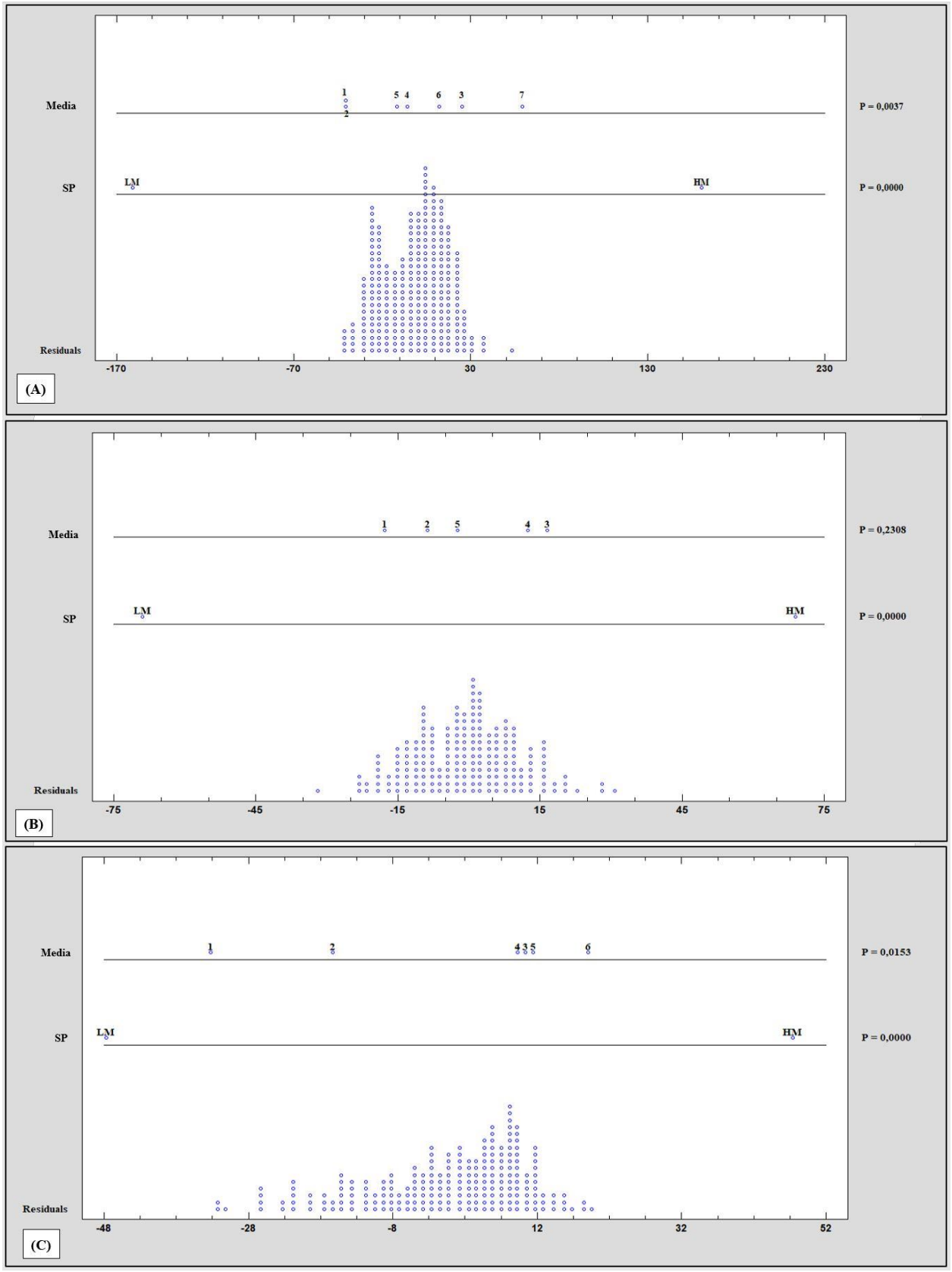


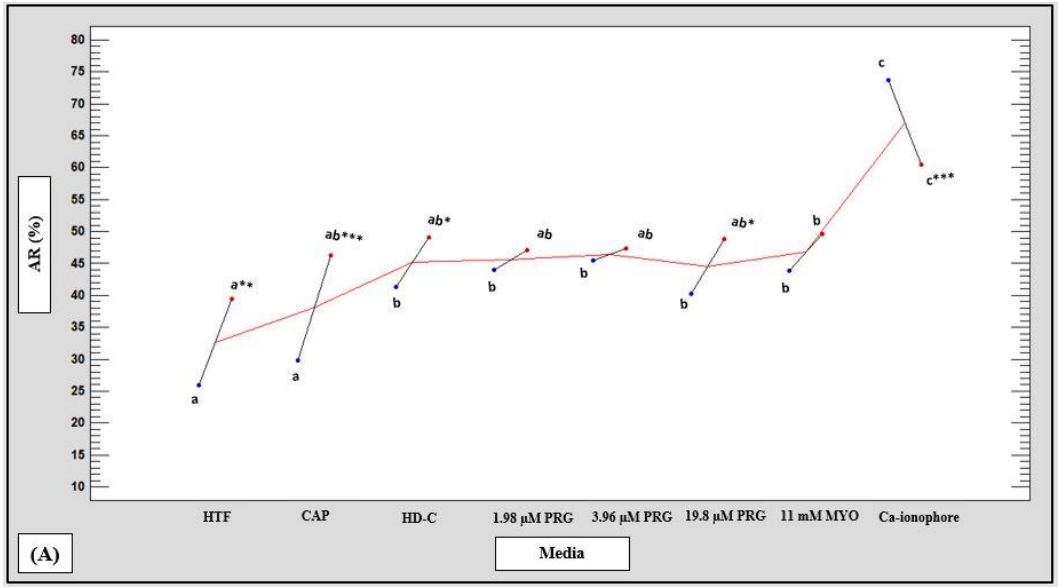
Figure 4.55 Multifactorial graphical ANOVA of the pooled data from the high motile (HM) and low motile (LM) sperm subpopulations percentage intact mitochondrial membrane potential (MMP). **(A)** Multifactorial graphical ANOVA displaying the significant interactions of subpopulations and media on MMP intactness percentages after treatment with HTF (1), CAP (2), HD-C (3), 1.98 µM PRG (4), 3.96 µM PRG (5), 19.8 µM PRG (6) and 11 mM MYO (7). **(B)** Multifactorial graphical ANOVA displaying the significant interactions of subpopulations and media on MMP intactness percentages after treatment HTF (1), CAP (2), 20 nM DOPA (3), 100 nM DOPA (4) and 1 µM DOPA (5). **(C)** Multifactorial graphical ANOVA displaying the significant interactions of subpopulations and media on MMP intactness percentages after treatment with HTF (1), CAP (2), 50 ng/mL PRL (3) 100 ng/mL PRL (4), 250 ng/mL PRL (5) and 500 ng/mL PRL (6) ***Note:*** The Graphical ANOVA plot shows the effects of each factor scaled so that they can be compared to the variability of the residuals. For each factor, the deviations of the adjusted level means from the estimated grand mean are displayed. Any factor that shows considerably larger variability than the residuals is likely to be an important factor. Student-Newman-Keuls was used for the post hoc test. **Abbreviations:** ANOVA, analysis of variance; CAP, capacitating-HTF; HD-C, HD capacitation medium; HM, high motile subpopulation; HTF, human tubal fluid; LM, low motile subpopulation; MMP, mitochondrial membrane intactness; MYO, myo-inositol; PRG, progesterone; PRL, prolactin and SP, subpopulations.

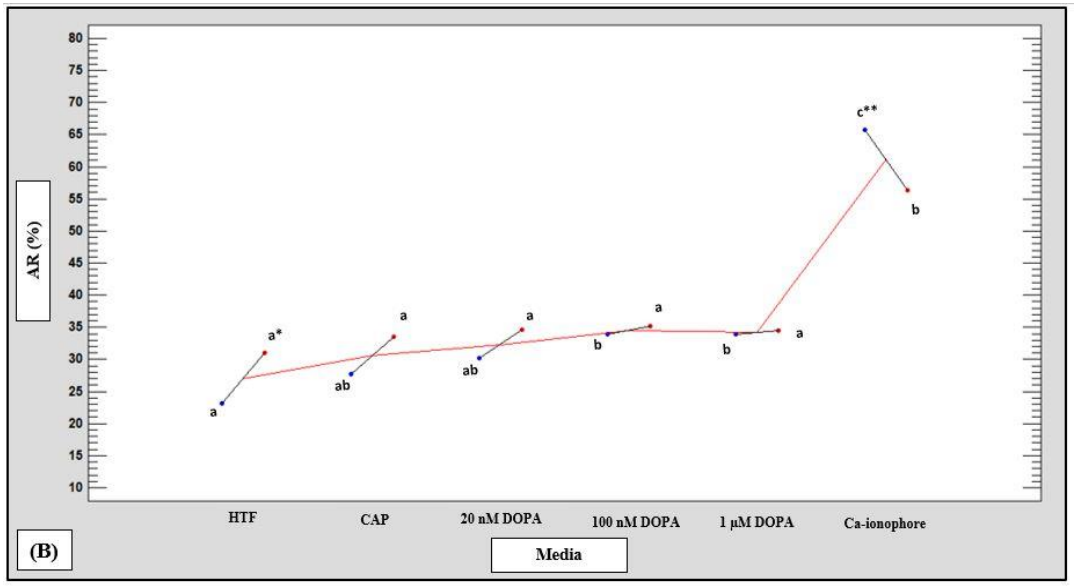
4.2.9 | Acrosome Reaction

Seen in Supplementary tables 5 - 7 and Figure 4.56 are the percentages acrosome reacted spermatozoa for both subpopulations after treatment with HTF, CAP, HD-C, progesterone, myo-inositol, dopamine, prolactin and calcium-ionophore. LM subpopulations had significantly higher percentages of spontaneous and induced acrosome reaction after treatment with HTF, CAP, HD-C, progesterone (Figure 4.56 A) and prolactin (Figure 4.56 C). In contrast, HM subpopulations had significantly higher percentages of calcium-ionophore induced acrosome reaction. After treatment with progesterone (1.98 µM and 3.96 µM), myo-inositol and dopamine, percentage acrosome reaction in the HM subpopulation could be increased to the point that significant differences between subpopulations were no longer observed (Figure 4.56 A & B).

Significant difference between media was more isolated to the HM subpopulations as compared to the LM subpopulations; however, for both subpopulations calcium ionophore consistently obtained significantly (p <0.001) higher percentages of acrosome reacted spermatozoa as compared to other media. Both progesterone and myo-inositol increased acrosome reaction in HM subpopulations as compared to HTF and CAP, whereas in LM subpopulations only myo-inositol increased acrosome reaction as compared to HTF (Figure 4.56 A). Furthermore both 100 nM and 1 µM dopamine significantly (p <0.001) increased acrosome reaction in HM subpopulations as compared to HTF, whereas dopamine did not induce acrosome reaction in the LM subpopulations (Figure 4.56 B). Moreover, prolactin did not stimulate acrosome reaction in either of the subpopulations (Figure 4.56 C).

Data for acrosome reactions were pooled and analysed in a multifactorial ANOVA to determine significant interactions between media, subpopulations and acrosome reaction (Figure 4.57). Subpopulations had a significant (p <0.001) interaction on percentage acrosome reaction (Figure 4.57 A & C). HM subpopulations presented with significantly lower percentages of acrosome reaction as compared to LM subpopulations. Furthermore, compared to HTF and CAP, progesterone and myo-inositol significantly (p <0.001) increased the acrosome reaction (Figure 4.57 A). In addition, 100 nM and 1 µM dopamine significantly (p <0.001) increased acrosome reaction as compared to HTF (Figure 4.57 B).





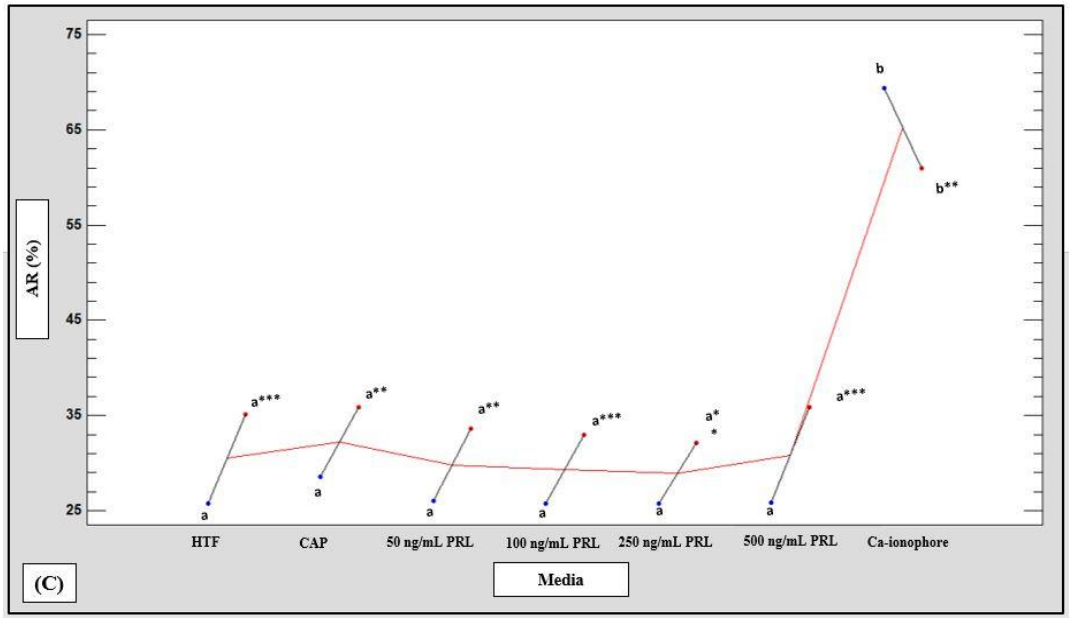


Figure 4.56 Multivariable chart displaying the percentage acrosome reaction (AR, %) of the low (LM, red dots) and high (HM, blue dots) motile sperm subpopulation after incubation in various media. **(A)** Comparison of percentage AR for the LM and HM motile sperm subpopulation after incubation in HTF, CAP, HD-C, PRG (1.98 µM, 3.96 µM and 19.8 µM), MYO (11 mM) and Ca-ionophore (1 mM). **(B)** Comparison of percentage AR for the LM and HM motile sperm subpopulation after incubation in HTF, CAP and DOPA (20 nM, 100 nM and 1 µM) and Ca-ionophore (1 mM). **(C)** Comparison of percentage AR for the LM and HM motile sperm subpopulation after incubation in PRL (50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL) and Ca-ionophore (1 mM) (n = 20). ***Note*:** Compared to the HM subpopulation, the LM subpopulation had significantly higher percentages of AR for HTF, CAP, HD-C, 19.8 µM PRG, 50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL PRL. Ca-ionophore in both subpopulations had significantly greater percentages of AR compared to all media. HM subpopulations in HTF had significantly lower percentages of AR compared to HD-C, all concentrations of PRG, MYO, 100 nM and 1µM DOPA. Values labelled with different letters (a, b, c) were significantly different between the various media for individual subpopulations (one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions). One-way ANOVA for parametric distributions was used or Kruskal-Wallis test for non-parametric distributions. Values with asterisks were significantly different between the HM and LM subpopulations for individual media (**p* <0.05, ***p* <0.01 and ****p* <0.001). Student's t-test was used or the Mann-Whitney test when normal distribution was void. **Abbreviations:** AR, acrosome reaction; Ca-ionophore, calcium-ionophore; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitation media; HTF, human tubal fluid; HM, high motile subpopulation; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin.

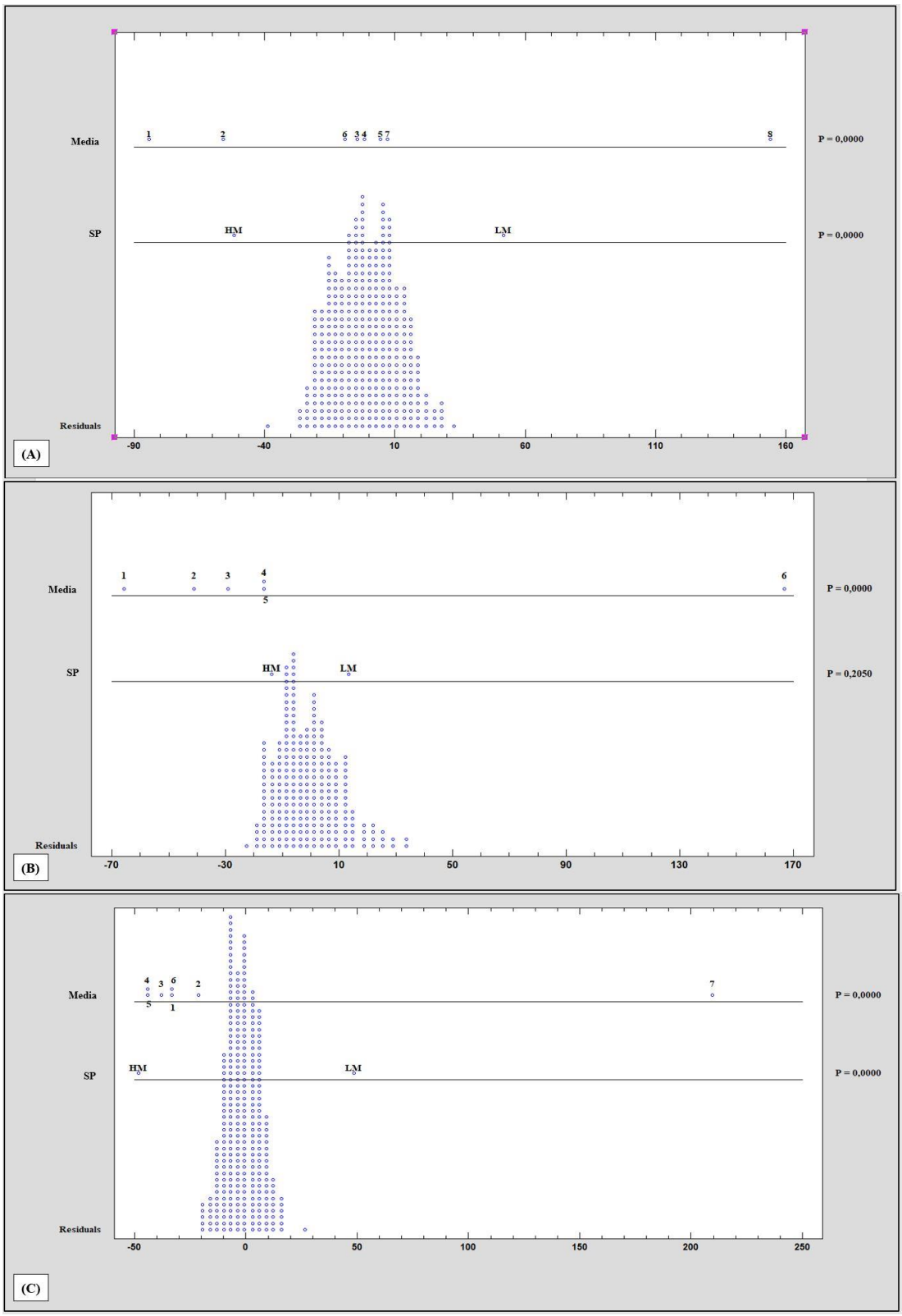


Figure 4.57 Multifactorial graphical ANOVA of the pooled data from the high motile (HM) and low motile (LM) sperm subpopulations percentage acrosome reaction (AR). **(A)** Multifactorial graphical ANOVA displaying the significant interactions of subpopulations and media on AR percentages after treatment with HTF (1), CAP (2), HD-C (3), 1.98 µM PRG (4), 3.96 µM PRG (5), 19.8 µM PRG (6), 11 mM MYO (7) and Ca-ionophore (8). **(B)** Multifactorial graphical ANOVA displaying the significant interactions of subpopulations and media on AR percentages after treatment with HTF (1), CAP (2), 20 nM DOPA (3) 100 nM DOPA (4), 1 µM DOPA (5) and Ca-ionophore (6). **(C)** Multifactorial graphical ANOVA displaying the significant interactions of subpopulations and media on AR percentages after treatment with HTF (1), CAP (2), 50 ng/mL PRL (3) 100 ng/mL PRL (4), 250 ng/mL PRL (5), 500 ng/mL PRL (6) and Ca-ionophore (7). ***Note:*** The Graphical ANOVA plot shows the effects of each factor scaled so that they can be compared to the variability of the residuals. For each factor, the deviations of the adjusted level means from the estimated grand mean are displayed. Any factor that shows considerably larger variability than the residuals is likely to be an important factor. Student-Newman-Keuls was used for the post hoc test. **Abbreviations:** ANOVA, analysis of variance; AR, acrosome reaction; CAP, capacitating-HTF; Ca-ionophore, calcium ionophore; DOPA, dopamine; HD-C, HD capacitation medium; HM, high motile subpopulation; HTF, human tubal fluid; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin and SP, subpopulations.

4.3 | Results of Phase Three of the Study (CASA and FAST)

4.3.1 | Basic Semen Analysis

Illustrated in Table 4.13 are the average basic semen analysis results of the selected semen samples used for the third phase of the study. The mean values of all the semen parameters were above the lower reference values according to the WHO laboratory manual (WHO, 2021). As the main aim of the third phase of the study was to determine the effects of selected media on flagellar characteristics of different functional quality of spermatozoa, selected semen samples had a large variation in quality of semen parameters, thereby resulting in the large range of values as indicated in Table 4.13.

Table 4.13 Basic semen parameters of selected semen samples used in this investigation (mean ± SD) (n=40).

	Mean	±	SD	95% C.I		
Total Motility (%)	65.1	±	22.5	55.1	-	75.0
Progressive motility (%)	46.0	±	22.0	36.2	-	55.8
Viscosity (cP)	8.5	±	11.0	3.6	-	13.3
Mucus penetration (10^6/ejaculate)	30.8	±	25.7	18.8	-	42.8
Concentration (10^6/ejaculate)	95.7	±	62.9	66.3	-	125.2
Concentration (10^6/mL)	69.7	±	74.3	36.7	-	102.7
Volume (mL)	2.9	±	1.2	2.3	-	3.4
Vitality (%)	66.6	±	15.9	59.5	-	73.7
Normal (%)	4.5	±	4.2	2.6	-	6.4
Head defects (%)	89.1	±	9.4	84.8	-	93.3
Midpiece defects (%)	54.8	±	15.3	47.8	-	61.7
Tail defects (%)	39.1	±	17.1	31.3	-	46.8
Cytoplasmic droplets (%)	13.5	±	8.6	9.6	-	17.5
TZI	2.1	±	0.2	2.0	-	2.1
MAI	2.6	±	0.5	2.4	-	2.8
DI	2.5	±	0.4	2.3	-	2.7
Normal head size (%)	16.3	±	10.0	11.7	-	20.8
Micro (%)	3.4	±	2.9	2.0	-	4.7
Macro (%)	23.3	±	9.2	19.1	-	27.5
Normal head shape (%)	81.4	±	7.7	77.9	-	84.9

Note: The mean semen parameters fall above the lower reference limits as recommended by WHO (2021). **Abbreviations:** C.I, confidence interval; cP, centipoise; DI, deformity index; SD, standard deviation; TZI, teratozoospermic index; MAI, multiple abnormalities index.

4.3.2 | Whole Sperm Subpopulation Motility Parameters

Significant differences between the subpopulations motility parameters were observed with the HM subpopulation displaying significantly improved results as compared to the LM subpopulation. However, dopamine and CAP were able to improve LM subpopulations until some significant differences were no longer observed. Significant differences between the media were predominantly observed in the LM subpopulations for dopamine and prolactin, but in the HM subpopulations for HD-C, progesterone and myo-inositol. Thus, similar to the results observed in both phases one and two of the study. It should be noted that due to time limitations during the analysis, subpopulations were only exposed to the media for 10-15 minutes. As such, LM subpopulations were not improved to the extent observed in phases two of the study where exposure was >30 minutes. Nevertheless, media displayed a similar trend as observed in phases two of the investigation.

Progesterone, HD-C and myo-inositol appeared to predominantly have an effect on the HM subpopulations velocity and progressivity groups (Table 4.14). Whereas dopamine and prolactin appeared to affect all motility parameters of LM subpopulations as was observed in phases two of the investigation (Table 4.15). In the HM subpopulation, myo-inositol decreased the medium speed groups as compared to all media, while increasing the RP group as compared to HD-C. However, in the HM subpopulation, CAP, dopamine and prolactin all significantly increased medium speeds as compared to HTF. Interestingly, HD-C appeared to increase the slow speeds as compared to HTF. In the LM subpopulations, dopamine increased the progressive motility as compared to HTF and 500 ng/mL prolactin. This was further observed in total motility where dopamine was significantly higher as compared to 500 ng/mL prolactin. In fact, it was noted that majority of differences were observed between 500 ng/mL prolactin and dopamine of which the former was significantly lower. Nonetheless dopamine in the LM subpopulation, significantly increased progressive and velocity groups as compared to HTF and frequently as compared to 500 ng/mL prolactin (Supplementary Figure 1).

4.3.3 | Whole Sperm Subpopulation Kinematic Parameters

From here on forward for better understanding of kinematic parameters – curvilinear velocity (VCL), average path velocity (VAP) and straight-line velocity (VSL) will be grouped as "velocity" kinematics; linearity (LIN) and straightness (STR) as "linear"; and amplitude of lateral head displacement (ALH), wobble (WOB) and beat cross frequency (BCF) as "vigour" kinematics.

4.3.3.1 | Velocity

Significant differences between the subpopulations' velocity kinematic parameters were observed with the HM subpopulation displaying significantly higher values as compared to the LM subpopulation (Table 4.14 & 4.15). However, CAP, myo-inositol, dopamine and 250 ng/mL prolactin improved LM subpopulations until some significant differences were no longer observed. Furthermore, the majority of significant differences in media appeared to only be displayed in the HM subpopulations as compared to LM subpopulations. In the HM subpopulations, HD-C decreased the motile velocity kinematics as compared to HTF and myo-inositol, while CAP and dopamine increased them. In LM subpopulations, CAP and dopamine increased motile velocity as compared to HTF, however 500 ng/mL prolactin had decreased values as compared to dopamine and CAP (Supplementary Figure 2). In contrast to motile velocity kinematics, HD-C and progesterone increased the NP velocity kinematics of the HM subpopulation as compared to CAP and myo-inositol, whereas no effect was seen for prolactin and dopamine or in LM subpopulations (Supplementary Figure 3). In HM subpopulations, CAP, HD-C, myo-inositol and dopamine influenced (both decreased and increased) MP velocity kinematics as compared to HTF (Supplementary Figure 4). Moreover, dopamine had significantly higher values as compared to 500 ng/mL prolactin. Both CAP and HD-C had lower RP velocity kinematics as compared to HTF in the HM subpopulation. Whereas in LM subpopulations, dopamine increased values as compared to HTF, CAP and prolactin (Supplementary Figure 5).

4.3.3.2 | Linear

Significant differences between the subpopulations linear kinematic parameters were observed with the HM subpopulation displaying significantly decreased linear swimming patterns as compared to the LM subpopulation (Table 4.14 & 4.15). In the HM subpopulation,

250 ng/mL prolactin decreased motile linear kinematics as compared to HTF, whereas both dopamine and 500 ng/mL decreased the LM subpopulations (Supplementary Figure 2). Compared to dopamine and CAP, 250 ng/mL prolactin decreased HM subpopulation NP linear kinematics, whereas HD-C, dopamine and 500 ng/mL prolactin decreased the LM subpopulations as compared to HTF (Supplementary Figure 3). CAP, myo-inositol, dopamine and prolactin decreased HM subpopulation MP linear kinematics as compared to HTF. In the LM subpopulation both CAP and myo-inositol decreased MP linear kinematics as compared to HTF (Supplementary Figure 4). Furthermore, compared to HTF the HM subpopulation RP linear kinematics were decreased by CAP, dopamine and prolactin. In the LM subpopulation, myo-inositol decreased RP linear kinematics as compared to HTF, whereas CAP increased them (Supplementary Figure 5).

4.3.3.3 | Vigour

Significant differences between the subpopulations vigour kinematic parameters were observed with the HM subpopulation displaying significantly increased vigour swimming patterns as compared to the LM subpopulation (Table 4.14 & 4.15). CAP, myo-inositol, dopamine and prolactin decreased the HM subpopulation motile vigour kinematics while increasing ALH. In the LM subpopulations, only dopamine and 250 ng/mL prolactin increased motile ALH as compared to 500 ng/mL prolactin (Supplementary Figure 2). No effects were seen in either subpopulations NP vigour kinematic parameters (Supplementary Figure 3). CAP, HD-C, progesterone, myo-inositol, dopamine and prolactin decreased HM subpopulation MP vigour kinematics, however CAP, myo-inositol, dopamine and 250 ng/mL prolactin increased MP ALH (Supplementary Figure 4). CAP and myo-inositol decreased LM subpopulation vigour kinematics. RP vigour kinematics decreased in the HM subpopulations by CAP, dopamine and prolactin, whereas in LM subpopulations CAP, dopamine and 250 ng/mL prolactin decreased values. In contrast, RP ALH was increased in both subpopulations by dopamine (Supplementary Figure 5).

Table 4.14 Comparison of SCA motility and kinematic parameters (mean ± SD) (n=20) for whole sperm motility subpopulations treated with HTF, CAP, HD-C, 3.96 µM progesterone and 11 mM myo-inositol.

	HTF				CAP				HD-C				3.96 µM Progesterone				11 mM Myo-inositol				
	Mean	±	SD	SP *p*	Mean	±	SD	SP *p*	Mean	±	SD	SP *p*	Mean	±	SD	SP *p*	Mean	±	SD	SP *p*	ANOVA
Tot. Mot (%)																					
HM	**70.2**	±	**19.8*****	**<0.001**	**68.9**	±	**19.6*****	**<0.001**	**70.5**	±	**17.2*****	**<0.001**	**71.0**	±	**20.2*****	**<0.001**	**71.6**	±	**20.6*****	**<0.001**	0.995
LM	**36.3**	±	**18.5*****		**39.1**	±	**18.1*****		**32.7**	±	**15.4*****		**35.7**	±	**24.3*****		**39.5**	±	**19.8*****		0.798
Prog (%)																					
HM	**54.5**	±	**25.6*****	**<0.001**	**55.9**	±	**24.1*****	**<0.001**	**47.1**	±	**17.1*****	**<0.001**	**54.7**	±	**20.8*****	**<0.001**	**57.9**	±	**23.7*****	**<0.001**	0.62
LM	**21.3**	±	**15.3*****		**28.3**	±	**17.5*****		**19.5**	±	**14.6*****		**23.4**	±	**21.1*****		**28.7**	±	**21.6*****		0.407
RP (%)																					
HM	**27.0**	±	**14.8**$^{ab\ ***}$	**<0.001**	**29.0**	±	**14.7**^{ab***}	**<0.001**	**18.2**	±	**10.2**^{a***}	**<0.001**	**23.9**	±	**10.8**^{ab***}	**<0.001**	**30.6**	±	**13.9**^{b***}	**<0.001**	**0.029**
LM	**10.3**	±	**9.5*****		**11.4**	±	**8.3*****		**5.9**	±	**5.0*****		**8.0**	±	**7.4*****		**11.0**	±	**7.5*****		0.134
MP (%)																					
HM	**27.5**	±	**12.5*****	**<0.001**	**26.9**	±	**12.1***	**0.0105**	**28.9**	±	**11.3*****	**<0.001**	**30.8**	±	**12.7****	**0.001**	**27.2**	±	**13.2***	**0.016**	0.854
LM	**11.0**	±	**6.6*****		**16.9**	±	**11.5***		**13.6**	±	**10.9*****		**15.3**	±	**14.1****		**17.6**	±	**15.8***		0.6052
NP (%)																					
HM	15.7	±	11.4^{ab}	0.6455	13.0	±	9.2^{a}	0.417	**23.4**	±	**16.5**^{b**}	**0.008**	**16.3**	±	**6.7**^{a*}	**0.0699**	13.7	±	6.5^{a}	0.1176	**0.023**
LM	15.0	±	7.4		10.9	±	4.9		**13.1**	±	**8.5****		**12.3**	±	**6.3***		10.8	±	4.8		0.3045
Rapid (%)																					
HM	**37.9**	±	**23.9****	**0.001**	**45.8**	±	**22.5*****	**<0.001**	**30.2**	±	**18.7*****	**<0.001**	**39.8**	±	**18.2****	**0.0012**	**48.9**	±	**22.6*****	**<0.001**	0.06
LM	**14.6**	±	**13.3****		**20.7**	±	**13.8*****		**11.5**	±	**6.9*****		**16.3**	±	**16.2****		**20.8**	±	**15.0*****		0.2013
Medium (%)																					
HM	**20.9**	±	**9.7**$^{a\ ***}$	**<0.001**	12.4	±	5.1^{b}	0.0582	**22.6**	±	**11.2**^{a***}	**<0.001**	**20.2**	±	**8.4**^{a***}	**<0.001**	**12.0**	±	**5.1**^{b*}	**0.0304**	**<0.001**
LM	**8.6**	±	**4.8*****		9.4	±	7.1		**9.6**	±	**9.8*****		**9.3**	±	**9.1*****		**9.8**	±	**8.8***		0.993
Slow (%)																					
HM	11.3	±	7.5^{a}	0.516	10.7	±	7.1^{ab}	0.2133	17.8	±	14.8^{b}	0.0547	11.0	±	5.8^{a}	0.5975	10.7	±	5.1^{ab}	0.2182	**0.053**
LM	13.1	±	6.2		9.1	±	3.9		11.5	±	7.8		10.1	±	5.4		8.8	±	4.6		0.2175
Motile VCL (µm/s)																					
HM	**150.7**	±	**40.1**$^{ab\ ***}$	**<0.001**	**167.7**	±	**33.9**^{a*}	**0.0317**	**134.4**	±	**31.6**^{b*}	**0.0402**	**155.0**	±	**30.5**^{ab**}	**0.0028**	**166.1**	±	**33.1**^{a**}	**0.0046**	**0.017**
LM	**111.1**	±	**28.2**$^{a\ ***}$		**144.2**	±	**32.5**^{b*}		**114.3**	±	**28.0**^{a*}		**121.3**	±	**36.2**^{ab**}		**135.0**	±	**32.3**^{ab**}		**0.005**
Motile VAP (µm/s)																					

HM	**91.6**	±	**21.3*****	**<0.001**	87.3	±	15.7	0.2438	**79.9**	±	**14.7***	**0.0292**	**86.9**	±	**13.0***	**0.0111**	**92.4**	±	**14.2*****	**<0.001**	0.116
LM	**65.8**	±	**15.6*****		81.3	±	16.0		**67.4**	±	**19.9***		**70.7**	±	**23.9***		**74.0**	±	**17.5*****		0.081
Motile VSL (µm/s)																					
HM	**68.7**	±	**17.5**^{a***}	**<0.001**	58.9	±	11.3^{ab}	0.7251	**55.4**	±	**11.7**^{b*}	**0.0276**	**59.6**	±	**8.5**^{ab**}	**0.0053**	**64.7**	±	**9.9**^{ab***}	**<0.001**	**0.008**
LM	**47.0**	±	**12.6*****		56.6	±	12.2		**45.1**	±	**16.4***		**46.8**	±	**17.4****		**47.8**	±	**12.7*****		0.1
Motile STR (%)																					
HM	67.9	±	8.6	0.1848	63.8	±	6.4	0.2179	**65.3**	±	**7.1****	**0.0022**	**64.4**	±	**4.2****	**0.0016**	**65.0**	±	**6.7***	**0.0304**	0.5278
LM	63.3	±	9.1		61.3	±	6.0		**57.2**	±	**8.3****		**57.9**	±	**7.5****		**60.3**	±	**7.3***		0.091
Motile LIN (%)																					
HM	43.8	±	7.7	0.2912	37.4	±	6.2	0.8653	**42.8**	±	**8.6***	**0.0161**	39.8	±	5.4	0.1761	**40.5**	±	**8.2***	**0.0284**	0.0689
LM	40.6	±	8.9		37.0	±	8.1		**35.4**	±	**9.9***		35.9	±	9.9		**34.7**	±	**7.4***		0.248
Motile WOB (%)																					
HM	60.0	±	4.5^{a}	0.5059	53.8	±	4.8^{b}	0.703	**61.5**	±	**6.9**^{a*}	**0.0143**	57.4	±	4.7^{ac}	0.5699	56.6	±	7.1^{bc}	0.0658	**<0.001**
LM	58.6	±	8.5		54.6	±	8.0		**54.6**	±	**9.8***		55.7	±	9.7		53.4	±	5.8		0.379
Motile ALH (µm)																					
HM	1.81	±	0.5^{a}	0.083	**2.4**	±	**0.6**^{b*}	**0.0397**	1.9	±	0.6^{a}	0.2152	**2.1**	±	**0.5**^{ab*}	**0.0128**	2.1	±	0.7^{ab}	0.2673	**0.022**
LM	1.5	±	0.45		**1.9**	±	**0.6***		1.7	±	0.5		**1.7**	±	**0.5***		1.9	±	0.5		0.056
Motile BCF (Hz)																					
HM	41.4	±	8.4	0.1018	37.4	±	4.1	0.7932	36.5	±	4.5	0.1051	**38.2**	±	**4.6*****	**0.0283**	37.0	±	7.0	0.9573	0.1612
LM	37.2	±	7.7		37.8	±	5.8		33.4	±	7.2		**33.8**	±	**7.3*****		37.1	±	6.2		0.128
NP VCL (µm/s)																					
HM	**64.5**	±	**12.4**^{a*}	**0.0114**	60.3	±	9.1^{a}	0.1758	**67.7**	±	**12.7**^{ab***}	**<0.001**	**74.7**	±	**16.2**^{b***}	**<0.001**	61.4	±	13.4^{a}	0.7148	**0.006**
LM	**54.8**	±	**10.1***		56.6	±	6.9		**53.1**	±	**13.5*****		**53.9**	±	**12.3*****		61.0	±	12.6		0.216
NP VAP (µm/s)																					
HM	**38.8**	±	**7.6**^{ab*}	**0.0481**	35.2	±	10.4^{a}	0.1964	**43.8**	±	**8.5**^{b***}	**<0.001**	**43.8**	±	**10.9**^{ab***}	**<0.001**	36.1	±	11.2^{a}	0.1221	**0.014**
LM	**33.1**	±	**9.8***		29.2	±	7.4		**29.3**	±	**12.7*****		**29.0**	±	**11.2*****		33.1	±	10.8		0.526
NP VSL (µm/s)																					
HM	22.2	±	6.3	0.4209	**22.9**	±	**9.2***	**0.036**	**24.2**	±	**7.5*****	**<0.001**	**23.9**	±	**8.5*****	**<0.001**	**22,0**	±	**7.3***	**0.0237**	0.868
LM	20.3	±	8.4		**16.5**	±	**5.5***		**15.3**	±	**6.5*****		**15.0**	±	**6.5*****		**17.2**	±	**5.0***		0.09
NP STR (%)																					
HM	53.5	±	10.2	0.9461	54.4	±	13.7	0.1885	**53.5**	±	**10.1****	**0.0029**	52.4	±	10.7	0.0505	51.5	±	10.9	0.3835	0.938
LM	53.3	±	12.5^{a}		49.7	±	6.5^{ab}		**44.2**	±	**6.5**^{b**}		46.4	±	7.7^{ab}		50.1	±	9.8^{ab}		**0.021**
NP LIN (%)																					

HM	35.6	±	10.9	0.3391	**37.3**	±	**15.9***	**0.0322**	**35.7**	±	**10.1****	**0.0018**	**34.1**	±	**13.3***	**0.0392**	**34.7**	±	**11.3***	**0.0394**	0.9856
LM	33.6	±	13.8		**28.2**	±	**8.0***		**25.3**	±	**9.3****		**26.0**	±	**10.5***		**27.8**	±	**8.8***		0.105
NP WOB (%)																					
HM	58.6	±	9.1	0.3328	54.9	±	13.7	0.168	**60.6**	±	**9.3****	**0.0097**	**58.0**	±	**11.4***	**0.0414**	53.9	±	13.0	0.0636	0.358
LM	55.2	±	11.9		49.6	±	9.0		**49.3**	±	**14.3****		**48.9**	±	**15.7***		50.1	±	11.1		0.515
MP VCL (µm/s)																					
HM	150.2	±	37.7[a]	0.5698	180.3	±	35.7[b]	0.3039	141.7	±	29.9[a]	0.1676	165.9	±	29.6[ab]	0.3866	182.4	±	32.9[b]	0.1338	**<0.001**
LM	141.2	±	27.9		166.3	±	34.8		155.6	±	34.5		156.5	±	36.7		167.2	±	29.6		0.093
MP VAP (µm/s)																					
HM	88.6	±	15.1	0.2539	93.7	±	15.8	0.2582	83.3	±	11.6	0.148	90.2	±	12.6	0.9695	95.1	±	12.4	0.1965	0.062
LM	83.6	±	12.0		88.3	±	14.1		88.9	±	12.2		90.4	±	15.5		89.7	±	13.5		0.546
MP VSL (µm/s)																					
HM	59.1	±	11.2[a]	0.2445	49.9	±	7.2[b]	0.7883	52.6	±	8.9[b]	0.6262	52.2	±	5.6[ab]	0.5474	50.9	±	10.2[b]	0.5161	**0.0311**
LM	55.2	±	11.7		49.3	±	8.3		50.4	±	7.1		54,0	±	12.1		48.1	±	8.3		0.096
MP STR (%)																					
HM	69.7	±	13.6[a]	0.3037	56.6	±	10.3[b]	0.4452	64.8	±	11.8[ab]	0.2034	61.4	±	8.4[ab]	0.8411	56.6	±	11.3[b]	0.5161	**0.001**
LM	68.0	±	11.7[a]		59.1	±	10.3[ab]		59.2	±	11.7[ab]		62.2	±	14.0[ab]		57.6	±	10.7[b]		**0.05**
MP LIN (%)																					
HM	46.7	±	12.9[a]	0.7454	34.1	±	8.1[b]	0.5268	43.3	±	12.0[ab]	0.1647	37.7	±	7.5[ab]	0.3063	34.2	±	10.1[b]	0.5884	**<0.001**
LM	45.5	±	12.4[a]		35.8	±	9.3[b]		38.4	±	9.6[ab]		41.3	±	13.5[ab]		34.5	±	9.7[b]		**0.016**
MP WOB (%)																					
HM	63.0	±	7.4[a]	0.871	55.5	±	4.0[b]	0.4912	62.5	±	7.5[ac]	0.2885	57.7	±	4.4[bc]	0.3297	56.2	±	6.3[b]	0.4169	**<0.001**
LM	62.9	±	8.0[a]		56.6	±	5.9[b]		60.3	±	5.3[a]		61.6	±	7.6[a]		56.7	±	6.3[b]		**0.005**
MP ALH (µm)																					
HM	2.0	±	0.6[a]	0.8497	2.6	±	0.7[b]	0.4811	2.0	±	0.6[ac]	0.176	2.3	±	0.5[abc]	0.4567	2.5	±	0.8[bc]	0.6738	**0.006**
LM	2.0	±	0.6		2.5	±	0.6		2.4	±	0.7		2.2	±	0.7		2.4	±	0.5		0.483
MP BCF (Hz)																					
HM	49.2	±	12.2[a]	0.2779	41.0	±	5.5[b]	0.1773	39.2	±	5.9[b]	0.9947	41.7	±	5.1[ab]	0.4988	42.3	±	5.6[ab]	0.2688	**0.0283**
LM	44.9	±	12.1		43.6	±	6.1		39.1	±	8.6		39.9	±	8.6		40.1	±	6.9		0.1604
RP VCL (µm/s)																					
HM	208.0	±	35.2	0.2192	211.9	±	21.6	0.7454	190.9	±	22.6	0.8661	202.9	±	20.0	0.8967	207.2	±	18.7	0.3425	0.1238
LM	190.9	±	18.4		224.5	±	60.6		193.3	±	36.1		202.0	±	22.1		200.5	±	24.8		0.1138
RP VAP (µm/s)																					

HM	136.1	±	37.0		0.0792	110.9	±	15.4		0.0514	114.7	±	21.7		0.3116	122.1	±	17.5		0.8221	125.6	±	28.3		0.0917	0.0833
LM	117.7	±	35.4			150.4	±	72.2			127.9	±	39.4			126.0	±	27.7			114.0	±	35.6			0.1516
RP VSL (µm/s)																										
HM	119.8	±	39.6[a]		0.1014	**89.6**	±	**16.0[b*]**		**0.0398**	95.7	±	24.4[b]		0.3835	103.5	±	22.2[ab]		0.9328	106.4	±	30.4[ab]		0.0766	**0.0363**
LM	101.2	±	38.6			**133.4**	±	**76.4[*]**			109.2	±	43.6			109.1	±	29.7			94.0	±	38.6			0.1926
RP STR (%)																										
HM	83.1	±	4.1[a]		0.963	77.8	±	3.0[b]		0.0908	80.6	±	5.7[ab]		0.6939	80.8	±	5.6[ab]		0.2506	81.1	±	4.9[ab]		0.1091	**0.019**
LM	83.2	±	5.6[a]			81.6	±	8.8[ab]			80.6	±	7.9[ab]			82.9	±	5.2[a]			78.2	±	4.0[b]			**0.0336**
RP LIN (%)																										
HM	53.9	±	9.3		0.2923	43.6	±	7.5		0.0834	50.8	±	13.0		0.2817	51.9	±	13.0		0.6733	51.4	±	13.5		0.0766	0.06
LM	53.8	±	16.6			54.4	±	20.5			55.5	±	14.2			53.7	±	13.3			45.5	±	10.0			0.27
RP WOB (%)																										
HM	62.6	±	7.7[a]		0.3059	**53.7**	±	**7.0[b*]**		**0.0483**	60.6	±	10.9[a]		0.1651	61.0	±	10.6[ab]		0.5	60.6	±	12.0[ab]		0.196	**0.05**
LM	62.3	±	14.4			**63.1**	±	**16.8[*]**			65.6	±	11.2			62.2	±	11.5			55.9	±	8.6			0.205
RP ALH (µm)																										
HM	2.1	±	0.5[a]		0.193	**2.9**	±	**0.6[b*]**		**0.0498**	2.4	±	0.8[a]		0.4825	2.4	±	0.8[ab]		0.1913	2.3	±	0.7[ab]		0.1493	**0.012**
LM	2.3	±	0.8			**2.3**	±	**0.9[*]**			2.2	±	0.9			2.2	±	0.7			2.6	±	0.6			0.483
RP BCF (Hz)																										
HM	39.9	±	11.4		0.7212	37.4	±	6.8		0.9242	32.9	±	7.6		0.1363	35.1	±	7.1		0.8743	37.6	±	9.5		0.6328	0.116
LM	38.6	±	10.8			37.1	±	11.8			35.9	±	13.1			35.5	±	10.8			40.5	±	9.0			0.623

Note: The HM sperm subpopulation had significantly higher values for various motility and kinematic parameters after exposure to different media, as compared to the LM subpopulations. However, media was able to improve the LM subpopulations' MP and RP kinematics until significant differences between subpopulations were no longer observed. Significant differences between media were predominantly seen in the HM subpopulations, however differences between media were observed in the LM subpopulation for motile VCL, NP STR, MP STR, LIN and WOB, and RP STR. **Abbreviations:** ALH, amplitude of lateral head displacement; ANOVA, analysis of variance between media for individual subpopulations and parameters; BCF, beat cross frequency; CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; HM, high motile subpopulation; LIN, linearity; LM, low motile subpopulation; MP, medium-progressive; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; Prog, progressive; RP, rapid-progressive; SD, standard deviation; STR, straightness; Tot. Mot; total motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble. [a, b,c,d] Values labelled with different superscript letters in the same row were significantly different between the various media for individual subpopulations and SCA parameters. Values labelled in bold with an asterisk in the same column were significantly different between the HM and LM subpopulations for individual media and SCA parameters (**p* <0.05, ***p* <0.01 and ****p* <0.001).

Table 4.15 Comparison of SCA motility and kinematic parameters (mean ± SD) (n=20) for whole sperm motility subpopulations treated with HTF, CAP, HD-C, 1 µM dopamine, 250 ng/mL prolactin and 500 ng/mL prolactin.

	HTF				CAP				1 µM Dopamine				250 ng/mL prolactin				500 ng/mL prolactin				
	Mean	**±**	**SD**	**SP *p***	**Mean**	**±**	**SD**	**SP *p***	**Mean**	**±**	**SD**	**SP *p***	**Mean**	**±**	**SD**	**SP *p***	**Mean**	**±**	**SD**	**SP *p***	**ANOVA**
Tot. Mot (%)																					
HM	**59.2**	**±**	**20.7*****	**<0.001**	**61.0**	**±**	**17.1*****	**<0.001**	**64.0**	**±**	**17.3*****	**<0.001**	**65.5**	**±**	**14.4*****	**<0.001**	**57.9**	**±**	**14.0*****	**<0.001**	0.537
LM	**32.2**	**±**	**15.3$^{ab\ ***}$**		**37.5**	**±**	**18.3$^{ab\ ***}$**		**41.2**	**±**	**12.2$^{a\ ***}$**		**30.6**	**±**	**13.4$^{ab\ ***}$**		**27.0**	**±**	**12.0$^{b\ ***}$**		**0.014**
Prog (%)																					
HM	**44.6**	**±**	**25.2*****	**<0.001**	**49.7**	**±**	**21.1*****	**<0.001**	**52.5**	**±**	**19.5*****	**<0.001**	**51.4**	**±**	**21.1*****	**<0.001**	**45.2**	**±**	**13.5*****	**<0.001**	0.6679
LM	**20.5**	**±**	**12.9$^{ac\ ***}$**		**28.9**	**±**	**16.6$^{bc\ ***}$**		**33.4**	**±**	**12.9$^{b\ ***}$**		**24.5**	**±**	**13.2^{abc} *****		**16.8**	**±**	**13.4$^{a\ ***}$**		**<0.001**
RP (%)																					
HM	**20.2**	**±**	**16.2***	**0.0273**	**22.3**	**±**	**11.0*****	**<0.001**	**23.3**	**±**	**13.9****	**0.0048**	**22.1**	**±**	**12.8*****	**<0.001**	**22.2**	**±**	**10.2*****	**<0.001**	0.957
LM	**9.3**	**±**	**6.6^{ab*}**		**10.8**	**±**	**9.6$^{ab\ ***}$**		**13.2**	**±**	**7.8$^{a\ **}$**		**7.9**	**±**	**7.2$^{b\ ***}$**		**6.1**	**±**	**4.6^{b***}**		**0.0207**
MP (%)																					
HM	**24.4**	**±**	**10.9*****	**<0.001**	**27.4**	**±**	**13.1***	**0.0102**	**29.3**	**±**	**11.2****	**0.0062**	**29.4**	**±**	**11.3*****	**<0.001**	**23.0**	**±**	**8.8*****	**<0.001**	0.217
LM	**11.2**	**±**	**8.8$^{a\ ***}$**		**18.1**	**±**	**9.6$^{bc\ *}$**		**20.2**	**±**	**8.8$^{b\ **}$**		**16.7**	**±**	**9.5$^{ab\ ***}$**		**10.7**	**±**	**10.2$^{ac\ ***}$**		**0.002**
NP (%)																					
HM	14.6	±	6.0	0.1647	11.3	±	6.8	0.1048	**12.7**	**±**	**6.6***	**0.0189**	**14.1**	**±**	**8.5*****	**<0.001**	12.7	±	6.1	0.1749	0.536
LM	11.7	±	7.6^{a}		8.5	±	6.3^{ab}		**7.8**	**±**	**6.8$^{ab\ *}$**		**6.1**	**±**	**4.6$^{b\ ***}$**		10.2	±	5.9^{ab}		**0.046**
Rapid (%)																					
HM	**30.3**	**±**	**20.7****	**0.006**	**42.5**	**±**	**20.8*****	**<0.001**	**43.9**	**±**	**19.5*****	**<0.001**	**43.0**	**±**	**21.8*****	**<0.001**	**35.2**	**±**	**14.5*****	**<0.001**	0.097
LM	**13.9**	**±**	**8.7$^{ac\ **}$**		**20.0**	**±**	**16.4$^{bc\ ***}$**		**25.3**	**±**	**14.9$^{b\ ***}$**		**16.2**	**±**	**7.9$^{abc\ ***}$**		**11.5**	**±**	**10.1$^{a\ ***}$**		**0.0067**
Medium (%)																					
HM	**18.5**	**±**	**8.5$^{a\ ***}$**	**<0.001**	9.2	±	5.5^{b}	0.7871	11.4	±	7.8^{b}	0.0865	**12.8**	**±**	**7.1$^{b\ *}$**	**0.0209**	**13.1**	**±**	**6.5$^{b\ ***}$**	**<0.001**	**<0.001**
LM	**8.4**	**±**	**6.6*****		10.3	±	7.1		9.8	±	8.1		**9.0**	**±**	**8.4***		**7.0**	**±**	**8.9*****		0.671
Slow (%)																					
HM	10.5	±	6.1	0.6136	9.4	±	5.7	0.0632	**8.7**	**±**	**5.2***	**0.0422**	**9.7**	**±**	**6.6***	**0.0146**	9.5	±	5.9	0.5205	0.906
LM	9.8	±	6.4		7.2	±	5.0		**6.1**	**±**	**6.0***		**5.4**	**±**	**4.3***		8.5	±	5.0		0.053
Motile VCL (µm/s)																					
HM	139.0	±	39.2^{a}	0.1454	**166.1**	**±**	**33.4$^{b\ *}$**	**0.0362**	170.1	±	35.1^{b}	0.503	153.0	±	34.1^{ab}	0.1087	**154.5**	**±**	**28.6$^{ab\ **}$**	**0.0011**	**0.03**

LM	123.5	±	29.7^{ac}		**142.2**	±	**39.4$^{ab\ *}$**		162.2	±	41.9^{b}		138.4	±	22.1^{ab}		**119.3**	±	**36.5$^{c\ **}$**		**0.0014**
Motile VAP (µm/s)																				
HM	**80.5**	**±**	**25.3***	**0.0366**	84.0	±	12.3	0.8567	84.2	±	15.1	0.4886	75.8	±	16.8	0.7758	**77.3**	**±**	**12.7****	**0.0036**	0.0675
LM	**64.8**	**±**	**17.7$^{a\ *}$**		83.1	±	20.2^{b}		83.0	±	25.3^{b}		74.5	±	12.8^{ab}		**63.1**	**±**	**14.8$^{a\ **}$**		**<0.001**
Motile VSL (µm/s)																				
HM	55.9	±	26.2	0.1521	52.7	±	8.5	0.2336	50.7	±	11.3	0.7159	45.9	±	12.5	0.481	**51.6**	**±**	**11.9*****	**<0.001**	0.3198
LM	43.1	±	16.2^{ac}		57.8	±	17.5^{b}		52.7	±	19.9^{ab}		47.9	±	15.3^{abc}		**38.9**	**±**	**11.5$^{c\ ***}$**		**0.002**
Motile STR (%)																				
HM	62.9	±	12.9	0.7424	59.9	±	6.9	0.1433	59.2	±	8.4	0.8901	57.2	±	5.9	0.0665	**63.4**	**±**	**7.7****	**0.0031**	0.0715
LM	61.2	±	10.0		63.7	±	10.0		59.5	±	9.9		60.0	±	12.4		**56.5**	**±**	**7.8****		0.205
Motile LIN (%)																				
HM	38.6	±	11.9^{a}	0.339	**33.9**	**±**	**7.1$^{ab\ **}$**	**0.0037**	32.5	±	7.5^{ab}	0.8418	**30.3**	**±**	**6.8$^{b\ *}$**	**0.0476**	34.3	±	8.2^{ab}	0.3851	**0.030**
LM	35.4	±	9.6^{ab}		**41.0**	**±**	**8.2$^{a\ **}$**		33.4	±	9.6^{b}		**35.9**	**±**	**10.6$^{ab\ *}$**		33.2	±	6.6^{b}		**0.037**
Motile WOB (%)																				
HM	57.3	±	6.9^{a}	0.1272	**52.2**	**±**	**5.4$^{b\ ***}$**	**<0.001**	51.6	±	5.6^{b}	0.6773	49.9	±	7.3^{b}	0.063	51.0	±	7.0^{b}	0.3418	**0.003**
LM	54.0	±	7.1		**58.6**	**±**	**5.6*****		52.4	±	7.2		54.4	±	8.0		54.7	±	7.0		0.057
Motile ALH (µm)																				
HM	1.9	±	0.5^{a}	0.3772	**2.3**	**±**	**0.6$^{b\ *}$**	**0.0127**	2.5	±	0.5^{b}	0.4693	**2.3**	**±**	**0.5$^{b\ *}$**	**0.0106**	**2.2**	**±**	**0.5$^{ab\ **}$**	**0.0076**	**0.009**
LM	2.0	±	0.6^{ab}		**1.9**	**±**	**0.6$^{ab\ *}$**		2.4	±	0.6^{bc}		**1.9**	**±**	**0.3$^{b\ *}$**		**1.8**	**±**	**0.6$^{a\ **}$**		**0.0085**
Motile BCF (Hz)																				
HM	42.5	±	8.0^{ac}	0.2136	38.8	±	4.5^{b}	0.177	40.9	±	6.3^{ab}	0.7872	40.1	±	4.9^{ab}	0.5929	**44.0**	**±**	**4.0$^{c\ *}$**	**0.0112**	**0.0073**
LM	39.8	±	6.3		37.4	±	6.8		40.7	±	7.1		41.6	±	8.5		**40.9**	**±**	**7.2***		0.343
NP VCL (µm/s)																				
HM	**68.2**	**±**	**10.4****	**0.0026**	60.7	±	12.2	0.8424	65.0	±	16.6	0.4384	**67.8**	**±**	**12.8****	**0.0016**	**70.8**	**±**	**23.1****	**0.0047**	0.314
LM	**56.9**	**±**	**13.7****		59.9	±	13.4		65.5	±	21.0		**51.6**	**±**	**16.8****		**55.6**	**±**	**7.6****		0.5434
NP VAP (µm/s)																				
HM	**40.5**	**±**	**7.3***	**0.0377**	35.7	±	14.6	0.753	**34.4**	**±**	**9.1***	**0.0456**	35.2	±	10.1	0.0523	37.2	±	15.1	0.1423	0.428
LM	**33.0**	**±**	**10.8***		34.8	±	10.8		**30.0**	**±**	**11.6***		30.4	±	15.9		31.4	±	8.4		0.742
NP VSL (µm/s)																				
HM	21.6	±	7.1	0.2249	24.9	±	15.1	0.4764	**19.7**	**±**	**7.5****	**0.0094**	16.9	±	5.9	0.1649	20.1	±	9.4	0.1294	0.103
LM	18.9	±	7.1		23.0	±	13.1		**14.1**	**±**	**5.5****		17.7	±	15.0		15.7	±	5.8		0.093
NP STR (%)																				

HM	49.6	±	10.5^{ab}	0.6098	56.3	±	16.5^{a}	0.9472	53.2	±	10.4^{a}	0.0518	42.9	±	10.2^{b}	0.681	48.1	±	12.6^{ab}	0.188	**0.008**
LM	50.1	±	12.0^{ac}		56.6	±	14.0^{c}		45.7	±	11.7^{ab}		45.2	±	23.9^{ab}		43.2	±	10.5^{b}		**0.0356**
NP LIN (%)																					
HM	31.7	±	11.8^{ab}	0.9318	37.9	±	19.4^{a}	0.8425	**32.2**	**±**	**10.0^{ab} ****	**0.0075**	24.2	±	10.2^{b}	0.865	27.4	±	14.0^{ab}	0.896	**0.017**
LM	31.4	±	11.3^{ac}		36.5	±	18.5^{c}		**23.0**	**±**	**10.4^{b} ****		28.9	±	23.9^{ab}		26.6	±	10.4^{ab}		**0.0243**
NP WOB (%)																					
HM	56.9	±	8.7	0.6484	55.1	±	16.5	0.4867	52.8	±	10.4	0.0519	48.9	±	12.7	0.599	48.8	±	15.8	0.3308	0.161
LM	55.1	±	16.7		55.5	±	13.9		46.1	±	10.3		51.9	±	22.2		53.1	±	11.4		0.366
MP VCL (µm/s)																					
HM	146.7	±	30.1^{a}	0.4666	**184.0**	**±**	**30.2^{bc} ****	**0.0096**	188.0	±	39.0^{b}	0.1771	166.7	±	23.9^{abc}	0.2203	161.5	±	34.7^{ac}	0.7267	**<0.001**
LM	152.1	±	25.0		**161.7**	**±**	**33.2****		178.6	±	55.9		157.8	±	23.0		157.6	±	38.8		0.3584
MP VAP (µm/s)																					
HM	**84.6**	**±**	**13.9^{a} ***	**0.0273**	**93.4**	**±**	**10.4^{b} ***	**0.0167**	93.7	±	12.5^{b}	0.0781	82.0	±	7.9^{a}	0.3638	81.9	±	9.3^{a}	0.6035	**<0.001**
LM	**78.0**	**±**	**9.1***		**86.4**	**±**	**12.2***		86.1	±	15.3		84.6	±	10.8		80.2	±	11.4		0.081
MP VSL (µm/s)																					
HM	**52.6**	**±**	**16.2^{a} ***	**0.031**	48.0	±	6.6^{a}	0.7378	43.8	±	8.2^{ab}	0.5973	**40.9**	**±**	**5.4^{b} ***	**0.0161**	46.8	±	7.5^{a}	0.1299	**0.0061**
LM	**42.7**	**±**	**13.1***		47.1	±	11.2		43.4	±	11.7		**48.9**	**±**	**12.8***		43.0	±	9.3		0.303
MP STR (%)																					
HM	64.8	±	16.0^{a}	0.1103	54.1	±	10.6^{b}	0.2289	49.8	±	12.2^{b}	0.7962	52.2	±	9.2^{b}	0.1319	59.7	±	10.8^{a}	0.4113	**0.0021**
LM	56.7	±	14.9		58.2	±	12.8		52.3	±	14.4		58.2	±	15.3		56.9	±	12.1		0.629
MP LIN (%)																					
HM	**42.1**	**±**	**15.1^{a} ***	**0.0472**	31.2	±	8.5^{bc}	0.5434	28.3	±	9.3^{b}	0.6138	**28.2**	**±**	**7.8^{b} ***	**0.0151**	34.2	±	10.5^{ac}	0.6898	**0.0027**
LM	**33.2**	**±**	**13.6***		34.9	±	12.0		30.1	±	13.4		**36.5**	**±**	**12.3***		33.3	±	12.2		0.559
MP WOB (%)																					
HM	**60.9**	**±**	**9.0^{a} ***	**0.032**	53.8	±	4.6^{b}	0.2533	53.0	±	6.0^{b}	0.9504	**51.3**	**±**	**5.8^{b} ****	**0.0037**	54.1	±	8.0^{b}	0.7072	**0.0021**
LM	**54.4**	**±**	**10.4***		56.7	±	6.8		52.8	±	10.7		**57.2**	**±**	**8.1****		54.6	±	10.6		0.547
MP ALH (µm)																					
HM	2.2	±	0.6^{a}	0.0707	**2.8**	**±**	**0.6^{b} ***	**0.0413**	2.8	±	0.7^{bc}	0.7267	**2.6**	**±**	**0.5^{bc} ***	**0.0357**	2.3	±	0.8^{ac}	0.7733	**0.003**
LM	2.5	±	0.6		**2.4**	**±**	**0.7***		2.7	±	0.9		**2.3**	**±**	**0.6***		2.4	±	0.7		0.295
MP BCF (Hz)																					
HM	48.3	±	11.1^{a}	0.093	41.2	±	4.5^{b}	0.9709	40.8	±	9.5^{b}	0.7924	43,0	±	6.9^{ab}	0.7943	46.8	±	8.3^{a}	0.2405	**0.0269**
LM	42.9	±	9.3		42.2	±	5.3		41.6	±	9.9		43.6	±	8.4		43.9	±	10.0		0.903
RP VCL (µm/s)																					

HM	189.5	±	31.6	0.6733	**201.1**	±	**17.4***	**0.0476**	204.7	±	22.5	0.8361	**191.4**	±	**23.5****	**0.0047**	**191.7**	±	**24.1****	**0.0036**	0.0605
LM	188.9	±	22.8^{a}		**193.1**	±	**38.7**$^{ab\ *}$		206.2	±	24.4^{d}		**172.8**	±	**15.9**$^{bc\ **}$		**171.2**	±	**19.0**$^{c\ **}$		**<0.001**
RP VAP (µm/s)																					
HM	109.8	±	30.8	0.2141	**102.2**	±	**15.3****	**0.0096**	98.7	±	9.1	0.8053	94.8	±	17.2	0.8919	95.5	±	20.3	0.5117	0.1257
LM	99.9	±	14.8^{a}		**137.3**	±	**48.3**$^{b\ **}$		108.4	±	33.3^{a}		90.8	±	18.5^{a}		91.5	±	20.9^{a}		**<0.001**
RP VSL (µm/s)																					
HM	91.9	±	32.7	0.2015	**80.0**	±	**16.1****	**0.0043**	76.6	±	10.6	0.8973	72.5	±	16.9	0.4628	74.5	±	21.3	0.5929	0.1028
LM	81.0	±	17.0^{a}		**122.2**	±	**53.7**$^{b\ **}$		86.0	±	34.1^{a}		69.1	±	21.2^{a}		73.3	±	22.7^{a}		**<0.001**
RP STR (%)																					
HM	78.8	±	7.0^{a}	0.8221	**76.0**	±	**3.5**$^{b\ ***}$	**<0.001**	76.2	±	4.6^{ab}	0.8951	74.2	±	4.2^{b}	0.4628	75.5	±	6.2^{b}	0.4156	**0.034**
LM	79.1	±	7.3^{a}		**84.9**	±	**9.5**$^{b\ ***}$		75.9	±	6.4^{a}		74.2	±	8.9^{a}		77.1	±	6.6^{a}		**<0.001**
RP LIN (%)																					
HM	46.4	±	12.6^{a}	0.5826	**40.7**	±	**6.6**$^{b\ ***}$	**<0.001**	38.8	±	6.7^{b}	0.8235	37.7	±	6.1^{b}	0.8811	38.4	±	10.3^{b}	0.1736	**0.0316**
LM	44.3	±	10.8^{a}		**63.0**	±	**22.6**$^{b\ ***}$		40.8	±	10.8^{a}		40.3	±	11.4^{a}		43.1	±	12.0^{a}		**0.0023**
RP WOB (%)																					
HM	56.4	±	10.6^{a}	0.4848	**51.8**	±	**6.1**$^{ab\ ***}$	**<0.001**	49.6	±	6.1^{b}	0.3391	49.5	±	5.5^{b}	0.1662	49.4	±	9.8^{b}	0.0761	**0.022**
LM	54.2	±	8.7^{a}		**70.9**	±	**18.5**$^{b\ ***}$		51.9	±	9.6^{a}		52.9	±	9.5^{a}		53.9	±	11.1^{a}		**0.0028**
RP ALH (µm)																					
HM	2.4	±	0.5^{a}	0.1706	**2.7**	±	**0.5**$^{ab\ ***}$	**<0.001**	3.0	±	0.5^{b}	0.1139	**2.8**	±	**0.4**$^{b\ *}$	**0.0324**	**2.7**	±	**0.6**$^{b\ *}$	**0.0146**	**0.008**
LM	2.8	±	0.7^{ac}		**1.8**	±	**1.0**$^{b\ ***}$		2.7	±	0.6^{c}		**2.5**	±	**0.4**$^{ac\ *}$		**2.3**	±	**0.6**$^{ab\ *}$		**0.0031**
RP BCF (Hz)																					
HM	41.8	±	10.1	0.374	**42.3**	±	**9.4***	**0.0184**	42.5	±	5.2	0.8763	42.4	±	3.5	0.1346	45.8	±	6.0	0.2557	0.3489
LM	38.9	±	9.7^{ab}		**31.0**	±	**16.8**$^{b\ *}$		42.2	±	10.5^{ac}		46.9	±	11.3^{c}		43.0	±	9.6^{ac}		**0.0101**

Note: The HM sperm subpopulation had significantly higher values for various motility and kinematic parameters after exposure to different media, as compared to the LM subpopulations. However, media was able to improve various NP, MP and RP kinematics of the LM subpopulations until significant differences between subpopulations were no longer observed. Significant differences between media were predominantly observed for the LM subpopulation's motility percentages, motile and RP kinematics parameters, and for the HM subpopulation's MP kinematic parameters. **Abbreviations:** ALH, amplitude of lateral head displacement; ANOVA, analysis of variance between media for individual subpopulations and parameters; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HTF, human tubal fluid; HM, high motile subpopulation; LIN, linearity; LM, low motile subpopulation; MP, medium-progressive; NP, non-progressive; PRL, prolactin; Prog, progressive; RP, rapid-progressive; SD, standard deviation; STR, straightness; Tot. Mot; total motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble. $^{a, b,c,d}$ Values labelled with different superscript letters in the same row were significantly different between the various media for individual subpopulations and SCA parameters. Values labelled in bold with an asterisk in the same column were significantly different between the HM and LM subpopulations for individual media and SCA parameters (*p <0.05, **p <0.01 and ***p <0.001).

4.3.4 | SCA Individual Sperm Kinematic Parameters

After applying the flush technique, kinematic parameters for 100 individual spermatozoa from both the HM and LM subpopulations were recorded after 10-15 minutes with the use SCA®. For a better understanding of kinematic parameters – curvilinear velocity (VCL), average path velocity (VAP) and straight-line velocity (VSL) will be grouped as "velocity" kinematics; linearity (LIN) and straightness (STR) as "linear"; and amplitude of lateral had displacement (ALH), wobble (WOB) and beat cross frequency (BCF) as "vigour" kinematics.

4.3.4.1 | Velocity

No significant differences were observed between the individual sperm of subpopulations apart from prolactin where HM individual sperm had significantly increased velocity kinematics as compared to the LM individual sperm (Table 4.16 & Table 4.17). CAP, progesterone and myo-inositol increased velocity in HM individual sperm as compared to HTF. In contrast, for the LM individual sperm HD-C decreased the velocity kinematic parameters. No significant differences between the media were further observed (Supplementary Figure 6).

4.3.4.2 | Linear

The LM individual sperm yielded significantly higher linear parameters for 250 ng/mL prolactin as compared to the HM individual sperm (Table 4.16 & Table 4.17). Compared to HTF, HM individual sperm linear kinematics were decreased by CAP, HD-C, progesterone and myo-inositol. No significant differences between the media were observed in the LM individual sperm (Supplementary Figure 6).

4.3.4.3 | Vigour

Compared to HM individual sperm, the LM individual sperm had significantly higher vigour parameters after being flushed with 250 ng/mL prolactin, however the HM yielded significantly higher values for ALH (Table 4.16 & Table 4.17). In addition, the HM individual sperm had significantly higher values for BCF after being flushed with 500 ng/mL prolactin. Compared to HTF in the HM individual sperm – CAP, progesterone and myo-inositol decreased the vigour kinematics parameters, however increased ALH. In the LM individual sperm HD-C increased vigour parameters as compared to CAP and progesterone but decreased ALH as compared to CAP (Supplementary Figure 6).

Table 4.16 Comparison of the various SCA kinematic parameters (mean ± SD) of different speed groups for individual spermatozoa from the high motile (HM) (n=406) and low motile (LM) (n=167) subpopulations treated with HTF, CAP, HD-C, 3.96 µM progesterone and 11 mM myo-inositol.

	HTF				CAP				HD-C				PRG				MYO				
	Mean	**±**	**SD**	**SP *p***	**Mean**	**±**	**SD**	**SP *p***	**Mean**	**±**	**SD**	**SP *p***	**Mean**	**±**	**SD**	**SP *p***	**Mean**	**±**	**SD**	**SP *p***	**ANOVA**
Elongation																					
HM	66.0	±	8.1^{ac}	0.4543	69.0	±	6.9^{b}	0.9644	65.6	±	6.8^{c}	0.0169	69.1	±	5.5^{b}	0.7869	68.4	±	7.0^{ab}	0.9706	**<0.001**
LM	67.4	±	8.0		68.6	±	6.1		69.6	±	7.7		68.8	±	4.2		68.0	±	7.9		0.869
VCL (µm/s)																					
HM	164.1	±	60.3^{a}	0.9293	219.4	±	61.6^{b}	0.2168	152.8	±	58.3^{a}	0.3706	205.5	±	63.8^{bc}	0.3015	190.3	±	69.0^{c}	0.7490	**<0.001**
LM	165.4	±	60.9^{ab}		203.1	±	67.2^{a}		138.8	±	54.9^{b}		183.6	±	63.2^{a}		178.3	±	73.2^{a}		**0.004**
VAP (µm/s)																					
HM	81.1	±	20.2^{a}	0.5607	93.8	±	25.8^{b}	0.4195	82.2	±	21.9^{a}	0.3163	93.5	±	23.4^{b}	0.3775	90.5	±	25.0^{b}	0.4967	**<0.001**
LM	78.4	±	20.5		90.0	±	22.3		74.8	±	19.4		85.4	±	22.3		87.1	±	24.6		0.05
VSL (µm/s)																					
HM	53.6	±	14.7^{a}	0.3416	55.4	±	25.9^{a}	0.7911	44.9	±	14.6^{b}	0.3083	53.5	±	15.3^{a}	0.0743	51.8	±	18.3^{a}	0.4694	**0.001**
LM	49.8	±	20.6		53.5	±	21.5		41.7	±	17.4		48.8	±	15.1		49.8	±	14.8		0.139
STR (%)																					
HM	67.3	±	15.3^{a}	0.2476	59.0	±	18.8^{b}	0.9567	56.8	±	19.2^{b}	0.9069	59.7	±	18.6^{b}	0.3514	58.1	±	17.9^{b}	0.8501	**0.006**
LM	62.5	±	22.8		59.4	±	20.6		56.3	±	20.0		58.3	±	16.4		59.0	±	16.1		0.831
LIN (%)																					
HM	35.7	±	12.8^{a}	0.0845	26.1	±	12.3^{b}	0.6759	32.5	±	13.3^{ac}	0.9808	27.9	±	10.3^{b}	0.6446	29.2	±	11.8^{bc}	0.8789	**<0.001**
LM	30.5	±	12.1		28.8	±	16.9		32.6	±	14.5		28.8	±	11.5		30.9	±	11.1		0.757
WOB (%)																					
HM	52.3	±	11.2^{a}	0.4017	43.8	±	10.9^{b}	0.3716	56.7	±	10.6^{c}	0.8567	47.5	±	10.5^{d}	0.8176	49.8	±	10.3^{d}	0.552	**<0.001**
LM	50.1	±	10.6^{ab}		47.0	±	13.8^{a}		56.9	±	10.4^{b}		48.9	±	11.0^{a}		52.2	±	10.6^{ab}		**0.011**
ALH (µm)																					
HM	2.8	±	1.1^{a}	0.8846	3.7	±	1.2^{b}	0.3256	2.6	±	1.1^{a}	0.3558	3.4	±	1.2^{bc}	0.8654	3.2	±	1.3^{c}	0.9263	**<0.001**
LM	2.8	±	1.1^{ab}		3.5	±	1.4^{a}		2.4	±	1.1^{b}		3.2	±	1.2^{ab}		3.1	±	1.4^{ab}		**0.018**
BCF (Hz)																					
HM	41.9	±	11.5^{a}	0.4627	35.1	±	8.7^{b}	0.336	39.0	±	11.6^{a}	0.2159	40.2	±	11.8^{a}	0.2031	37.8	±	12.2^{ab}	0.6979	**0.005**

LM	43.9	±	12.0		37.3	±	8.2		42.1	±	12.4		36.7	±	10.2		36.9	±	14.4		0.074					

Note: Significant differences between media was observed in both subpopulations however predominantly in the HM subpopulation. **Abbreviations:** ALH, amplitude of lateral head displacement; ANOVA, analysis of variance between media for individual subpopulations and parameters; BCF, beat cross frequency; CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; HM, high motile subpopulation; LIN, linearity; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; SCA, sperm class analyser; SD, standard deviation; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble. [a, b,c,d] Values labelled with different superscript letters in the same row were significantly different between the various media for individual subpopulations and SCA parameters. Values labelled in bold with an asterisk in the same column were significantly different between the HM and LM subpopulations for individual media and SCA parameters (**p* <0.05, ***p* <0.01 and ****p* <0.001).

Table 4.17 Comparison of the various SCA kinematic parameters (mean ± SD) of different speed groups for individual spermatozoa from the low motile (LM) (n=60) and high motile (HM) (n=185) subpopulations after being treated with HTF, CAP, 1 µM dopamine, 250 ng/mL prolactin and 500 ng/mL prolactin.

	HTF				**CAP**				**1 µM DOPA**				**250 ng/mL PRL**				**500 n g/mL PRL**				
	Mean	±	**SD**	**SP *p***	**Mean**	±	**SD**	**SP *p***	**Mean**	±	**SD**	**SP *p***	**Mean**	±	**SD**	**SP *p***	**Mean**	±	**SD**	**SP *p***	**ANOVA**
Elongation																					
HM	68.1	±	6.3	0.6853	**70.9**	±	**3.1****	**0.0038**	70.8	±	3.8	0.5258	69.0	±	5.2	0.2718	68.2	±	5.9	0.7227	0.061
LM	69.1	±	4.2		**66.6**	±	**6.3****		70.0	±	4.9		71.0	±	6.9		71.6	±	5.0		0.254
VCL (µm/s)																					
HM	160.7	±	50.2	0.8046	196.0	±	48.0	0.3493	191.3	±	50.8	0.8904	**184.2**	±	**44.0****	**0.0045**	188.4	±	52.7	0.0504	0.056
LM	165.7	±	55.2		180.8	±	30.8		193.4	±	48.4		**149.9**	±	**49.3****		177.2	±	80.8		0.202
VAP (µm/s)																					
HM	79.8	±	19.6	0.5782	89.5	±	12.9	0.8932	91.0	±	17.3	0.5951	88.3	±	18.6	0.3256	84.8	±	17.2	0.0930	0.079
LM	84.1	±	20.0		88.9	±	14.5		88.3	±	15.9		82.5	±	17.7		90.2	±	36.0		0.841
VSL (µm/s)																					
HM	45.3	±	15.7	0.9572	52.4	±	16.3	0.8901	47.4	±	12.9	0.9091	44.0	±	12.8	0.4731	**49.2**	±	**17.4***	**0.0168**	0.113
LM	44.9	±	19.7		53.3	±	17.8		47.0	±	11.4		47.2	±	18.5		**38.0**	±	**16.3***		0.291
STR (%)																					
HM	57.2	±	15.9	0.6601	58.6	±	15.9	0.7415	52.9	±	13.3	0.7418	51.2	±	15.8	0.1962	57.9	±	17.0	0.7935	0.133
LM	54.3	±	21.5		60.5	±	19.1		54.2	±	13.8		58.3	±	21.6		44.2	±	14.4		0.218
LIN (%)																					
HM	28.9	±	8.7	0.9838	27.2	±	7.6	0.2942	25.6	±	6.6	0.5054	**24.8**	±	**8.4***	**0.0481**	26.9	±	8.5	0.2470	0.263
LM	28.9	±	11.3		30.4	±	11.5		25.4	±	8.2		**33.6**	±	**16.4***		23.3	±	7.3		0.207
WOB (%)																					
HM	51.3	±	11.0	0.7013	47.4	±	8.8	0.2637	49.0	±	8.7	0.4363	**48.8**	±	**7.4****	**0.0022**	46.5	±	7.6	0.4852	0.253

	LM	53.8	±	11.3		49.6	±	6.1		47.0	±	7.9		**57.1**	±	**10.8****		53.4	±	8.6		0.052
ALH (µm)																						
	HM	2.8	±	1.0	0.6135	3.2	±	0.9	0.5587	3.2	±	1.0	0.6062	**3.2**	±	**0.8****	**0.0024**	3.2	±	1.1	0.0508	0.258
	LM	3.0	±	1.1		3.1	±	0.7		3.3	±	0.9		**2.4**	±	**1.0****		3.0	±	1.5		0.312
BCF (Hz)																						
	HM	39.4	±	9.8	0.6497	41.6	±	10.3	0.7194	38.7	±	13.3	0.7691	**35.8**	±	**10.6*****	**<0.001**	**41.0**	±	**12.5**	**0.0048**	0.164
	LM	37.8	±	7.9		40.3	±	11.5		38.0	±	11.1		**48.8**	±	**14.6*****		**38.1**	±	**13.7**		0.131

Note: HM subpopulations had significantly higher values for elongation in CAP medium, VCL and ALH in 250 ng/mL prolactin and VSL and BCF in 500 ng/mL prolactin. Significant differences between media were only observed in the HM subpopulations for area. **Abbreviations:** ALH, amplitude of lateral head displacement; ANOVA, analysis of variance between media for individual subpopulations and parameters; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HTF, human tubal fluid; HM, high motile subpopulation; LIN, linearity; LM, low motile subpopulation; PRL, prolactin; SCA, sperm class analyser; SD, standard deviation; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble. $^{a, b,c,d}$ Values labelled with different superscript letters in the same row were significantly different between the various media for individual subpopulations and SCA parameters. Values labelled in bold with an asterisk in the same column were significantly different between the HM and LM subpopulations for individual media and SCA parameters (*p <0.05, **p <0.01 and ***p <0.001).

4.3.5 | FAST Individual Sperm Kinematic Parameters

After individual spermatozoa were captured in SCA®, fields were saved as AVI files and analysed in the Flagellar Analysis and Sperm Tracking (FAST) programme to determine the flagellar and CASA parameters of individual spermatozoa. Spermatozoa with distinct and clear tangent angles, 35 µm< analysed flagellum length >50 µm, maximum analysed flagellum <60 µm and frames captured >60 were further selected for statistical analysis. For better understanding of kinematic parameters – curvilinear velocity (VCL), average path velocity (VAP) and straight-line velocity (VSL) will be grouped as "velocity" kinematics; linearity (LIN) and straightness (STR) as "linear"; and amplitude of lateral had displacement (ALH), wobble (WOB) and beat cross frequency (BCF) as "vigour" kinematics. FAST flagellar parameters including mean power, maximum analysed flagellar length, flagellar arc-wavelength (fAWL), flagellar arc-wave speed (fAWS), flagellar beat frequency (fBF) and track centroid speed (TCS) will further be grouped as "flagellar parameters".

4.3.5.1 | Flagellar Parameters

Seen in Figure 4.58 – 4.60 are the typical flagellar tracks observed for the various media for both HM and LM individual sperm. Compared to the LM individual sperm, the HM individual sperm had significantly higher values for mean power in CAP medium, fAWS in 500 ng/mL prolactin and fBF in myo-inositol (Supplementary Table 8 & 9). No other significant differences between subpopulation individual sperm were observed for flagellar parameters. Furthermore, significant differences between the media were predominantly seen in the HM individual sperm as compared to the LM individual sperm (Supplementary Figure 7). Compared to HTF in the HM individual sperm, CAP, progesterone and myo-inositol presented with significantly higher mean power values, whereas HD-C showed lower values in both subpopulations' individual sperm. In addition, HD-C had lower values as compared to the other media for TCS and fAWL in HM individual sperm, and for fAWS of both subpopulations. Moreover, both CAP and 500 ng/mL prolactin had higher fAWL values as compared to HTF in the HM individual sperm.

To determine the interaction of media as the main factor on FAST flagellar parameters, data for subpopulations were pooled and analysed in a mixed model ANOVA (Figures 4.61 – 4.63). Media had a significant (p <0.01) interaction on TCS, fAWL and fAWS. For individual effects of the media HD-C had significantly lower TCS, fAWL and fAWS values as compared to HTF, CAP, progesterone and myo-inositol (Figure 4.62 A & C, 4.63 A). Myo-inositol had

lower fAWL values as compared to CAP, whereas progesterone had lower fAWS values as compared to CAP (Figure 4.62 C & 4.63 A). Furthermore, both HD-C and progesterone had lower fBF values as compared to myo-inositol (Figure 4.63 C).

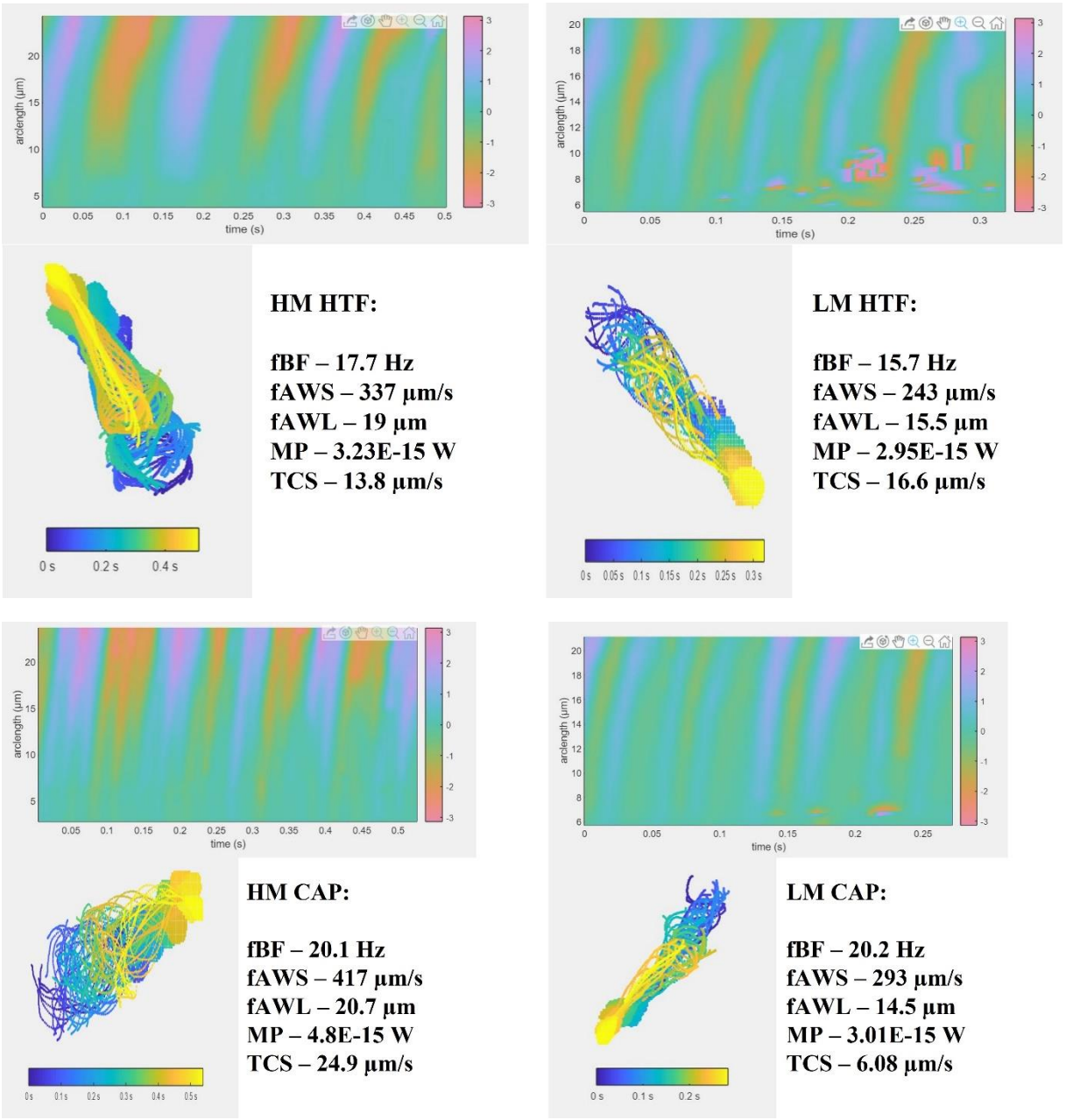


Figure 4.58 Typical Flagellar tangent angles and swimming tracks of both HM and LM individual spermatozoa in HTF and CAP media. **Abbreviations:** CAP, non-capacitating HTF; fAWL, flagellar arc-wavelength; fAWS, flagellar arc-wave speed; fBF, flagellar beat frequency; HM, high motile, HTF, human tubal fluid; LM, low motile; MP, mean power; TCS, track centroid speed.

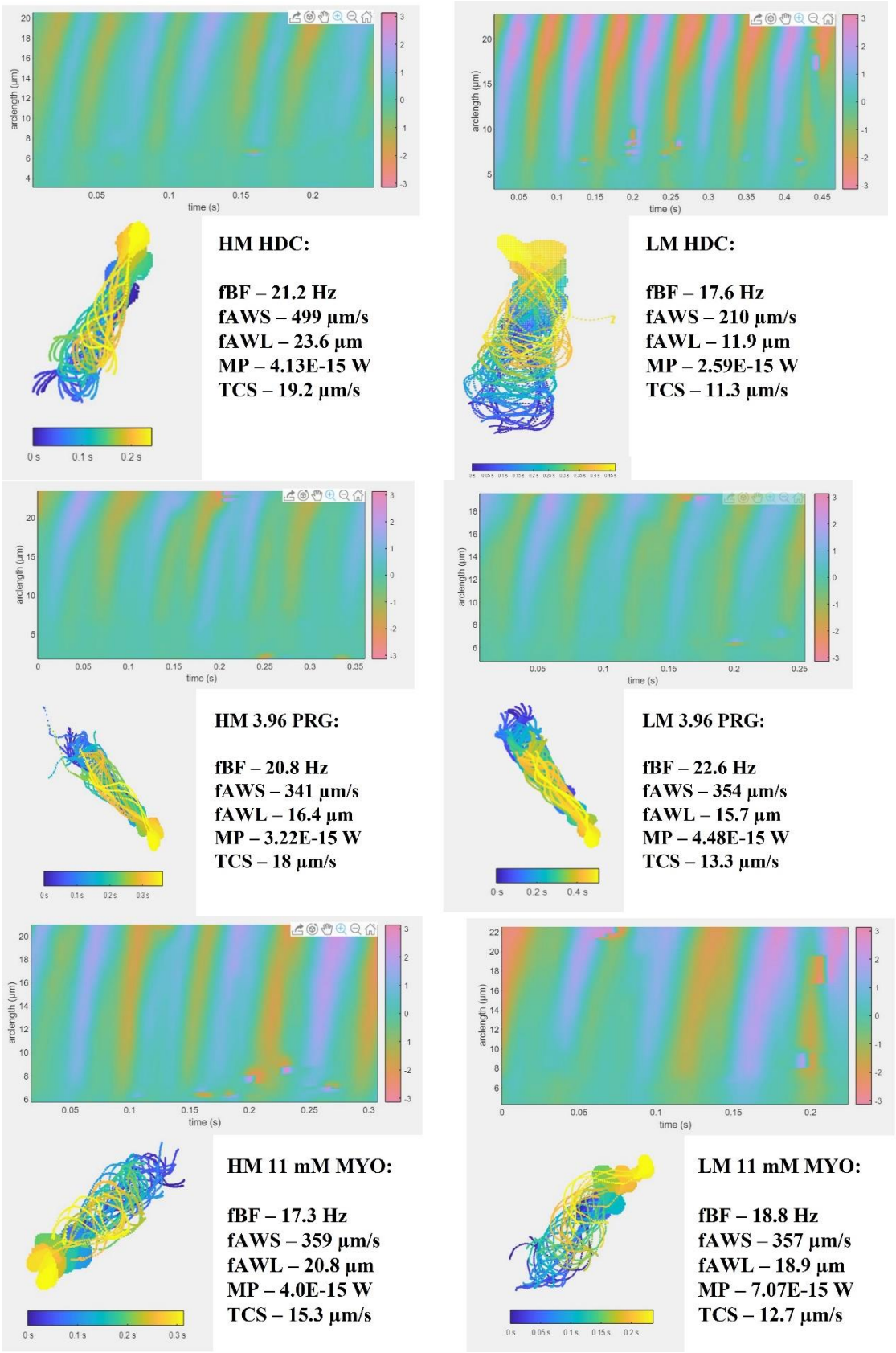
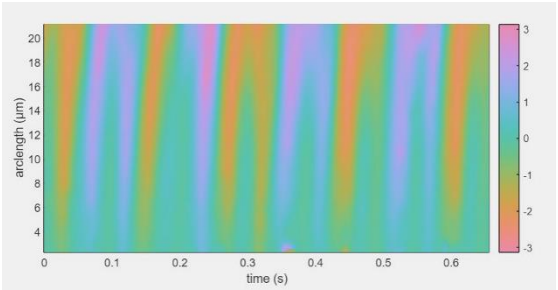
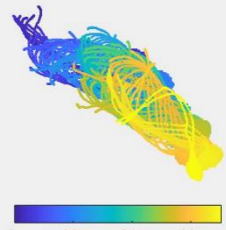


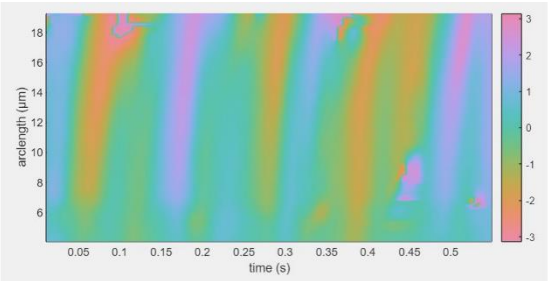
Figure 4.59 Typical Flagellar tangent angles and swimming tracks of both HM and LM individual spermatozoa in HDC, 3.96 µM PRG and 11 mM MYO. **Abbreviations:** fAWL, flagellar arc-wavelength; fAWS, flagellar arc-wave speed; fBF, flagellar beat frequency; HDC, HD-capacitating medium; HM, high motile, HTF, human tubal fluid; LM, low motile; MP, mean power; MYO, myo-inositol; PRG, progesterone; TCS, track centroid speed.

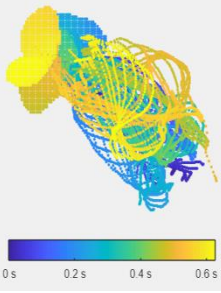




HM 1 µM DOPA:

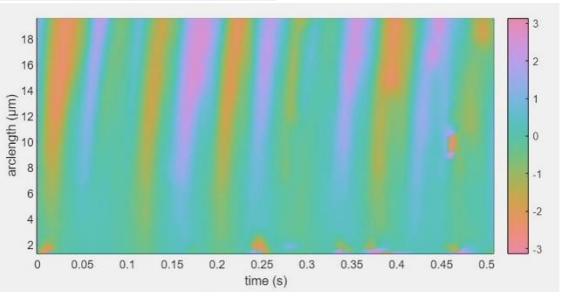
fBF – 15.1 Hz
fAWS – 363 µm/s
fAWL – 24.1 µm
MP – 4.87E-15 W
TCS – 11.4 µm/s

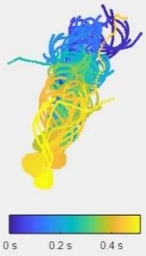




LM 1 µM DOPA:

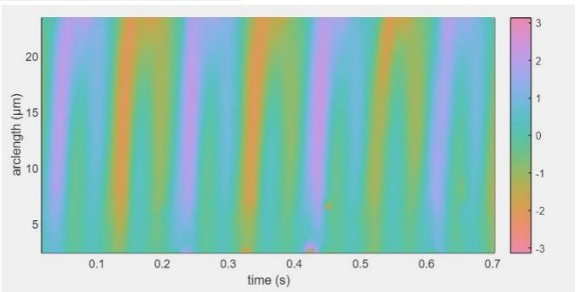
fBF – 21.2 Hz
fAWS – 450 µm/s
fAWL – 21.3 µm
MP – 2.63E-15 W
TCS – 12.9 µm/s

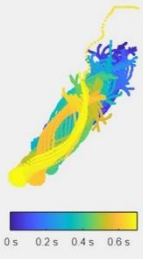




HM 250 ng/mL PRL:

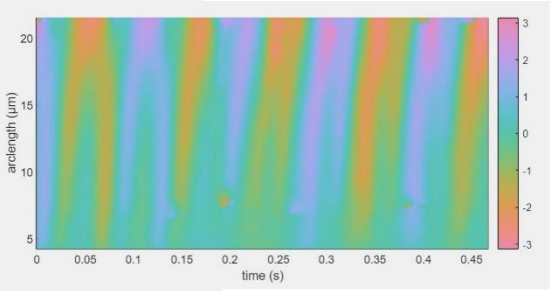
fBF – 19.4 Hz
fAWS – 429 µm/s
fAWL – 22.1 µm
MP – 4.48E-15 W
TCS – 21.4 µm/s

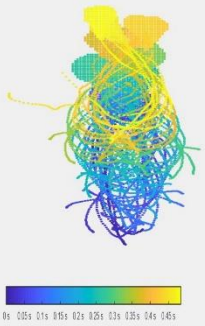




LM 250 ng/mL PRL:

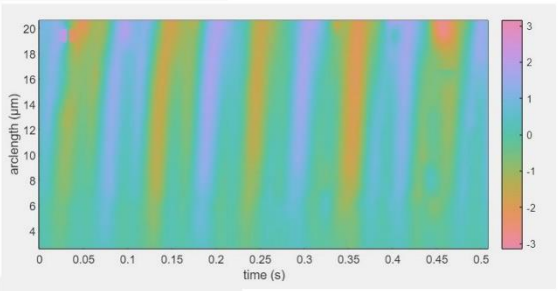
fBF – 15.7 Hz
fAWS – 422 µm/s
fAWL – 26.9 µm
MP – 1.75E-14 W
TCS – 10.9 µm/s

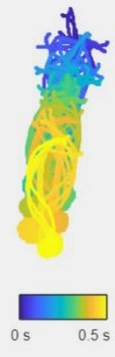




HM 500 ng/mL PRL:

fBF – 19.1 Hz
fAWS – 359 µm/s
fAWL – 18.7 µm
MP – 4.19E-15 W
TCS – 12.9 µm/s





LM 500 ng/mL PRL:

fBF – 24.6 Hz
fAWS – 513 µm/s
fAWL – 20.9 µm
MP – 7.38E-15 W
TCS – 16.9 µm/s

Figure 4.60 Typical Flagellar tangent angles and swimming tracks of both HM and LM individual spermatozoa in 1 µM DOPA, 250 ng/mL and 500 ng/mL PRL. **Abbreviations:** DOPA, dopamine; fAWL, flagellar arc-wavelength; fAWS, flagellar arc-wave speed; fBF, flagellar beat frequency; HM, high motile, LM, low motile; MP, mean power; PRL, prolactin; TCS, track centroid speed.

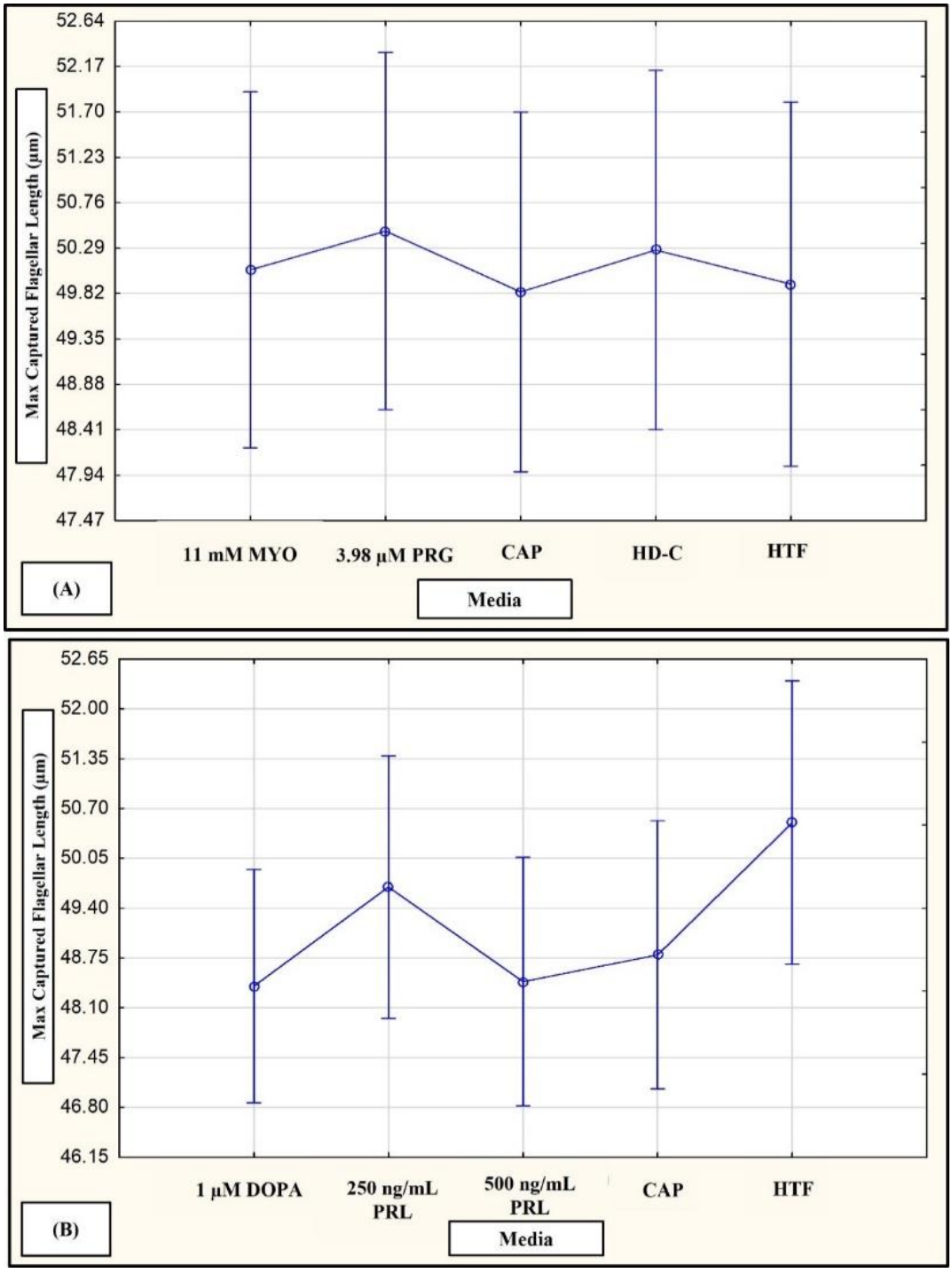


Figure 4.61 Pooled data of both high motile (HM) and low motile (LM) subpopulation individual spermatozoa as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on FAST flagellar parameters**. (A)** Effects of HTF, CAP, HD-C, 3.96 µM PRG and 11 mM MYO on maximum analysed flagellar length (F (4,524) = 0.35, p = 0.84)**. (B)** Effect of HTF, CAP, 1 µM DOPA, 250 ng/mL PRL and 500 ng/mL PRL on maximum analysed flagellar length (F (4,22) = 1.38, p = 0.27). ***Note:*** Media as a fixed factor had no significant effect on max analysed flagellar length. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations**: CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HTF, human tubal fluid; MYO, myo-inositol; PRG, progesterone; PRL, prolactin.

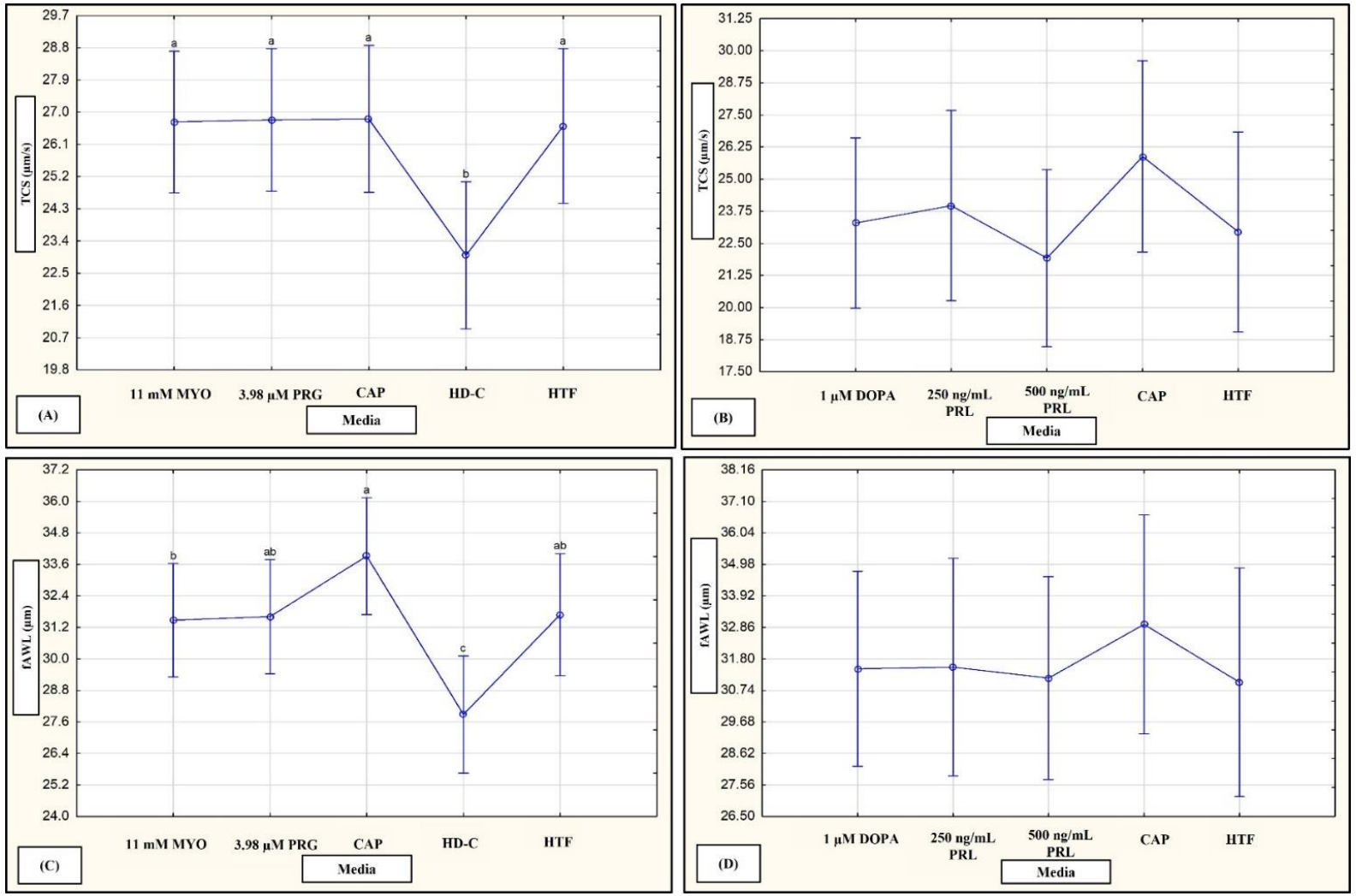


Figure 4.62 Pooled data of both high motile (HM) and low motile (LM) subpopulation individual spermatozoa as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on FAST flagellar parameters. **(A)** Effects of HTF, CAP, HD-C, 3.96 µM PRG and 11 mM MYO on FAST TCS ($F(4,531) = 3.48$, p <0.01). **(B)** Effect of HTF, CAP, 1 µM DOPA, 250 ng/mL PRL and 500 ng/mL PRL on FAST TCS ($F(4,23) = 0.90$, $p = 0.48$). **(C)** Effects of HTF, CAP, HD-C, 3.96 µM PRG and 11 mM MYO on fAWL ($F(4,529) = 5.83$, p <0.01). **(D)** Effect of HTF, CAP, 1 µM DOPA, 250 ng/mL PRL and 500 ng/mL PRL on fAWL ($F(4,23) = 0.25$, $p = 0.91$). ***Note:*** Media as a fixed factor had a significant effect on FAST TCS values where HD-C had significantly lower values as compared to HTF, CAP, PRG and MYO. Media as a fixed factor had a significant effect on FAST fAWL values where HD-C had significantly lower values as compared to HTF, CAP, PRG and MYO. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** CAP, capacitating-HTF; DOPA, dopamine; fAWL, flagellar arc-wavelength; HD-C, HD capacitating medium; HTF, human tubal fluid; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; TCS, track centroid speed.

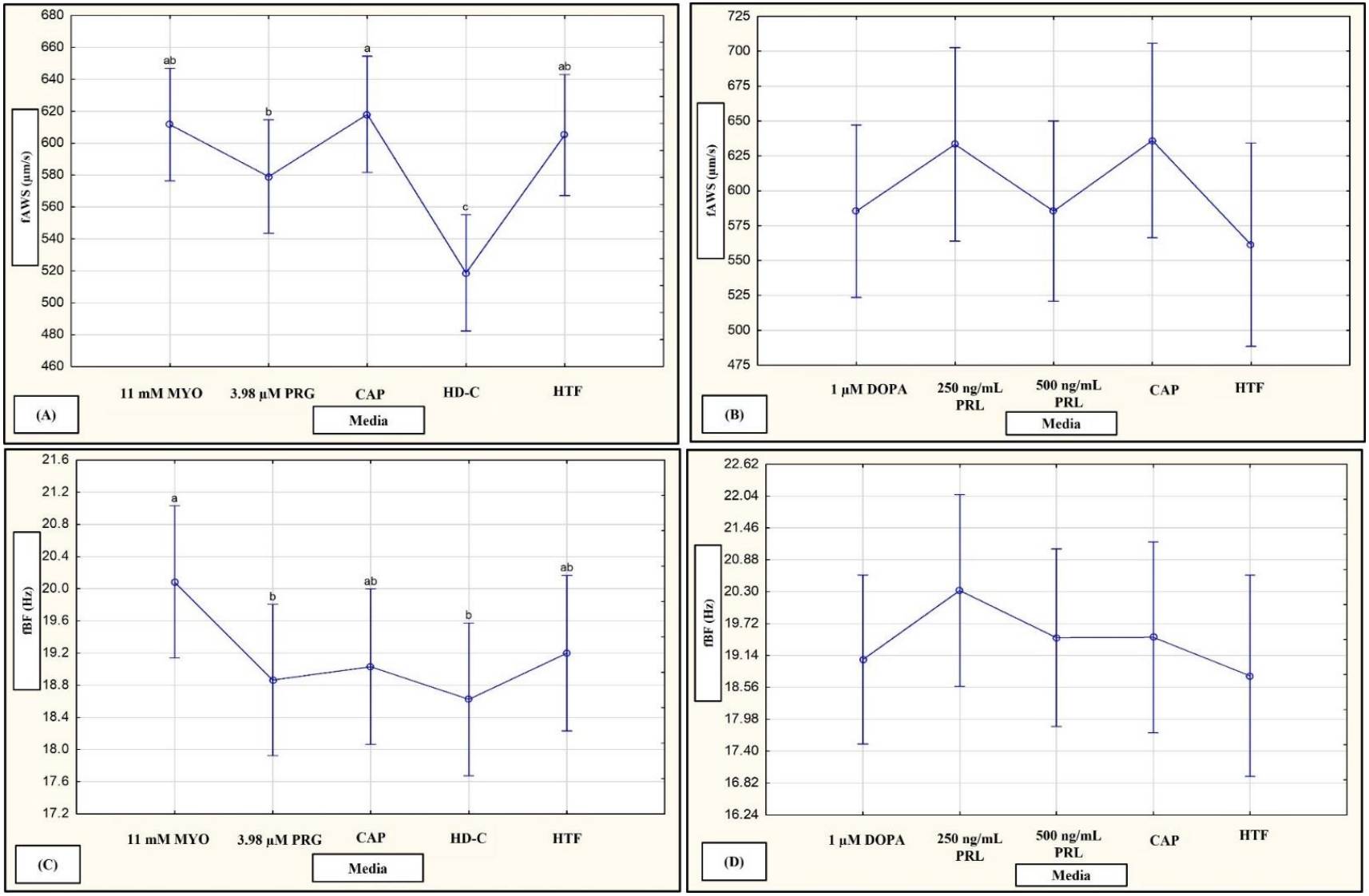


Figure 4.63 Pooled data of both high motile (HM) and low motile (LM) subpopulation individual spermatozoa as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on FAST flagellar parameters. **(A)** Effects of HTF, CAP, HD-C, 3.96 µM PRG and 11 mM MYO on fAWS ($F(4,529) = 8.00$, p <0.01). **(B)** Effect of HTF, CAP, 1 µM DOPA, 250 ng/mL PRL and 500 ng/mL PRL on fAWS ($F(4,23) = 1.19$, $p = 0.34$). **(C)** Effects of HTF, CAP, HD-C, 3.96 µM PRG and 11 mM MYO on fBF ($F(4,528) = 2.00$, $p = 0.09$). **(D)** Effect of HTF, CAP, 1 µM DOPA, 250 ng/mL PRL and 500 ng/mL PRL on fBF ($F(4,23) = 0.51$, $p = 0.73$). ***Note:*** Media also had a signficant effect on FAST fAWS values with HD-C being significantly lower than HTF, CAP, PRG and MYO. Media as a fixed factor had no significant effect on FAST fBF values Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** CAP, capacitating-HTF; DOPA, dopamine; fAWS, flagellar arc-wave speed; fBF, flagellar beat frequency; HD-C, HD capacitating medium; HTF, human tubal fluid; MYO, myo-inositol; PRG, progesterone; PRL, prolactin.

4.3.5.2 | Velocity

Compared to the LM individual sperm, the HM individual sperm presented with significantly higher values for velocity kinematic parameters after being flushed with progesterone and 250 ng/mL prolactin (Supplementary Table 8 and 9). CAP, progesterone and myo-inositol increased the HM individual sperm velocity kinematics as compared to HTF. Whereas HD-C appeared to decrease velocity kinematics in both subpopulations as compared to other media (Supplementary Figure 8). To determine the interaction of media as the main factor on FAST velocity kinematic parameters, data for subpopulations were pooled and analysed in a mixed model ANOVA (Figures 4.64 & 4.65). Media had a significant (p <0.01) interaction on VCL and VSL. For individual effects of the media, HD-C had significantly lower VCL (Figure 4.64 A) and VSL (Figure 4.65 C) values as compared to all media – whereas progesterone and myo-inositol had significantly higher values than HD-C and HTF, but lower than CAP. In addition, HD-C VAP values were significantly lower than myo-inositol and CAP (Figure 4.65 A).

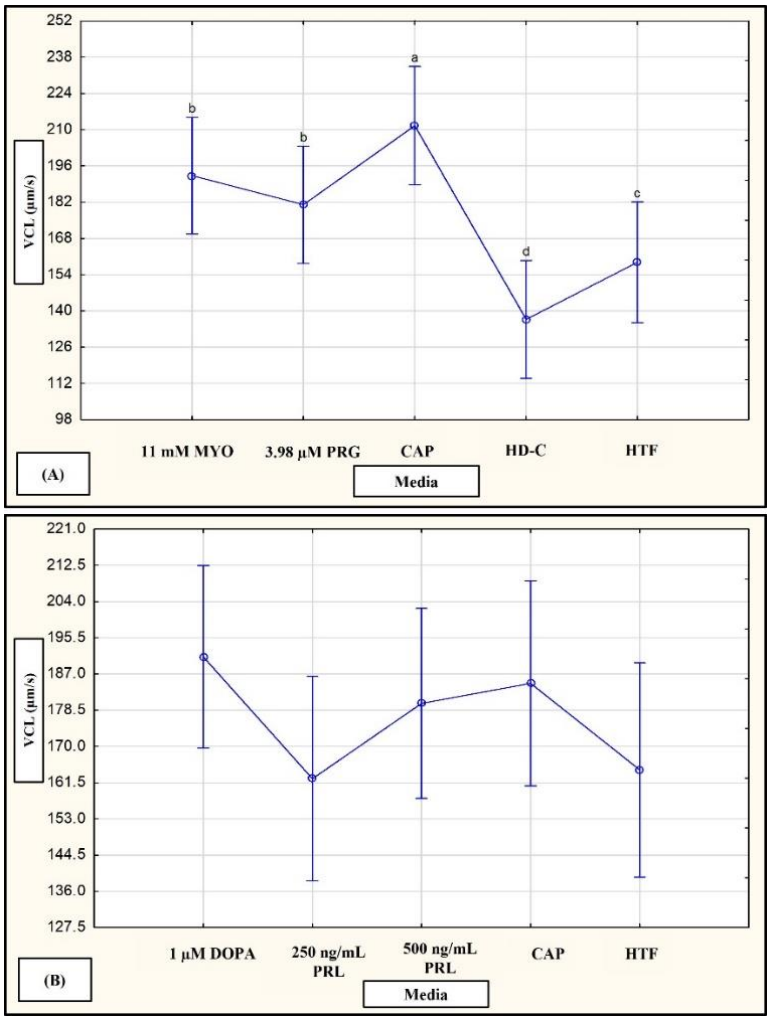


Figure 4.64 Pooled data of both high motile (HM) and low motile (LM) subpopulation individual spermatozoa as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on FAST velocity kinematic parameters. **(A)** Effects of HTF, CAP, HD-C, 3.96 µM PRG and 11 mM MYO on VCL (F (4,525) = 20.15, p <0.01). **(B)** Effect of HTF, CAP, 1 µM DOPA, 250 ng/mL PRL and 500 ng/mL PRL on VCL (F (4,23) = 1.60, p = 0.21). ***Note:*** Media as a fixed factor had a significant effect on FAST VCL values where HD-C had significantly lower values as compared to HTF, CAP, PRG and MYO and PRG and MYO had higher values that HTF and HD-C, however lower than CAP. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations**: CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HTF, human tubal fluid; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; VCL, curvilinear velocity.

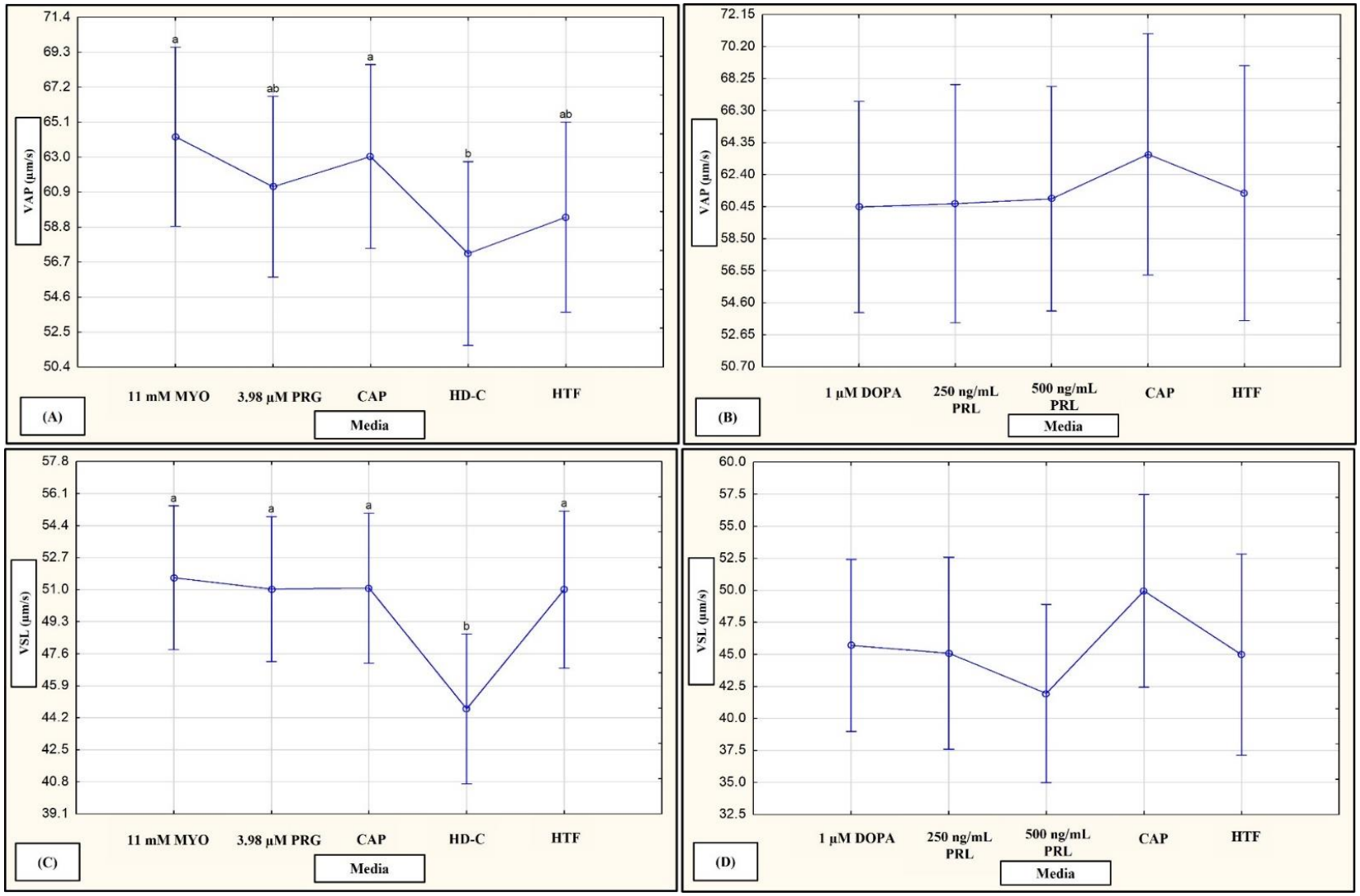


Figure 4.65 Pooled data of both high motile (HM) and low motile (LM) subpopulation individual spermatozoa as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on FAST velocity kinematic parameters. **(A)** Effects of HTF, CAP, HD-C, 3.96 µM PRG and 11 mM MYO on VAP (F (4,528) = 2.09, p = 0.08). **(B)** Effect of HTF, CAP, 1 µM DOPA, 250 ng/mL PRL and 500 ng/mL PRL on VAP (F (4,23) = 0.14, p = 0.97). **(C)** Effects of HTF, CAP, HD-C, 3.96 µM PRG and 11 mM MYO on VSL (F (4,531) = 2.80, p = 0.03). **(D)** Effect of HTF, CAP, 1 µM DOPA, 250 ng/mL PRL and 500 ng/mL PRL on VSL (F (4,23) = 0.84, p = 0.51). ***Note:*** Media as a fixed factor had a significant effect on FAST VSL values where HD-C had significantly lower values as compared to HTF, CAP, PRG and MYO and PRG and MYO. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HTF, human tubal fluid; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; VAP, average path velocity; VSL, straight-line velocity.

4.3.5.3 | Linear

After being flushed with 500 ng/mL prolactin, the HM individual sperm yielded significantly higher values for linear kinematic parameters as compared to the LM individual sperm (Supplementary Table 9). CAP, progesterone and myo-inositol decreased the HM individual sperm linear kinematics parameters as compared to HTF. No significant differences between the media were observed in the LM individual sperm (Supplementary Figure 8). To determine the interaction of media as the main factor on FAST linear kinematic parameters, data for subpopulations were pooled and analysed in a mixed model ANOVA (Figures 4.66). Media had a significant (p <0.01) interaction on LIN. For individual effects of the media, CAP, progesterone and myo-inositol had significantly lower LIN values as compared to HD-C. In addition, CAP and myo-inositol had lower LIN values as compared to HTF, whereas CAP additionally had lower values as compared to progesterone (Figure 4.66 C).

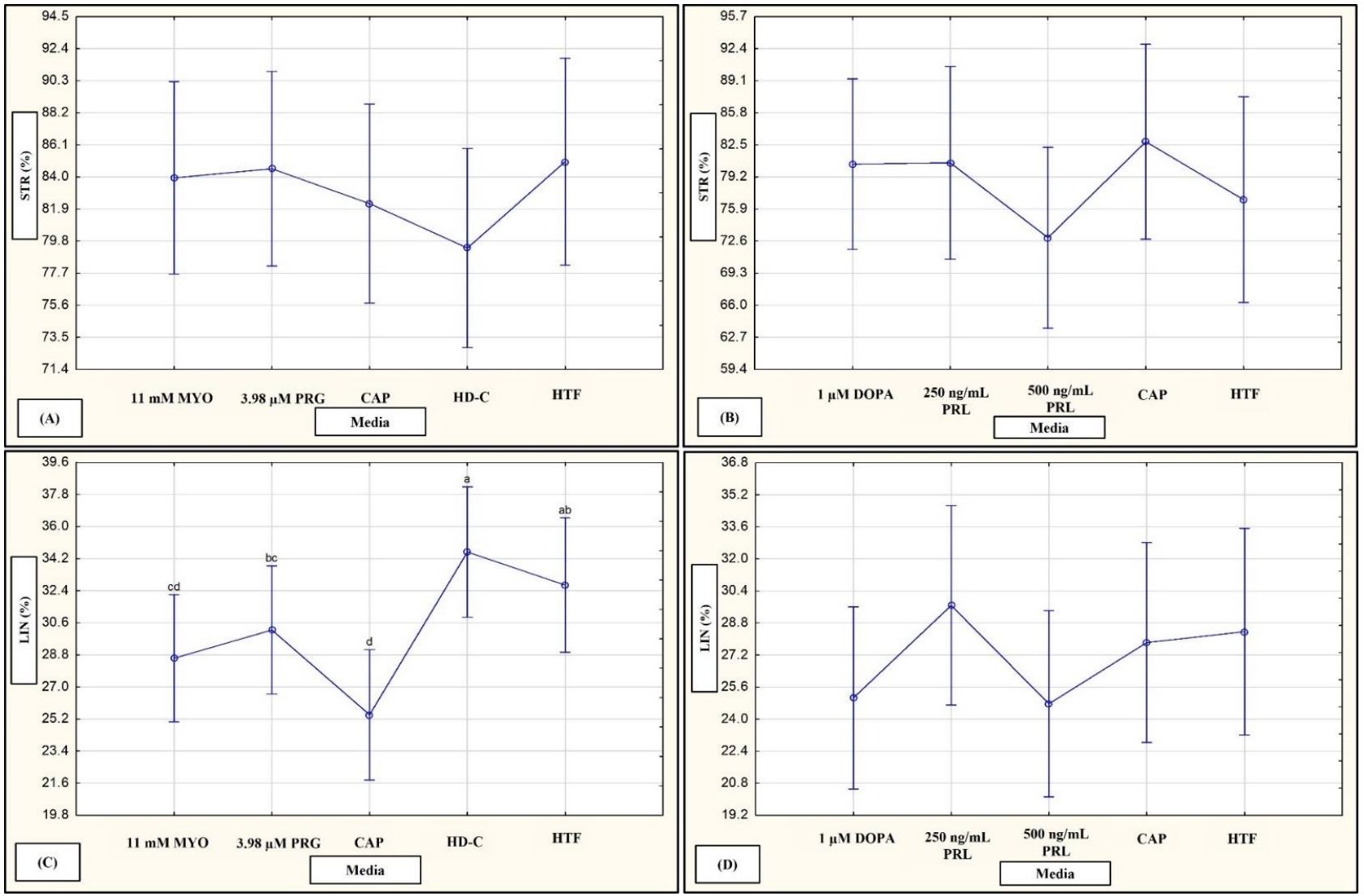


Figure 4.66 Pooled data of both high motile (HM) and low motile (LM) subpopulation individual spermatozoa as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on FAST linear kinematic parameters. **(A)** Effects of HTF, CAP, HD-C, 3.96 µM PRG and 11 mM MYO on STR (F (4,529) = 0.82, p = 0.52). **(B)** Effect of HTF, CAP, 1 µM DOPA, 250 ng/mL PRL and 500 ng/mL PRL on STR (F (4,23) = 0.75, p = 0.57). **(C)** Effects of HTF, CAP, HD-C, 3.96 µM PRG and 11 mM MYO on LIN (F (4,527) = 7.93, p <0.01). **(D)** Effect of HTF, CAP, 1 µM DOPA, 250 ng/mL PRL and 500 ng/mL PRL on LIN (F (4,23) = 1.42, p = 0.26). ***Note:*** Media as a fixed factor had a significant effect on FAST LIN values where CAP and MYO had significantly lower values as compared to HTF and HD-C, and PRG had lower values that HD-C. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations**: CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HTF, human tubal fluid; LIN, linearity; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; STR, straightness.

4.3.5.4 | Vigour

HM individual sperm had significantly higher vigour parameters as compared to the LM individual sperm after being flushed with progesterone and 250 ng/mL prolactin (Supplementary Table 8 and 9). However, LM individual sperm had significantly higher WOB values after being flushed with prolactin. In the HM individual sperm - CAP, progesterone, myo-inositol, dopamine and prolactin increased vigour kinematics as compared to HTF, whereas HD-C decreased values. Likewise, HD-C decreased the LM individual sperm ALH, however 250 ng/mL prolactin and HD-C increased WOB (Supplementary Figure 8). To determine the interaction of media as the main factor on FAST vigour kinematic parameters, data for subpopulations were pooled and analysed in a mixed model ANOVA (Figures 4.67 & 4.68). Media had a significant (p <0.01) interaction on WOB, ALHavg and ALHmax. For individual effects of the media, HD-C had significantly higher WOB values as compared to all media. In addition, myo-inositol and CAP had lower WOB values as compared to HTF (Figure 4.67 A). Both HTF and HD-C had lower ALH values as compared to CAP, progesterone and myo-inositol (Figure 4.67 C & 4.68 A). Furthermore, HTF and CAP had higher BCF values as compared to HD-C (Figure 4.68 C).

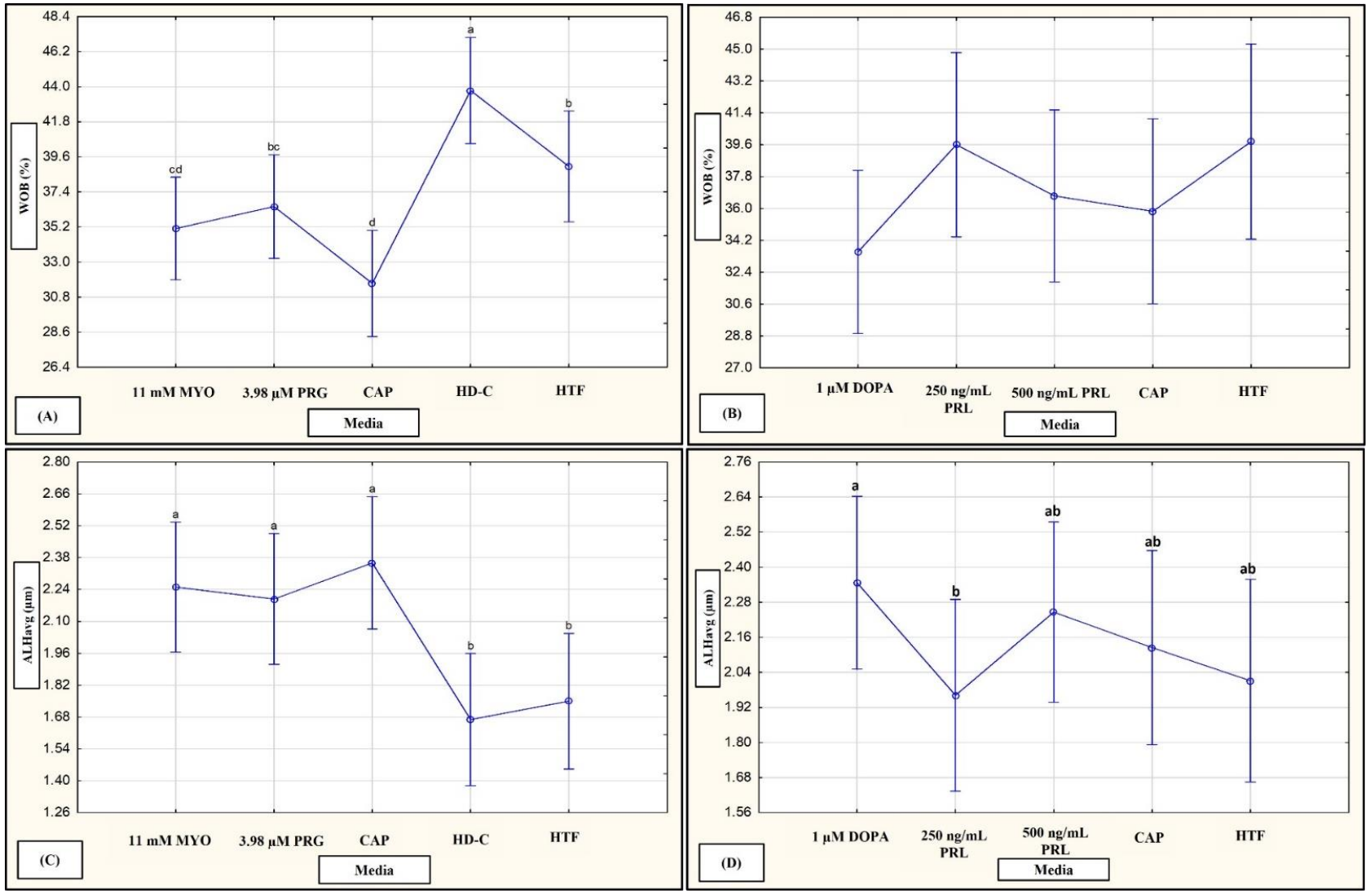


Figure 4.67 Pooled data of both high motile (HM) and low motile (LM) subpopulation individual spermatozoa as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on FAST vigour kinematic parameters. **(A)** Effects of HTF, CAP, HD-C, 3.96 µM PRG and 11 mM MYO on WOB (F (4,529) = 11.64, p <0.01)**. (B)** Effect of HTF, CAP, 1 µM DOPA, 250 ng/mL PRL and 500 ng/mL PRL on WOB (F (4,23) = 1.19, p = 0.34). **(C)** Effects of HTF, CAP, HD-C, 3.96 µM PRG and 11 mM MYO on ALHavg (F (4,526) = 11.18, p <0.01). **(D)** Effect of HTF, CAP, 1 µM DOPA, 250 ng/mL PRL and 500 ng/mL PRL on ALHavg (F (4,22) = 1.66, p = 0.19). ***Note:*** Media as a fixed factor had a significant effect on FAST WOB values where HD-C had significantly higher values as compared to all media and MYO and CAP had lower values as compared to HTF and HD-C. Media additionally had a significant effect on ALHavg values with CAP, PRG and MYO having significantly higher values as compared to HD-C and HTF. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations**: ALHavg, average amplitude of lateral head displacement; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HTF, human tubal fluid; LIN, linearity; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; WOB, wobble.

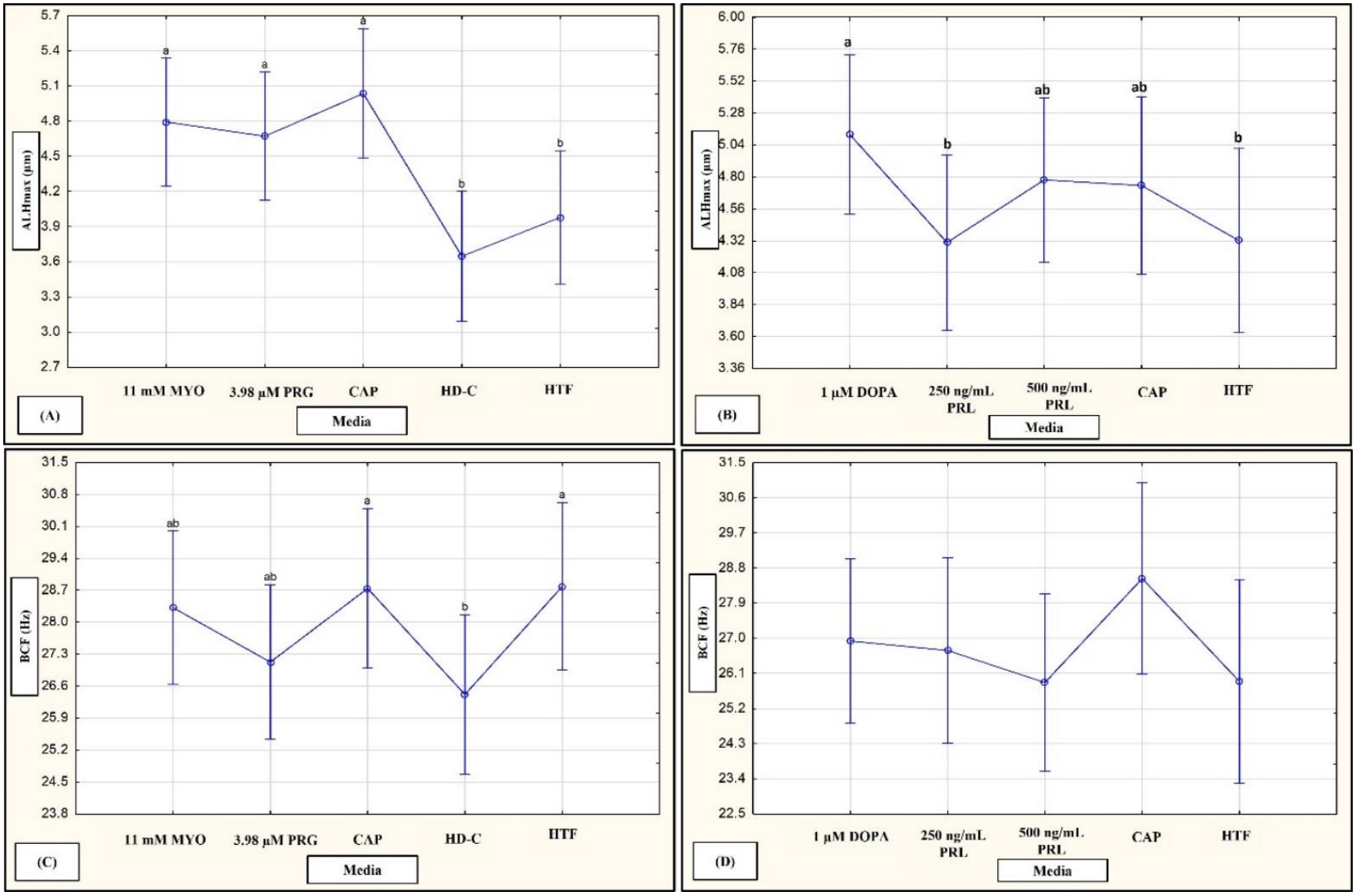


Figure 4.68 Pooled data of both high motile (HM) and low motile (LM) subpopulation individual spermatozoa as analysed in a mixed model repeated measures ANOVA to determine significant (p <0.01) interactions between media as a fixed factor on FAST vigour kinematic parameters. **(A)** Effects of HTF, CAP, HD-C, 3.96 µM PRG and 11 mM MYO on ALHmax (F (4,526) = 11.13, p <0.01). **(B)** Effect of HTF, CAP, 1 µM DOPA, 250 ng/mL PRL and 500 ng/mL PRL on ALHmax (F (4,22) = 1.98, p = 0.13). **(C)** Effects of HTF, CAP, HD-C, 3.96 µM PRG and 11 mM MYO on BCF (F (4,531) = 1.88, p = 0.11). **(D)** Effect of HTF, CAP, 1 µM DOPA, 250 ng/mL PRL and 500 ng/mL PRL on BCF (F (4,22) = 0.84, p = 0.51). ***Note:*** Media as a fixed factor had a significant effect on FAST ALHmax values with CAP, PRG and MYO having significantly higher values as compared to HD-C and HTF. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different (p <0.01). The Fisher LSD was used for the post hoc test and reports. **Abbreviations:** ALHmax, maximum amplitude of lateral head displacement; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HTF, human tubal fluid; LIN, linearity; MYO, myo-inositol; PRG, progesterone; PRL, prolactin.

4.3.6 | ANOVA Between SCA Whole Subpopulations, FAST Individual Sperm and SCA Individual Sperm Kinematic Parameters

Illustarted in Tables 4.18 are the ANOVA results for the SCA whole sperm subpopulation, SCA individual spermatozoa and FAST individual spermatozoa for each of the kinematic parameters. One-way ANOVA analysis was applied to determine whether significant differences between the SCA and FAST programme kinematic parameters exist. Theoretically only kinematic parameters including VCL, VSL and LIN should be compared between software as these parameters are followed point by point and thus should be similar between software provided the same frames per second were identical in each program. However, in this study we decided to compare and correlate all the various kinematics to see if any new information could be derived or interesting correlations could be revealed amongst the software and parameters. Both FAST and SCA individual spermatozoa had significantly higher values for VCL in both sperm subpopulations as compared to the VCL of the SCA whole sperm subpopulation. This was further observed for LIN and the HM subpopulation VSL values, however the whole motile sperm subpopulation had greater values as compared to individual spermatozoa. All three analysis methods differed significantly from one another for BCF, WOB and the LM subpopulations VAP values. Furthermore, FAST individual sperm analysis had significantly lower values as compared to SCA individual and whole sperm subpopulation analysis for HM subpopulation VAP values, however higher values were observed for STR. Moreover, the SCA individual spermatozoa values for ALH were significantly higher as compared to FAST and SCA whole sperm subpopulations.

Table 4.18 ANOVA comparison between the average SCA parameters of the whole high sperm subpopulation, and SCA and FAST parameters of individual spermatozoa from both the HM and LM sperm subpopulation (mean ± SD) after treatment with HTF.

		Programme						ANOVA
		SCA Whole Sperm Subpopulation		SCA individual Sperm		FAST individual Sperm		
		Mean ± SD		Mean ± SD		Mean ± SD		
VCL (µm/s)								
	HM	139.4 ± 34.0[a]		164.9 ± 53.4[b]		168.6 ± 56.8[b]		**<0.001**
	LM	118.9 ± 27.9[a]		153.3 ± 55.3[b]		165.6 ± 58.2[b]		**<0.001**
VAP (µm/s)								
	HM	82.2 ± 19.4[a]		81.7 ± 19.8[a]		60.4 ± 16.7[b]		**<0.001**
	LM	67.1 ± 15.2[a]		76.6 ± 20.9[b]		58.3 ± 14.8[c]		**<0.001**
VSL (µm/s)								
	HM	60.5 ± 18.6[a]		51.1 ± 15.2[b]		51.4 ± 15.6[b]		**<0.001**
	LM	47.0 ± 13.4		47.7 ± 17.4		49.7 ± 17.2		0.245

STR (%)												
	HM	68.7	±	10.6[a]	63.9	±	17.7[a]	86.9	±	21.1[b]		**<0.001**
	LM	64.6	±	9.7[a]	62.7	±	20.2[a]	85.6	±	22.7[b]		**<0.001**
LIN (%)												
	HM	43.5	±	9.8[a]	33.3	±	12.3[b]	33.0	±	12.4[b]		**<0.001**
	LM	39.8	±	9.1[a]	33.0	±	13.0[b]	32.1	±	12.0[b]		**<0.001**
WOB (%)												
	HM	59.5	±	5.8[a]	51.7	±	10.6[b]	38.3	±	11.8[c]		**<0.001**
	LM	57.3	±	6.9[a]	52.2	±	10.4[b]	38.0	±	11.7[c]		**<0.001**
ALH (µm)												
	HM	1.8	±	0.5[a]	2.8	±	1.0[b]	1.9	±	0.8[a]		**<0.001**
	LM	1.8	±	0.5[a]	2.7	±	1.1[b]	1.9	±	0.8[a]		**<0.001**
BCF (Hz)												
	HM	44.3	±	7.9[a]	41.4	±	11.4[b]	27.4	±	7.1[c]		**<0.001**
	LM	41.8	±	7.6[a]	39.6	±	13.2[b]	28.4	±	6.9[c]		**<0.001**

Note: The SCA parameters of the whole sperm subpopulation were significantly different from that of the SCA and FAST individual sperm parameters for the HM subpopulation VCL, VSL and LIN - in addition to LM subpopulation VCL and LIN. FAST individual sperm parameters were significantly different from SCA whole subpopulations and individual sperm for HM VAP and both subpopulations STR values. Kinematic parameters of the different programmes were significantly different to each other for LM VAP, both subpopulations WOB and ALH. SCA individual sperm parameters were significantly different from the whole sperm subpopulations and FAST parameters for both subpopulations ALH values. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; FAST, flagellar analysis and sperm tracking; HTF, human tubal fluid; HM, high motile subpopulation; LIN, linearity; LM, low motile subpopulation; SD, standard deviation; SCA, sperm class analyser; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble. [a, b,c,] Values labelled with different superscript letters in the same row for individual parameters were significantly different between the whole subpopulation and individual sperm for FAST and SCA (p <0.05).

4.3.7 | Correlation and Multiple Regression Analysis of FAST Parameters with SCA Parameters

FAST parameters were grouped as flagellar parameters and kinematic parameters. The flagellar parameters included the analysed flagellar length, maximum analysed flagellar length, fAWL, fAWS, Fbf, mean power and TCS values. Kinematic parameters were further grouped as velocity, linear and vigour subsets. Curvilinear velocity, VAP and VSL are grouped as "velocity" kinematics; LIN and STR as "linear"; and ALH, WOB and BCF as "vigour" kinematics parameters. The SCA whole sperm subpopulation parameters are divided into motility and kinematic groups. Motility groups include the different motility parameters, and the kinematic group includes the different kinematic parameters for the different progressive speed groups (average, rapid, medium and slow). Kinematics are further categorized into velocity (VCL, VAP and VSL), linear (LIN and STR) and vigour (WOB, BCF and ALH) groups. The SCA individual sperm parameters are grouped as kinematic parameters which are further categorized into velocity (VCL, VAP and VSL), linear (LIN and STR) and vigour (WOB, BCF and ALH) groups. For grouped parameters, the highest significant correlation was selected to represent the group,

however individual correlations for each parameter are displayed as a heatmap in Supplementary Table 10 and 11.

4.3.7.1 | FAST Correlations with the SCA Whole Sperm Subpopulation

FAST flagellar parameters had more correlations with the SCA whole sperm subpopulation kinematic parameters as compared to the percentage population motility parameters (Table 4.19 & Supplementary Table 10). Nevertheless, FAST flagellar parameters appeared to negatively correlate with the medium and slow velocity speed groups, but positively correlate with the rapid speed group. Similar observations were seen for progressive groups where rapid progressive (RP) speed groups had positive correlations with FAST flagellar parameters, but non-progressive (NP) and medium progressive (MP) groups had negative correlations. From the flagellar parameters, TCS appeared to display the best correlations with the motility parameters of the SCA whole sperm subpopulations. The majority of the flagellar parameters positively correlated with the motile and RP speed group kinematic parameters, of which equal correlations were observed with the velocity and vigour groups. Of the flagellar parameters, both TCS and mean power appeared to display most correlations with the kinematic parameters.

FAST velocity kinematic parameters generally had a positive correlation with the progressive, RP and rapid speeds as compared to other motility parameters. Likewise, FAST velocity kinematics positively correlated with the velocity and vigour kinematics of the motile, RP and MP speed groups. In contrast, FAST velocity kinematics had majority negative correlations with the NP speed group kinematic parameters. FAST linear kinematics frequently correlated with the SCA whole sperm subpopulation kinematic parameters as compared to motility parameters. Of these correlations, majority were isolated to the motile, MP and NP speed groups, however the MP speed group appeared to be mostly negative. FAST vigour kinematics appeared to typically have a positive correlation with the rapid and RP speed groups in addition to negatively correlating with the MP speed groups as compared to other motility parameters. Furthermore, the FAST vigour parameters had positive correlations with the velocity and vigour kinematics of the motile, RP and MP speed groups, however negatively correlated with their linear kinematics.

Table 4.19 Significant (p <0.05) correlations between individual FAST parameters and the whole SCA sperm subpopulation parameters for both the high motile (HM) and low motile (LM) sperm subpopulations in HTF.

FAST individual sperm parameters			Motility Percentages									Kinematic Parameters											
												Motile			RP			MP			NP		
			Immotile	Motile	Prog	RP	MP	NP	Rapid	Medium	Slow	Velocity	Linear	Vigour	Velocity	Linear	Vigour	Velocity	Linear	Vigour	Velocity	Linear	Vigour
Flagella Parameters	Analysed FL	HM										0.17		0.13	0.15	0.12	0.14	0.10					
		LM																			0.22		
	Max FL	HM										0.14			0.13		-0.13	0.14			-0.11	-0.17	
		LM						-0.24		-0.17		0.26		0.21				0.17					
	fAWL	HM				0.15	-0.13		0.11	-0.1	-0.11	0.14	0.10				0.12	0.11					
		LM							0.16					0.21	0.16		0.26		-0.16	-0.22	0.16		
	fAWS	HM				0.20	-0.16	-0.12	0.14	-0.12	-0.18	0.13					0.15						
		LM								-0.16				0.21	-0.18	-0.17	0.29			-0.18		0.19	
	fBF	HM									-0.13										-0.1		
		LM													-0.22								
	Mean power	HM	0.12	-0.12		0.15	-0.19	-0.1	0.26	-0.37	-0.18	0.29	-0.29	-0.33	0.21	-0.26	0.24	0.36	-0.32	0.31	-0.34	-0.26	-0.34
		LM							0.19	-0.16		0.23	-0.22	-0.35	0.17	-0.24	0.30	0.32	-0.25	0.35			
	TCS	HM	-0.11	0.11	0.21	0.21	-0.18		0.16		-0.15	0.27	0.13	0.11	0.18	0.11		0.19	0.11	0.13			
		LM	-0.17	0.17	0.24	0.21	0.19		0.19			0.18	0.26	0.21			0.26						
Kinematic Parameters	Velocity: VCL	HM	0.10	-0.1	0.15	0.27	-0.35		0.40	-0.45	-0.32	0.47	-0.34	0.48	0.31	-0.33	0.35	0.48	-0.39	0.42	-0.3	-0.25	-0.3
		LM			0.30	0.46		-0.17	0.46	-0.16		0.48		0.42	0.33		0.32	0.38	-0.23	0.33			
	Velocity: VAP	HM			0.17	0.16	-0.22		0.20	-0.15	-0.26	0.25	-0.11	0.22	0.11	-0.13	0.13	0.29	-0.15	0.17	-0.13	-0.18	
		LM			0.16	0.16			0.20			0.30		0.28				0.28	-0.21	0.26			
	Velocity: VSL	HM	-0.11	0.11	0.21	0.20	-0.18		0.15		-0.16	0.27	0.15	0.12	0.17	0.11	0.11	0.17	0.13	0.15		0.11	
		LM	-0.16	0.16	0.24	0.23	0.16		0.19			0.18	0.23	0.21			0.28				-0.19		
	Linear: STR	HM								0.16		0.14	0.27	0.20		0.21	-0.11	-0.23	0.26	-0.26	0.17	0.26	0.18
		LM			0.15								0.30				0.18	0.18	0.22		-0.2	0.17	
	Linear: LIN	HM	-0.12	0.12		-0.1	0.17		-0.26	0.38	0.17	-0.34	0.40	0.42	-0.24	0.34	-0.32	-0.45	0.42	-0.43	0.29	0.30	0.30
		LM						0.20	-0.2	0.32	0.16	-0.3	0.29	0.21	-0.35			-0.3	0.27	-0.2	0.20	0.26	
	Vigour: WOB	HM		0.09		-0.18	0.20		-0.28	0.33	0.16	-0.32	0.26	0.34	-0.26	0.24	-0.27	-0.36	0.28	-0.3	0.19	0.14	0.22
		LM			-0.21	-0.36			-0.35			-0.32		-0.26	-0.27		-0.24	-0.25		-0.2			
	Vigour: ALHavg	HM	0.11	-0.11		0.15	-0.27		0.32	-0.37	-0.28	0.20	-0.39	0.45	0.27	-0.36	0.34	0.47	-0.43	0.45	-0.27	-0.32	-0.27
		LM			0.24	0.41		-0.22	0.43	-0.32		0.52		0.47	0.27		0.32	0.39	-0.27	0.40			
	Vigour: ALHmax	HM	0.10	-0.1		0.14	-0.23		0.30	-0.37	-0.24	0.38	-0.39	0.44	0.29	-0.34	0.32	0.46	-0.43	0.45	-0.3	-0.34	-0.3
		LM			0.19	0.37		-0.23	0.40	-0.35		0.50	-0.19	0.46	0.27	-0.16	0.29	0.43	-0.32	0.43		-0.19	
	Vigour: BCF	HM			0.17	0.18	-0.18		0.20	-0.14	-0.12	0.17			0.13		0.11	0.19			-0.17		
		LM	-0.17	0.17		0.18				0.20											-0.18		
	Vigour: MAD	HM								-0.13		-0.13	-0.21	-0.19		-0.18	-0.16	0.19	-0.2	-0.21		-0.15	
		LM											-0.16			-0.19					0.18		

Note: SCA whole subpopulation parameters are divided into motility and kinematic groups. Motility groups include the different motility parameters, and the kinematic group includes the different kinematic parameters for the different progressive speed groups (average, rapid, medium, and slow). Kinematics are further categorized into velocity (VCL, VAP and VSL), linear (LIN and STR) and vigour (WOB, BCF and ALH) groups. FAST parameters are grouped in flagellar parameters and kinematic parameters. The FAST flagellar parameters correlated with the whole SCA sperm subpopulation kinematics, whereas FAST kinematic parameters correlated with both SCA motility and kinematic parameters. **Abbreviations:** ALH, amplitude of lateral head displacement; avg, average; BCF, beat cross frequency; FAST, flagellar analysis and sperm tracking; fAWL, flagellar arc-wavelength; fAWS, flagellar arc-wave speed; fBF, flagellar beat frequency; FL, flagellar length; high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MAD, mean angle displacement; max, maximum; MP, medium progressive; NP, non-progressive; RP, rapid progressive; SCA, sperm class analyser; STR, straightness; TCS, track centroid speed; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

4.3.7.2 | FAST Correlations with the SCA Individual Sperm Parameters

FAST flagellar parameters correlated better with the RP and MP speed groups of the SCA individual sperm parameters as compared to the NP speed groups (Table 4.20 & Supplementary table 11). From these correlations TCS appeared to have the highest correlations with RP velocity kinematics (HM, 0.79 and LM, 0.72) and MP velocity kinematics (HM, 0.81 and LM, 0.67). The flagellar parameters further appeared to correlate better with the vigour kinematics of RP speed groups but correlated with both velocity and vigour kinematics of the MP speed groups. Furthermore, flagellar parameters additionally appeared to have many negative correlations with the NP speed group kinematic parameters whereas more positive correlations were observed between flagellar parameters and the MP and RP speed groups.

FAST velocity kinematic parameters positively correlated with HM subpopulation progressive speed groups' velocity and vigour kinematics, whereas the LM subpopulation presented with negative correlations for the NP speed group vigour kinematics. In addition, FAST velocity kinematics appeared to have higher correlation coefficients with the RP and MP speed groups as compared to the NP speed groups. Moreover, from the FAST parameters, VCL appeared to negatively correlate with all the SCA speed groups linear kinematics. FAST linear kinematic parameters consistently presented with positive correlations to the linear kinematics of all speed groups for SCA individual sperm parameters. Furthermore, the MP speed groups of SCA had more correlations with FAST linear kinematics as compared to the RP and NP speed groups. In addition, the MP speed group correlation coefficients further appeared to be higher than the other speed groups.

FAST vigour kinematic parameters correlated more with RP and MP speed SCA individual sperm kinematics as compared to the NP speed group. Both ALH and WOB of the FAST vigour kinematics correlated with velocity, linear and vigour parameters of all speed groups for SCA. Albeit ALH negatively correlated with the linear parameters of which higher coefficients were observed in MP speed groups. In addition, both SCA velocity and vigour kinematics appeared to positively correlate with FAST ALH and WOB parameters, however higher coefficients were once again seen for the MP speed groups. In contrast, MAD of the FAST vigour kinematics consistently presented with negative correlations towards the various kinematics of the RP and MP speed groups for SCA.

Table 4.20 Significant (p <0.05) correlations between individual FAST parameters and the individual SCA sperm parameters for both the high motile (HM) and low motile (LM) sperm subpopulations in HTF.

FAST indidvual sperm parameters				SCA Individual Sperm Paramaters														
				RP					MP					NP				
				Elongation	Area	Velocity	Linear	Vigour	Elongation	Area	Velocity	Linear	Vigour	Elongation	Area	Velocity	Linear	Vigour
Flagellar Parameters		Analysed FL																
			HM			-0.27	0.35	0.39					0.15		0.33			
			LM		0.32			-0.32										
		Max FL																
			HM		0.20		0.17	0.23	0.17		0.20					0.54	-0.38	
			LM					-0.29			0.23							
		fAWL																
			HM			0.19		0.18		0.16	0.31	-0.24	-0.34		0.55	0.60	0.47	0.38
			LM								0.52	-0.46	0.56					-0.44
		fAWS																
			HM					0.23				-0.14	-0.28		0.54	0.49		
			LM															-0.52
		fBF																
			HM		-0.16		-0.19	-0.22		-0.26	-0.27	0.15	-0.3					-0.56
			LM					0.26			-0.42	0.53	-0.46					
		Mean power																
			HM			0.35	-0.21	0.19		-0.19	0.41	-0.34	0.34			-0.36	-0.37	-0.51
			LM				-0.3				0.34	-0.32	0.36		-0.37			-0.58
		TCS																
			HM			0.79	0.45	0.35			0.81	0.61	0.32		0.41	0.53	0.35	
			LM			0.72	0.55				0.67	0.48	0.35		-0.4			-0.47
Kinematic Parameters	Velocity	VCL																
			HM			0.88	-0.6	0.78			0.94	-0.81	0.90			0.80	-0.5	0.63
			LM			0.74	-0.59	0.79			0.82	-0.67	0.83			0.53	-0.46	-0.62
		VAP																
			HM			0.76	0.23	0.29			0.37		0.30			0.74		0.39
			LM		-0.26	0.59		0.26			0.59		0.44			0.37		-0.38
		VSL																
			HM			0.8	0.47	0.38			0.86	0.67	0.38		0.42	0.63	0.41	0.34
			LM			0.79	0.54				0.74	0.55	0.33			0.55	0.46	-0.48
	Linear	STR																
			HM			-0.3	0.57	-0.26			0.60	0.78	-0.55			-0.34	0.57	
			LM		0.29		0.50				0.56	0.75	-0.47				0.46	
		LIN																
			HM			-0.59	0.88	0.76			-0.8	0.94	-0.81			0.54	0.8	0.47
			LM			0.54	0.83	0.58			-0.73	0.92	0.75			0.60	0.88	0.54
	Vigour	WOB																
			HM			-0.49	0.77	0.75			-0.65	0.72	0.83			0.55	0.47	0.75
			LM			0.47	0.72	0.67			-0.71	0.70	0.80			0.44	0.69	0.78
		ALHavg																
			HM			0.72	-0.61	0.70			0.87	-0.82	0.88			0.82	-0.4	0.76
			LM			0.60	-0.67	0.67			0.82	-0.75	0.85			0.46	-0.39	0.68
		ALHmax																
			HM			0.70	-0.6	0.63			0.84	-0.8	0.84			0.75	-0.5	0.64
			LM		-0.37	0.57	-0.71	0.64			0.77	-0.72	0.77			0.43	-0.43	0.62
		BCF																
			HM			0.27	0.24	0.37	-0.19				-0.27			-0.6		-0.59
			LM										0.25			-0.38		
		MAD																
			HM			-0.34	-0.41	-0.3			-0.41	-0.35	-0.25					
			LM			-0.54	-0.58				-0.34	-0.44	0.24					

Note: SCA individual sperm parameters are divided into kinematic groups. Kinematic groups include the different kinematic parameters for the different progressive speed groups (average, rapid, medium, and slow). Kinematics are further categorized into velocity (VCL, VAP and VSL), linear (LIN and STR) and vigour (WOB, BCF and ALH) groups. FAST parameters are grouped in flagellar parameters and kinematic parameters. The FAST flagellar parameters correlated with the whole SCA sperm subpopulation kinematics, whereas FAST kinematic parameters correlated with both SCA motility and kinematic parameters. **Abbreviations:** ALH, amplitude of lateral head displacement; avg, average; BCF, beat cross frequency; FAST, flagellar analysis and sperm tracking; fAWL, flagellar arc-wavelength; fAWS, flagellar arc-wave speed; fBF, flagellar beat frequency; FL, flagellar length; high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MAD, mean angle displacement; max, maximum; MP, medium progressive; NP, non-progressive; RP, rapid progressive; SCA, sperm class analyser; STR, straightness; TCS, track centroid speed; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

4.3.7.3 | FAST Linear Regression Analysis with the SCA Individual Sperm Parameters

Illustrated in Table 4.20 is the R-squared (R2) and beta coefficients (b*) from the multiple regression analysis used to determine the relationship between a group of FAST parameters (4 independent variables) and the SCA individual and whole sperm subpopulations motility and kinematic parameters (69 dependent variables). The selected FAST group comprised of FAST flagellar parameters namely, fBF, fAWS, fAWL and analysed flagellar length. Mean power was excluded from the group as it did not sufficiently represent entire sperm tail length. Data from the FAST grouping with significant (p <0.05) predictor variables were displayed in Table 4.20.

The selected FAST group had less predictive power on the whole sperm subpopulation parameters as compared to the SCA individual sperm parameters which had more significant correlations. Furthermore, the R-squared values were additionally lower for that of the SCA whole sperm subpopulations (range: 0.02-0.04) as compared to the SCA individual sperm parameters (range: 0.05-0.25). From the significant parameters of the SCA whole sperm subpopulation – the FAST group had the greatest predictive power over (0.04) the percentage rapid-progressive (RP) spermatozoa, however remaining low. As such, FAST fBF, fAWS, fAWL and analysed FL could account for at least 4% of variation observed in the RP speed groups of the whole sperm subpopulations. In addition, the FAST parameters could account for 3% of variation observed in non-progressive (NP), slow, non-progressive STR and 2% of variation observed for rapid, motile VSL and ALH values.

FAST parameters appeared to have more significant correlations with the medium progressive (MP) speed group parameters of SCA individual sperm parameters as compared to RP and NP speed groups. That said, the NP had the least number of significant correlations as compared to the other speed groups. FAST parameters could account for ≥20% of the variation observed for NP ALH, MP ALH and RP ALH. Whereas ≥10% of the variation could be accounted for in NP STR and WOB, MP VCL, VAP, STR, LIN, WOB and BCF, and RP VCL and WOB. From the individual beta* coefficients, fBF (positive) had the greatest effect on NP parameters, analysed FL (positive) had the greatest effect on MP parameters and fBF (both positive and negative) had the greatest effect on RP parameters.

Table 4.20 Significant beta coefficients (b*) and R-squared (R2) values from the multiple linear regression analysis used to determine the relationship between selected FAST parameters and motility and kinematic parameters of SCA whole sperm subpopulations and in dividual spermatozoa in HTF.

SCA CASA VARIABLES		R	R2	b*			
				fBF	fAWL	fAWS	Analysed FL
WHOLE SCA	**RP**	0.20	0.04				
	NP	0.16	0.03				
	Rapid	0.16	0.02				
	Slow	0.18	0.03				
	Motile VSL	0.15	0.02				
	Motile ALH	0.15	0.02				
	NP STR	0.17	0.03				-0.35
SCA INDIVIDUAL SPERM	**NP STR**	0.38	0.14	5.41	-0.18	3.81	
	NP WOB	0.43	0.18				
	NP ALH	0.44	0.20				-0.05
	MP area	0.24	0.06				
	MP VCL	0.42	0.18				
	MP VAP	0.33	0.11				
	MP STR	0.34	0.11				
	MP LIN	0.36	0.13				0.72
	MP WOB	0.40	0.16				0.67
	MP ALH	0.48	0.23			0.09	
	MP BCF	0.37	0.14				
	RP area	0.23	0.05				0.20
	RP VCL	0.33	0.11	7.04	-0.23	5.06	-2.77
	RP LIN	0.30	0.09	-1.56	0.04	-0.83	0.74
	RP WOB	0.37	0.13	-1.38	0.04	-0.91	0.83
	RP ALH	0.39	0.25	0.14	-0.01	0.11	-0.05
	RP BCF	0.30	0.09	-1.58	0.07	-1.06	0.03

Note: The selected FAST group comprised of FAST flagellar parameters namely, fBF, fAWS, fAWL and analysed flagellar length. Mean power was excluded from the group due to values being too small to include for proper analysis. Data from the FAST grouping with significant (p <0.05) predictor variables were displayed. FAST group had greater predictive power over the SCA individual sperm parameters as compared to the whole sperm subpopulation. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; FAST, flagellar analysis and sperm tracking; fAWL, flagellar arc-wavelength; fAWS, flagellar arc-wave speed; fBF, flagellar beat frequency; FL, flagellar length; HTF, human tubal fluid; LIN, linearity; MP, medium progressive; NP, non-progressive; RP, rapid progressive; SCA, sperm class analyser; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

CHAPTER FIVE: Discussion

5.1 | Introduction

The existence of sperm selection mechanisms throughout nature has stimulated considerable theoretical interest and debate for many years (Holt and Van Look, 2004). Such insights into selected spermatozoa will ultimately help to develop useful tests for semen quality assessment, which, despite many years of work, have yet to be developed (Sakkas *et al*., 2015; Nagata *et al*., 2018). The primary objective of clinical and agricultural andrologists is to answer the question "How fertile is this semen sample?" or "How can one improve this semen sample?". Although the ability to distinguish problematic samples is usually possible, the prediction of fertility is not precise (Nagata *et al*., 2018). If natural selection mechanisms can differentiate between spermatozoa of different quality, a thorough understanding of these mechanisms can assist in clarifying which of the many laboratory tests is most likely to be helpful in determining fertility (Nagata *et al*., 2018). As such, observations strongly suggest that some aspects of sperm quality *per se* determine fertilisation success (Flint *et al*., 2003). In human semen, the cell population is heterogeneous, with different levels of maturity that influence both functional quality and fertilizing ability (Santolaria *et al*., 2016). Distinct techniques can be used to fractionate human spermatozoa from individual semen samples, resulting in a considerable improvement in the quality of spermatozoa recovered in the pellet (Buffone *et al*., 2004; Sousa *et al*., 2011). There remains, however, the question of whether it is possible to improve these different subpopulations or whether they respond to various stimuli in the same way.

The present study evaluated an extensive set of functional and structural parameters of two sperm motility subpopulations (HM, high motile and LM, low motile) generated via double density gradient centrifugation (DGC) of healthy donor semen samples. In addition to collectively comparing a large number of diverse characteristics between motility subpopulations, a new approach of grouping variables and correlating subpopulations' results to the standard semen analysis was explored. Thus, providing possible insights into a select group of semen characteristics that could improve its predictive value on the quality of a HM sperm subpopulation. Furthermore, this study investigated the effects of specific biological substances found in follicular fluid (FF) on two human sperm motility subpopulations to determine whether spermatozoa with different functional qualities display similar responses to various stimuli. Concomitantly, we wanted to determine whether these biological substances could improve a

LM sperm subpopulation to display the same characteristics of the HM subpopulation or at least to the point where significant difference no longer exist. Finally, the study explored the flagellar characteristics of spermatozoa to determine if it provide more valuable insight into sperm functionality as compared to only using computer aided sperm analysis (CASA). This warranted an investigation into whether selected concentrations of specific biological substances found in FF have an effect or varying effects on flagellar characteristic of different sperm motility subpopulations.

5.2 | Vitality

Intact and functioning sperm plasma membranes are essential for protection against the harsh environment of the vagina and oxidative stress (Talwar and Hayatnagarkar, 2015). Vitality of spermatozoa used for intracytoplasmic sperm injection (ICSI) is a crucial factor for successful fertilization as well as establishment and outcome of a pregnancy in assisted reproductive technique (ART) cycles (Stalf, *et al*., 2005). In the female reproductive tract, sperm cells are activated by so-called capacitation factors, resulting in reorienting and modifying plasma membrane molecules, which links the integrity of the plasma membrane to various sperm functional characteristics (Flesch and Gadella, 2000; Zhou *et al*., 2010). Thus, damage to the sperm plasma membrane renders irreversible impairment of sperm function (Aurich, 2005). Numerous studies have reported higher percentages of viable, mature, motile, and morphologically normal spermatozoa in the recovered HM subpopulation with increased chromatin integrity, intact MMP and less ROS; however, these comparisons were made to neat semen samples (Chen and Bongso, 1999; Marchetti *et al*., 2002; Ricci *et al*., 2009; Highland *et al*., 2016; Lestari *et al*., 2018; Pérez-Cerezales *et al.*, 2018; Muratori *et al*., 2019). In agreement, our study found that the HM subpopulation comprised of significantly higher percentages of viable spermatozoa as compared to the LM subpopulation.

5.2.1 | Progesterone

Follicular fluid contains progesterone in varying concentrations throughout the female reproductive tract (Machado *et al*., 2019). Progesterone's activation of the principal Ca^{2+} channel of sperm (CatSper), located near the principal piece of the sperm flagellum, is essential for sperm function and may have an active role in cell cycles and survival (Rahman *et al*., 2014; Mannowetz *et al*., 2017; Ghanbari *et al*., 2018; Machado *et al*., 2019; Berendsen *et al*., 2020). In agreement with the findings of Contreras (2009), individual progesterone concentrations

investigated in the current study had no significant effect on sperm vitality percentages for either sperm motility subpopulations; however, collectively with media considered as the main factor, progesterone preserved sperm vitality when compared to HTF controls. Our results of media as a main factor thereby agrees with Ghanbari *et al.* (2018) who demonstrated that inhibition of CatSper channels in progesterone-treated sperm had reduced vitality percentages and increased mortality rates as compared to controls. Given that CatSper is the main Ca^{2+} pathway in human sperm, it is plausible that progesterone through CatSper activation is responsible for calcium homeostasis and therefore may have an active role in vitality (Ghanbari *et al*., 2018).

5.2.2 | Myo-inositol

As a precursor to secondary messengers in the cellular signal transduction system, myo-inositol plays an essential role in morphogenesis, cytogenesis, membrane formation/growth, and lipid production (Condorelli *et al*., 2011; Palmieri *et al*., 2016; Qamar *et al*., 2019). Qamar *et al*. (2019) demonstrated that after supplementation with myo-inositol, dog sperm vitality percentages where significantly higher in treated samples as compared to controls following cryopreservation. Our collective results with media as the main factor therefore agree with these findings. This study found that, compared to HTF, CAP, HD-C and progesterone, myo-inositol was able to significantly preserve sperm vitality percentages, likely due to its ability to aid in the production of lipids and maintain intact plasma membranes of spermatozoa (Qamar *et al*., 2019). In addition, the higher concentrations of myo-inositol within the seminiferous tubules as compared to seminal plasma (Condorelli *et al.*, 2011) also suggest that the antioxidant properties of inositol contribute to maintaining vitality.

5.2.3 | HD-C

HD-C medium is a sperm capacitation medium that contains progesterone and myo-inositol. Like progesterone and myo-inositol, HD-C had no significant effect on sperm vitality percentages of individual subpopulations, while collectively with media as the main factor, HD-C improved vitality percentages as compared to HTF. By incorporating the antioxidant and lipid production properties of myo-inositol, in addition to calcium homeostasis of progesterone, the media was able to integrate various essential aspects involved in maintaining and preserving the sperm plasma membrane (Condorelli *et al.*, 2011; Ghanbari *et al*., 2018; Qamar *et al*., 2019).It is therefore plausible that the HD-C media could be used for preserving spermatozoa in clinical aspects to ensure that membranes remain intact prior to processing or after cryo-preservation.

5.2.4 | Dopamine

Dopamine receptors have been reported in spermatozoa, semen and oviductal fluids, thereby linking them to the reproductive process (Ramirez *et al*., 2009). Similar to Cariati and colleagues (2016), this study did not find any significant differences between dopamine concentrations and HTF vitality percentages. However, 20 nM dopamine did improve the LM subpopulations until significant differences between HM and LM subpopulations were no longer observed. In contrast, Ramirez *et al*. (2009) found that after 3 hours of incubation, low levels of dopamine could induce the activation of D2-receptors in boar spermatozoa, thereby maintaining sperm viability. Through stimulation of D1-like receptors, adenylyl cyclase is activated through stimulating GTP-binding regulatory protein, leading to increased cyclic AMP abundance and cyclic AMP-dependent protein kinase (PKA) (Ramírez *et al.*, 2009). In contrast, D2-like receptors, which are found in human spermatozoa except for the acrosome, inhibits adenylyl cyclase by binding to inhibitory GTP-binding regulatory proteins and decreases PKA activity (Ramírez *et al.*, 2009; DeCarlo *et al*., 2019). It is therefore plausible that the improvement in the LM subpopulations may be through activation of D2-like receptors by dopamine, thereby maintaining levels of calcium essential for cell survival. Furthermore, considering that incubation time in this study was only 30 minutes, it is likely that longer incubation periods such as Ramírez *et al.* (2009) may have resulted in significant differences as compared to controls.

5.2.5 | Prolactin

Prolactin is a peptide hormone that modulates more than 300 biological functions and is produced by the pituitary lactotrophs and closely regulated by dopamine (DeCarlo *et al*., 2019). Through immunocytochemical analyses, prolactin receptors (PRLR) have been identified on the post-acrosomal regions of sperm head, neck, midpiece, and principal piece of the sperm tail (Pujianto *et al*., 2010). Pujianto and colleagues (2010) showed that prolactin has the potential to inhibit sperm capacitation while simultaneously acting as a pro-survival factor. This occurrence is suggested to be mediated by prolactin's ability to prevent spermatozoa from entering the default pathway of cellular death (Pujianto *et al*., 2010). Despite not having a significant effect on individual subpopulations in our study, when media was considered as the main factor prolactin did significantly improve vitality percentages as compared to HTF, thereby agreeing with Pujianto and colleagues (2010). Interestingly, prolactin possibly displays a dose-dependent

effect as 100 ng/mL resulted in significantly (p <0.01) improved vitality percentages as compared to a higher concentration of 500 ng/mL.

5.3 | Motility and Kinematics

Motility is a common characteristic of spermatozoa throughout the animal kingdom (Holt and Van Look, 2004), and is vital for sperm transport within the reproductive tract and for egg penetration in species with internal fertilization (Fujihara *et al.,* 2018). Therefore, spermatozoa with normal flagellar function can cross the uterine tubal junction and enter the oviduct, while for those spermatozoa with abnormal flagellar function such progression through the female reproductive tract is problematic (Suarez, 2016). In many ways motility initiates and integrates the biochemical events occurring in the spermatozoa (Martínez-Pastor *et al.,* 2011). Moreover, sperm samples are heterogeneous, thus spermatozoa with different motility co-exist in the same ejaculate. Whilst exploiting the heterogeneity of semen samples, the analysis of sperm subpopulations based on motility characteristics may help to assess the status of semen samples and its fertility potential (Martínez-Pastor *et al.,* 2011).

Several studies have compared the functionality of human sperm subpopulations but are limited in the number of functional and structural parameters investigated. Nonetheless, the latter studies reported higher motility and kinematic parameters in HM subpopulations as compared to LM subpopulations for both human and bull semen (Gil-Guzman *et al*., 2001; Ollero *et al*., 2001; Buffone *et al*., 2014; Capra *et al*., 2017; Takeshima *et al*., 2017; Caballero-Campo *et al*., 2020). In this study, significant differences between the two motility subpopulations were observed amongst various motility and kinematic parameters. Compared to the LM subpopulations, the HM subpopulations contained spermatozoa with significantly higher percentages of total motility, progressive motility, velocity and progressivity speed groups. Our results are in accordance with Chantler *et al.* (2004), who found that the bottom sperm fraction yielded significantly higher values for total motility. Additional studies have also found that the bottom pellet is characterized by a subpopulation enriched in highly motile and morphologically normal cells with better kinetic properties as compared to the semen sample and remaining supernatants (Henkel and Schill, 2003; Piomboni *et al*., 2006; Sousa *et al.,* 2011). This study similarly observed that HM subpopulations had higher values for various kinematic parameters; however, interestingly the significant difference between subpopulations were predominantly isolated to the kinematic parameters of the total motile, medium and non-progressive speed groups.

5.3.1 | Progesterone

As expected, and in accordance with literature (Calogero *et al*., 2000; Ghanbari *et al*., 2018), significant effects of progesterone on sperm motility and kinematics were observed. Progesterone mainly had a stimulatory effect on kinematic parameters; however, it appeared to also improve the LM subpopulation's motility percentages as compared to HTF for the rapid and medium velocity and progressivity speed groups. In contrast, progesterone displayed minor improvements on the already superior HM subpopulations, which presented with significantly higher motility percentages as compared to LM subpopulations. Servin-Vences *et al*. (2012) found that the elevation of Ca^{2+} concentrations by caged progesterone treatment resulted in sperm flagellar beat becoming more vigorous with increased curvature. In addition to this, it was observed that some spermatozoa did not show a significant increase in flagellar curvature despite the apparent increase in Ca^{2+} concentrations (Servin-Vences *et al*., 2012). This was thought to be partially due to the heterogeneity of human sperm responsiveness and certain undesirable features of the caged progesterone (Servin-Vences *et al*., 2012). It may be plausible that, due to HM subpopulations already comprising of highly functional spermatozoa, progesterone could not improve motility percentages any further, whereas in LM subpopulations which comprised of less functional spermatozoa, increased Ca^{2+} could ultimately increase the percentages of motility. However, it should be noted that as sperm motility depends on a change in membrane potential, observations in LM subpopulations may be a result of either intracellular pH or proper balancing of intracellular ions and not necessarily activation of progesterone CatSper Ca^{2+} influx (Nowicka-Bauer and Szymczak-Cendlak, 2021).

Interestingly, progesterone appeared to influence the rapid progressive and medium progressive kinematics of the HM subpopulations, whereas in the LM subpopulations it only had an effect on the medium progressive kinematics. These observations may therefore further substantiate the previously mentioned effects that the results seen in the LM subpopulations may be a consequence of balancing of intracellular ions. Nonetheless, results observed in the HM subpopulations agree with Uhler *et al*. (1992) who found that progesterone decreased linearity of progression and straightness of motile spermatozoa, but also increased VCL. In addition, Sumigama *et al*. (2015) reported that in macaque spermatozoa, ALH increased proportionally with increased progesterone concentrations, with maximum responsiveness being achieved at 10 µM. They further reported a dose-dependent increase in VCL starting at concentrations of 1 µM. These findings suggest that the expression of the progesterone receptor on human spermatozoa

is the major factor determining the biological responsiveness of individual spermatozoa, as progesterone actively triggers a Ca^{2+} influx into through the activation of CatSper channels (Sumigama *et al*., 2015). This is reiterated in a study done by Mannowetz *et al.* (2017), where it was shown that progesterone causes the activation of CatSper and resulted in hyperactivation and increased motility.

Furthermore, spermatozoa respond differently to progesterone under very viscous and less viscous media (Pérez-Cerezales *et al*., 2016). When in less viscous media such as that used in this study, progesterone treatment in mouse spermatozoa seemed to reduce LIN, STR and WOB and increase ALH (thus creating a hyperactive-like swimming pattern, known to facilitate spermatozoon progression under viscous conditions) (Pérez-Cerezales *et al*., 2016). Under viscous conditions, opposite affects were noticed. In addition to viscosity, when a spermatozoon swims towards a higher concentration of progesterone, its motility becomes linearized, propagating the cell towards the higher concentrations (Pérez-Cerezales *et al*., 2016). One could additionally speculate that the lack of responsiveness to progesterone in the LM subpopulations could further be a result of decrease in capability of the less motile spermatozoa to respond to chemo-attractants and gradients. Moreover, asthenozoospermic samples have been shown to have a down regulation in CatSper channels, which may additionally be the case of the observed low motile subpopulations (Jin *et al*., 2021). Furthermore, this could additionally explain the differences observed between the concentrations of progesterone used in our study, however it was clear that 3.96 μM progesterone had the greatest effect on both subpopulations.

5.3.2 | Myo-inositol

According to Governini *et al*. (2020), treatment with myo-inositol significantly increased sperm motility and oxygen consumption, which agrees with our findings. Myo-inositol significantly improved the rapid and rapid progressive speed percentages while decreasing the slow and medium speed percentages. As such, the concentration of myo-inositol investigated was able to increase originally low progressive motile spermatozoa into higher progressive speed groups. Similar to progesterone, this study found that the majority of improvements was observed in LM subpopulations as compared to HM subpopulation motility percentages. This further substantiates our hypothesis that due to the HM subpopulations already comprising of highly functional spermatozoa, myo-inositol could not improve percentages any further, whereas in LM subpopulations increased intracellular Ca^{2+} could ultimately increase the percentages of

motility. Despite the lack of statistical significance, in comparison to the other media, 11 mM myo-inositol seemed to have a trend of expressing higher values for the various motility parameters in both sperm subpopulations. In addition, this study found that myo-inositol had majority of significant effects on the motile and medium progressive kinematics of both subpopulations and that myo-inositol could further improve LM subpopulations until significant differences between subpopulations were no longer observed. This further indicates that the myoinositol improves the less motile sperm subpopulations and increased progressive speed groups. This could further be indicative of increased oxidative phosphorylation efficiency and ATP production by the mitochondrial sheaths that wound around the axoneme in addition to the increase of intracellular Ca^{2+} (Dcunha *et al*., 2020).

Previous studies have demonstrated similar effects of myo-inositol on improving sperm parameters such as motility, morphology, and quality, both *in vitro* and *in vivo* (Carlomagno *et al.,* 2011; Condorelli *et al*., 2011; Condorelli *et al*., 2012; Calogero *et al*., 2015; Montanino *et al.,* 2016; Dinkova *et al.,* 2017). An investigation by Palmiere *et al.* (2016) indicated that total sperm motility increased significantly from 46.5% to 50.2% in fresh samples after the addition of myo-inositol, which is in agreement with the current study as myo-inositol seemed to improve various motility parameters compared to the negative control (HTF). Palmiere *et al.* (2016) further found that a significant increase was observed in sperm progressivity from 47.7% to 56.9% after myo-inositol treatment. Artini *et al.* (2017) also reported an improvement in progressive motility in both normospermia and oligoasthenoteratozoospermic (OAT) subjects after myo-inositol treatment. Condorelli *et al.* (2012) treated OAT and normozoospermic men with 2 mg/mL of myo-inositol for 2 hours and found that myo-inositol significantly improved the total and progressive motility of the spermatozoa from OAT (30.0% to 44.0% and 20.0% to 34.0%, respectively) and normozoospermic patients (51.0% to 68.0% and 25.0% to 44.0%, respectively).

Our observations as well as those from previous studies indicate that treatment with myo-inositol can possibly increase subfertile samples to be of the same quality as that of a fertile sample or alternatively in close range in terms of sperm motility. In addition to this, Condorelli *et al.* (2012) suggested that the sperm motility of patients with abnormal sperm parameters (such as motility, morphology, concentration, and progressive motility) can be enhanced by incubation with myo-inositol, and this seems to be paralleled by an increase in the proportion of spermatozoa with high mitochondrial membrane potential (MMP). Korosi *et al.* (2017) tested the combination

of oral supplementation and semen incubation with myo-inositol in OAT men before ICSI and found significantly higher fertilization indexes and good quality embryos in the treated groups as compared to the controls.

5.3.3 | HD-C

HD-C had less of an effect on the motility parameters of individual subpopulations; however, HD-C predominantly improved the LM subpopulations' progressive motility and velocity speed groups. In contrast to both progesterone and myo-inositol, HD-C was able to increase and improve both subpopulations' medium and rapid progressive speed group kinematics. By containing both progesterone and myo-inositol the medium is capable of targeting various motility groups and fertilizing capabilities of spermatozoa. In highly functional and structurally normal spermatozoa, progesterone in HD-C can bind to the CatSper channels and associated proteins and thereby mediate progesterone-induced Ca^{2+} influx, regulating the motility and kinematics (Ren and Xia, 2010; Diao *et al*., 2019; Zhang *et al.*, 2020). In contrast, in the LM subpopulations which are known to comprise of less motile and morphologically abnormal spermatozoa, myo-inositol in the medium can increase intracellular Ca^{2+} release through inositol-gated channels due to the activation of protein kinase PKC (Calogero *et al.,* 2015). Furthermore, this investigation observed that HD-C significantly decreased linearity and increased velocity and vigour kinematics such as ALH, thereby forming motility patterns closely mimicking that of hyperactivation (Mortimer, 1997). It is plausible that the combination of progesterone and myo-inositol could increase both the intracellular Ca^{2+} influx in addition to increasing oxidative phosphorylation efficiency and ATP production which would ultimately explain the motility patterns observed from HD-C medium (Dcunha *et al*., 2020).

5.3.4 | Dopamine

A study conducted by Singh *et al*. (2013) demonstrated that when rats were treated with dopamine after being impaired by ethinyl estradiol, sperm motility, MMP and ROS levels improved significantly. These findings are consistent with previous studies (Cariati *et al*., 2016; Urra *et al*., 2014), which noted that dopamine improved sperm motility parameters following 30 minutes of incubation. In the current study, we observed that dopamine had significant effects on both subpopulations' motility percentages. Dopamine was able to increase progressive speed group percentages and decrease slow and medium speed group percentages to ultimately increase the rapid velocity speed percentages. In addition, this study observed that unlike myo-inositol

and progesterone, dopamine was capable of improving all the progressive speed groups' kinematics, specifically the LM subpopulations NP kinematic parameters. Portions of both female and male reproductive tracts, especially the ovary, the isthmus of the oviduct, and the vas deferens, are heavily innervated by sympathetic adrenergic inputs (Schuh *et al*., 2006). Presence of different catecholaminergic receptors in sperm have been observed, including the presence of α2- and β-adrenergic receptors in mouse and human sperm, and the dopamine type 2 receptor (DRD2) in testis, spermatogenic cells and sperm of rat, human, bull, mouse and boar sperm (Urra *et al*., 2014). As such, reports have indicated that adenosine and catecholamine agonists can stimulate sperm by increasing the percentage of motile cells, intracellular cAMP content, protein tyrosine phosphorylation, capacitation, or fertilizing ability of mouse, bull, and human sperm (Schuh *et al*., 2006).

Urra *et al*. (2014) found that after boar sperm was treated for 3 hours with 100 nM dopamine a significant decrease in the number of dead sperm was observed as compared to the controls. They further noted that dopamine promoted a significant increase in proteins phosphorylated on tyrosine (Urra *et al*., 2014). Our study hereby agrees with these finding as we observed that 20 nM dopamine improved the LM subpopulations vitality percentages, and that dopamine could further increase and improve the various motility parameters. Dopamine may therefore display a possible protective effect on sperm whilst acting as a modulating agent in sperm capacitation (Urra *et al*., 2014). Catecholamines are present throughout the entire passage of sperm along the male and female reproductive tracts and may have a biphasic effect at varying concentrations (Urra *et al*., 2014). While low concentrations of dopamine promote viability and motility, high dopamine concentrations dramatically affect sperm motility without altering viability. However, sperm could experience a decrease in the capability to undergo capacitation (Urra *et al*., 2014). It is plausible that the higher concentrations of dopamine function in immobilizing sperm in the oviductal reservoirs, thereby promoting selection of the best sperm until ovulation when catecholamine levels fall (Urra *et al*., 2014). Consequently, this pause in motility will permit other factors present in oviduct and follicular fluid to stimulate sperm motility and capacitation, thus enhancing the oocyte penetration in the appropriate segment (Urra *et al*., 2014).

5.3.5 | Prolactin

The role of prolactin in reproductive physiology of the male is unclear, however, normal levels of prolactin may be important for the proper functioning of the male accessory glands (Segal *et al*., 1978). Hyperprolactinemia was found in infertile patients with defective sperm production and conditions were improved following prolactin suppression (Segal *et al*., 1978). In contrast, physiologic concentrations of prolactin added *in vitro* to normal semen stimulated adenyl cyclase, rate of fructose utilization and glucose oxidation (Segal *et al*., 1978). As such, semen samples with sperm motility of <30% had lower prolactin content as compared to those with high motility (Segal *et al*., 1978). Furthermore, Stovall and Shabanowitz (1991) found that prolactin did not increase motility rates at initial incubations, however, it kept motility steady over a period as compared to controls in which sperm motility decreased. In our study we observed that prolactin predominantly improved the LM subpopulations' motility percentages, whereas little to no effect was observed in the HM subpopulations. Considering that human spermatozoa are transcriptionally and translationally silent cells and depend on posttranslational modifications for their functional activation, it may be plausible that prolactin may aid in these signals in a LM subpopulation which may be less responsive to signals from biological components such as progesterone due to protein deletions or alterations (Amaral *et al*., 2014; Pujianto *et al*., 2010).

The functional significance of prolactin has been unclear although positive correlations with sperm motility have been observed in certain studies (Pujianto *et al*., 2010). In addition to improving the LM subpopulations motility percentages, this study observed that prolactin, like dopamine, could improve the kinematic parameters of all progressivity groups; however, improvements were once again isolated to the LM subpopulation. It is possible that the LM subpopulations had altered, or compromised energy production capabilities as compared to the HM subpopulations, which could also contribute to the already significantly lower motility percentages observed in LM subpopulations as compared to HM subpopulations. Considering that reports have indicated that prolactin may increase sperm motility by increasing oxygen uptake by spermatozoa (Gonzales *et al*., 1989), it may be plausible that the LM subpopulations experienced such an effect when treated with prolactin and resulting in our observations. In contrast, the HM subpopulations already comprised of functionally and structurally sound spermatozoa, thus this subpopulation may already have functional oxidative phosphorylation and were unaffected by prolactin treatment.

5.4 | Hyperactivation

Functional spermatozoa are critical for successful fertilization and often is characterized by high motility and normal morphology (Nagata *et al.,* 2018). Moreover, sperm hyperactivated motility, a state in which spermatozoa will exhibit vigorous and high amplitude flagellar beating, is vital for both the detachment from the oviductal wall and for penetration of the zona pellucida (ZP), subsequently resulting in fertilization (Holt and Van Look, 2004; Armon and Eisenbach, 2011). Capacitation is a complex physiological process involving biochemical, biophysical and metabolic modifications of the spermatozoon (Esteves *et al.,* 2015). These changes result in altered plasma membrane architecture and permeability that modulate flagellar activity and leads to hyperactivation (Esteves *et al.,* 2015). One interesting consequence of capacitation is the apparent activation, or unmasking, of receptors that respond to chemical stimuli from oocytes or thermal gradients within the oviduct (Fabro *et al.,* 2002; Bahat *et al*., 2003). Once the sperm reservoir has been established, only a small proportion of spermatozoa undergo capacitation at any given time (Ho and Suarez, 2001; Jin and Yang, 2017). These spermatozoa, representing a selected population from the original ejaculate, are therefore subjected to further stringent selection. In mammals only about 10% of the capacitated spermatozoa are responsive to chemotactic or thermotactic signals and they remain responsive for a limited period (Ho and Suarez, 2001; Jin and Yang, 2017).

This study observed that the percentage hyperactivation for the HM subpopulation was significantly higher compared to the LM subpopulation for the control (non-capacitating HTF) and each of the hyperactivating media used namely, capacitating medium, 5 mM caffeine and 2 mM procaine. Since kinematic parameters such as VCL, LIN and ALH are used in a sort function to classify spermatozoa as being hyperactivated (van der Horst and du Plessis, 2017), lower percentages achieved for these parameters in the LM subpopulation can further be related to the low percentage of hyperactivation reported for this subpopulation. Our findings are in agreement with Ollero *et al*. (2000) who demonstrated that the HM bottom pellet yielded better hyperactivation results as compared to the LM subpopulations. Ollero *et al*. (2000) isolated four sperm subpopulations, which revealed different membrane lipid composition and varying degrees of sperm maturation and normal morphology. It is likely that the LM subpopulations examined in this study contained less mature and more abnormal spermatozoa with protein aberrations related to tyrosine phosphorylation as in cases of asthenozoospermia, where spermatozoa are incapable of hyperactivation due to the impairment of tyrosine phosphorylation

(Wang *et al.* 2021). Furthermore, Buffone *et al.* (2004) indicated that three Percoll-separated sperm subsets showed different abilities to develop hyperactivated motility when they were incubated under capacitating conditions for 5 and 18 hours. After 5 hours, the percentage of hyperactivated cells in LM subpopulations was significantly lower in comparison to the HM subpopulations (Buffone *et al.*, 2004). Furthermore, our study showed significant differences in percentage hyperactivation according to both media used and the time intervals investigated - with procaine, caffeine and CAP displaying better hyperactivation percentages as compared to non-capacitating HTF. This investigation observed that subpopulations had lower percentages of hyperactivation at the 60-minute interval as compared to the 5, 15 and 30-minute intervals. This could be an outcome of peak hyperactivation being reached between 15-30 minutes' incubation. These observations agree with findings of Ntanjana (2014), who indicated that the maximum hyperactivation of spermatozoa in semen was seen after 15-30 minutes in both procaine and caffeine. In addition, it was found that significant differences in overall percentage motility, sperm kinematic parameters and hyperactivation exist among the sperm subpopulations (Ntanjana, 2014).

5.4.1 | Progesterone

The role of progesterone in sperm function has been investigated with *in vitro* data supporting it having an effect on chemo-attraction, capacitation, modulation of hyperactivated motility and the acrosome reaction (Teves *et al.*, 2006). Studies have shown that even at very low concentrations, progesterone induces sperm hyperactivation, thereby significantly increasing the number of spermatozoa moving with hyperactivated motility (Uhler *et al*, 1992; Sueldo *et al,* 1993). This effect was observed by Uhler *et al.* (1992) on capacitated cells where hyperactivation appeared after 10 minutes incubation with 3.1 ng/mL progesterone. Higher concentrations did not result in an additional increase of the percentage of hyperactivated cells, thus Uhler *et al.* (1992) stated that the effect of progesterone is transient. Increased hyperactivated motility has also been shown by some investigators (Calogero *et al.*, 1996; Contreras and Llanos, 2001) at concentrations varying from as low as 0.1 µM (Uhler *et al.*, 1992) to as high as 31 µM, (Yang *et al.*, 1994), whereas some studies observed no effect after progesterone exposure (Luconi *et al*., 2004; Tantibhedhyangkul *et al*., 2014; Rehfeld *et al.*, 2018).

Hyperactivation induced by progesterone displayed early stimulatory effects after 5 minutes like that observed for motility in the current study. Typical steroid signalling pathways

are time-consuming due to hormones binding to genomic receptors, initiating translocation to the nucleus, and subsequently altering gene expression (Mannowetz *et al*., 2017). Through bypassing this long process progesterone quickly stimulates Ca^{2+} influx (Mannowetz *et al*., 2017) that is sustained for several minutes (Calogero *et al*., 2000), thus explaining our findings for both motility and hyperactivation results. Progesterone is well-documented to induce hyperactivation and is suggested to elicit different effects at varying concentrations as mentioned (Calogero *et al*., 2000; Alasmari *et al*., 2013; Tantibhedhyangkul *et al*., 2014; Achikanu *et al*., 2018; Ghanbari *et al*., 2018; Berendsen *et al*., 2020). In agreement, this study observed that 3.96 µM progesterone significantly induced higher percentages of hyperactivation compared to 1.98 µM and 19.8 µM progesterone. Additionally, LM subpopulations exhibited a small, gradual increase in hyperactivation followed by a gradual decrease, despite remaining significantly lower than HM subpopulations. In contrast, HM subpopulations experienced a sudden increase followed by a sudden decrease.

Progesterone-induced Ca^{2+}-influx followed by Ca^{2+} oscillations in the intracellular compartment could be responsible for observed motility and hyperactivation patterns in HM subpopulations. Thus, prolonging motility regulation and assisting in repeated alteration of activated and hyperactive motility (Achikanu *et al*., 2018). Furthermore, Ca^{2+} store utilization may support flagellar activity and possibly even hyperactivation in CatSper-null spermatozoa (Alasmari *et al*., 2013), which may also explain the improved motility parameters seen in the LM subpopulations. Furthermore, it has been proposed that the Ca^{2+} concentration induced oscillation is due to Ca^{2+} release from stores localized at the base of the flagellum, rather than influx through the plasma membrane (Kirkman-Brown *et al*., 2004). The latter situation may be true in the case of the LM subpopulations whereas HM subpopulations could additionally receive the progesterone-induced Ca^{2+} influx by CatSper channels (Ren and Xia, 2010; Diao *et al*., 2019; Zhang *et al.,* 2020).

5.4.2 | Myo-inositol

Previous studies on myo-inositol in FF have suggested a role of this sugar compound in chemotaxis and human sperm thermotaxis through activation of Phospholipase-C, resulting in the production of inositol triphosphate (IP3) (Condorelli *et al*., 2012). IP3 is a secondary messenger which is involved in signal transduction, ultimately leading to the release of intracellular Ca^{2+} that is needed for the regulation of sperm motility and capacitation, including

hyperactivation (Fujinoki, 2013). In males, myo-inositol is mainly produced by Sertoli cells in response to follicle-stimulating hormone (FSH) and has been suggested to play a role in the osmoregulation of seminal fluid, since both hypo- and hyper-osmotic media have been found to significantly decrease sperm progressive motility and velocities (Bevilacqua *et al.*, 2015; Condorelli *et al.*, 2017).

Myo-inositol induced hyperactivation in both sperm subpopulations, as well as when media was considered as the main factor. By activating Phospholipase-C as previously mentioned, myo-inositol results in production of IP3 which opens Ca^{2+} channels and regulates the intracellular Ca^{2+} stores in the sperm plasma membrane, mitochondria, acrosome and neck region (Korosi *et al.*, 2017). The subsequent regulation and rise of cytosolic Ca^{2+} and increase of mitochondrial Ca^{2+} stimulates oxidative mechanisms and ATP production that improve mitochondrial function, prevent apoptosis, and assist in sperm capacitation (Korosi *et al.*, 2017; Governini *et al.*, 2020; De Luca *et al.*, 2021). As such, myo-inositol was able to target and induce sperm hyperactivation in spermatozoa of varying functional qualities by regulating the levels of intracellular Ca^{2+} which in turn regulates sperm motility, capacitation, and acrosome reaction, all of which occur in spermatozoa at the plasma membrane and mitochondria level (Palmieri *et al.*, 2016). These findings can further be related to the fact that myo-inositol preserved and maintained intact plasma membranes as was observed for sperm vitality which is essential for sperm functionality.

5.4.3 | HD-C

Due to both progesterone and myo-inositol playing a role in sperm functions such as capacitation and hyperactivation (Teves *et al.*, 2006; Korosi *et al.*, 2017), it was anticipated that HD-C would induce sperm hyperactivation in both sperm subpopulations, as reported in our study. These results are related to the ability of HD-C to significantly increase sperm velocity, while decreasing linearity and increasing ALH – all functional changes in sperm swimming characteristics required for spermatozoa to be classified as being hyperactivated (Mortimer, 1997).

The most convincing evidence for the role of ion channels in sperm motility comes from characterization of novel class Ca^{2+} channels known as the CatSper family, the expression of which is confined to the testes (Rezaian *et al.*, 2009). Four members of the family have been described, and two of them, CatSpers 1 and 2, localized at the flagella (Rezaian *et al.*, 2009). It

has been demonstrated that knockout mice lacking CatSper 1 gene resulted in infertility, mostly due to a lack of sperm motility and hyperactivation (Rezaian *et al*., 2009). In addition, both CatSper 1- and 2-knockout mice presented with identical phenotypes, due to all four subunits of the family collaborating with each other to form a complex functional channel (Rezaian *et al*., 2009). As the LM subpopulations are characterised by morphologically abnormal and immature spermatozoa (Chen and Bongso, 1999; Marchetti *et al*., 2002; Ricci *et al*., 2009; Highland *et al*., 2016; Lestari *et al*., 2018; Pérez-Cerezales *et al.*, 2018; Muratori *et al*., 2019), their CatSper channels may be altered or contain deletions. It is thus plausible that the progesterone in HD-C may have a more pronounced effect in inducing hyperactivation in the HM subpopulations which have more functional and morphologically normal spermatozoa. On the other hand, myo-inositol in HD-C may have resulted in the LM subpopulations' sperm hyperactivation due to increased oxidative phosphorylation efficiency and ATP production by the mitochondrial sheaths in addition to the increase of intracellular Ca^{2+} (Dcunha *et al*., 2020).

5.4.4 | Dopamine

Catecholamines exert their actions on different parameters of sperm physiology, including the induction of capacitation in mouse, hamster, and bull sperm, as well as *in vitro* stimulation of motility in hamster sperm and promoting the acrosome reaction in hamster and bovine sperm (Cornett and Meizel, 1978; Bavister *et al*., 1979; Cornett *et al*., 1979; Meizel and Working, 1980; Way and Killian, 2002; Adeoya-Osiguwa *et al*., 2006; Urra *et al*., 2014). In the stimulation of D1-like receptors, adenylyl cyclase is activated through stimulating GTP-binding regulatory protein, leading to increased cAMP abundance and cAMP-dependent protein kinase (PKA) (Ramírez *et al.*, 2009). In contrast, D2-like receptors, which are found in human spermatozoa, inhibits adenylyl cyclase by binding to inhibitory GTP-binding regulatory proteins, decreasing PKA activity (Ramírez *et al.*, 2009; DeCarlo *et al*., 2019).

This investigation observed that 1 µM dopamine significantly increased sperm hyperactivation in both subpopulations and further displayed a sustained increase and gradual decrease in hyperactivation patterns. In agreement, Urra *et al.* (2014) reported increased sperm capacitation *in vitro* after dopamine stimulation, possibly due to increased protein-tyrosine phosphorylation and sperm motility. Likewise, Ramirez *et al*. (2009) observed that 100 nM and 10 µM dopamine increased the phosphorylation signal in spermatozoa, mainly in proteins with a molecular mass between ∼40 and ∼58 kDa. In contrast, spermatozoa incubated with high

concentrations of 1 mM dopamine showed a great decrease in tyrosine phosphorylation (Ramirez *et al*., 2009). Thus, higher dopamine concentrations may inhibit motility and tyrosine phosphorylation indicating that dopamine may have a biphasic effect at varying concentrations (Ramírez *et al.*, 2009; Urra *et al*., 2014; Ramírez-Reveco *et al*., 2017). Given that spermatozoa contain both β- and α-adrenergic receptors, it is plausible that high doses activate inhibitory β-adrenergic receptors, whereas low concentrations stimulate α-adrenergic receptors (Way and Killian, 2002).

5.4.5 | Prolactin

Prolactin appears to modulate male reproduction by regulating receptor levels for other hormones in the testis and accessory sex organs (Smith Luqman, 1982). Prolactin has therefore been associated with many of the biochemical changes in spermatozoa which are known to be associated with the process of capacitation (Shah and Sheth, 1979). It is necessary for spermatozoa to acquire this fertilizing capacity before they are used for IVF (Stovall and Shabanowitz, 1991). In this study, we observed that prolactin significantly increased and maintained hyperactivation in both subpopulations. Prolactin inducible protein (PIP) is a 17 kDa protein expressed in human body fluids and interacts with several other proteins and contributes to multifaceted molecular functions in diverse classes of biological processes (Tomar *et al*., 2016). Similar to our findings, Tomar *et al*. (2016) observed that this complex acts as an inducer of *in vitro* sperm capacitation. In addition, *in vitro* studies with prolactin showed that added prolactin increased oxygen uptake by spermatozoa when acetate, glucose, lactate, or pyruvate were used as substrates and prolactin also increased glycolysis (Smith Luqman, 1982). Therefore, prolactin may have provided the additional increase of ATP production required for hyperactivation in both the sperm subpopulations. In addition, it is plausible that prolactin induces hyperactivation as a result of dose-dependent increases of cAMP levels and utilization of fructose and glucose was demonstrated after exposure to bovine prolactin (Stovall and Shabanowitz, 1991).

Fukuda *et al*. (1989) demonstrated that prolactin may additionally play a role in the biological processes of spermatozoa by shortening the optimal preincubation period for spermatozoa to acquire capacitation and by maintaining their motility and ability to attach to the oocyte during IVF. They observed that when preincubated for only 15 or 30 minutes in modified Krebs-Ringer bicarbonate solution (mKRB) with prolactin (50 ng/mL and 100 ng/mL),

significantly higher fertilization rates were yielded in IVF (Fukuda *et al*., 1989). As such, it was suggested that the results are relevant to the clinical use of prolactin for infertile patients presenting with oligozoospermia or asthenozoospermia (Fukuda *et al*., 1989).

5.5 | Reactive Oxygen Species and Mitochondrial Membrane Potential

Mitochondria are unique organelles, which participate in various cellular functions and recently its connections to other organelles have gained much attention, as it may help to integrate distinct cellular functions (Rowland and Voeltz, 2012; Amaral *et al.,* 2013). Mitochondria participate in many crucial processes such as the production of ATP and calcium homoeostasis, with both processes depending on the electric nature of the MMP which can be harnessed to sequester Ca^{2+} (Nichols and Ferguson, 2002; Zhang *et al.,* 2016). Besides its involvement in ATP synthesis, mitochondria also promote the production of reactive oxygen species (ROS), which function in signalling pathways, but can result in oxidative damage if produced in an unchecked manner (Wagner *et al,* 2017). Mitochondria are the main source of sperm-produced ROS, and controlled ROS levels are necessary for proper sperm functions such as motility, capacitation, acrosome reaction (AR), hyperactivation and fertilizing ability (Moraes and Meyers, 2018). Spermatozoa are, however, negatively affected by extensive ROS produced locally, or by ROS formed in leukocytes present in semen (Wagner *et al.,* 2017). Excess ROS may result in a decrease in viability, motility, MMP, and increases in DNA damage, morphology defects and lipid peroxidation, possibly resulting in an apoptosis-like phenomena (Kothari *et al*., 2010; Mahfouz *et al.,* 2010; Aitken *et al.,* 2012; Bui *et al.,* 2018). These observations indicate that active sperm mitochondria are required for fertilization. Defects in sperm mitochondrial ultrastructure appear to be associated with decreased sperm motility in humans (Pelliccione *et al.,* 2011; Wang *et al.,* 2018). Additionally, the activity of sperm mitochondrial enzymes, including ETC complexes, correlates with sperm parameters, such as concentration, vitality, motility function and male infertility (Ramio-Lluch *et al*., 2011; Wang *et al.,* 2018).

This investigation observed significant differences in the percentage of positive ROS and intact MMP spermatozoa between the LM and HM subpopulations. The HM subpopulations comprised of significantly higher percentages of intact MMP spermatozoa with decreased levels of ROS, as compared to LM subpopulations. Reports have indicated the presence of damaged and altered mitochondrial proteins that are frequently being observed and associated with low motility sperm subpopulations (Amaral *et al.,* 2014). Our results therefore agree with these

findings as the LM subpopulations presented with lower percentages of intact MMP and higher percentages of ROS. The diminished MMP intactness in LM subpopulations can possibly be partly responsible for the lower percentage hyperactivation and motility parameters found within this subpopulation, as sperm quality, particularly motility, is positively correlated with the enzymatic activity of the ETC complexes and the expression of ETC subunits (Wang *et al.,* 2018). As a result, the LM subpopulations may have altered ATP production thus resulting in decreased motility percentages.

Decreased MMP has been associated with elevated ROS generation by sperm mitochondria, creating a state of oxidative stress and a consequent loss of functional competence (Lobacio *et al*., 2015; Voncina *et al.,* 2016). This explains the significantly higher percentages of positive ROS spermatozoa found within the LM subpopulations as compared to the HM subpopulations. Studies have found that ROS levels vary in ejaculates, and when spermatozoa are separated by Percoll gradients, the low-density top fraction has a more prominent number of positive ROS cells than the high-density bottom fraction (Koppers *et al*., 2008; Aitken *et al*., 2013). Various investigations have shown that spermatozoa collected from the bottom pellet of HM spermatozoa possess less DNA damage (Larson *et al*., 1999; Sakkas *et al*., 1999) and produce less ROS than those recovered from the top fractions, where abnormal spermatozoa are predominantly found (Ollero *et al*., 2001; du Plessis *et al.,* 2015). Additionally, the recovered bottom pellets were shown to possess more spermatozoa with functional mitochondria (Donnelly *et al.,* 2000; Marchetti *et al.,* 2002; Sousa *et al.,* 2011) and decreased amounts of spermatozoa with apoptotic and necrotic markers (Ricci *et al.,* 2009). Outcomes of two reports using fluorescence-activated cell sorting (FACS) with different mitochondrial probes suggest that subpopulations of spermatozoa with higher MMP might have an enhanced fertilization potential (Sousa *et al.,* 2011). Likewise, others have shown evidence that these spermatozoa also have more motile and morphologically normal gametes, with a higher capacity to respond to induced AR (Gallon *et al.,* 2006). Positive correlations between sperm MMP and other parameters known to be required for sperm functionality (motility, viability, capacitation status, acrosome and chromatin integrity), may therefore suggest that mitochondrial status reflects the state of other fundamental attributes, and is thus a good candidate as a criterion to segregate more functional sperm subpopulations (Sousa *et al.,* 2011).

5.5.1 | Progesterone

In essence, progesterone functions by regulating gene expression via two nuclear progesterone receptors; however, actions of progesterone independent of gene regulation have been observed for decades (Price and Dai, 2015). The discovery of a mitochondrial progesterone receptor, PR-M, has opened the possibility of direct, ligand-dependent modulation of mitochondrial activity by progesterone (Price and Dai, 2015). Progesterone activates protein tyrosine kinase, enabling phosphorylation of proteins in the tail and head of the sperm, and thereby increases sperm motility and sperm acrosome response. However, in addition, motility requires energy and energy supply is the responsibility of mitochondria (Baranizadeh *et al*., 2021). Progesterone therefore appears to bind to mitochondrial membrane receptors to increase MMP, oxygen consumption and cellular respiration, thus resulting in increased ATP production and sperm motility (Baranizadeh *et al*., 2021). Similarly, Tantibhedhyangkul *et al*. (2014) reported a progestin-dependent increase in MMP which is presumably associated with increased cellular respiration. Fan *et al*. (2013) reported that progesterone induction can inhibit the relative activity of mitochondria, keep mitochondria potential at a more balanced level and further reduce the production of ROS.

In this study we observed no significant effects of progesterone on either the MMP or ROS levels of the two sperm subpopulations; however, with media as the main factor, progesterone reduced ROS levels in spermatozoa. In a study by Tantibhedhyangkul *et al*. (2014), spermatozoa were incubated in progestin for 90 minutes, rather than 30 minutes as in the current study, which is likely to be explanation for our results observed for MMP. There is further speculation that increased progesterone concentrations in the female reproductive tract may stimulate mitochondrial progesterone receptors, increasing mitochondrial ATP with an additional alteration of nutrient availability from glucose to lactate (Tantibhedhyangkul *et al*., 2014). Progesterone may therefore have a dual effect on spermatozoa by increasing the motility through not only the influx of intracellular Ca^{2+}, but also increasing ATP production and maintaining MMP, which consequently keeps ROS levels in check.

5.5.2 | Myo-inositol

High values of MMP are an indication of mitochondria with good integrity and optimal levels of activity, which are associated with high cell viability (Condorelli *et al*., 2017). At a functional level, myo-inositol acts directly on mitochondria by increasing the membrane

potential and further has antioxidant properties (Marchetti *et al.,* 2004; Condorelli *et al.,* 2011; Marchetti *et al*., 2012; Artini *et al.,* 2017). Studies have shown that myo-inositol did not affect the mitochondrial function of spermatozoa isolated from normozoospermic men, whereas it significantly increased the number of spermatozoa with high MMP in OAT patients (Condorelli *et al.,* 2011). From reported studies, the effect of myo-inositol seems to be not only related to an improvement of mitochondria, but in addition to the reduction of semen amorphous material related to increasing semen viscosity which frequently impairs male fertility (Condorelli *et al.,* 2011). Additionally, the percentage of spermatozoa with high MMP levels is directly related to the fertilization rate after IVF with embryo transfer (Marchetti *et al*., 2002; Condorelli *et al.,* 2012; Dinkova *et al*., 2017).

This study observed that myo-inositol improved the MMP of the LM subpopulation and displayed the largest effect on MMP when media was considered as the main factor. Consequently, our study also observed a decrease in ROS percentages when media was considered as the main factor. Because myo-inositol preserves vitality and MMP function, it reduces and controls ROS levels, improving ATP production and reducing oxidative stress that alters capacitation. As oxidative stress has been observed in 30–80% of infertile patients, multi-antioxidant supplementation is an effective treatment that helps to improve male fertility parameters (De Luca *et al*., 2021). This may also explain our observation that myo-inositol significantly improved LM subpopulation parameters, whereas in HM subpopulations only subtle enhancements were seen.

5.5.3 | HD-C

HD-C did not display significant effects on MMP for either of the subpopulations; however, a significant decrease in ROS percentages were observed when media was considered as the main factor. This could further be a contribution of myo-inositol's antioxidant interactions within the media. HD-C can display both progesterone and myo-inositol's beneficial effects due to its composition. Subfertile spermatozoa produce large amounts of ROS, which impair various functional characteristics of spermatozoa and further prevent sperm capacitation (Korosi *et al*., 2017; Governini *et al*., 2020; De Luca *et al*., 2021). By containing myo-inositol, HD-C counteracts these detrimental effects, improving sperm functionality such as motility, hyperactivation, MMP and reducing ROS. In contrast, progesterone may additionally have contributed to the results as a progesterone receptor on sperm mitochondria have been identified.

As such, progesterone has been suggested to increase MMP, increase oxygen consumption and cellular respiration (Baranizadeh *et al*., 2021).

5.5.4 | Dopamine

The term oxidative stress describes a complex phenomenon, that can be defined simplistically as an excess of ROS in tissues, resulting from either increased ROS generation or decreased ROS degradation (Zeng *et al*., 2009). Many studies have implicated oxygen radicals and other oxygen-derived species as important causative agents in a wide range of diseases and disorders such as Parkinson's disease, which is associated with non-production of dopamine and can be treated with L-dopa (Yen and Hsieh, 1997). It has been suggested that the antioxidant activity of dopamine and its related compounds correspond to the number of hydroxyl groups and its position on the phenolic ring (Yen and Hsieh, 1997). This is consistent with the hypothesis that phenols with two hydroxyl groups in the 1,2 positions can be a good antioxidant and by donating a hydrogen or an electron, can become a stable radical through an intramolecular hydrogen bond (Yen and Hsieh, 1997).

Dopamine improved ROS levels in the LM subpopulation to an extent that no significant difference between the two subpopulations could be observed. Likewise, this trend was observed for MMP intactness in the LM subpopulations. Low concentrations of dopamine or dopamine agonists have been suggested to act on the D1-like receptors, thereby decreasing oxidative stress in peripheral blood lymphocytes and kidneys (Zeng *et al*., 2009). Infertility in humans is associated with complex disturbances in hormones as well as ROS generation and disposal, which may benefit from dopamine by virtue of its antioxidant properties and free radical scavenging potential (Singh *et al*., 2013).

5.5.5 | Prolactin

Prolactin has been suggested to affect a range of sperm processes *in vitro* such as Ca^{2+} binding, maintaining motility and attachment, and reducing capacitation time (Binart *et al*., 2003). No significant effect was observed for the HM subpopulation’s MMP and ROS percentages after prolactin exposure. In contrast, higher concentrations of prolactin (250 ng/mL and 500 ng/mL) did increase MMP values and reduced ROS, which may reflect increased energy metabolism in the LM subpopulations that correlate with motility and hyperactivation observations. It is suggested that in the absence of pro-survival factors such as prolactin,

spermatozoa enter the default apoptotic pathway associated with ROS generation by mitochondria (Bibov *et al*., 2018). Thus, it is plausible that prolactin's pro-survival factors may in part be related to preserving MMP intactness of spermatozoa and subsequently decreasing ROS levels.

A key enzyme promoting sperm survival is phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) which, if activated and in a phosphorylated state, permits downstream effector kinases, such as AKT (protein kinase B), to remain phosphorylated and therefore maintains the gametes in an active, viable state (Aitken, 2017). The downstream targets of AKT will include molecules such as BCL2-associated death promoter (BAD), which, when dephosphorylated, forms pro-apoptotic pores in the mitochondrial membrane and fosters pore formation by BAK/BAX (Aitken, 2017). Spermatozoa possess numerous receptors for pro-survival hormones, such as prolactin and insulin which if activated by the appropriate ligand, ensure the stimulation of PI3K activity and their continued survival (Aitken, 2017). Conversely, if PI3K activity is suppressed with an inhibitor, the gametes rapidly exhibit an increase in mitochondrial ROS generation as they are forced into a state of apoptosis (Aitken, 2017). Prolactin may therefore act through PI3K, preserving sperm viability through maintaining intact MMP and reduced ROS levels.

5.6 | Acrosome Reaction

Ejaculated mammalian spermatozoa should reside in the female genital tract for several hours before gaining the ability to fertilize the egg (Henkel, 2012). As soon as spermatozoa move out of the ejaculate and pass the cervical mucus, they undergo several biochemical changes collectively called capacitation (Ickowicz *et al*., 2012). The capacitated spermatozoon penetrates the cumulus oophrous of the ovum and binds to the ZP with its plasma membrane intact (Esteves *et al.,* 2015). After binding, the spermatozoon undergoes an exocytotic process called the acrosome reaction (AR), an essential event required for fertilization (Chavez *et al*., 2018). AR enables passage of the spermatozoa through the ZP and its subsequent fusion with the egg oolema (Ickowicz *et al.,* 2012). The preparatory alterations during *in vivo* capacitation include many internal and external sperm surface modifications, such as the removal of seminal plasma proteins/glycoproteins, as well as modification and reorganization of the sperm plasma membrane molecules (Abou-haila and Tulsiani, 2009; Esteves *et al.,* 2015; Ostermeier *et al.,* 2018).

This study observed that after incubation with Ca-ionophore, the HM subpopulations presented with significantly higher percentages of induced AR, as compared to the LM subpopulation. In addition, the LM subpopulations had higher percentages of spontaneous AR as compared to the HM subpopulations. These results indicate that a subpopulation of highly functional spermatozoa such as that of the HM subpopulation comprised of spermatozoa which were more reactive and responsive to induced AR as compared to the LM subpopulations. A previous study has shown that subpopulations of spermatozoa with higher MMP also have more motile and morphologically normal gametes, with a higher capacity to respond to induced AR (Gallon *et al.,* 2006). This was evident in the HM subpopulation which also presented with increased percentages of intact MMP and motility. Other studies have also indicated that the bottom sperm fraction seems to be enriched in viable spermatozoa (Marchetti *et al.,* 2002; Ricci *et al*., 2009) and responds better to induced capacitation (Buffone *et al*., 2004; Chantler *et al.,* 2004; Buffone *et al.,* 2009). This may ultimately be a result of intact plasma membranes and functional proteins which respond to various stimuli resulting in the increased Ca^{2+} influx required for capacitation (Santos, 2018).

5.6.1 | Progesterone

The physiological activation of AR by progesterone is well documented for humans, but less so between different sperm subpopulations (Calogero *et al*., 2000; Tantibhedhyangkul *et al*., 2014; Ghanbari *et al*., 2018; Denisenko *et al*., 2021). In our study, we observed that progesterone only induced AR in HM subpopulations but had no effect on the LM subpopulations. Contreras (1999) indicated that 1.0 µg/mL of progesterone increased AR only on the sperm population with normal morphology or in the group with high motility, thus corresponding to our observations in the functional HM subpopulation. Keshtgar *et al* (2018) found that progesterone (1 µM) induced AR, whereas NNC 55-0396 (NNC) and zinc inhibited the process. Further studies have also shown that GABA and progesterone (2.5 µM/L) improve sperm motility and induce AR in patients with varicocele (Mahran and Kholef, 2014). In addition, Rehfeld *et al.* (2018) and Mata-Martínez *et al*. (2018) also found that progesterone not only induces sperm AR, but increases sperm movement through viscous medium.

To successfully achieve capacitation by CatSper, a quadrilateral arrangement of four pore-forming α -subunits needs to develop (Chung *et al*., 2014; Nowicka-Bauer and Szymczak-Cendlak, 2021). This formation structurally organizes unique Ca^{2+} signalling domains along the

flagellum (Chung *et al*., 2014; Nowicka-Bauer and Szymczak-Cendlak, 2021). The absence or alteration of genes that prevent the formation of this heterotetrameric CatSper complex, will thereby prevent capacitation (Chung *et al*., 2014; Nowicka-Bauer and Szymczak-Cendlak, 2021). Both the hyperactivation and AR percentages in the LM subpopulations could thus be explained in this way. It is plausible that due to LM subpopulations containing significantly higher percentages of immature and morphologically abnormal spermatozoa, that there may be alterations or deletions in the CatSper subunits. Thus, LM subpopulations cannot form a functional CatSper channel to permit the required Ca^{2+} influx needed for capacitation. Moreover, it is reported that despite progesterone-induced Ca^{2+} increases, less than 50% of spermatozoa actually undergo AR (Tantibhedhyangkul et al., 2014).

5.6.2 | Myo-inositol

Myo-inositol induced AR in both subpopulations, as well as when media was considered as the main factor. By activating phospholipase-C, myo-inositol results in the production of inositol triphosphate, which opens Ca^{2+} channels and regulates the intracellular Ca^{2+} stores in the sperm plasma membrane, mitochondria, acrosome and neck region (Korosi *et al*., 2017). The subsequent regulation and rise of cytosolic Ca^{2+} and increase of mitochondrial Ca^{2+} further stimulates oxidative mechanisms and ATP production that improve mitochondrial function, prevent apoptosis and assist in sperm capacitation (Korosi *et al*., 2017; Governini *et al*.,2020; De Luca *et al*., 2021).

Myo-inositol is mainly produced by Sertoli cells in response to follicle-stimulating hormone (FSH) and is involved in processes that include the regulation of motility, capacitation and acrosome reaction (Palmieri et al., 2016; Condorelli *et al*., 2017; Vazquez-Levin and Verón, 2020). Calogero *et al*. (2015) found that AR was significantly increased in men with idiopathic infertility after three months' treatment with oral myo-inositol which agrees with our findings that the LM subpopulations had increased percentages of AR after treatment. Furthermore, Santoro *et al*. (2021) observed that OAT patients treated *in vivo* and *in vitro* with myo-inositol resulted in increased motility, viability, Bcl-2 phosphorylation and cholesterol efflux of spermatozoa. In addition, elevated glucose-6-phosphate dehydrogenase activity as well as triglycerides levels and lipase activity after myo-inositol treatment highlighted an enhancement of energy expenditure, all of which correspond with capacitation (Santoro *et al*., 2021).

5.6.3 | HD-C

At first, after penetrating the cumulus oophorus of the ovum, the spermatozoon binds to the zona pellucida with its plasma membrane intact, then subsequently undergoes the acrosome reaction (Condorelli *et al*., 2017). These events are permitted through inositols and particularly by increasing intracellular Ca^{2+} release through inositol-gated channels because of the protein kinase PKC activation (Condorelli *et al*., 2017). However, only capacitated spermatozoa show adequate motility and undergo the acrosome reaction, thus acquiring fertilizing capacity (Palmieri *et al*., 2016). Our investigation observed that HD-C medium could significantly induce sperm acrosome reaction in the HM subpopulations, but not in the LM subpopulation. This increase in AR of the HM subpopulations may be a result of both the CatSper activation by progesterone in addition to IP3 by myo-inositol, both which will result in increased Ca^{2+} influx essential for inducing the AR (Santos, 2018). In contrast, the result observed in the LM subpopulations could be due to this subpopulation already contained significantly higher percentages of spontaneous acrosome reacted spermatozoa. In addition, the LM subpopulations most probably comprised of spermatozoa with increased protein malformations which prevent the proper signal transduction pathways from taking place and thus prevent spermatozoa from undergoing capacitation (Buffone *et al*., 2005). Alternatively, this subpopulation may have Ca^{2+} depletion as semen of men with hypomotility have been observed to have significantly lower Ca^{2+} levels as compared to men with normal motility (Harchegani *et al*., 2019).

5.6.4 | Dopamine

Information on the actions of catecholamines on sperm capacitation is lacking; however, norepinephrine and epinephrine were reported to induce capacitation in mouse, hamster and bull sperm (Schuh *et al*., 2006). Catecholamines were also reported to increase spontaneous acrosome reaction and *in vitro* fertilization rates (Schuh *et al*., 2006; Ramirez *et al*., 2009). This study observed that dopamine had no effect on the LM subpopulation's acrosome reaction, whereas higher concentrations of dopamine (100 nM and 1µM) could significantly induce acrosome reaction in HM subpopulations. The LM subpopulations comprised of significantly higher percentages of spontaneous acrosome reacted spermatozoa, and considering that this event is irreversible, it may therefore explain our observations that dopamine had no significant effect (Fraser, 1998). Nevertheless, catecholamines may still be essential in the preparation of the sperm membranes for fertilization either through capacitation or acrosome reaction (Way and Killian,

2002). Urra *et al*. (2014) observed a decrease in acrosome integrity of stallion sperm after 6 hours' incubation in high concentrations of dopamine (1 mM), thereby agreeing with our findings observed in the HM subpopulations which comprised of less spontaneous acrosome reactions as compared to LM subpopulations.

In contrast, Way and Killian (2002) observed no effect of dopamine on acrosome reaction status of spermatozoa. Changes in tyrosine phosphorylation following changes in the levels of cAMP and activation of PKA regulate sperm functions such as the initiation and regulation of motility and the regulation of sperm capacitation and the acrosome reaction (Ramirez *et al*., 2009). Ramirez *et al*. (2009) observed that dopamine influenced boar sperm capacitation, specifically tyrosine phosphorylation, but not the acrosomal reaction. They therefore suggested that observed increases in the proportion of spontaneous acrosomal reactions, are likely associated with toxicity (Ramirez *et al*., 2009).

5.6.5 | Prolactin

Prolactin is reported to reduce the time taken to achieve capacitation (Binart *et al*., 2003). Despite observing significantly increased hyperactivation percentages after prolactin treatment, no significant effects of prolactin were observed on acrosome reaction percentages for either subpopulation. Agreeing with our findings, Stovall and Shabanowitz (1991) observed no significant effects of prolactin concentrations (0 ng/mL – 200 ng/mL) on sperm acrosome reaction. Furthermore, the lack of effect on AR may also reflect its suggested inhibitory effect on the phosphotyrosine expression in spermatozoa (Puijinto *et al*., 2010).

In vitro incubation of human spermatozoa with bovine or ovine prolactin was observed to increase cyclic AMP levels, fructose utilization, glucose oxidation, ATPase activity and decrease tetracycline binding of spermatozoa (Chan *et al*., 1984), all of which are associated with the process of capacitation. However, Chan *et al.* (1984) also found no effect of prolactin on sperm fertilizing capacity. It was thus suggested that the high concentrations of seminal plasma prolactin may over saturate spermatozoa and consequently the spermatozoa became refractory to the exogenous prolactin added into the culture medium (Chan *et al*., 1984). Prolactin may therefore initiate or enhance the process of capacitation but may not directly induce the acrosome reaction (Smith and Luqman, 1982).

5.7 | Chromatin Intactness and Maturity

Many studies have demonstrated that sperm DNA damage is responsible for majority of cases of human reproduction failure (Castro *et al.*, 2018). Sperm chromatin defects can arise from multiple causes, which are associated with decreased fertilization rate, poor ART outcomes, and a higher incidence of pregnancy loss (Pourmasumi *et al*., 2019). Sperm chromatin transports half of the genomic material to offspring and the integrity of sperm chromatin has fundamental importance for balanced transmission to future generations (Pourmasumi *et al*., 2019). The sperm nuclear genome includes a central compact toroid comprised of protamine-bound DNA that is both transcriptionally and translationally silent (Esteves *et al*., 2017). The peripheral compartment, composed of histone-bound DNA, retains the nucleosomal structure and contains promoters for developmentally critical genes, microRNAs, and signalling factors (Esteves *et al*., 2017). This genome is highly sensitive to the environment and is maintained through the retention of histones, the compaction of significant portions of the genome by protamines, DNA methylation, and covalent histone modifications (Esteves *et al*., 2017). Therefore, understanding the complex packaging of mammalian sperm chromatin and assessment of DNA integrity could potentially provide a benchmark in clinical infertility (Kumaresan *et al*., 2020).

Studies have observed that the recovered bottom pellets of spermatozoa after DGC possess more spermatozoa with functional mitochondria (Donnelly *et al.,* 2000; Marchetti *et al.,* 2002; Sousa *et al.,* 2011) and lower amounts of spermatozoa with apoptotic and necrotic markers (Ricci *et al.,* 2009). In addition, higher numbers of spermatozoa with chromatin integrity has also been reported for the pellet (Donnelly *et al.,* 2000; Sakkas *et al.,* 2000; Marchetti *et al.,* 2002), although this has been contradicted by others (Sakkas *et al*., 2000). Agreeing to these findings, our study observed that the HM subpopulations comprised of significantly higher percentages of chromatin intact and mature spermatozoa as compared to the LM subpopulation. Presence of immature cells, cytoplasmic droplets and alterations in sperm head shape have been associated with sperm DNA integrity and protamine deficiency (Kumaresan *et al*., 2020). Thus, it comes as no surprise that the LM subpopulation, which contained higher percentages of morphologically abnormal spermatozoa, contain higher numbers of immature spermatozoa with abnormal chromatin. DNA damage commonly occurs due to substances such as ROS, therefore the significantly higher percentages of ROS in the LM subpopulation may have contributed to the decreased percentages of intact chromatin (Kumaresan *et al*., 2020). In addition, the decreased chromatin maturation during spermiogenesis may further contribute to our observations in the LM subpopulations.

5.8 | Correlations Between Subpopulations and Basic Semen Analysis

Male factor has been implicated in about 50% of the infertile cases and an estimated 15% of men with normal basic semen analysis have been associated with infertility (Pourmasumi *et al*., 2019). Success rates of ART depend mostly on the structural and functional integrity of the gametes and current techniques employed in the field of andrology can be improved by the addition of a new tests for assessment of semen quality (Pourmasumi *et al*., 2019). Furthermore, in the era of ICSI, the value of proper male evaluation and treatment is overlooked since ICSI may give the couple a baby without the need of explaining the nature or cause of underlying male infertility (Esteves *et al*., 2017). Several reference values have been published in the World Health Organization (WHO) manual defining optimal sperm parameters; however, confusion persists about the different purposes of semen analysis and the issue relevant to the usefulness and significance of basic semen analysis parameters (Björndahl, 2010, Patel *et al*., 2017; Milachich and Dyulgerova-Nikolova, 2020). Human semen is known to contain a distinct heterogeneous cell population with varying maturation, functionality, and fertilizing ability (Alvarez *et al*., 2003; Sousa *et al*., 2011; García-Peiró *et al*., 2012; Ramone *et al*., 2014; Santolaria *et al*., 2016). Subpopulations are often ignored in BSA, which could impede an accurate assessment of sperm quality, especially since overlaps have previously been observed between the semen characteristics of both fertile and infertile men (Lewis, 2007; Hamada *et al*., 2012; Bompart *et al*., 2018; Oehninger and Ombelet, 2019).

Reports have indicated that HM subpopulations appeared to contain higher concentrations of proteins essential for sperm functionality and spermatogenesis, whereas LM subpopulations consistently presented with alterations in proteins (Amaral *et al*., 2014; D'Amours *et al*., 2018) and gene expression profiles associated with male infertility (Caballero-Campo *et al*., 2020). These differences in the two sperm subpopulations were found to closely coincide with those seen in normospermic samples compared to asthenozoospermic samples (Parte *et al*., 2012; Amaral *et al.*, 2014; Freitas *et al*., 2019; Martin-Hidalgo *et al*., 2020). Notably, the LM subpopulation in our study also presented with similar structural and functional complications to what have been found in subfertile semen samples in terms of levels of immature spermatozoa, ROS, motility parameters (Marchetti *et al*., 2002; Kothari *et al*., 2010; Aitken, 2017), hyperactivation (Travis *et al*., 2016) and DNA fragmentation (Marzano *et al*., 2019; Oseguera-López *et al*., 2019). Additionally, normozoospermic samples tend to be more homogeneous compared to heterogeneous asthenozoospermic and subfertile samples (Punjabi *et al*., 2019;

Caballero-Campo *et al*., 2020), which closely corresponds to our observations that the HM subpopulations displayed more homogeneity in sperm function and structural parameters compared to the LM subpopulation. This heterogeneous trend of characteristics could therefore be an attribute of the varying degrees of alterations in proteins found in the LM subpopulation that affect spermatogenesis and ultimately sperm structure and function.

According to the World Health Organization guidelines (WHO, 2010), clinical human semen analysis is considered an important initial test in evaluating male infertility and is partly based on analysis of sperm viability, morphology, concentration and motility (Yoon *et al*., 2020). Sperm motility is frequently utilized for the assessment of semen quality due to its general positive correlation with MMP, concentration, viability (Ramió-Lluch *et al*., 2011; Wang *et al.,* 2018) and fertilising capacity (Cejko *et al*., 2019), while progressive motility is suggested as the most important motility percentage to evaluate in ART programs (Boshoff *et al*., 2018; Gonzalez-Castro and Carnevale, 2019; Ibănescu *et al*., 2020). From the multiple regression analysis of the HM subpopulation, our study observed that collectively semen total motility, mucus penetration, progressive motility, and viscosity presented with better predictive value towards the HM subpopulation motility and kinematic parameters as compared to semen morphology parameters. Furthermore, from these combined semen parameters it appeared that semen viscosity had the most predictive impact on the motility and kinematic parameters. From the individual correlations of both subpopulations, no relationship between the percentage total motility of the basic semen analysis and either of the sperm subpopulations' motility percentages were found; however, an inverse relationship was observed with the HM subpopulation's immature spermatozoa and the LM subpopulation's induced acrosome reaction.

Additionally, progressive motility from the basic semen analysis had a positive correlation with the LM subpopulation's rapid vigour kinematic parameters. Furthermore, the semen morphology parameters presented with numerous correlations to the LM subpopulation motility and kinematic parameters, immature spermatozoa, positive ROS and MMP. Considering the strong correlations found between the percentage of diverse subpopulations and sperm quality and fertility (Henkel and Schill, 2003; Quintero-Moreno *et al*., 2003; Buffone *et al*., 2004; Dorado *et al*., 2011; Jayaraman *et al*., 2012; Peña *et al*., 2012; Santolaria *et al*., 2016; Ibănescu *et al*., 2018). Higher concentrations of spermatozoa in the LM subpopulation of the semen sample, can thus result in the basic semen analysis reflecting the quality of LM subpopulations despite the presence of a significantly improved HM subpopulation. As such, consequently

resulting in skewed basic semen analysis results and fertility diagnosis. Separating sperm subpopulation with elevated levels of ROS and immature spermatozoa from semen may therefore improve sperm motility and help maintain membrane and chromatin integrity.

5.9 | FAST and CASA

Flagellar and Sperm Tracking (FAST) is a quantitative method to assess several sperm flagellar characteristics and was developed by the School of Mathematics and Centre for Human Reproductive Science, Institute of Metabolism and Systems Research, at the University of Birmingham (Gallagher *et al*., 2019, van der Horst, 2020). This program characterizes the flagellar wave in terms of the tangent angles θ (*s*, *t*) (Gallagher *et al*., 2019). The analysis region is restricted to the section of the flagellum with 10 µm< *s* <30 µm ensuring a wave developed and is further large enough to contain all the relevant information and small enough to allow for fair comparisons between all cells (Gallagher *et al*., 2019). FAST flagellar analysis provides a whole range of new sperm motion parameters in addition to the standard kinematic parameters, including energy expenditure per sperm in watts (van der Horst, 2020).

In contrast to the second phase of the study, we observed very few significant differences between the media for FAST parameters. As individual spermatozoa were selected with the best distinct tangent angle and further refined according to analysed flagellar length and captured number of frames, it is likely that the spermatozoa used for statistical analysis had similar values or values in close range. As such, major differences between media and subpopulations were not observed. This is further explained as the program characterizes the flagellar wave in terms of the tangent angles θ (*s*, *t*) (Gallagher *et al*., 2019). Nevertheless, progesterone, myo-inositol, prolactin and dopamine did appear to increase some of the velocity and vigour kinematics while decreasing the linear kinematics, thereby agreeing with the second phase of the study. Despite having similar FAST parameters to HTF - CAP, progesterone and myo-inositol increased the HM subpopulation mean power outputs which could be related to the increased VCL and ALH values observed for these media. In addition to the decrease in WOB and LIN, it is plausible that progesterone and myo-inositol may be inducing the early stages of hyperactivation in the HM subpopulation.

Thus, increased mean power output could relate to this physiological characteristic of spermatozoa, however this needs to be further explored. In contrast, the LM subpopulation did not display such differences between progesterone and myo-inositol and therefore further

demonstrates that this subpopulation may have altered protein channels essential for motility and hyperactivation regulation. Dopamine and prolactin did not significantly alter the subpopulations' flagellar parameters which could be due to longer incubation periods required for these media. Nevertheless, it appears that both progesterone and myo-inositol could be good candidates for further investigation into the flagellar characteristics of the subpopulations and their relation for functional parameters.

Similar to our results of the media and subpopulations, Ghallagher *et al*. (2019) observed a median fAWL of 31 µM and fBF of 19 Hz for spermatozoa in diluted media. The various types of flagellar movements are a direct reflection of the intimate relationships between the spermatozoa and their environment (Dresdner and Katz, 1981). The resistance of the adjoining fluid to the movements of a spermatozoon results in an expenditure of hydrodynamic energy, thus the rate at which such energy is expended can therefore be a fundamental component of the energy budget of a spermatozoon (Dresdner and Katz, 1981). In contrast to Ghallagher *et al*. (2019), which observed a median power output of $8.4x10^{-15}$ watt, this study found that spermatozoa presented with higher mean power values when in HTF as compared to their study. This may be a consequence of Ghallagher using diluted semen whereas our study had washed sperm subpopulations, thus the viscosity of the media may differ resulting in different mean power outputs.

When the SCA kinematic parameters for both the individual spermatozoa and whole sperm subpopulations were compared with FAST, FAST individual kinematic parameters differed significantly from SCA for BCF, WOB, VAP and STR. However, no differences were observed for SCA individual sperm VSL, LIN and VCL which is expected if both software had the same frames per second – as the kinematics are followed on a point-to-point basis. The FAST parameters further differed from the SCA whole sperm subpopulations for VCL, VAP, VSL, STR, LIN, WOB and BCF which we expected to observe as this takes the average of the whole subpopulation into account. However, this raises the question as to whether FAST parameters may provide information into the whole sperm subpopulation instead of just the individual spermatozoa?

FAST flagellar parameters appeared to have more correlations with the SCA individual sperm kinematic parameters as compared to SCA whole sperm subpopulations. This may be because identical spermatozoa were selected with SCA individual parameters, whereas the whole subpopulations contained a larger average of the separated motility subpopulations. The lack of

correlations and low values of those present further means that a large percentage of information contributing the various parameters may still be unaccounted for. In addition to this, FAST may therefore actually be providing additional information as compared to SCA especially in the case for the whole subpopulation. However, the exact significance and relation of this information remains elusive. Nevertheless, TCS and mean power did display majority of correlations with the SCA whole sperm subpopulations parameters and could thus provide some information into what the motility parameters of the whole sperm subpopulation may be. Moreover, these correlations further explain our hypothesis that mean power may indicate the early stages of hyperactivation. This can be observed by the positive correlations of mean power with VCL and ALH, and negative correlation with LIN.

FAST parameters appeared to be positively correlated with the RP and MP speed groups but were negatively correlated with NP speed groups. As motile spermatozoa were selected with distinct flagellar movements, this may be a consequence of our observations. Albeit FAST parameters may reflect and therefore provide insight into the RP and MP speed groups of sperm motility subpopulations. In addition, kinematic groups (linear, velocity and vigour) all appeared to correlate with the respective groups in the SCA individual sperm parameters. Moreover, a grouping of FAST parameters (fBF, fAWL, fAWS and analysed flagellar length) had no predictive power on the motility and kinematic parameters of the whole sperm subpopulation. However, the predictive flagellar group of the FAST parameters could account for at least 10-20% of the variation in some of the kinematic parameters of the individual SCA sperm parameters. Moreover, the flagellar group had more predictive value towards the MP and RP speed groups as compared to the NP speed group.

The small R-squared values once again further substantiate the fact that additional information could be provided with flagellar parameters of FAST, however the physiological and biological relevance of this information needs to be further investigated. Interestingly, this study found that FAST kinematics which are measured differently to SCA kinematics and thus theoretically should not be the same did in fact have some correlations to one another. For example, VAP and the derivatives of VAP for FAST appeared to correlate with the velocity (VCL, VAP and VSL) and vigour (WOB, ALH and BCF) groups of SCA individual spermatozoa. It may be that despite VAP and its derivatives (e.g., WOB) being calculated differently, possible deviations from the standard point may therefore explain the correlations between the two software. The VAP of FAST and SCA may therefore not be exactly the same,

but close in values. It could therefore be plausible that FAST may additionally provide more information than SCA alone, considering that the software investigates the flagellar characteristics which further correlate with various speed groups as previously mentioned. However, such information could further be explored and eluded through creating in-depth statistical models instead of correlations as done in this study.

CHAPTER SIX: Conclusion

Our study has confirmed that spermatozoa of the HM sperm subpopulation present with significantly improved functional and structural parameters while displaying a more homogenous pattern in parameter values amongst individual samples. Thereby the HM sperm subpopulation closely mimics the quality of spermatozoa observed in that of potentially fertile semen samples as classified by WHO. In addition, the LM sperm subpopulations displayed significantly lower sperm functionality with a more heterogenous pattern amongst individual samples that closely mimics the quality of a sub-fertile semen sample. We therefore propose that neat semen samples be separated into sperm subpopulations before analysis for either clinical or research purposes. It is further suggested that the quantification of functional and structural sperm parameters for individual sperm subpopulations may provide a more accurate reflection of sperm and semen quality which could consequently improve the prediction and diagnosis of the fertilization potential of the whole ejaculate, especially in sub-fertile semen cases. Our investigation also suggests that sperm motility subpopulations could potentially be utilised as a prospective research model of sperm physiology for investigating "fertile" and "sub-fertile" samples. Moreover, when focusing on an individual semen trait as a possible predictor for male fertility, such as progressive motility, this may result in an over- or underestimated prediction. As such, using a combined group of related semen traits may elude more information into a specific group and even sub-group of the functional and structural variables of either sperm motility subpopulations relating to fertility. The grouped combinations of traits may compensate for an individual trait of poor quality, thereby producing more accurate estimations of overall functional quality of the spermatozoa.

Our results further indicate that the diverse functional capabilities of spermatozoa and subpopulations should be considered when selecting an appropriate medium to enhance the individual sperm attributes. As such, progesterone appears to be a suitable medium for improving sperm progressivity and motility parameters while inducing hyperactivation for clinical scenarios, whereas HD-C has the capability of improving both the HM and LM sperm subpopulations functional quality. Both dopamine and myo-inositol could be used for their antioxidant properties before the processing and separation of semen samples, thereby preserving sperm motility, DNA and MMP integrity and reducing ROS. Prolactin could additionally be used as a pro-survival factor for sample storage in ART techniques, however, further

investigations on such factors need to be explored. It is suggested that one should consider the heterogeneity of human ejaculates before selection of appropriate media, as to ascertain the selection of an appropriate medium which contains various biological substances and suitable concentrations thereof in order to target each of the sperm subpopulations. Since the LM subpopulation closely mimics the functionality of subfertile semen samples, it is conceivable that such media can be utilised in clinical scenarios to enhance the functionality of spermatozoa before ART treatment. Alternatively, exposing subfertile samples to a selection of the substances investigated could potentially increase the quality of retrieved spermatozoa so that more affordable ART techniques can be utilised, e.g., intrauterine insemination (IUI) instead of intra-cytoplasmic sperm injection (ICSI).

Finally, our study observed that FAST analysis may provide additional information into the sperm flagellar waveforms as compared to the conventional SCA kinematic parameters. FAST flagellar parameters such as mean power and TCS may provide some information on the whole sperm subpopulations progressive speed groups and FAST flagellar parameters have more predictive power on individual SCA sperm kinematic parameters, specifically of the rapid progressive and medium progressive speed groups. However, additional investigations need to be performed to determine whether the media or the biological substances it contain may have different effects on flagellar waveforms when spermatozoa are exposed to different viscosities. Furthermore, the various FAST parameters need to be explored to determine where other parameters may provide more information on sperm subpopulations as compared to the selected flagellar parameter model used in this study or whether the flagellar parameters may correlate with other functional paramaters.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1 Percentage vitality results for LM and HM sperm subpopulations at 5, 30 and 60 minutes after treatment with HTF, CAP, HD-C, progesterone, myo-inositol, dopamine and prolactin (Mean ± SD) (n=20).

Medium and Subpopulation	5 minutes		30 minutes		60 minutes		
	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	TP *p*
HTF							
HM	**64.7 ± 14.4****	**0.002**	**64.9 ± 17.7****	**0.001**			0.975
LM	**50.0 ± 13.5****		**47.1 ± 12.7****				0.477
CAP							
HM	**67.0 ± 13.9****	**0.001**	**66.0 ± 15.9*****	**<0.001**			0.829
LM	**50.6 ± 14.4****		**48.0 ± 13.6*****				0.561
HD-C							
HM	**67.9 ± 13.0****	**0.001**	**68.9 ± 13.7****	**0.001**			0.806
LM	**52.2 ± 13.9****		**52.6 ± 14.6****				0.928
1.98 µM PRG							
HM	**65.6 ± 15.4****	**0.004**	**67.0 ± 14.9****	**0.001**			0.99
LM	**51.8 ± 14.2****		**54.9 ± 11.9****				0.442
3.96 µM PRG							
HM	**66.3 ± 12.1*****	**<0.001**	**65.6 ± 15.4***	**0.015**			0.66
LM	**50.9 ± 13.1*****		**56.5 ± 12.4***				0.159
19.8 µM PRG							
HM	**64.5 ± 13.0***	**0.01**	**67.0 ± 12.3****	**0.003**			0.58
LM	**53.5 ± 13.3***		**55.2 ± 11.8****				0.652
11 mM MYO							
HM	**69.1 ± 14.7****	**0.002**	**70.8 ± 15.6****	**0.001**			0.489
LM	**54.2 ± 15.3****		**55.3 ± 13.4****				0.81
HTF							
HM	**70.7 ± 7.9**$^{*** *}$	**<0.001**	**64.6 ± 6.3**$^{** *}$	**0.003**			**0.01**
LM	**59.2 ± 8.6*****		**53.6 ± 14.1**$^{** *}$				0.13
CAP							
HM	**70.0 ± 12.1***	**0.032**	64.0 ± 10.1	0.071			0.088
LM	**62.3 ± 9.6***		57.2 ± 13.1				0.167
20 nM DOPA							
HM	68.7 ± 10.8	0.165	**66.1 ± 9.1***	**0.043**			0.415
LM	64.2 ± 9.3		**58.9 ± 12.3***				0.143
100 nM DOPA							
HM	**70.7 ± 10.0****	**0.003**	**67.6 ± 11.7***	**0.04**			0.381
LM	**59.8 ± 11.4****		**56.9 ± 12.6***				0.715
1 µM DOPA							

HM	**69.5**	±	**7.9****	**0.002**	**69.3**	±	**9.0****	**0.001**		0.955
LM	**60.6**	±	**8.7****		**58.7**	±	**9.4****			0.51
HTF										
HM	**62.6**	±	**8.9*****	**<0.001**	**62.8**	±	**9.7*****	**<0.001**	**60.5** ± **10.9*****	**<0.001** 0.975
LM	**45.8**	±	**11.7*****		**46.5**	±	**11.5*****		**43.8** ± **12.0*****	0.477
CAP										
HM	**65.2**	±	**7.8*****	**<0.001**	**66.7**	±	**7.0*****	**<0.001**	**65.5** ± **8.3*****	**<0.001** 0.829
LM	**52.2**	±	**9.5*****		**48.4**	±	**10.7*****		**45.1** ± **11.3*****	0.561
50 ng/mL PRL										
HM	**63.6**	±	**9.0****	**0.001**	**65.9**	±	**8.9*****	**<0.001**	**63.7** ± **9.7*****	**<0.001** 0.806
LM	**51.8**	±	**10.3****		**49.9**	±	**12.6*****		**45.6** ± **11.2*****	0.928
100 ng/mL PRL										
HM	**65.8**	±	**9.0*****	**<0.001**	**67.4**	±	**9.0*****	**<0.001**	**62.8** ± **9.8***	**0.015** 0.99
LM	**50.3**	±	**9.9*****		**53.1**	±	**8.8*****		**51.5** ± **14.9***	0.442
250 ng/mL PRL										
HM	**63.7**	±	**8.4****	**0.001**	**62.3**	±	**7.5*****	**<0.001**	**64.0** ± **8.7****	**0.001** 0.66
LM	**50.3**	±	**12.4****		**50.7**	±	**10.6*****		**50.7** ± **12.4****	0.159
500 ng/mL PRL										
HM	**64.1**	±	**10.1****	**0.001**	**61.2**	±	**11.7****	**0.002**	**62.3** ± **11.1****	**0.001** 0.58
LM	**50.4**	±	**14.4****		**46.6**	±	**13.6****		**47.7** ± **12.3****	0.652

Note: The HM subpopulation had significantly higher percentages (%) of viable spermatozoa as compared to the LM subpopulation for all media and time points. However, at 5 minutes 20 nM DOPA enhanced sperm viability in the LM subpopulation until significant differences between the two subpopulations were no longer observed. This trend was further observed for CAP at 30 minutes. Percentage viable spermatozoa was additionally significantly lower at 30 minutes as compared to 5 minutes for the HM subpopulation in HTF. Values labelled in bold with an asterisk in the same column were significantly different between the HM and LM subpopulations for individual media and time points (**p* <0.05, ***p* <0.01 and ****p* <0.001). Values labelled in bold with an asterisk in the same row were significantly different between time points for individual media and subpopulations (**p* <0.05, ***p* <0.01 and ****p* <0.001). **Abbreviations:** CAP, capacitating-HTF, DOPA, dopamine; HD-C, HD Capacitating medium; HM, high motile; HTF, human tubal fluid; LM, low motile; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; SP *p*, significant differences between subpopulations; TP *p*, significant differences between time points.

Supplementary Table 2 Comparison of mean SCA motility and kinematic parameters at 5 and 30 minutes for both the low motile (LM) and high motile (HM) sperm subpopulations after treatment with HTF, CAP, HD-C, 1.98 µM progesterone, 3.96 µM progesterone, 19.8 µM progesterone and 11 mM myo-inositol (mean ± SD) (n = 20).

		HTF			CAP			HD-C			1.98 µM PRG			3.96 µM PRG			19.8 µM PRG			11 mM MYO			
		Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	ANOVA
Motile (%)																							
5 min	HM	**66.9 ± 21.8*****	**<0.001**	0.552	**67.7 ± 20.0****	**0.009**	0.484	**66.1 ± 20.2****	**0.001**	0.835	**67.1 ± 19.0****	**0.005**	0.633	**70.7 ± 19.2*****	**<0.001**	0.734	**69.9 ± 19.9*****	**<0.001**	0.673	**74.8 ± 15.8*****	**<0.001**	0.233	0.784
	LM	**32.5 ± 24.2*****		0.462	**48.6 ± 23.1****		0.629	**41.9 ± 19.3****		0.871	**47.0 ± 25.5****		0.950	**45.1 ± 24.7*****		0.542	**44.1 ± 25.6*****		0.799	**45.6 ± 23.8*****		0.897	0.558
30 min	HM	**62.4 ± 19.9*****	**<0.001**		**71.8 ± 19.2*****	**<0.001**		**64.6 ± 21.0****	**0.002**		**64.0 ± 23.0***	**0.018**		**70.6 ± 23.1****	**0.006**		**68.4 ± 18.9****	**0.002**		**67.1 ± 21.1****	**0.005**		0.768
	LM	**26.8 ± 18.5*****			**44.9 ± 27.0*****			**40.8 ± 20.7****			**46.5 ± 24.5***			**49.8 ± 25.6****			**45.5 ± 26.3****			**45.9 ± 25.6****			0.139
Prog (%)																							
5 min	HM	**48.8 ± 22.1****	**0.001**	0.585	**56.8 ± 21.4****	**0.001**	0.414	**54.2 ± 21.2****	**0.005**	0.564	**55.8 ± 22.0***	**0.014**	0.734	**60.1 ± 22.2*****	**<0.001**	0.964	**57.8 ± 21.3****	**0.001**	0.669	**63.3 ± 19.9*****	**<0.001**	0.146	0.524
	LM	**21.1 ± 21.9****		0.235	**34.5 ± 22.3****		0.987	**32.8 ± 19.8****		0.490	**35.6 ± 26.5***		0.843	**35.1 ± 23.0*****		0.734	**32.5 ± 24.4****		0.669	**34.2 ± 22.5*****		0.769	0.600
30 min	HM	**44.7 ± 20.5*****	**<0.001**		**61.1 ± 21.4*****	**<0.001**		**50.0 ± 21.2****	**0.006**		**53.3 ± 25.0***	**0.020**		**59.8 ± 25.4****	**0.005**		**55.2 ± 19.7****	**0.003**		**53.8 ± 22.4***	**0.024**		0.332
	LM	**12.4 ± 11.4**$^{***\,a}$			**34.4 ± 25.2**$^{***\,b}$			**28.1 ± 20.2**$^{**\,b}$			**34.9 ± 26.1**$^{*\,b}$			**37.5 ± 25.3**$^{**\,b}$			**33.6 ± 25.6**$^{**\,b}$			**36.2 ± 25.6**$^{*\,b}$			**0.040**
RP (%)																							
5 min	HM	**20.2 ± 12.5**$^{**\,ab}$	**0.001**	0.869	**28.2 ± 15.6**$^{**\,ac}$	**0.004**	0.396	**17.9 ± 9.7**$^{*\,b}$	**0.026**	0.564	**25.9 ± 14.3**$^{*\,abc}$	**0.036**	0.285	**30.5 ± 15.2**$^{***\,c}$	**<0.001**	0.838	**27.6 ± 14.5**$^{**\,ac}$	**0.001**	0.787	**31.2 ± 16.5**$^{**\,c}$	**0.004**	0.771	**0.039**
	LM	**8.0 ± 10.7****		0.452	**15.2 ± 12.4****		0.742	**11.6 ± 10.0***		0.741	**17.4 ± 16.5***		0.921	**15.4 ± 12.4*****		0.578	**13.7 ± 13.2****		0.787	**17.8 ± 13.4****		0.647	0.293
30 min	HM	**20.8 ± 10.8*****	**<0.001**		**32.2 ± 15.7****	**0.007**		**19.5 ± 9.8***	**0.014**		**31.2 ± 17.3****	**0.007**		**31.4 ± 17.1****	**0.008**		**28.8 ± 15.8***	**0.019**		29.7 ± 18.4	0.099		0.293
	LM	**4.7 ± 5.3**$^{***\,a}$			**18.2 ± 15.8**$^{**\,b}$			**12.2 ± 12.6**$^{*\,ab}$			**17.0 ± 13.6**$^{**\,b}$			**18.2 ± 14.3**$^{**\,b}$			**17.5 ± 14.8**$^{*\,b}$			20.6 ± 18.2^{b}			**0.027**
MP (%)																							
5 min	HM	**28.7 ± 14.0****	**0.002**	0.286	28.2 ± 15.8	0.081	0.482	**36.3 ± 15.2****	**0.003**	0.305	**29.9 ± 14.6***	**0.012**	0.059	**29.7 ± 14.2***	**0.015**	0.753	**30.4 ± 12.1****	**0.005**	0.266	**32.1 ± 14.3**$^{***\,*}$	**<0.001**	**0.042**	0.675
	LM	**13.1 ± 12.7****		0.154	19.4 ± 11.9		0.352	**21.3 ± 12.2****		0.170	**18.6 ± 13.9***		0.854	**19.7 ± 12.6***		0.927	**18.8 ± 13.1****		0.266	**16.4 ± 12.0*****		0.972	0.618
30 min	HM	**23.8 ± 11.0*****	**<0.001**		**28.9 ± 12.8*****	**<0.001**		**30.5 ± 16.4****	**0.004**		22.2 ± 11.3	0.259		**28.3 ± 13.9***	**0.029**		**26.4 ± 11.5****	**0.005**		**24.1 ± 10.8**$^{**\,*}$	**0.009**		0.327
	LM	**7.8 ± 6.7*****			**16.2 ± 10.4*****			**15.9 ± 10.2****			17.9 ± 13.6			**19.3 ± 12.8***			**16.0 ± 11.8****			**15.5 ± 9.7****			0.098
NP (%)																							
5 min	HM	18.0 ± 11.6	0.054	0.880	10.9 ± 5.2	0.132	0.666	11.9 ± 6.2	0.152	0.313	11.4 ± 6.3	0.549	0.708	10.6 ± 5.0	0.462	0.901	12.1 ± 8.6	0.888	0.46	11.5 ± 7.2	0.652	0.820	**0.040**
	LM	11.4 ± 5.6		0.339	14.0 ± 7.0		0.242	9.0 ± 5.0		0.262	11.3 ± 8.1		0.684	10.1 ± 6.7		0.296	11.6 ± 8.0		0.651	11.4 ± 4.5		0.292	0.354
30 min	HM	17.7 ± 11.6	0.327		10.7 ± 6.8	0.511		14.6 ± 8.2	0.325		10.7 ± 5.0	0.633		10.8 ± 5.4	0.466		13.2 ± 7.8	0.606		13.2 ± 11.0	0.319		0.096
	LM	14.3 ± 10.4			11.7 ± 6.3			12.7 ± 8.6			11.6 ± 7.2			12.2 ± 7.4			12.2 ± 7.6			9.7 ± 5.8			0.716
Rapid (%)																							
5 min	HM	**37.2 ± 20.9****	**0.005**	0.977	**48.6 ± 20.5***	**0.002**	0.123	**50.0 ± 21.5****	**0.009**	0.614	**50.0 ± 20.6***	**0.013**	0.827	**54.3 ± 23.7*****	**<0.001**	0.867	**51.7 ± 23.0****	**0.001**	0.714	**57.1 ± 19.7*****	**<0.001**	0.188	0.164
	LM	**17.0 ± 20.0****		0.268	**28.5 ± 20.6***		0.991	**30.6 ± 19.5****		0.444	**31.5 ± 25.3***		0.734	**31.0 ± 21.1*****		0.751	**28.0 ± 23.0****		0.714	**29.2 ± 20.2*****		0.680	0.524
30 min	HM	**36.9 ± 18.7*****	**<0.001**		**56.0 ± 22.2*****	**<0.001**		**46.7 ± 21.3****	**0.006**		**47.9 ± 24.0***	**0.017**		**55.6 ± 26.0****	**0.004**		**49.3 ± 20.0****	**0.004**		**48.9 ± 21.7***	**0.018**		0.180
	LM	**9.2 ± 9.3**$^{***\,a}$			**29.4 ± 23.4**$^{***\,b}$			**25.5 ± 19.1**$^{**\,b}$			**29.6 ± 24.0**$^{*\,b}$			**33.2 ± 23.9**$^{**\,b}$			**29.1 ± 23.3**$^{**\,b}$			**31.9 ± 23.5**$^{*\,b}$			**0.020**
Medium (%)																							
5 min	HM	**16.6 ± 10.9****	**0.002**	0.571	11.4 ± 6.3	0.170	0.130	**8.6 ± 4.8***	**0.035**	0.927	9.2 ± 4.9	0.112	0.938	9.1 ± 6.1	0.287	0.380	10.3 ± 6.8	0.121	0.796	9.8 ± 4.9	0.191	0.503	0.095
	LM	**7.0 ± 3.9****		0.649	9.2 ± 4.4		0.463	**5.5 ± 3.0***		0.427	7.0 ± 4.1		0.784	7.3 ± 4.2		0.667	7.5 ± 6.0		0.983	8.2 ± 5.8		0.897	0.336
30 min	HM	**13.5 ± 8.4***	**0.028**		8.8 ± 5.1	0.702		8.4 ± 4.7	0.262		9.1 ± 4.6	0.340		7.2 ± 3.3	0.573		10.2 ± 6.3	0.146		9.8 ± 8.3	0.544		0.073
	LM	**6.6 ± 5.3***			8.2 ± 4.5			6.6 ± 4.6			8.3 ± 6.9			7.8 ± 4.3			7.9 ± 5.9			7.6 ± 4.8			0.915

Slow (%)																				
5 min	**HM**	13.1 ± 8.1	0.220	0.734	**7.7 ± 3.9***	**0.034**	0.610	7.5 ± 4.3	0.235	0.387	7.9 ± 4.7	0.698	0.716	7.3 ± 4.0	0.429	0.637	7.9 ± 6.3	0.656	0.716	8.0 ± 4.9	0.489	0.955	0.196
	LM	8.5 ± 4.7		0.359	**10.9 ± 5.5***		0.115	5.8 ± 4.0		0.141	8.5 ± 6.7		0.852	6.8 ± 5.2		0.163	8.6 ± 7.0		0.640	8.2 ± 4.0		0.174	0.118
30 min	**HM**	12.0 ± 8.3	0.497		7.0 ± 4.4	0.278		9.5 ± 5.1	0.325		7.0 ± 3.8	0.496		9.3 ± 8.9	0.829		8.9 ± 6.8	0.981		8.4 ± 5.1	0.249		0.251
	LM	10.9 ± 9.1			8.5 ± 4.4			8.7 ± 6.8			8.5 ± 5.9			8.8 ± 5.6			9.0 ± 7.8			6.4 ± 4.7			0.575
Motile VCL (µm/s)																							
5 min	**HM**	112.7 ± 24.5[a]	0.066	0.692	**130.0 ± 21.5**[** b]	**0.005**	0.342	139.3 ± 22.6[b]	0.157	0.238	**133.4 ± 23.0**[* b]	**0.038**	0.550	**140.2 ± 27.9**[* b]	**0.043**	0.558	**137.0 ± 25.2**[* b]	**0.015**	0.318	**135.8 ± 22.1**[** b]	**0.001**	0.281	**0.018**
	LM	97.9 ± 18.1[a]		0.105	**110.0 ± 24.1**[** ab]		0.433	127.2 ± 26.3[b]		0.227	**116.6 ± 28.3**[* ab]		0.980	**123.0 ± 27.9**[* b]		0.685	**117.0 ± 27.1**[* ab]		0.558	**113.2 ± 22.7**[** ab]		0.734	**0.034**
30 min	**HM**	**115.7 ± 22.0**[**]	**0.001**		**136.6 ± 24.7**[**]	**0.010**		130.6 ± 18.8	0.068		129.2 ± 23.1	0.122		135.3 ± 28.2	0.066		**129.5 ± 24.1***	**0.038**		128.0 ± 21.2	0.152		0.185
	LM	**88.1 ± 19.4**[** a]			**115.9 ± 26.0**[** b]			116.9 ± 22.9[b]			116.4 ± 30.5[b]			119.6 ± 27.4[b]			**112.0 ± 29.7*** b**			116.0 ± 31.1[b]			**0.026**
Motile VAP (µm/s)																							
5 min	**HM**	**61.5 ± 13.4**[**]	**0.008**	0.727	**67.5 ± 13.2**[**]	**0.003**	0.395	63.3 ± 8.1	0.112	0.983	62.9 ± 10.5	0.162	0.437	**69.5 ± 13.9**[**]	**0.002**	0.690	**67.8 ± 12.3**[**]	**0.002**	0.473	**69.4 ± 12.2**[***]	**<0.001**	0.735	0.202
	LM	**49.6 ± 9.5**[**]		0.208	**56.1 ± 10.7**[**]		0.489	58.4 ± 9.5		0.441	57.6 ± 12.9		0.949	**57.2 ± 11.8**[**]		0.892	**55.7 ± 11.7**[**]		0.824	**56.8 ± 12.0**[***]		0.548	0.384
30 min	**HM**	**59.9 ± 12.3**[**]	**0.002**		**70.7 ± 12.5**[**]	**0.002**		63.3 ± 7.8	0.024		**66.9 ± 13.2***	**0.031**		**67.8 ± 14.4***	**0.019**		**65.0 ± 13.5***	**0.044**		68.1 ± 12.9	0.066		0.201
	LM	**46.5 ± 10.6**[**]			**58.1 ± 12.6**[**]			55.8 ± 10.2			**57.9 ± 13.7***			**57.7 ± 13.3***			**56.5 ± 13.5***			59.5 ± 16.9			0.120
Motile VSL (µm/s)																							
5 min	**HM**	47.6 ± 13.3	0.006	0.763	**53.5 ± 14.8**[**]	**0.005**	0.689	44.4 ± 10.7	0.183	0.834	47.6 ± 11.1	0.263	0.166	**53.9 ± 13.2**[**]	**0.002**	0.606	**51.8 ± 11.7**[**]	**0.002**	0.645	**53.1 ± 12.6***	**0.014**	0.945	0.127
	LM	35.0 ± 9.8		0.288	**42.5 ± 10.2**[**]		0.625	39.7 ± 9.1		0.939	43.3 ± 13.4		0.799	**41.4 ± 12.4**[**]		0.671	**40.3 ± 11.2**[**]		0.527	**43.3 ± 13.5***		0.504	0.386
30 min	**HM**	**45.6 ± 10.5**[**]	**0.002**		**55.2 ± 12.6***	**0.014**		43.7 ± 6.4	0.184		**52.9 ± 13.6***	**0.010**		**51.9 ± 12.9***	**0.030**		50.1 ± 12.6	0.071		53.4 ± 13.9	0.154		0.052
	LM	**32.4 ± 11.6**[**]			**44.0 ± 12.8***			39.5 ± 10.9			**42.4 ± 12.9***			**43.1 ± 13.5***			42.7 ± 13.7			46.5 ± 17.4			0.090
Motile STR (%)																							
5 min	**HM**	**73.7 ± 10.4*** a**	**0.041**	0.431	75.8 ± 9.8[a]	0.166	0.746	65.9 ± 7.6[b]	0.867	0.938	71.4 ± 8.4[a]	0.823	0.215	**74.8 ± 7.5*** a**	**0.046**	0.486	**73.8 ± 7.1*** a**	**0.029**	0.828	73.3 ± 8.4[a]	0.767	0.756	**0.014**
	LM	**65.9 ± 9.7***		0.452	72.1 ± 8.3		0.609	65.5 ± 7.2		0.842	69.6 ± 11.9		0.668	**69.2 ± 10.7***		0.735	**68.4 ± 8.6***		0.439	71.4 ± 11.4		0.549	0.310
30 min	**HM**	**71.0 ± 8.3*** ab**	**0.041**		75.0 ± 7.3[a]	0.090		66.1 ± 6.5[b]	0.986		74.8 ± 8.3[a]	0.051		73.3 ± 7.1[a]	0.221		73.4 ± 5.8[a]	0.305		74.1 ± 7.9[a]	0.771		**0.007**
	LM	**64.3 ± 9.6***			70.8 ± 8.8			66.1 ± 12.1			69.5 ± 9.4			70.2 ± 9.4			70.7 ± 10.6			71.6 ± 12.4			0.311
Motile LIN (%)																							
5 min	**HM**	**45.0 ± 10.1*** a**	**0.015**	0.103	43.3 ± 10.9[a]	0.412	0.611	32.5 ± 6.4[b]	0.819	0.449	36.8 ± 7.7[bc]	0.570	0.065	**40.5 ± 8.2*** ac**	**0.046**	0.531	39.5 ± 6.6[ac]	0.075	0.968	40.4 ± 8.3[ac]	0.553	0.436	**< 0.001**
	LM	**36.6 ± 7.5*** ab**		0.722	40.9 ± 8.5[a]		0.467	32.0 ± 6.4[b]		0.409	38.2 ± 7.6[ab]		0.697	**35.4 ± 8.4*** ab**		0.525	36.0 ± 6.2[ab]		0.157	38.9 ± 9.0[ab]		0.784	**0.018**
30 min	**HM**	39.8 ± 6.9[ab]	0.163		41.8 ± 8.2[a]	0.262		34.0 ± 4.8[b]	0.973		41.1 ± 7.0[a]	0.065		39.4 ± 6.9[a]	0.312		39.4 ± 4.4[a]	0.710		42.3 ± 7.7[a]	0.297		**0.010**
	LM	36.7 ± 6.9			39.2 ± 6.9			34.1 ± 8.3			37.4 ± 6.1			36.9 ± 6.8			38.8 ± 6.7			39.6 ± 8.8			0.252
Motile WOB (%)																							
5 min	**HM**	56.8 ± 7.5[a]	0.102	0.123	54.2 ± 8.4[ac]	0.819	0.847	47.5 ± 4.3[b]	0.649	0.083	50.2 ± 4.7[bc]	0.333	0.061	52.5 ± 6.8[ac]	0.095	0.892	51.6 ± 4.7[c]	0.437	0.970	52.7 ± 5.8[ac]	0.633	0.244	**0.004**
	LM	52.9 ± 5.2[ab]		0.527	53.7 ± 6.4[a]		0.574	48.2 ± 4.0[b]		0.339	51.6 ± 4.6[ab]		0.804	49.2 ± 6.2[ab]		0.395	50.6 ± 4.0[ab]		0.089	51.8 ± 6.3[ab]		0.580	**0.022**
30 min	**HM**	53.4 ± 4.2	0.497		53.7 ± 5.9	0.524		49.9 ± 3.1	0.862		53.2 ± 4.6	0.650		52.3 ± 4.7	0.228		51.6 ± 2.8	0.275		54.6 ± 5.0	0.211		0.061
	LM	54.7 ± 3.9[a]			52.7 ± 4.6[ab]			49.6 ± 4.7[b]			51.9 ± 4.3[ab]			50.6 ± 4.4[ab]			52.8 ± 4.5[ab]			52.8 ± 4.4[ab]			**0.024**
Motile ALH (µm)																							
5 min	**HM**	2.9 ± 0.7[a]	0.572	0.201	3.5 ± 0.7[b]	0.064	0.219	4.2 ± 0.8[b]	0.444	0.253	3.9 ± 0.8[b]	0.050	0.343	4.0 ± 0.9[b]	0.351	0.676	3.9 ± 0.8[b]	0.116	0.450	**3.8 ± 0.7*** b**	**0.013**	0.217	**< 0.001**
	LM	2.8 ± 0.4[a]		0.213	3.1 ± 0.7[ac]		0.188	4.0 ± 1.0[b]		0.189	3.4 ± 0.9[abc]		0.843	3.7 ± 0.9[bc]		0.709	3.5 ± 0.8[abc]		0.510	**3.2 ± 0.7*** ac**		0.720	**< 0.001**
30 min	**HM**	**3.3 ± 0.7**[**]	**0.002**		3.8 ± 0.8	0.146		3.9 ± 0.6	0.145		3.6 ± 0.6	0.364		3.9 ± 0.9	0.234		3.7 ± 0.7	0.111		3.5 ± 0.6	0.333		0.103
	LM	**2.6 ± 0.5**[** a]			17.1 ± 64.7[b]			3.6 ± 0.7[b]			3.4 ± 0.9[b]			3.6 ± 0.8[b]			3.3 ± 0.9[b]			3.3 ± 0.9[b]			**0.012**

Motile BCF (Hz)																						
5 min	HM	**22.3 ± 2.9**** a	**0.005**	**0.024**	21.5 ± 3.5ac	0.082	0.343	17.8 ± 2.6b	0.848	0.616	19.3 ± 2.7bc	0.668	0.877	**20.2 ± 3.0* ac**	**0.032**	0.234	**19.5 ± 2.5* bc**	**0.023**	0.517	20.2 ± 3.0abc	0.775	0.935	**< 0.001**
	LM	**19.2 ± 2.9***		0.082	19.8 ± 2.8		0.759	17.6 ± 2.2		0.481	18.8 ± 3.9		0.314	**18.5 ± 2.5***		0.572	**17.8 ± 2.4***		0.751	19.6 ± 3.4		0.751	0.119
30 min	HM	**20.0 ± 2.6* ** ab**	**0.012**		20.5 ± 3.2a	0.446		17.4 ± 2.0b	0.666		19.4 ± 3.2ab	0.101		19.2 ± 3.1ab	0.205		19.0 ± 2.7ab	0.131		20.2 ± 3.2a	0.741		**0.043**
	LM	**17.7 ± 2.9***			19.1 ± 3.7			17.0 ± 3.0			17.7 ± 3.6			18.0 ± 3.0			17.5 ± 3.8			19.8 ± 3.6			0.127
NP VCL (µm/s)																						
5 min	HM	61.5 ± 4.0	0.501	0.819	61.9 ± 7.7	0.370	0.417	64.5 ± 4.9	0.695	0.541	62.4 ± 6.2	0.345	0.742	62.5 ± 6.9	0.918	0.802	64.3 ± 8.6	0.100	0.814	63.1 ± 5.4	0.345	0.650	0.728
	LM	61.0 ± 5.5		0.329	60.1 ± 5.4		0.333	63.4 ± 10.1		0.393	60.3 ± 8.1		0.586	62.7 ± 6.7		0.433	60.4 ± 7.2		0.769	62.1 ± 6.9		0.397	0.677
30 min	HM	61.0 ± 7.4	0.267		**63.8 ± 8.0***	**0.011**		63.2 ± 6.8	0.316		61.7 ± 8.5	0.911		62.0 ± 7.3	0.656		61.4 ± 13.7	0.181		61.1 ± 8.1	0.552		0.939
	LM	57.7 ± 8.7			**58.7 ± 4.3***			61.0 ± 5.9			61.4 ± 5.2			61.0 ± 8.4			61.1 ± 7.2			64.2 ± 9.5			0.287
NP VAP (µm/s)																						
5 min	HM	33.3 ± 3.6	0.615	0.144	33.6 ± 7.6	0.750	0.982	32.0 ± 3.1	0.658	0.942	31.2 ± 4.4	0.964	0.382	33.6 ± 4.5	0.813	0.524	34.6 ± 6.0	0.270	0.223	32.5 ± 5.3	0.75	0.82	0.455
	LM	32.5 ± 4.8		0.944	32.9 ± 5.9		0.334	32.8 ± 7.1		0.85	31.3 ± 6.4		0.700	33.3 ± 3.8		0.856	32.1 ± 4.6		0.223	31.8 ± 4.7		0.599	0.893
30 min	HM	30.8 ± 5.5	0.430		33.6 ± 6.7	0.202		31.9 ± 4.2	0.801		32.7 ± 6.6	0.691		32.9 ± 6.9	0.401		32.4 ± 5.5	0.623		32.3 ± 7.4	0.812		0.9
	LM	32.3 ± 5.3			31.5 ± 3.7			31.5 ± 4.8			32.0 ± 5.8			32.9 ± 8.1			33.2 ± 5.0			32.8 ± 7.5			0.951
NP VSL (µm/s)																						
5 min	HM	**22.7 ± 5.9****	0.090	**0.031**	23.4 ± 8.1	0.741	0.340	18.0 ± 2.8	0.640	0.744	20.0 ± 4.8	0.857	0.653	21.7 ± 5.9	0.841	0.238	22.4 ± 7.2	0.159	0.224	20.6 ± 6.7	0.930	0.634	0.118
	LM	19.2 ± 5.4		0.975	22.7 ± 7.2		0.134	18.7 ± 5.1		0.466	19.7 ± 5.9		0.236	21.3 ± 5.8		0.547	19.8 ± 4.9		0.418	20.3 ± 6.3		0.619	0.344
30 min	HM	**18.4 ± 4.9****	0.704		21.4 ± 6.0	0.364		17.7 ± 2.9	0.854		19.8 ± 6.3	0.401		19.7 ± 5.0	0.859		20.2 ± 4.3	0.597		20.7 ± 6.2	0.733		0.383
	LM	19.1 ± 5.8			19.9 ± 5.0			17.4 ± 4.7			17.6 ± 6.0			20.1 ± 7.7			20.9 ± 4.6			21.4 ± 7.4			0.251
NP STR (%)																						
5 min	HM	63.4 ± 12.0	0.080	0.060	63.5 ± 11.8	0.668	0.223	53.7 ± 5.9	0.657	0.378	57.9 ± 11.7	0.551	0.870	61.4 ± 11.7	0.948	0.192	61.7 ± 12.3	0.361	0.214	57.9 ± 11.4	0.695	0.785	0.087
	LM	55.5 ± 12.4		0.837	63.7 ± 10.7		0.440	54.8 ± 7.9		0.287	60.2 ± 12.8		0.106	61.2 ± 13.1		0.426	58.6 ± 10.1		0.606	59.3 ± 11.7		0.901	0.209
30 min	HM	56.1 ± 9.1	0.938		59.5 ± 10.1	0.888		51.9 ± 5.8	0.893		57.4 ± 10.5	0.307		56.7 ± 12.3	0.684		57.9 ± 6.6	0.375		58.7 ± 8.4	0.740		0.288
	LM	56.4 ± 11.2			58.2 ± 11.9			51.5 ± 9.7			52.7 ± 15.0			58.2 ± 11.7			60.1 ± 8.9			59.7 ± 9.8			0.117
NP LIN (%)																						
5 min	HM	**37.8 ± 10.7**** a**	0.127	**0.021**	38.4 ± 12.8a	0.980	0.132	27.7 ± 4.3b	0.363	0.692	31.6 ± 9.3ab	0.631	0.832	36.6 ± 11.3a	0.485	0.142	35.6 ± 11.0a	0.413	0.236	32.7 ± 10.8ab	0.652	0.364	**0.023**
	LM	32.3 ± 8.9		0.835	38.3 ± 12.1		0.175	29.3 ± 5.8		0.615	33.0 ± 9.7		0.219	34.3 ± 10.8		0.609	33.2 ± 7.6		0.442	33.1 ± 10.2		0.993	0.163
30 min	HM	**30.0 ± 7.3****	0.268		33.4 ± 8.2	0.874		27.1 ± 4.3	0.943		32.2 ± 8.5	0.316		32.0 ± 9.3	0.809		31.6 ± 6.2	0.146		33.4 ± 9.3	0.905		0.224
	LM	33.5 ± 10.2			33.9 ± 9.2			28.0 ± 8.5			29.4 ± 9.8			32.7 ± 9.8			35.0 ± 7.1			33.1 ± 9.9			0.201
NP WOB (%)																						
5 min	HM	53.5 ± 7.2	0.803	0.182	54.8 ± 10.8	0.863	0.429	49.5 ± 3.7	0.264	0.868	50.6 ± 6.2	0.667	0.255	54.1 ± 7.9	0.691	0.626	54.4 ± 8.0	0.564	0.107	51.0 ± 7.2	0.819	0.622	0.166
	LM	54.1 ± 6.9		0.267	55.3 ± 9.1		0.454	51.4 ± 5.9		0.948	51.5 ± 7.4		0.700	53.2 ± 7.3		0.810	53.2 ± 5.1		0.585	51.5 ± 8.1		0.997	0.516
30 min	HM	**50.4 ± 5.5***	**0.007**		52.6 ± 6.6	0.888		49.7 ± 4.3	0.478		53.0 ± 7.3	0.818		53.0 ± 7.3	0.742		50.8 ± 6.4	0.085		52.2 ± 8.5	0.811		0.621
	LM	**57.0 ± 7.2***			52.2 ± 7.7			51.2 ± 7.4			52.5 ± 8.8			53.7 ± 7.9			54.2 ± 6.3			51.5 ± 9.2			0.376
MP VCL (µm/s)																						
5 min	HM	124.7 ± 24.8a	0.926	0.285	138.8 ± 23.9ab	0.138	0.196	156.7 ± 22.6b	0.307	0.359	149.8 ± 23.0b	0.111	0.103	155.2 ± 31.7b	0.072	0.507	151.1 ± 26.5b	0.154	0.290	**148.8 ± 20.8** b**	**0.004**	0.304	**0.003**
	LM	123.9 ± 18.0		0.527	127.8 ± 25.5		0.118	148.7 ± 22.7		0.121	138.0 ± 24.6		0.564	139.4 ± 26.2		0.383	140.5 ± 22.0		0.33	**129.7 ± 21.3****		0.240	**0.025**
30 min	HM	133.7 ± 22.0	0.063		149.0 ± 28.2	0.186		146.5 ± 39.0	0.070		137.8 ± 24.0	0.542		149.3 ± 28.2	0.825		143.1 ± 23.2	0.197		142.1 ± 22.3	0.593		0.499
	LM	119.8 ± 17.9			138.9 ± 21.0			139.3 ± 25.3			142.5 ± 27.1			147.3 ± 33.2			133.3 ± 26.4			138.1 ± 25.3			0.077

MP VAP (µm/s)																					
5 min	HM	**64.7 ± 10.1***	**0.036**	0.836	**67.6 ± 9.4***	**0.034**	0.581	67.8 ± 7.5	0.174	0.554	64.1 ± 11.9	0.335	0.466	**70.5 ± 12.5****	**0.003**	0.629	**69.5 ± 9.7****	**0.006**	0.229	**70.8 ± 7.7*****	**<0.001**	0.229	0.207
	LM	**57.9 ± 7.4***		0.520	**62.0 ± 8.0***		0.279	63.4 ± 5.3		0.704	62.8 ± 6.8		0.091	**60.5 ± 8.2****		0.192	**62.2 ± 7.0****		0.561	**61.7 ± 8.7*****		0.413	0.439
30 min	HM	**65.4 ± 7.3****	**0.003**		69.2 ± 10.0	0.061		65.2 ± 16.4	0.058		66.7 ± 9.1	0.917		68.9 ± 9.2	0.161		65.7 ± 10.8	0.105		67.7 ± 9.2	0.159		0.798
	LM	**55.6 ± 9.6**$^{** a}$			64.3 ± 6.4^{ab}			63.3 ± 5.8^{b}			67.0 ± 9.4^{b}			64.5 ± 11.5^{b}			60.7 ± 9.0^{b}			63.8 ± 8.3^{b}			**0.009**
MP VSL (µm/s)																					
5 min	HM	**45.1 ± 9.2***	**0.045**	0.575	46.4 ± 9.2	0.253	0.590	40.7 ± 5.9	0.089	0.875	**42.3 ± 6.2***	0.882	**0.045**	**47.2 ± 8.3****	**0.003**	0.350	**46.0 ± 7.4****	**0.007**	0.107	46.1 ± 6.1	0.063	0.156	**0.009**
	LM	**39.0 ± 6.8***		0.884	43.6 ± 7.1		0.849	37.1 ± 6.1		0.142	42.0 ± 6.9		0.430	**39.1 ± 9.3****		0.216	**40.0 ± 6.7****		0.366	40.7 ± 10.8		0.542	0.204
30 min	HM	**43.6 ± 5.0****	**0.002**		45.0 ± 7.3	0.373		40.4 ± 4.9	0.921		**46.0 ± 5.8***	0.209		45.0 ± 6.9	0.259		42.5 ± 6.6	0.868		44.0 ± 5.8	0.441		0.116
	LM	**37.8 ± 6.4****			43.2 ± 6.4			40.2 ± 6.2			43.6 ± 6.8			42.4 ± 8.3			42.1 ± 8.7			42.4 ± 7.3			0.252
MP STR (%)																					
5 min	HM	70.3 ± 12.8^{a}	0.542	0.490	68.8 ± 10.8^{a}	0.739	0.293	59.4 ± 8.3^{b}	0.796	0.517	**63.9 ± 7.4**$^{* ab}$	0.287	**0.025**	67.7 ± 9.2^{ab}	0.364	0.362	66.1 ± 10.3^{ab}	0.645	0.411	66.0 ± 7.5^{ab}	0.191	0.686	**0.024**
	LM	67.7 ± 9.8		0.944	69.9 ± 11.0		0.595	59.6 ± 9.3		0.270	66.9 ± 10.7		0.724	64.8 ± 12.2		0.665	64.8 ± 8.9		0.263	66.6 ± 16.2		0.330	0.193
30 min	HM	67.5 ± 9.1	0.993		65.7 ± 9.0	0.294		60.1 ± 6.4	0.292		**69.1 ± 7.2***	0.194		65.7 ± 9.6	0.307		62.5 ± 14.0	0.074		65.1 ± 7.2	0.525		0.084
	LM	67.5 ± 7.8			68.4 ± 7.8			63.9 ± 13.1			65.8 ± 8.8			66.4 ± 11.5			68.3 ± 11.6			66.8 ± 9.1			0.839
MP LIN (%)																					
5 min	HM	42.4 ± 13.0^{a}	0.103	0.187	39.0 ± 12.5^{ab}	0.761	0.112	28.6 ± 7.7^{d}	0.679	0.195	**31.8 ± 6.5**$^{* dc}$	0.243	**0.033**	34.6 ± 9.3^{abc}	0.359	0.645	34.3 ± 9.1^{bc}	0.542	0.833	35.0 ± 8.0^{ac}	0.684	0.742	**0.003**
	LM	35.7 ± 8.4^{ab}		0.890	40.1 ± 11.7^{a}		0.291	27.7 ± 7.0^{b}		0.391	34.7 ± 9.2^{ab}		0.964	31.9 ± 11.0^{ab}		0.414	32.7 ± 9.5^{ab}		0.312	35.8 ± 12.5^{ab}		0.783	**0.008**
30 min	HM	37.4 ± 8.7	0.968		34.2 ± 10.0	0.054		29.3 ± 4.4	0.756		**37.4 ± 7.8***	0.146		34.2 ± 10.6	0.700		33.6 ± 5.0	0.390		34.7 ± 6.7	0.952		0.068
	LM	35.4 ± 6.9			36.9 ± 7.9			31.7 ± 10.5			34.5 ± 8.2			33.3 ± 9.9			35.7 ± 10.0			34.9 ± 8.7			0.068
MP WOB (%)																					
5 min	HM	**56.1 ± 8.9**$^{* a}$	**0.048**	0.159	52.9 ± 9.8^{ac}	0.956	0.210	45.4 ± 5.5^{b}	0.770	0.183	**46.8 ± 4.9**$^{** bd}$	0.243	**0.015**	48.5 ± 6.6^{bcd}	0.157	0.904	49.4 ± 6.2^{dc}	0.258	0.945	50.3 ± 6.4^{ad}	0.904	0.555	**0.003**
	LM	**50.0 ± 7.5**$^{* ab}$		0.862	53.0 ± 8.9^{a}		0.267	42.7 ± 11.1^{b}		0.187	48.8 ± 6.4^{ab}		0.351	46.5 ± 8.7^{ab}		0.382	47.3 ± 5.6^{ab}		0.374	50.9 ± 9.0^{a}		0.612	**0.005**
30 min	HM	52.3 ± 5.4	0.214		49.6 ± 7.3	0.691		47.7 ± 3.9	0.776		**51.7 ± 5.8****	0.699		49.4 ± 7.6	0.388		49.5 ± 4.8	0.859		50.7 ± 5.4	0.576		0.304
	LM	49.5 ± 6.9			50.4 ± 6.3			47.1 ± 6.5			50.5 ± 5.7			47.3 ± 6.9			49.1 ± 7.7			49.6 ± 6.6			0.522
MP ALH (µm)																					
5 min	HM	3.6 ± 0.9^{a}	0.728	0.281	3.9 ± 0.9^{ac}	0.322	0.114	4.8 ± 0.9^{b}	0.602	0.723	4.5 ± 0.8^{bc}	0.196	0.053	4.6 ± 1.1^{bc}	0.285	0.664	4.5 ± 1.0^{bc}	0.561	0.430	**4.3 ± 0.8**$^{* bc}$	**0.025**	0.638	**0.002**
	LM	3.7 ± 0.6^{a}		0.762	3.6 ± 0.9^{a}		0.116	4.6 ± 0.9^{b}		0.567	4.2 ± 0.9^{ab}		0.609	4.3 ± 1.0^{ab}		0.429	4.3 ± 0.8^{ab}		0.289	**3.7 ± 0.9**$^{* a}$		0.111	**0.002**
30 min	HM	3.9 ± 0.8	0.253		4.4 ± 0.9	0.241		4.7 ± 0.6	0.404		4.0 ± 0.8	0.274		4.5 ± 1.0	0.857		4.2 ± 0.8	0.387		4.2 ± 0.7	0.924		0.110
	LM	3.6 ± 0.7			4.0 ± 0.8			4.4 ± 1.1			4.3 ± 0.9			4.5 ± 1.1			4.0 ± 0.9			4.2 ± 0.9			0.084
MP BCF (Hz)																					
5 min	HM	**22.0 ± 3.4**$^{* ** a}$	**0.017**	**0.009**	20.3 ± 3.7^{ac}	0.290	0.089	17.2 ± 2.8^{b}	0.667	0.540	17.6 ± 2.6^{b}	0.97	0.243	18.8 ± 2.7^{bc}	0.234	0.127	18.3 ± 2.7^{bc}	0.116	0.664	19.3 ± 2.7^{bc}	0.487	0.132	**< 0.001**
	LM	**19.4 ± 2.2**$^{* ab}$		0.312	19.1 ± 3.7^{ab}		0.700	16.8 ± 2.3^{a}		0.843	17.6 ± 3.6^{ab}		0.586	18.0 ± 2.2^{ab}		0.117	17.1 ± 2.5^{a}		0.577	**20.0 ± 4.0**$^{* b}$		**0.044**	**0.006**
30 min	HM	**19.2 ± 2.3****	0.442		18.6 ± 3.5	0.690		16.4 ± 1.7	0.904		18.5 ± 2.5	0.08		17.8 ± 3.4	0.346		17.9 ± 2.7	0.991		18.1 ± 2.6	0.629		0.165
	LM	18.4 ± 2.8			19.7 ± 8.0			16.9 ± 4.4			17.0 ± 3.0			16.5 ± 3.8			17.5 ± 2.8			**17.6 ± 3.4***			0.292
RP VCL (µm/s)																					
5 min	HM	137.2 ± 18.2^{a}	0.501	0.951	145.9 ± 16.1^{ab}	0.601	0.315	152.2 ± 11.3^{ab}	0.104	0.301	148.2 ± 15.6^{ab}	0.597	0.662	154.4 ± 18.4^{b}	0.401	0.665	153.0 ± 19.6^{b}	0.118	0.280	147.7 ± 14.3^{ab}	0.514	0.801	**0.041**
	LM	133.6 ± 13.5		0.432	143.3 ± 17.4		0.468	144.9 ± 14.1		0.683	142.8 ± 18.7		0.947	149.5 ± 20.2		0.961	142.6 ± 23.7		0.865	144.9 ± 15.2		0.794	0.33
30 min	HM	137.5 ± 13.3	0.226		150.9 ± 17.1	0.495		148.4 ± 9.1	0.304		150.2 ± 16.0	0.163		152.0 ± 18.0	0.595		146.9 ± 17.7	0.554		146.6 ± 14.1	0.501		0.124
	LM	130.2 ± 18.9			147.2 ± 18.5			142.3 ± 21.8			142.4 ± 20.6			149.3 ± 16.1			143.6 ± 17.7			143.7 ± 13.8			0.116

RP VAP (µm/s)																							
5 min	**HM**	79.6 ± 11.7	0.105	0.837	79.8 ± 12.3	0.214	0.264	71.2 ± 17.7	0.692	0.183	77.2 ± 10.9	0.530	0.296	**81.3 ± 11.7****	**0.005**	0.954	**80.8 ± 10.0***	**0.027**	0.701	**81.3 ± 10.3***	**0.042**	0.492	0.139
	LM	73.5 ± 11.1		0.861	75.4 ± 11.2		0.419	72.7 ± 7.6		0.964	75.2 ± 9.8		0.779	**72.6 ± 8.1****		0.047	**74.4 ± 8.7***		0.275	**74.5 ± 11.8***		0.181	0.947
30 min	**HM**	78.8 ± 8.8	0.084		83.7 ± 10.9	0.075		78.1 ± 6.7	0.069		**80.7 ± 10.5***	**0.035**		81.1 ± 11.4	0.344		79.6 ± 10.9	0.334		83.4 ± 10.3	0.118		0.519
	LM	72.9 ± 9.3			78.0 ± 9.8			72.8 ± 9.2			**74.4 ± 8.8***			78.0 ± 9.6			76.9 ± 6.3			78.8 ± 7.9			0.171
RP VSL (µm/s)																							
5 min	**HM**	71.7 ± 11.7	0.166	0.920	71.9 ± 12.5	0.226	0.369	66.3 ± 7.6	0.422	0.359	68.6 ± 10.5	0.928	0.289	**72.3 ± 11.1****	**0.004**	0.782	**71.9 ± 8.8***	**0.039**	0.617	72.7 ± 10.2	0.061	0.437	0.436
	LM	66.4 ± 11.1		0.895	67.6 ± 11.5		0.329	64.2 ± 7.6		0.921	68.8 ± 8.6		0.326	**63.8 ± 7.4****		0.036	**66.7 ± 7.4***		0.485	66.4 ± 12.2		0.150	0.643
30 min	**HM**	71.3 ± 9.1	0.108		**75.1 ± 10.8***	**0.013**		68.7 ± 7.0	0.154		**72.0 ± 10.4***	**0.049**		73.2 ± 11.8	0.242		70.5 ± 10.0	0.371		75.2 ± 10.5	0.166		0.383
	LM	65.1 ± 11.1			**69.1 ± 9.1***			64.5 ± 9.4			**66.3 ± 8.3***			69.4 ± 9.5			68.1 ± 6.3			71.1 ± 7.5			0.334
RP STR (%)																							
5 min	**HM**	90.5 ± 2.0^{a}	0.580	0.595	89.9 ± 2.6^{a}	0.318	0.836	87.4 ± 2.2^{b}	0.480	0.378	88.5 ± 1.9^{ab}	0.199	0.570	88.7 ± 2.1^{ab}	0.211	0.753	88.8 ± 1.6^{ab}	0.868	0.386	89.1 ± 2.4^{ab}	0.484	0.364	**< 0.001**
	LM	89.9 ± 3.1		0.458	89.2 ± 2.6		0.717	86.5 ± 7.8		0.974	89.6 ± 3.2		0.427	87.8 ± 2.5		0.364	88.9 ± 3.4		0.609	88.6 ± 3.0		0.089	0.112
30 min	**HM**	90.1 ± 2.4	0.340		89.8 ± 2.7	0.270		87.8 ± 1.9	0.809		88.9 ± 2.2	0.986		88.5 ± 1.8	0.993		89.2 ± 1.6	0.221		89.8 ± 2.3	0.764		**0.024**
	LM	88.8 ± 4.7			88.9 ± 2.5			88.2 ± 3.5			88.9 ± 2.5			88.5 ± 2.4			88.5 ± 2.3			90.0 ± 2.2			0.334
RP LIN (%)																							
5 min	**HM**	54.4 ± 7.3^{a}	0.123	0.624	50.9 ± 8^{ac}	0.214	0.985	44.9 ± 5.9^{b}	0.734	0.160	47.4 ± 5.1^{bc}	0.264	0.715	**48.3 ± 6.4**$^{* bc}$	**0.029**	0.975	49.1 ± 4.7^{ab}	0.889	0.936	50.4 ± 6.1^{ab}	0.071	0.271	**< 0.001**
	LM	50.5 ± 8.0		0.965	48.0 ± 8.0		0.743	45.6 ± 5.9		0.704	49.9 ± 8.7		0.299	**44.2 ± 5.7***		0.060	48.8 ± 9.3		0.936	**46.7 ± 7.1***		**0.043**	0.112
30 min	**HM**	53.2 ± 6.2^{a}	0.604		50.9 ± 6.2^{ab}	0.270		47.3 ± 3.9^{b}	0.138		48.1 ± 6.1^{ab}	0.848		48.3 ± 5.1^{ab}	0.776		49.0 ± 3.7^{ab}	0.466		52.4 ± 6.2^{ab}	0.369		**0.005**
	LM	51.5 ± 11.6			48.7 ± 6.8			46.5 ± 8.2			47.8 ± 4.5			47.8 ± 6.6			48.7 ± 5.6			**50.8 ± 5.0***			0.112
RP WOB (%)																							
5 min	**HM**	59.6 ± 7.1^{a}	0.123	0.659	56.2 ± 7.5^{ac}	0.459	0.849	51.0 ± 5.5^{b}	0.811	0.121	53.2 ± 4.6^{bc}	0.271	0.265	**54.0 ± 6.0**$^{* bc}$	**0.022**	0.869	54.3 ± 4.1^{bc}	0.993	0.829	**56.1 ± 5.5**$^{* ac}$	**0.040**	0.387	**0.011**
	LM	55.7 ± 7.3		0.895	54.7 ± 5.9		0.896	51.5 ± 5.4		0.673	55.3 ± 7.4		0.296	**50.0 ± 5.4**$^{* *}$		**0.029**	54.4 ± 8.3		0.829	**52.3 ± 6.5**$^{* *}$		**0.038**	0.054
30 min	**HM**	58.6 ± 5.5^{a}	0.661		56.5 ± 5.5^{ac}	0.414		53.7 ± 3.4^{b}	0.098		54.8 ± 4.5^{bc}	0.303		54.3 ± 4.7^{bc}	0.890		54.6 ± 3.4^{bcd}	0.512		57.5 ± 5.6^{ad}	0.367		**0.013**
	LM	57.3 ± 10.0			54.9 ± 7.3			52.4 ± 7.1			53.4 ± 4.0			**54.0 ± 6.4***			54.7 ± 5.3			**56.1 ± 4.3***			0.127
RP ALH (µm)																							
5 min	**HM**	3.3 ± 0.6^{a}	0.843	0.254	3.8 ± 0.8^{b}	0.968	0.506	4.3 ± 0.7^{b}	0.274	0.658	4.1 ± 0.7^{b}	0.244	0.853	4.1 ± 0.8^{b}	0.594	0.735	4.1 ± 0.8^{b}	0.568	0.735	3.9 ± 0.6^{b}	0.666	0.988	**< 0.001**
	LM	3.4 ± 0.7		0.570	3.8 ± 0.7		0.227	4.0 ± 0.8		0.497	3.8 ± 0.9		0.593	4.3 ± 1.0		0.902	4.0 ± 1.0		0.913	4.0 ± 0.7		0.666	0.154
30 min	**HM**	3.5 ± 0.5^{a}	0.841		4.0 ± 0.7^{ab}	0.703		4.2 ± 0.5^{b}	0.942		4.1 ± 0.6^{b}	0.298		4.2 ± 0.7^{b}	0.870		4.1 ± 0.6^{b}	0.794		3.9 ± 0.7^{ab}	0.967		**0.017**
	LM	3.4 ± 1.0			4.0 ± 0.6			4.2 ± 0.7			3.9 ± 0.7			4.2 ± 0.7			4.0 ± 0.8			3.9 ± 0.7			0.059

RP BCF (Hz)																			
5 min	HM	**25.7 ± 2.6*** [a]	0.105	**0.034**	24.1 ± 3.0[ac]	0.189	0.191	20.5 ± 3.4[b]	0.342	0.925	22.1 ± 2.9[bc]	0.461	0.761	22.6 ± 3.0[bc]	0.256	0.163	22.1 ± 2.7[bc]	0.774	0.332	22.6 ± 2.8[bc]	0.138	0.871	**< 0.001**
	LM	24.0 ± 2.7		0.662	22.8 ± 3.3		0.666	21.6 ± 3.5		0.061	22.8 ± 3.7		0.209	21.5 ± 3.0		0.432	21.8 ± 5.0		0.844	20.0 ± 6.1		0.098	0.097
30 min	HM	**23.7 ± 2.7***	0.778		22.8 ± 3.4	0.622		20.4 ± 2.8	0.407		21.8 ± 3.5	0.700		20.7 ± 4.9	0.733		21.4 ± 2.2	0.823		22.8 ± 3.3	0.723		0.097
	LM	23.3 ± 5.3			21.3 ± 5.7			19.6 ± 2.7			20.7 ± 5.0			20.8 ± 3.4			21.6 ± 2.0			23.1 ± 3.4			0.110

Note: High motile (HM) subpopulations had significantly higher percentages for various motility and kinematic parameters at 5 and 30 when exposed in HTF, as compared to the low motile (LM) subpopulations. After 5 min and 30 min exposure in CAP, HD-C, PRG and MYO - various LM motility and kinematic parameters could be enhanced to the point that no significant difference between subpopulations could further be seen. Compared to HTF at 30 min - CAP, HD-C, PRG and MYO significantly enhanced progressive motility, rapid and RP speed in the LM subpopulation. For HM subpopulations at 5 min - 3.96 µM PRG and 11 mM MYO significantly enhanced the RP speed group. CAP, HD-C, PRG and MYO enhanced motile VCL of HM subpopulations at 5 min, whereas HD-C and 3.96 µM PRG enhanced LM subpopulation as compared to HTF. However, at 30 min CAP, HD-C, PRG and MYO enhanced motile VCL of LM subpopulations. At 5 min, HD-C, PRG and MYO increased HM subpopulation MP VCL as compared to HTF. For the LM subpopulation at 30 min - HD-C, PRG and MYO increased MP VAP as compared to HTF. At 5 min, 3.96 µM and 19.8 µM PRG increased RP VCL in HM subpopulations as compared to HTF. In HM subpopulations at 5 min HD-C significantly decreased motile STR percentages as compared to HTF, CAP, PRG and MYO - whereas at 30 min as compared to CAP, PRG and MYO. Compared to HTF and CAP both HD-C and 1.98 µM PRG decreased motile LIN at 5 min in the HM subpopulation, however HD-C decreased motile LIN at 30 min as compared to all media. In LM subpopulations, HD-C decreased motile LIN at 5 min as compared to CAP. At 5 min HD-C decreased the HM subpopulation NP LIN as compared to HTF, CAP, 3.96 µM and 19.8 µM PRG. HD-C also decreased MP STR at 5 min as compared to HTF and CAP; and decreased MP LIN as compared to HTF, CAP, 3.96 µM and 19.8 µM PRG, and MYO. Additionally, 1.98 µM PRG also decreased MP LIN as compared to HTF and CAP; and 19.8 µM PRG as compared to HTF. In LM subpopulations, HD-C decreased MP LIN as compared to CAP; and decreased HM subpopulation RP STR and RP LIN at 5 min as compared to HTF and CAP. Whereas 1.98 µM and 3.96 µM PRG decreased RP LIN as compared to HTF. At 30 min HD-C decreased RP STR as compared to HTF. For HM subpopulations at 5 min, HD-C decreased motile WOB as compared to HTF, CAP, 3.96 µM and 19.8 µM PRG and MYO - whereas 1.98 µM and 19.8 µM PRG decreased values as compared to HTF. For LM subpopulations at 5 min and 30 min, HD-C decreased motile WOB as compared to CAP and HTF, respectively. At 5 min, CAP, HD-C, PRG and MYO increased HM subpopulation motile ALH as compared to HTF. In LM subpopulations at 5 min, HD-C increased motile ALH as compared to HTF, CAP and MYO - whereas 3.96 µM PRG increased values as compared HTF. At 30 min all media increased motile ALH as compared to HTF. For HM subpopulations at 5 min, HD-C decreased BCF as compared to HTF, CAP and 3.96 µM PRG - whereas 1.98 µM and 19.8 µM PRG decreased values as compared to HTF. At 5 min, HD-C decreased MP WOB of HM subpopulations as compared to HTF, CAP, 19.8 µM PRG and MYO - whereas 19.8 µM PRG decreased values as compared to HTF. In the LM subpopulation, HD-C decreased MP WOB as compared to HTF and MYO. At 5 min, HD-C increased HM subpopulation MP ALH as compared to HTF and CAP, whereas PRG and MYO increased values as compared to HTF. In LM subpopulations, HD-C increased MP ALH as compared to HTF, CAP and MYO. For MP BCF in HM subpopulations, HD-C and 1.98 µM PRG decreased values as compared to HTF and CAP - whereas 3.96 µM and 19.8 µM PRG, and MYO decreased values as compared to HTF. In LM subpopulations, HD-C and 19.8 µM PRG decreased values as compared to MYO. For HM subpopulations, at 5 min HD-C decreased RP WOB as compared to HTF, CAP and MYO - whereas PRG as compared to HTF. For 30 min, HD-C decreased values as compared to HTF, CAP and MYO - whereas 1.98 µM and 3.96 µM PRG decreased values as compared to HTF and MYO; and 19.8 µM PRG as compared to HTF. For RP ALH at 5 min in HM subpopulations all media increased values as compared to HTF. At 30 min, HD-C and PRG increased values as compared to HTF. For BCF at 5 min in HM subpopulations, HD-C decreased values as compared to HTF and CAP - whereas PRG and MYO as compared to HTF. **Abbreviations:** ALH, average path velocity; BCF, beat cross frequency; CAP, capacitating-HTF; HD-C, HD capacitation medium; HTF, human tubal fluid; HM, high motile subpopulation; LIN, linearity; LM, low motile subpopulation; MP, medium-progressive; min, minutes; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; Prog, progressive; RP, rapid-progressive; SD, standard deviation; SP p, t-test between subpopulations; STR, straightness; TP p, t test between time points; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble. [a, b,c,d] Values labelled with different superscript letters in the same row were significantly different between the various media for individual subpopulations and SCA parameters. One-way ANOVA was used for parametric distributions or Kruskal-Wallis test for non-parametric distributions. Values labelled in bold with an asterisk in the same column were significantly different between the HM and LM subpopulations for individual media and SCA parameters (**p* <0.05, ***p* <0.01 and ****p* <0.001). Student's t-test was used or the Mann-Whitney test when normal distribution was void. Values labelled in bold and red with an asterisk in the same column were significantly different between the 5 and 30 minutes for individual subpopulations and media (**p* <0.05, ***p* <0.01 and ****p* <0.001). Student's t-test was used or the Mann-Whitney test when normal distribution was void.

Supplementary Table 3 Comparison of mean SCA motility and kinematic parameters at 5 and 30 minutes for both the low motile (LM) and high motile (HM) sperm subpopulations after treatment with HTF, CAP, 20 nM, 100 nM and 1 µM dopamine (mean ± SD) (n = 20).

		HTF			CAP			20 nM DOPA			100 nM DOPA			1 µM DOPA			
		Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	ANOVA
Prog (%)																	
5 min	HM	**44.5 ± 23.5*****	**<0.001**	0.251	**51.4 ± 21.1*****	**<0.001**	0.944	**57.4 ± 27.1*****	**<0.001**	0.415	**54.5 ± 22.2*****	**<0.001**	0.318	57.0 ± 25.0	0.174	0.204	0.306
	LM	**18.4 ± 15.7*****		0.497	**26.7 ± 17.3*****		0.190	**29.2 ± 17.5*****		0.849	**28.7 ± 18.2***		0.550	31.1 ± 25.5		0.968	0.140
30 min	HM	**37.0 ± 22.5*****	**<0.001**		**51.0 ± 25.4*****	**<0.001**		**51.4 ± 25.6****	**0.003**		**48.1 ± 24.7*****	**0.011**		47.4 ± 27.6	0.968		0.257
	LM	**14.9 ± 14.3**$^{***\,a}$			**21.7 ± 20.0**$^{***\,ab}$			**30.3 ± 21.0**$^{**\,b}$			**30.7 ± 20.0**$^{*\,ab}$			30.5 ± 22.0^{b}			**0.017**
NP (%)																	
5 min	HM	16.3 ± 8.3^{a}	0.101	0.574	16.0 ± 12.2^{ac}	0.946	0.388	8.6 ± 4.5^{b}	0.287	0.692	11.1 ± 5.4^{bc}	0.758	0.503	9.2 ± 7.3^{c}	0.290	0.290	**< 0.001**
	LM	13.4 ± 12.5		0.976	14.2 ± 7.9		0.286	10.4 ± 7.2		0.421	11.7 ± 8.1		0.828	10.2 ± 4.5		0.899	0.441
30 min	HM	14.3 ± 8.4	0.522		13.0 ± 9.6	0.854		9.1 ± 4.8	0.100		10.9 ± 7.5	0.665		11.5 ± 8.8	0.653		0.305
	LM	13.7 ± 10.8			12.3 ± 9.0			12.1 ± 7.6			11.5 ± 6.4			10.0 ± 6.0			0.920
Motile (%)																	
5 min	HM	**60.8 ± 24.0*****	**<0.001**	0.170	**67.4 ± 19.6*****	**<0.001**	0.854	**66.0 ± 26.2*****	**<0.001**	0.388	**65.6 ± 22.5*****	**<0.001**	0.377	66.2 ± 22.3	0.273	0.222	0.875
	LM	**31.8 ± 24.5*****		0.646	**40.9 ± 21.9*****		0.306	**39.6 ± 21.2*****		0.667	**40.3 ± 22.3*****		0.781	41.4 ± 26.4		0.857	0.583
30 min	HM	**51.3 ± 24.4****	**0.001**		**64.0 ± 26.6*****	**<0.001**		**60.5 ± 27.2***	**0.011**		**59.0 ± 25.8***	**0.015**		57.9 ± 24.9	0.857		0.521
	LM	**28.5 ± 20.5****			**34.0 ± 25.0*****			**42.4 ± 23.6***			**42.2 ± 22.6***			40.5 ± 23.9			0.167
Rapid (%)																	
5 min	HM	**29.9 ± 19.8**$^{***\,a}$	**<0.001**	0.873	**40.9 ± 22.3**$^{***\,ab}$	**<0.001**	0.557	**50.0 ± 27.0**$^{***\,b}$	**<0.001**	0.520	**47.1 ± 21.0**$^{***\,b}$	**<0.001**	0.491	49.3 ± 24.2^{b}	0.240	0.301	**0.012**
	LM	**11.6 ± 10.3**$^{***\,a}$		0.711	**19.6 ± 14.2**$^{***\,b}$		0.313	**22.2 ± 13.4**$^{***\,b}$		0.933	**22.8 ± 16.0**$^{***\,b}$		0.657	27.9 ± 23.6^{b}		0.818	**0.027**
30 min	HM	**30.8 ± 20.8*****	**<0.001**		**45.0 ± 26.1*****	**<0.001**		**45.3 ± 24.7*****	**<0.001**		**42.9 ± 23.6****	**0.007**		41.7 ± 26.8	0.818		0.211
	LM	**11.3 ± 11.9**$^{***\,a}$			**16.8 ± 16.0**$^{***\,ac}$			**22.5 ± 15.4**$^{***\,bc}$			**24.8 ± 17.1**$^{**\,bc}$			29.0 ± 23.9^{b}			**0.005**
Medium (%)																	
5 min	HM	**18.1 ± 14.3**$^{**\,**\,a}$	**0.007**	**0.009**	13.4 ± 9.6^{ab}	0.273	0.148	9.4 ± 7.9^{b}	0.961	0.786	10.2 ± 5.2^{ab}	0.107	0.207	9.4 ± 8.0^{b}	0.295	0.295	**0.046**
	LM	**9.2 ± 7.7****		0.197	10.7 ± 7.2		0.133	9.4 ± 7.4		0.522	8.0 ± 6.5		0.543	5.5 ± 5.0		0.180	0.086
30 min	HM	**9.6 ± 7.9****	0.165		9.3 ± 7.9	0.560		9.1 ± 8.0	0.265		8.7 ± 6.5	0.812		7.5 ± 7.0	0.180		0.876
	LM	6.7 ± 5.6			7.7 ± 6.8			10.8 ± 7.7			8.6 ± 5.7			6.9 ± 4.6			0.130

Slow (%)																
5 min	HM	12.9 ± 7.8[a]	0.148	0.327	13.1 ± 12.0[a]	0.839	0.304	6.5 ± 3.9[b]	0.289	0.719	7.1 ± 4.9[b]	0.273	0.684	7.5 ± 6.0[b]	0.594	0.594	**0.001**
	LM	11.0 ± 11.0		0.968	10.6 ± 6.2		0.256	8.0 ± 5.8		0.528	9.5 ± 7.3		0.796	8.0 ± 4.2		0.779	0.533
30 min	HM	10.9 ± 6.9	0.689		9.7 ± 7.0	0.823		**6.2 ± 3.2***	**0.036**		7.4 ± 4.5	0.451		8.4 ± 6.1	0.654		0.194
	LM	10.6 ± 8.7			9.5 ± 7.9			**9.0 ± 5.7***			8.6 ± 5.5			7.7 ± 5.0			0.656
RP (%)																
5 min	HM	**18.9 ± 14.4*** **	**<0.001**	0.443	**25.2 ± 17.8*** **	**<0.001**	0.778	**27.5 ± 17.7****	**0.003**	0.907	**28.4 ± 15.1****	**0.001**	0.497	30.5 ± 20.3	0.946	0.946	0.160
	LM	**6.6 ± 7.3*** **		0.856	**10.7 ± 8.9*** **		0.248	**12.8 ± 8.6****		0.771	**13.1 ± 11.7****		0.606	16.2 ± 17.5		0.834	0.056
30 min	HM	**15.0 ± 11.2**[a]**	**0.006**		**26.5 ± 17.8*** [b]**	**<0.001**		**28.7 ± 17.9*** [b]**	**<0.001**		**25.4 ± 16.6**[b]**	**0.006**		30.2 ± 19.9[b]	0.834		**0.017**
	LM	**6.3 ± 7.5****			**9.4 ± 11.9*** **			**13.4 ± 11.6*** **			**13.9 ± 9.8****			14.4 ± 13.9			0.056
MP (%)																
5 min	HM	**25.7 ± 18.7****	**0.003**	0.727	**26.3 ± 17.7***	**0.024**	0.977	**29.9 ± 15.9****	**0.002**	0.106	**26.6 ± 14.7****	**0.002**	0.516	**26.5 ± 15.6****	**0.004**	0.060	0.908
	LM	**11.8 ± 9.4****		0.194	**15.9 ± 11.0***		0.236	**16.5 ± 10.3****		0.915	**15.6 ± 7.9****		0.910	**14.9 ± 11.3****		0.704	0.501
30 min	HM	**22.0 ± 13.6*** **	**<0.001**		**24.5 ± 15.1****	**0.002**		22.7 ± 14.8	0.127		22.7 ± 12.5	0.114		19.7 ± 15.3	0.704		0.836
	LM	**8.6 ± 7.8*** [a]**			**12.3 ± 10.2**[ab]**			16.9 ± 11.5[ab]			16.8 ± 12.2[ab]			16.1 ± 11.9[b]			**0.035**
Motile VCL (µm/s)																
5 min	HM	**101.0 ± 21.0*** [a]**	**0.015**	0.144	**115.5 ± 26.2*** [b]**	**0.026**	0.083	**137.2 ± 29.0*** [c]**	**<0.001**	0.393	**134.1 ± 22.7**[c]**	**0.005**	0.299	**132.6 ± 25.4*** [c]**	**0.027**	0.865	**< 0.001**
	LM	**87.6 ± 16.3*** [a]**		0.554	**100.9 ± 18.1*** [b]**		0.909	**107.3 ± 18.0*** [bc]**		0.872	**115.4 ± 21.8**[c]**		0.599	**114.9 ± 29.1*** [c]**		0.862	**< 0.001**
30 min	HM	**110.7 ± 24.9*** [a]**	**<0.001**		**128.9 ± 27.3*** [b]**	**<0.001**		**130.8 ± 23.5*** [b]**	**<0.001**		**129.4 ± 27.2**[b]**	**0.009**		131.2 ± 31.2[ab]	0.089		**0.038**
	LM	**84.5 ± 19.3*** [a]**			**100.2 ± 18.4*** [b]**			**108.1 ± 17.6*** [bc]**			**111.7 ± 17.4**[bc]**			116.3 ± 28.6[c]			**< 0.001**
Motile VAP (µm/s)																
5 min	HM	**56.4 ± 9.6*** [a]**	**<0.001**	0.320	**59.1 ± 11.6*** [ab]**	**0.012**	0.565	**63.7 ± 10.1*** [ab]**	**<0.001**	0.836	**63.5 ± 9.7**[b]**	**0.002**	0.272	64.9 ± 11.3[b]	0.467	0.392	**0.022**
	LM	**45.9 ± 10.5*** [a]**		0.147	**51.6 ± 8.5*** [b]**		0.244	**53.7 ± 5.6*** [bc]**		0.85	**55.8 ± 6.9**[c]**		0.267	55.5 ± 14.1[bc]		0.897	**< 0.001**
30 min	HM	**53.4 ± 10.9*** [a]**	**<0.001**		**60.9 ± 10.4*** [b]**	**<0.001**		**63.1 ± 9.0*** [b]**	**<0.001**		**60.6 ± 8.7**[b]**	**0.004**		61.8 ± 13.9[b]	0.064		**0.018**
	LM	**41.8 ± 9.1*** [a]**			**48.6 ± 9.0*** [b]**			**53.3 ± 7.7*** [b]**			**53.5 ± 7.5**[b]**			54.9 ± 11.6[b]			**< 0.001**
Motile VSL (µm/s)																
5 min	HM	**46.0 ± 10.7** * **	**0.001**	**0.014**	**47.4 ± 13.6***	**0.023**	0.734	**49.2 ± 11.0***	**0.018**	0.541	48.9 ± 10.2	0.076	0.677	**51.3 ± 12.7***	**0.013**	0.403	0.582
	LM	**34.7 ± 12.7**[a]**		0.121	**38.4 ± 10.8*** [ab]**		0.442	**41.7 ± 7.1*** [b]**		0.963	42.6 ± 8.3[b]		0.197	**41.5 ± 14.0*** [ab]**		0.916	**0.039**
30 min	HM	**38.4 ± 10.5** * [a]**	**0.005**		**47.3 ± 11.1*** [b]**	**<0.001**		**50.3 ± 9.4**[b]**	**0.002**		**47.1 ± 7.5**[b]**	**0.004**		48.0 ± 14.6[b]	0.071		**0.002**
	LM	**29.5 ± 10.5*** [a]**			**36.1 ± 9.8*** [b]**			**41.6 ± 9.8**[b]**			**39.9 ± 9.2**[b]**			41.2 ± 110[b]			**< 0.001**
Motile STR (%)																
5 min	HM	**57.8 ± 8.5** * ***	**0.036**	**<0.001**	53.7 ± 7.7	0.140	0.436	49.5 ± 8.2	0.992	0.282	49.6 ± 6.2	0.959	0.612	**51.9 ± 7.7***	**0.034**	0.327	0.410
	LM	**54.0 ± 8.4***		0.145	52.9 ± 7.0		0.368	52.6 ± 6.6		0.793	51.8 ± 6.2		0.375	**50.8 ± 7.3***		0.734	0.623
30 min	HM	**49.9 ± 4.9*** [a]**	0.298		**49.2 ± 6.6*** [ab]**	**0.047**		50.6 ± 5.5[b]	0.255		49.0 ± 4.8[ab]	0.124		48.7 ± 5.9[ab]	0.465		**0.019**
	LM	50.9 ± 8.1			**50.0 ± 6.0***			51.0 ± 6.6			50.2 ± 5.4			49.6 ± 5.5			0.060
Motile LIN (%)																
5 min	HM	**78.7 ± 10.7** * *** [a]**	**0.042**	**<0.001**	76.8 ± 12.3[ab]	0.284	0.097	74.2 ± 9.4[b]	0.229	0.367	73.8 ± 8.1[b]	0.442	0.818	76.1 ± 9.0[ab]	0.233	0.115	**0.020**
	LM	**70.7 ± 15.1***		0.080	70.9 ± 12.0		0.230	74.2 ± 8.8		0.568	73.7 ± 10.3		0.235	70.1 ± 10.5		0.952	0.828
30 min	HM	**68.3 ± 9.6*** **	0.650		74.3 ± 9.8	0.489		76.7 ± 7.3	0.809		74.8 ± 4.8	0.716		72.5 ± 12.1	0.993		0.317
	LM	64.8 ± 12.8			68.8 ± 9.1			73.7 ± 10.9			70.9 ± 11.4			71.1 ± 8.9			0.365

Motile WOB (%)																	
5 min	**HM**	**47.6 ± 12.2**$^{***\,a}$	0.115	**<0.001**	**43.1 ± 11.5**$^{*\,ab}$	0.695	**0.032**	39.1 ± 11.4^{b}	0.079	0.299	38.3 ± 7.8^{b}	0.205	0.703	41.2 ± 10.1^{b}	0.630	0.218	**0.001**
	LM	40.2 ± 13.0		0.201	39.7 ± 10.5		0.128	41.0 ± 8.9		0.389	40.1 ± 8.5		0.328	37.6 ± 10.1		0.504	0.613
30 min	**HM**	**35.5 ± 7.3*****	0.606		**38.1 ± 9.0***	0.687		40.1 ± 7.6	0.819		37.8 ± 5.2	0.422		36.8 ± 9.1	0.478		0.765
	LM	34.4 ± 9.1			36.5 ± 7.9			39.5 ± 9.7			37.1 ± 8.5			36.8 ± 7.3			0.929
Motile ALH (µm)																	
5 min	**HM**	**2.6 ± 0.8**$^{**\,a}$	0.655	**0.003**	**3.0 ± 0.8**$^{*\,a}$	0.367	**0.006**	**3.8 ± 1.0**$^{**\,b}$	**0.001**	0.780	**3.7 ± 0.6**$^{*\,b}$	**0.030**	0.727	3.6 ± 0.8^{b}	0.092	0.550	**< 0.001**
	LM	2.4 ± 0.5^{a}		0.313	2.8 ± 0.6^{b}		0.447	**3.0 ± 0.6**$^{**\,b}$		0.622	**3.2 ± 0.8**$^{*\,b}$		0.734	3.2 ± 0.8^{b}		0.551	**< 0.001**
30 min	**HM**	**3.2 ± 0.7**$^{***\,**}$	**<0.001**		**3.6 ± 0.9**$^{***\,*}$	**<0.001**		**3.7 ± 0.7****	**0.001**		**3.7 ± 0.7***	**0.020**		3.7 ± 0.9	0.105		0.148
	LM	**2.5 ± 0.5**$^{***\,a}$			**2.9 ± 0.5**$^{***\,b}$			**3.0 ± 0.6**$^{**\,b}$			**3.3 ± 0.6**$^{*\,b}$			3.3 ± 0.8^{b}			**< 0.001**
Motile BCF (Hz)																	
5 min	**HM**	**23.2 ± 4.4*****	0.206	**<0.001**	**23.2 ± 4.5***	0.060	**0.015**	21.3 ± 4.3	0.728	0.230	20.7 ± 3.8	0.951	0.268	**21.9 ± 3.6***	0.121	**0.024**	0.128
	LM	**21.6 ± 4.3****		**0.004**	20.9 ± 3.7		0.233	20.9 ± 2.9		0.298	20.7 ± 3.2		0.071	20.3 ± 3.5		0.502	0.758
30 min	**HM**	**18.9 ± 2.8*****	0.382		**20.4 ± 3.0***	0.464		20.0 ± 2.8	0.981		19.7 ± 2.7	0.392		**19.7 ± 2.8***	0.927		0.398
	LM	**18.0 ± 4.0****			19.8 ± 3.0			20.0 ± 2.8			19.0 ± 3.1			19.6 ± 2.9			0.179
NP VCL (µm/s)																	
5 min	**HM**	**60.9 ± 7.7****	**0.007**	0.250	59.4 ± 10.4	0.372	0.706	60.0 ± 9.9	0.108	0.757	64.6 ± 13.5	0.138	0.447	59.1 ± 10.0	0.832	0.911	0.350
	LM	**55.3 ± 10.1****		0.111	58.5 ± 11.9		0.254	**56.0 ± 7.5****		**0.006**	59.6 ± 9.4		0.415	59.5 ± 10.4		0.358	0.433
30 min	**HM**	58.4 ± 7.4	0.631		58.3 ± 10.9	0.289		61.6 ± 7.7	0.472		62.2 ± 7.7	0.685		58.8 ± 8.1	0.276		0.311
	LM	57.6 ± 8.0			61.4 ± 9.5			**62.1 ± 7.4****			61.4 ± 4.9			61.2 ± 6.6			0.244
NP VAP (µm/s)																	
5 min	**HM**	**33.9 ± 6.1****	**0.001**	0.076	**32.9 ± 7.4****	0.190	**0.008**	30.2 ± 7.1	0.885	0.389	29.7 ± 6.0	0.069	0.780	32.7 ± 9.1	0.428	0.179	0.185
	LM	**28.7 ± 5.2****		0.059	30.4 ± 10.0		0.968	30.2 ± 5.6		0.920	33.9 ± 7.4		0.502	30.9 ± 6.3		0.258	0.166
30 min	**HM**	31.2 ± 6.8	0.675		**27.2 ± 7.1****	0.188		32.0 ± 7.0	0.472		29.5 ± 5.9	0.100		29.4 ± 7.3	0.741		0.138
	LM	29.2 ± 6.9			29.9 ± 6.8			30.1 ± 5.0			32.4 ± 5.9			28.7 ± 6.9			0.326
NP VSL (µm/s)																	
5 min	**HM**	**24.7 ± 8.1**$^{***\,*}$	**<0.001**	**0.014**	**22.9 ± 9.4***	0.367	**0.035**	21.1 ± 8.1	0.898	0.805	**18.1 ± 7.4****	**0.005**	0.745	22.9 ± 11.8	0.299	0.123	0.122
	LM	**17.1 ± 5.0**$^{***\,a}$		0.767	20.1 ± 9.2^{ab}		0.905	20.8 ± 7.2^{ab}		0.749	**24.4 ± 7.6**$^{**\,*\,b}$		**0.042**	19.8 ± 8.2^{ab}		0.225	**0.030**
30 min	**HM**	**19.6 ± 8.9***	0.675		**18.0 ± 6.4***	0.485		21.4 ± 7.8	0.928		18.7 ± 4.6	0.645		18.2 ± 8.4	0.647		0.492
	LM	17.4 ± 6.1			19.3 ± 6.9			20.2 ± 5.9			**19.6 ± 8.0***			17.1 ± 6.9			0.401
NP STR (%)																	
5 min	**HM**	**68.4 ± 14.0**$^{*\,*}$	**0.015**	**0.038**	66.8 ± 17.5	0.186	0.406	65.3 ± 13.8	0.849	0.558	**56.8 ± 18.1***	**0.016**	0.292	65.1 ± 21.3	0.382	0.186	0.152
	LM	**57.4 ± 15.7***		0.733	61.2 ± 11.3		0.650	64.5 ± 16.4		0.728	**67.7 ± 11.8**$^{*\,*}$		**0.021**	60.3 ± 16.4		0.413	0.140
30 min	**HM**	**59.4 ± 15.6***	0.413		63.0 ± 14.2	0.392		63.0 ± 12.7	0.999		61.1 ± 8.0	0.420		57.6 ± 16.8	0.805		0.588
	LM	55.9 ± 14.1			59.6 ± 13.4			63.0 ± 12.0			**58.3 ± 15.0***			56.4 ± 16.4			0.411
NP LIN (%)																	
5 min	**HM**	**41.3 ± 14.4**$^{*\,*}$	**0.013**	**0.015**	**39.1 ± 15.2***	0.421	**0.048**	35.2 ± 13.3	0.473	0.665	**29.7 ± 13.8****	**0.002**	0.857	39.7 ± 20.4	0.265	0.081	0.073
	LM	**31.7 ± 10.3***		0.640	35.1 ± 15.0		0.749	38.1 ± 14.7		0.172	**41.4 ± 10.9**$^{**\,*}$		**0.018**	34.0 ± 14.4		0.185	0.116
30 min	**HM**	**33.3 ± 17.0***	0.984		**31.3 ± 11.6***	0.844		34.4 ± 12.9	1		30.3 ± 5.8	0.462		30.9 ± 15.0	0.358		0.794
	LM	30.3 ± 10.6			31.9 ± 10.9			33.0 ± 11.3			**32.5 ± 13.5***			28.9 ± 11.9			0.734

NP WOB (%)																		
30 min 5 min	**HM**	**56.3 ± 10.5**$^{*\,a}$	0.221	**0.028**	**56.1 ± 10.5**$^{**\,ab}$	0.134	**0.003**	50.6 ± 10.8^{a}	0.181	0.621	**47.6 ± 11.6**$^{**\,ab}$	**0.001**	0.842	55.9 ± 14.3^{b}	0.375	0.124	**0.023**
	LM	52.7 ± 9.5		0.742	51.7 ± 9.8		0.378	54.6 ± 9.9		0.055	**57.2 ± 7.2****		0.052	52.6 ± 10.8		0.129	0.287
	HM	**52.7 ± 12.9***	0.617		**46.8 ± 10.3****	0.372		52.2 ± 9.8	0.617		47.9 ± 6.6	0.104		50.1 ± 10.9	0.407		0.193
	LM	50.6 ± 10.6			49.3 ± 9.0			49.1 ± 9.6			53.1 ± 9.9			47.4 ± 11.3			0.422
MP VCL (µm/s)																		
30 min 5 min	**HM**	**109.5 ± 22.9**$^{***\,a}$	0.873	**<0.001**	123.5 ± 35.1^{a}	0.603	0.081	**149.2 ± 37.4**$^{**\,b}$	**0.002**	0.345	148.4 ± 28.6^{b}	0.105	0.443	145.1 ± 26.5^{b}	0.863	0.806 <	**0.001**
	LM	108.2 ± 18.3^{a}		0.288	116.2 ± 23.5^{a}		0.203	**121.2 ± 22.3**$^{**\,ab}$		0.740	134.7 ± 30.3^{bc}		0.672	143.6 ± 30.6^{c}		0.343 <	**0.001**
	HM	**130.4 ± 19.9**$^{**\,***}$	**0.007**		**139.9 ± 29.7***	**0.043**		**139.5 ± 34.6***	**0.018**		143.8 ± 31.4	0.124		143.1 ± 30.8	0.368		0.541
	LM	**114.0 ± 17.7**$^{**\,a}$			**124.0 ± 18.8**$^{*\,ab}$			**119.0 ± 23.5**$^{*\,ab}$			131.0 ± 23.1^{ab}			135.1 ± 30.3^{b}			**0.022**
MP VAP (µm/s)																		
30 min 5 min	**HM**	**58.7 ± 8.3***	**0.028**	0.881	60.0 ± 9.7	0.245	0.751	**63.1 ± 7.4****	**0.003**	0.916	64.3 ± 9.3	0.337	0.290	64.0 ± 7.0	0.199	0.161	0.068
	LM	**54.1 ± 5.8**$^{*\,a}$		0.427	57.2 ± 6.7^{ab}		0.710	**57.2 ± 5.7**$^{**\,ab}$		0.923	60.7 ± 9.4^{a}		0.074	61.0 ± 8.9^{b}		0.363	**0.013**
	HM	**59.1 ± 7.4****	**0.005**		**60.8 ± 8.3***	**0.048**		**62.9 ± 8.5****	**0.005**		**61.0 ± 6.8***	**0.016**		60.8 ± 9.0	0.414		0.603
	LM	**52.4 ± 7.5**$^{**\,a}$			**56.5 ± 5.0**$^{*\,ab}$			**57.1 ± 4.7**$^{**\,ab}$			**56.3 ± 7.4**$^{*\,ab}$			58.8 ± 8.1^{b}			**0.041**
MP VSL (µm/s)																		
30 min 5 min	**HM**	**45.3 ± 9.7****	0.131	**0.002**	44.8 ± 11.1	0.162	0.248	41.0 ± 6.8	0.814	0.178	40.9 ± 10.0	0.999	0.662	41.4 ± 9.9	0.223	0.450	0.280
	LM	40.8 ± 10.7		0.209	39.6 ± 10.5		0.636	41.5 ± 7.9		0.899	40.9 ± 7.9		0.129	38.1 ± 8.5		0.567	0.737
	HM	**37.2 ± 7.1**$^{**\,a}$	1		40.6 ± 8.5^{ab}	0.323		44.6 ± 10.3^{b}	0.961		40.4 ± 4.8^{ab}	0.114		39.5 ± 8.0^{ab}	0.962		**0.030**
	LM	37.2 ± 7.1			38.3 ± 6.7			41.8 ± 7.3			36.3 ± 9.3			39.6 ± 9.0			0.159
MP STR (%)																		
30 min 5 min	**HM**	**57.2 ± 11.2**$^{***\,a}$	0.582	**<0.001**	**54.1 ± 11.7**$^{*\,a}$	0.174	**0.037**	46.8 ± 9.8^{b}	0.083	0.073	47.1 ± 9.5^{b}	0.315	0.300	48.7 ± 9.3^{b}	0.651	0.999 <	**0.001**
	LM	53.1 ± 11.7		0.31	52.7 ± 9.1		0.702	51.6 ± 8.3		0.563	48.6 ± 9.8		0.404	45.9 ± 7.6		0.175	0.074
	HM	**47.9 ± 6.4*****	0.092		**47.2 ± 9.1***	0.914		50.1 ± 13.1	0.490		45.8 ± 6.5	0.414		45.3 ± 6.5	0.335		0.317
	LM	48.1 ± 7.5			48.3 ± 7.3			51.5 ± 8.4			46.2 ± 7.5			47.3 ± 10.3			0.182
MP LIN (%)																		
30 min 5 min	**HM**	**77.7 ± 13.2**$^{***\,a}$	0.309	**<0.001**	**75.9 ± 15.5**$^{*\,a}$	0.349	**0.018**	**65.5 ± 12.3**$^{*\,b}$	**0.032**	0.160	64.4 ± 12.0^{b}	0.357	0.410	65.2 ± 15.5^{b}	0.459	0.389 <	**0.001**
	LM	75.4 ± 15.6^{a}		0.112	69.5 ± 17.0^{ab}		0.202	**71.5 ± 11.4**$^{*\,ab}$		0.853	68.2 ± 14.4^{ab}		0.599	63.3 ± 14.1^{b}		0.328	**0.046**
	HM	**64.6 ± 12.0*****	0.278		**67.4 ± 12.1***	0.794		71.0 ± 12.0	0.211		67.7 ± 9.8	0.837		65.2 ± 11.7	0.328		0.268
	LM	71.0 ± 12.4			67.8 ± 10.6			73.5 ± 13.3			64.5 ± 16.3			68.5 ± 11.8			0.170
MP WOB (%)																		
30 min 5 min	**HM**	**47.1 ± 15.1**$^{***\,a}$	0.218	**<0.001**	**43.7 ± 16.4**$^{*\,ab}$	0.656	**0.026**	33.2 ± 13.0^{b}	0.064	0.357	32.3 ± 12.7^{b}	0.367	0.600	34.1 ± 14.0^{b}	0.388	0.135	**0.001**
	LM	42.5 ± 15.9^{a}		0.100	39.5 ± 14.4^{a}		0.092	39.4 ± 11.4^{a}		0.957	35.4 ± 12.8^{b}		0.477	31.3 ± 12.0^{ab}		0.734	**0.046**
	HM	**32.7 ± 9.5*****	0.923		**33.8 ± 11.8***	0.664		38.1 ± 16.8	0.184		32.4 ± 8.2	0.859		31.2 ± 9.3	0.734		0.323
	LM	35.9 ± 9.7			34.7 ± 9.6			40.0 ± 12.2			31.9 ± 11.3			34.6 ± 13.0			0.251
MP ALH (µm)																		
30 min 5 min	**HM**	**2.9 ± 1.0**$^{***\,a}$	0.804	**<0.001**	**3.3 ± 1.3**$^{*\,a}$	0.774	**0.040**	**4.3 ± 1.4**$^{**\,b}$	**0.009**	0.517	4.3 ± 1.0^{b}	0.172	0.600	4.2 ± 1.0^{b}	0.872	0.744 <	**0.001**
	LM	3.0 ± 0.9^{a}		0.064	3.2 ± 1.0^{ab}		0.139	**3.4 ± 0.9**$^{**\,ab}$		0.953	3.9 ± 1.1^{ab}		0.773	4.2 ± 1.0^{b}		0.323 <	**0.001**
	HM	**3.9 ± 0.8**$^{*\,***}$	**0.032**		**4.0 ± 1.1***	0.160		4.1 ± 1.4	0.069		4.3 ± 1.0	0.327		4.3 ± 1.1	0.251		0.746
	LM	**3.4 ± 0.7***			3.6 ± 0.7			3.4 ± 0.9			4.0 ± 0.9			3.9 ± 1.1			0.131

MP BCF (Hz)																			
5 min	**HM**	**22.7 ± 4.1**$^{***\ a}$	0.716	**<0.001**	**23.2 ± 5.6**$^{*\ a}$	0.123	**0.003**	19.3 ± 4.8^{b}	0.406	0.930	18.9 ± 3.8^{b}	0.540	0.319	19.8 ± 4.0^{b}	0.118	0.142	**0.001**		
	LM	**22.2 ± 4.7**$^{**\ a}$		**0.007**	21.0 ± 4.3^{ab}		0.147	20.3 ± 3.6^{ab}		0.524	19.6 ± 4.6^{ab}		0.066	18.1 ± 3.2^{b}		1	**0.015**		
30 min	**HM**	**18.1 ± 3.1*****	0.925		**18.9 ± 4.1***	0.911		19.2 ± 5.1	0.282		18.0 ± 2.3	0.427		18.0 ± 4.4	1		0.725		
	LM	**18.5 ± 3.8****			19.1 ± 4.3			19.7 ± 3.8			17.2 ± 4.3			18.6 ± 4.0			0.304		
RP VCL (µm/s)																			
5 min	**HM**	**125.9 ± 17.7**$^{**\ a}$	0.576	**0.001**	**137.5 ± 16.2**$^{**\ b}$	0.718	**0.006**	**151.7 ± 19.4**$^{***\ bc}$	**<0.001**	0.399	151.7 ± 23.5^{c}	0.353	0.778	150.2 ± 22.7^{c}	0.089	0.401	**< 0.001**		
	LM	129.0 ± 17.3^{a}		0.339	135.3 ± 20.6^{ab}		0.729	**132.3 ± 15.2**$^{***\ a}$		0.163	146.2 ± 16.0^{b}		0.093	139.4 ± 20.7^{ab}		0.261	**0.024**		
30 min	**HM**	**144.1 ± 18.7**$^{*** **}$	**<0.001**		**151.6 ± 17.6**$^{*** **}$	**<0.001**		146.6 ± 20.1	0.251		150.0 ± 21.3	0.058		155.6 ± 19.9	0.262		0.320		
	LM	**122.9 ± 11.2**$^{***\ a}$			**133.3 ± 18.0**$^{***\ ab}$			139.7 ± 20.9^{b}			140.2 ± 23.3^{b}			146.1 ± 18.7^{b}			**0.005**		
RP VAP (µm/s)																			
5 min	**HM**	73.3 ± 9.9	0.243	0.600	74.4 ± 10.6	0.270	0.580	**76.3 ± 9.3****	**0.004**	0.877	77.7 ± 11.2	0.201	0.884	76.8 ± 9.6	0.702	0.459	0.532		
	LM	69.0 ± 13.5		0.178	71.1 ± 9.4		0.329	**69.4 ± 6.5****		0.204	73.2 ± 6.5		0.258	75.6 ± 12.6		0.537	0.275		
30 min	**HM**	**74.8 ± 9.2*****	**<0.001**		**75.9 ± 8.5****	**0.010**		75.6 ± 8.7	0.161		**76.2 ± 10.0***	0.017		78.9 ± 9.7	0.064		0.616		
	LM	**63.7 ± 8.7**$^{***\ a}$			**70.0 ± 6.4**$^{**\ b}$			72.3 ± 7.1^{b}			**70.0 ± 7.0**$^{*\ b}$			73.6 ± 9.3^{b}			**0.002**		
RP VSL (µm/s)																			
5 min	**HM**	67.8 ± 9.8	0.058	0.921	68.4 ± 10.5	0.141	0.765	68.4 ± 9.9	0.073	0.467	70.1 ± 11.5	0.183	0.969	69.0 ± 9.6	0.909	0.545	0.945		
	LM	61.2 ± 12.0		0.277	64.1 ± 9.2		0.682	62.8 ± 7.4		0.207	65.7 ± 6.2		0.398	68.7 ± 13.0		0.369	0.301		
30 min	**HM**	**67.5 ± 8.6****	**0.001**		**67.6 ± 8.3***	**0.049**		68.0 ± 8.2	0.265		**68.7 ± 9.8***	0.025		70.7 ± 9.3	0.065		0.715		
	LM	**57.1 ± 9.7**$^{**\ a}$			**63.1 ± 6.5**$^{*\ b}$			65.5 ± 7.0^{b}			**63.1 ± 6.2**$^{*\ b}$			65.7 ± 8.5^{b}			**0.006**		
RP STR (%)																			
5 min	**HM**	**92.2 ± 3.3**$^{*\ a}$	0.102	**0.012**	**91.0 ± 2.5**$^{**\ ab}$	0.154	**0.001**	89.1 ± 3.1^{b}	0.292	0.444	90.0 ± 2.5^{b}	0.628	0.961	89.5 ± 2.4^{b}	0.349	0.736	**0.001**		
	LM	90.3 ± 4.0		0.356	89.7 ± 3.0		0.910	90.1 ± 3.1		0.84	89.5 ± 2.4		0.869	90.3 ± 3.2		0.130	0.889		
30 min	**HM**	**90.0 ± 2.2***	0.371		**88.7 ± 2.1****	0.154		89.7 ± 2.0	0.436		89.8 ± 1.9	0.620		89.3 ± 2.2	0.748		0.282		
	LM	89.0 ± 3.8			89.9 ± 3.3			90.2 ± 2.8			89.5 ± 2.3			89.1 ± 1.6			0.604		
RP LIN (%)																			
5 min	**HM**	**55.2 ± 8.9**$^{*\ a}$	0.071	**0.011**	**50.8 ± 7.1**$^{**\ ab}$	0.352	**0.005**	46.8 ± 8.4^{b}	0.190	0.377	48.1 ± 7.6^{b}	0.477	0.930	48.4 ± 7.4^{b}	0.411	0.343	**0.003**		
	LM	49.7 ± 11.0		0.358	48.9 ± 7.3		0.897	49.0 ± 9.0		0.804	46.3 ± 5.6		0.423	**50.2 ± 7.9***		**0.032**	0.474		
30 min	**HM**	**48.2 ± 6.4***	0.521		**45.7 ± 4.9****	0.098		47.5 ± 5.1	0.597		47.1 ± 4.4	0.975		46.6 ± 5.3	0.640		0.555		
	LM	46.8 ± 6.9			48.6 ± 6.7			48.5 ± 7.1			46.7 ± 6.5			**45.9 ± 4.6***			0.566		
RP WOB (%)																			
5 min	**HM**	**59.4 ± 7.6**$^{**\ a}$	0.053	**0.010**	**55.4 ± 6.6**$^{*\ ab}$	0.471	**0.013**	51.9 ± 7.5^{b}	0.204	0.304	53.1 ± 7.1^{b}	0.346	0.954	53.6 ± 6.9^{b}	0.435	0.330	**0.004**		
	LM	54.4 ± 9.7		0.421	54.0 ± 6.7		0.875	53.9 ± 8.4		0.796	51.3 ± 5.1		0.350	**55.2 ± 7.1***		**0.031**	0.476		
30 min	**HM**	**53.2 ± 6.0****	0.597		**51.3 ± 4.5***	0.122		52.6 ± 4.7	0.650		52.2 ± 4.1	0.877		51.9 ± 5.0	0.643		0.732		
	LM	52.2 ± 5.7			53.7 ± 6.0			53.4 ± 6.2			51.9 ± 6.4			**51.2 ± 4.5***			0.589		
RP ALH (µm)																			
5 min	**HM**	**3.0 ± 0.7**$^{***\ a}$	0.416	**<0.001**	**3.4 ± 0.7**$^{***\ b}$	0.734	**<0.001**	**3.9 ± 0.9**$^{*\ b}$	**0.014**	0.981	3.9 ± 0.7^{b}	0.817	0.712	**3.8 ± 0.8**$^{*\ b}$	0.078	**0.021**	**< 0.001**		
	LM	3.2 ± 0.8^{a}		0.133	3.6 ± 0.9^{ab}		0.847	**3.4 ± 0.6**$^{*\ ab}$		0.144	3.9 ± 0.8^{b}		0.645	**3.4 ± 0.6**$^{**\ b}$		**0.005**	**0.031**		
30 min	**HM**	**3.9 ± 0.8**$^{* ***}$	**0.018**		**4.1 ± 0.7**$^{** ***}$	**0.007**		3.9 ± 0.7	0.253		4.0 ± 0.6	0.060		**4.2 ± 0.6**$^{** *}$	**0.005**		0.311		
	LM	**3.4 ± 0.4***			**3.5 ± 0.8****			3.7 ± 0.8			3.8 ± 0.9			**3.9 ± 0.7**$^{** **}$			0.152		

RP BCF (Hz)																
5 min	HM	**26.0 ± 3.4****	0.500	**<0.001**	24.7 ± 4.7	0.307	0.055	23.5±4.6	0.911	0.235	23.3 ± 4.1	0.327	0.265	**24.2±3.8****	0.610	**0.009**	0.153
	LM	**25.1 ± 5.5***		**0.039**	23.4 ± 4.6		1	23.3±3.4		0.161	23.4 ± 3.3		0.116	**24.8±4.6***		**0.046**	0.486
30 min	HM	**21.8 ± 4.3****	0.950		22.6 ± 2.9	0.453		22.1±2.9	0.756		21.9 ± 3.7	0.882		**21.5±3.1****	0.406		0.855
	LM	**21.7 ± 3.5***			23.4 ± 4.3			21.8±3.9			21.8 ± 2.7			**22.3±3.4***			0.531

Note: High (HM) subpopulations had significantly higher percentages for various motility parameters at 5 min and 30 min when exposed in HTF, as compared to the LM subpopulations. After exposure in CAP and DOPA - various LM motility and kinematics parameters could be enhanced to the point that no significant difference between subpopulations could further be seen. Compared to HTF for the LM subpopulation, 20 nM and 1 µM DOPA significantly enhanced progressive motility (30 min); CAP and all DOPA concentrations enhanced rapid speed (5 min, 30 min); and 1 µM DOPA enhanced MP speed (30 min). Compared to HTF, in the HM subpopulation DOPA significantly increased rapid speed groups (5 min), however decreased non-progressive motility (5 min); medium- (5 min) and slow speed groups (5 min). In addition, CAP and DOPA further increased RP speed groups at 30 min. At 30 min, 20 nM DOPA increased motile STR as compared to HTF - whereas at 5 min both 20 nM and 100 nM DOPA decreased motile LIN as compared to HTF. All DOPA concentrations decreased MP STR and MP LIN as compared to HTF and CAP at 5 min for HM subpopulations - whereas for LM subpopulations 1 µM DOPA decreased MP LIN. All DOPA concentrations decreased RP STR and RP LIN as compared to HTF at 5 min for HM subpopulations. At 5 min for HM subpopulations CAP increased motile VCL as compared to HTF, whereas DOPA concentrations increased values as compared to both HTF and CAP. At 30 min CAP, 20 nM and 100 nM DOPA increased values as compared to HTF. In LM subpopulations at 5 min, CAP and 20 nM DOPA increased motile VCL as compared to HTF - whereas 100 nM and 1 µM DOPA increased values as compared to HTF and CAP. At 30 min CAP, 20 nM and 100 nM DOPA increased values as compared to HTF, however 1 µM DOPA increased values as compared to HTF and CAP. At 5 min for HM subpopulations, 100 nM and 1 µM DOPA increased motile VAP as compared to HTF - where at 30 min all media increased values as compared to HTF. For LM subpopulations at 5 min, CAP, 20 nM and 1 µM DOPA increased motile VAP as compared to HTF - whereas 100 nM DOPA increased values as compared to HTF and CAP. At 30 min, all media increased motile VAP as compared to HTF. For motile VSL at 30 min in HM subpopulations, all media increased values as compared to HTF. For LM subpopulations at 5 min, 20 nM and 100 nM DOPA increased values as compared to HTF - and at 30 min all media increased values as compared to HTF. At 5 min for LM subpopulations, 100 nM DOPA increased NP VSL as compared to HTF. For HM subpopulations at 5 min, DOPA increased MP VCL as compared to HTF and CAP - whereas for LM subpopulations 100 nM DOPA increased values as compared to HTF and CAP; and 1 µM DOPA as compared to HTF, CAP and 20 nM DOPA. At 30 min for LM subpopulations, 1 µM DOPA increased MP VCL as compared to HTF. At 30 min, 1 µM DOPA increased MP VAP in HM subpopulations as compared to HTF and 100 nM DOPA, for LM subpopulations as compared to HTF. At 30 min for the LM subpopulation 20 nM DOPA increased MP VSL as compared to HTF. All media increased RP VCL at 5 min in HM subpopulations, however 100 nM and 1 µM DOPA increased values as compared to both HTF and CAP. In the LM subpopulation only 100 nM DOPA increased values as compared HTF and 20 nM DOPA. At 30 min, all DOPA concentrations increased RP VCL as compared to HTF. For RP VAP and RP VSL at 30 min for LM subpopulations all media increased values as compared to HTF. All DOPA concentrations decreased motile WOB in the HM subpopulation as compared to HTF. At 5 min for HM subpopulations, DOPA increased motile ALH as compared to HTF and CAP. In LM subpopulations at 5 min and 30 min, all media increased motile ALH as compared to HTF. For NP WOB at 5 min for HM subpopulations, 1 µM DOPA decreased values as compared to HTF and 20 nM DOPA. At 5 min, DOPA decreased MP WOB as compared to HTF in HM subpopulations, and in LM subpopulations 100 nM DOPA decreased values as compared to HTF, CAP and 20 nM DOPA. For ALH of HM subpopulations, DOPA increased MP ALH as compared to HTF and CAP, whereas in LM subpopulations 1 µM DOPA increased MP ALH as compared to HTF. DOPA further decreased MP BCF in HM subpopulations as compared to HTF and CAP, but in LM subpopulations 1 µM DOPA decreased MP BCF as compared to HTF. DOPA decreased RP WOB as compared to HTF and all media increased RP ALH as compared to HTF. In LM subpopulations 100 nM and 1 µM DOPA increased RP ALH as compared to HTF. **Abbreviations:** ALH, average path velocity; BCF, beat cross frequency; CI, confidence interval; CAP, capacitating-HTF; DOPA; dopamine; HTF, human tubal fluid; HM, high motile subpopulation; LIN, linearity; LM, low motile subpopulation; MP, medium-progressive; min, minutes; NP, non-progressive; Prog, progressive; RP, rapid-progressive; SD, standard deviation; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble. [a, b,c,d] Values labelled with different superscript letters in the same row were significantly different between the various media for individual subpopulations and SCA parameters (one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions). Values labelled in bold with an asterisk in the same column were significantly different between the HM and LM subpopulations for individual media and SCA parameters (*p <0.05, **p <0.01 and ***p <0.001) (Student's t-test or the Mann-Whitney test when normal distribution was void). Values labelled in bold and red with an asterisk in the same column were significantly different between the 5 and 30 minutes for individual subpopulations and media (*p <0.05, **p <0.01 and ***p <0.001) (Student's t-test or the Mann-Whitney test when normal distribution was void).

Supplementary Table 4. Comparison of mean SCA motility and kinematic parameters at 5, 30 and 60 minutes for both the low motile (LM) and high motile (HM) sperm subpopulations after treatment with HTF, CAP, 50 ng/mL, 100 ng/mL, 250 ng/mL, and 500 ng/mL prolactin (mean ± SD) (n = 20).

		HTF			CAP			50 ng/mL PRL			100 ng/mL PRL			250 ng/mL PRL			500 ng/mL PRL			
		Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	Mean ± SD	SP *p*	TP *p*	ANOVA
Prog (%)																				
5 min	HM	**33.3 ± 21.1*****	**<0.001**	0.184	**42.2 ± 21.5*****	**<0.001**	0.515	**39.5 ± 20.3****	**0.002**	0.625	**36.9 ± 19.7***	**0.011**	0.710	**38.2 ± 19.1****	**0.001**	0.801	**36.3 ± 21.4*****	**<0.001**	0.524	0.757
5 min	LM	**10.0 ± 9.8**$^{***\,a}$		0.542	**15.0 ± 11.8**$^{***\,ab}$		0.641	**22.7 ± 14.1**$^{**\,b}$		0.779	**23.4 ± 15.3**$^{*\,b}$		0.435	**20.9 ± 15.4**$^{**\,b}$		0.752	**17.9 ± 13.6**$^{***\,ab}$		0.711	**0.005**
30 min	HM	**27.7 ± 19.5*****	**<0.001**		**37.0 ± 22.2****	**0.008**		**37.5 ± 19.1****	**0.003**		32.6 ± 19.3	0.241		34.6 ± 20.6	0.054		**34.5 ± 23.2****	**0.002**		0.636
30 min	LM	**9.4 ± 9.7**$^{***\,a}$			**19.0 ± 17.4**$^{**\,ab}$			**21.5 ± 19.5**$^{**\,ab}$			25.9 ± 19.9^{b}			23.3 ± 20.4^{ab}			**16.3 ± 13.9**$^{**\,ab}$			**0.024**
60 min	HM	**22.1 ± 17.7****	**0.002**		**34.8 ± 21.7****	**0.004**		**34.0 ± 15.0****	**0.006**		**33.3 ± 17.0****	**0.009**		**35.9 ± 15.6****	**0.003**		**29.1 ± 18.0***	**0.011**		0.155
60 min	LM	**7.0 ± 8.2**$^{**\,a}$			**16.8 ± 13.1**$^{**\,ab}$			**19.2 ± 13.2**$^{**\,ab}$			**19.2 ± 14.6**$^{**\,b}$			**19.3 ± 16.6**$^{**\,ab}$			**14.4 ± 14.7**$^{*\,ab}$			**0.044**
NP (%)																				
5 min	HM	**28.2 ± 19.1***	**0.048**	0.793	26.5 ± 20.9	0.805	0.602	27.9 ± 16.1	0.084	0.950	29.1 ± 19.2	0.146	0.716	30.6 ± 21.1	0.108	0.869	29.0 ± 19.6	0.087	0.885	0.988
5 min	LM	**18.8 ± 15.2***		0.481	24.1 ± 17.7		0.461	20.0 ± 14.7		0.619	21.1 ± 15.4		0.551	19.8 ± 12.3		0.801	18.3 ± 13.6		0.617	0.807
30 min	HM	**28.3 ± 16.0****	**0.007**		30.1 ± 18.2	0.053		**28.5 ± 19.7***	**0.026**		**33.7 ± 23.6***	**0.014**		28.4 ± 23.2	0.149		32.2 ± 27.0	0.327		0.950
30 min	LM	**16.8 ± 13.3****			19.9 ± 12.5			**16.9 ± 14.5***			**17.4 ± 13.1***			17.5 ± 13.7			21.8 ± 15.7			0.766
60 min	HM	**25.1 ± 15.6***	**0.014**		**32.1 ± 15.2****	**0.004**		29.7 ± 20.5	0.300		**33.0 ± 19.0****	**0.003**		27.3 ± 17.3	0.062		**30.1 ± 21.3***	**0.049**		0.780
60 min	LM	**13.7 ± 11.7***			**18.6 ± 14.4****			20.8 ± 13.3			**17.3 ± 10.1****			17.9 ± 12.6			**18.5 ± 10.9***			0.631
Motile (%)																				
5 min	HM	**61.5 ± 23.5*****	**<0.001**	0.128	**68.7 ± 22.0*****	**<0.001**	0.952	**67.4 ± 21.1*****	**<0.001**	0.842	**65.9 ± 22.4****	**0.001**	0.998	**68.7 ± 21.6*****	**<0.001**	0.613	**65.2 ± 22.2*****	**<0.001**	0.655	0.873
5 min	LM	**28.8 ± 19.5*****		0.364	**39.1 ± 19.8*****		0.822	**42.7 ± 19.4*****		0.738	**44.5 ± 20.6****		0.370	**40.8 ± 20.2*****		0.783	**36.2 ± 15.7*****		0.541	0.077
30 min	HM	**56.0 ± 23.7*****	**<0.001**		**67.1 ± 21.9*****	**<0.001**		**66.0 ± 22.4*****	**<0.001**		**66.3 ± 20.9*****	**<0.001**		**63.0 ± 24.6****	**0.002**		**66.8 ± 23.8*****	**<0.001**		0.544
30 min	LM	**26.2 ± 18.8*****			**38.1 ± 20.8*****			**38.3 ± 21.1*****			**43.3 ± 21.5*****			**40.9 ± 20.5****			**38.1 ± 15.6*****			0.072
60 min	HM	**47.2 ± 20.1**$^{***\,a}$	**<0.001**		**66.9 ± 20.3**$^{***\,b}$	**<0.001**		**63.8 ± 16.6**$^{***\,b}$	**<0.001**		**66.3 ± 18.9**$^{***\,b}$	**<0.001**		**63.2 ± 19.4**$^{***\,b}$	**<0.001**		**59.2 ± 20.7**$^{***\,ab}$	**<0.001**		**0.024**
60 min	LM	**20.7 ± 17.3**$^{***\,a}$			**35.4 ± 18.6**$^{***\,b}$			**40.0 ± 16.7**$^{***\,b}$			**36.5 ± 15.2**$^{***\,b}$			**37.2 ± 16.3**$^{***\,b}$			**32.8 ± 15.2**$^{***\,b}$			**0.010**
Rapid (%)																				
5 min	HM	**15.9 ± 18.0***	**0.025**	0.570	21.1 ± 24.2	0.152	0.943	20.4 ± 21.2	0.060	0.725	16.9 ± 20.5	0.485	0.863	**21.0 ± 23.9***	**0.020**	0.752	19.5 ± 26.0	0.416	0.769	0.946
5 min	LM	**4.7 ± 6.2***		0.804	7.4 ± 8.5		0.870	10.0 ± 12.9		0.935	11.5 ± 15.0		0.513	**9.0 ± 12.8***		0.718	8.8 ± 10.1		0.990	0.694
30 min	HM	**13.5 ± 15.6***	**0.029**		19.8 ± 25.9	0.172		21.8 ± 21.9	0.118		17.3 ± 21.7	0.707		20.4 ± 20.1	0.090		20.3 ± 23.6	0.159		0.816
30 min	LM	**4.4 ± 6.9***			9.0 ± 13.4			11.4 ± 15.7			13.4 ± 16.9			12.1 ± 16.9			8.8 ± 9.8			0.390
60 min	HM	10.8 ± 12.2	0.063		18.6 ± 22.6	0.104		16.7 ± 19.1	0.403		14.1 ± 18.1	0.425		16.4 ± 18.6	0.166		15.3 ± 20.2	0.217		0.856
60 min	LM	3.5 ± 4.4			7.6 ± 11.0			10.5 ± 13.0			8.2 ± 10.8			9.6 ± 11.1			9.2 ± 13.1			0.626

Medium (%)																				
5 min	HM	**22.9 ± 16.5*****	**<0.001**	0.279	**27.1 ± 20.2****	**0.005**	0.850	26.4 ± 19.0	0.064	0.798	27.2 ± 20.5	0.065	0.546	24.2 ± 17.8	0.108	0.651	**23.5 ± 17.1***	**0.018**	0.745	0.937
	LM	**8.0 ± 9.7*****		0.574	**11.3 ± 11.5****		0.784	16.4 ± 15.4		0.780	15.8 ± 13.9		0.865	15.4 ± 14.5		0.704	**12.2 ± 11.3***		0.267	0.177
30 min	HM	**19.8 ± 13.3*****	**<0.001**		24.0 ± 19.8	0.072		22.9 ± 19.0	0.110		21.5 ± 16.7	0.194		23.0 ± 19.1	0.170		**20.3 ± 15.5***	**0.041**		0.998
	LM	**6.7 ± 8.0*****			13.5 ± 14.0			14.3 ± 12.8			15.7 ± 14.5			15.8 ± 15.4			**12.5 ± 9.4***			0.121
60 min	HM	**15.6 ± 13.8*****	**<0.001**		**25.1 ± 15.6****	**0.008**		**23.6 ± 16.8***	**0.038**		**26.1 ± 19.1***	**0.017**		**28.3 ± 20.9****	**0.007**		**20.6 ± 13.5****	**0.001**		0.239
	LM	**5.2 ± 8.6**$^{***\,a}$			**13.3 ± 9.5**$^{**\,b}$			**13.8 ± 10.8**$^{*\,b}$			**13.6 ± 15.6**$^{*\,ab}$			**12.4 ± 12.1**$^{**\,b}$			**8.0 ± 8.1**$^{**\,ab}$			**0.05**
Slow (%)																				
5 min	HM	22.7 ± 16.7	0.083	0.896	20.5 ± 17.0	0.926	0.776	20.7 ± 13.5	0.298	0.857	21.9 ± 15.4	0.224	0.559	23.5 ± 16.9	0.080	0.572	22.2 ± 15.1	0.076	0.761	0.985
	LM	16.1 ± 13.2		0.515	20.4 ± 16.0		0.311	16.3 ± 12.2		0.618	17.2 ± 13.5		0.617	16.4 ± 9.7		0.515	15.2 ± 11.3		0.875	0.792
30 min	HM	**22.7 ± 13.8***	**0.047**		23.2 ± 15.0	0.056		21.3 ± 17.9	0.083		**27.5 ± 21.5***	**0.019**		19.6 ± 18.4	0.415		26.2 ± 23.0	0.261		0.676
	LM	**15.2 ± 12.3***			15.6 ± 11.8			13.0 ± 11.5			**14.1 ± 10.4***			12.9 ± 10.2			16.8 ± 12.6			0.827
60 min	HM	**20.8 ± 13.7***	**0.037**		**23.2 ± 12.4***	**0.029**		23.4 ± 17.9	0.108		**26.0 ± 18.1***	**0.019**		18.5 ± 13.2	0.422		23.3 ± 18.7	0.405		0.772
	LM	**12.0 ± 9.3***			**14.4 ± 12.4***			15.8 ± 13.2			**14.6 ± 9.0***			15.2 ± 11.6			15.6 ± 9.2			0.912
RP (%)																				
5 min	HM	**9.8 ± 10.4****	**0.004**	0.626	11.5 ± 14.8	0.133	0.999	**11.8 ± 12.3***	**0.033**	0.962	9.3 ± 11.2	0.144	0.911	**11.1 ± 11.6***	**0.027**	0.870	10.3 ± 12.8	0.169	0.869	0.979
	LM	**2.2 ± 3.7****		0.994	4.3 ± 6.0		0.861	**4.3 ± 5.0***		0.370	4.8 ± 7.1		0.467	**5.6 ± 9.3***		0.788	4.5 ± 5.9		0.845	0.775
30 min	HM	7.8 ± 9.4	0.065		11.6 ± 14.4	0.142		12.8 ± 13.5	0.155		10.5 ± 12.8	0.369		12.6 ± 13.5	0.139		**12.1 ± 13.9***	**0.023**		0.782
	LM	2.1 ± 3.2			5.4 ± 8.5			7.3 ± 11.6			7.6 ± 10.7			7.5 ± 11.2			**4.1 ± 6.2***			0.596
60 min	HM	7.2 ± 8.2	0.115		11.6 ± 14.5	0.335		11.9 ± 13.9	0.606		9.1 ± 10.2	0.091		**10.9 ± 10.3***	**0.049**		10.4 ± 12.6	0.118		0.874
	LM	2.1 ± 2.9			5.4 ± 7.3			7.8 ± 9.2			4.8 ± 7.5			**6.1 ± 8.2***			5.4 ± 10.6			0.489
MP (%)																				
5 min	HM	**23.5 ± 15.3*****	**<0.001**	0.128	**30.7 ± 14.6*****	**<0.001**	0.192	**27.7 ± 13.8***	**0.019**	0.312	**27.5 ± 14.5***	**0.021**	0.381	**25.7 ± 11.7****	**0.004**	0.521	**25.9 ± 12.0*****	**<0.001**	0.138	0.577
	LM	**7.8 ± 9.2**$^{***\,a}$		0.447	**10.7 ± 10.3**$^{***\,ab}$		0.617	**18.4 ± 12.8**$^{*\,b}$		0.253	**18.6 ± 12.2**$^{*\,b}$		0.539	**15.3 ± 11.7**$^{**\,ab}$		0.766	**13.4 ± 12.2**$^{***\,ab}$		0.425	**0.007**
30 min	HM	**19.9 ± 12.2*****	**<0.001**		**25.4 ± 14.4****	**0.005**		**24.3 ± 11.2****	**0.003**		22.2 ± 12.2	0.330		22.0 ± 12.0	0.101		**22.4 ± 13.2****	**0.008**		0.748
	LM	**7.3 ± 7.8*****			**13.6 ± 12.7****			**14.2 ± 12.1****			18.3 ± 14.8			15.9 ± 13.3			**12.2 ± 10.9****			0.057
60 min	HM	**14.9 ± 12.8****	**0.002**		**23.2 ± 12.6****	**0.001**		**22.1 ± 11.0****	**0.002**		**24.2 ± 13.4***	**0.025**		**25.0 ± 11.3****	**0.003**		**18.7 ± 8.3****	**0.004**		0.075
	LM	**4.9 ± 5.9****			**11.4 ± 7.5****			**11.4 ± 7.9****			**14.3 ± 13.8***			**13.2 ± 11.2****			**8.9 ± 10.6****			0.055
Motile VCL (µm/s)																				
5 min	HM	111.8 ± 17.3	0.086	0.825	124.5 ± 22.1	0.144	0.998	125.2 ± 20.5	0.533	0.182	122.6 ± 22.0	0.920	0.899	**125.9 ± 22.2***	**0.026**	**0.638**	126.1 ± 26.0	0.196	0.674	0.189
	LM	102.4 ± 19.6		0.243	113.7 ± 28.0		0.923	121.0 ± 26.3		0.607	121.9 ± 26.7		0.885	**111.4 ± 21.4***		0.546	116.7 ± 23.6		0.974	0.074
30 min	HM	**108.8 ± 17.8**$^{**\,a}$	**0.007**		124.8 ± 23.7^{b}	0.148		**135.6 ± 20.2**$^{**\,b}$	**0.002**		121.3 ± 20.1^{b}	0.812		**132.0 ± 22.7**$^{*\,b}$	**0.040**		127.2 ± 22.4^{b}	0.164		**0.001**
	LM	**92.8 ± 20.3**$^{**\,a}$			114.9 ± 21.6^{b}			**118.1 ± 16.9**$^{**\,b}$			119.6 ± 30.5^{b}			**118.6 ± 21.1**$^{*\,b}$			116.5 ± 29.5^{b}			**0.001**
60 min	HM	**111.2 ± 17.9**$^{*\,a}$	**0.017**		125.0 ± 20.4^{ab}	0.052		130.7 ± 15.7^{b}	0.429		124.2 ± 17.9^{ab}	0.440		130.7 ± 25.6^{b}	0.054		121.1 ± 20.5^{ab}	0.476		**0.034**
	LM	**97.7 ± 17.5***			111.9 ± 22.3			125.2 ± 25.7			117.3 ± 34.2			113.8 ± 26.7			114.8 ± 31.9			0.092
Motile VAP (µm/s)																				
5 min	HM	57.6 ± 9.0	0.067	0.661	60.1 ± 10.4	0.343	0.855	61.2 ± 9.6	0.217	0.535	59.2 ± 9.9	0.691	0.791	**60.0 ± 8.6***	**0.017**	0.370	59.5 ± 10.2	0.167	0.991	0.872
	LM	52.8 ± 8.7		0.199	55.3 ± 10.6		0.798	58.0 ± 7.8		0.280	58.1 ± 10.0		0.863	**54.2 ± 7.8***		0.163	56.4 ± 9.3		0.856	0.275
30 min	HM	**54.9 ± 11.1**$^{*\,a}$	**0.026**		58.6 ± 10.0^{ab}	0.563		63.3 ± 8.1^{b}	0.073		58.0 ± 8.1^{ab}	0.535		63.6 ± 9.8^{b}	0.103		59.8 ± 8.5^{ab}	0.055		**0.014**
	LM	**48.2 ± 8.3**$^{*\,a}$			57.1 ± 8.2^{b}			59.3 ± 6.8^{b}			60.0 ± 13.4^{b}			59.2 ± 8.2^{b}			55.0 ± 11.6^{b}			**<0.001**
60 min	HM	57.0 ± 12.1	0.118		60.3 ± 11.8	0.362		64.0 ± 8.8	0.502		59.9 ± 10.0	0.741		63.7 ± 11.5	0.148		59.4 ± 10.4	0.213		0.329
	LM	51.3 ± 9.3			56.9 ± 10.7			62.9 ± 14.9			58.5 ± 14.7			58.6 ± 12.8			54.5 ± 13.3			0.140

Motile VSL (µm/s)																			
5 min	**HM**	45.1 ± 11.9	0.118	0.498	47.3 ± 11.0	0.248	0.690	48.1 ± 10.2	0.094	0.681	46.0 ± 9.0	0.223	0.690	**46.7±6.7***	**0.032**	0.542	45.7 ± 9.7	0.171	0.970	0.908
	LM	40.0 ± 10.3		0.458	42.2 ± 10.3		0.690	43.3 ± 9.3		0.270	43.1 ± 7.1		0.416	**41.8±8.3***		0.336	41.5 ± 11.2		0.547	0.854
30 min	**HM**	40.9 ± 12.9	0.172		44.5 ± 9.6	0.267		47.9 ± 8.2	0.368		44.4 ± 8.4	0.567		48.2±11.6	0.494		**45.5 ± 8.9***	**0.013**		0.142
	LM	36.1 ± 10.0[a]			42.6 ± 10.4[ab]			45.6 ± 9.4[b]			46.3 ± 13.8[b]			46.0±10.9[b]			**38.5 ± 9.9*** [ab]**			**0.004**
60 min	**HM**	43.5 ± 11.7	0.166		45.8 ± 11.9	0.682		50.3 ± 9.9	0.431		46.7 ± 10.5	0.965		50.3±11.1	0.338		**46.2 ± 11.1***	**0.036**		0.359
	LM	37.8 ± 11.0			44.3 ± 10.3			49.1 ± 15.9			43.1 ± 14.4			46.3±14.3			**38.8 ± 10.0***			0.073
Motile STR (%)																			
5 min	**HM**	75.3 ± 10.9	0.343	0.330	77.2 ± 7.9	0.147	0.180	76.8 ± 7.0	0.082	0.340	75.9 ± 6.9	0.126	0.543	76.2±6.4	0.306	0.524	74.6 ± 7.8	0.523	0.807	0.887
	LM	73.2 ± 11.9		0.597	73.6 ± 8.7		0.642	72.5 ± 9.7		0.632	72.8 ± 6.8		0.476	74.0±8.2		0.761	71.9 ± 11.9		0.500	0.979
30 min	**HM**	70.8 ± 11.0	0.921		73.7 ± 7.2	0.307		73.8 ± 7.5	0.370		73.7 ± 7.1	0.974		74.0±9.4	0.387		73.3 ± 6.8	0.070		0.784
	LM	70.4 ± 13.2			71.7 ± 9.9			74.8 ± 9.8			73.8 ± 9.8			75.8±10.1			68.4 ± 11.2			0.150
60 min	**HM**	73.0 ± 8.0	0.166		73.2 ± 8.2	0.770		75.8 ± 6.9	0.678		74.5 ± 6.2	0.405		76.2±6.2	0.917		74.3 ± 6.9	0.088		0.668
	LM	69.5 ± 11.2			74.1 ± 7.3			74.8 ± 8.3			70.4 ± 10.6			75.9±11.1			68.9 ± 10.0			0.132
Motile LIN (%)																			
5 min	**HM**	42.4 ± 11.2	0.769	0.446	41.4 ± 10.6	0.574	0.357	40.9 ± 10.4	0.550	0.632	40.0 ± 7.4	0.363	0.558	40.1±7.1	0.724	0.758	38.8 ± 7.4	0.613	0.711	0.820
	LM	41.4 ± 11.0		0.551	39.8 ± 8.3		0.892	38.2 ± 7.7		0.522	37.9 ± 8.2		0.533	39.4±6.5		0.423	37.6 ± 8.9		0.568	0.636
30 min	**HM**	38.7 ± 11.0	0.475		37.2 ± 5.7	0.434		37.9 ± 8.6	0.265		38.2 ± 7.6	0.433		38.5±8.5	0.269		37.5 ± 6.7	0.405		0.987
	LM	39.2 ± 9.8			39.0 ± 9.5			40.6 ± 8.0			40.4 ± 10.0			41.4±9.2			35.6 ± 8.6			0.321
60 min	**HM**	39.6 ± 7.2	0.328		38.2 ± 7.9	0.420		39.9 ± 7.7	0.943		37.9 ± 5.9	0.946		39.7±6.8	0.274		39.0 ± 6.1	0.060		0.927
	LM	38.3 ± 7.1			40.2 ± 7.2			40.1 ± 7.2			37.8 ± 8.2			42.6±8.9			35.2 ± 6.2			0.060
Motile WOB (%)																			
5 min	**HM**	54.1 ± 8.4	0.885	0.704	51.3 ± 9.1	0.699	0.636	51.2 ± 8.9	0.665	0.649	51.1 ± 6.4	0.906	0.648	50.6±6.0	0.733	1	49.9 ± 5.9	0.636	0.655	0.488
	LM	54.5 ± 7.8		0.878	52.3 ± 7.4		0.889	51.2 ± 6.5		0.631	50.8 ± 7.2		0.555	51.2±5.5		0.247	50.7 ± 5.7		0.830	0.365
30 min	**HM**	52.4 ± 7.9	0.574		**49.3 ± 6.0***	**0.048**		49.3 ± 7.3	0.077		49.9 ± 6.6	0.228		50.6±6.7	0.228		49.4 ± 5.6	0.459		0.599
	LM	53.6 ± 7.0			**53.3 ± 7.4***			52.9 ± 6.2			53.0 ± 7.5			52.9±6.4			50.7 ± 6.4			0.731
60 min	**HM**	52.7 ± 6.0	0.704		50.3 ± 7.0	0.254		51.0 ± 6.3	0.678		49.4 ± 5.2	0.226		50.6±6.1	0.083		50.9 ± 5.2	0.466		0.661
	LM	53.5 ± 4.9			53.0 ± 7.4			51.8 ± 5.5			52.3 ± 6.3			54.4±7.2			49.7 ± 5.2			0.263
Motile ALH (µm)																			
5 min	**HM**	3.1 ± 0.6	0.883	0.834	3.4 ± 0.8	0.495	0.796	3.4 ± 0.8	0.514	0.104	3.4 ± 0.7	0.539	0.619	3.5±0.8	0.216	0.424	3.5 ± 0.8	0.596	0.676	0.335
	LM	3.0 ± 0.7		0.152	3.3 ± 0.8		0.752	3.6 ± 0.9		0.573	3.5 ± 0.8		0.877	3.2±0.6		0.499	3.4 ± 0.7		0.973	0.131
30 min	**HM**	**3.1 ± 0.5*** [a]**	**0.027**		3.6 ± 0.7[ab]	0.236		**3.8 ± 0.7*** [b]**	**0.023**		3.5 ± 0.6[b]	0.639		3.7±0.7[b]	0.129		3.7 ± 0.7[b]	0.382		**0.005**
	LM	**2.7 ± 0.6*** [a]**			3.4 ± 0.7[b]			**3.4 ± 0.6*** [b]**			3.4 ± 0.9[b]			3.4±0.7[b]			3.5 ± 0.9[b]			**0.006**
60 min	**HM**	3.2 ± 0.5	0.054		3.5 ± 0.6	0.088		3.7 ± 0.5	0.615		3.6 ± 0.5	0.707		3.7±0.7	0.164		3.5 ± 0.5	0.963		0.057
	LM	2.9 ± 0.3[a]			3.2 ± 0.6[ab]			3.6 ± 0.6[b]			3.5 ± 1.0[b]			3.3±0.7[b]			3.5 ± 0.8[b]			**0.029**
Motile BCF (Hz)																			
5 min	**HM**	**22.8 ± 4.3***	**0.01**	0.144	**23.1 ± 3.6***	**0.028**	0.090	**22.9 ± 3.6****	**0.002**	0.075	**22.5 ± 2.5**** *a*	**0.002**	**0.053**	**22.9±3.3***	**0.026**	0.124	**21.8 ± 2.8***	**0.028**	0.408	0.799
	LM	**20.2 ± 3.9***		0.625	**20.9 ± 2.4***		0.588	**19.7 ± 2.7****		0.311	**19.7 ± 3.2****		0.405	**20.9±2.8***		0.595	**19.9 ± 2.8***		0.074	0.577
30 min	**HM**	20.7 ± 4.0	0.240		21.1 ± 2.6	0.341		20.9 ± 2.9	0.773		**20.9 ± 2.8*** *ab***	0.268		21.1±3.2	0.331		**20.9 ± 2.5****	**0.003**		0.998
	LM	19.7 ± 3.9			20.2 ± 3.7			20.8 ± 3.3			19.9 ± 3.0			20.2±3.1			**18.1 ± 3.4****			0.129
60 min	**HM**	20.7 ± 3.7	0.088		21.4 ± 3.2	0.469		**21.3 ± 2.7***	**0.024**		**20.9 ± 2.0*** *b***	**0.011**		**21.8±2.5***	**0.049**		**21.0 ± 1.9*****	**<0.001**		0.838
	LM	18.9 ± 4.0			20.7 ± 3.4			**19.6 ± 2.8***			**18.7 ± 3.0***			**20.0±2.8***			**17.7 ± 3.1*****			0.075

NP VCL (µm/s)																			
5 min	**HM**	85.7 ± 22.4	0.174	0.850	96.3 ± 33.2	0.564	0.679	94.8 ± 34.2	0.524	0.874	91.6 ± 31.9	0.606	0.638	95.3±29.8	0.322	0.658	96.5 ± 35.9	0.303	0.749 0.822
	LM	78.8 ± 23.5		0.674	91.0 ± 31.6		0.892	89.9 ± 36.8		0.545	87.6 ± 33.6		0.980	86.8±26.4		0.809	86.5 ± 30.9		0.917 0.801
30 min	**HM**	85.8 ± 25.1	0.066		95.5 ± 32.5	0.475		99.3 ± 41.3	0.742		91.7 ± 29.3	0.431		98.2±33.2	0.392		89.8 ± 27.0	0.949	0.778
	LM	73.2 ± 19.9			87.1 ± 29.8			91.2 ± 34.2			85.8 ± 36.1			89.8±27.4			90.3 ± 31.0		0.415
60 min	**HM**	89.3 ± 20.4	0.054		103.5 ± 28.3	0.087		94.4 ± 28.5	0.502		99.3 ± 27.2	0.184		102.7±32.6	0.188		96.7 ± 26.5	0.164	0.608
	LM	75.6 ± 20.7			88.3 ± 23.8			101.2 ± 32.2			86.0 ± 33.1			89.4±27.3			84.5 ± 26.5		0.174
NP VAP (µm/s)																			
5 min	**HM**	41.1 ± 11.3	0.773	0.858	42.7 ± 13.6	0.790	0.800	42.8 ± 13.8	0.629	0.933	42.1 ± 14.4	0.660	0.694	43.7±11.9	0.242	0.911	41.8 ± 13.9	0.711	0.795 0.988
	LM	40.6 ± 10.5		0.613	43.6 ± 10.8		0.870	41.3 ± 13.5		0.521	40.4 ± 12.3		0.949	39.8±11.0		0.469	41.3 ± 12.3		0.621 0.909
30 min	**HM**	40.3 ± 12.2	0.419		42.0 ± 13.7	0.967		41.7 ± 15.7	0.853		40.2 ± 14.0	0.882		43.8±14.0	0.645		40.8 ± 12.1	0.832	0.954
	LM	37.4 ± 11.8			42.2 ± 11.0			44.0 ± 11.0			40.4 ± 13.7			43.0±10.2			41.5 ± 10.0		0.448
60 min	**HM**	42.3 ± 11.5	0.274		44.8 ± 13.7	0.197		43.2 ± 13.9	0.574		44.0 ± 14.1	0.287		45.3±14.3	0.504		43.5 ± 12.5	0.215	0.986
	LM	38.7 ± 11.0			41.9 ± 13.0			45.7 ± 11.9			39.2 ± 14.4			43.8±13.0			38.2 ± 14.3		0.419
NP VSL (µm/s)																			
5 min	**HM**	27.6 ± 8.8	0.759	0.707	29.3 ± 8.5	0.949	0.632	29.6 ± 9.0	0.344	0.636	28.1 ± 9.3	0.232	0.715	30.5±8.0	0.143	0.767	27.7 ± 9.1	0.951	0.737 0.827
	LM	28.5 ± 10.3		0.538	29.1 ± 10.2		0.632	27.1 ± 9.4		0.499	25.7 ± 9.2		0.788	26.9±8.9		0.509	27.2 ± 10.0		0.865 0.862
30 min	**HM**	25.9 ± 9.5	0.848		26.8 ± 9.7	0.726		27.0 ± 10.8	0.503		26.8 ± 10.4	0.952		28.7±9.7	0.995		26.2 ± 9.4	0.718	0.952
	LM	25.2 ± 11.8			27.8 ± 8.9			30.1 ± 8.2			27.0 ± 11.0			28.7±11.0			26.6 ± 6.8		0.720
60 min	**HM**	27.9 ± 8.3	0.438		29.0 ± 10.0	0.339		28.9 ± 10.0	0.964		29.4 ± 10.7	0.198		30.3±9.8	0.988		28.3 ± 9.3	0.287	0.984
	LM	25.6 ± 10.1			26.3 ± 9.2			29.5 ± 8.7			24.8 ± 10.7			30.9±13.6			24.7 ± 11.0		0.373
NP STR (%)																			
5 min	**HM**	66.0 ± 8.8	0.578	0.558	**68.9 ± 9.0**[a]	0.392	**0.050**	68.8 ± 8.2	0.286	0.095	66.0 ± 7.8	0.259	0.557	69.5±9.0	0.097	0.145	66.2 ± 8.3	0.736	0.357 0.474
	LM	67.9 ± 13.7		0.348	66.0 ± 13.8		0.379	64.3 ± 10.2		0.408	62.5 ± 12.8		0.781	64.6±11.0		0.827	65.2 ± 12.4		0.457 0.769
30 min	**HM**	63.3 ± 8.4	0.517		**63.4 ± 7.8**[ab]	0.686		63.0 ± 10.2	0.163		64.1 ± 8.0	0.969		65.4±8.1	0.775		62.2 ± 13.5	0.915	0.912
	LM	61.4 ± 19.5			64.6 ± 12.3			67.8 ± 12.9			64.0 ± 10.1			65.1±15.6			63.3 ± 8.5		0.849
60 min	**HM**	65.1 ± 8.7	0.565		**63.5 ± 8.6**[b]	0.339		65.6 ± 8.3	0.489		63.4 ± 8.1	0.548		65.3±7.2	0.631		63.0 ± 7.1	0.512	0.872
	LM	63.0 ± 12.7			60.5 ± 12.2			63.3 ± 11.1			61.7 ± 9.5			67.2±15.3			61.0 ± 11.0		0.602
NP LIN (%)																			
5 min	**HM**	36.2 ± 13.5	0.209	0.281	33.9 ± 7.1	0.657	0.061	34.0 ± 8.8	0.775	0.219	32.8 ± 7.8	1	0.408	35.8±9.4	0.592	0.127	31.8 ± 5.8	0.357	0.877 0.508
	LM	38.5 ± 12.1		0.296	35.2 ± 12.2		0.459	33.6 ± 7.9		0.244	32.0 ± 11.4		0.701	32.8±8.4		0.587	33.9 ± 9.6		0.257 0.340
30 min	**HM**	31.9 ± 7.7	0.602		30.1 ± 5.3	0.093		**29.6 ± 9.7**[*]	**0.025**		30.2 ± 8.5	0.324		32.0±8.9	0.173		30.9 ± 10.4	0.629	0.911
	LM	33.6 ± 13.4			35.4 ± 13.8			**36.3 ± 10.1**[*]			32.8 ± 8.9			34.8±11.6			32.2 ± 7.6		0.795
60 min	**HM**	32.5 ± 6.5	0.641		29.9 ± 6.4	0.675		33.3 ± 8.6	0.539		29.9 ± 7.2	0.942		30.8±7.0	0.106		30.7 ± 5.9	0.608	0.579
	LM	33.7 ± 8.9			31.1 ± 9.4			31.5 ± 8.8			30.4 ± 6.4			36.2±12.5			29.3 ± 10.0		0.270
NP WOB (%)																			
5 min	**HM**	50.5 ± 7.4	0.116	0.695	47.3 ± 8.3	0.111	0.465	47.6 ± 8.3	0.333	0.312	47.8 ± 8.0	0.496	0.551	49.3±7.5	0.951	0.343	46.4 ± 6.2	0.060	0.895 0.477
	LM	54.3 ± 8.8		0.463	51.8 ± 10.8		0.615	49.9 ± 7.3		0.279	49.7 ± 10.2		0.712	48.1±8.8		0.389	50.3 ± 7.9		0.269 0.262
30 min	**HM**	49.1 ± 7.2	0.366		**46.5 ± 7.7**[*]	**0.033**		**44.8 ± 9.4**[**]	**0.009**		45.5 ± 9.9	0.112		47.5±9.0	0.201		47.3 ± 8.3	0.322	0.587
	LM	51.4 ± 9.7			**53.1 ± 12.3**[*]			**52.0 ± 9.0**[**]			49.8 ± 8.5			50.8±7.9			49.7 ± 8.1		0.810
60 min	**HM**	48.9 ± 5.9	0.247		45.4 ± 6.7	0.109		48.5 ± 7.6	0.828		45.3 ± 7.9	0.431		**45.7±7.1**[*]	**0.039**		47.0 ± 6.1	0.850	0.407
	LM	51.5 ± 7.3			49.7 ± 9.2			48.0 ± 7.5			47.8 ± 7.4			**51.6±9.6**[*]			46.0 ± 11.4		0.338

MP VCL (µm/s)																				
5 min	**HM**	122.6 ± 23.6	0.29	0.531	128.7 ± 27.9	0.757	0.314	135.7±21.9	0.699	0.107	134.0 ± 26.2	0.378	0.685	135.2 ± 24.7	0.121	0.444	133.5 ± 25.3	0.879	0.373	0.338
	LM	130.5 ± 22.6		0.087	131.6 ± 31.8		0.282	133.0±25.5		0.656	141.3 ± 29.9		0.919	123.9 ± 23.1		0.419	134.8 ± 29.0		0.069	0.438
30 min	**HM**	125.3 ± 17.4[a]	0.081		139.5 ± 21.8[ab]	0.359		**149.5±26.5[** b]**	**0.003**		135.5 ± 21.8[ab]	0.693		143.9 ± 24.9[ab]	0.152		140.3 ± 26.4[ab]	0.484		**0.020**
	LM	114.4 ± 20.4			133.6 ± 17.5			**126.3±22.5[**]**			138.5 ± 28.1			125.9 ± 35.1			133.6 ± 34.8			0.121
60 min	**HM**	129.7 ± 20.0	0.930		136.0 ± 21.4	0.061		**146.4±20.3[*]**	**0.033**		139.8 ± 12.1	0.760		140.5 ± 19.1	0.533		**129.9 ± 20.3[*]**	**0.026**		0.061
	LM	129.0 ± 24.7[ac]			123.0 ± 17.4[c]			**129.1±25.7[* ac]**			142.2 ± 30.6[ab]			135.9 ± 23.0[abc]			**160.3 ± 38.6[*b]**			**0.030**
MP VAP (µm/s)																				
5 min	**HM**	65.7 ± 9.6	0.902	0.538	66.1 ± 7.9	0.667	0.720	67.9±8.3	0.535	0.321	68.0 ± 8.2	0.530	0.669	67.5 ± 10.3	0.321	0.386	67.8 ± 7.9	0.651	0.908	0.899
	LM	67.3 ± 9.6		0.082	64.9 ± 10.6		0.910	66.3±9.6		0.925	69.8 ± 10.7		0.325	64.7 ± 7.7		0.374	66.6 ± 9.9		0.605	0.548
30 min	**HM**	64.6 ± 13.5	0.283		67.1 ± 10.0	0.724		70.1±7.3	0.113		67.4 ± 8.0	0.966		71.2 ± 10.4	0.294		66.9 ± 7.0	0.932		0.217
	LM	60.7 ± 6.9			66.0 ± 11.5			65.8±10.5			67.6 ± 11.4			68.3 ± 7.6			66.6 ± 14.9			0.363
60 min	**HM**	68.7 ± 12.7	0.817		68.5 ± 10.0	0.244		71.6±8.2	0.082		69.6 ± 7.2	0.280		70.6 ± 8.1	0.366		67.6 ± 7.5	0.424		0.789
	LM	67.6 ± 12.4			64.5 ± 9.7			67.1±8.8			73.2 ± 11.2			67.6 ± 11.0			71.0 ± 15.6			0.350
MP VSL (µm/s)																				
5 min	**HM**	55.4 ± 12.9	0.485	0.466	53.2 ± 12.1	0.883	0.830	53.9±13.2	0.782	0.664	54.5 ± 10.5	0.837	0.468	53.7 ± 13.2	0.625	0.458	54.9 ± 12.0	0.073	0.750	0.991
	LM	52.5 ± 11.7		0.472	52.7 ± 13.1		0.641	52.5±12.9		0.865	53.9 ± 10.1		0.887	53.4 ± 10.7		0.930	48.7 ± 11.3		0.777	0.741
30 min	**HM**	50.9 ± 17.6	0.365		52.8 ± 11.7	0.550		53.7±11.4	0.826		52.8 ± 11.8	0.876		54.0 ± 11.6	0.991		53.2 ± 11.3	0.30		0.998
	LM	49.0 ± 10.3			50.1 ± 16.7			52.3±14.3			52.3 ± 12.5			54.5 ± 10.5			50.1 ± 12.3			0.823
60 min	**HM**	56.1 ± 13.6	0.777		55.0 ± 11.7	0.836		56.8±11.5	0.804		57.3 ± 12.2	0.206		58.1 ± 12.5	0.181		55.8 ± 11.0	0.351		0.981
	LM	54.2 ± 13.5			55.2 ± 11.7			54.6±14.2			53.9 ± 10.7			54.5 ± 11.3			51.7 ± 12.4			0.988
MP STR (%)																				
5 min	**HM**	**83.9 ± 11.6[*]**	**0.027**	0.143	80.3 ± 13.0	0.903	0.896	79.4±12.0	0.815	0.680	80.5 ± 9.6	0.865	0.546	79.4 ± 11.8	0.388	0.539	81.0 ± 11.5	0.173	0.643	0.798
	LM	**78.1 ± 9.3[*]**		0.879	80.6 ± 13.2		0.272	79.7±13.3		0.949	79.2 ± 11.1		0.768	82.3 ± 11.5		0.821	75.1 ± 14.5		0.961	0.519
30 min	**HM**	76.2 ± 18.3	0.821		79.0 ± 10.6	0.807		76.8±12.6	0.531		78.0 ± 12.2	0.814		76.6 ± 13.2	0.593		79.3 ± 11.5	0.503		0.953
	LM	80.0 ± 11.1			75.6 ± 15.4			79.1±11.8			77.9 ± 11.9			80.7 ± 9.9			76.3 ± 13.3			0.757
60 min	**HM**	81.4 ± 9.9	0.656		80.6 ± 10.5	0.270		79.7±11.3	0.497		81.6 ± 11.1	0.055		81.4 ± 11.4	0.492		82.4 ± 9.2	0.105		0.979
	LM	79.0 ± 11.4			84.6 ± 8.3			80.5±13.1			76.4 ± 13.9			80.3 ± 9.9			75.2 ± 15.0			0.339
MP LIN (%)																				
5 min	**HM**	49.3 ± 13.3	0.225	0.435	47.0 ± 16.0	0.705	0.326	44.1±13.7	0.694	0.622	45.7 ± 12.3	0.468	0.677	43.9 ± 13.7	0.510	0.656	45.9 ± 13.5	0.220	0.443	0.791
	LM	44.5 ± 10.4		0.771	45.6 ± 14.6		0.747	42.7±10.5		0.756	43.2 ± 11.4		0.885	47.4 ± 11.0		0.872	41.1 ± 12.3		0.425	0.558
30 min	**HM**	44.0 ± 15.8	0.567		41.0 ± 10.5	0.915		40.5±12.8	0.195		43.1 ± 11.6	0.681		41.8 ± 12.2	0.101		42.2 ± 11.9	0.910		0.945
	LM	46.6 ± 12.0			40.6 ± 12.8			44.4±9.2			41.6 ± 12.1			47.0 ± 15.3			41.6 ± 12.5			0.450
60 min	**HM**	46.5 ± 11.5	0.417		44.2 ± 12.4	0.422		42.2±11.6	0.436		43.1 ± 10.0	0.212		44.5 ± 11.5	0.901		**46.6 ± 11.1[*]**	**0.015**		0.803
	LM	43.9 ± 10.9			47.6 ± 11.9			45.7±15.1			43.0 ± 13.2			45.1 ± 14.6			**36.1 ± 10.8[*]**			0.297
MP WOB (%)																				
5 min	**HM**	56.8 ± 9.9	0.553	0.705	56.0 ± 13.1	0.612	0.199	53.5±10.8	0.702	0.587	55.0 ± 10.8	0.718	0.468	53.1 ± 10.4	0.482	0.996	54.6 ± 10.1	0.281	0.345	0.846
	LM	55.0 ± 8.2		0.775	54.0 ± 11.7		0.514	52.5±7.8		0.536	53.3 ± 8.5		0.640	55.6 ± 7.8		0.822	53.0 ± 9.9		0.187	0.852
30 min	**HM**	54.2 ± 12.1	0.571		50.5 ± 8.1	0.420		50.7±9.9	0.131		52.9 ± 8.1	0.706		52.8 ± 10.4	0.369		51.2 ± 9.2	0.850		0.763
	LM	56.2 ± 9.1			52.6 ± 8.3			54.5±6.6			51.9 ± 9.4			56.2 ± 13.1			52.5 ± 10.1			0.567
60 min	**HM**	55.6 ± 9.6	0.441		53.2 ± 9.9	0.653		51.4±8.5	0.310		51.7 ± 6.6	0.311		53.0 ± 8.5	0.843		**55.1 ± 8.9[*]**	**0.018**		0.612
	LM	53.9 ± 7.2			54.7 ± 9.9			55.0±12.0			54.9 ± 11.1			53.8 ± 13.7			**46.8 ± 8.7[*]**			0.385

MP ALH (µm)																			
5 min	HM	3.1 ± 0.9	0.069	0.339	3.4 ± 1.1	0.660	0.239	3.6 ± 0.9	0.604	0.094	3.6 ± 0.8	0.423	0.448	3.6±1.0	0.633	0.184	3.6 ± 1.0	0.319	0.234	0.415
	LM	3.7 ± 0.8		0.365	3.6 ± 1.0		0.251	3.8 ± 1.0		0.615	3.9 ± 1.0		0.678	3.4±0.9		0.279	3.9 ± 1.0		0.066	0.590
30 min	HM	4.8 ± 6.6	0.713		3.8 ± 0.8	0.817		**4.1 ± 1.0***	**0.017**		3.8 ± 0.8	0.791		4.0±0.9	0.234		4.0 ± 1.0	0.565		0.198
	LM	3.3 ± 0.8			3.8 ± 0.7			**3.5 ± 0.6***			3.9 ± 1.0			3.7±1.0			3.8 ± 1.2			0.198
60 min	HM	3.4 ± 0.7	0.824		3.7 ± 0.8	0.185		4.1 ± 0.8	0.075		3.9 ± 0.5	0.442		3.9±0.7	0.551		**3.6 ± 0.6*****	**<0.001**		0.062
	LM	3.5 ± 0.8[a]			3.4 ± 0.6[a]			3.6 ± 1.0[a]			4.1 ± 1.0[ab]			3.9±0.6[a]			**4.7 ± 1.0***** [b]			**0.001**
MP BCF (Hz)																			
5 min	HM	**25.0 ± 5.0****	**0.007**	0.062	24.0 ± 5.5	0.240	0.407	23.3 ± 4.5	0.063	0.265	23.0 ± 3.6	0.118	0.395	23.2±6.4	0.376	0.372	23.2 ± 4.8	0.078	0.336	0.762
	LM	**20.7 ± 4.1****		0.164	21.9 ± 4.8		0.366	20.9 ± 4.2		0.499	21.3 ± 4.6		0.162	22.5±5.0		0.248	20.6 ± 4.8		0.459	0.732
30 min	HM	21.6 ± 4.9	0.598		22.1 ± 4.3	0.264		21.3 ± 4.2	0.826		21.7 ± 3.5	0.131		21.1±4.6	0.977		21.4 ± 3.6	0.181		0.979
	LM	20.7 ± 4.5			20.4 ± 4.9			21.7 ± 3.8			19.8 ± 4.6			21.0±4.5			20.1 ± 8.6			0.882
60 min	HM	22.5 ± 4.9	0.595		23.4 ± 4.6	0.551		22.0 ± 4.4	0.219		**21.8 ± 3.5***	**0.033**		22.2±4.1	0.120		**22.2 ± 2.9*****	**<0.001**		0.894
	LM	21.4 ± 6.2			22.5 ± 3.9			20.1 ± 4.2			**18.2 ± 5.7***			19.8±5.0			**17.9 ± 3.3*****			0.079
RP VCL (µm/s)																			
5 min	HM	141.0 ± 14.0	0.796	0.143	149.6 ± 19.3	0.356	0.226	**143.8 ± 26.9***[a]*	0.715	**0.030**	152.7 ± 20.6	0.786	0.174	157.1±30.2	0.329	0.441	153.7 ± 20.8	0.220	0.398	0.277
	LM	147.0 ± 25.9		0.889	142.8 ± 18.4		0.210	147.1 ± 22.7		0.086	153.1 ± 19.3		0.723	146.9±20.5		0.831	147.5 ± 29.0		0.931	0.933
30 min	HM	147.1 ± 14.5	0.566		157.9 ± 27.0	0.157		**158.9 ± 26.3***[ab]*	0.125		157.8 ± 22.5	0.549		159.2±21.4	0.222		154.8 ± 22.4	0.755		0.700
	LM	143.0 ± 20.3			143.3 ± 22.6			145.2 ± 21.4			152.0 ± 27.2			150.9±27.8			151.5 ± 34.2			0.908
60 min	HM	151.4 ± 13.2	0.587		164.2 ± 18.2	0.378		**167.8 ± 22.0***[b]*	0.670		169.1 ± 28.3	0.850		168.3±28.0	0.208		163.7 ± 20.2	0.469		0.390
	LM	146.8 ± 24.3			156.9 ± 19.6			163.9 ± 25.4			160.1 ± 26.1			153.5±21.9			169.0 ± 57.7			0.737
RP VAP (µm/s)																			
5 min	HM	81.2 ± 13.6	0.438	0.351	80.7 ± 13.3	0.378	0.210	84.9 ± 12.8	0.365	0.156	82.2 ± 13.2	0.983	0.108	**82.4±15.8***	**0.019**	0.065	84.6 ± 12.4	0.114	0.194	0.924
	LM	77.4 ± 15.2		0.606	75.8 ± 10.9		0.137	80.7 ± 13.3		0.057	80.0 ± 12.1		0.170	**72.1±7.8***		0.140	78.2 ± 15.3		0.915	0.600
30 min	HM	85.5 ± 12.6	0.065		81.3 ± 14.4	0.441		84.6 ± 11.9	0.399		82.9 ± 11.5	0.682		86.4±12.7	0.180		83.4 ± 11.6	0.334		0.878
	LM	75.7 ± 11.4			77.3 ± 10.7			80.6 ± 15.0			85.0 ± 15.2			82.5±20.2			77.7 ± 18.6			0.724
60 min	HM	88.4 ± 13.7	0.316		90.1 ± 17.2	0.803		92.1 ± 10.7	0.890		91.1 ± 12.2	0.849		94.3±15.4	0.227		91.5 ± 13.9	0.469		0.925
	LM	82.0 ± 14.8			88.4 ± 15.6			92.9 ± 15.6			92.2 ± 16.6			85.9±15.7			88.7 ± 28.5			0.751
RP VSL (µm/s)																			
5 min	HM	75.0 ± 14.2	0.410	0.565	73.4 ± 13.4	0.380	0.277	78.3 ± 13.2	0.331	0.295	74.2 ± 12.5	0.851	0.180	**73.4±14.3*** *a*	**0.010**	**0.045**	76.6 ± 12.2	0.087	0.283	0.850
	LM	69.3 ± 15.4		0.791	69.1 ± 11.5		0.065	73.6 ± 14.5		0.111	72.2 ± 11.9		0.185	**64.3±6.6***		0.125	70.8 ± 14.6		0.482	0.588
30 min	HM	77.7 ± 14.2	0.132		72.6 ± 13.5	0.395		75.7 ± 11.9	0.570		74.1 ± 10.6	0.547		**77.1±12.0***[ab]*	0.222		75.7 ± 11.6	0.261		0.879
	LM	69.1 ± 11.7			68.6 ± 8.7			72.9 ± 15.2			77.1 ± 15.0			74.8±19.3			69.4 ± 17.3			0.690
60 min	HM	80.7 ± 13.6	0.201		81.0 ± 17.1	0.867		82.4 ± 9.7	0.744		81.1 ± 10.8	0.626		**85.2±14.7***[b]*	0.223		82.7 ± 14.0	0.694		0.950
	LM	73.0 ± 13.4			79.9 ± 15.0			83.9 ± 14.5			83.8 ± 16.1			77.3±14.0			79.3 ± 25.1			0.686
RP STR (%)																			
5 min	HM	**91.9 ± 3.1***	**0.043**	0.507	90.6 ± 2.8	0.985	0.357	**91.9 ± 3.5***[a]*	0.389	**0.019**	90.0 ± 4.1	0.917	0.628	88.9±4.2	0.715	0.538	90.4 ± 3.4	0.713	0.928	0.090
	LM	**89.3 ± 3.8***		0.324	90.5 ± 3.4		0.389	90.7 ± 4.7		0.922	89.9 ± 2.9		0.835	89.1±2.1		0.461	89.9 ± 3.2		0.842	0.860
30 min	HM	90.6 ± 3.7	0.760		89.1 ± 2.5	0.739		**89.2 ± 2.9***[b]*	0.391		89.2 ± 2.5	0.289		89.0±3.4	0.251		90.5 ± 2.1	0.220		0.376
	LM	91.1 ± 2.9			88.7 ± 3.6			90.1 ± 3.2			90.4 ± 3.2			90.4±3.0			89.0 ± 4.3			0.562
60 min	HM	91.0 ± 2.4	0.105		89.5 ± 3.4	0.650		**89.3 ± 2.9***[b]*	0.393		88.9 ± 2.7	0.190		90.1±2.3	0.945		90.1 ± 3.2	0.718		0.529
	LM	88.9 ± 3.2			90.1 ± 2.6			90.3 ± 2.7			90.7 ± 3.6			90.0±2.1			89.5 ± 4.2			0.856

RP LIN (%)																			
5 min	**HM**	54.9 ± 9.8	0.057	0.997	50.6 ± 10.7	0.859	0.612	57.4±15.2	0.199	0.097	50.6 ± 9.5	0.786	0.671	48.8±10.8	0.591	0.513	51.8 ± 10.8	0.561	0.924	0.217
	LM	48.7 ± 12.2		0.898	49.8 ± 10.3		0.686	51.1±11.2		0.959	48.4 ± 7.7		0.398	45.1±5.9		0.089	49.4 ± 11.0		0.832	0.781
30 min	**HM**	54.9 ± 12.0	0.558		47.7 ± 8.5	0.515		49.6±8.5	0.676		48.1 ± 7.1	0.354		50.0±8.7	0.589		50.8 ± 8.3	0.252		0.312
	LM	50.1 ± 12.9			49.9 ± 8.1			50.8±7.9			52.9 ± 11.8			50.7±8.5			46.9 ± 8.6			0.810
60 min	**HM**	54.6 ± 9.9	0.503		51.5 ± 12.0	0.756		50.6±8.8	0.199		49.2 ± 6.1	0.345		52.6±9.9	0.951		52.1 ± 10.3	0.290		0.791
	LM	51.4 ± 12.0			52.9 ± 8.6			51.8±6.7			54.2 ± 12.7			51.2±4.3			48.0 ± 3.0			0.786
RP WOB (%)																			
5 min	**HM**	59.2 ± 9.0	0.086	0.977	55.4 ± 10.4	0.818	0.614	61.8±14.2	0.193	0.153	55.7 ± 9.0	0.722	0.761	54.3±10.1	0.576	0.520	56.8 ± 10.3	0.518	0.875	0.296
	LM	54.1 ± 11.3		0.812	54.5 ± 9.7		0.629	55.9±9.9		0.911	53.4 ± 7.1		0.335	50.3±5.7		0.066	54.3 ± 10.4		0.881	0.786
30 min	**HM**	59.9 ± 10.8	0.412		53.1 ± 8.1	0.405		55.2±8.1	0.751		53.6 ± 6.7	0.407		55.7±8.1	0.857		55.7 ± 8.1	0.271		0.318
	LM	54.6 ± 12.5			55.9 ± 8.5			56.1±7.0			57.9 ± 10.8			55.8±7.8			52.3 ± 7.2			0.788
60 min	**HM**	59.6 ± 9.5	0.651		56.8 ± 11.2	0.755		56.3±8.3	0.369		55.1 ± 5.6	0.529		58.0±10.1	0.951		57.3 ± 9.5	0.277		0.862
	LM	57.4 ± 12.3			58.2 ± 8.2			57.1±5.9			59.3 ± 12.0			56.6±4.1			53.5 ± 2.2			0.677
RP ALH (µm)																			
5 min	**HM**	3.3 ± 0.7	0.385	0.431	4.9 ± 4.3	0.307	0.675	**3.4±1.1**^{a}	0.165	**0.018**	**3.7 ± 0.8**^{a}	0.213	**0.026**	4.1±1.3	0.677	0.983	3.7 ± 0.9	0.793	0.313	0.179
	LM	4.0 ± 0.9		0.401	3.5 ± 1.0		0.556	3.9±0.9		0.018	4.0 ± 1.0		0.989	3.9±0.9		0.883	3.8 ± 1.2		0.733	0.832
30 min	**HM**	3.6 ± 0.8	0.725		4.1 ± 1.0	0.582		**4.1±0.8**^{b}	0.250		**4.2 ± 0.9**^{ab}	0.573		4.1±0.9	0.636		4.0 ± 0.8	0.780		0.556
	LM	3.5 ± 0.8			3.9 ± 1.0			3.7±0.7			3.9 ± 1.1			4.0±1.0			3.9 ± 1.0			0.886
60 min	**HM**	3.6 ± 0.7	0.987		4.1 ± 0.9	0.489		**4.3±0.9**^{b}	0.512		**4.5 ± 0.8**^{b}	0.975		4.1±0.8	0.903		4.3 ± 1.2	0.690		0.197
	LM	3.6 ± 1.0			3.8 ± 0.8			4.2±0.8			4.0 ± 1.2			4.1±0.7			4.2 ± 1.2			0.709
RP BCF (Hz)																			
5 min	**HM**	**27.1 ± 5.0*****	**<0.001**	0.452	25.2 ± 3.2	0.745	0.289	**25.7±3.9**^{a}	0.059	**0.039**	**26.6 ± 4.7**^{a}	0.217	**0.010**	23.5±4.6	0.817	0.342	25.3 ± 5.4	0.18	0.324	0.253
	LM	**16.3 ± 9.0**$^{***\,a\,a}$		**0.040**	26.8 ± 6.3^{b}		0.102	22.2±6.3^{b}		0.799	23.3 ± 4.4^{b}		0.383	23.1±4.4^{b}		0.687	22.9 ± 3.7^{b}		0.724	**0.020**
30 min	**HM**	25.5 ± 6.4	0.847		23.0 ± 4.0	0.959		**22.8±3.3**^{b}	0.609		**22.7 ± 3.0**^{b}	0.606		23.3±4.0	0.444		23.0 ± 3.5	0.269		0.450
	LM	**24.9 ± 6.4**^{b}			21.2 ± 8.0			23.5±4.6			23.2 ± 2.6			22.2±3.7			22.3 ± 6.3			0.450
60 min	**HM**	24.5 ± 4.6	0.630		23.8 ± 4.0	0.314		**23.1±4.0**^{ab}	0.630		**23.2 ± 3.0b**	0.207		**25.3±4.2***	**0.038**		23.4 ± 5.2	0.320		0.689
	LM	**25.8 ± 7.1**^{ab}			25.5 ± 3.8			22.2±5.9			21.2 ± 4.4			**21.7±2.8***			21.1 ± 4.8			0.165

Note: High motile (HM) subpopulations had significantly higher percentages for various motility parameters at 5, 30 and 60 min when exposed in HTF, as compared to the low motile (LM) subpopulations. After exposure in CAP and PRL - various LM motility and kinematic parameters could be enhanced to the point that no significant difference between subpopulations could further be seen. In comparison to HTF - 50 ng/mL, 100 ng/mL and 250 ng/mL PRL significantly enhanced progressive motility at 5 min for the LM subpopulation. However, only 100 ng/mL PRL enhanced progressive motility at 30 min and 60 min. Furthermore, CAP and PRL concentrations enhanced total motility (60 min); CAP, 50 ng/mL and 250 ng/mL PRL enhanced medium speeds (60 min); and 50 ng/mL and 100 ng/mL PRL enhanced MP speed groups (5 min). Compared to HTF, in HM subpopulations CAP, 50 ng/mL, 100 ng/mL and 250 ng/mL PRL all significantly increased total motility at 60 min. For better understanding of kinematic parameters – VCL, VAP and VSL will be grouped as "velocity" kinematics; LIN and STR as "linearity"; and ALH, WOB and BCF as "vigour" kinematics. CAP and 100 ng/mL PRL increased LM subpopulation motile velocity kinematics until differences between subpopulations were no longer seen. No significant differences between subpopulations were seen for NP and MP velocity kinematics. However, for MP velocity kinematics, 50 ng/mL PRL significantly increased HM subpopulations (30 min and 60 min) as compared to LM subpopulations and 500 ng/mL PRL increased LM subpopulations as compared to HM subpopulations (60 min). For RP velocity kinematics, 250 ng/mL PRL significantly increased HM subpopulations as compared to LM subpopulations. CAP and PRL increased motile VCL at 30 min for both subpopulations, as compared to HTF. At 60 min for HM subpopulations, 50 ng/mL and 250 ng/mL PRL increased motile VCL as compared to HTF. At 30 min 50 ng/mL and 250 ng/mL increased motile VAP in HM subpopulations as compared to HTF, however all media increased LM subpopulations as compared to HTF. For motile VSL at 30 min, 50 ng/mL, 100 ng/mL and 250 ng/mL PRL all increased LM subpopulation values as compared to HTF. At 30 min, 50 ng/mL increased MP VCL in HM subpopulations as compared to HTF - and at 60 min 500 ng/mL increased LM subpopulation values as compared to HTF, CAP and 50 ng/mL PRL. At 30 min for RP ALH, PRL increased values in HM subpopulations as compared to HTF and increased values in LM subpopulations as compared to HTF and CAP. At 60 min for LM subpopulations, PRL increased RP ALH as compared to HTF. At 60 min for the LM subpopulation, 500 ng/mL PRL increased MP ALH as compared to HTF, CAP, 50 ng/mL and 250 ng/mL PRL. At 5 min in the LM subpopulation, all media increased RP BCF as compared to HTF**. Abbreviations:** ALH, average path velocity; BCF, beat cross frequency; CAP, capacitating-HTF; HTF, human tubal fluid; HM, high motile subpopulation; LIN, linearity; LM, low motile subpopulation; MP, medium-progressive; min, minutes; NP, non-progressive; PRL, prolactin; Prog, progressive; RP, rapid-progressive; SD, standard deviation; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble. [a, b,c,d] Values labelled with different superscript letters in the same row were significantly different between the various media for individual subpopulations and SCA parameters (one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions). Values labelled in bold with an asterisk in the same column were significantly different between the HM and LM subpopulations for individual media and SCA parameters (*p <0.05, **p <0.01 and ***p <0.001) (Student's t-test or the Mann-Whitney test when normal distribution was void). [a, b] Values labelled in bold and red with different superscript letters in the same column were significantly different between time points for individual subpopulations and media (one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions).

Supplementary Table 5. Reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and acrosome reaction (AR) results of both the high (HM) motile and low (LM) motile subpopulations after exposure to various HTF, CAP, progesterone and myo-inositol (Mean ± SD) (n = 20).

	HTF		CAP		HD-C		1.98 µM PRG		3.96 µM PRG		19.8 µM PRG		11 mM MYO		1 mM Ca-ionophore		
	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	ANOVA
ROS																	
HM	42.7 ± 12.6**	**0.0032**	41.0 ± 10.5*	**0.0225**	34.8 ± 13.9*	**0.0138**	35.4 ± 13.6*	**0.0423**	36.9 ± 12.8*	**0.0305**	33.1 ± 12.8*	**0.0133**	34.0 ± 11.2**	**0.0082**			0.124
LM	55.7 ± 13.5**		50.9 ± 15.3*		46.1 ± 14.2*		44.8 ± 14.6*		46.9 ± 15.3*		44.4 ± 14.7*		44.8 ± 13.2**				0.13
MMP																	
HM	55.7 ± 16.4***	**<0.001**	50.7 ± 19.0*	**0.0265**	62.5 ± 16.1**	**0.0016**	58.3 ± 19.3**	**0.0022**	58.5 ± 13.2***	**<0.001**	59.6 ± 13.4***	**<0.001**	65.0 ± 16.5*	**0.0263**			0.403
LM	29.5 ± 20.7$^{a\ ***}$		34.8 ± 23.7$^{ab\ *}$		42.8 ± 17.3$^{ab\ **}$		37.7 ± 20.3$^{ab\ **}$		35.7 ± 18.7$^{ab\ ***}$		41.8 ± 18.6$^{ab\ ***}$		50.5 ± 22.8$^{b\ *}$				**0.048**
AR																	
HM	25.9 ± 12.8$^{a\ **}$	**0.0021**	29.8 ± 11.7$^{a\ ***}$	**<0.001**	41.3 ± 13.0$^{b\ *}$	**0.0376**	44.1 ± 15.1^{b}	0.4374	45.5 ± 12.3^{b}	0.5358	40.2 ± 11.7$^{b\ *}$	**0.0104**	43.9 ± 18.2^{b}	0.2262	73.7 ± 10.7$^{c\ ***}$	**<0.001**	**<0.001**
LM	39.4 ± 12.2$^{a\ **}$		46.3 ± 10.9$^{ab\ ***}$		49.1 ± 12.3$^{ab\ *}$		47.0 ± 10.8^{ab}		47.3 ± 8.1^{ab}		48.8 ± 10.6$^{ab\ *}$		49.6 ± 13.8^{b}		60.4 ± 13.0$^{c\ ***}$		**<0.001**

Note: Compared to the HM subpopulation, the LM subpopulation had significantly higher percentages of positive ROS and spontaneous AR, and lower percentages of intact MMP. Differences between the media was predominantly seen in LM subpopulations. Values labelled with different letters (a, b, c) were significantly different between the various media for individual subpopulations (one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions). One-way ANOVA for parametric distributions was used or Kruskal-Wallis test for non-parametric distributions. Values with asterisks were significantly different between the HM and LM subpopulations for individual media (*p <0.05, **p <0.01 and ***p <0.001). Student's t-test was used or the Mann-Whitney test when normal distribution was void.
Abbreviations: AR, acrosome reaction; Ca-ionophore, calcium-ionophore; CAP, capacitating-HTF; HD-C, HD capacitation media; HTF, human tubal fluid; HM, high motile subpopulation; LM, low motile subpopulation; MMP, mitochondrial membrane potential; MYO, myo-inositol; PRG, progesterone; ROS, reactive oxygen species; SP *p*, significant differences between subpopulations.

Supplementary Table 6. Reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and acrosome reaction (AR) results of both the high (HM) motile and low (LM) motile subpopulations after exposure to various HTF, CAP and dopamine (Mean ± SD) (n = 20).

	HTF		CAP		20 nM DOPA		100 nM DOPA		1 µM DOPA		1 mM Ca-ionophore		
	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	ANOVA
ROS													
HM	31.6 ± 10.6*	**0.0137**	36.1 ± 12.5	0.3743	26.1 ± 12.8*	**0.0219**	31.6 ± 12.9	0.0855	31.3 ± 12.1	0.1208			0.16
LM	41.5 ± 13.4*		39.4 ± 10.2		35.7 ± 12.6*		39.6 ± 15.6		37.0 ± 12.8				0.693
MMP													
HM	59.2 ± 11.3**	**0.0024**	59.6 ± 6.7***	**<0.001**	64.2 ± 10.6**	**0.0024**	61.7 ± 9.8*	**0.0135**	58.7 ± 11.5	0.0927			0.408
LM	47.0 ± 12.5**		49.2 ± 10.0***		52.0 ± 13.0**		53.2 ± 10.9*		52.0 ± 13.0				0.469
AR													
HM	23.1 ± 10.3$^{a\,*}$	**0.0324**	27.7 ± 9.4^{ab}	0.0519	30.2 ± 11.4^{ab}	0.0949	34.0 ± 14.0^{b}	0.769	34.0 ± 9.6^{b}	0.8503	65.7 ± 8.0$^{c\,**}$	**0.0025**	**<0.001**
LM	31.0 ± 12.6$^{a\,*}$		33.6 ± 10.7^{a}		34.6 ± 10.6^{a}		35.2 ± 11.8^{a}		34.6 ± 10.6^{a}		56.4 ± 10.5$^{b\,**}$		**<0.001**

Note: Compared to the HM subpopulation, the LM subpopulation had significantly higher percentages of positive ROS and spontaneous AR, and lower percentages of intact MMP. Differences between the media was predominantly seen in LM subpopulations. Values labelled with different letters (a, b, c) were significantly different between the various media for individual subpopulations (one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions). One-way ANOVA for parametric distributions was used or Kruskal-Wallis test for non-parametric distributions. Values with asterisks were significantly different between the HM and LM subpopulations for individual media (*p <0.05, **p <0.01 and ***p <0.001). Student's t-test was used or the Mann-Whitney test when normal distribution was void.
Abbreviations: AR, acrosome reaction; Ca-ionophore, calcium-ionophore; DOPA, dopamine; CAP, capacitating-HTF; HTF, human tubal fluid; HM, high motile subpopulation; LM, low motile subpopulation; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; SP p, significant differences between subpopulations.

Supplementary Table 7. Reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and acrosome reaction (AR) results of both the high (HM) motile and low (LM) motile subpopulations after exposure to various HTF, CAP and prolactin (Mean ± SD) (n = 20).

	HTF		CAP		50 ng/mL PRL		100 ng/mL PRL		250 ng/mL PRL		500 ng/mL PRL		1 mM Ca-ionophore		
	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	ANOVA
ROS															
HM	39.8 ± 12.9$^{a\,**}$	**0.0094**	37.4 ± 13.4	0.091	31.8 ± 12.6	0.2374	32.6 ± 12.4	0.0657	31.6 ± 12.0	0.0983	28.7 ± 10.4*	**0.0168**			0.083
LM	51.2 ± 12.0$^{a\,**}$		44.2 ± 9.7^{ab}		36.5 ± 10.9^{b}		41.4 ± 15.1^{b}		39.3 ± 15.1^{b}		37.9 ± 11.4$^{b\,*}$				0.008
MMP															
HM	61.1 ± 10.9$^{a\,**}$	**0.0019**	61.5 ± 15.1	0.0763	66.3 ± 14.8	0.0688	67.2 ± 12.2*	**0.0367**	66.5 ± 10.3	0.168	68.4 ± 13.1	0.0762			0.4
LM	50.8 ± 10.1$^{a\,**}$		57.7 ± 6.7^{ab}		61.2 ± 9.1^{b}		60.0 ± 6.8$^{ab\,*}$		61.3 ± 11.7^{b}		61.8 ± 12.3^{b}				0.012
AR															
HM	26.0 ± 9.6$^{a\,***}$	**<0.001**	28.5 ± 9.3$^{a\,**}$	**0.0046**	26.0 ± 9.8$^{a\,**}$	**0.0039**	25.7 ± 6.5$^{a\,***}$	**<0.001**	25.7 ± 7.4$^{a\,**}$	**0.0059**	25.8 ± 8.5$^{a\,***}$	**<0.001**	69.4 ± 8.4$^{b\,**}$	**0.0022**	**<0.001**
LM	35.1 ± 6.7$^{a\,***}$		35.9 ± 6.7$^{a\,**}$		33.6 ± 6.2$^{a\,**}$		33.0 ± 5.6$^{a\,***}$		32.1 ± 7.2$^{a\,**}$		35.9 ± 7.1$^{a\,***}$		61.0 ± 8.6$^{b\,**}$		**<0.001**

Note: Compared to the HM subpopulation, the LM subpopulation had significantly higher percentages of positive ROS and spontaneous AR, and lower percentages of intact MMP. Differences between the media was predominantly seen in LM subpopulations. Values labelled with different letters (a, b, c) were significantly different between the various media for individual subpopulations (one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions). One-way ANOVA for parametric distributions was used or Kruskal-Wallis test for non-parametric distributions. Values with asterisks were significantly different between the HM and LM subpopulations for individual media (**p* <0.05, ***p* <0.01 and ****p* <0.001). Student's t-test was used or the Mann-Whitney test when normal distribution was void.
Abbreviations: AR, acrosome reaction; Ca-ionophore, calcium-ionophore; CAP, capacitating-HTF; HTF, human tubal fluid; HM, high motile subpopulation; LM, low motile subpopulation; MMP, mitochondrial membrane potential; PRL, prolactin; ROS, reactive oxygen species; SP p, significant differences between subpopulations.

Supplementary Table 8: Comparison of the various FAST kinematic parameters (mean±SD) of the individual spermatozoa from the low motile (LM) and high motile (HM) subpopulations after being flushed with HTF, CAP, HD-C, 3.96 µM progesterone and 11 mM myo-inositol.

	HTF		CAP		HD-C		PRG		MYO		
	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	ANOVA
Mean power (Watt)											
HM	1.7E-14 ± 7.4E-15^{a}	0.37	2.8E-14 ± 1.7E-14^{b}	0.1016	1.7E-14 ± 1.7E-14^{a}	0.5068	2.2E-14 ± 1.4E-14^{c}	0.3438	2.6E-14 ± 2.2E-14^{bc}	0.0687	**<0.001**
LM	1.5E-14 ± 7.2E-14^{ab}		2.7E-14 ± 2.8E-14^{a}		1.3E-14 ± 7.7E-15^{b}		2.0E-14 ± 1.3E-14^{a}		1.9E-14 ± 1.1E-14^{a}		**0.014**
Max FL (µm)											
HM	50.6 ± 5.2	0.9019	50.9 ± 4.2	0.5109	**50.6 ± 4.0***	**0.0476**	50.4 ± 4.7	0.1415	50.5 ± 4.8	0.0836	0.983
LM	50.7 ± 3.4		51.1 ± 4.1		**52.2 ± 3.6***		51.6 ± 4.4		52.2 ± 4.9		0.571
TCS (µm/s)											
HM	28.4 ± 8.9^{a}	0.4385	27.2 ± 9.2^{a}	0.9873	23.3 ± 7.6^{b}	0.6071	27.9 ± 8.2^{a}	0.1343	27.3 ± 8.9^{a}	0.5311	**<0.001**
LM	26.0 ± 10.2		27.2 ± 8.6		22.4 ± 8.1		25.7 ± 7.1		26.2 ± 8.2		0.253
fAWL (µm)											
HM	32.3 ± 6.8^{ac}	0.9691	33.4 ± 9.2^{a}	0.4807	29.0 ± 6.1^{b}	0.1876	32.1 ± 8.9^{ac}	0.3167	30.7 ± 9.0^{bc}	0.0502	**0.001**
LM	32.4 ± 8.0		35.1 ± 11.5		28.1 ± 7.8		31.2 ± 10.1		33.4 ± 8.6		0.058
fAWS (µm/s)											
HM	628.3 ± 134.8^{a}	0.3371	616.8 ± 133.6^{a}	0.8309	535.6 ± 134.2^{b}	0.5164	621.3 ± 148.2^{a}	0.5521	583.9 ± 140.7^{a}	0.6729	**<0.001**
LM	597.3 ± 141.3^{ab}		622.6 ± 128.9^{a}		517.3 ± 132.3^{b}		605.6 ± 118.2^{a}		588.8 ± 133.5^{a}		**0.024**
fBF (Hz)											
HM	19.6 ± 3.1	0.1611	19.1 ± 4	0.6231	18.6 ± 3.6	0.9835	20 ± 4.8	0.278	**19.7 ± 4.1***	**0.0225**	0.112
LM	18.6 ± 2.8		18.7 ± 4.1		18.6 ± 3.2		20.2 ± 3.8		**18.2 ± 4.2***		0.154
VCL (µm/s)											
HM	169.7 ± 62.0^{a}	0.939	224.1 ± 60.3^{b}	0.2373	155.8 ± 60.4^{a}	0.1664	209.9 ± 66.0^{bc}	0.1084	195.4 ± 68.8^{c}	0.2086	**<0.001**
LM	168.8 ± 60.9^{ab}		208.7 ± 67.1^{a}		138.3 ± 51.1^{b}		190.7 ± 61.9^{a}		179.5 ± 74.5^{a}		**0.001**
VAP (µm/s)											
HM	63.4 ± 17.9^{ab}	0.3328	65.0 ± 19.9^{ab}	0.5571	59.5 ± 18.6^{a}	0.5117	**69.6 ± 23.2^{b}** *	**0.0323**	63.3 ± 17.1^{ab}	0.1793	**0.015**
LM	59.6 ± 15.8		63.1 ± 17.2		56.5 ± 13.3		**59.8 ± 16.9***		60.6 ± 17.7		0.651
VSL (µm/s)											
HM	55.5 ± 15.9^{a}	0.1144	53.3 ± 18.6^{a}	0.2327	44.9 ± 14.8^{b}	0.8438	52.7 ± 15.6^{a}	0.5809	52.4 ± 17.6^{a}	0.4092	**<0.001**
LM	49.0 ± 20.5		50.9 ± 17.1		44.3 ± 15.3		51.0 ± 15.1		49.6 ± 15.2		0.487

STR (%)											
HM	89.3 ± 19.0	0.3773	84.7 ± 24.8	0.4787	79.0 ± 24.3	0.919	81.8 ± 27.3	0.6577	83.8 ± 21.3	0.6267	0.066
LM	81.1 ± 30.0		82.0 ± 23.8		79.4 ± 23.8		87.0 ± 19.7		85.0 ± 22.0		0.663
LIN (%)											
HM	35.4 ± 12.0^{a}	0.1213	25.0 ± 9.3^{b}	0.8657	32.4 ± 13.7^{ac}	0.3941	27.1 ± 10.9^{b}	0.2427	29.1 ± 11.7^{bc}	0.3186	**<0.001**
LM	29.8 ± 12.3		26.5 ± 11.1		34.9 ± 14.6		29.4 ± 12.0		30.9 ± 11.4		0.114
WOB (%)											
HM	39.8 ± 11.0^{a}	0.3749	30.5 ± 10.1^{b}	0.5109	41.7 ± 14.0^{a}	0.4738	35.1 ± 11.7^{c}	0.5495	35.4 ± 12.7^{c}	0.3186	**<0.001**
LM	37.9 ± 11.6^{ab}		33.0 ± 12.6^{a}		43.8 ± 12.0^{b}		34.0 ± 13.0^{a}		37.5 ± 12.5^{ab}		**0.007**
ALH (µm)											
HM	1.8 ± 0.8^{a}	0.7974	2.5 ± 0.8^{b}	0.3547	1.9 ± 0.9^{a}	0.2007	2.3 ± 0.9^{bc}	0.6639	2.3 ± 1.0^{c}	0.6485	**<0.001**
LM	1.9 ± 0.8^{ab}		2.3 ± 0.8^{a}		1.6 ± 0.6^{b}		2.3 ± 0.8^{a}		2.2 ± 1.1^{a}		**0.003**
ALHmax (µm)											
HM	4.1 ± 1.5^{a}	0.8889	5.3 ± 1.5^{b}	0.2789	4.2 ± 1.9^{a}	0.0851	4.9 ± 1.6^{bc}	0.8023	4.9 ± 1.8^{c}	0.5406	**<0.001**
LM	4.1 ± 1.4^{ab}		4.9 ± 1.6^{a}		3.4 ± 1.2^{b}		4.9 ± 1.6^{a}		4.6 ± 1.8^{a}		**0.001**
BCF (Hz)											
HM	27.9 ± 7.9^{ab}	0.3030	29.0 ± 7.7^{a}	0.4946	26.0 ± 6.6^{b}	0.7219	**30.0 ± 8.3^{a}** *	0.0119	27.7 ± 7.2^{ab}	0.2741	**0.005**
LM	29.7 ± 6.7		27.8 ± 4.7		26.5 ± 6.6		**26.3 ± 6.2***		26.1 ± 7.3		0.163
MAD (degrees)											
HM	218.4 ± 45.3	0.857	225.9 ± 42.4	0.6052	222.5 ± 41.0	0.6416	212.7 ± 45.6	0.1717	221.0 ± 44.6	0.6774	0.422
LM	216.7 ± 45.1		219.9 ± 45.6		215.7 ± 47.6		224.7 ± 43.6		227.8 ± 39.1		0.769

Note: Compared to LM subpopulations, the HM subpopulation yielded significantly higher values for motile fBF in MYO. Significant differences between media were observed in both subpopulations, however predominantly in the HM subpopulation**. Abbreviations**: ALH, amplitude of lateral head displacement; ALHmax, maximum amplitude of lateral head displacement; BCF, beat cross frequency; CAP, capacitating-HTF; FAST, flagellar analysis and sperm tracking; fAWL, flagellar arc-wavelength; fAWS, flagellar arc-wave speed; fBF, flagellar beat frequency; HD-C, HD capacitating medium; HTF, human tubal fluid; HM, high motile subpopulation; Hz, hertz; LIN, linearity; LM, low motile subpopulation; MAD, mean angle displacement; Max FL, maximum analysed flagellum; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; SD, standard deviation; STR, straightness; TCS, track centroid speed; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; Watt, wattage; WOB, wobble$^{\cdot\ a,\ b,c,d}$ Values labelled with different superscript letters in the same row were significantly different between the various media for individual subpopulations and FAST parameters. Values labelled in bold with an asterisk in the same column were significantly different between the HM and LM subpopulations for individual media and FAST parameters (*p <0.05, **p <0.01 and ***p <0.001).

Supplementary Table 9: Comparison of the various FAST kinematic parameters (mean±SD) of the individual spermatozoa from the low motile (LM) and high motile (HM) subpopulations after being treated with HTF, CAP, 1 µM dopamine, 250 ng/mL prolactin and 500 ng/mL prolactin.

	HTF		CAP		1 µM DOPA		250 ng/mL PRL		500 ng/mL PRL		
	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	Mean ± SD	SP *p*	ANOVA
Mean power (Watt)											
HM	2.9E-14 ± 3.7E-14	0.9563	**3.8E-14 ± 2.3E-14***	**0.0135**	3.4E-14 ± 3.1E-14	0.7425	2.7E-14 ± 2.3E-14	0.9851	2.8E-14 ± 2.1E-14	0.6840	0.413
LM	2.0E-14 ± 1.2E-14		**2.0E-14 ± 1.6E-14***		3.1E-14 ± 2.7E-14		2.7E-14 ± 2.1E-14		2.6E-14 ± 1.8E-14		0.622
Max FL (µm)											
HM	49.7 ± 5.2	0.3512	49.2 ± 4.2	0.6473	47.8 ± 3.8	0.4571	48.6 ± 4.3	0.2464	48.4 ± 3.9	0.7227	0.411
LM	51.5 ± 4.2		48.5 ± 4.3		48.8 ± 5.3		50.3 ± 4.8		47.9 ± 4.4		0.402
TCS (µm/s)											
HM	24.2 ± 8.7	0.8744	27.3 ± 8.4	0.7552	24.4 ± 6.4	0.5389	23.1 ± 6.9	0.1895	24.6 ± 8.5	0.0504	0.183
LM	23.7 ± 9.1		26.4 ± 8.5		23.2 ± 6.1		26.2 ± 8.8		19.1 ± 8.0		0.171
fAWL (µm)											
HM	29.0 ± 5.4[a]	0.1690	34.3 ± 6.3[b]	0.1980	32.0 ± 8.5[abc]	0.7472	31.1 ± 6.7[ac]	0.5805	34.1 ± 10.6[bc]	0.0930	**0.038**
LM	32.5 ± 9.2		31.5 ± 5.6		32.8 ± 5.7		32.3 ± 6.0		28.3 ± 9.7		0.531
fAWS (µm/s)											
HM	577.0 ± 136.8	0.6838	678.7 ± 127.0	0.1661	610.9 ± 173.7	0.7398	614.0 ± 149.7	0.2852	**654.0 ± 146.0***	**0.0168**	0.056
LM	556.2 ± 111.9		609.7 ± 173.5		595.2 ± 90.8		663.1 ± 125.0		**529.3 ± 170.4***		0.148
fBF (Hz)											
HM	20.0 ± 3.7	0.1486	20.0 ± 3.0	0.7059	19.4 ± 4.1	0.5138	19.8 ± 3.2	0.2873	19.8 ± 4.0	0.51	0.947
LM	17.9 ± 4.2		19.2 ± 3.8		18.6 ± 3.8		20.9 ± 3.9		19.5 ± 4.7		0.466
VCL (µm/s)											
HM	160.1 ± 48.1	0.7065	195.5 ± 50.0	0.2472	189.8 ± 52.1	0.4294	**183.1 ± 41.8****	**0.002**	187.5 ± 52.1	0.2470	0.058
LM	167.4 ± 56.0		176.0 ± 29.3		196.7 ± 49.3		**145.1 ± 48.3****		165.2 ± 71.5		0.094
VAP (µm/s)											
HM	59.8 ± 16.3	0.5226	63.5 ± 13.9	0.8879	62.3 ± 17.7	0.6603	57.7 ± 20.1	0.0863	57.6 ± 17.0	0.4852	0.452
LM	63.5 ± 18.3		64.3 ± 15.8		60.1 ± 13.0		64.4 ± 15.4		61.9 ± 23.2		0.963
VSL (µm/s)											
HM	47.3 ± 17.8	0.9691	52.6 ± 16.6	0.9155	47.4 ± 11.5	0.8649	45.4 ± 12.4	0.4832	47.3 ± 17.6	0.0508	0.335
LM	47.5 ± 17.7		51.9 ± 15.4		46.8 ± 13.5		48.5 ± 17.7		36.2 ± 15.1		0.158

STR (%)											
HM	80.0 ± 22.4	0.4539	84.0 ± 22.0	0.7354	80.3 ± 23.3	0.8843	83.8 ± 22.3	0.6748	**84.1 ± 24.7****	**0.0048**	0.878
LM	75.8 ± 20.7		83.7 ± 24.4		81.3 ± 25.3		78.2 ± 27.9		**61.0 ± 20.7****		0.162
LIN (%)											
HM	29.9 ± 10.5	0.8891	27.4 ± 7.6	0.2941	26.1 ± 7.1	0.6013	25.7 ± 8.1	0.0662	26.2 ± 9.2	0.4495	0.275
LM	30.5 ± 11.3		30.6 ± 10.9		24.9 ± 8.4		35.7 ± 16.5		23.9 ± 10.1		0.145
WOB (%)											
HM	38.8 ± 11.5	0.6964	34.4 ± 10.8	0.2581	34.7 ± 12.1	0.5049	**32.6 ± 12.1****	**0.0013**	**32.5 ± 10.4***	**0.0325**	0.67
LM	40.5 ± 12.1^{ab}		36.9 ± 8.3^{ab}		32.4 ± 10.3^{a}		**46.9 ± 13.4**$^{b\,**}$		**40.8 ± 13.9**$^{ab\,*}$		**0.036**
ALH (µm)											
HM	1.9 ± 0.6	0.3997	2.2 ± 0.6	0.2276	2.3 ± 0.8	0.6695	**2.3 ± 0.7*****	**<0.001**	2.3 ± 0.8	0.6354	0.065
LM	2.1 ± 0.9		2.0 ± 0.5		2.4 ± 0.7		**1.6 ± 0.7*****		2.2 ± 0.9		0.079
ALHmax (µm)											
HM	4.1 ± 1.4^{a}	0.6027	4.9 ± 1.2^{ab}	0.3636	5.1 ± 1.4^{b}	0.8180	**4.9 ± 1.4**$^{b\,*}$	**0.0103**	4.9 ± 1.7^{b}	0.7167	**0.047**
LM	4.4 ± 1.8		4.5 ± 1.3		5.2 ± 1.0		**3.7 ± 1.3***		4.7 ± 1.6		0.106
BCF (Hz)											
HM	25.9 ± 5.9	0.9687	27.5 ± 6.0	0.3407	26.2 ± 6.9	0.4829	26.6 ± 7.1	0.8275	27.2 ± 6.4	0.1616	0.83
LM	25.9 ± 2.8		29.6 ± 5.4		27.7 ± 6.3		27.1 ± 7.8		24.3 ± 5.2		0.295
MAD (degrees)											
HM	231.0 ± 46.3	0.8080	220.0 ± 48.3	0.9172	221.6 ± 37.5	0.0536	235.5 ± 43.8	0.0502	229.3 ± 36.9	0.2174	0.441
LM	226.7 ± 42.8		220.9 ± 53.2		196.6 ± 54.5		213.8 ± 36.7		243.5 ± 27.8		0.095

Note: Compared to LM subpopulations, the HM subpopulation yielded significantly higher values for mean power, fAWS, VCL, STR and ALH. Significant differences between media were observed in the HM subpopulations for ALH and fAWL, and in the LM subpopulations for WOB. **Abbreviations:** ALH, amplitude of lateral head displacement; ALHmax, maximum amplitude of lateral head displacement; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; FAST, flagellar analysis and sperm tracking; fAWL, flagellar arc-wavelength; fAWS, flagellar arc-wave speed; fBF, flagellar beat frequency; HTF, human tubal fluid; HM, high motile subpopulation; Hz, hertz; LIN, linearity; LM, low motile subpopulation; MAD, mean angle displacement; Max FL, maximum analysed flagellum; PRL, prolactin; SD, standard deviation; STR, straightness; TCS, track centroid speed; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; Watt, wattage; WOB, wobble. $^{a,\,b,c,d}$ Values labelled with different superscript letters in the same row were significantly different between the various media for individual subpopulations and FAST parameters. Values labelled in bold with an asterisk in the same column were significantly different between the HM and LM subpopulations for individual media and FAST parameters (*p <0.05, **p <0.01 and ***p <0.001).

Supplementary Table 10: Heatmap of the correlations between individual FAST parameters and the whole SCA sperm subpopulation parameters for both the high motile (HM) and low motile (LM) sperm subpopulations in HTF.

				FAST Individual Spermatozoa																
				Flagellar Parameters							Velocity			Linear		Vigour				
				FL	Max FL	fAWL	fAWS	fBF	Mean power	TCS	VCL	VAP	VSL	STR	LIN	WOB	ALH avg	ALH max	BCF	MAD
Whole SCA Sperm Subpopulation	Motility Parameters	Immotile																		
			HM	-0.01	0,00	-0.01	0.01	0.04	0.12	-0.11	0.1	-0.01	-0.11	-0.07	-0.12	-0.09	0.11	0.1	-0.04	0.04
			LM	-0.03	0.08	-0.05	0.03	0.07	0.02	-0.17	-0.14	-0.1	-0.16	-0.13	-0.06	0.1	-0.08	-0.03	-0.17	0.1
		Motile																		
			HM	0.01	0,00	0.01	-0.01	-0.04	-0.12	0.11	-0.1	0.01	0.11	0.07	0.12	0.09	-0.11	-0.1	0.04	-0.04
			LM	0.03	-0.08	0.05	-0.03	-0.07	-0.02	0.17	0.14	0.1	0.16	0.13	0.06	-0.1	0.08	0.03	0.17	-0.1
		Prog																		
			HM	-0.01	0.01	0.08	0.07	0.01	0.04	0.21	0.15	0.17	0.21	0.06	-0.01	-0.06	0.08	0.05	0.17	-0.07
			LM	0.05	0.02	0.09	0.02	-0.06	0.06	0.24	0.3	0.16	0.24	0.15	-0.03	-0.21	0.24	0.19	0.15	-0.12
		RP																		
			HM	0.03	-0.02	0.15	0.2	0.05	0.15	0.21	0.27	0.16	0.2	0.06	-0.1	-0.18	0.15	0.14	0.18	0.01
			LM	-0.04	0.07	0.15	0.07	-0.08	0.14	0.21	0.46	0.16	0.23	0.11	-0.13	-0.36	0.41	0.37	0.18	-0.12
		MP																		
			HM	-0.07	0.04	-0.1	-0.12	-0.05	-0.1	0.06	-0.04	0.06	0.06	-0.02	0.04	0.06	-0.01	-0.03	0.03	-0.06
			LM	0.08	-0.04	-0.02	-0.04	-0.01	-0.02	0.19	0.11	0.11	0.16	0.1	0.1	-0.05	0.1	0.07	0,00	-0.08
		NP																		
			HM	-0.02	-0.05	-0.13	-0.16	-0.09	-0.19	-0.18	-0.35	-0.22	-0.18	0.02	0.17	0.2	-0.27	-0.23	-0.18	0.05
			LM	0.01	-0.24	-0.04	-0.12	-0.09	-0.12	-0.05	-0.17	-0.07	-0.06	0.01	0.2	0.1	-0.22	-0.23	0.12	-0.04
		Rapid																		
			HM	-0.01	-0.01	0.11	0.14	0.05	0.26	0.16	0.4	0.2	0.15	-0.05	-0.26	-0.28	0.32	0.3	0.2	0.08
			LM	0,00	0.07	0.16	0.06	-0.09	0.19	0.19	0.46	0.2	0.19	0.04	-0.2	-0.35	0.43	0.4	0.12	-0.07
		Medium																		
			HM	0,00	0.02	-0.1	-0.12	-0.05	-0.37	-0.01	-0.45	-0.15	0.01	0.16	0.38	0.33	-0.37	-0.37	-0.14	-0.13
			LM	0.13	-0.15	-0.04	-0.03	-0.01	-0.1	0.1	-0.04	-0.01	0.1	0.12	0.16	0.04	-0.05	-0.11	0.01	-0.05
		Slow																		
			HM	0,00	-0.04	-0.11	-0.18	-0.13	-0.18	-0.15	-0.32	-0.26	-0.16	0.06	0.17	0.16	-0.28	-0.24	-0.12	0.05
			LM	-0.05	-0.17	-0.06	-0.16	-0.1	-0.16	0.01	-0.16	-0.06	0.01	0.11	0.32	0.11	-0.32	-0.35	0.2	-0.14

Whole SCA Sperm Subpopulation	Motile/Average	Velocity	VCL	HM	0.04	0.06	0.1	0.11	0.06	0.29	0.15	0.47	0.24	0.14	-0.1	-0.34	-0.32	0.39	0.38	0.17	0.11
				LM	0.02	0.18	0.14	0.04	-0.09	0.23	0.16	0.48	0.3	0.13	-0.08	-0.3	-0.32	0.52	0.5	0.03	0,00
			VAP	HM	0.17	0.14	0.11	0.1	0.04	0.13	0.27	0.29	0.25	0.25	0,00	-0.11	-0.14	0.2	0.21	0.17	-0.03
				LM	-0.02	0.26	0.04	-0.08	-0.06	0.11	0.08	0.38	0.21	0.06	-0.08	-0.26	-0.27	0.41	0.4	0.04	0.01
			VSL	HM	0.17	0.08	0.14	0.13	0,00	-0.01	0.27	0.07	0.14	0.27	0.14	0.12	0.01	-0.04	-0.03	0.14	-0.13
				LM	-0.04	0.21	0.03	-0.04	-0.05	0.01	0.18	0.31	0.13	0.18	0.1	-0.06	-0.19	0.27	0.22	0.12	-0.09
		Linear	STR	HM	-0.03	-0.1	0.1	0.07	-0.06	-0.25	0.12	-0.3	-0.11	0.14	0.27	0.37	0.21	-0.37	-0.39	0.05	-0.21
				LM	-0.05	0.05	0.08	0.14	0.02	-0.12	0.26	0.06	0.01	0.23	0.3	0.26	-0.05	-0.02	-0.11	0.09	-0.16
			LIN	HM	0.05	-0.04	0.07	0.02	-0.08	-0.29	0.13	-0.34	-0.1	0.15	0.25	0.4	0.26	-0.39	-0.4	0.03	-0.21
				LM	-0.04	0.08	-0.08	-0.02	0.04	-0.22	0.1	-0.08	-0.1	0.09	0.21	0.29	0.03	-0.13	-0.19	0.07	-0.1
		Vigour	WOB	HM	0.13	0.03	0,00	-0.05	-0.08	-0.33	0.11	-0.39	-0.06	0.12	0.2	0.42	0.34	-0.38	-0.38	-0.04	-0.19
				LM	-0.06	0.1	-0.2	-0.16	-0.01	-0.35	-0.11	-0.23	-0.17	-0.14	-0.02	0.21	0.16	-0.18	-0.21	-0.1	0.07
			ALH	HM	-0.06	0.03	0.04	0.07	0.09	0.31	0.04	0.48	0.22	0.03	-0.19	-0.41	-0.34	0.45	0.44	0.1	0.15
				LM	0.14	0.21	0.21	0.21	0.05	0.32	0.21	0.42	0.28	0.21	0.03	-0.18	-0.26	0.47	0.46	0.01	0.03
			BCF	HM	-0.1	-0.07	0.02	0.02	-0.05	-0.25	0.04	-0.28	-0.12	0.05	0.17	0.28	0.19	-0.35	-0.35	0.05	-0.14
				LM	0.05	-0.08	-0.05	-0.16	-0.03	-0.13	-0.05	-0.14	-0.08	0.02	0.1	0.11	0.09	-0.21	-0.27	0.08	-0.04
	Rapid Progressive	Velocity	VCL	HM	0.12	0.13	0.02	0.02	0.03	0.21	0.09	0.31	0.09	0.07	-0.04	-0.24	-0.26	0.27	0.29	0.12	0.1
				LM	-0.03	-0.01	0.16	-0.06	-0.22	0.17	-0.04	0.33	0.11	0,00	-0.11	-0.35	-0.27	0.27	0.27	0.14	-0.07
			VAP	HM	0.15	0.13	0.02	-0.01	-0.01	0,00	0.18	0.07	0.11	0.17	0.05	0.03	-0.02	0.01	0.04	0.13	-0.06
				LM	-0.11	0.05	-0.05	-0.18	-0.16	-0.09	-0.14	0.13	0.05	-0.04	-0.14	-0.2	-0.02	0.08	0.09	0.08	-0.1
			VSL	HM	0.14	0.11	0.03	-0.01	-0.01	-0.05	0.16	-0.01	0.05	0.15	0.07	0.09	0.02	-0.07	-0.04	0.12	-0.07
				LM	-0.1	0.04	-0.07	-0.16	-0.12	-0.13	-0.13	0.07	0.02	-0.02	-0.07	-0.15	0,00	0.01	0.01	0.08	-0.14
		Linear	STR	HM	0.08	0.01	0.02	-0.02	-0.07	-0.26	0.1	-0.33	-0.13	0.09	0.21	0.34	0.24	-0.36	-0.34	0.02	-0.18
				LM	0,00	0.04	-0.02	-0.04	-0.04	-0.15	0.09	-0.05	0.04	0.08	0.12	0.12	0.08	-0.13	-0.16	0.11	-0.19
			LIN	HM	0.12	0.06	0.02	-0.04	-0.06	-0.22	0.11	-0.26	-0.03	0.11	0.12	0.29	0.23	-0.29	-0.27	0.05	-0.16
				LM	-0.1	0.05	-0.12	-0.17	-0.07	-0.24	-0.1	-0.09	0.01	-0.03	-0.05	-0.02	0.14	-0.13	-0.13	0.06	-0.1
		Vigour	WOB	HM	0.14	0.08	-0.01	-0.04	-0.05	-0.24	0.12	-0.25	0.01	0.11	0.1	0.28	0.25	-0.28	-0.25	0.04	-0.16
				LM	-0.15	0.06	-0.16	-0.2	-0.05	-0.22	-0.12	-0.05	0.03	-0.04	-0.1	-0.03	0.14	-0.08	-0.07	0.07	-0.07
			ALH	HM	-0.09	-0.01	-0.04	0.02	0.06	0.24	0,00	0.35	0.13	-0.01	-0.11	-0.32	-0.27	0.34	0.32	0.02	0.14
				LM	0.14	0.11	0.26	0.29	0.02	0.3	0.26	0.32	0.14	0.28	0.18	-0.02	-0.24	0.32	0.29	0.05	-0.03
			BCF	HM	-0.13	-0.13	0.12	0.15	0.02	-0.02	0.05	-0.01	-0.01	0.04	0.04	0.03	0,00	-0.11	-0.12	0.11	-0.02
				LM	0.04	-0.08	0.02	-0.1	-0.08	0.02	0.04	-0.06	0,00	0.07	0.12	0.14	0.06	-0.14	-0.19	0.08	-0.06

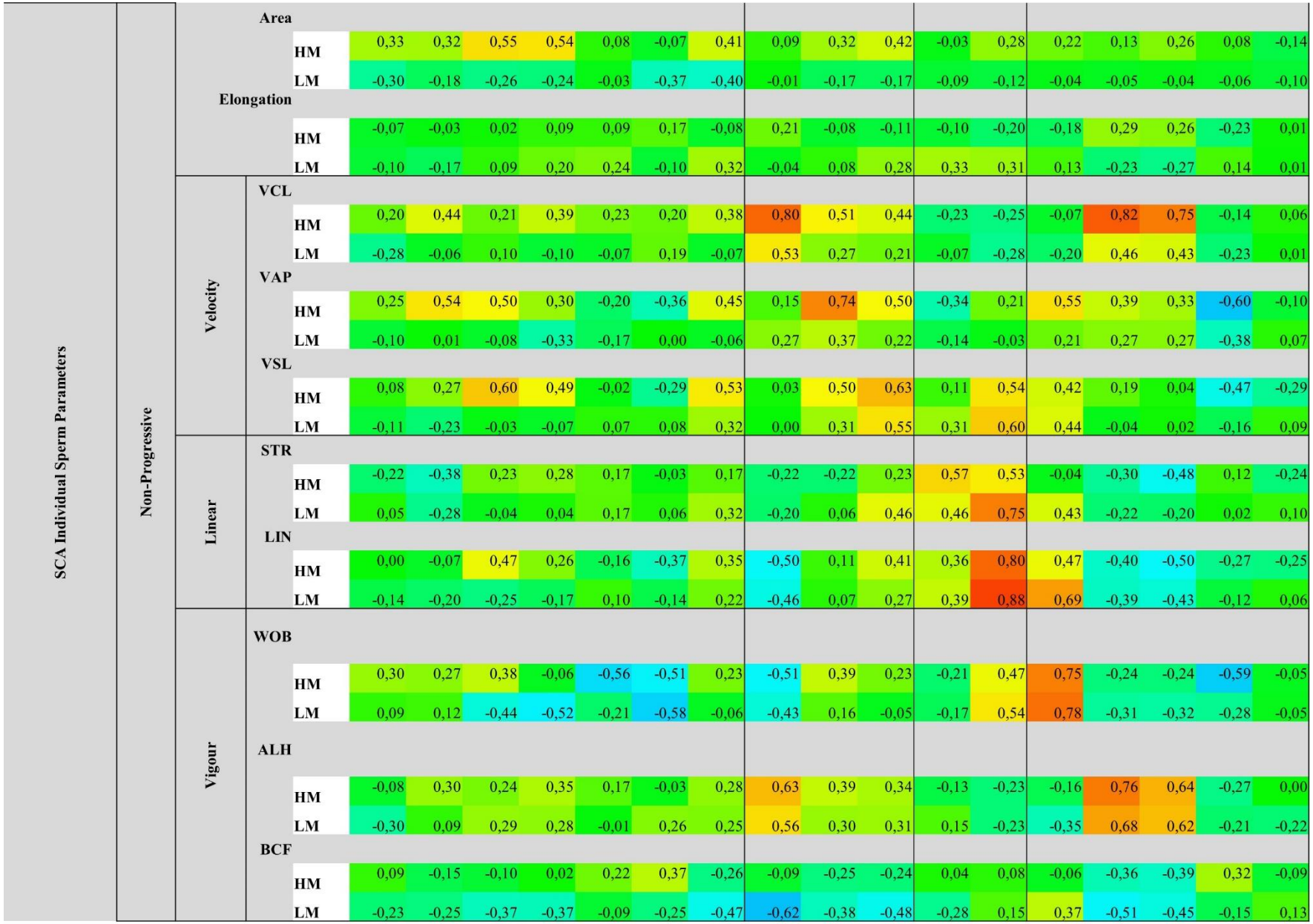
Whole SCA Sperm Subpopulation																				
Medium Progressive	Velocity	VCL	HM	0.01	0.07	0.01	0.01	0.04	0.36	-0.01	0.48	0.17	-0.03	-0.23	-0.45	-0.36	0.47	0.46	0.11	0.19
			LM	0.01	0.08	0.08	0.02	-0.03	0.32	0.06	0.38	0.28	0.05	-0.16	-0.3	-0.25	0.39	0.43	0.01	0,00
		VAP	HM	0.1	0.14	0.07	0.05	0.03	0.28	0.19	0.44	0.29	0.17	-0.13	-0.29	-0.26	0.36	0.35	0.19	0.04
			LM	-0.04	0.17	-0.04	-0.15	-0.04	0.14	-0.03	0.3	0.14	0.02	-0.11	-0.25	-0.22	0.3	0.33	0.03	-0.03
		VSL	HM	0.07	-0.01	0.11	0.08	-0.05	-0.24	0.15	-0.27	-0.04	0.16	0.21	0.35	0.23	-0.33	-0.33	0.03	-0.19
			LM	-0.06	0.03	-0.13	-0.08	0.06	-0.16	0.04	-0.03	-0.13	0.06	0.18	0.12	-0.07	-0.06	-0.08	0.02	-0.08
	Linear	STR	HM	0.01	-0.06	0.07	0.01	-0.06	-0.32	0.08	-0.39	-0.15	0.1	0.26	0.42	0.28	-0.43	-0.43	-0.03	-0.2
			LM	-0.05	-0.05	-0.09	0.01	0.06	-0.25	0.06	-0.22	-0.2	0.05	0.22	0.27	0.12	-0.27	-0.32	0.02	-0.08
		LIN	HM	0.03	-0.05	0.08	0.03	-0.06	-0.3	0.11	-0.35	-0.09	0.13	0.23	0.41	0.28	-0.4	-0.41	-0.01	-0.2
			LM	-0.03	-0.01	-0.16	-0.07	0.07	-0.25	-0.01	-0.23	-0.21	0,00	0.19	0.22	0.08	-0.26	-0.29	0.01	-0.05
	Vigour	WOB	HM	0.07	0.01	0.07	0.03	-0.06	-0.27	0.13	-0.32	-0.04	0.15	0.21	0.39	0.28	-0.35	-0.37	0,00	-0.21
			LM	-0.05	0.05	-0.22	-0.17	0.03	-0.29	-0.1	-0.22	-0.21	-0.06	0.1	0.15	0.1	-0.22	-0.23	-0.03	-0.02
		ALH	HM	0,00	0.07	-0.04	-0.02	0.06	0.31	-0.06	0.42	0.17	-0.08	-0.26	-0.43	-0.3	0.45	0.45	0.02	0.19
			LM	0.11	0.14	0.15	0.18	0.09	0.35	0.12	0.33	0.26	0.11	-0.08	-0.2	-0.2	0.4	0.43	-0.07	0.05
		BCF	HM	-0.02	-0.05	0.01	0.03	-0.04	-0.26	0.04	-0.33	-0.14	0.05	0.18	0.32	0.22	-0.4	-0.38	0.02	-0.13
			LM	0.02	-0.08	-0.04	-0.18	-0.06	-0.23	-0.04	-0.15	-0.09	0.01	0.09	0.15	0.09	-0.22	-0.27	0.1	-0.08
Non-Progressive	Velocity	VCL	HM	0.01	-0.02	-0.05	-0.05	-0.01	-0.06	-0.07	-0.06	0.04	-0.05	-0.09	0,00	0.07	0.03	0.03	-0.17	0.06
			LM	0.22	-0.02	0.03	0.07	0,00	0.04	-0.1	-0.07	-0.02	-0.13	-0.16	-0.1	0.05	0.12	0.12	-0.18	0.17
		VAP	HM	0.03	-0.04	-0.04	-0.05	-0.05	-0.24	-0.01	-0.21	0,00	0.02	0.04	0.17	0.18	-0.14	-0.14	-0.17	0.02
			LM	0.15	0.06	0.05	0.02	-0.08	-0.07	-0.14	-0.08	-0.07	-0.19	-0.2	-0.03	0.06	0.08	0.07	-0.16	0.18
		VSL	HM	0,00	-0.11	0.04	0.01	-0.1	-0.34	0.01	-0.3	-0.13	0.04	0.17	0.29	0.19	-0.27	-0.3	-0.08	-0.09
			LM	0.1	0.08	0.16	0.13	-0.01	0.01	0.03	-0.02	-0.07	-0.01	0.03	0.2	-0.01	0.03	-0.04	-0.08	0.03
	Linear	STR	HM	-0.07	-0.17	0.09	0.06	-0.09	-0.23	0.07	-0.25	-0.18	0.1	0.26	0.29	0.11	-0.32	-0.34	0.05	-0.15
			LM	-0.03	-0.03	0.13	0.19	0.05	-0.04	0.1	-0.08	-0.06	0.05	0.17	0.23	0.02	-0.11	-0.19	0.02	-0.1
		LIN	HM	0,00	-0.11	0.06	0.04	-0.08	-0.26	0.09	-0.24	-0.12	0.11	0.23	0.3	0.14	-0.29	-0.31	0.02	-0.12
			LM	0.02	0.07	0.13	0.09	-0.06	-0.11	0.07	-0.04	-0.06	0.05	0.13	0.26	0.03	-0.07	-0.18	0.06	-0.07
	Vigour	WOB	HM	0.02	-0.06	-0.02	-0.03	-0.07	-0.34	0.07	-0.3	-0.07	0.09	0.18	0.3	0.22	-0.27	-0.3	-0.1	-0.05
			LM	0.11	0.07	0.05	-0.02	-0.08	-0.14	-0.09	-0.1	-0.08	-0.13	-0.08	0.13	0.08	-0.03	-0.09	-0.05	0.11

Note: SCA whole subpopulation parameters are divided into motility and kinematic groups. Motility groups include the different motility parameters, and the kinematic group includes the different kinematic parameters for the different progressive speed groups (average, rapid, medium, and slow). Kinematics are further categorized into velocity (VCL, VAP and VSL), linear (LIN and STR) and vigour (WOB, BCF and ALH) groups. FAST parameters are grouped in flagellar parameters and kinematic parameters. The FAST flagellar parameters correlated with the whole SCA sperm subpopulation kinematics, whereas FAST kinematic parameters correlated with both SCA motility and kinematic parameters. **Abbreviations:** ALH, amplitude of lateral head displacement; avg, average; BCF, beat cross frequency; FAST, flagellar analysis and sperm tracking; fAWL, flagellar arc-wavelength; fAWS, flagellar arc-wave speed; fBF, flagellar beat frequency; FL, flagellar length; high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MAD, mean angle displacement; max, maximum; MP, medium progressive; NP, non-progressive; RP, rapid progressive; SCA, sperm class analyser; STR, straightness; TCS, track centroid speed; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

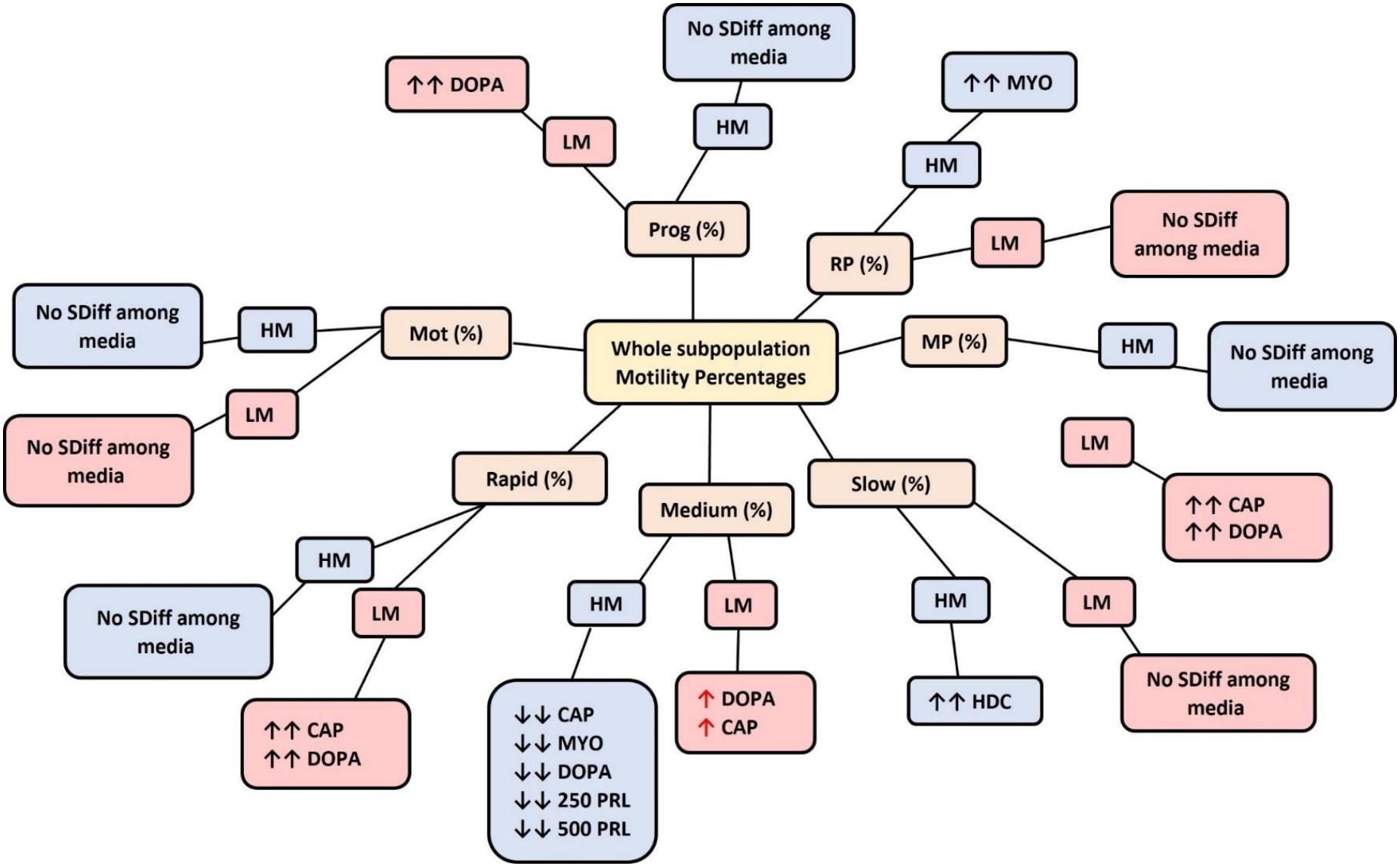
Supplementary Table 11: Heatmap of the correlations between individual FAST parameters and the individual SCA sperm parameters for both the high motile (HM) and low motile (LM) sperm subpopulations in HTF.

					Flagellar Parameters							Kinematic Parameters									
												Velocity			Linear		Vigour				
					Analysed FL	Max FL	fAWL	fAWS	fBF	Mean power	TCS	VCL	VAP	VSL	STR	LIN	WOB	ALHavg	ALHmax	BCF	MAD
SCA Individual Sperm Parameters	Rapid Progressive		Area																		
				HM	0,13	0,20	0,05	-0,08	-0,16	-0,03	0,04	0,00	0,04	0,02	-0,02	0,04	0,07	-0,02	-0,03	0,08	-0,16
				LM	0,32	0,04	0,07	0,04	-0,04	0,23	0,01	-0,21	-0,26	-0,08	0,29	0,16	0,02	-0,21	-0,37	0,16	0,00
			Elongation																		
				HM	-0,10	-0,06	0,05	-0,04	-0,11	0,14	-0,11	0,02	-0,06	-0,08	-0,03	-0,05	-0,03	0,03	0,03	0,02	-0,07
				LM	0,15	0,25	-0,16	-0,09	0,20	-0,05	-0,12	0,14	-0,11	-0,10	0,05	-0,23	-0,25	0,14	0,13	-0,24	-0,02
		Velocity	VCL																		
				HM	-0,27	-0,11	0,19	0,11	-0,12	0,35	0,16	0,88	0,35	0,17	-0,30	-0,59	-0,49	0,72	0,70	0,02	0,18
				LM	-0,09	0,16	0,05	-0,13	-0,17	0,18	0,10	0,74	0,20	0,12	-0,11	-0,53	-0,44	0,60	0,57	0,06	-0,05
			VAP																		
				HM	0,05	0,15	0,11	0,11	-0,01	0,31	0,58	0,46	0,76	0,61	-0,21	0,04	0,18	0,42	0,47	0,12	-0,06
				LM	-0,07	0,20	0,09	-0,10	-0,11	0,15	0,22	0,39	0,59	0,37	-0,25	-0,07	0,14	0,44	0,39	0,13	-0,03
			VSL																		
				HM	0,07	0,15	0,18	0,12	-0,12	-0,01	0,79	0,27	0,70	0,80	0,12	0,45	0,38	0,12	0,12	0,27	-0,34
				LM	0,11	-0,01	0,17	0,05	-0,19	-0,19	0,72	-0,01	0,59	0,79	0,19	0,54	0,47	-0,20	-0,28	-0,06	-0,54
		Linear	STR																		
				HM	0,08	-0,03	0,12	0,02	-0,19	-0,21	0,40	-0,37	0,07	0,41	0,57	0,60	0,38	-0,58	-0,60	0,24	-0,41
				LM	0,22	-0,19	0,12	0,13	-0,09	-0,24	0,55	-0,33	0,08	0,54	0,50	0,64	0,38	-0,62	-0,66	-0,14	-0,58
			LIN																		
				HM	0,35	0,17	-0,03	-0,02	-0,05	-0,15	0,45	-0,60	0,23	0,47	0,41	0,88	0,77	-0,61	-0,57	0,16	-0,39
				LM	0,10	-0,16	0,08	0,12	0,04	-0,30	0,39	-0,59	0,20	0,41	0,22	0,83	0,72	-0,67	-0,71	-0,08	-0,34
		Vigour	WOB																		
				HM	0,39	0,23	-0,15	-0,05	0,09	-0,15	0,35	-0,52	0,29	0,38	0,10	0,76	0,75	-0,39	-0,33	0,08	-0,30
				LM	0,00	-0,04	-0,07	0,06	0,14	-0,23	0,06	-0,52	0,20	0,12	-0,18	0,58	0,67	-0,35	-0,37	-0,04	0,03
			ALH																		
				HM	-0,29	-0,12	0,18	0,06	-0,22	0,19	0,05	0,78	0,19	0,05	-0,26	-0,60	-0,55	0,70	0,63	-0,04	0,20
				LM	0,01	0,28	0,03	-0,06	-0,20	0,22	0,15	0,79	0,26	0,17	-0,14	-0,52	-0,43	0,67	0,64	0,00	-0,01
			BCF																		
				HM	0,08	0,06	0,16	0,23	0,08	0,02	0,04	-0,24	0,03	0,03	0,06	0,21	0,27	-0,43	-0,33	0,37	-0,08
				LM	-0,32	-0,29	-0,05	0,17	0,26	-0,19	-0,13	-0,35	-0,13	-0,12	-0,01	0,23	0,22	-0,55	-0,36	0,24	-0,01

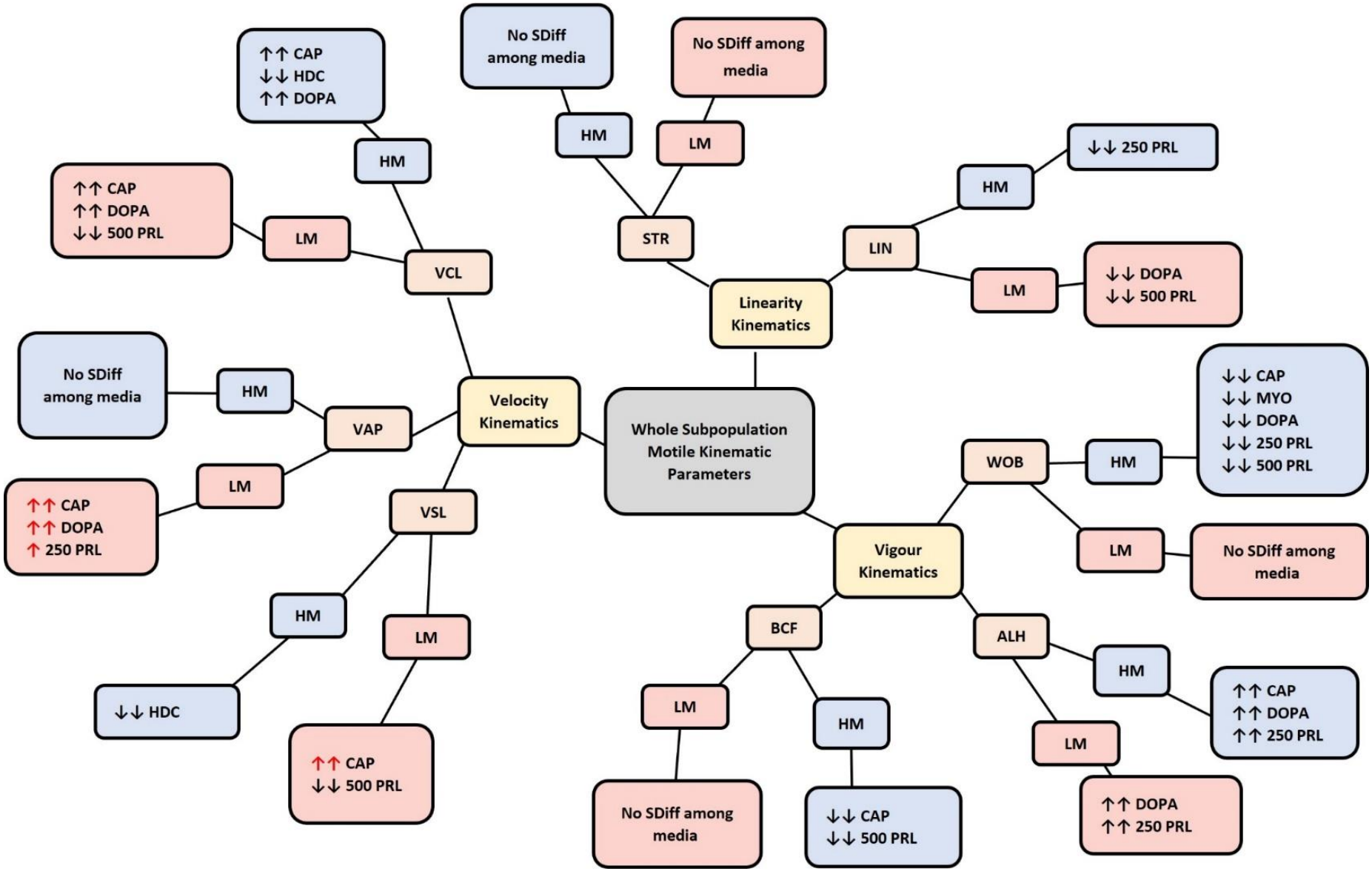
SCA Individual Sperm Parameters	Medium Progressive																			
		Area																		
			HM	0,12	0,07	0,16	-0,08	-0,26	-0,19	0,03	0,02	-0,01	0,01	0,07	0,05	-0,01	0,03	-0,04	-0,01	-0,03
			LM	-0,02	-0,02	0,02	-0,10	-0,01	-0,10	0,02	0,10	0,08	-0,01	0,03	-0,06	-0,18	0,12	0,07	-0,07	0,00
		Elongation																		
			HM	0,03	0,17	0,06	0,09	-0,01	0,11	-0,05	0,09	0,03	-0,01	-0,08	-0,03	-0,02	0,11	0,11	-0,19	-0,04
			LM	0,08	-0,02	0,11	0,13	-0,02	0,16	0,12	-0,05	-0,01	0,08	0,06	0,08	0,02	-0,06	-0,04	-0,11	0,07
		Velocity	VCL																	
			HM	-0,09	0,09	0,31	0,11	-0,22	0,41	-0,16	0,94	0,37	-0,22	-0,53	-0,80	-0,65	0,87	0,84	0,01	0,11
			LM	-0,12	0,13	0,52	0,11	-0,42	0,34	-0,11	0,82	0,44	-0,16	-0,52	-0,73	-0,71	0,82	0,77	-0,10	0,24
			VAP																	
			HM	-0,02	0,20	0,20	-0,06	-0,27	0,32	0,04	0,77	0,62	-0,02	-0,48	-0,55	-0,30	0,75	0,75	-0,11	-0,05
			LM	0,02	0,23	0,40	0,02	-0,38	0,29	0,07	0,62	0,59	0,05	-0,44	-0,51	-0,36	0,66	0,64	-0,18	0,19
			VSL																	
			HM	0,07	0,04	-0,05	-0,01	0,10	-0,15	0,81	-0,33	0,26	0,86	0,60	0,70	0,46	-0,43	-0,43	0,09	-0,41
			LM	0,05	0,18	-0,17	0,08	0,29	-0,04	0,67	-0,14	0,28	0,74	0,56	0,64	0,41	-0,20	-0,22	0,09	-0,34
		Linear	STR																	
			HM	0,06	-0,08	-0,20	-0,09	0,15	-0,32	0,61	-0,74	-0,13	0,67	0,78	0,91	0,59	-0,81	-0,80	0,11	-0,35
			LM	0,12	-0,03	-0,41	0,04	0,53	-0,31	0,48	-0,60	-0,17	0,55	0,75	0,87	0,62	-0,72	-0,71	0,18	-0,44
			LIN																	
			HM	0,11	-0,03	-0,24	-0,14	0,13	-0,34	0,54	-0,81	-0,08	0,60	0,69	0,94	0,72	-0,82	-0,80	0,00	-0,32
			LM	0,12	-0,02	-0,46	-0,03	0,49	-0,32	0,46	-0,67	-0,11	0,52	0,69	0,92	0,70	-0,75	-0,72	0,16	-0,37
		Vigour	WOB																	
			HM	0,15	0,09	-0,34	-0,28	0,04	-0,24	0,32	-0,70	0,12	0,38	0,30	0,75	0,83	-0,53	-0,49	-0,27	-0,25
			LM	0,14	0,05	-0,39	-0,21	0,25	-0,02	0,35	-0,62	0,04	0,33	0,33	0,75	0,80	-0,54	-0,50	-0,04	-0,15
			ALH																	
			HM	-0,10	0,07	0,34	0,08	-0,30	0,34	-0,23	0,90	0,30	-0,28	-0,55	-0,81	-0,67	0,88	0,84	-0,02	0,13
			LM	-0,07	0,13	0,56	0,11	-0,46	0,36	-0,07	0,83	0,44	-0,10	-0,47	-0,73	-0,71	0,85	0,77	-0,10	0,24
			BCF																	
			HM	0,04	-0,07	-0,20	0,05	0,28	-0,12	0,25	-0,56	-0,20	0,26	0,39	0,55	0,43	-0,67	-0,62	0,20	-0,05
			LM	0,13	-0,04	-0,42	-0,01	0,43	0,03	-0,02	-0,63	-0,20	0,00	0,22	0,53	0,59	-0,72	-0,61	0,25	-0,11



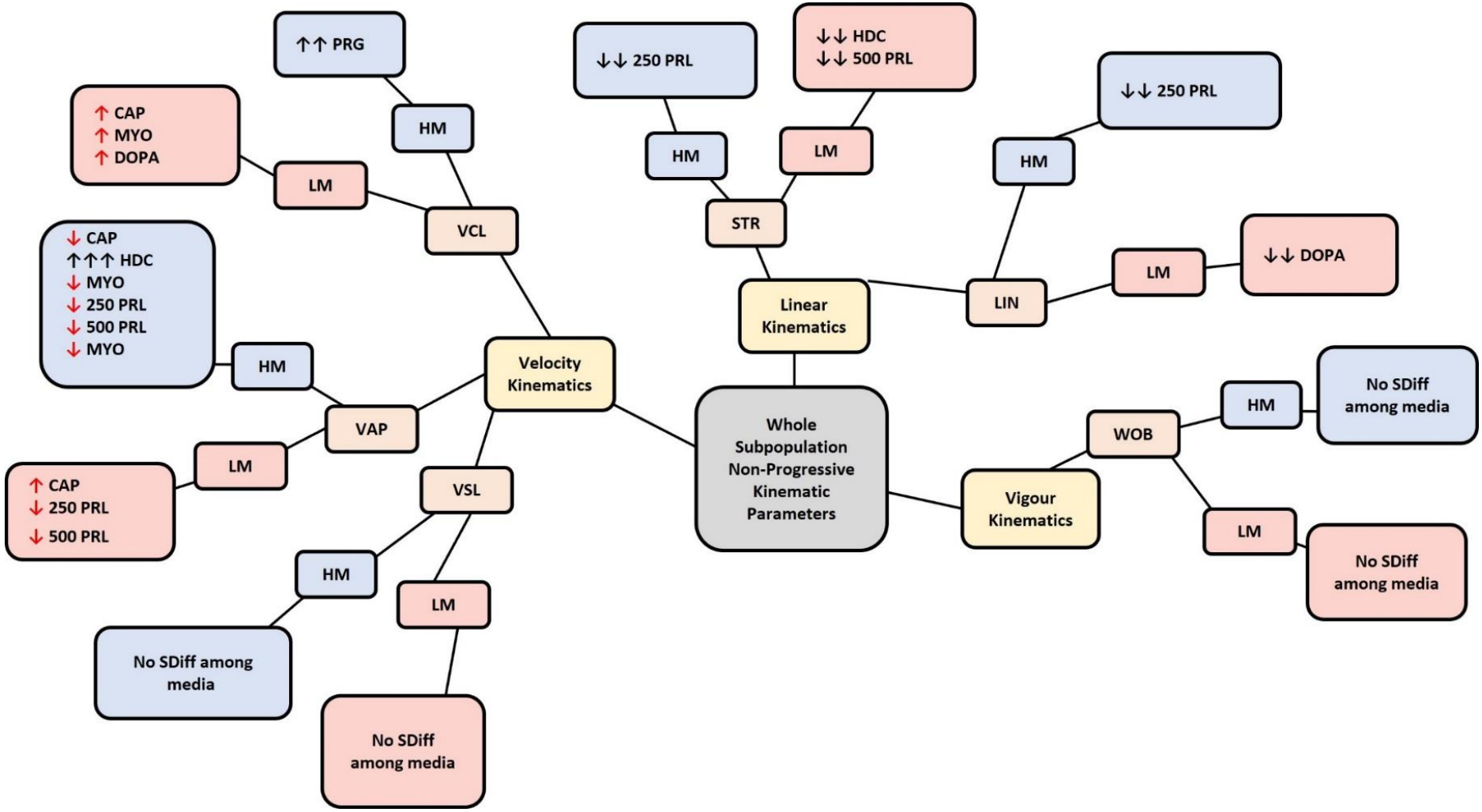
Note: SCA individual sperm parameters are divided into kinematic groups. Kinematic groups include the different kinematic parameters for the different progressive speed groups (average, rapid, medium, and slow). Kinematics are further categorized into velocity (VCL, VAP and VSL), linear (LIN and STR) and vigour (WOB, BCF and ALH) groups. FAST parameters are grouped in flagellar parameters and kinematic parameters. The FAST flagellar parameters correlated with the whole SCA sperm subpopulation kinematics, whereas FAST kinematic parameters correlated with both SCA motility and kinematic parameters. **Abbreviations:** ALH, amplitude of lateral head displacement; avg, average; BCF, beat cross frequency; FAST, flagellar analysis and sperm tracking; fAWL, flagellar arc-wavelength; fAWS, flagellar arc-wave speed; fBF, flagellar beat frequency; FL, flagellar length; high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MAD, mean angle displacement; max, maximum; MP, medium progressive; NP, non-progressive; RP, rapid progressive; SCA, sperm class analyser; STR, straightness; TCS, track centroid speed; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.



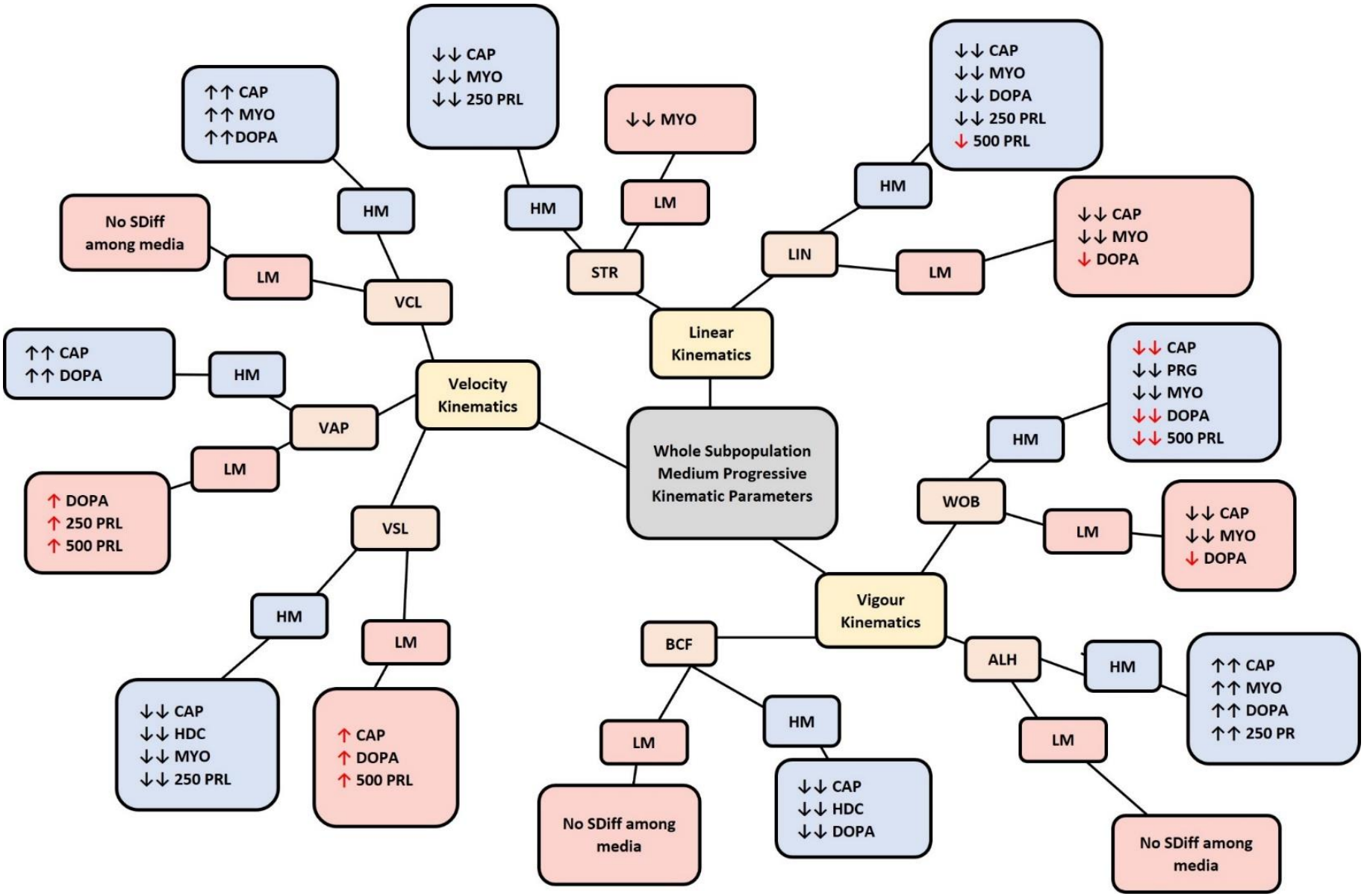
Supplementary Figure 1. Bubble diagram displaying the various motility parameters of spermatozoa for both subpopulations after being flushed with various media. ***Note:*** Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values until significant differences between subpopulations were no longer observed. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. Student's *t*-test or the Mann-Whitney test when normal distribution was void in addition to one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions; $p < 0.05$. **Abbreviations:** CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LM, low motile subpopulation; Mot, total motility; MP, medium progressive; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; PRL, prolactin; Prog, progressive motility; RP, rapid progressive; SDiff; significant difference.



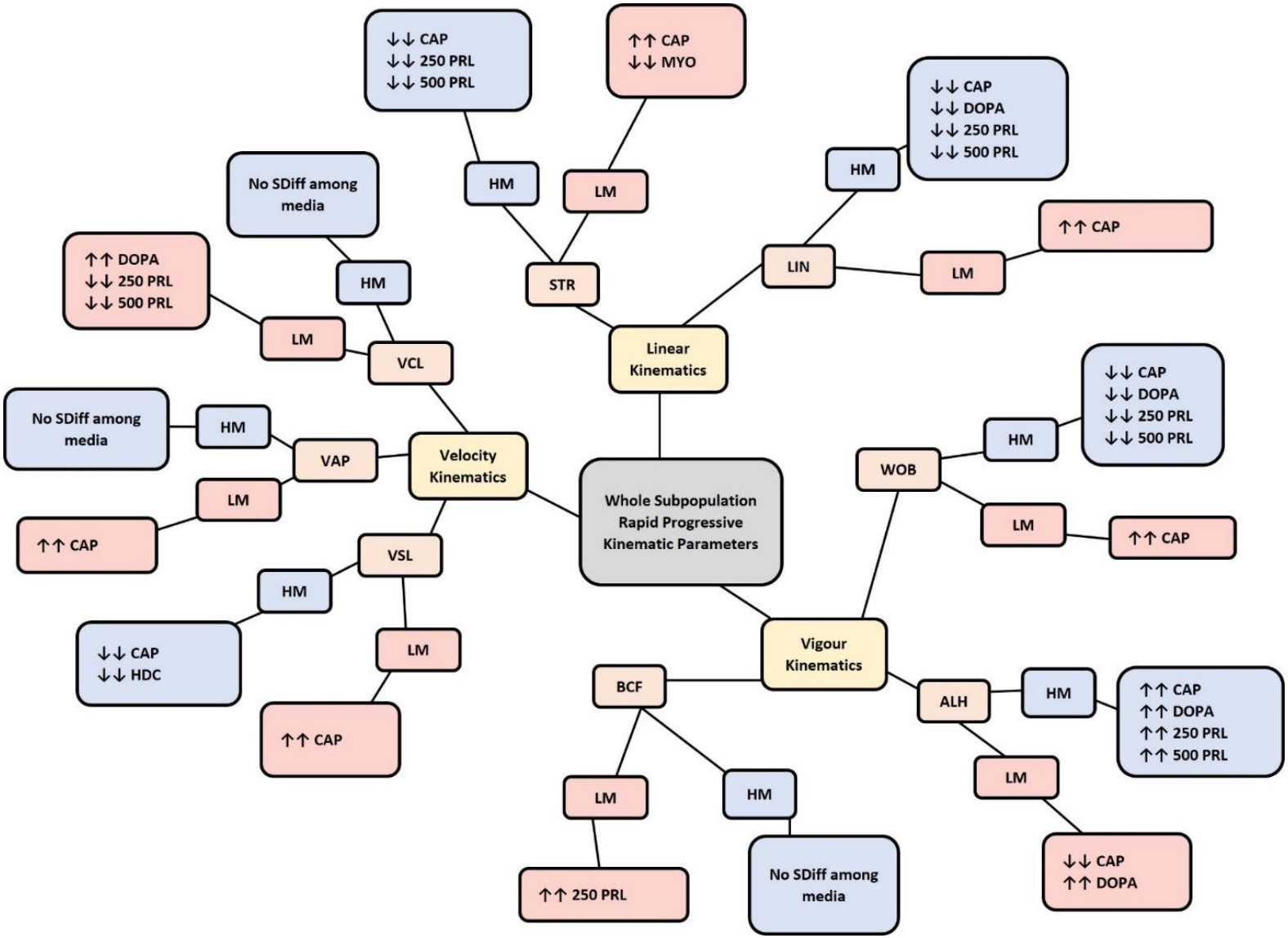
Supplementary Figure 2. Bubble diagram displaying the various motile/average kinematic parameters of spermatozoa for both subpopulations after being flushed with various media ***Note:*** Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values until significant differences between subpopulations were no longer observed. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. Student's *t*-test or the Mann-Whitney test when normal distribution was void in addition to one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions; $p < 0.05$. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; SDiff, significant differences; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.



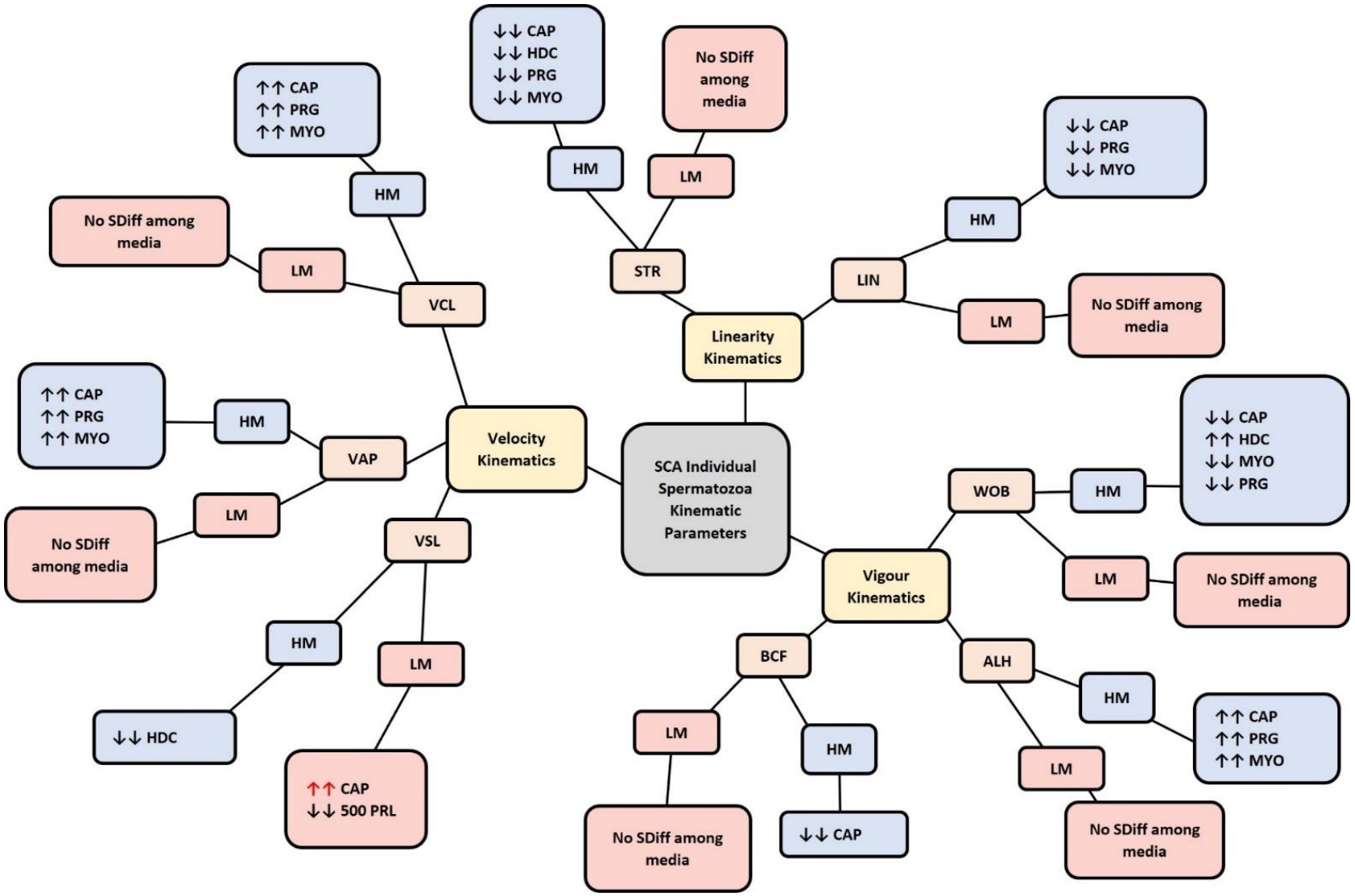
Supplementary Figure 3. Bubble diagram displaying the various non-progressive speed kinematic parameters of spermatozoa for both subpopulations after being flushed with various media ***Note:*** Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values until significant differences between subpopulations were no longer observed. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. Student's *t*-test or the Mann-Whitney test when normal distribution was void in addition to one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions; $p < 0.05$. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; SDiff, significant differences; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.



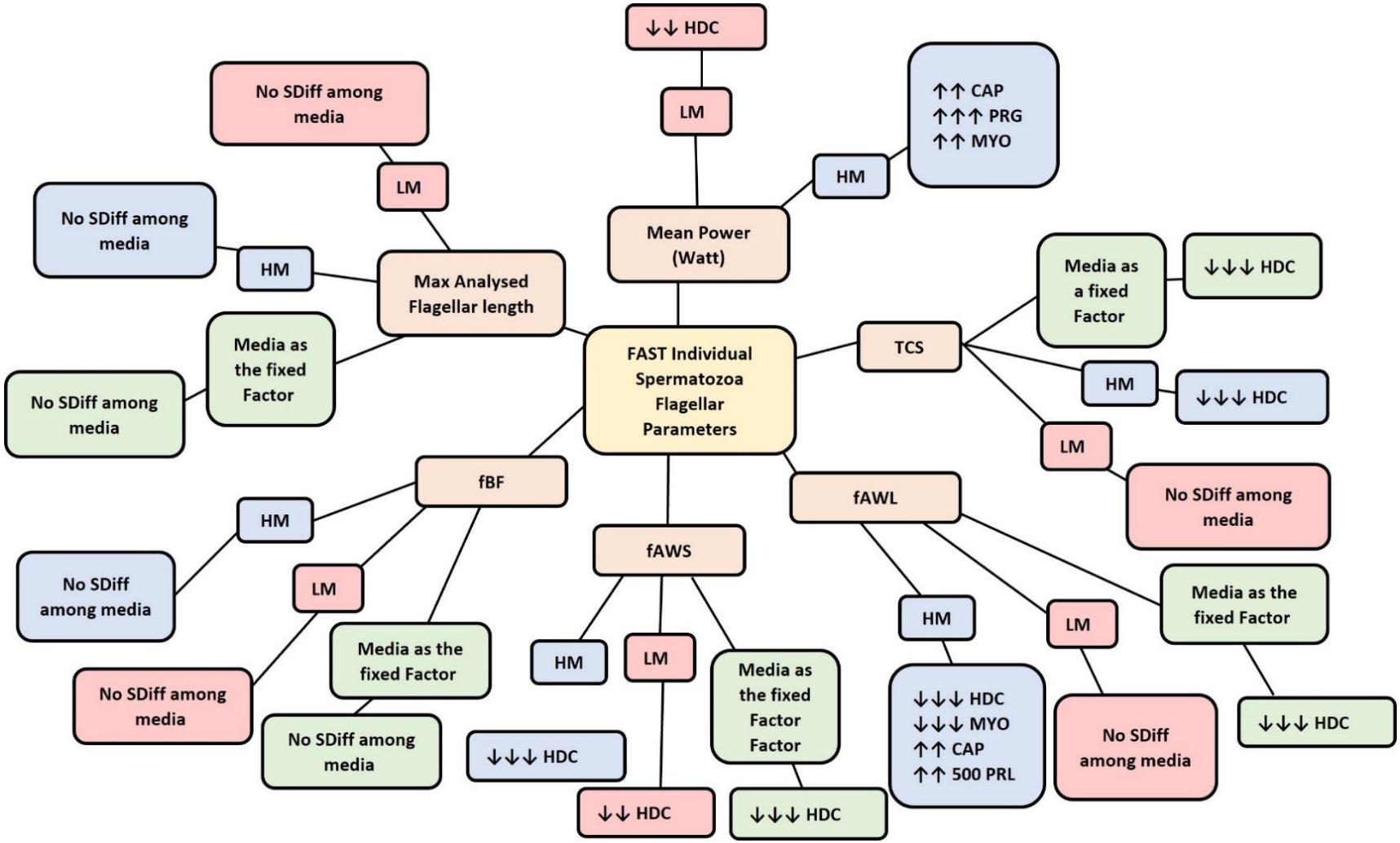
Supplementary Figure 4. Bubble diagram displaying the various medium progressive speed kinematic parameters of spermatozoa for both subpopulations after being flushed with various media ***Note:*** Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values until significant differences between subpopulations were no longer observed. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. Student's *t*-test or the Mann-Whitney test when normal distribution was void in addition to one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions; $p < 0.05$. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; SDiff, significant differences; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.



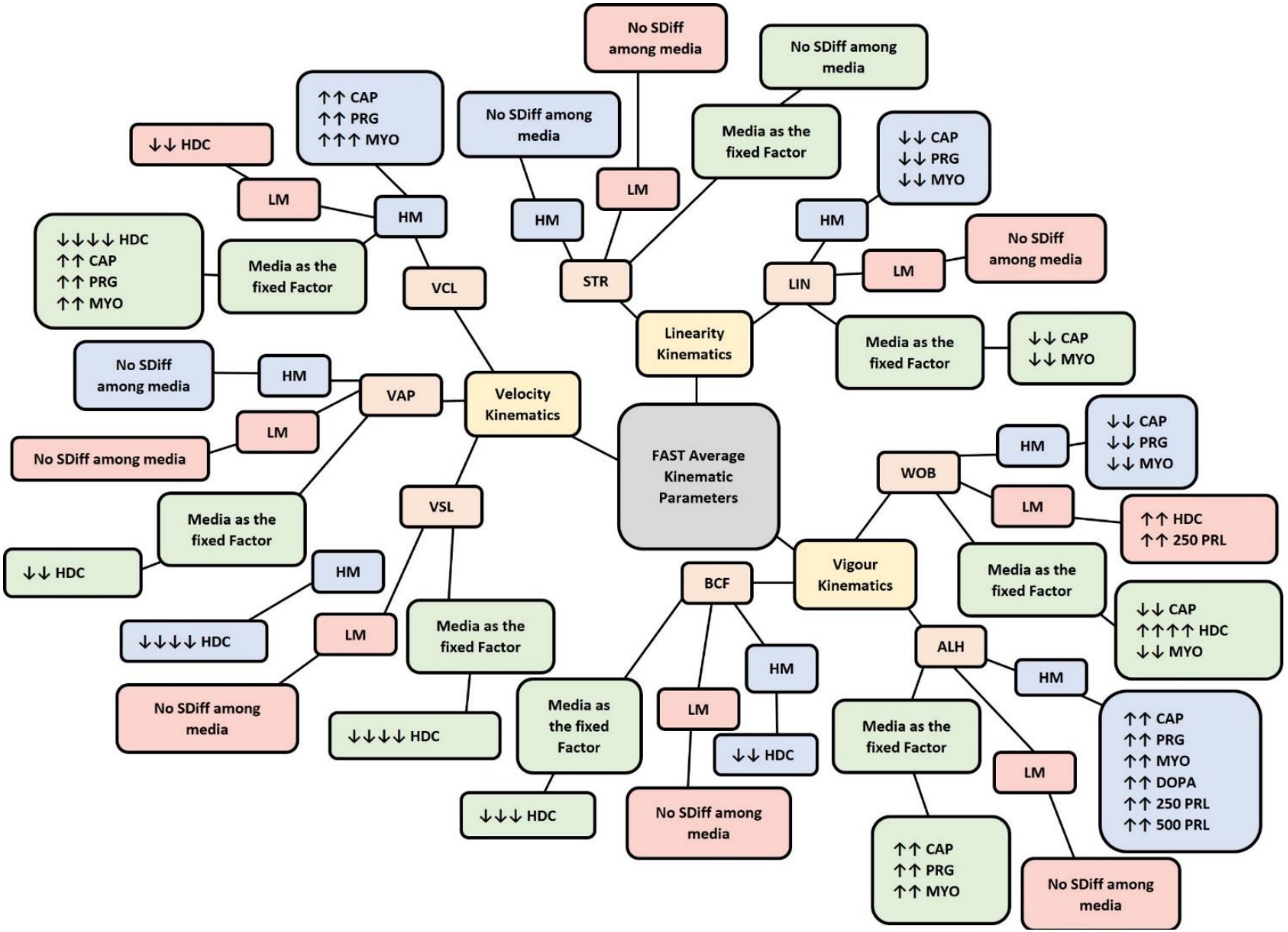
Supplementary Figure 5. Bubble diagram displaying the various rapid progressive speed kinematic parameters of spermatozoa for both subpopulations after being flushed with various media ***Note:*** Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values until significant differences between subpopulations were no longer observed. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. Student's *t*-test or the Mann-Whitney test when normal distribution was void in addition to one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions; $p < 0.05$. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; SDiff, significant differences; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.



Supplementary Figure 6. Bubble diagram displaying the various motile/average kinematic parameters of individual spermatozoa for both subpopulations after being flushed with various media and being analyzed with SCA. ***Note:*** Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values until significant differences between subpopulations were no longer observed. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. Student's *t*-test or the Mann-Whitney test when normal distribution was void in addition to one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions; $p < 0.05$. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; SDiff, significant differences; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.



Supplementary Figure 7. Bubble diagram displaying the various flagellar parameters of individual spermatozoa for both subpopulations after being flushed with various media and being analyzed with FAST. ***Note:*** Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values until significant differences between subpopulations were no longer observed. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. Student's *t*-test or the Mann-Whitney test when normal distribution was void in addition to one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions; $p < 0.05$. **Abbreviations:** CAP, capacitating-HTF; DOPA, dopamine; fAWL, flagellar arc-wavelength; fAWS, flagellar arc-wave speed; fBF, flagellar beat frequency; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; SDiff, significant differences; TCS, track centroid speed.



Supplementary Figure 8. Bubble diagram displaying the various FAST kinematic parameters of individual spermatozoa for both subpopulations after being flushed with various media and being analyzed with FAST ***Note:*** Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values until significant differences between subpopulations were no longer observed. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. Student's *t*-test or the Mann-Whitney test when normal distribution was void in addition to one-way ANOVA for parametric distributions or Kruskal-Wallis test for non-parametric distributions; $p < 0.05$. **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; SDiff, significant differences; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

APPENDICES

Improved Sperm Function in Human Sperm Subpopulations: A Model for Studying Subfertility

Shannen Keyser, Gerhard van der Horst, and Liana Maree

Background: Infertility is affecting one in four couples in developing countries, with the male factor being implicated in about half of these cases. In approximately 15% of the presumed sub- or infertile males, their semen analyses do not reveal obvious abnormalities (idiopathic), thereby conveying the importance of sperm functional testing. Furthermore, human semen is heterogeneous in nature, including subpopulations with different degrees of maturation and fertilizing ability. Evaluating the quality of such sperm subpopulations can reveal the functional capabilities of sperm in semen samples and may provide a model for studying sperm functionality in subfertile males.

Main Questions: This study aimed to determine and compare the functional characteristics of two sperm subpopulations found within the same human semen samples. Concomitantly, the improvement of such functional parameters was evaluated after exposure to various capacitating media and chemicals.

Experimental Design: Normospermic donor semen samples (n = 20) were separated into two fractions via 45% and 90% discontinuous AllGrad gradient centrifugation. These sperm fractions were assessed for their sperm functional characteristics, including evaluation of sperm vitality (BrightVit), CASA motility (SCA, Microptic SL, Barcelona), kinematics, hyperactivation (2 μM procaine, 5 μM caffeine), acrosome reaction (1 mM calcium ionophore), reactive oxygen species (ROS), and mitochondrial membrane potential (MMP) in human tubal fluid (HTF). Subsequently, sperm fractions were exposed to capacitating media, progesterone (1.98, 3.96, 19.8 μM), and myoinositol (11 mM), and sperm functionality was again assessed over time.

Main Results: AllGrad fractionation resulted in two distinctive sperm subpopulations, with the more motile (bottom) sperm fraction observed to have significantly higher percentages of sperm vitality, motility, swimming speed, hyperactivation,

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induced acrosome reaction, ROS negative, and intact MMP ($P < 0.05$) than the less motile (top) sperm fraction. After a 30 min exposure to capacitating media, progesterone, and myoinositol, there was a notable improvement in both sperm fractions' functional characteristics. Furthermore, after exposure, no differences were apparent between the two sperm fractions in terms of percentage sperm motility and hyperactivation, as well as sperm kinematics and acrosome reaction ($P > 0.05$) for most media.

Conclusions: Selected sperm functional parameters in lower quality sperm subpopulations could be improved to the level of a higher quality sperm subpopulation, separated from the same semen samples. If the lower quality sperm subpopulation mimics the sperm population in subfertile males, this can be used as a model to study such subpopulations and possibly broaden the potential treatment regimens and sperm isolation procedures for ART.

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Progesterone, Myo-Inositol, Dopamine and Prolactin Present in Follicular Fluid Have Differential Effects on Sperm Motility Subpopulations

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Abstract: Considering the challenges surrounding causative factors in male infertility, rather than relying on standard semen analysis, the assessment of sperm subpopulations and functional characteristics essential for fertilization is paramount. Furthermore, the diagnostic value of sperm interactions with biological components in the female reproductive tract may improve our understanding of subfertility and provide applications in assisted reproductive techniques. We investigated the response of two sperm motility subpopulations (mimicking the functionality of potentially fertile and sub-fertile semen samples) to biological substances present in the female reproductive tract. Donor semen was separated via double density gradient centrifugation, isolated into high (HM) and low motile (LM) sperm subpopulations and incubated in human tubal fluid (HTF), capacitating HTF, HD-C medium, progesterone, myo-inositol, dopamine and prolactin. Treated subpopulations were evaluated for vitality, motility percentages and kinematic parameters, hyperactivation, positive reactive oxygen species (ROS), intact mitochondrial membrane potential (MMP) and acrosome reaction (AR). While all media had a significantly positive effect on the LM subpopulation, dopamine appeared to significantly improve both subpopulations' functional characteristics. HD-C, progesterone and myo-inositol resulted in increased motility, kinematic and hyperactivation parameters, whereas prolactin and myo-inositol improved the LM subpopulations' MMP intactness and reduced ROS. Furthermore, progesterone, myo-inositol and dopamine improved the HM subpopulations' motility parameters and AR. Our results suggest that treatment of sub-fertile semen samples with biological substances present in follicular fluid might assist the development of new strategies for IVF treatment.

Keywords: progesterone; myo-inositol; dopamine; prolactin; sperm subpopulations; functional characteristics; double density gradient centrifugation; CASA; high motile; low motile

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1. Introduction

The aim of male fertility evaluations is to detect possible abnormalities in reproductive system and to determine which cases may require assisted reproductive technologies (ART) as treatment for low sperm count or quality [1,2]. Despite the increased use of ART, 68.5% of in vitro fertilization (IVF) cycles do not result in a live birth [3] and overall pregnancies per aspiration have only increased by 0.2% as compared to rates in 2012 [4]. Consequently, alternative approaches and interventions that may improve not only conventional sperm parameters, but also the genetic material of spermatozoa and thereby ART success rates [2], should be explored. Ideally, such approaches and interventions would be applicable in cases where fertility issues can be corrected without the need of costly ART cycles.

Spermatozoa must be functionally and structurally intact to traverse the female reproductive tract, respond to various chemo- and thermotactic signals and ultimately achieve successful fertilization. As a result, basic semen parameters alone cannot predict the fertility

potential of ejaculates; additional functional tests should be explored, including how spermatozoa respond to various biological substances found within the female reproductive tract [5]. The functionality of spermatozoa is closely associated with posttranslational modifications initiated by secondary messengers, as these cells are transcriptionally and translationally silent [6–9]. Mature spermatozoa with a high fertility potential typically exhibit progressive motility, normal morphology, viability, ability to undergo capacitation (hyperactivation and acrosome reaction), intact mitochondrial membranes (MMP), normal levels of reactive oxygen species (ROS) and intact DNA [10].

Due to the fact that signaling events in human spermatozoa remain poorly understood, along with the identities of many molecules that stimulate them [11], the current study aimed to establish the unique and collective biological effects of various components present in follicular fluid (FF) on human sperm motility subpopulations. It is well known that FF contains some metabolites critical for oocyte growth and development, forming a microenvironment that plays a key role in fertilization, implantation and early embryo development [12]. In the female reproductive tract and FF, substances such as progesterone, myo-inositol, dopamine, and prolactin are assisted by cyclic AMP (cAMP)/cAMP dependent protein kinase A (PKA) pathways to emanate the physiological changes that allow spermatozoa to fertilize oocytes [7,13–21].

In human spermatozoa, progesterone-induced Ca^{2+} influx is mediated through CatSper channels and its associated proteins, thereby regulating capacitation, hyperactivation and the acrosome reaction [14,19,22,23]. Moreover, it has been proposed that only capacitated human spermatozoa are able to participate in chemotactic swimming by using progesterone gradients in close proximity to the oocyte [18]. In human epididymal and testicular spermatozoa, CatSper currents showed sensitivity to progesterone early in sperm development and peaks when spermatozoa are ejaculated [24].

Myo-inositol is involved in numerous biological processes such as protein binding to the cell surface, cell signaling, vesicle trafficking, membrane excitability, regulation of channel opening and intracellular Ca^{2+} signaling [25]. Myo-inositol is an important precursor for signaling pathways which form secondary messengers that modulate protein phosphorylation and intracellular Ca^{2+} concentrations [7]. As such, myo-inositol present in the female reproductive tract plays a vital role in improving sperm quality, including motility, viability and cholesterol efflux, which regulates sperm capacitation [26,27].

High concentrations of catecholamines have been detected in both human semen and the oviduct [13,28]. Dopamine type 1-like receptors (DRD1) activate adenylyl cyclase which results in increasing cAMP accumulation and activating PKA, whereas dopamine type 2-like receptors (DRD2) inhibits adenylyl cyclase, thereby decreasing PKA activity [13]. Dopamine appears to act physiologically as a regulator of viability, fertility and sperm motility [13,29]. Dopamine-induced activation of DRD2 in boar sperm seems to have a beneficial effect on cell viability [16].

Prolactin plays an important role in female reproduction but prolactin receptors (PRL-R) have been detected in male reproductive organs, with a positive modulatory effect on various aspects of testicular function [30]. Pro-survival mechanisms of prolactin on spermatozoa have been suggested; however, it remains unclear whether prolactin has a stimulatory, inhibitory or no measurable effect on sperm capacitation [6].

Due to practical and ethical considerations, information about human sperm interaction with oviductal and follicular fluid contents is scarce in comparison with rodents and other mammalian species [18]. In this study, a comprehensive set of parameters and advanced statistical approaches were utilized to investigate and compare functional and structural sperm characteristics between two sperm motility subpopulations (HM: high motile and LM: low motile) after exposure to various concentrations of progesterone, myo-inositol, dopamine and prolactin. We hypothesize that these biological substances found within the female reproductive tract will differentially improve/change sperm function of the two sperm subpopulations. These subpopulations were used as a direct mimicking model for "fertile" and "sub-fertile" semen samples [5,31,32], respectively, to determine

whether the selected substances could enhance sperm quality and further be utilized in ART to improve sub-fertile samples and possibly result in successful fertilization.

2. Materials and Methods

2.1. Sample Collection and Standard Semen Analysis

One hundred and sixty human semen samples from forty-four donors were obtained via masturbation as part of a donor program (Division of Medical Physiology, Department of Biomedical Sciences, Stellenbosch University and Department of Medical Bioscience, University of the Western Cape) after two to three days of sexual abstinence. Donor samples were incubated permitting liquefaction (30–60 min at 37 °C in a 5% CO_2 regulated incubator) and subsequently processed as recommended by the World Health Organization (WHO) [33]. Semen volume, pH and viscosity were assessed, as well as several sperm parameters, including total motility, progressive motility, sperm concentration, total number of spermatozoa, mucus penetration, vitality and morphology (analyzed with Sperm Class Analyser® (SCA®) computer-aided sperm analysis (CASA) system, version 6.2 (Microptic S.L., Barcelona, Spain)). A minimal cut-off point for percentage total sperm motility of 15% was used, as the basis of the study was to determine how various concentrations of media can improve different qualities of spermatozoa. The study was approved by the ethical boards of the University of the Western Cape (code BM20/9/14), and Stellenbosch University (code N14/06/074). The Helsinki Declaration [34] governing research on humans has been adhered to and each human donor gave written consent.

2.2. Preparation of Sperm Subpopulations

Two motility sperm subpopulations (highly motile spermatozoa, HM; less motile spermatozoa, LM) were prepared from semen samples through double density gradient centrifugation (DGC) with the use of AllGrad® 90/45% and AllGrad Wash® (Delfran, Johannesburg, South Africa). Equal volumes (300 µL) of semen and preheated (37 °C) density gradient 90–45% were layered in an Eppendorf tube, and centrifuged at room temperature (RT) for 20 min at 500× *g*. Resultant top coats consisting of seminal plasma were discarded, and remaining intermediate layers and bottom pellets were separated into Eppendorf tubes as top (LM subpopulation) and bottom (HM subpopulation) subpopulations, respectively. Subpopulations were re-suspended in 300 µL AllGrad Wash® and centrifuged at 500× *g* for 10 min. Washed pellets were resuspended in the various media and final sperm concentrations adjusted to 15–25 × 10^6/mL before being assessed.

2.3. Preparation of Media

Four of the five different media (containing progesterone, myo-inositol, dopamine and prolactin) were prepared at various concentrations in capacitating HTF (constituted of non-capacitating HTF supplemented with 0.105 g $NaHCO_3$, 1.1915 g HEPES and 0.6 mL NaOH), whereas non-capacitating HTF (HTF) and capacitating HTF (CAP) [35] functioned as the negative and positive controls, respectively. All media were void of 1% human serum albumin (HSA) supplementation, as samples were not exposed to extensive incubation hours and the study aimed to determine the individual effects of the media alone on sperm function. Due to the negative effect that time has on sperm functionality, selected media was grouped together and groups were assessed on separate samples and occasions; however, controls were included throughout. To this effect, HD-C, myo-inositol and progesterone were grouped and analyzed together, after which dopamine concentrations and prolactin concentrations were respectively grouped and analyzed individually for each group. Furthermore, due to time constraints, the investigation of functional parameters was additionally grouped and analyzed on different samples and occasions as follows: vitality was assessed on 20 samples; motility and hyperactivation were assessed together on 20 samples; and MMP, ROS and AR were assessed together on 20 samples. For the dopamine group, vitality, motility and hyperactivation could be analyzed together on the same 20 samples due to fewer concentrations being investigated.

2.3.1. HD-C Medium

HD Sperm Capacitation™ medium (HD-C, HDSC-0005) purchased from Delfran Pty Ltd. (Delfran, Johannesburg, South Africa) comprised of a mixture of NaCl, KCl, $MgSO_4.7H_2O$, KH_2PO_4, Na_2HPO_4, $NaHCO_3$, $CaCl_2$, glucose, progesterone and myo-inositol for inducing hyperactivation in spermatozoa.

2.3.2. Progesterone

Progesterone in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Cape Town, South Africa) was prepared in CAP to yield three working solutions of 1.98 µM, 3.96 µM and 19.8 µM (volume/volume). Selected concentrations were based on previous investigations in addition to physiological concentrations in the female reproductive tract [36–38].

2.3.3. Myo-Inositol

Myo-inositol (MW = 180.16 g/mol) was weighed out and dissolved in CAP (mass/volume) to yield a working solution of 11 mM. This selected concentration was based on previous investigations in addition to physiological concentrations in the female reproductive tract [36–38].

2.3.4. Dopamine

Dopamine hydrochloride (H8502, Sigma-Aldrich, Cape Town, South Africa) was weighed out and dissolved in CAP (mass/volume) to yield a stock solution of 1 mM dopamine, which was further diluted to three working solutions of 20 nM, 100 nM and 1 µM (volume/volume). Selected concentrations were adjusted from previous investigation and physiological concentrations found within the female reproductive tract [13,29,39,40].

2.3.5. Prolactin

Hydrolyzed Prolactin (L7009, Sigma Aldrich, Cape Town, South Africa) was prepared in 4 mM HCl (mass/volume) to a stock solution of 10 µg/mL and was diluted in CAP to yield four working solutions of 50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL. Selected concentrations were adjusted from previous investigations [6,41–43].

2.4. Viscosity

Using the viscosity evaluation technique described by Rijnders et al. [44], 3 µL semen aliquots were loaded into preheated (37 °C) four-chamber, 20 µm-depth Leja slides (Leja Products B.V., Nieuw Vennep, The Netherlands) and the filling time was recorded in seconds (s). Viscosity in centipoise (cP) was subsequently determined by using the following equation:

$$y = 0.34x + 1.34 \quad (1)$$

where y = viscosity in cP and x = filling time in s.

2.5. Sperm Morphology

Semen aliquots (300 µL) were centrifuged in AllGrad Wash® at RT for 20 min at 500× *g* and subsequent pellets re-suspended in HTF. Morphology smears were prepared (15 µL) and dried slides stained with SpermBlue® fixative and stain mixture (Microptic S.L., Barcelona, Spain) as described by van der Horst and Maree and modified by Microptic [45,46]. Coverslips were mounted with DPX mounting medium (Sigma Aldrich, Cape Town, South Africa) and 100 spermatozoa were analyzed with the Morphology module of the SCA® using brightfield optics, an acA1300-200uc camera, a blue filter and a 60× objective on a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa).

2.6. Sperm Vitality

All reagents and equipment were heated to 37 °C prior to use. Subpopulation vitality smears were prepared after 5 and 30 min incubation in the various media, with

the additional time point of 60 min for prolactin. Semen samples and treated sperm subpopulations were stained in suspension with BrightVit (Microptic S.L., Barcelona, Spain), and smears prepared following the staining and preparation technique proposed by Microptic [46]. Dried vitality smears were mounted with a coverslip using DPX mounting medium and viewed with brightfield optics and a 100× oil immersion objective. Percentages of viable spermatozoa was calculated after at least 100 spermatozoa per slide were manually assessed.

2.7. Sperm Motility, Concentration and Mucous Penetration

Total sperm motility, progressive motility, concentration and mucus penetration were assessed with the Motility module of the SCA® and data captured with a Basler acA1300-200uc digital camera (Microptic S.L., Barcelona, Spain) attached to a Nikon Eclipse 50i microscope with a 10x positive phase contrast objective, green filter and heated stage. Preheated (37 °C) four or eight-chamber, 20 µm-depth Leja slides were loaded with 2–3 µL of semen or treated subpopulations and at least two fields with 200 motile spermatozoa were analysed at 50 frames per second (f/s).

After incubation (5, 30 and 60 min) in the different media, motility percentages assessed for treated subpopulations included total motility, progressive motility, rapid-, medium- and non-progressive motility, as well as rapid-, medium- and slow-swimming spermatozoa. In addition, eight kinematic parameters, including curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF), were recorded for the average motile sperm population as well as various subpopulations for progressiveness. ALH was measured as half the width of the VCL track and not as the full VCL wave or doubling of riser values (risers' method) as described by Mortimer [35,47]. Kinematic parameter cut-off values for mucus penetration were VAP > 25 µm/s, STR > 80% and 7.5 µm < ALH > 2.5 µm [48,49].

2.8. Hyperactivation

Applying the flush technique described by van der Horst et al. [50], each chamber of a 20 µm-depth, eight-chamber Leja slide was loaded with 0.5 µL sperm preparation (HM or LM sperm subpopulations suspended in HTF) and flushed with 1.5 µL of preheated media as mentioned in Section 2.3. Percentages of hyperactivation (using cut-off values: VCL > 150 µm/s; LIN < 50%; ALH > 7 µm (3.5 for SCA®)) [47] of at least 200 motile spermatozoa was assessed after 5, 15, 30, 45 and 60 min exposure to each treatment, for each sperm subpopulation using the Motility module of SCA® and equipment describe in the previous section.

2.9. Reactive Oxygen Species

Subpopulations were incubated for 30 min in media mentioned in Section 2.3, after which 20 µM Dihydroethidium (DHE, excitation = 518 nm and emission = 605 nm, Molecular Probes, Eugene, OR, USA) was used to detect spermatozoa positive for reactive oxygen species (ROS). Treated subpopulations were stained in the dark for 15 min in suspension (180 µL) with 20 µL of DHE at 37 °C. Following incubation, 5–10 µL of suspension was placed on a clean slide with a coverslip, and immediately analyzed using a 100× oil immersion objective and triband filter (MXU440) (excitation wavelengths: 457 nm = blue, 530 nm = green and 628 nm = red) on a Nikon Eclipse 50i fluorescence microscope. Percentages of spermatozoa positive for ROS were calculated after manual assessment of at least 100 spermatozoa.

2.10. Mitochondrial Membrane Potential ($\Delta\Psi m$)

The protocol of the Mitochondria Staining Kit (CS0390, Sigma Aldrich, Cape Town, South Africa) to assess mitochondrial membrane potential (MMP) was optimized for this specific study. Subpopulations were incubated for 30 min in the media mentioned in

Section 2.3, followed by staining (1:1 ratio) in the dark at 37 °C for 20 min in suspension (200 µL) with the MMP staining solution (160 µL dH_2O, 40 µL JC-5 buffer and 1 µL frozen MMP 200x stock solution). After incubation, suspensions were centrifuged at 500× *g* for 5 min at 5–7 °C and washed pellets re-suspended in 200 µL JC-1 buffer (80 µL JC-5 buffer and 320 µL dH_2O), prepared and cooled on ice before use. Suspensions were centrifuged for a second time as described above and subsequent pellets were re-suspended in the remaining 200 µL JC-1 buffer. A single drop of 5–10 µL of suspension was placed on a clean slide and covered with a coverslip. Slide preparations were immediately analyzed using the same equipment as described in Section 2.9, and the percentage of spermatozoa with intact MMP calculated after manual assessment of at least 100 spermatozoa.

2.11. Acrosome Reaction

The acrosome reaction was determined with the use of the FluAcro protocol described by Microptic [46]. Subpopulations were incubated at 37 °C for 3 h in preheated (37 °C) capacitating medium (1 mL HAMS-F10 and 0.03 g HSA; Sigma-Aldrich, Cape Town, South Africa). After incubation, subpopulations were treated with 10 µL of each of the following media: 1 mM Ca-ionophore made up in DMSO (yielding a final concentration of 0.01 mM); capacitating and non-capacitating HTF; HD-C; progesterone; myo-inositol; dopamine; and prolactin. Samples were incubated in the media for 15 min after which reactions were terminated with 100 µL 70% ethanol. Two 5 µL drops of each suspension were placed on a clean slide and left to air dry before fixation in 95% ethanol (United Scientific, Cape Town, South Africa) at 4 °C for 30 min. Fixed slides were stained in a dark room for 30–40 min with 80 µL fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA; Sigma-Aldrich, Cape Town, South Africa) on each drop, and dipped twice in dH_2O to remove excess stain. Slides were subsequently counterstained for 7 min with 5 µL Hoechst (H33258, Sigma-Aldrich, Cape Town, South Africa) on each drop, followed by destaining in dH_2O, and further left to air dry before viewing. The acrosome status of at least 100 spermatozoa per sample was manually assessed using a 40× quartz fluorescence objective and a triband fluorescence filter (MXU440) on a Nikon Eclipse 50i epi-fluorescence microscope.

2.12. Statistical Analysis

MedCalc statistical software version 14.8.1 (Mariakerke, Gent, Belgium) was used to calculate basic summary statistics, and results are presented as mean ± standard deviation in all the tables. Treated sperm subpopulations were compared using the Student's t-test or the Mann–Whitney test when normal distribution was lacking. Where applicable, the one-way analysis of variance (ANOVA) for parametric distributions or the Kruskal–Wallis test for non-parametric distributions were used to compare various subpopulations, time points and media. Significance was determined at a level of $p < 0.05$ with the Student–Newman–Keuls post hoc test. Additional analyses, such as multifactorial ANOVA and the creation of multivariable charts, were performed with Statgraphics® Centurion XVII (Statgraphics Technologies, Inc., The Plains, VA, USA). More advanced statistics were calculated by performing a mixed model repeated measures ANOVA with STATISTICA, version 10 (StatSoft Inc., Tulsa, OK, USA). Subpopulations, media and time were used as fixed factors and samples as the random factors. The Fisher LSD was used for the post hoc test and reports based on third-order effects (interactions amongst subpopulations, media and time). If third-order effects were absent, reports were based on interactions between fixed factors (subpopulations and media, subpopulations and time or media and time). If none of the fixed factors (subpopulations, media or time) were included in interactions, only the main effects were reported.

3. Results

3.1. Bubble Diagrams and Quantitative Results

To indicate the comprehensive effects of the media on sperm subpopulations, and collectively, with media as a fixed factor, the results are summarized and displayed as

bubble diagrams. Figure 1 displays the overall effects of each medium on functional parameters, including vitality, mitochondrial membrane potential (MMP), positive reactive oxygen species (ROS), hyperactivation (HA) and acrosome reaction (AR). Illustrated in Figures 2 and 3, respectively, are the effects of each medium on motility percentages and average kinematic parameters. Individual functional parameters are considered in more detail under the subsequent subheadings; however, bubble diagrams will be referred back to. Results for sperm functional parameters are depicted as figures in the text but detailed tables with the actual data are presented as supplementary material and will be referred to as such.

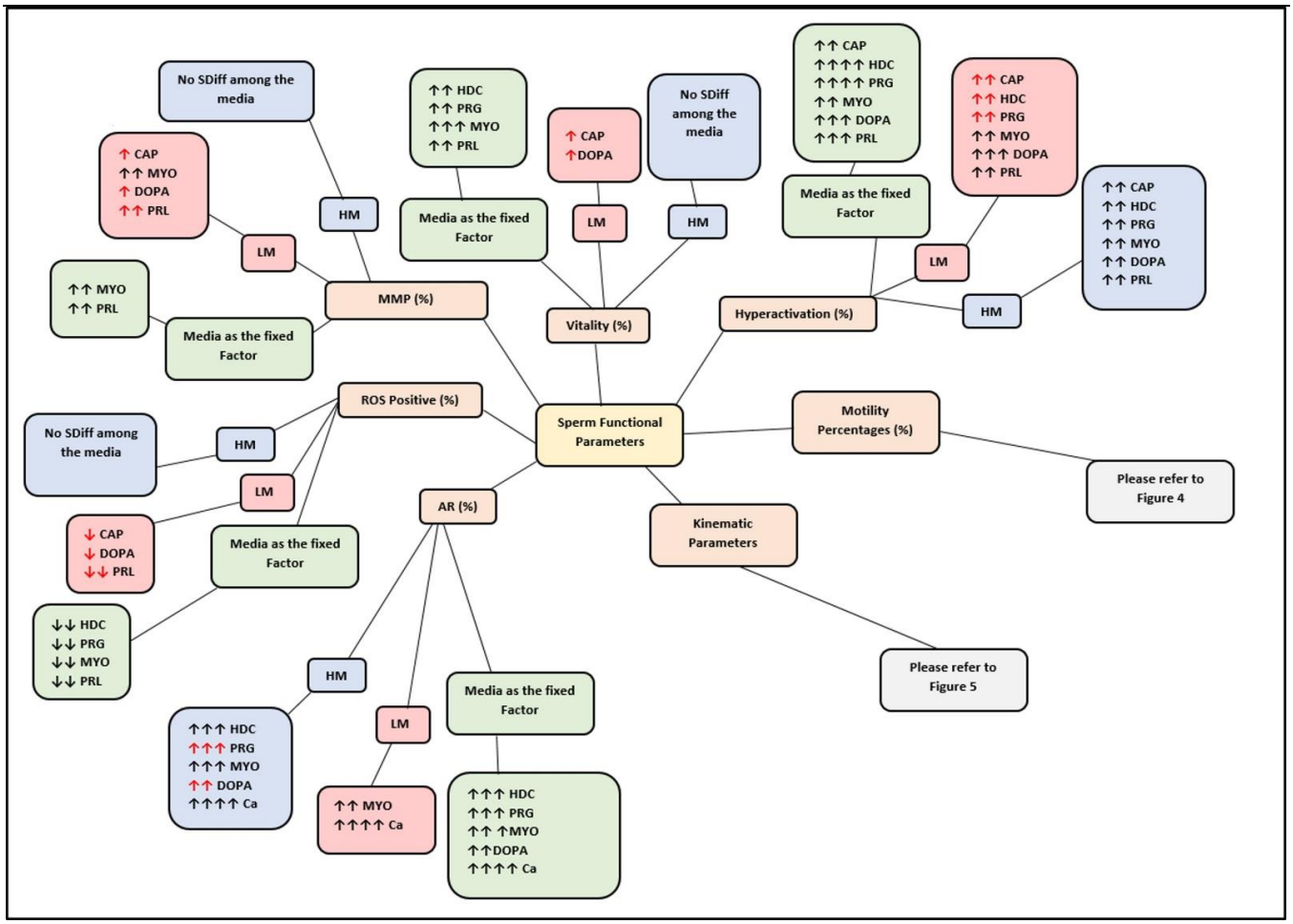

Figure 1. Bubble diagram displaying the overall effect of media on various functional parameters of spermatozoa for both subpopulations, and collectively with media as fixed factor. Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values resulting in elimination of significant differences between subpopulations. Two arrows (↑↑ or ↓↓) indicate significant differences between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate significant differences between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate significant differences between the medium and HTF, CAP and other media. Student's *t*-test or the Mann–Whitney test (for non-parametric data) in addition to one-way ANOVA (for parametric distributions) or Kruskal–Wallis test (for non-parametric distributions) were used throughout unless indicated differently; $p < 0.05$. AR, acrosome reaction; CAP, capacitating-HTF; Ca-ionophore, calcium-ionophore; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LM, low motile subpopulation; MMP, mitochondrial membrane potential; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; ROS, positive reactive oxygen species; SDiff, significant difference.

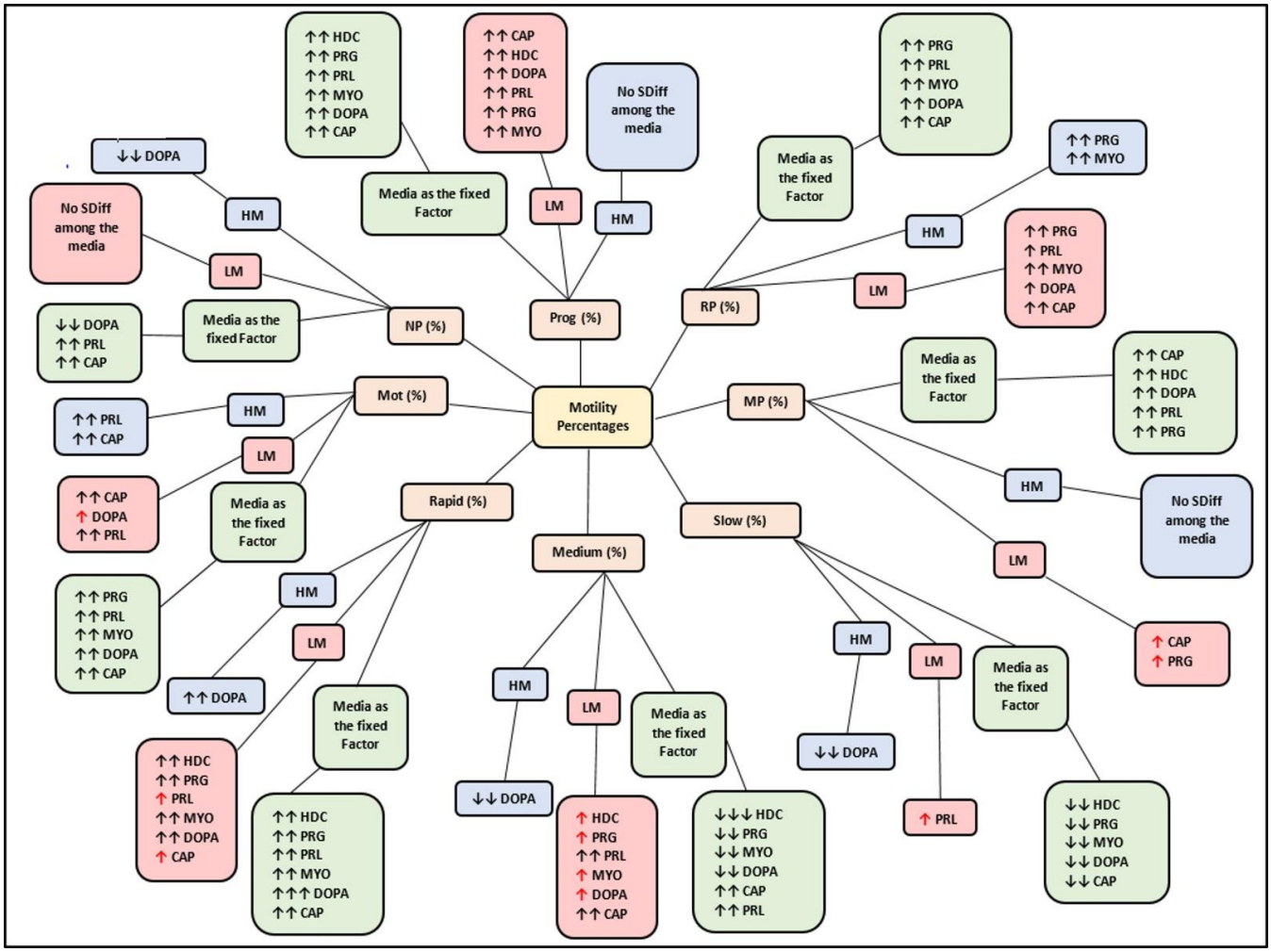

Figure 2. Bubble diagram displaying the overall effect of media on various motility percentages of spermatozoa for both subpopulations, and collectively with media as fixed factor. Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values resulting in elimination of significant differences between subpopulations. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LM, low motile subpopulation; Mot, total motility; MP, medium progressive; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; PRL, prolactin; Prog, progressive motility; RP, rapid progressive; SDiff, significant difference.

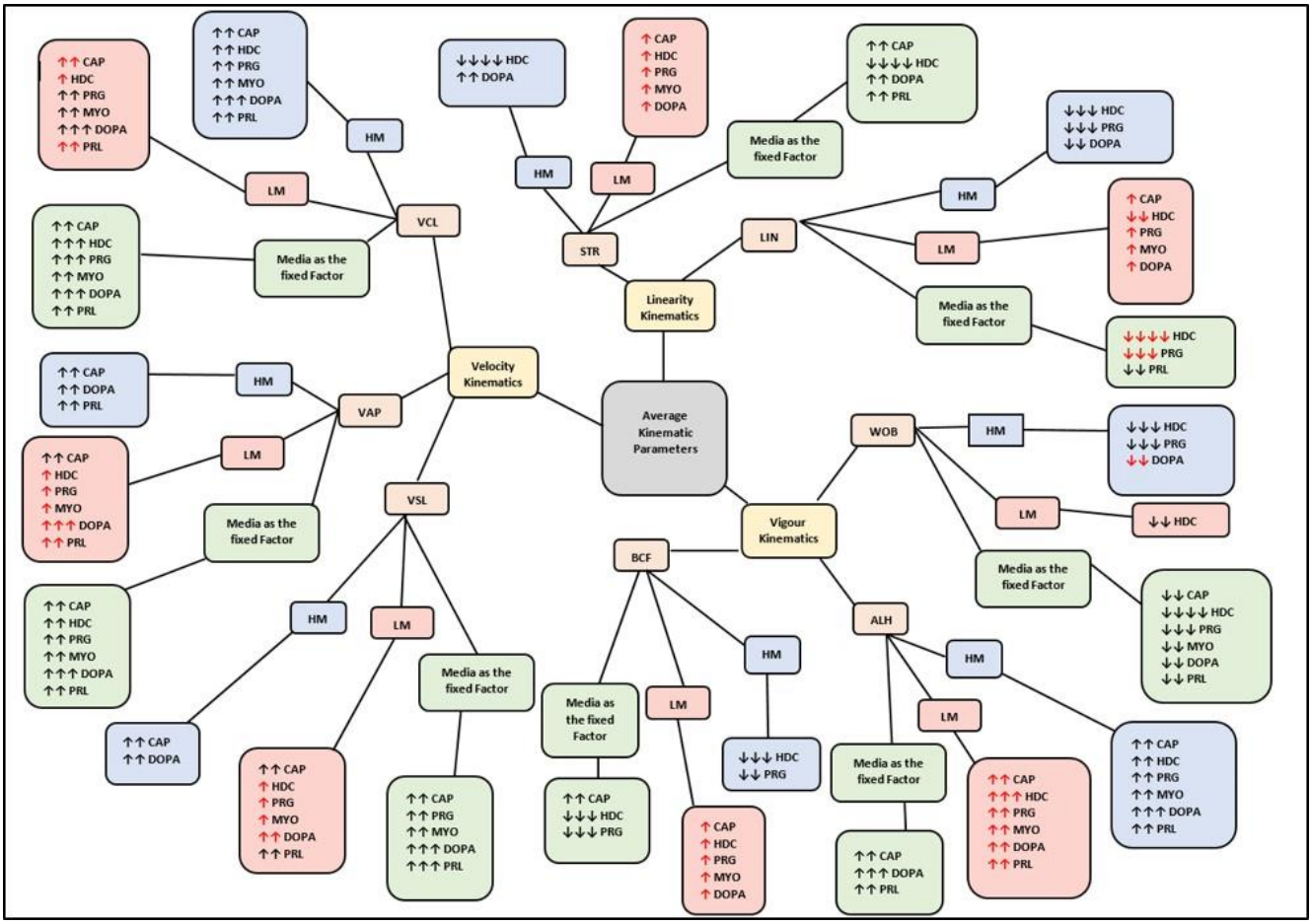

Figure 3. Bubble diagram displaying the overall effect of media on various motile/average kinematic parameters of spermatozoa for both subpopulations, and collectively with media as fixed factor. Red arrows (↑ or ↓) indicate a change in subpopulation (LM or HM) values resulting in elimination of significant differences between subpopulations. Two arrows (↑↑ or ↓↓) indicate a significant difference between the medium and HTF. Three arrows (↑↑↑ or ↓↓↓) indicate a significant difference between the medium and both HTF and CAP. Four arrows (↑↑↑↑ or ↓↓↓↓) indicate a significant difference between the medium and HTF, CAP and other media. ALH, amplitude of lateral head displacement; BCF, beat cross frequency; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LIN, linearity; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

3.2. Standard Semen Analysis

Average semen parameters of donor samples used in this investigation are displayed in Table 1. Mean values ranged above lower reference values as recommended by the WHO laboratory manual [33], with the exclusion of progressive motility falling in the 2.5th percentile.

Table 1. Basic semen parameters (mean ± SD) of donor samples used in this investigation (n = 160).

	Mean ± SD	**95% C.I**
Total Mot (%)	54.5 ± 18.5	51.7–57.3
Prog Mot (%)	27.3 ± 16.5	24.8–29.8
MPT (10^6/ejaculate)	28.3 ± 27.0	24.2–32.4
pH	7.4 ± 0.2	7.4–7.5
Viscosity (cP)	10.8 ± 11.1	9.1–12.5
Volume (mL)	3.2 ± 2.9	2.8–3.7
Conc (10^6/mL)	58.6 ± 42.8	52.2–65.1
Conc (10^6/ejaculate)	161.3 ± 143.5	139.6–183.0
Vitality (%)	69.9 ± 10.7	68.3–71.6
Normal (%)	5.7 ± 4.6	5.0–6.4

C.I, confidence interval; Conc, concentration; cP, centipoise; MPT, mucous penetration test; Prog Mot, progressive motility; SD, standard deviation; Total Mot, total motility.

3.3. Vitality

High motile (HM) subpopulations had significantly ($p < 0.05$, $p < 0.01$ and $p < 0.001$) higher vitality percentages as compared to low motile (LM) subpopulations (Figure 4A–C). However, after 5 min incubation, 20 nM dopamine improved the LM vitality percentages (Figure 1) to the extent that significant ($p = 0.1653$) differences between subpopulations were eliminated (Figure 4B). No significant differences among media was observed for individual subpopulations' vitality percentages. Furthermore, only HTF vitality percentages of the HM subpopulations were significantly ($p = 0.01$) lower at 30 min as compared to 5 min incubation (Figure 4B).

To determine whether combined factors (subpopulations, time and media) had an interaction on vitality percentages, data were analyzed in a mixed model repeated measures ANOVA. A significant effect ($p < 0.01$) was only observed for media as fixed factor, as illustrated in Figure 5A,B. For individual effects of media, HD-C ($p < 0.01$), progesterone (1.98 µM, $p = 0.03$; 3.96 µM, $p = 0.03$ and 19.8 µM, $p = 0.02$; Figure 5A) and prolactin (50 ng/mL, 100 ng/mL and 250 ng/mL, $p < 0.01$; Figure 5B) significantly improved percentages of viable spermatozoa as compared to HTF. In addition, myo-inositol ($p < 0.01$) significantly improved vitality as compared to both HTF and CAP. Prolactin appeared to display a possible dose-dependent trend as 100 ng/mL resulted in significantly ($p < 0.01$) higher vitality percentages as compared to 500 ng/mL (Figure 5B).

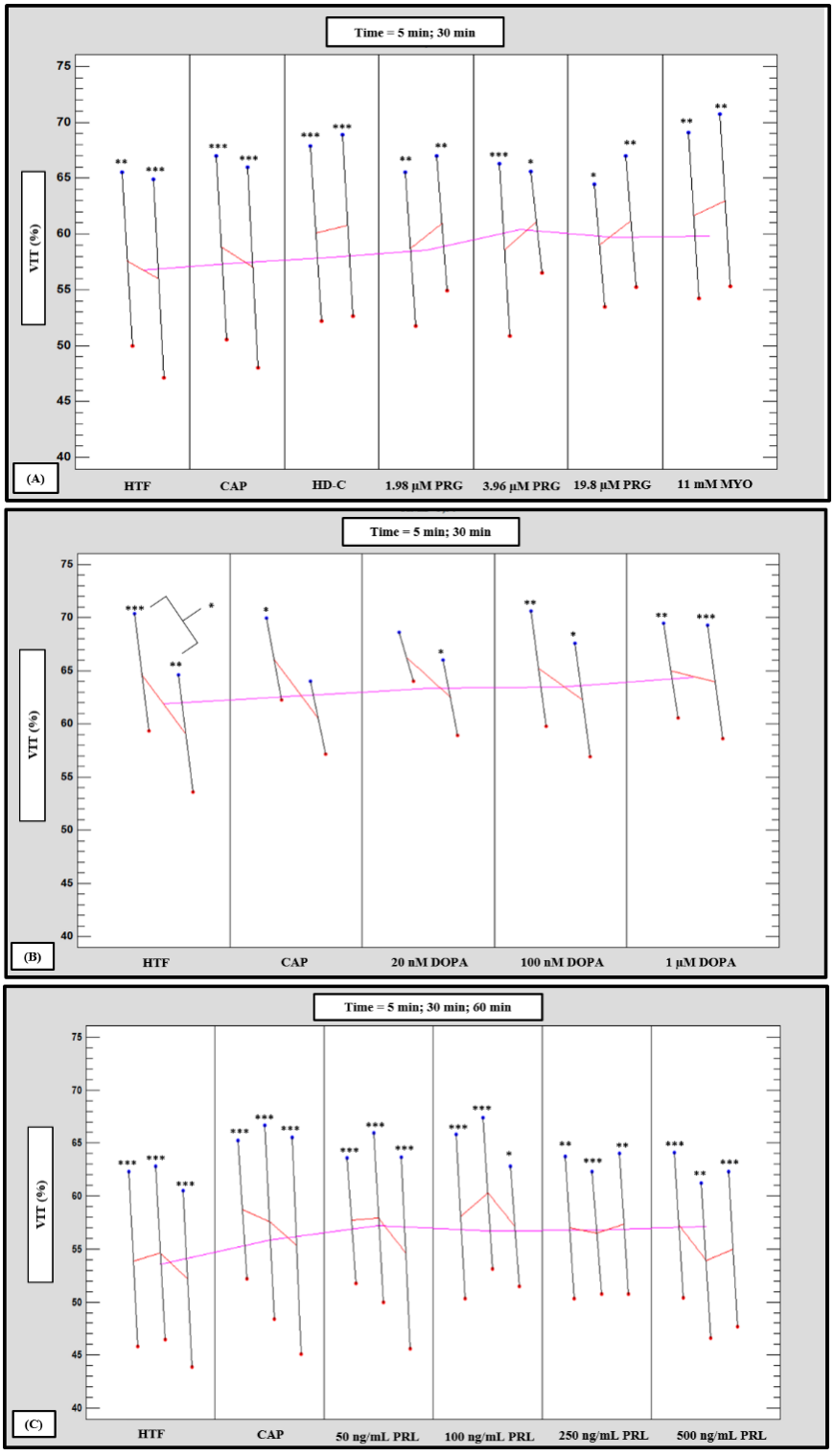

Figure 4. Multivariable chart comparing the mean percentage viable spermatozoa of high motile (HM, blue dots) and low motile (LM, red dots) sperm subpopulations at different time points after treatment with: (**A**) HTF, CAP, HD-C, PRG and MYO; (**B**) HTF, CAP and DOPA; and (**C**) HTF, CAP and PRL. Significant differences between subpopulations for individual media and time points are indicated as an asterisk (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitating medium; HM, high motile subpopulation; HTF, human tubal fluid; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; VIT, vitality.

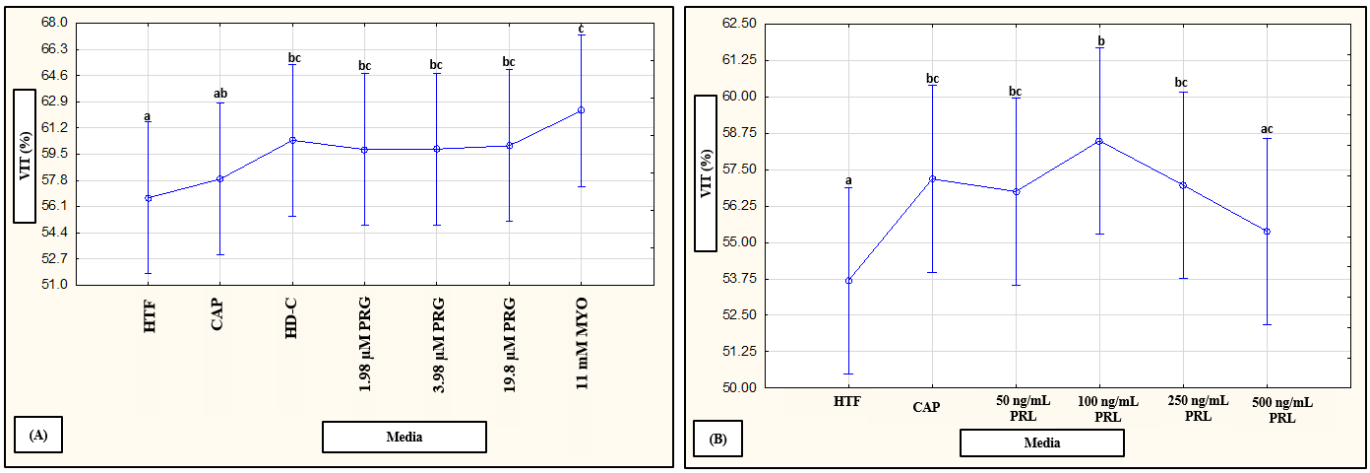

Figure 5. Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 min) as analyzed in a mixed model repeated measures ANOVA to determine significant ($p < 0.01$) interactions between media as fixed factor on percentage viable spermatozoa. Effect of (**A**) HTF, CAP, HD-C, PRG and MYO ($F(6540) = 3.29$, $p < 0.01$), and (**B**) HTF, CAP and PRL ($F(5630) = 4.26$, $p < 0.01$) on percentage viable spermatozoa. Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different ($p < 0.01$). The Fisher LSD was used for the post hoc test and reports. CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; MYO, myo-inositol; PRL, prolactin; PRG, progesterone; VIT, vitality.

3.4. Motility and Kinematic Parameters

Compared to the LM subpopulations, the HM subpopulations frequently displayed significantly higher percentages for various motility parameters at 5, 30 and 60 min incubation in HTF (Supplementary Tables S1–S3). After incubation in various media, motility percentages were improved in the LM subpopulations to the extent that significant differences between subpopulations were eliminated (Figure 2). This was mainly observed for motility speed groups (rapid, medium and slow) and progressivity speed groups (RP and MP) for various media, but predominantly for higher concentrations of dopamine (100 nM and 1 µM) and prolactin (100 ng/mL and 500 ng/mL).

For the HM subpopulations, various media improved motility percentages as compared to HTF (Supplementary Tables S1–S3). Percentages of rapid and RP speeds were increased by CAP, progesterone, myo-inositol and dopamine—whereas only CAP and prolactin increased percentages of total motility (Figure 2). For the LM subpopulations, prolactin increased both total motility and slow speed groups, while all media improved progressive, RP, MP, rapid and medium speed groups of this subpopulation (Figure 2). Whereas higher concentrations of dopamine (1 µM) and progesterone (3.96 µM) displayed larger stimulatory effects on motility percentages of subpopulations, prolactin varied in its effects among concentrations by displaying a possible biphasic or dose-dependent response (Supplementary Tables S1–S3).

No interactions of media, subpopulations and time points on motility percentages were revealed in a mixed model repeated measures ANOVA; however, media as fixed factor had a significant ($p < 0.01$) effect on various motility percentages (see Figure 6A–C, Figure 7A–C and Figure 8A–C for examples). Compared to HTF, all media increased the percentages of progressive motility (Figures 2, 6A, 7A and 8A), rapid and RP speed groups (Figures 2, 6B, 7B and 8B) and MP speed groups (Figures 2, 6C, 7C and 8C), while decreasing the percentages of slow speed groups (Figure 2). In contrast, CAP and prolactin appeared to increase the percentage of medium speed groups (Figure 2). A dose-dependent response for prolactin was observed again as the highest concentration (500 ng/mL), often presented with decreased motility percentages as compared to lower concentrations (100 ng/mL and 250 ng/mL).

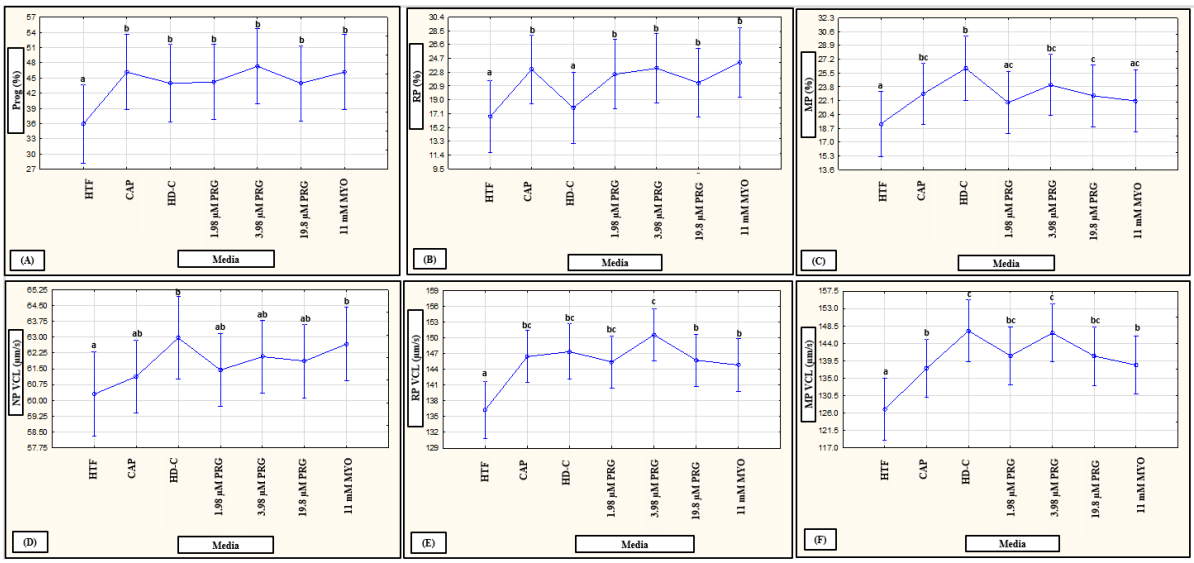

Figure 6. Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 min) as analyzed in a mixed model repeated measures ANOVA to determine the effect of media as a fixed factor. Effect of HTF, CAP, HD-C, PRG and MYO on: (**A**) progressivity (F (6527) = 3.54, $p < 0.01$); (**B**) rapid progressive (RP) speed groups (F (6528) = 2.92, $p < 0.01$) (**C**) medium progressive (MP) speed groups (F (6527) = 5.40, $p < 0.01$); (**D**) non-progressive (NP) VCL (F (6529) = 1.13, $p = 0.34$); (**E**) RP VCL (F (6524) = 7.15, $p = 0.34$); and (**F**) MP VCL (F (6520) = 6.16, $p = 0.34$). Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different ($p < 0.01$). The Fisher LSD was used for the post hoc test and reports. CAP, capacitating-HTF; HD-C, HD capacitating medium; HTF, human tubal fluid; MP, medium progressive; MYO, myo-inositol; NP, non-progressive; PRG, progesterone; Prog, progressive; RP, rapid progressive; VCL, curvilinear velocity.

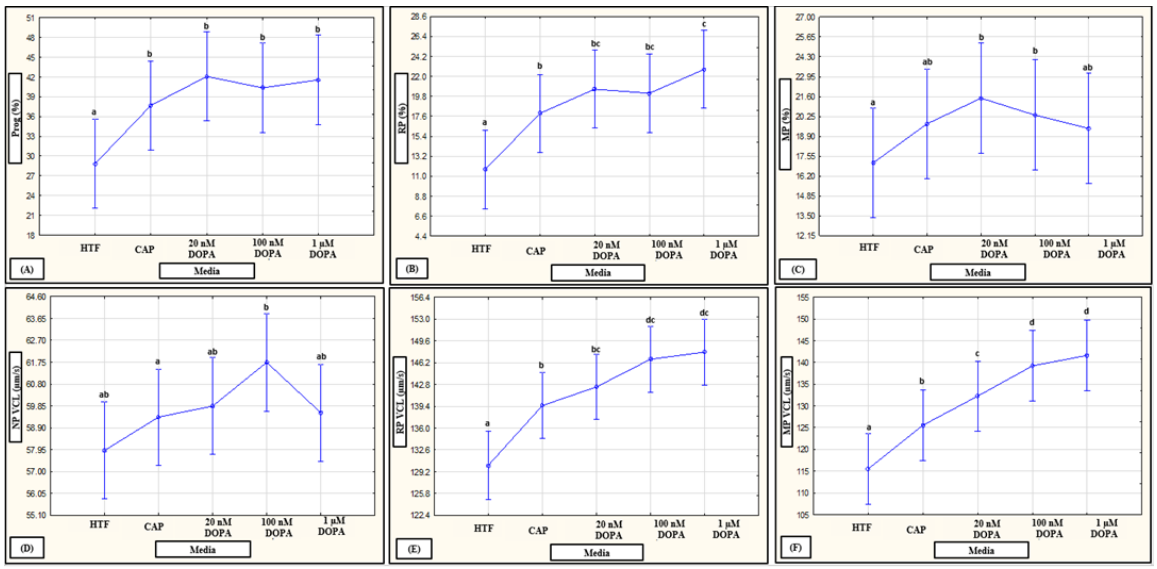

Figure 7. Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 min) as analyzed in a mixed model repeated measures ANOVA to determine the effect of media as a fixed factor. Effect of HTF, CAP and DOPA on: (**A**) progressivity (F (4452) = 11.41, $p < 0.01$); (**B**) rapid progressive (RP) speed groups (F (4452) = 14.67, $p < 0.01$); (**C**) medium progressive (MP) speed groups (F (4452) = 2.30, $p = 0.06$); (**D**) non-progressive (NP) VCL (F (4438) = 2.37, $p = 0.05$) (**E**) RP VCL (F (4438) = 23.82, $p = 0.34$); and (**F**) MP VCL (F (4421) = 15.24, $p = 0.34$). Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different ($p < 0.01$). The Fisher LSD was used for the post hoc test and reports. CAP, capacitating-HTF; DOPA, dopamine; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; Prog, progressive; RP, rapid progressive; VCL, curvilinear velocity.

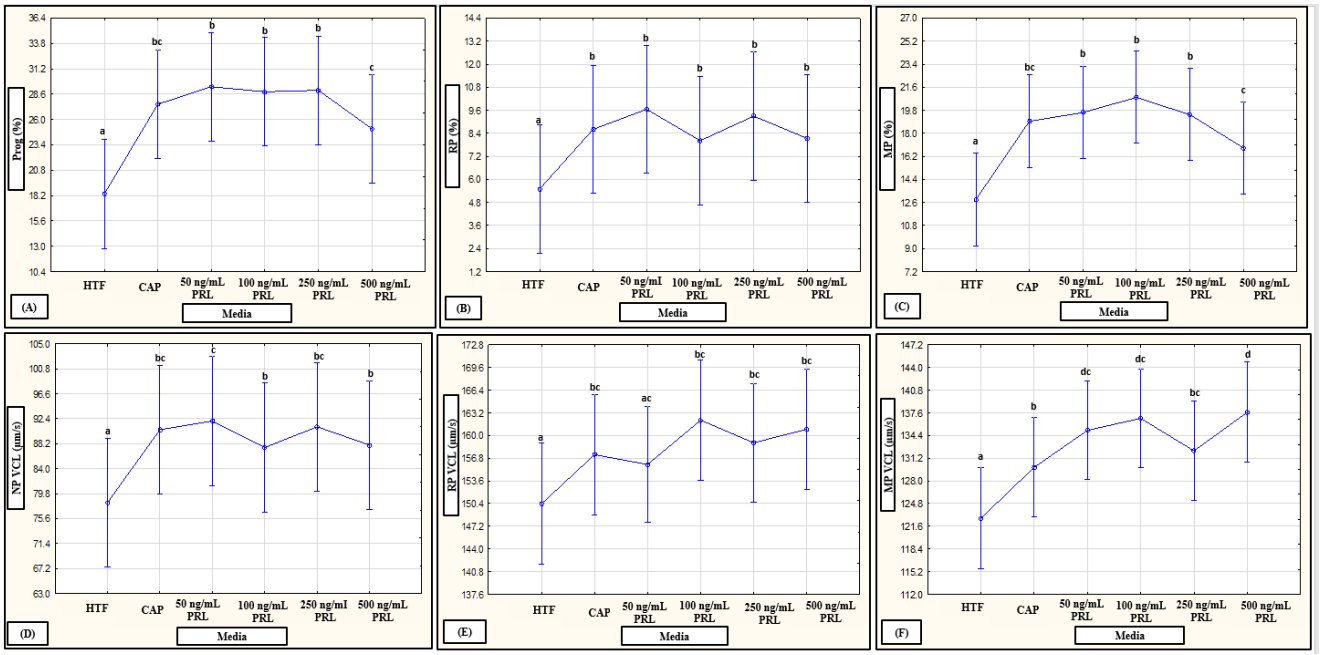

Figure 8. Pooled data of both high motile (HM) and low motile (LM) subpopulations and various time points (5, 30 min) as analyzed in a mixed model repeated measures ANOVA to determine the effect of media as a fixed factor. Effect of HTF, CAP and PRL on: (**A**) progressivity (F (5741) = 16.28, $p < 0.01$); (**B**) rapid progressive (RP) speed groups (F (5741) = 6.16, $p < 0.01$); (**C**) medium progressive (MP) speed groups (F (5741) = 13.25, $p < 0.01$); (**D**) non-progressive (NP) VCL (F (5730) = 12.61, $p < 0.01$); (**E**) RP VCL (F (5425) = 3.11, $p < 0.01$); and (**F**) MP VCL (F (5661) = 7.90, $p < 0.01$). Vertical bars denote 0.95 confidence intervals and bars labelled with different superscript letters (a, b and c) were significantly different ($p < 0.01$). The Fisher LSD was used for the post hoc test and reports. CAP, capacitating-HTF; HTF, human tubal fluid; MP, medium progressive; NP, non-progressive; Prog, progressive; PRL, prolactin; RP, rapid progressive; VCL, curvilinear velocity.

3.5. Hyperactivation

The HM subpopulation displayed significantly higher values for percentage hyperactivation as compared to the LM subpopulations at most time points when incubated in HTF (Supplementary Tables S4–S6). Exposure to different concentrations of the selected biological substances generally induced a higher percentage hyperactivation in both subppulations over time (Figure 1). However, the media had a more pronounced effect on the LM subpopulations which resulted in the elimination of the differences between the two subpopulations (Figure 1) at most time points (Supplementary Tables S4–S6). Increased concentrations of the substances did not necessarily have the same effect on the two subpopulations. For instance, dopamine significantly increased hyperactivation in the HM subpopulations as compared to HTF; however, higher dopamine concentrations appeared to increase hyperactivation in the LM subpopulations, as compared to HTF and CAP (Supplementary Table S5). Prolactin also resulted in an increase in percentage hyperactivation of both subpopulations as compared to HTF; however, less variation between concentrations and time points were observed in the LM subpopulations as was found in the HM subpopulation (Supplementary Table S6).

Individual fixed-factor effects as well as interactions between fixed factors as were highlighted with a mixed model repeated measures ANOVA are displayed in Figures 9–11. Both media and time as fixed factors had significant effects on hyperactivation percentages after exposure to HD-C, progesterone, myo-inositol (Figure 9) and prolactin (Figure 11). While CAP, progesterone, myo-inositol and prolactin induced hyperactivation, exposure to HD-C and 3.96 µM progesterone (Figure 9A) and all concentrations of prolactin (Figure 11A) resulted in significantly higher percentages. An increase in percentage hyperactivation was observed from 5 min to 15 min after exposure to media, where after a significant

decline was observed between 30–45 min, indicating an early stimulatory effect of media on hyperactivation (Figures 9B and 11C).

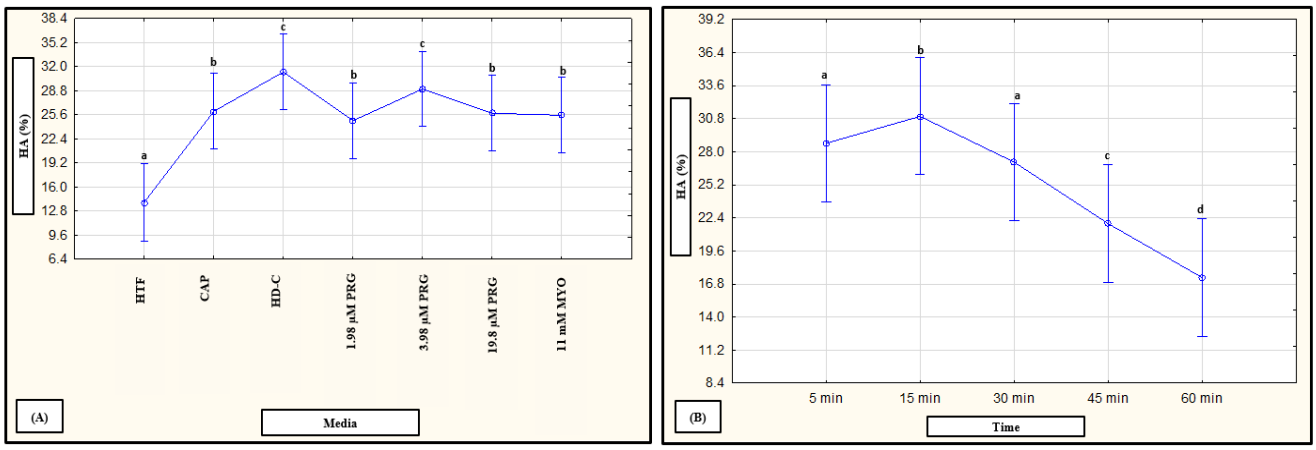

Figure 9. Mixed model repeated measures ANOVA to determine the effect media and time as individual fixed factors on percentage hyperactivation. (**A**) Effect of HTF, CAP, HD-C, PRG and MYO ($F(6, 1252) = 33.06$, $p < 0.01$). (**B**) Effect of time (5, 15, 30, 45 and 60 min) for pooled data of HTF, CAP, HD-C, PRG and MYO ($F(4, 1250) = 60.35$, $p < 0.01$). Vertical bars denote 0.95 confidence intervals and bars labelled with different letters were significantly different ($p < 0.01$). The Fisher LSD was used for the post hoc test and reports. CAP, capacitating-HTF; HD-C, HD capacitating medium; HA, hyperactivation; HTF, human tubal fluid; MYO, myo-inositol; PRG, progesterone.

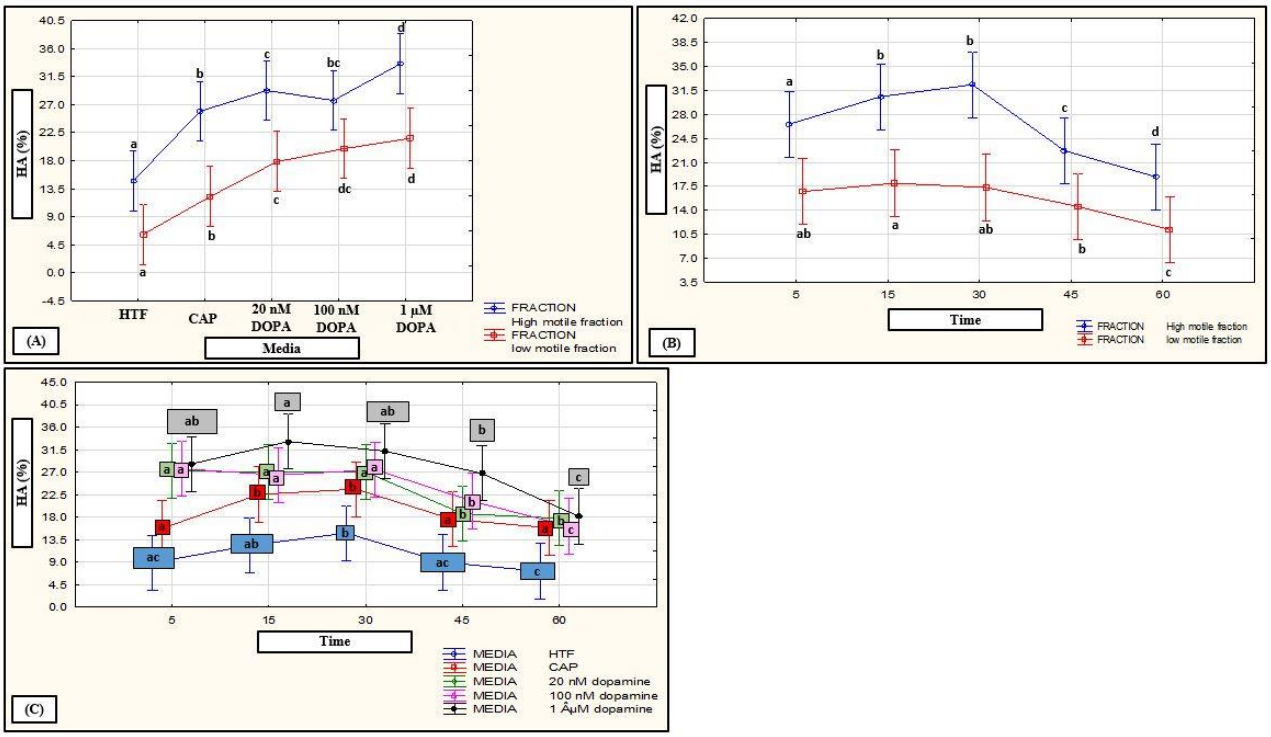

Figure 10. Mixed model repeated measures ANOVA to determine interactions between media and time as individual fixed factors and combined on percentage hyperactivation. (**A**) Interaction of media (HTF, CAP and DOPA) on HM and LM subpopulations ($F(4, 931) = 2.45$, $p = 0.04$). (**B**) Interaction of time (5, 15, 30, 45 and 60 min) on HM and LM subpopulations for pooled data of HTF, CAP and DOPA ($F(4, 931) = 3.87$, $p < 0.01$). (**C**) Interaction of media (HTF, CAP and DOPA) and time (5, 15, 30, 45 and 60 min) ($F(16, 931) = 1.77$, $p = 0.03$). Vertical bars denote 0.95 confidence intervals and bars labelled with different letters were significantly different ($p < 0.01$). The Fisher LSD was used for the post hoc test and reports. CAP, capacitating-HTF; DOPA; dopamine; HA, hyperactivation; HM, high motile; HTF, human tubal fluid; LM, low motile.

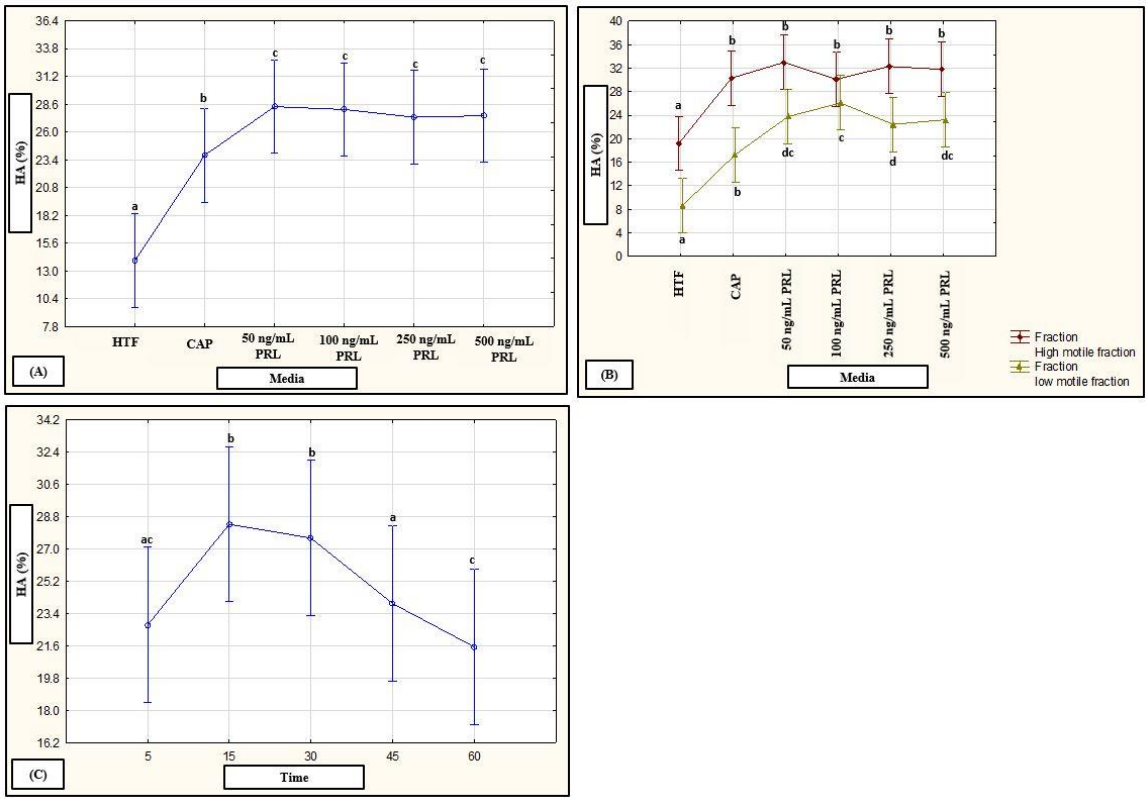

Figure 11. Mixed model repeated measures ANOVA to determine the effect of media and time as fixed factors as well as interactions between media and subpopulations on percentage hyperactivation. (**A**) Effect of HTF, CAP and PRL ($F(5, 1357) = 52.15$, $p < 0.01$). (**B**) Interaction between media (HTF, CAP and PRL) and subpopulations ($F(5, 1357) = 3.66$, $p < 0.01$). (**C**) Effect of time (5, 15, 30, 45 and 60 min) for pooled data of HTF, CAP and PRL ($F(4, 1357) = 18.09$, $p < 0.01$). Vertical bars denote 0.95 confidence intervals and bars labelled with different letters were significantly different ($p < 0.01$). The Fisher LSD was used for the post hoc test and reports. CAP, capacitating-HTF; HA, hyperactivation; HM, high motile; HTF, human tubal fluid; LM, low motile; PRL, prolactin.

Fixed factor interactions were found for "subpopulations and media" after exposure to dopamine and prolactin as well as for "subpopulations and time" after exposure to dopamine (Figure 10A,B and Figure 11B). While exposure to all concentrations of dopamine resulted in significantly higher percentages hyperactivation (compared to HTF and CAP), and 1 μM dopamine resulted in the highest hyperactivation percentages in both subpopulations, there was no difference in the percentage hyperactivation induced by 100 nM and 1 μM dopamine in the LM subpopulations (Figure 10A). A similar trend was seen after prolactin exposure, with significantly higher percentages of hyperactivation found compared to HTF in the HM subpopulations, as well as compared to HTF and CAP in the LM subpopulations; however, the highest concentrations of prolactin (250 ng/mL and 500 ng/mL) did not induce significantly higher percentages hyperactivation (Figure 11B). In terms of exposure time (Figure 10B), the LM subpopulations displayed a delayed or prolonged response to dopamine (insignificant increase in hyperactivation from 5 to 30 min with significant decrease at 60 min), whereas the HM subpopulations displayed a rapid response (increase in hyperactivation from 5 to 15 min with significant decrease at 45 min). Illustrated in Figure 10C, a fixed-factor interaction was also found for "media and time" after exposure to dopamine. HTF, CAP and dopamine resulted in an increased percentage hyperactivation from 5 to 30 min.

3.6. Reactive Oxygen Species

Significantly higher percentages of ROS positive spermatozoa ($p < 0.05$ and $p < 0.01$) were recorded for the LM subpopulations compared to the HM subpopulations after

exposure to all media (Figure 12A–C). However, exposure to 100 nM and 1 µM dopamine, as well as 50 ng/mL, 100 ng/mL and 250 ng/mL prolactin reduced ROS levels of the LM subpopulations which eliminated these differences between subpopulations (Figure 1). In addition, only prolactin in the LM subpopulations significantly decreased ROS levels ($p = 0.008$) as compared to HTF (Figure 12C). After data for subpopulations was pooled in a multifactorial ANOVA, significant interactions between media and ROS percentages were seen for HD-C, progesterone and myo-inositol ($p = 0.0059$), as well as for prolactin ($p = 0.002$), but not dopamine (Supplementary Figure S1A,B).

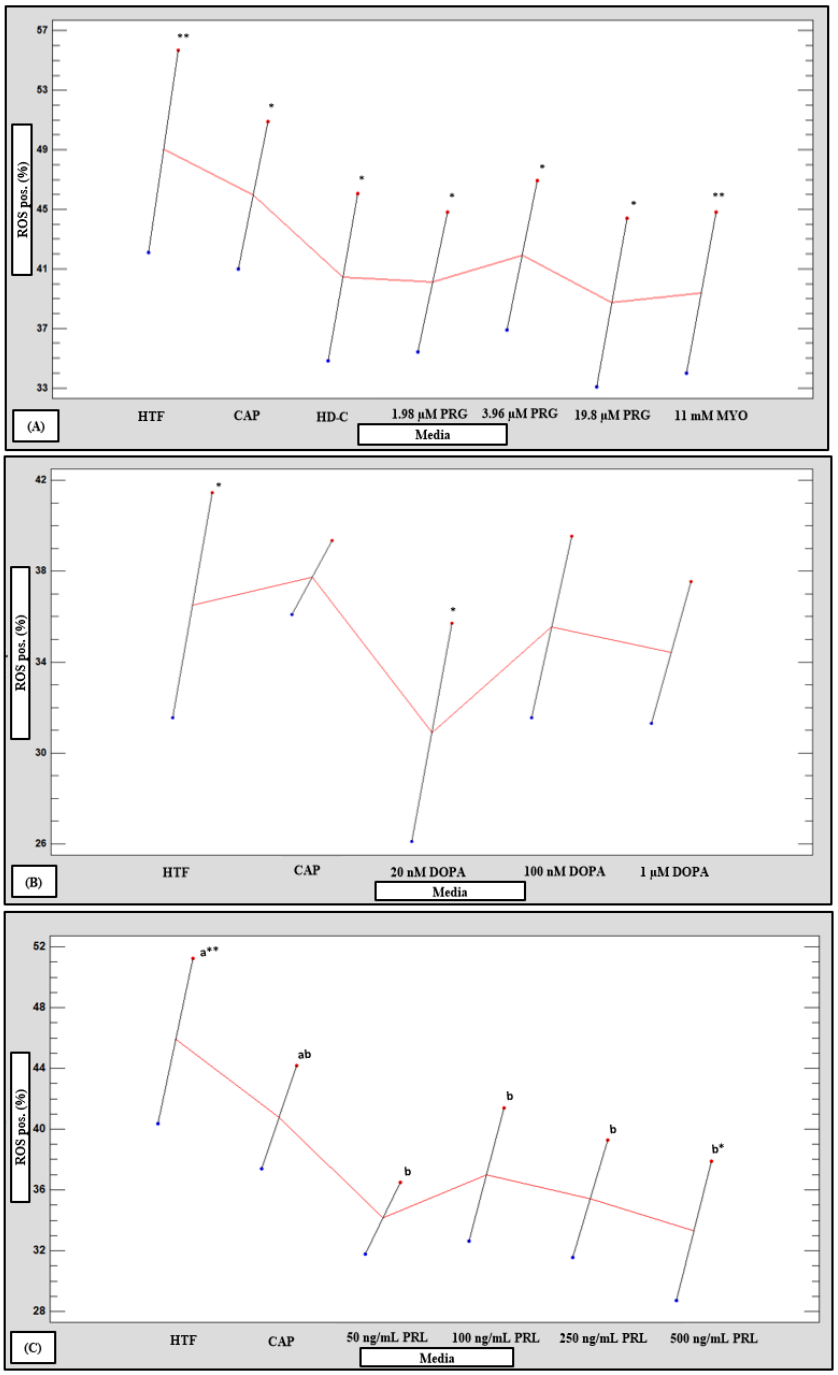

Figure 12. Multivariable chart comparing the mean percentage positive reactive oxygen species spermatozoa of high motile (HM, blue dots) and low motile (LM, red dots) sperm subpopulations

after treatment with various media (n = 20). Comparison of HM and LM subpopulations after treatment with: (**A**) HTF, CAP, HD-C, PRG and MYO; (**B**) HTF, CAP and DOPA; and (**C**) HTF, CAP and PRL. Vertical bars labelled with different letters (a, b, c) were significantly different between media for individual subpopulations ($p < 0.05$). Vertical bars labelled with an asterisk were significantly different between subpopulations for individual media (* $p < 0.05$, ** $p < 0.01$). CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitation medium; HTF, human tubal fluid; HM, high motile subpopulation; LM, low motile subpopulation; MYO, myo-inositol; PRG, progesterone; PRL, prolactin; ROS pos., positive reactive oxygen species.

3.7. Mitochondrial Membrane Potential

HM subpopulations had significantly higher percentages of spermatozoa with intact MMP ($p < 0.05$, $p < 0.01$ and $p < 0.001$) compared to the LM subpopulations after exposure to all media (Figure 13A–C). However, 1 µM dopamine, as well as 50 ng/mL, 250 ng/mL and 500 ng/mL prolactin were able to maintain and increase MMP intactness in the LM subpopulations to the extent that differences between subpopulations were eliminated (Figure 1). No significant differences between media were seen in the HM subpopulations; however, in the LM subpopulations, myo-inositol ($p = 0.048$) as well as 50 ng/mL, 250 ng/mL and 500 ng/mL prolactin ($p = 0.012$) all significantly maintained higher percentages of intact MMP as compared to HTF (Figure 13A–C). Once data were pooled and analyzed in the multifactorial ANOVA, a significant interaction was observed between media and percentages of MMP intactness (Supplementary Figure S2A,B). Compared to HTF, 500 ng/mL prolactin maintained significantly higher MMP percentages ($p = 0.0037$). Myo-inositol also significantly maintained a higher percentage MMP intactness ($p = 0.0153$) as compared to both HTF and CAP.

3.8. Acrosome Reaction

The LM subpopulations revealed significantly higher percentages of spontaneous and induced acrosome reaction compared to the HM subpopulations after treatment with HTF, CAP, HD-C, progesterone (Figure 14A) and prolactin (Figures 1 and 14C). In contrast, the HM subpopulations had significantly higher percentages of Ca-ionophore induced acrosome reaction (Figure 14A–C) compared to the LM subpopulations. Nonetheless, progesterone, myo-inositol and dopamine increased the percentage of acrosome reacted spermatozoa in the HM subpopulations (Figures 1 and 14A,B). In the LM subpopulations, only myo-inositol significantly increased the percentage of acrosome reacted spermatozoa as compared to HTF (Figure 14A).

For both subpopulations, Ca-ionophore consistently obtained significantly higher percentages of acrosome reacted spermatozoa ($p < 0.001$) as compared to all other media used (Figure 14A–C, Supplementary Figure S3A–C). Once data were pooled in a multifactorial ANOVA, significant interactions between media and acrosome reaction were observed. Compared to HTF and CAP, progesterone and myo-inositol significantly increased the percentage acrosome reacted spermatozoa ($p < 0.001$) (Supplementary Figure S3A). Compared to HTF, 100 nM and 1 µM dopamine significantly increased the percentage acrosome reacted spermatozoa ($p < 0.001$) (Supplementary Figure S3B).

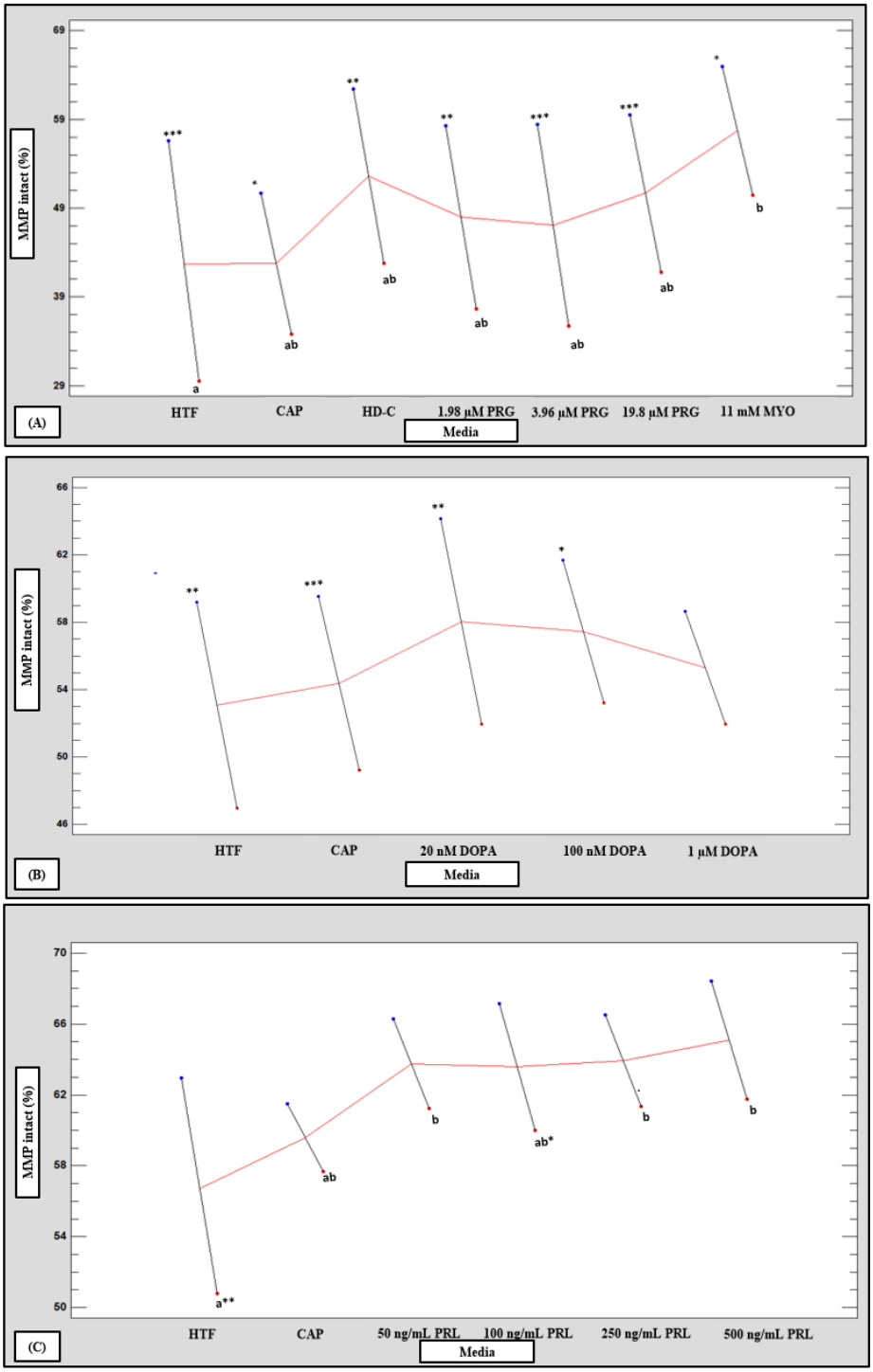

Figure 13. Multivariable chart comparing the mean percentage intact mitochondrial membrane potential (MMP) of the low (LM, red dots) and high (HM, blue dots) motile sperm subpopulation after incubation in various media (n = 20). Comparison of percentage intact MMP of LM and HM subpopulations after treatment with: (**A**) HTF, CAP, HD-C, PRG and MYO; (**B**) HTF, CAP and DOPA; and (**C**) HTF, CAP and PRL. Values labelled with different letters (a, b, c) were significantly different between the various media for individual subpopulations. Values with asterisks were significantly different between the HM and LM subpopulations for individual media (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitation medium; HTF, human tubal fluid; HM, high motile subpopulation; LM, low motile subpopulation; MMP, mitochondrial membrane potential; MYO, myo-inositol; PRG, progesterone; PRL, prolactin.

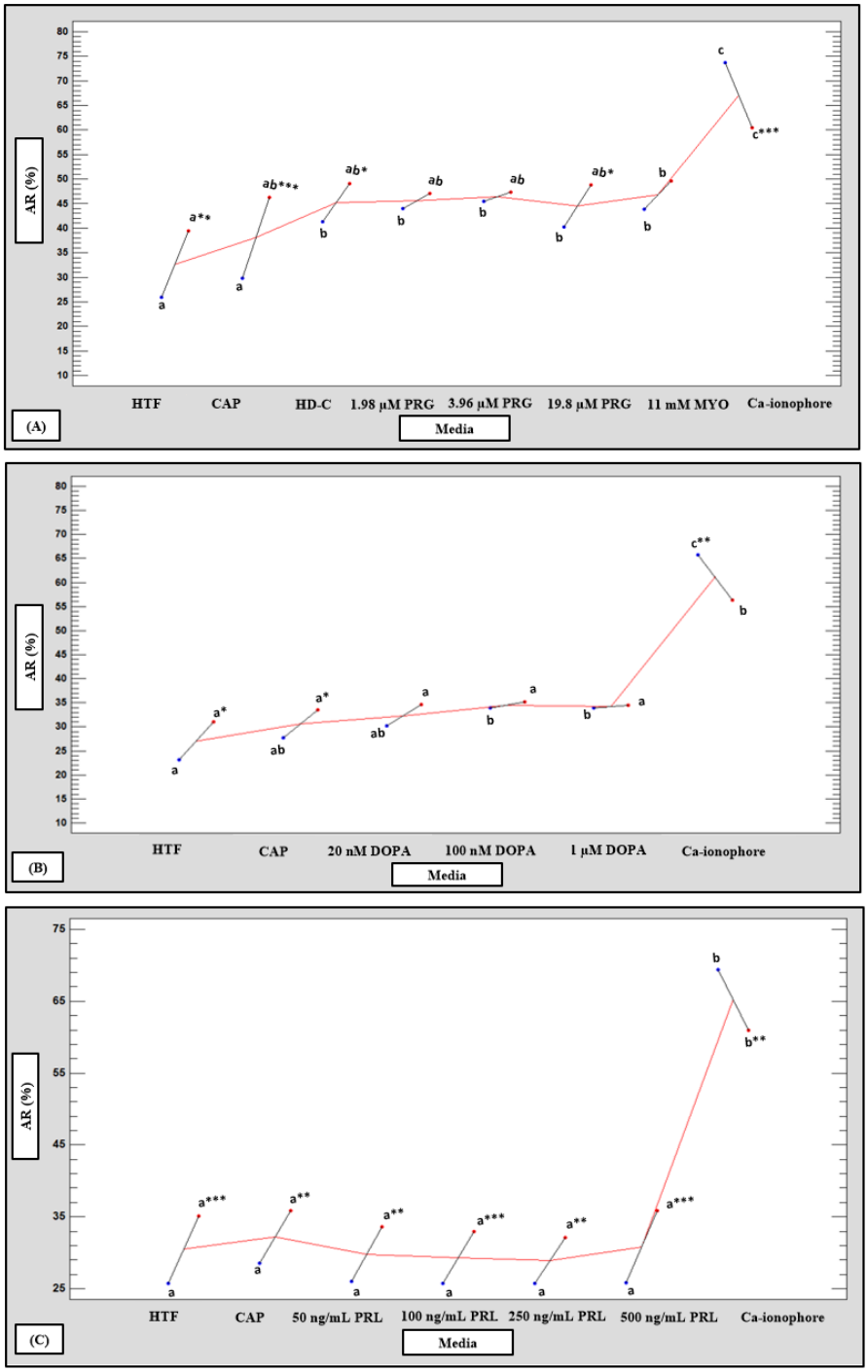

Figure 14. Multivariable chart displaying the percentage acrosome reaction (AR) of the low (LM, **red** dots) and high (HM, blue dots) motile sperm subpopulation after incubation in various media ($n = 20$). Comparison of percentage AR for the LM and HM motile sperm subpopulation after incubation in: (**A**) HTF, CAP, HD-C, PRG, MYO and Ca-ionophore; (**B**) HTF, CAP, DOPA and Ca-ionophore; and (**C**) HTF, CAP, PRL and Ca-ionophore. Values labelled with different letters (a, b, c) were significantly different between the various media for individual subpopulations. Values with asterisks were significantly different between the HM and LM subpopulations for individual media (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). AR, acrosome reaction; Ca-ionophore, calcium-ionophore; CAP, capacitating-HTF; DOPA, dopamine; HD-C, HD capacitation media; HTF, human tubal fluid; HM, high motile subpopulation; LM, low motile subpopulation; MMP, mitochondrial membrane potential; MYO, myo-inositol; PRG, progesterone; PRL, prolactin.

4. Discussion

This study investigated the effects of specific biological substances found in follicular fluid (FF) on two human sperm motility subpopulations. Our results confirm that HM and LM subpopulations are diverse in their structural and functional characteristics and indicate that these subpopulations respond differently after short-term exposure to various concentrations of the biological substances tested. While the HM subpopulations' functional status was often not significantly affected by such exposure, progesterone, myo-inositol and dopamine improved the HM subpopulations' motility parameters and acrosome reaction. In contrast, all the biological substances investigated were capable of improving the LM subpopulations' reduced functional capabilities to some extent (as compared to HM subpopulations) and, in many instances, such improvements eliminated the initial differences found between the two subpopulations in HTF.

4.1. Progesterone

Progesterone in FF varies in concentrations throughout the female reproductive tract, and is well known to effect spermatozoa [21] due to progesterone-sensitive CatSper Ca^{2+} channels located near the principal piece of the flagellum [21,51–54]. Ghanbari et al. [53] observed a reduction in sperm vitality after CatSper channels of progesterone-stimulated sperm were inhibited. In the current study, progesterone had no effect on percentage vitality of individual sperm subpopulations, which agrees with the findings reported by Contreras et al. [55]. However, with media considered as a fixed factor, exposure to progesterone increased the overall sperm vitality percentages compared to the control medium.

Progesterone increased the rapid speed and rapid progressivity (RP) groups, with increased percentages reported for both subpopulations. In accordance with our results, Ghanbari et al. [53] found that progesterone increased progressive motility, whereas Pujianto et al. [56] reported that progesterone displayed a dose-dependent response with increased motility percentages at higher concentrations (500 ng/mL). In the current study, 3.96 µM progesterone seems to have the largest stimulatory effect on both subpopulations' motility percentages as well as collectively with media as fixed factor. Progesterone increased the velocity kinematics of the RP and MP groups, but linear kinematics decreased. Calogero et al. [57] similarly reported that progesterone increased BCF and VCL, but decreased LIN and STR of motile spermatozoa. Furthermore, the observed improvements in the LM subpopulations' motility parameters may be a result of either intracellular pH or proper balancing of intracellular ions, and not necessarily activation of progesterone CatSper Ca^{2+} influx [58].

Progesterone is well documented to induce hyperactivation and elicits different effects at varying concentrations [53,54,57,59–61]. Similarly, we observed that exposure to 3.96 µM progesterone induced higher hyperactivation percentages compared to 1.98 µM and 19.8 µM progesterone. Whereas typical steroid signaling pathways are time-consuming due to hormones binding to genomic receptors, progesterone bypasses this process by binding to sperm plasma membrane receptors and causes a rapid Ca^{2+} influx that is sustained for several minutes [52,57]. This effect of progesterone is in agreement with our findings, with an increased percentage hyperactivation observed after 5 min of exposure. However, time differences for subpopulations were evident, with the LM subpopulations exhibiting a small, gradual increase and decrease in percentage hyperactivation after progesterone exposure, while the HM subpopulations revealed a sudden increase and decrease in this sperm parameter. Progesterone-induced Ca^{2+}-influx followed by Ca^{2+} oscillations in the intracellular compartment could be responsible for the observed motility and hyperactivation patterns in the HM subpopulations, by prolonging motility regulation and assisting in repeated alteration of activated and hyperactive motility [61]. Genomic errors at CatSper subunit loci are reported in infertile men; it has been suggested this prevents functional channel expression and completely compromises fertilization in vivo and in vitro [62]. This could be a plausible causative effect of the lower percentage of hyperactivation and only small increases found in the LM subpopulations as compared to the HM subpopulations.

The physiological activation of AR by progesterone is well documented for humans, but less so between different sperm subpopulations [53,57,60,63]. Our results indicate that progesterone only induced AR in the HM subpopulations, but values remained in the range of 20–50%, and thus significantly lower than the 60–75% AR reported after Ca-ionophore exposure. Similarly, Tantibhedhyangkul et al. [60] reported that despite progesterone-induced Ca^{2+} increases, less than 50% of spermatozoa actually undergo AR. The seemingly inability of progesterone to rapidly induce higher hyperactivation and AR percentages in the LM subpopulations could be related to slowness or deficiency in the formation of Ca^{2+} signaling domains. To successfully achieve capacitation by CatSper, a quadrilateral arrangement of four pore-forming α-subunits needs to develop, which structurally organizes unique Ca^{2+} signaling domains along the flagellum [58,64]. The absence or alteration of genes that prevent the formation of this heterotetrametric CatSper complex will thereby prevent capacitation [58,64].

Even though Tantibhedhyangkul et al. [60] reported a progestin-dependent increase in MMP after 90 min incubation, the MMP levels of both subpopulations did not significantly change after 30 min progesterone exposure in the current study—the difference possibly being related to exposure time. It was speculated that increased progesterone concentrations in the female reproductive tract may stimulate mitochondrial progesterone receptors (PR-M), increasing mitochondrial ATP [60]. Furthermore, Fan et al. [65] reported that progesterone reduced ROS production as well as balanced MMP in spermatozoa. Our results indicated no effect of progesterone exposure on ROS levels in the two subpopulations; however, when media was considered as a fixed factor, progesterone significantly reduced ROS positive spermatozoa.

4.2. Myo-Inositol

As a precursor to secondary messengers in cellular signal transduction systems, myo-inositol plays an essential role in morphogenesis, cytogenesis, membrane formation/growth and lipid production [66–68]. Dog sperm with myo-inositol supplementation reportedly had higher vitality percentages as compared to controls, which is consistent with our results [68]. Myo-inositol displayed the greatest effect on preserving sperm vitality in both the LM subpopulation and collectively with media as fixed factor. The ability to maintain and preserve sperm vitality may be a result of myo-inositol's role in lipid production, thereby maintaining intact plasma membranes [68].

Similar to progesterone, myo-inositol generally improved sperm motility, but had a more substantial effect on the LM subpopulations' motility parameters (rapid and RP speed percentages) as well as kinematic parameters (MP and NP speed groups). According to Governini et al. [69], treatment with myo-inositol significantly increased sperm motility and oxygen consumption in oligoasthenozoospermic patients. In addition, Artini et al. [70] observed an increase in progressive motility of both normospermic and oligoasthenospermic semen after treatment with myo-inositol (15 µL/mL). These observations in LM subpopulations and infertile/subfertile males after myo-inositol exposure may be indicative of its ability to increase oxidative phosphorylation efficiency and ATP production by the mitochondria [71].

Myo-inositol induced hyperactivation and AR in both subpopulations, as well as when media was considered as a fixed factor. By activating phospholipase-C, myo-inositol results in production of inositol triphosphate, which opens Ca^{2+} channels and regulates intracellular Ca^{2+} stores in the sperm plasma membrane, mitochondria, acrosome and neck. Ultimately, myo-inositol thus indirectly stimulates oxidative mechanisms and ATP production, which improve mitochondrial function, prevent apoptosis and assists sperm capacitation [69,72,73].

Furthermore, myo-inositol also improved MMP of the LM subpopulation and had the largest effect on improving MMP and decreasing ROS percentages when media was considered as a fixed factor. It is reported that elevated ROS levels compromise sperm quality due to lipid peroxidation and loss of membrane fluidity, mitochondrial dysfunction,

altered morphology, reduced vitality and other changes that result in the failure to fertilize [73]. Supplementation with myo-inositol thus seems to be beneficial, since it preserves vitality and MMP function, reduces and controls ROS levels, improves ATP production and reduces oxidative stress that alters capacitation. As oxidative stress has been observed in 30–80% of infertile patients, multi-antioxidant supplementation is an effective treatment that helps to improve male fertility parameters [73].

4.3. HD-C Medium

HD-C medium is a sperm capacitation medium containing both progesterone and myo-inositol. Exposure of sperm subpopulations to HD-C resulted in similar effects on preserving sperm vitality as progesterone and myo-inositol when media was considered as a fixed factor. HD-C exposure also displayed a similar pattern to progesterone and myo-inositol media on its own, with improvement in motility percentages (mainly rapid and MP speed groups) and kinematic parameters (increased NP velocity kinematics and decreased linear and vigor kinematics). The great effect on inducing hyperactivation and the increases observed in the AR of the HM subpopulation after HD-C exposure are possibly due to the presence of progesterone in the medium, and are in accordance with the literature [69,72,73].

Although HD-C did not affect MMP, a significant decrease in ROS percentages was evident when media was considered as a fixed factor. These observations could be a result of myo-inositol's antioxidant interactions within the media. Subfertile spermatozoa produce large amounts of ROS, which impair various functional characteristics of spermatozoa and prevent sperm capacitation [69,72,73]. HD-C exposure counteracts these detrimental effects by improving sperm functionality, such as motility and hyperactivation, and reducing ROS. In addition, myo-inositol can probably improve the functionality of spermatozoa which may have altered CatSper channels and therefore do not respond to progesterone [62]. HD-C thus has the ability to interact with various functional sperm qualities and therefore augment the different fertilizing properties.

4.4. Dopamine

Dopamine is present in semen and oviduct fluids, and dopamine receptors have been reported in spermatozoa [13]. In accordance with Cariati and colleagues [74], we observed no significant effect of dopamine on sperm vitality percentages in subpopulations. However, dopamine improved the LM subpopulations' vitality percentages to the extent that differences between the subpopulations were eliminated. Ramirez et al. [13] found that after a 3 h incubation in low levels of dopamine, the activation of DRD2-receptors in boar spermatozoa maintained sperm viability. As subpopulations were only incubated for 30 min in the current study, longer incubation time may have resulted in higher vitality percentages as compared to controls.

Our findings showed that dopamine improved motility percentages of both subpopulations and with media as a fixed factor. These findings are consistent with previous studies [29,74], which reported that dopamine could enhance sperm motility parameters following 30 min of incubation. Singh et al. [75] demonstrated that after being impaired by ethinyl estradiol, rats treated with dopamine had significantly improved sperm motility, MMP and ROS levels.

In addition, higher dopamine concentrations increased sperm hyperactivation percentages (1 μM) in both subpopulations, as well as acrosome reaction percentages (100 nM and 1 μM), but only in the HM subpopulation. Our results are in agreement with Urra et al. [29], who reported increased sperm capacitation in vitro after dopamine stimulation, possibly due to increased protein–tyrosine phosphorylation and sperm motility. The apparent inability of dopamine to increase acrosome reaction in the LM subpopulations could be related to the fact that protein–tyrosine phosphorylation may be altered in LM subpopulations or subfertile males [76]. Interestingly, much higher dopamine concentrations (1 mM) have been reported to decrease both aspects; thus, dopamine may have a biphasic effect at

varying concentrations [13,16,29]. Urra et al. [29] observed a decrease in acrosome integrity of stallion sperm after 6 hours' incubation in high concentrations of dopamine (1 mM). Given that spermatozoa contain both β- and α-adrenergic receptors, it is plausible that high doses activate inhibitory β-adrenergic receptors, whereas low concentrations stimulate α-adrenergic receptors [77].

Our results confirmed that dopamine decreased ROS but improved MMP of the LM subpopulation. Since human infertility is associated with complex disturbances in hormones as well as ROS generation and disposal, exposure of sperm to dopamine may enhance its antioxidant and free radical scavenging properties [75].

4.5. Prolactin

Prolactin receptors are located on the post-acrosomal region of the sperm head, neck, midpiece and principal piece of the sperm tail; however, the exact role of the hormone in sperm functionality is disputed in the literature [6]. With media considered as a fixed factor, prolactin exposure preserved sperm vitality percentages. Pujianto and colleagues [6] reported that prolactin may have the potential to inhibit sperm capacitation while simultaneously acting as a pro-survival factor that prevents spermatozoa from entering the default apoptotic pathway associated with ROS generation by mitochondria [78].

Serum prolactin levels are suggested to keep the male reproductive tract healthy, and prolactin is believed to improve sperm motility by increasing oxygen uptake by spermatozoa [79]. We observed that prolactin largely increased motility percentages in the LM subpopulations and when media was considered as a fixed factor. In contrast to the other media, prolactin did not appear to affect the kinematic parameters of the individual subpopulations. A possible explanation for prolactin mainly affecting the LM subpopulation could be that LM subpopulations, similar to asthenozoospermic males, may have compromised energy metabolism [80] as compared to HM subpopulations, and that prolactin may have a greater impact on improving mitochondrial ATP production in spermatozoa exhibiting low levels of motility.

Prolactin has been suggested to affect a range of processes in vitro such as Ca^{2+} binding, maintaining mobility and attachment, as well as reducing capacitation time [81]. We observed that prolactin exposure significantly increased and maintained hyperactivation in both subpopulations. It is plausible that prolactin induces hyperactivation, as a dose-dependent increases of cAMP levels as well as utilization of fructose and glucose were demonstrated after exposure to bovine prolactin [82]. In contrast, no effect was found on subpopulation AR, as was also reported by Stovall and Shabanowitz [82] for similar prolactin concentrations (0–200 ng/mL) on sperm AR. The lack of effect on AR may reflect its suggested inhibitory effect on phosphotyrosine expression in spermatozoa [6]. While the HM subpopulation did not respond to prolactin exposure, the higher concentrations of prolactin (250 ng/mL and 500 ng/mL) did increase MMP values and reduce ROS in the LM subpopulation, which may reflect an increased energy metabolism in the LM subpopulations, as well as prolactin's pro-survival effect [6,78].

5. Conclusions

This study highlighted the diverse functional characteristics of sperm subpopulations and recommends that their unique functional capabilities be considered when selecting an appropriate medium to enhance individual sperm attributes. As such, progesterone could be a potential medium to assist in inducing hyperactivation or improve progressivity in clinical scenarios, while HD-C has the potential to improve both HM and LM subpopulations' sperm qualities. Dopamine and myo-inositol's antioxidant capabilities could be utilized in semen to preserve MMP and vitality before processing/separation of semen samples, thereby increasing overall sperm motility while reducing ROS and subsequently DNA damage. Future investigations on possible pro-survival substances such as prolactin may allow for possible storage of spermatozoa under ART conditions, or, alternatively, be used as cultivation media for the processing of samples. Moreover, due

to the heterogeneity of human ejaculates, it may be necessary to use media that contain a combination of biological substances in concentrations that are targeted for each subpopulation. Since the LM subpopulation closely mimics the functionality of sub-fertile semen samples, it is plausible that such media can be utilized in clinical scenarios to enhance the functionality of spermatozoa before ART treatment. Alternatively, exposing sub-fertile samples to a selection of the substances investigated could potentially increase the quality of retrieved spermatozoa so that more affordable ART techniques can be utilized; for example, intrauterine insemination (IUI) instead of intracytoplasmic sperm injection (ICSI).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/life11111250/s1, Supplementary Table S1. Comparison of mean SCA motility and kinematic parameters at 5 and 30 min for both the low motile (LM) and high motile (HM) sperm subpopulations after treatment with HTF, CAP, HD-C, 1.98 μM progesterone, 3.96 μM progesterone, 19.8 μM progesterone and 11 mM myo-inositol (mean ± SD) (n = 20). Supplementary Table S2. Comparison of mean SCA motility and kinematic parameters at 5 and 30 min for both the low motile (LM) and high motile (HM) sperm subpopulations after treatment with HTF, CAP, 20 nM, 100 nM and 1 μM dopamine (mean ± SD) (n = 20). Supplementary Table S3. Comparison of mean SCA motility and kinematic parameters at 5, 30 and 60 min for both the low motile (LM) and high motile (HM) sperm subpopulations after treatment with HTF, CAP, 50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL prolactin (mean ± SD) (n = 20). Supplementary Table S4. Percentage hyperactivation in both low motile and high motile sperm subpopulations after treatment with HTF, CAP, HD-C, 1.98 μM progesterone, 3.96 μM progesterone, 19.8 μM progesterone and 11 mM myo-inositol at 5, 15, 30, 45 and 60 min (mean ± SD) (n = 20). Supplementary Table S5. Percentage hyperactivation in both low motile and high motile sperm subpopulations after treatment with HTF, CAP, 20 nM, 100 nM and 1 μM dopamine (mean ± SD) (n = 20). Supplementary Table S6. Percentage hyperactivation in both low motile and high motile sperm subpopulations after treatment with HTF, CAP, 50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL prolactin at 5, 15, 30, 45 and 60 min (mean ± SD) (n = 20). Supplementary Figure S1. Multifactorial graphical ANOVA of the pooled data from the high motile (HM) and low motile (LM) sperm subpopulations percentage reactive oxygen species (ROS) positive spermatozoa. Supplementary Figure S2. Multifactorial graphical ANOVA of the pooled data from the high motile (HM) and low motile (LM) sperm subpopulations percentage intact mitochondrial membrane potential (MMP). Supplementary Figure S3. Multifactorial graphical ANOVA of the pooled data from the high motile (HM) and low motile (LM) sperm subpopulations percentage acrosome reaction (AR).

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Institutional Review Board Statement: The study was approved by the ethical boards of the University of the Western Cape (code BM20/9/14), and Stellenbosch University (code N14/06/074). The Helsinki Declaration [32] governing research on humans has been adhered to and each human donor gave written consent.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Conflicts of Interest: G.v.d.H is a senior consultant to Microptic SL (Barcelona, Spain), from which the SCA® system was purchased. This has no bearing on the outcome of the results.

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New approaches to define the functional competency of human sperm subpopulations and its relationship to semen quality

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Abstract

Background: This study aimed at comparing a comprehensive set of functional and structural sperm characteristics between sperm motility fractions and correlating results to the standard semen parameters. By grouping related variables, our objective was to establish the predictive power of semen parameters and whether they accurately reflect the functionality of sperm motility fractions or merely a small set of parameters within individual fractions.

Materials and methods: Donor semen samples (n = 55) were separated via double density gradient centrifugation, isolating a high (HM) and low motile (LM) sperm fraction. Fractions were evaluated for percentage vitality, chromatin integrity, mature spermatozoa, motility and kinematic parameters, hyperactivation, positive reactive oxygen species, intact mitochondrial membrane potential and acrosome reaction.

Results: HM fractions had significantly ($p < 0.001$) enhanced percentages of induced acrosome reaction (HM, 55.6 ± 14.3%; LM, 25.0 ± 16.5%), motility and kinematic parameters, hyperactivation, vitality (HM, 70.4 ± 9.7%; LM, 47.9 ± 10.3%), mitochondrial membrane intactness (HM, 67.2 ± 10.4%; LM, 44.7 ± 15.0%) and mature spermatozoa (HM, 83.4 ± 10.0%; LM, 64.6 ± 8.2%) with intact chromatin (HM, 80.5 ± 8.1%; LM, 71.3 ± 8.0%). Various sperm morphology abnormalities correlated with LM fractions' grouped motility parameters (range, 0.46 to 0.51; range -0.4 to -0.75), whereas combined semen traits of total motility, progressive motility, viscosity and mucus penetration correlated with HM fractions' grouped motility parameters (range, 0.44 to 0.84).

Conclusion: Collectively, total and progressive motility, viscosity and mucus penetration may represent a reliable grouping of semen parameters for predicting the quality of HM sperm fractions. Separating the same donor semen samples into two significantly diverse motility sperm fractions could be a potential model in mimicking the qualities of fertile and sub-fertile males' sperm populations and could be used for future research on the improvement of sperm subpopulations from males with different fertility statuses.

Keywords

CASA ; Differential gradient centrifugation ; Hyperactivation ; Reactive oxygen species ; Sperm motility subpopulations

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IMPROVED SPERM FUNCTION IN HUMAN SPERM SUBPOPULATIONS: A MODEL FOR STUDYING SUBFERTILITY

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INTRODUCTION

Infertility is affecting one in four couples in developing countries, with a male factor being implicated in about half of these cases. In approximately 15% of the presumed sub- or infertile males, their semen analyses do not reveal obvious abnormalities (idiopathic), thereby conveying the importance of sperm functional testing. Furthermore, human semen is heterogeneous in nature, including subpopulations with different degrees of maturation and fertilizing ability. Evaluating the quality of such sperm subpopulations can reveal the functional capabilities of sperm in a semen sample and may provide a model for studying sperm functionality in sub-fertile males. This study aimed to determine and compare the functional characteristics of two sperm subpopulations found within the same human semen samples. Concomitantly, the improvement of such functional parameters was evaluated after exposure to various capacitating media and chemicals.

MATERIALS AND METHODS

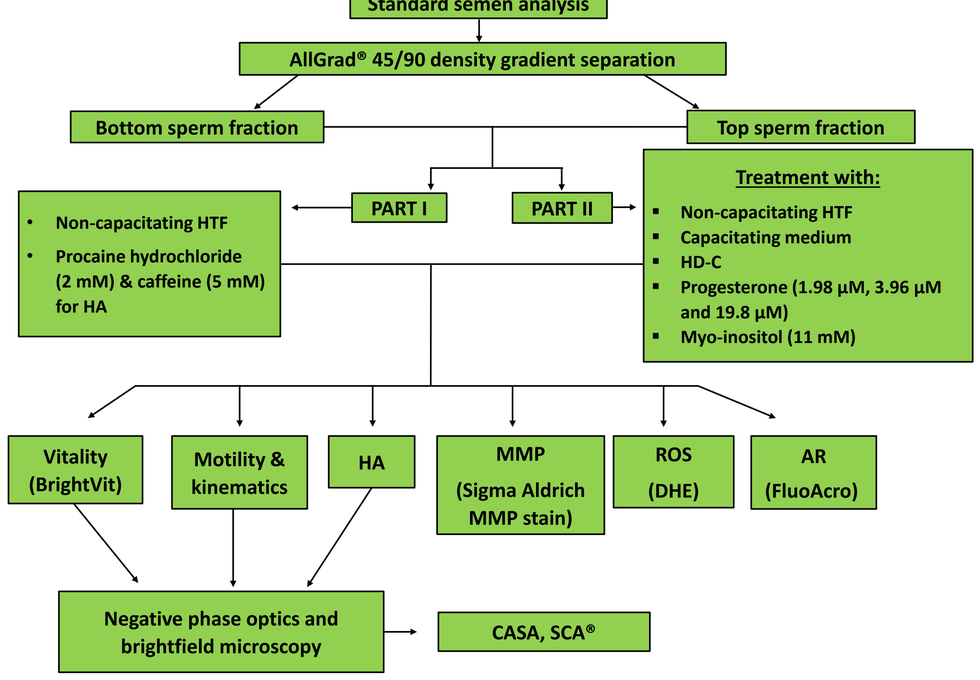


Figure 1. Schematic illustration of the study methodology.
HTF = human tubal fluid, HA = hyperactivation, HD-C = new capacitation medium , MMP = mitochondrial membrane potential, ROS = reactive oxygen species, DHE = dihydroethidium stain, AR = acrosome reaction, CASA = computer-aided sperm analysis and SCA® = Sperm Class Analyzer.

RESULTS

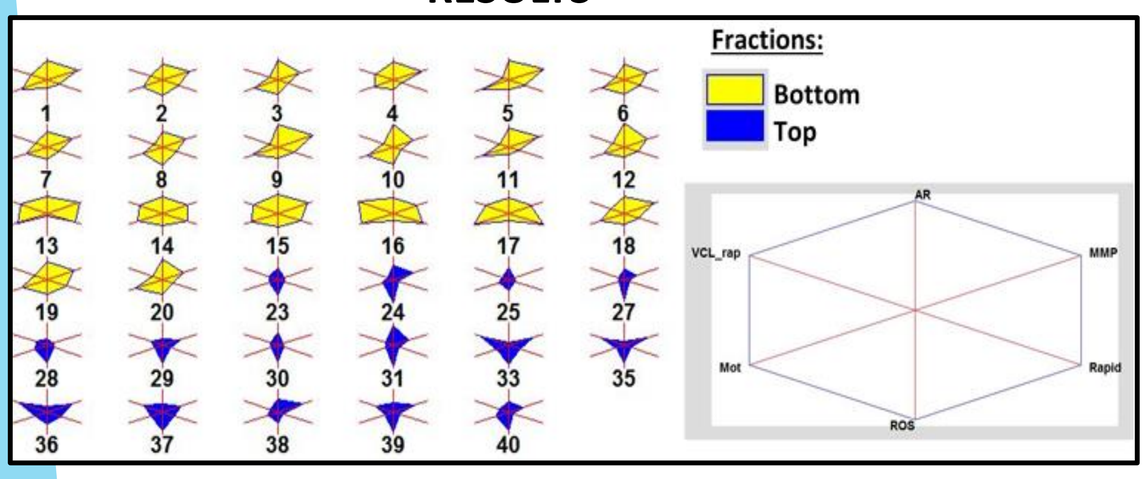


Figure 2: Star glyphs representing different parameters for individual samples top and bottom fractions (n=20). Acrosome reaction (AR), mitochondrial membrane potential (MMP), rapid swimming sperm (Rapid), reactive oxygen species (ROS), total motility (MOT) and rapid curvilinear velocity (VCL_rap) parameters of bottom and top fractions for individual semen samples. Star glyphs are designed to distinguish cases that have similar values for the six input variables.

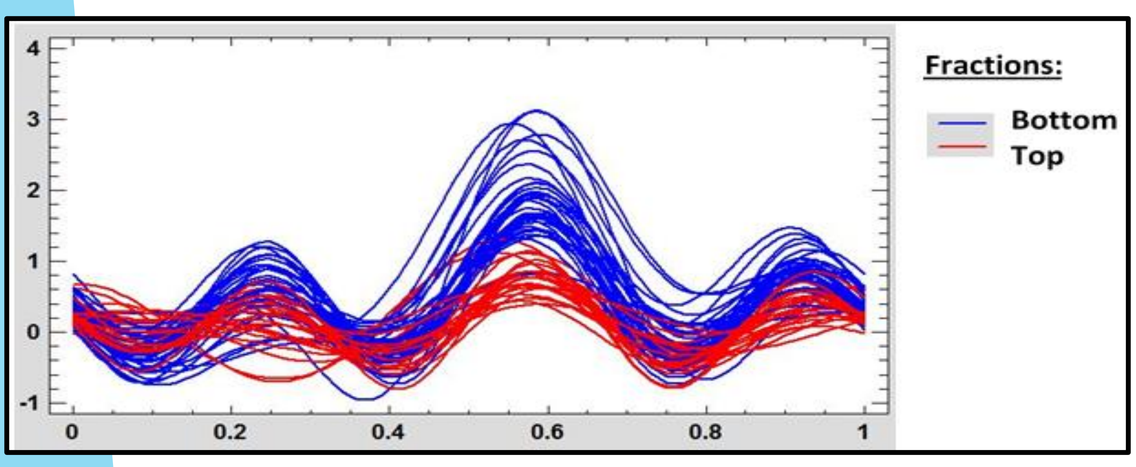
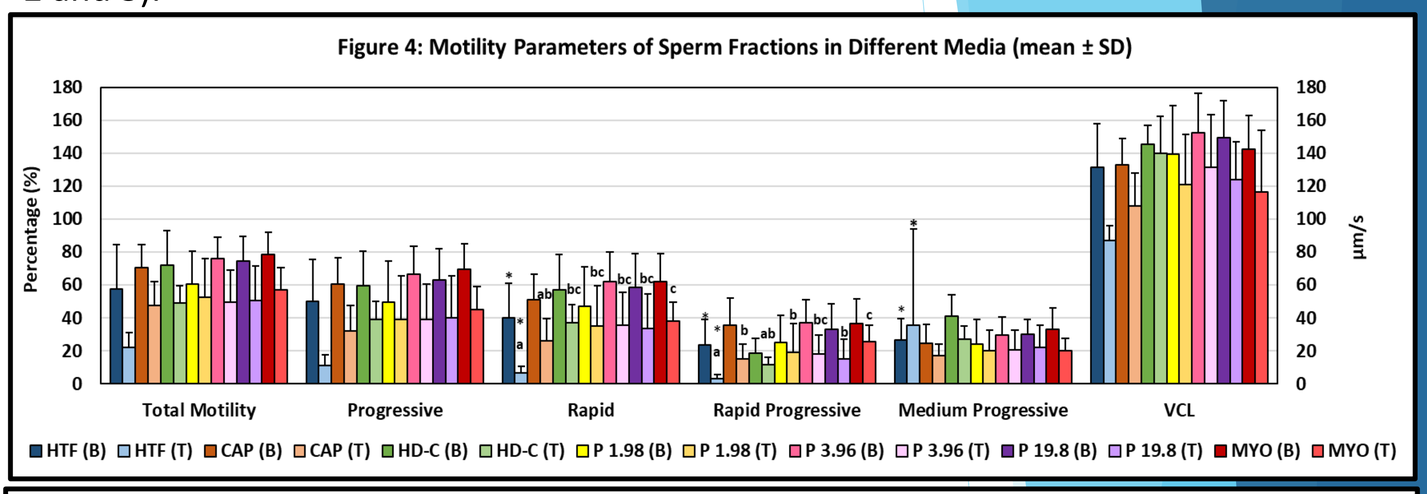
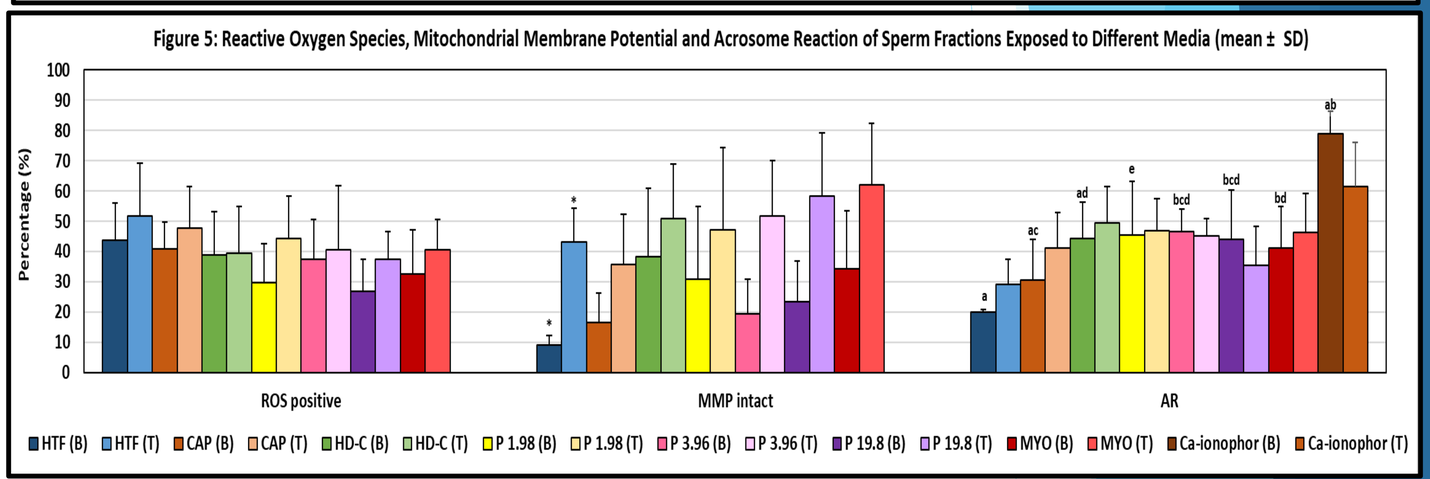


Figure 3: Andrews plot representing individual semen samples' bottom and top fractions (n=20). The Andrews plot is designed to distinguish cases that have similar values for six input variables namely percentage total motility, rapid progressive motility, rapid motility, rapid beat cross frequency, rapid DANCE (VCL x ALH) and acrosome reaction.

AllGrad fractionation resulted in two distinctive sperm subpopulations, with the more motile (bottom) sperm fraction observed to have significantly higher percentages sperm vitality, motility, swimming speed, hyperactivation, induced acrosome reaction, ROS negative and intact MMP ($P<0.05$) than the less motile (top) sperm fraction (Figure 2 and 3).





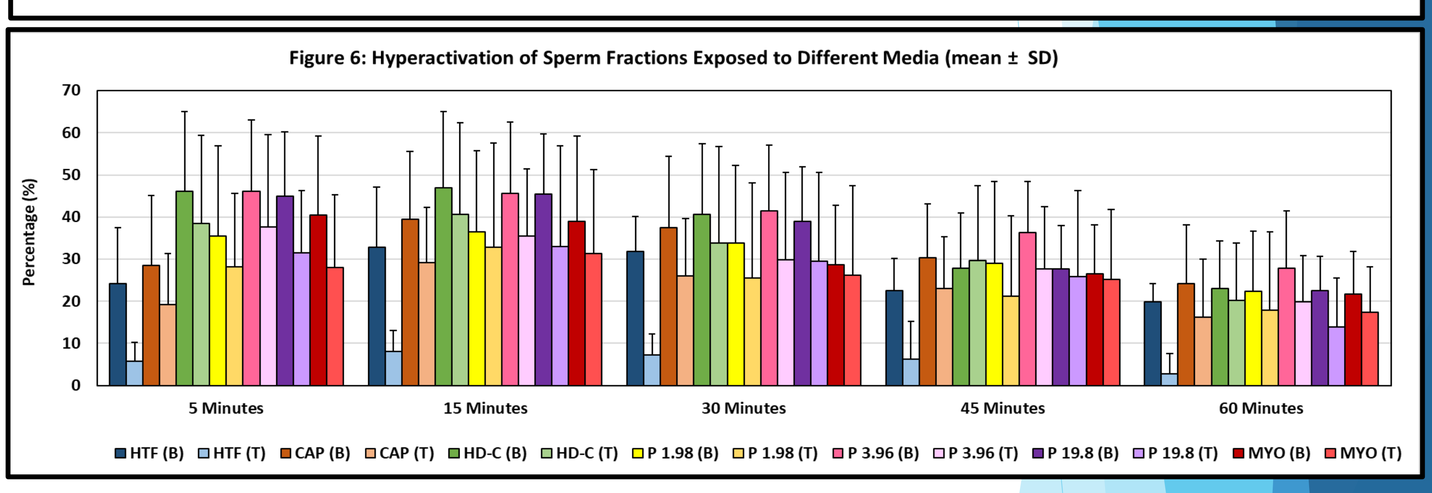


Figure 4, 5 & 6: Comparison of bottom (B) and top (T) sperm fractions after exposure to different media.
VCL = curvilinear velocity (n=13), ROS = percentage reactive oxygen species (n=5), MMP intact = mitochondrial membrane intactness (n=5), AR = acrosome reaction (n=9), HA = hyperactivation (n=13), HTF = non-capacitating human tubal fluid, CAP = capacitating HTF, HD-C = new capacitation medium , P = progesterone (1.98 µM, 3.96 µM and 19.8 µM), MYO = myo-inositol (11 mM) and Ca-ionophor = calcium ionophore (10 µM/l).
^{a,b,c,d} = significant differences by ANOVA for different media
* = significant differences by t-test for top and bottom fractions

The bottom fraction only had higher percentages ($p<0.05$) for percentage rapid, rapid progressive, medium progressive swimming sperm (Fig. 4) and MMP intactness (Fig. 5) in HTF. Furthermore, significant differences for percentage rapid, rapid progressive swimming sperm (Fig. 4) and acrosome reaction (Fig. 5) occurred among the different media. No significant differences were apparent among the various media used for inducing hyperactivation or between the two fractions (Fig. 6).

DISCUSSION

Selected sperm functional parameters in lower quality sperm subpopulations could be improved to the level of a higher quality sperm subpopulation, separated from the same semen samples. If the lower quality sperm subpopulation mimics the sperm population in sub-fertile males, this can be used as a model to study such subpopulations and possibly broaden the potential treatment regimens and sperm isolation procedures for ART.

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PROGESTERONE AND MYO-INOSITOL IMPROVE SPERM FUNCTIONALITY IN SUBFERTILE HUMAN SPERM SUBPOPULATIONS

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INTRODUCTION

Infertility is affecting one in four couples in developing countries, with a male factor being implicated in 50% of these cases. The semen analyses of ±15% of sub- or infertile males do not reveal obvious abnormalities (idiopathic), thereby conveying the importance of sperm functional testing. Furthermore, human semen is heterogeneous in nature, including subpopulations with different degrees of maturation and fertilizing ability. Evaluating the quality of such sperm subpopulations will reveal their functional capabilities and may provide a model for studying sperm functionality in sub-fertile males. This study aimed to determine and compare the functional characteristics of two sperm subpopulations found within the same human semen samples. Concomitantly, the improvement of such functional parameters was evaluated after exposure to various capacitation media and chemicals.

MATERIALS AND METHODS

Part 1:

- Samples were separated into a highly motile bottom fraction and less motile top fraction through AllGrad® 45/90 double density centrifugation.
- Fractions were exposed to non-capacitation human tubal fluid (HTF) and several functional sperm parameters were evaluated, namely vitality (BrightVit), motility and kinematics (Sperm Class Analyzer®), hyperactivation (capacitation HTF, 2 mM procaine hydrochloride & 5 mM caffeine), chromatin maturity (aniline blue), chromatin fragmentation (toluidine blue), reactive oxygen species (ROS, dihydroethidium), mitochondrial membrane potential (MMP) intactness and acrosome reaction (AR) induced with 10 mM calcium ionophore (FluoAcro).

Part 2:

- Samples were separated into a highly motile bottom fraction and less motile top fraction through AllGrad® 45/90 double density centrifugation.
- Fractions were exposed to various media, namely non-capacitation HTF, capacitation HTF, HD Sperm Capacitation Plus, 3.96 µM progesterone and 11 mM myo-inositol.
- Fractions were incubated in media and several functional parameters were evaluated, namely vitality, motility and kinematics, hyperactivation, reactive oxygen species, mitochondrial membrane intactness and acrosome reaction (using similar protocols as for Part 1).

RESULTS

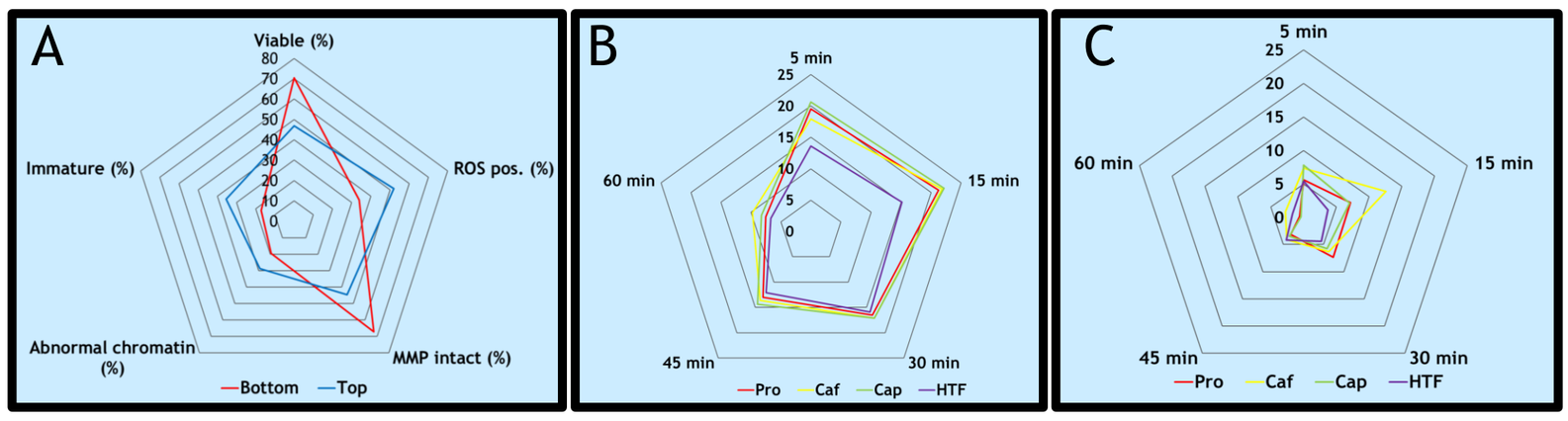


Figure 1: Radar plots comparing bottom and top fractions (n=20). **A)** Differences between bottom and top fractions for percentage immature spermatozoa, abnormal chromatin, intact MMP, viability and ROS positive spermatozoa. **B)** Bottom fraction hyperactivation (%) at five time points. **C)** Top fraction hyperactivation (%) at five time points. Pro, procaine ; Caf, caffeine, Cap, capacitation HTF, HTF, non-capacitation HTF.

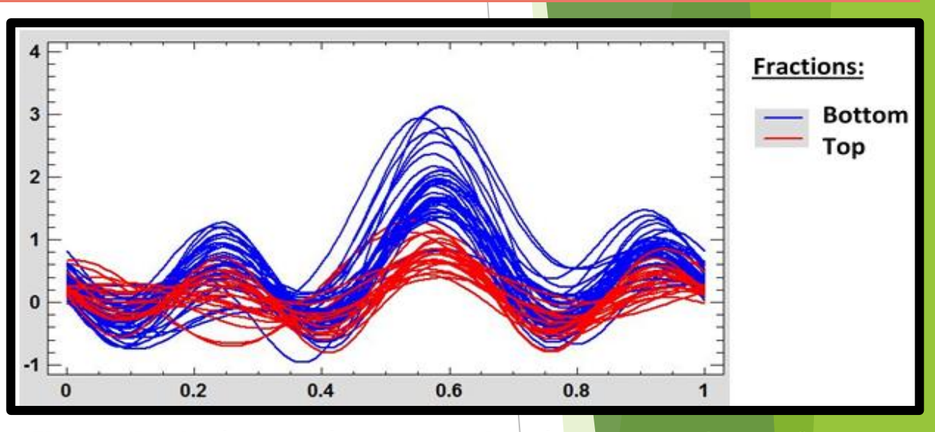
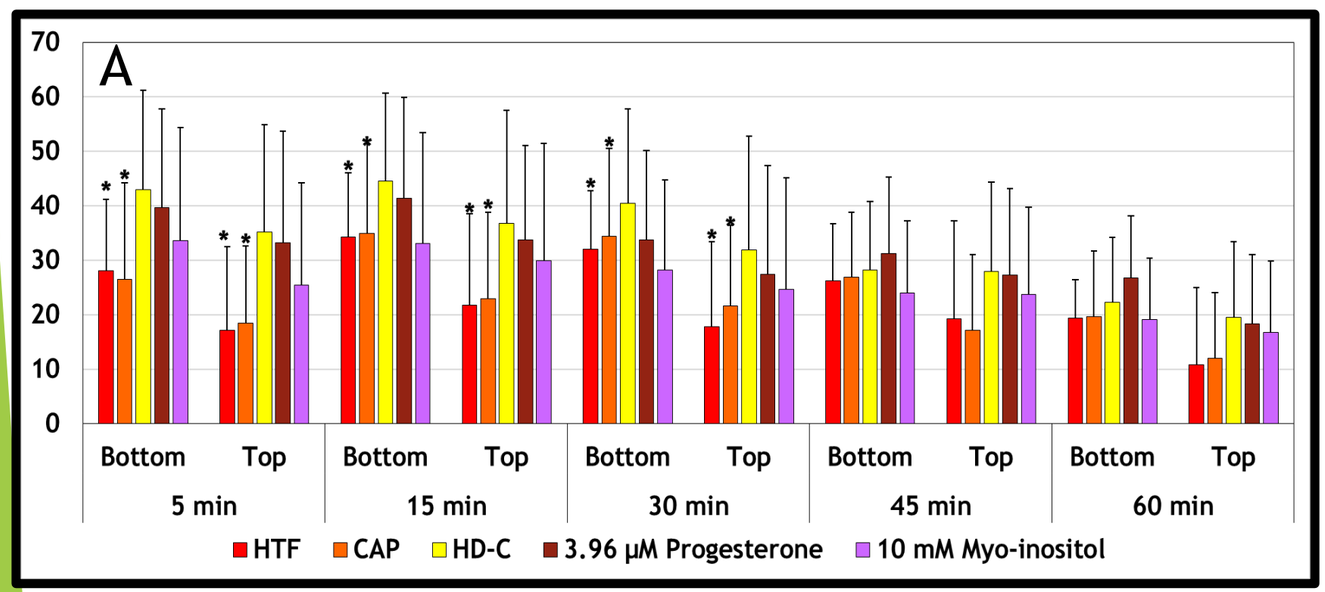
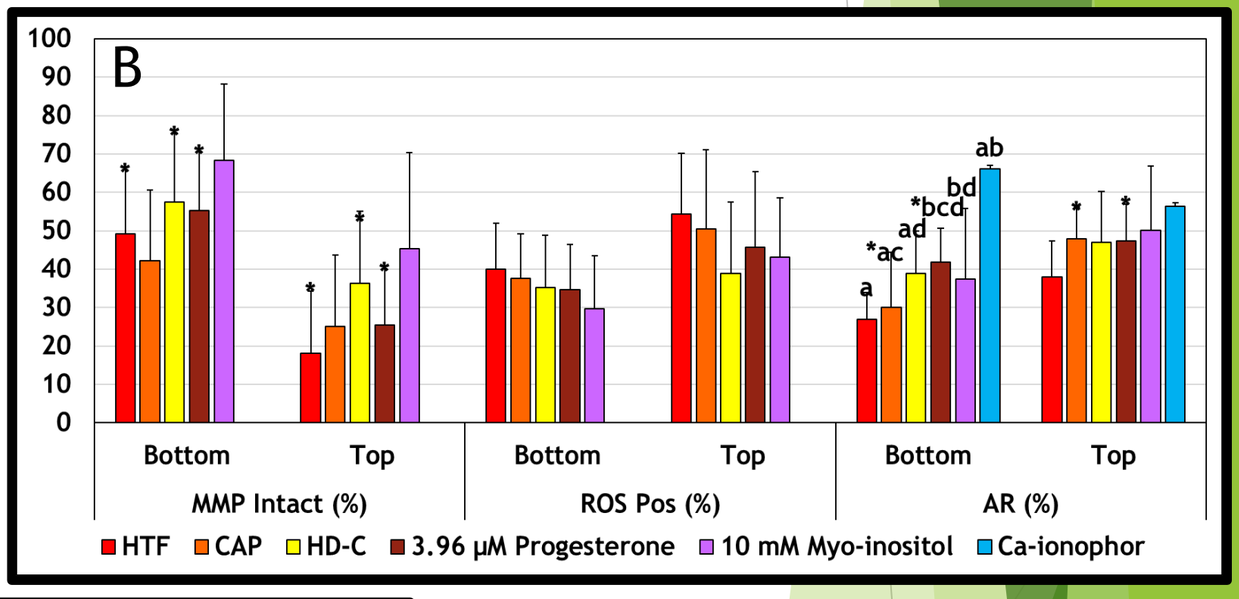


Figure 2: Andrews plot comparing bottom and top fractions (n=20). Each line represents an individual semen sample and the plot distinguishes cases with similar values for six input variables: total motility, rapid progressive motility, rapid motility, rapid beat cross frequency, rapid DANCE (VCL x ALH) and acrosome reaction. Clear separation is seen for bottom (blue) and top (red) fractions at several peaks and valleys.

AllGrad fractionation resulted in two distinctive sperm subpopulations, with the more motile (bottom) sperm fraction observed to have significantly higher percentages for sperm vitality, motility parameters, swimming speeds, hyperactivation, mature spermatozoa and normal chromatin structures, induced acrosome reaction, ROS negative spermatozoa and intact MMP ($P<0.05$) than the less motile (top) sperm fraction (Figure 1 and 2).





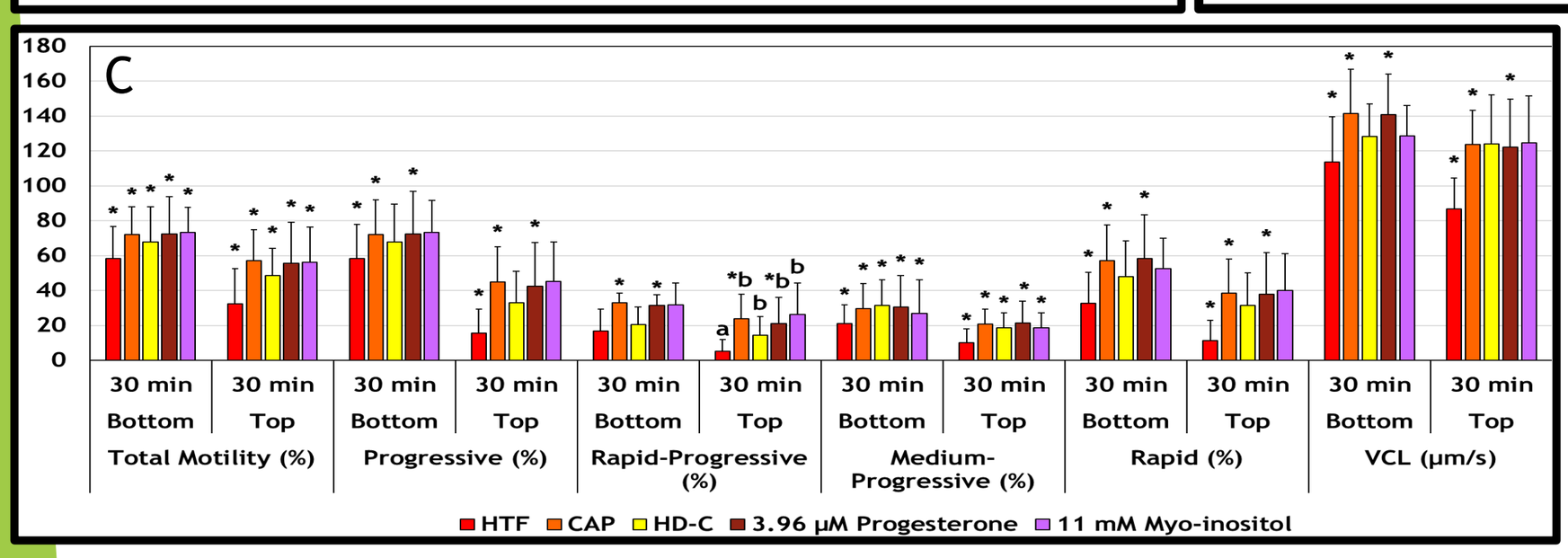


Figure 3: Comparison of bottom (B) and top (T) sperm fractions after exposure to different media.
A) Hyperactivation over a period of 60 minutes (n=15), HTF = non-capacitation human tubal fluid, CAP = capacitation HTF, HD-C = HD Sperm Capacitation Plus. **B)** ROS = reactive oxygen species (n=7), MMP intact = mitochondrial membrane intactness (n=7), AR = acrosome reaction (n=9), Ca-ionophore = calcium ionophore (10 mM). **C)** Motility parameters, VCL = curvilinear velocity (n=18).
[a,b,c,d] = significant differences by ANOVA for different media
* = significant differences by t-test for top and bottom fractions

No significant differences were apparent in percentage hyperactivation among the various media used or between the two fractions. However, a difference between the bottom and top fractions was seen for HTF and CAP medium at 5, 15 and 30 minutes (Figure 3A). Furthermore, significant differences for percentage rapid progressive swimming sperm (Figure C) and acrosome reaction (Figure B) occurred among the different media. The bottom fraction only had higher percentages ($p<0.05$) for all the motility parameters demonstrated in Figure 3C. In addition, the bottom fraction presented with higher percentages of intact MMP, induced AR and less ROS positive spermatozoa compared to the top fraction (Figure 3B).

DISCUSSION

Selected sperm functional parameters in lower quality sperm subpopulations could be improved to the level of a higher quality sperm subpopulation, separated from the same semen samples. If the lower quality sperm subpopulation mimics the sperm population in sub-fertile males, this can be used as a model to study such subpopulations and possibly broaden the potential treatment regimens and sperm isolation procedures for ART.

ACKNOWLEDGEMENT

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MICROPTIC AUTOMATIC DIAGNOSTIC SYSTEMS

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UNIVERSITY of the WESTERN CAPE

COMPARATIVE SPERMATOLOGY GROUP
UNIVERSITY OF THE WESTERN CAPE

DEPARTMENT OF MEDICAL BIOSCIENCE

PARTICIPANT INFORMATION SHEET

DONOR SEMEN FOR RESEARCH PURPOSES

PROJECT TITLE:
Sperm function in different human sperm sub-populations

INTRODUCTION

You have been requested to consider participating in a scientific study on male fertility. It is a principle of medical ethics that the human participants of a research protocol are informed of: the purpose and benefits of the project; the research methods to be used; the potential risks or hazards of participation; and the right to ask for further information at any time during the research process. Your choice to participate is a voluntary one, and you are free to withdraw from the research project at any time. Your signature at the end of this consent form will indicate that the principal investigator or laboratory director has answered all your questions, that you voluntarily consent to participate in this investigation and that the data generated may be used for scientific purposes.

1) PURPOSE OF THE RESEARCH STUDY

The main aim of this project is to investigate sperm function in human sperm subpopulations from healthy donors and subfertile/infertile men. Sperm functional testing will include both the use of standard protocols/techniques but also the development of new/novel analysis techniques. Concurrently, the effect of various physiological substances on the functional capabilities of selected sperm subpopulations will be evaluated.

2) ELIGIBILITY

The criteria for inclusion for donors will be: age (20-40 years); previous screening by the mentioned laboratory; and semen quality according to WHO criteria for human semen analysis (volume $\geq$ 1.5 ml, sperm concentration $\geq$ 15 x 10^6/ml and percentage total motility $\geq$ 40%). Participants will be excluded from the study if semen has indications of a reproductive tract infection or if they are taking any type of medication.

3) PROCEDURES

If you decide to take part in this research study, the following procedures will be part of the research project:

- Semen collection via masturbation after 2-3 days of sexual abstinence.
- Basic semen analysis, including assessment of semen pH, volume and viscosity, as well as sperm morphology, motility and concentration.
- Sperm structural and functional testing, including assessment of sperm kinematics, hyperactivation, flagellar analysis, chromatin maturity and fragmentation, acrosome reaction, mitochondrial membrane potential and reactive oxygen species.

4) RISKS
This is an *in vitro* study and thus is not invasive. The researchers will not use semen sample(s) for *in vivo* insemination, genetic manipulation, or for *in vitro* fertilization of human oocytes for the creation of embryos and embryonic stem cells. Any unused semen samples and sperm preparations will be disposed of in the correct manner (by incineration) once the data is captured as per the University of the Western Cape's biohazardous waste removal protocol.

5) BENEFITS
Participants will not have to incur any costs to take part in the study and transport costs will be covered by the Comparative Spermatology Laboratory at the University of the Western Cape. Participants will not receive any financial benefits from future commercial development and scientific patents of discoveries made through the use of data generated from this study.

6) USE OF DATA
The information as well as the data generated from your semen sample in our laboratory will be used for scientific purposes related to this study only.

7) NEW FINDINGS
You will be notified of any significant new developments that may cause you to change your mind about participating in the research study. The participants will be notified via publications and independently by the principal investigator in an understandable format and lay terms.

8) CONFIDENTIALITY
Information related to you will be treated in strict confidence to the extent provided by law. Your identity will be coded and will not be associated with any published results. Your code number and identity will be kept in a locked file of the manager of the Comparative Spermatology Laboratory at the University of the Western Cape.

9) FREEDOM TO WITHDRAW
Your participation in this study is voluntary and you may stop your participation at any time without prejudice and without affecting future health care.

10) REMOVAL FROM STUDY
It is possible that you may be removed from the research study by the researchers, if for example you have a reproductive tract infection or taken medication that can affected the quality of semen, e.g. antibiotics.

In the event of any problems or concerns, additional information can be obtained from:

Principal investigator
Ms Shannen Keyser skeyser@uwc.co.za 021 959 2433

Co-investigator & Supervisor
Dr Liana Maree lmaree@uwc.ac.za 021 959 2917

Biomedical Research Ethics Committee
BMREC
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Tel: 021 959 4111,
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UNIVERSITY of the WESTERN CAPE

COMPARATIVE SPERMATOLOGY GROUP
UNIVERSITY OF THE WESTERN CAPE

DEPARTMENT OF MEDICAL BIOSCIENCE

VOLUNTARY CONSENT FORM

DONOR SEMEN FOR RESEARCH PURPOSES

PROJECT TITLE:
Sperm function in different human sperm sub-populations

I have been informed about the above mentioned research project and I understand the purpose and procedures of the study.
I have been given an opportunity to answer questions about the study and have been given answers to my satisfaction.
I declare that my participation in this study is entirely voluntary and that I may withdraw at any time without affecting any treatment or care that I would usually be entitled to.
If I have any questions or concerns about my rights as a study participant, or if I am concerned about an aspect of the study or the researchers then I may contact the scientific investigators directly.
By signing this form, I agree to participate in this research project and that the data generated from my semen sample may be used for scientific purposes.
A signed copy of this consent form will be given to me.

Participant Details:

Name: _____ Surname: _____

Donor Code: _____

ID or Student number: _____

Cell phone number: _____

Email Address: _____

Referred to the program by: _____

Participant Banking details:

Name on account: _____

Bank: _____

Account Type: _____

Branch code: _____

Account number: _____

_____ _____

Signature of Participant **DATE**

I certify that the nature and purpose, the potential benefits, and possible risks associated with participation in this research study have been explained to the above individual and that any questions about this information have been answered.

_____ _____ _____

Print Name of Person Obtaining Consent (Principal Investigator or Designee) **Signature of Person Obtaining Consent (Principal Investigator or Designee)** **Date**