MODULATION OF HUMAN SPERM BY FOLLICULAR FLUID STEROID HORMONES

By

BENJAMIN GBENRO TAIWO

A thesis submitted to

The University of Birmingham

For the Degree of

DOCTOR OF PHILOSOPHY

UNIVERSITY OF BIRMINGHAM

College of Medical and Dental Sciences

Institute of Metabolism and Systems Research

August 2016

ABSTRACT

Recent detailed steroid hormone profiling of human follicular fluid has paved the way for research into the modulation of human sperm by physiological concentrations of follicular fluid steroid hormones. Synthetic human follicular fluid (shFF), a novel steroid hormone analogue of human follicular fluid was prepared based upon local data, consisting of 14 different steroid hormones including progesterone. The modulation of human sperm by shFF was then investigated with particular focus on sperm Ca^{2+} signalling, kinesis and acrosome reaction (AR).

Exposure of human spermatozoa (>2000 cells) to shFF stimulus at physiological and standard laboratory temperatures resulted in a rapid biphasic elevation in $[Ca^{2+}]_i$ characterised by an initial transient Ca^{2+} influx immediately followed by a sustained elevation of $[Ca^{2+}]_i$ for the duration of shFF exposure. Population fluorimetry studies conducted at 37°C revealed a complex dose-dependent $[Ca^{2+}]_i$ response amplitude following the sequential treatment of spermatozoa with increasing doses of shFF (0.001%, 0.01%, 0.1%, 1%, 10% and 100% shFF). A significant increase in the percentage of acrosome-reacted spermatozoa was observed in shFF-treated sperm ($P<0.05$) however, this was significantly lower than the % AR in spermatozoa treated with progesterone alone ($P<0.01$).

With regards to shFF-induced sperm kinesis, a significant reduction in sperm straight line velocity (VSL), curvilinear velocity (VCL) and linearity (LIN) was observed 5 minutes post-incubation with shFF ($P<0.05$). A potential inhibitory effect was also observed in the cervical-mucus penetrating capacity of spermatozoa constantly exposed to shFF, though this did not reach statistical significance. The study of shFF-induced chemotaxis

revealed a chemokinetic effect characterised by a significant inhibition of sperm migration up a gradient of shFF ($P<0.05$), possibly due to 'hyperactivated trapping'.

We conclude that the high concentration of progesterone (13.5μM) present in the shFF mixture is likely to be responsible for the biphasic sperm $[Ca^{2+}]_i$ influx characteristic of a progesterone stimulus. However, the data obtained from the sperm kinesis and AR experiments leads us to hypothesize that the other steroid hormones present in the shFF mixture exert antagonistic effects on progesterone-mediated physiological responses in human spermatozoa. The data presented in this study represents a significant step towards understanding the role of follicular fluid steroid hormones in the modulation of human sperm, and the knowledge obtained from further research could be applied towards the improvement of clinical ART diagnostics and gamete culture systems.

DEDICATION

To my family

ACKNOWLEDGEMENTS

My profound gratitude goes to Dr. Jackson Kirkman-Brown MBE for his supervision, support and friendship throughout my PhD. Your invaluable input and guidance made the completion of this work possible.

I am indeed grateful to Dr. João Correia for the tireless support and advice in the laboratory despite his other commitments. Obrigado!

I would like to thank Dr. Sarah Conner and Dr. Linda Lefievre for their quality advice and expert critique throughout my PhD. My gratitude also goes to Dr. Lorraine Frew for her immense help with donor recruitment during very crucial periods of my PhD research.

I also want to thank all the staff at the Birmingham Woman's Hospital Fertility Centre who were always friendly and supportive whenever I was working at the premises.

Many thanks go to my past and present colleagues in the reproductive biology and genetics (RBG) research group here at the University of Birmingham Medical School – Asma, Claudia, David Foo, Israa, Ling, Marie and Dr. Pete Hewett. I am grateful for the comradery and support during the time we've spent working together. It has been a pleasure working with you all.

Finally, I would like to thank my parents, and my siblings for their love, care, understanding and support.

GLOSSARY OF ABBREVIATIONS

2AG	2-arachidonyl glycerol
3βHSD	3β hydroxysteroid dehydrogenase
17βHSD	17β hydroxysteroid dehydrogenase
17OHP	17α-hydroxyprogesterone
17OHPreg	17-hydroxyprenenolone
AA	arachidonic acid
ABHD2	alpha/beta hydrolase domain containing protein
AC	adenylyl cyclase
AR	acrosome reaction
ART	assisted reproductive technology
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
Ca^{2+}	calcium ion
$[Ca^{2+}]_i$	intracellular calcium
cAMP	cyclic adenosine monophosphate
CASA	computer-assisted sperm analysis
CatSper	cation channels of sperm
CCD	charge-coupled device
CCE	capacitative calcium entry
CE	cholesterol ester
CICR	calcium-induced calcium release
COC	cumulus-oocyte complex
CYP1A	cytochrome p4501A
CYP1B	cytochrome p4501B
CYP11A	cholesterol monooxygenase or cytochrome p45011A
CYP17	17-monooxygenase
CYP19A	aromatase
CYP21	steroid 21-hydroxylase
DAG	diacylglycerol
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EBSS	Earle's balanced salts solution
sEBSS	supplemented Earle's balanced salts solution
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
ER	endoplasmic reticulum or estrogen receptor
ESHRE	European society for human reproduction and embryology
FF	follicular fluid
FITC-PSA	fluorescein isothiocyanate – conjugated *Pisum sativum* agglutinin
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
GCPR	G protein-coupled receptor

Gs	guanine-nucleotide binding protein
HCO^{3-}	hydrogen bicarbonate
HDL	high density lipoprotein
HFEA	human fertilisation and embryology authority
hFF	human follicular fluid
HOS	hypo-osmotic swelling
IP_3	inositol triphosphate
IP_3R	inositol triphosphate receptor
IVF	*in vitro* fertilisation
Kd	dissociation constant
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LED	light emitting diode
LH	luteinizing hormone
LHR	luteinizing hormone receptor
LIN	linearity
MAFP	methyl arachidonyl fluorophosphate
MC	methylcellulose
NCX	sodium-calcium exchanger
PBS	phosphate buffered saline
PDE	phosphodiesterase
PHN	posterior head-neck
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
PKA	protein kinase A
PMCA	plasma membrane calcium ATPase
RNE	redundant nuclear envelope
RT-PCR	real time polymerase chain reaction
SACY	soluble adenylyl cyclase
SERCA	sarco/endoplasmic reticulum calcium ATPase
shFF	synthetic human follicular fluid
SEM	standard error of the mean
SOCE	store-operated calcium entry
SPCA	secretory pathway calcium ATPase
SPINKL	serine protease inhibitor Kasal-type-like protein
SR-B1	scavenger receptor class B1
$TPMP^{+}$	triphenylmethylphosphonium
TRPC	transient receptor potential-canonical
VCL	curvilinear velocity
VSL	straight line velocity
WHO	world health organisation
ZIF	zona-binding inhibitory factor
ZP	zona pellucida

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

CHAPTER 1

THE SPERM'S JOURNEY TO THE OOCYTE – HUMAN SPERM INTERACTION WITH THE FEMALE REPRODUCTIVE TRACT

FOREWORD TO CHAPTER 1

The interaction of human spermatozoa with the female reproductive tract environment has been shown via several published studies to be important for successful fertilisation and embryonic development. This chapter summarises the current understanding of the physiological interactions and modulation of human gametes from gametogenesis all the way to fertilisation.

1.1 THE HUMAN SPERMATOZOON

1.1.1 Spermatogenesis

A mature haploid human male gamete or spermatozoon is the product of a process known as spermatogenesis. Spermatogenesis occurs in the epithelium of highly convoluted structures known as seminiferous tubules located inside the male testis (Dym, 1994) (Figure 1). This multi-phase differentiation process involves distinct changes in cell cycle dynamics and morphogenesis (White-Cooper, 2004). Spermatogenesis consists of three phases beginning with a mitotic proliferation phase, followed by two reductive divisions of meiosis, and a post-meiotic phase of spermiogenesis (Matson *et al.*, 2010). Prior to the initiation of spermatogenesis at puberty, embryonic primordial germ cells divide to form type A spermatogonia which in turn proceed to undergo mitotic proliferation. These spermatogonial cells are smaller than the primordial germ cells and are characterised by an ovoid nucleus (Zhang, 1999). Specialised cells located in the seminiferous epithelium known as Sertoli cells function to provide hormonal regulation, nourishment and structural support to the spermatogonia as they go through the different phases of spermatogenesis (Newton, 1993; Griswold, 1998) (Figure 1).

Detailed studies on the characterisation of the mammalian spermatogonial pathway have been carried out however little is known about the specific spermatogonial mechanisms in the human (Dym *et al.*, 2009). The mitotic proliferation of mammalian spermatogonia is characterised by different phases beginning with the division of a single type A spermatogonia (type A_S). This mitotic division is characterised by an incomplete cytokinesis, resulting in the formation of a syncytium of paired type A spermatogonia (A_{Pr}) separated by cytoplasmic bridges which facilitate synchronous maturation of the

A_{Pr} (Dym and Fawcett, 1971; Guraya, 1987; Zhang, 1999). This syncytium is maintained throughout spermatogenesis and the characteristic cytoplasmic bridges were found to be about 1μm in diameter (Dym and Fawcett, 1971). The A_{Pr} spermatogonia then undergo series of mitotic divisions to give rise to diploid type B spermatogonia.

Studies by Clermont (1963) on human spermatogenesis revealed two types of spermatogonial cells according to their nuclear staining pattern – the A_{dark} and A_{pale} spermatogonia. The A_{dark} spermatogonia were identified as reserve stem cells while the A_{pale} spermatogonia are suggested to be the renewing stem cells that go on to divide into type B spermatogonia (Clermont 1963; 1966). The last division of the mitotic proliferation phase occurs as the type B spermatogonia divide giving rise to diploid daughter cells (2n) that are committed to the formation of mature spermatozoa (Zhang, 1999; de Rooij and Russell, 2000). Each daughter cell known as a primary spermatocyte undergoes the first meiotic division resulting in a pair of haploid secondary spermatocytes (n) that go on to complete the second meiotic division (Dym, 1994; Gilbert, 2014). Haploid cells formed from the second meiotic division-spermatids, are still connected to one another via cytoplasmic bridges (Guraya, 1987; Zhang, 1999; Gilbert, 2014). Each spermatid is functionally diploid despite having a haploid nucleus as gene products made in one spermatid can readily diffuse into the cytoplasm of neighbouring spermatids via the cytoplasmic bridges (Braun *et al.*, 1989). Spermatids now located at the border of the seminiferous tubule lumen lose their cytoplasmic connections and differentiate into mature spermatozoa via the process of spermiogenesis (Gilbert, 2014). The first step in spermiogenesis is the formation of the acrosome from the Golgi apparatus, forming an acrosomal cap that covers the sperm

nucleus (Abou-Haila and Tulsiani, 2000). This is then followed by the condensation of the sperm nuclear chromatin characterised by the replacement of spermatogonial histones with the sperm-specific protamines (Govin *et al.*, 2004; Gill-Sharma *et al.*, 2011; Oliva and Castillo, 2011). This chromatin remodelling results in a transcriptional shutdown in the sperm nucleus (Govin *et al.*, 2004). The last stages of spermiogenesis involve the loss of the remaining cytoplasm and the ubiquitination of mitochondria (Guraya, 1987; Sun and Yang, 2010). Spermiogenesis prepares the sperm for the functions of motility and interaction with the female reproductive tract. These mature spermatozoa are then released into the lumen of the seminiferous tubule in a process known as spermiation.

The entire process of spermatogenesis results in the division of a single diploid spermatogonium into four haploid spermatozoa. In humans, this takes approximately 64 days (Heller and Clermont, 1963) and is regulated via an array of endocrine, autocrine and paracrine pathways. Follicle stimulating hormone (FSH) secreted by the Sertoli cells, luteinising hormone (LH) secreted by the Leydig cells, and testosterone are the principal hormonal messengers in the regulation of spermatogenesis (Holdcraft and Braun, 2004). They are important not only for the regulation of spermatogenesis, but also for the growth and function of the somatic cells types essential for proper testicular development (McLachlan *et al.*, 2002). FSH stimulates the proliferation of Sertoli cells which are essential for the maturation of germ cells. LH is produced in the anterior pituitary gland and its secretion is regulated by the release of gonadotropin releasing hormone (GnRH) from the hypothalamus. LH stimulates the Leydig cells to synthesize

testosterone which in turn acts through the testicular somatic cells to regulate germ cell differentiation (Holdcraft and Braun, 2004).

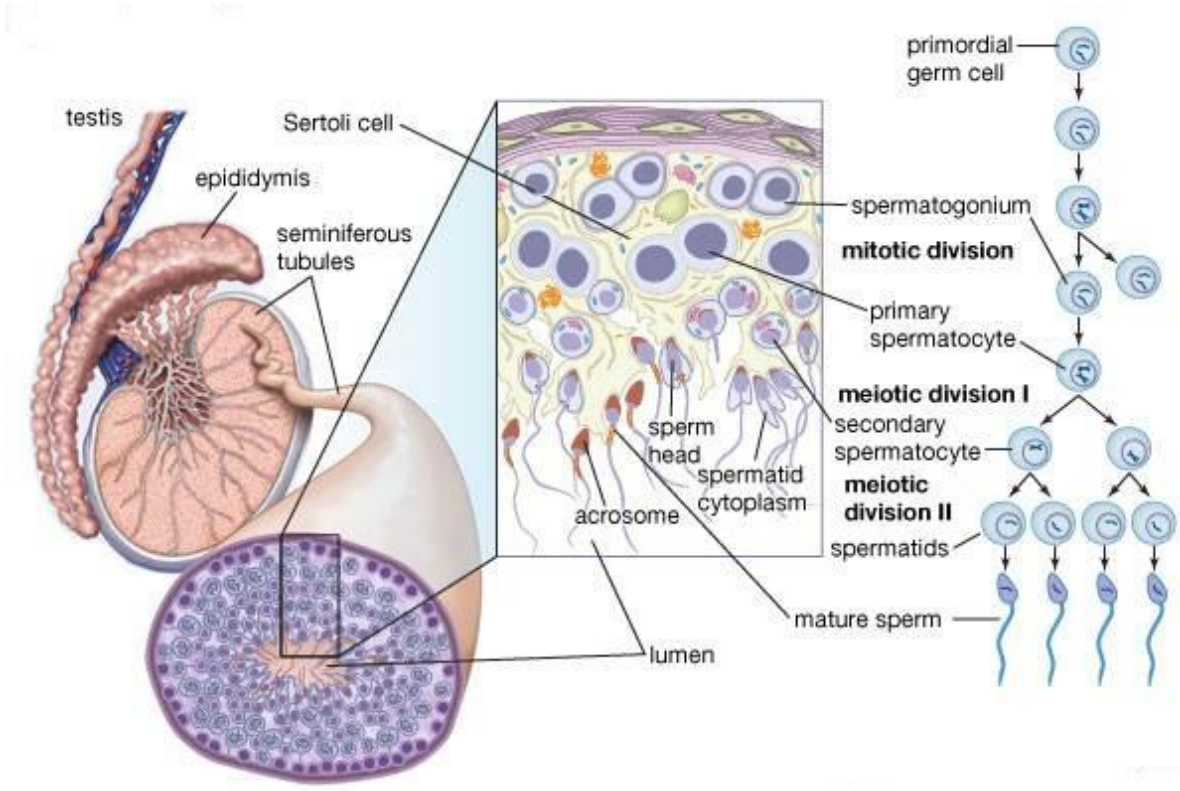


Figure 1 Mammalian spermatogenesis. The primordial germ cell located at the basal lamina of the seminiferous tubule undergoes mitotic divisions to give rise to spermatogonia (2n) which in turn mitotically divide to form primary spermatocytes (2n). The primary spermatocytes then enter a meiotic phase resulting in the formation of haploid spermatids (n). Spermatids are then remodelled into mature spermatozoa via the process of spermiogenesis which is characterised by acrosome formation, nuclear condensation and a loss of cytoplasm. The Sertoli cells or sustenacular cells provide nourishment and structural support as the germ cells undergo the different phases of spermatogenesis. Adapted from Encyclopaedia Britannica (2010).

1.1.2 Structure of a mature spermatozoon

The mature human spermatozoon-a terminally differentiated germ cell, possessing the same basic structure as all mammalian spermatozoa which comprises of a head and a tail (flagellum) (Figure 2). These components work together to ensure the delivery of an intact haploid genome into the oocyte during fertilisation. Spermatozoa of many mammalian species including humans have spatulate heads, with the exception of some rodents which have falciform heads (Toshimori, 2009). The head of a spermatozoon is structurally characterised by an acrosomal region and a nuclear region surrounded by small amounts of cytoskeleton and cytoplasm (Toshimori and Eddy, 2015). The nuclear region is enclosed in a nuclear envelope and contains tightly packaged chromatin as a result of the cellular remodelling that takes place during spermiogenesis in which nucleosomal histones from the spermatogenic germ cells are being replaced with sperm-specific DNA binding proteins called protamines (Lewis *et al.*, 2003; Rathke *et al.*, 2014; Sheng and Liang, 2014; Dogan *et al.*, 2015). This nuclear condensation along with the loss of cytoplasm and other organelles during spermiogenesis is believed to give the sperm cell its hydrodynamic properties which facilitate sperm interaction with the female tract and the penetration of oocyte vestments (Oliva, 2006; Rathke *et al.*, 2014).

Another component of the sperm head directly overlaying the nuclear region is the acrosome (Figure 2) which is a secretory vesicle tightly bound to the nuclear region via a network of proteins called the acroplaxome (Kierszenbaum *et al.*, 2003). The inner acrosomal membrane overlies the anterior outer membrane of the nuclear envelope while the outer acrosomal membrane is in close proximity to the inner surface of the sperm's plasma membrane (Toshimori and Eddy, 2015) (Figure 2). The acrosome contains a wide variety of hydrolytic enzymes (e.g. hyaluronidase) that are believed to

aid the penetration of the cumulus-oophorus and zona pellucida layers surrounding the oocyte (Zaneveld *et al.*, 1991; Lin *et al.*, 1994; Toshimori, 2009; Jones and Lopez, 2014). These include proteinases, acid phosphatases, phospholipases, neuraminidases, esterases, glycohydrolases, aryl sulfatases, beta-N-acetylglucosaminidase and collagenase (Yanagimachi, 1981; Tulsiani *et al.*, 1998).

The flagellum of mammalian spermatozoa including humans is structurally composed of four regions namely the connecting piece (neck), the middle piece, the principal piece and the end piece (Figure 2). The flagellar ultrastructure plays an indispensable role in the coordination of sperm motility and is characterised by an axial filament (axoneme) containing a conventional '9+2' microtubular arrangement that extends from the connecting piece all the way through to the end piece (Fawcett, 1975) (Figure 2). In the middle piece, the axial filament is surrounded by mitochondria and a ring of protein structures known as outer dense fibres (Fawcett, 1975; Baccetti and Afzelius, 1976). Outer dense fibres extend from the connecting piece to the annulus and principal piece (Fawcett, 1975; Turner, 2006). In the principal piece, the axial filament is enclosed in a fibrous sheath that terminates at the anterior end of the end piece leaving only the axial filament surrounded by plasma membrane to form the end piece segment (Turner, 2006; Toshimori and Eddy, 2015) (Figure 2).

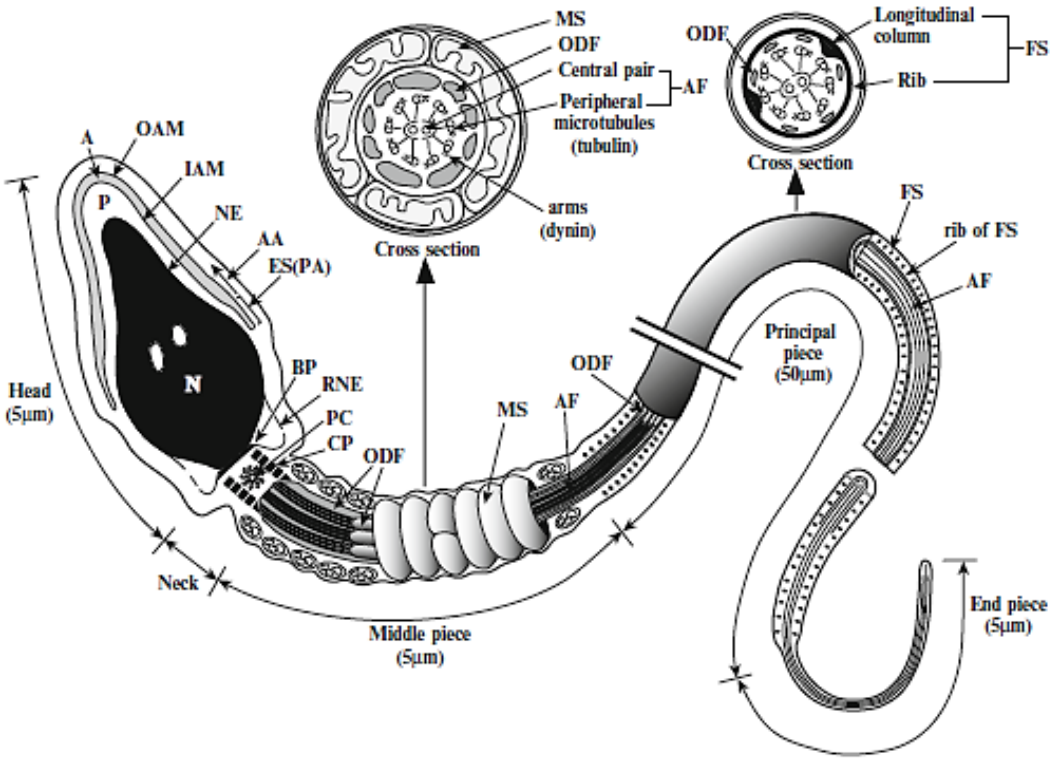


Figure 2 Schematic representations of spermatozoon head and tail components including flagellar ultrastructure. **A**: Acrosome, **P**: Perforatorium, **N**: Nucleus, **OAM**: Outer acrosomal membrane, **IAM**: Inner acrosomal membrane, **NE**: Nuclear envelope, **AA**: Anterior acrosome, **ES (PA)**: Equatorial segment (posterior acrosome), **BP**: Basal plate, **RNE**: Redundant nuclear envelope, **PC**: Proximal centriole, **CP**: Connecting piece, **ODF**: Outer dense fibre, **MS**: Mitochondrial sheath, **AF**: Axial filaments, **FS**: Fibrous sheath. Figure from Toshimori (2009).

1.1.3 Epididymal storage and transport

Following the process of spermiation, morphologically mature spermatozoa make their way from the testes into the epididymis (Figure 1). The mammalian epididymis is divided into four anatomical regions as first described by Benoit (1926) – the initial segment, the caput (head), the corpus (body), and the cauda (tail). Each region is characterised by the presence of a lumen and a polarised epithelium (Lasserre *et al.*, 2001; Dacheux *et al.*, 2005) (Figure 3). The movement of spermatozoa from the caput to the cauda occurs with the help of smooth muscle contractions within the epididymal wall, as well as pressure from fluid and spermatozoa entering the epididymis from the testis (Hinton, 2010).

The epididymis functions in sperm transport to the vas deferens, the initiation of sperm motility, the development of sperm fertilising capability, and the creation of a specialised environment in the epididymal lumen that aids the completion of the maturation process via the secretions and absorptive activity of the epididymal epithelium (Austin, 1985; Cooper, 2007; Robaire and Hinton, 2015). Sperm cells stored in the epididymal lumen prior to ejaculation are prepared for fertilisation via the regulation of essential factors including temperature, pH, oxygen tension and energy substrates (Dacheux *et al.*, 2005). These result in the production of forward progressive spermatozoa that are further equipped for interaction with the female reproductive tract.

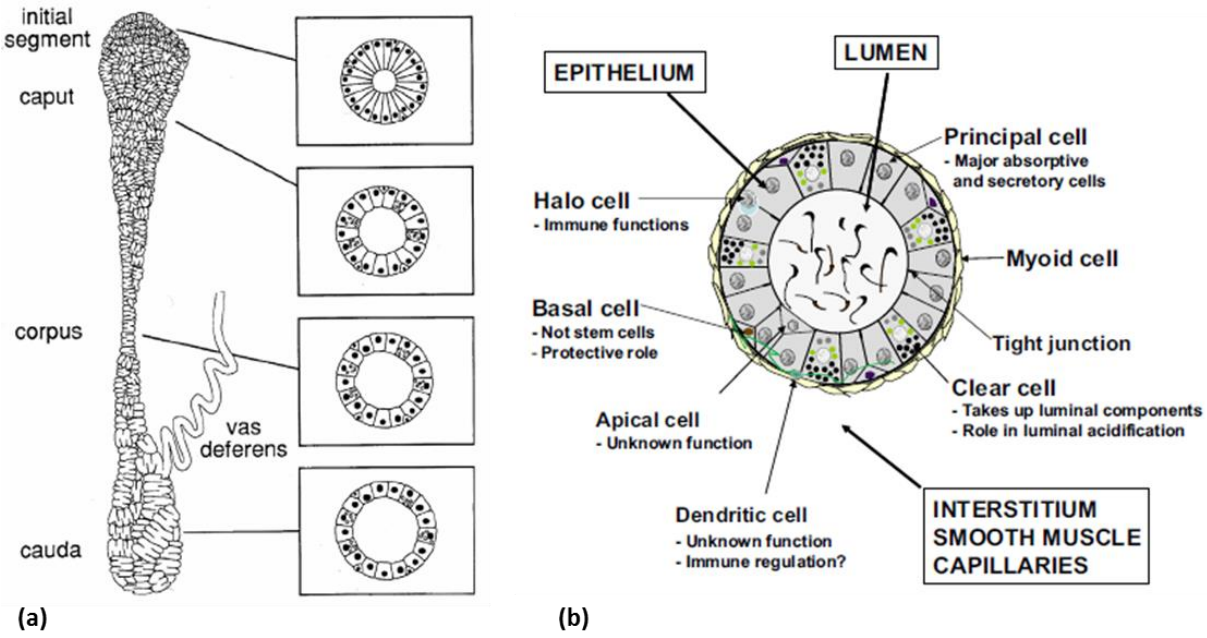


Figure 3 (a) Schematic representation of a mammalian epididymis showing the four different anatomical regions-the initial segment, caput, corpus and cauda (b) Schematic representations of the major cell types in an epididymal cross section as viewed under a microscope. Note the spermatozoa in the centre of the lumen. Adapted from Hinton (2010); Robaire and Hinton (2015).

1.1.4 Ejaculation and seminal components

Mature epididymal spermatozoa are expelled from the urethra of the male in a process known as ejaculation. A typical human male ejaculate appears in the form of seminal fluid (plasma) in which spermatozoa are suspended. It is estimated that spermatozoa accounts for about 1-5% of an ejaculate volume (Mortimer, 1994). The minimal seminal fluid parameters expected in a typical fertile male are an ejaculate volume of 1.5ml and a total sperm count of 39 million (Cooper *et al.*, 2010). Seminal plasma is a complex mixture made up of fluid secretions from accessory sex glands. The Cowper and Littre glands contribute the first 5% of the ejaculate while the second portion produced by the prostate gland accounts for about 15-30%. The ampulla and epididymis contribute small amounts of substrate secretions while the seminal vesicle secretions account for the remainder of the ejaculate volume (Owen and Katz, 2005). Biochemically, seminal plasma is made up of energy substrates (e.g. fructose), organic compounds (e.g. citric acid, ascorbic acid, amino acids, cytokines, lipids and hormones), ions (e.g. K^+, Zn^+, Na^+, Mg^{2+}, Cl^- and Ca^{2+}) and enzymes (e.g. acid phosphatase, aspartate aminotransferase and alanine aminotransferase) (Juyena and Stelletta, 2012). Refer to Owen and Katz (2009) for a complete review on seminal plasma constituents.

1.2 THE HUMAN OOCYTE

1.2.1 Oogenesis

The differentiation of a human female gamete or oocyte occurs via the process of oogenesis. In a similar manner to spermatogenesis, this differentiation process involves series of mitotic proliferations and meiotic divisions resulting in the formation of a haploid oocyte (Figure 4). Unlike spermatogenesis which begins at puberty and continues throughout adulthood, oogenesis involves stages of cell cycle arrest that extend from prenatal development through to puberty and adulthood (Figure 4). During mammalian oogenesis, the oocyte maintains a synchronous relationship within the ovarian follicle ensuring that the process of oocyte differentiation and follicle development (folliculogenesis) are integrated (see section 1.2.2).

The process of oogenesis begins in the foetal ovary where diploid primordial germ cells undergo series of mitotic proliferations resulting in the formation of oogonia. The first series of oogonial proliferations are characterised by complete mitotic divisions resulting in the formation of two separate daughter cells while subsequent mitotic divisions are incomplete resulting in the formation of daughter oogonia cells connected via cytoplasmic bridges (Gondos and Zamboni, 1969; Pepling and Spradling, 1998). These incomplete mitotic divisions result in the formation of an oogonial syncytium. This feature is conserved across species and is believed to facilitate intracellular communication required to initiate the meiotic phase of germ cell differentiation (Robinson and Cooley, 1996; Ventela, 2006). In the human female, these proliferations translate into a rapid increase in the germ cell population from 600,000 at 8 weeks of gestation to about 6 million at 20 weeks (Oktem and Oktay, 2008). Following this

peak, in addition to the initiation of meiosis, oogonial atresia becomes operational and continues throughout a female's lifetime (Oktem and Oktay, 2008). The centrally located oogonia of the syncytia exit the mitotic cycle and begin the first meiotic division, with the remaining oogonia undergoing atresia (Byskov and Andersen, 2013). This transition to meiosis marks the end of germ cell proliferation and they are now referred to as primary oocytes. During the diplotene stage of prophase I of the meiotic cycle, the primary oocytes become arrested (Sirard, 2001; Eppig *et al.*, 2004; Mehlmann, 2005). This occurs around birth and is maintained until shortly before the commencement of ovulation. The mechanisms that control the process of meiotic arrest are not properly understood however studies have shown that the regulation of meiotic arrest is dependent on high levels of adenosine-3', 5'-cyclic monophosphate (cAMP), brought about by a G protein coupled receptor activation of adenylyl cyclase within the oocyte (Cho *et al.*, 1974; Mehlmann, 2005; Liu *et al.*, 2013). The inactivation of a maturation promoting factor (MPF) has also been found to be crucial to the maintenance this meiotic arrest (Han and Conti, 2006; Conti, 2013).

Following the commencement of puberty with about 3000-4000 primary oocytes remaining, a surge of LH from the pituitary stimulates the resumption of meiosis prior to ovulation (Oktem and Oktay, 2008). This is morphologically characterised by the breakdown of the oocyte's nuclear envelope also known as the germinal vesicle (Neal and Baker, 1975; Lei *et al.*, 2001; Tripathi *et al.*, 2010). The germinal vesicle breakdown is immediately followed by a transition into metaphase I and subsequently the completion of the first meiotic division (Tripathi *et al.*, 2010). At this point, the primary oocyte will have undergone an asymmetric division resulting in two haploid daughter

cells with unequal cytoplasmic content – a secondary oocyte and the first polar body. The secondary oocyte then progresses into the second meiotic division and undergoes a second arrest at metaphase II. This state of arrest is also dependent on cAMP levels as well as the inhibition of an anaphase promoting complex (APC) resulting in MPF degradation (Tunquist and Maller, 2003; Homer, 2013). The oocyte is mature for ovulation at this stage. The fertilisation of an ovulated oocyte by a mature spermatozoon results in an intracellular calcium ($[Ca^{2+}]_i$) influx which in turn triggers the oocyte's release from meiotic arrest, the completion of the second meiotic division as well as the formation of a second polar body (Malcuit *et al.*, 2006) (Figure 4).

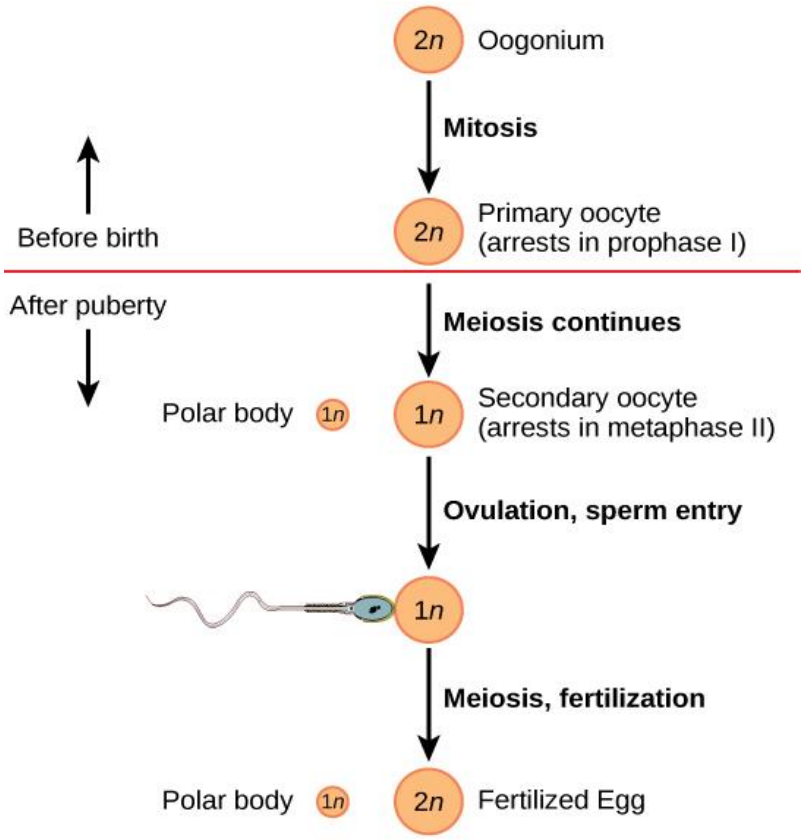


Figure 4 Mammalian oogenesis
Figure from Boundless (2014).

1.2.2 Folliculogenesis and the formation of follicular fluid

Folliculogenesis is the process by which an ovarian follicle develops from the primordial phase to the antral pre-ovulatory phase. This process begins with the recruitment of the follicle into the growing pool in the ovary, as well as the proliferation and differentiation of specialised somatic cells i.e. granulosa and theca cells. A developing follicle consists of an immature oocyte surrounded by a dense arrangement of granulosa and theca cells critical to the growth and maturation of the oocyte. Folliculogenesis and oogenesis are integrated processes central to the formation of follicular fluid in the female ovary, with the developmental phases of an ovarian follicle synchronised with that of the oocyte within. Studies have shown that the entire duration of post-pubertal human folliculogenesis from the primordial to the antral pre-ovulatory phase, is estimated to be more than 175 days (Gougeon, 1986).

Primordial follicle

The initial pool of ovarian follicles (primordial follicles) are present in the foetal ovary with a definitive stock of about 7 million follicles by the 20th week of gestation (Baker, 1963). These follicles undergo continuous depletion via atresia resulting in a pool of between 250,000 and 500,000 follicles at birth (Forabosco *et al.*, 1991; Gougeon *et al.*, 1994; Gougeon, 1996). Several studies have made an attempt to challenge the dogma of a finite primordial follicle population without conclusive evidence on the expansion of the primordial follicle pool via post-natal oogenesis (Reviewed by McLaughlin and McIver, 2009). Resting primordial follicles are quiescent with little or no physiological activity and have a typical diameter of approximately 30μm (Patrizio *et al.*, 2003; Carlsson, 2008). A Primordial follicle consists of a small immature oocyte surrounded by a single layer of flattened squamous granulosa cells resting on a basement membrane

and surrounding stromal cells (Gosden *et al.*, 2002) (Figure 5). As these follicles are released from their post-natal quiescent state and recruited into the growing pool, the granulosa cells surrounding the oocyte become enlarged and cuboid whilst maintaining a single layer around the oocyte (van Wezel and Rodgers, 1996; Eichenlaub-Ritter and Plancha, 2013). At this stage, the granulosa cells begin to express proliferation markers (Oktay *et al.*, 1995; Wandji *et al.*, 1996; Sobinoff *et al.*, 2011) leading to the following physiological changes: the initiation of paracrine signalling between the oocyte and granulosa cells (Vanderhyden *et al.*, 1992; Lanuza *et al.*, 1998; Li *et al.*, 2000), activation of oocyte gene transcription (Reviewed by McLaughlin and McIver, 2009) and the expression of the FSH receptor (Tisdall *et al.*, 1995; Oktay *et al.*, 1997; Meduri *et al.*, 2002).

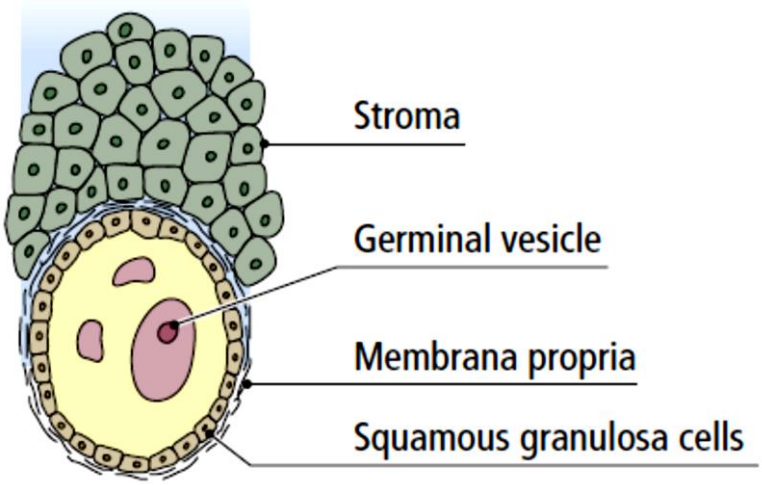


Figure 5 Primordial follicle
Figure from Johnson (2013).

Primary follicle

The appearance of primary follicles is the first sign of follicular activation and recruitment. The regular recruitment of follicles from the primordial follicle pool into a pool of growing primary follicles begins at puberty (Johnson, 2013). When the primordial follicle is activated, oocyte growth and follicular growth occur in tandem and independent of gonadotropins (Albertini, 2015). At this stage, the follicle has reached a diameter of approximately 60μm and this is largely due to growth of the primary oocyte within (Carlsson, 2008). Following the initiation of follicular growth, glycoproteins (ZP1, ZP2, ZP3) are secreted which then condense to form an acellular layer around the oocyte called the *zona pellucida* (ZP) (Wassarman *et al.*, 1996; Gosden *et al.*, 1997). The ZP layer is situated between the oocyte and the surrounding granulosa cells however, the origin of its constituent proteins remains controversial as ZP proteins have been identified within the oocyte, as well as in the granulosa cells of primordial follicles (Gook *et al.*, 2008).

Despite the formation of the ZP layer, the oocyte and surrounding granulosa cell layer still remain connected via cytoplasmic processes of granulosa origin which penetrate the ZP, forming gap junctions at the surface of the oocyte (Anderson and Albertini, 1976; Valdimarsson *et al.*, 1993; Figure 6). This allows for the transport of amino acids, nucleotides, meiotic regulation signals and lipid precursors to the oocyte (Eppig, 1991; Matzuk *et al.*, 2002; Carlsson, 2008). Homologous gap junctions are also present between granulosa cells as this forms this basis of an extensive nutritional network for the oocyte (Albertini *et al.*, 1975; Johnson, 2013). In addition to the intercellular communication and oocyte growth that occurs at this stage, the primary follicle also increases in size and cellular complexity via the condensation of fibroblast-like precursor

cells from the stroma to form a layer of spindle shaped theca cells (Gougeon, 2004; Johnson, 2013). Granulosa cell proliferation also begins, with the cells taking up a cuboidal shape (Albertini, 2015).

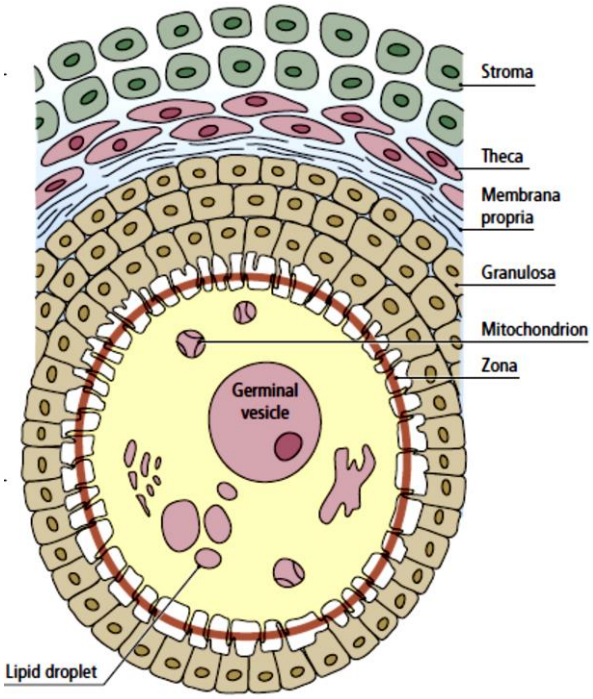


Figure 6 Primary follicle
Figure from Johnson (2013).

Secondary follicle

Accompanying the progression to secondary follicle stage is the continuous proliferation of granulosa cells that form multiple layers around the oocyte. These specialised granulosa cell layers are known as the cumulus oophorus, and together with the developing oocyte make up the cumulus-oocyte complex (Hennet and Combelles, 2012). Cells in the theca layer differentiate into two distinct cell layers – *theca interna* and *theca externa* (Figure 7). In humans, morphological studies have shown that distinct theca layers only appear when the follicle possesses 3 to 6 layers of granulosa cells (Gougeon, 1996). An intricate network of blood capillaries is present between these

two layers, facilitating blood circulation to and from the follicle (Young and McNeilly, 2010). In contrast, the granulosa cell layers are completely avascular (Jones and Lopez, 2014). With the oocyte having completed most of its growth at this stage, the follicle diameter now ranges approximately between 100 and 200μm (Carlsson, 2008). Inside the theca interna, some stromal precursor cells undergo morphological changes and assume the appearance of epithelioid cells (Gougeon, 1996; Gougeon, 2004). Following this morphological change, the secondary follicle is now regarded as a pre-antral follicle. Further proliferation of granulosa cells occurs, accompanied by an increased expression of the FSH receptor. However, the role of FSH in early and pre-antral follicular development is debated and remains unclear (Goldenberg *et al.*, 1976; McGee *et al.*, 1997; Oktay *et al.*, 1997; Oktay *et al.*, 1998; Gougeon, 2004).

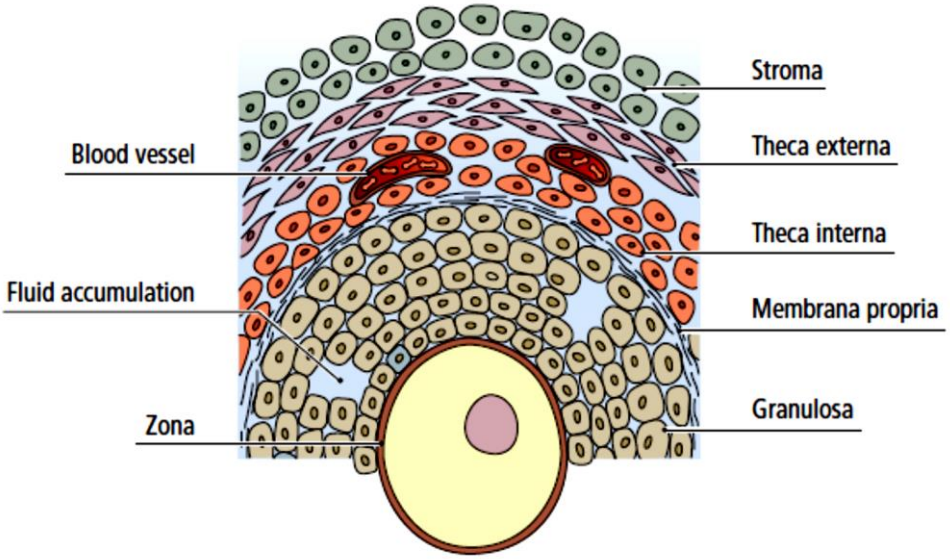


Figure 7 Secondary follicle
Figure from Johnson (2013).

Antral follicle and ovulation

A small amount of pre-antral secondary follicles that avoid atresia grow further to a more advanced phase to become tertiary follicles (Jones and Lopez, 2014). At this stage, the follicle consists of 5 distinct structural units: a fully developed oocyte encircled by a zona pellucida, 5-8 layers of granulosa cells, a basement membrane, a theca interna and a theca externa (Erickson, 2004). The onset of tertiary (antral) follicle development is marked by the formation of a fluid-filled cavity (antrum) within the granulosa cells in close proximity to the oocyte (Erickson, 2000; Johnson, 2013; Figure 8). This process begins with the formation of multiple small cavities which then coalesce to form a single antral cavity (Fahiminiya and Gerard, 2010). Studies carried out on mammalian species suggest that the antrum is fully formed when the follicle reaches a diameter of about 0.2mm (Turnbull *et al.*, 1977; McNatty *et al.*, 1979; Fahiminiya and Gerard, 2010). The precise mechanism behind the accumulation of follicular fluid in the antrum is yet to be determined. Clarke *et al.* (2006), based on their studies, proposed a hypothesis for this phenomenon. It is presumed that the follicular fluid is formed from a combination of endogenous secretions from within the follicle (granulosa and theca cell secretions), and the accumulation of water and electrolytes from blood plasma molecules through the blood-follicular barrier with the aid of an osmotic gradient. As reported by Clarke *et al.* (2006), possible candidates for the creation of this osmotic gradient necessary for the growth of the antrum are glycosaminoglycan, hyaluronan, chondroitin/dermatan sulphate and their associated molecules. The increase in antral volume is responsible for the continuous growth of the antral follicle and this is made possible via the action of FSH (Findlay and Drummond, 1999; Carlsson, 2008; Pangas and Rajkovic, 2015). During ovulation, in response to a surge in LH secretion from the pituitary, the dominant

antral (Graafian) follicle ruptures and releases its contents into the female reproductive tract - the cumulus-oocyte complex and follicular fluid. Following ovulation, residual granulosa and theca cells in the ruptured ovarian follicle differentiate to form the *corpus luteum*. The differentiation of these steroidogenic follicular cells is induced by the pre-ovulatory LH surge that is also responsible for ovulation (Niswender *et al.*, 2000). The *corpus luteum* functions primarily in the secretion of progesterone which is required to maintain a normal pregnancy in mammals.

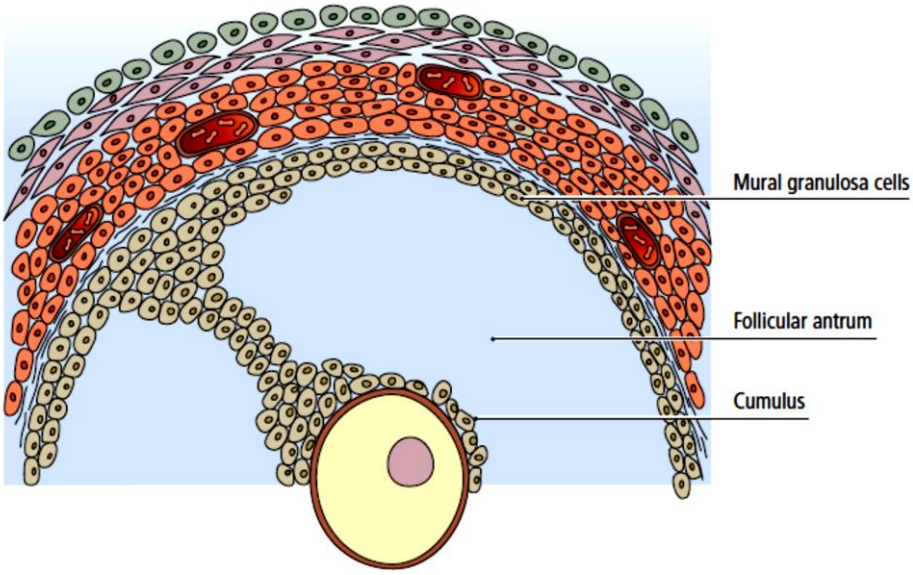


Figure 8 Antral follicle
Figure from Johnson (2013)

1.2.3 Composition of follicular fluid

Several studies have been carried out in an attempt to identify the constituents of follicular fluid (FF) however, the full biochemical composition of FF is yet to be established (Revelli *et al.*, 2009; Jarkovska *et al.*, 2010; Ambekar *et al.*, 2013). Nevertheless, FF has been found to be a rich source of steroid hormones (Table 1) especially progesterone, which has been widely studied (Calogero *et al.*, 2000; Baldi *et al.*, 2009; Wendler *et al.*, 2012). With regards to protein constituents, several studies have been carried out and more than a hundred proteins and amino acids have been identified in FF (see Table 1). However, there is insufficient and conflicting data amongst researchers as to the origin and function of these protein constituents. The main difficulty encountered in the proteomic analysis of complex biological fluids such as FF is that their protein composition is very complicated and dynamic and as a result, sensitive and high-resolving protein separation techniques are required (Revelli *et al.*, 2009). In addition to the proteomic and steroid hormone constituents of follicular fluid, other metabolites and growth factors have been found to be present. These include reactive oxygen species, vascular endothelial growth factor, transforming growth factor-beta family and interleukins (see Table 1). Data from several studies have correlated the presence of the above-mentioned biochemical substances with oocyte quality (Revelli *et al.*, 2009; Carpintero *et al.*, 2014) and fertilisation outcome (Attaran *et al.*, 2000; Zollner *et al.*, 2013).

Table 1 Biochemical constituents of follicular fluid.

FF Constituent	Sub category	Reference
1. Hormones	Gonadotropins • FSH and LH	Enien *et al.*, 1995 Cha *et al.*, 1986
	Growth Hormone (GH)	Lanzone *et al.*, 1996 Jia *et al.*, 1986
	Prolactin	Lindner *et al.*, 1988 Laufer *et al.*, 1984
	Steroid Hormones • Estrogens, Progesterone, Androgens and Corticoids	Tarlatzis *et al.*, 1993 Lee *et al.*, 1987 Messinis and Templeton, 1987 Uehara *et al.*, 1985
2. Growth factors of the transforming growth factor-beta (TGF-beta) super family.	Inhibin and Activin • Activins A and B • Inhibins A and B	Lau *et al.*, 1999 Wen *et al.*, 2006
	Anti-mullerian Hormone (AMH)	Cupisti *et al.*, 2007
	Bone morphogenetic protein-15 (BMP-15)	Wu *et al.*, 2007
3. Other growth factors and interleukins.	Insulin-like Growth Factors (IGF) • IGF I and II	Wang *et al.*, 2006 Jimena *et al.*, 1992 Oosterhuis *et al.*, 1998
	Interleukins (IL) • IL-1α, IL-1β, IL-2, IL-10 and 1L-12	Mendoza *et al.*, 1999 Ledee *et al.*, 2008 Bili *et al.*, 1998
4. Reactive Oxygen Species (ROS).	ROS and anti-oxidation factors	Agarwal *et al.*, 2003 Attaran *et al.*, 2000
	Nitric Oxide (NO)	Barrionuevo *et al.*, 2000 Manau *et al.*, 2000 Machado-Oliveira *et al.*, 2008
5. Vascular Endothelial Growth Factor (VEGF)	VEGF	Manau *et al.*, 2000 Monteleone *et al.*, 2008
6. Anti-apoptotic factors	Tumour Necrosis Factor (TNF) and Fas ligand (Fas-L)	Sarandakou *et al.*, 2003 Malamitsi-Puchner *et al.*, 2004
7. Proteins, peptides and amino-acids.	Proteins, peptides and amino-acids. Glycodelins	Angelucci *et al.*, 2006 Hanrieder *et al.*, 2008 Zamah *et al.*, 2015 Dell *et al.* 1995 Chiu *et al.*, 2003; 2007
8. Sugars	Hyaluronan and Myo-inositol	Suchanek *et al.*, 1994 Chiu *et al.*, 2002
9. Prostanoids	Prostaglandin-E_2 (PGE2) and Prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$)	Jeremy *et al.*, 1987

1.2.4 Illustrative steroidogenic pathways for follicular fluid

Human follicular development is regulated by autocrine, endocrine and paracrine factors, further enabled by the functional differentiation of the granulosa cell layer. Granulosa and theca cells in immature follicles up to the pre-antral stage of development secrete minimal steroid hormones and are considered to be undifferentiated (Wang *et al.*, 2012). In contrast, granulosa and theca cells from antral and pre-ovulatory follicles secrete large amounts of steroid hormones in response to gonadotropins. The biosynthesis of these steroid hormones takes place in the granulosa and theca cells of the antral/pre-ovulatory follicle before ovulation and in the *corpus luteum* after ovulation (Figure 10). These steroid hormones are produced from a primary steroid precursor - cholesterol, present in the blood stream (Miller, 1988; Payne and Hales, 2004; Hu *et al.*, 2010). Cholesterol is transported into the cytoplasm of the theca cells and undergoes conversion to Pregnenolone in the mitochondria via the action of the CYP11A enzyme located within (Sanderson, 2006). Pregnenolone, the secondary steroid precursor, undergoes series of conversions into androgens and progestogens via the action of steroid 17-monooxygenase (CYP17), 3β Hydroxysteroid Dehydrogenase (3βHSD) and 17β Hydroxysteroid Dehydrogenase (17βHSD) enzymes. (Hu *et al.*, 2010). Refer to Figure 10 for detailed steroid hormone biosynthesis pathways within the ovarian follicle. Furthermore, a group of follicular fluid steroid hormones known as corticosteroids play an important role in ovarian function. However unlike other steroid hormones present in follicular fluid (as shown in Figure 10), there is no evidence of their *de novo* synthesis within the ovarian follicle (Omura and Morohashi, 1995). Corticosteroids (e.g. cortisol) are believed to make their way into the cells of the ovarian follicle via the bloodstream following synthesis in the adrenal steroidogenic pathway

(Andersen, 2002) (see Figures 9 and 10). Following their arrival in the ovarian follicle, inert corticosteroids like cortisone undergo a reversible conversion into active cortisol in the granulosa cells via the action of 11β Hydroxysteroid Dehydrogenase enzymes (11βHSD). The regulation of this conversion process in the ovarian follicle is important for maintaining the physiological levels of corticosteroids in follicular fluid.

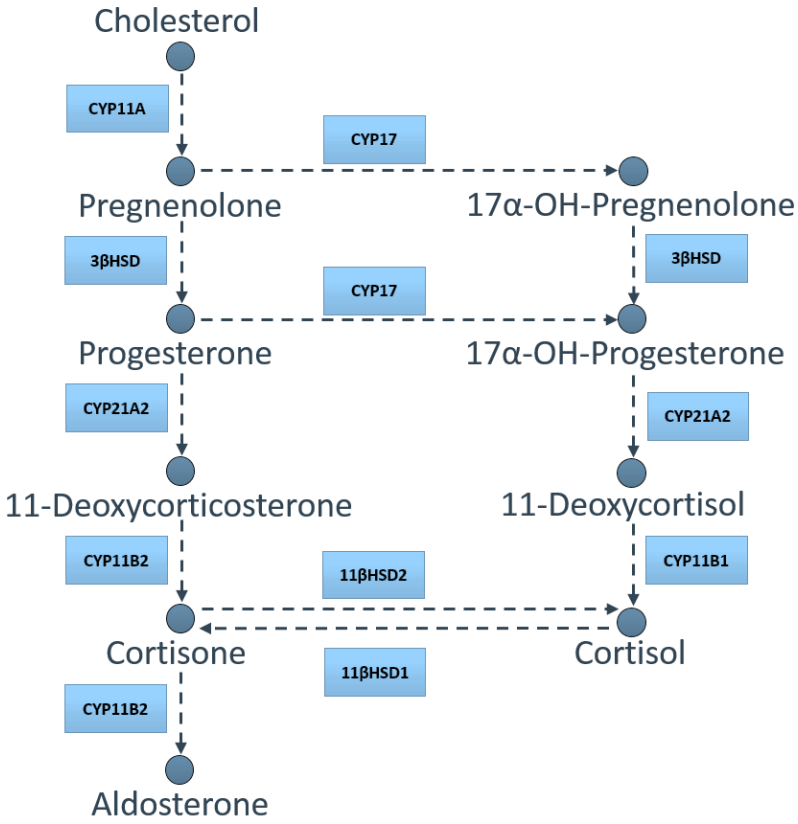


Figure 9 Major corticosteroid biosynthesis pathway in the adrenal cortex. Conversion enzymes are highlighted in blue shaded boxes. Cholesterol, the primary steroid precursor is converted into Pregnenolone, a secondary precursor which then undergoes further conversion into corticosteroids via the action of 3β Hydroxysteroid Dehydrogenase (3βHSD) and Steroid 21-Hydroxylase (CYP21) enzymes.

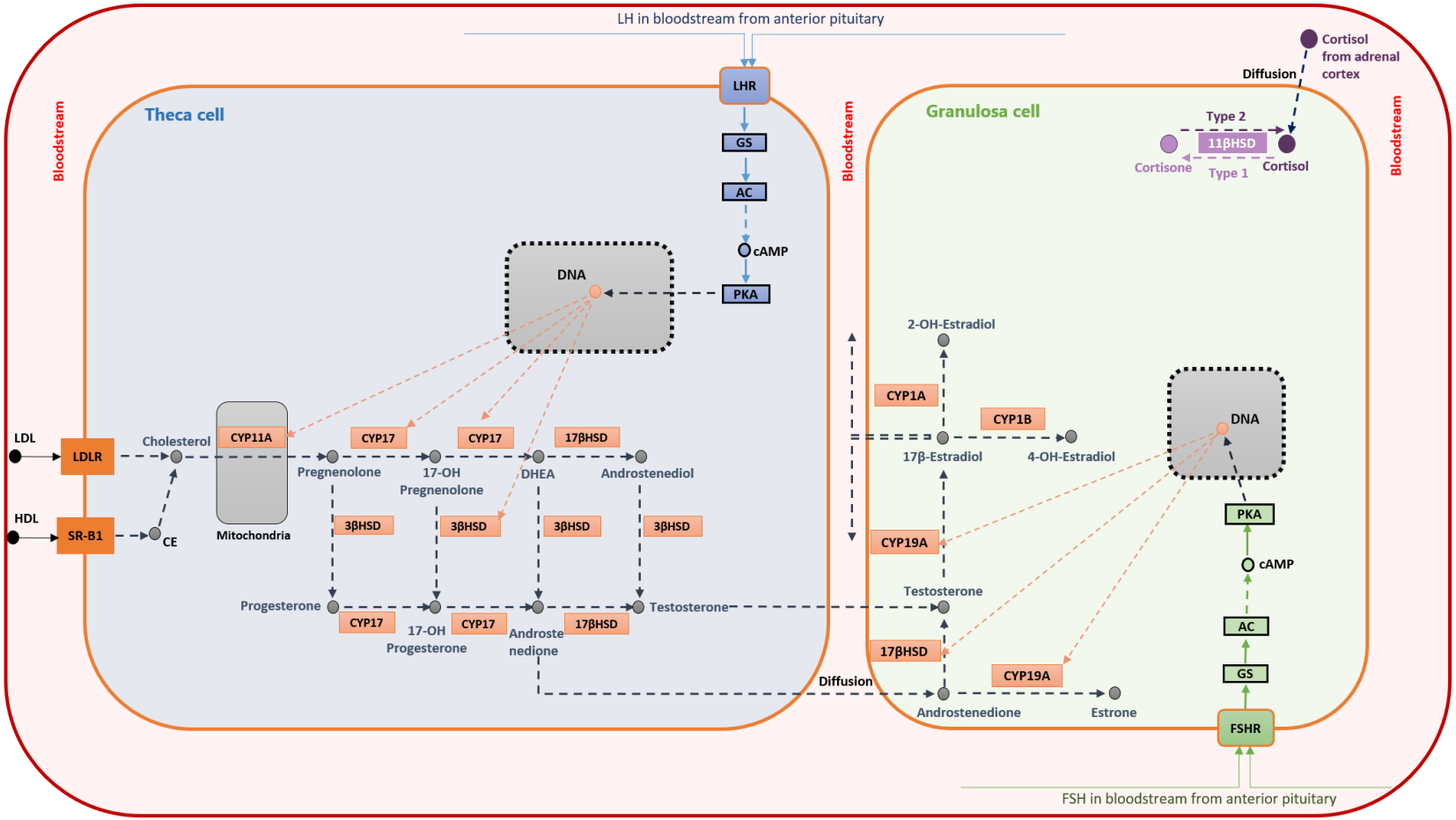


Figure 10 Major steroidogenic pathways in the granulosa and theca cell of the antral pre-ovulatory follicle. Conversion enzymes are highlighted in the pink shaded boxes. Cholesterol is converted to Pregnenolone in the mitochondria of the theca cell via the cholesterol monooxygenase enzyme (CYP11A). Testosterone and Androstenedione produced in the theca cell diffuse into the granulosa cell, and are converted into 17β-Estradiol and Estrone via the action of aromatase (CYP19A). Corticosteroids like cortisol diffuse into the granulosa cell from the bloodstream and are reversibly converted into biologically active cortisone via the action of 11β Hydroxysteroid Dehydrogenase.

1.3 SPERM MIGRATION AND INTERACTIONS WITHIN THE FEMALE REPRODUCTIVE TRACT

Mammalian sperm are incapable of fertilisation at the point of entry into the female reproductive tract (Florman *et al.*, 2007). In order to attain fertilising capacity, spermatozoa are reliant on spatio-temporal interactions with a complex female reproductive tract characterised by a complex biochemical environment and physiologically distinct regions such as the cervix, uterus and Fallopian tubes. An overview of these interactions is presented in this section (See Gaffney *et al.* (2011) and Suarez (2016) for comprehensive reviews on the interactions of spermatozoa with the female reproductive tract).

1.3.1 Sperm interaction with the cervix

Following ejaculation, sperm cells are normally shielded by seminal plasma with a pH ranging from neutral to slightly alkaline (Edström Hägerwall *et al.*, 2012). This serves to protect them from the acidic vaginal environment until they penetrate the cervical barrier which has a neutral pH (Kopito *et al.*, 1973; Gilks *et al.*, 1989; Suarez and Pacey, 2006). Migration of spermatozoa through the cervical canal is inhibited by a viscous mucus that serves as a filter through which seminal plasma and atypical sperm cells do not gain entry (Patrat and Serres, 2009; Curlin and Bursac, 2013). Passage of abnormal spermatozoa through the cervical mucus is hindered by means of a hydrodynamic effect where channels present within the mucus fibres coupled with the viscosity of the mucus, ensure that only normally formed and highly motile sperm cells are able to penetrate the cervical barrier (Gaffney *et al.*, 2011). The cervical canal is also characterised by the presence of epithelial crypts (Figure 11) which may also contribute to the cervical barrier by functioning as slow sperm release reservoirs (Croxatto, 1995; Suarez, 2002).

1.3.2 Sperm interaction with the uterus and Fallopian tubes

Sperm cells that have successfully made their way through the cervical canal into the uterus traverse the uterine cavity and make their way to the utero-tubal junctions aided by rapid peristaltic uterine muscle contractions (Kunz *et al.*, 1996; Kunz *et al.*, 2007) as well as by a number of other interaction mechanisms such as chemokinesis, chemotaxis and thermotaxis (see Chapter 4, section 4.1.1 for a detailed overview of sperm interaction mechanisms and taxes in the female reproductive tract).

As spermatozoa make their way through the utero-tubal junctions into the Fallopian tubes (oviducts), they come in contact with a family of proteins called the glycodelins. Sperm cells are exposed to glycodelins-A and F during their passage through the Fallopian tubes which function to inhibit sperm-zona-pellucida binding (Chiu *et al.*, 2007). When spermatozoa are in close proximity to the COC, they are further exposed to a glycodelin-C which in contrast functions to promote sperm-zona binding (Chiu *et al.*, 2007). Along the sperm's path further from the utero-tubal junction, a sperm 'reservoir' is formed in the Fallopian tubes where sperm cells interact with the tubal epithelia (Figure 11) (Lyons *et al.*, 2006). This sperm-tubal contact serves to preserve the sperm in a viable state whilst awaiting ovulation which in turn increases the chance of successful fertilisation (Suarez and Pacey, 2006). Similarly, sperm cells experience enhanced viability and motility when cultured with tubal epithelia *in vitro* (Kervancioglu *et al.*, 1994; Connolly, 2011). Studies carried out on mammalian species have shown the anatomical location of the sperm 'reservoir' to be the tubal isthmus, with much fewer sperm present in the tubal ampulla (Yanagimachi and Chang, 1963; Hunter, 1981; Hunter and Nichol, 1983; Baillie *et al.*, 1997; Lyons *et al.*, 2006). However, a visibly distinct sperm 'reservoir' is yet to be observed in the Fallopian tubes

of humans. It is believed that the retention of sperm in the tubal isthmus is a natural mechanism for the prevention of polyspermic fertilisation which may likely occur if the reservoir were situated in the tubal ampulla (Suarez, 2016). Sperm cells that make their way from the tubal reservoir proceed to undergo the completion of a major modification process in preparation for fertilisation – a process known as sperm capacitation.

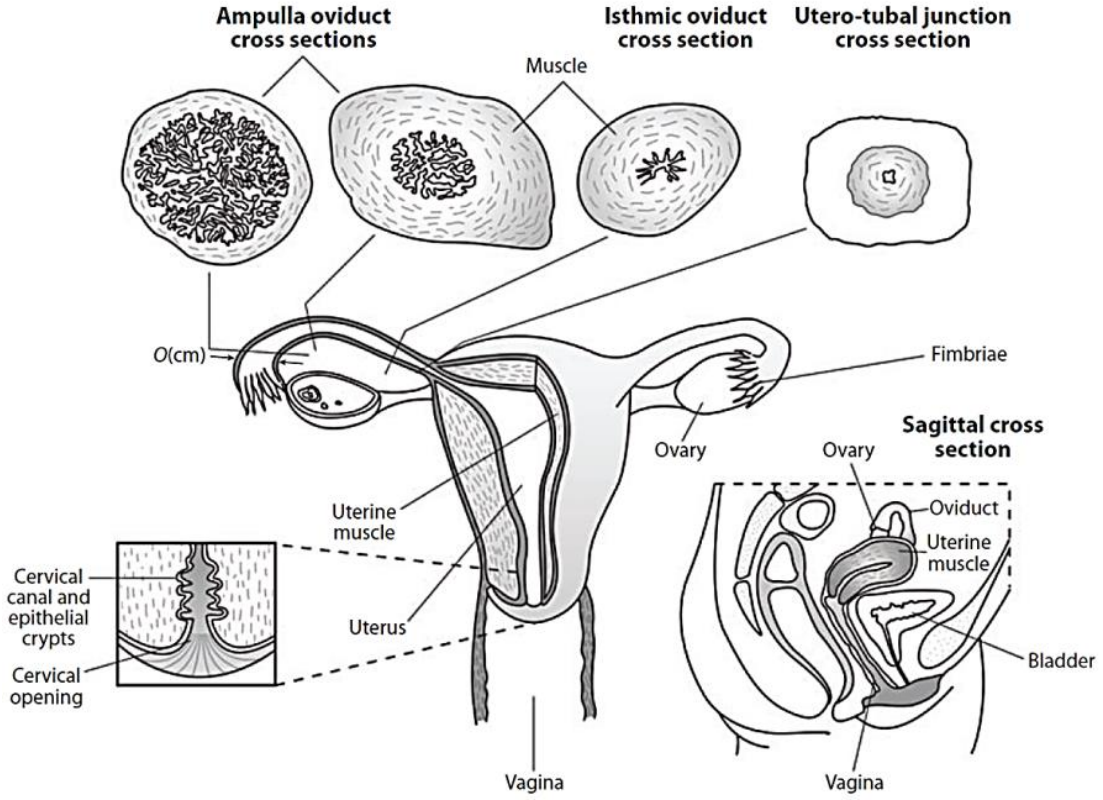


Figure 11 The path of sperm transport to the oocyte within the female reproductive tract. Millions of sperm cells are ejaculated and deposited at the cervical opening. Migration through the cervix is inhibited by a viscous cervical mucus - a substantial barrier to sperm progression, as well as numerous epithelial crypts (inset) which may act as slow release reservoirs. Cells that successfully penetrate the cervical canal progress through the uterus via the aid of peristaltic uterine muscle contractions. Further progression to the isthmic region of the oviduct occurs via a narrow lumen, the utero-tubal junction, approximately 300μm in diameter and filled with viscoelastic mucus. The diameter of the oviduct in proximity to the ovaries is in the order of centimetres (as indicated on the left-hand tube). During the ovulatory period in fertile human couples, a continuous flux of spermatozoa through the oviduct is observed. As indicated in the cross sections, the epithelium of the ampulla and isthmic regions of the oviduct are lined with motile cilia. As highlighted in the cross-sections, there are also convoluted epithelial folds within the ampulla region where the sperm may encounter the oocyte. Adapted from Gaffney *et al.* (2011).

1.3.3 Sperm Capacitation

Sperm capacitation is a further and final maturation process sperm cells have to undergo before they are rendered capable of fertilising an oocyte (Austin, 1951; Chang, 1951; Austin, 1952). This modification process involves a series of molecular mechanisms and signalling events which ultimately confer the following functional characteristics on to the sperm: (i) the sperm's ability to bind to the oocyte's *zona pellucida* and afterwards undergo the acrosome reaction (Saling *et al.*, 1979; Abou-haila and Tulsiani, 2009; Karasu *et al.*, 2012) (ii) a change to a state of hyperactivated motility - whiplash flagellar movements by the sperm crucial for the penetration of the oocyte vestments (Ho and Suarez, 2001b; Suarez and Pacey, 2006), and (iii) the capacity of the sperm to fuse with the oocyte (Marquez and Suarez, 2004; Suarez and Pacey, 2006). The process of sperm capacitation actually begins when spermatozoa interact with seminal fluid, and is completed *en route* to fertilisation in the Fallopian tubes (Visconti, 2009) thus rendering spermatozoa responsive to chemotactic signals encountered in the immediate surroundings of the oocyte (Eisenbach, 1999; Salicioni *et al.*, 2007). Sperm capacitation is characterised by the following key cellular changes: increase in intracellular calcium and subsequent activation of sperm motility, cholesterol efflux from the cell membrane, tyrosine phosphorylation and loss of decapacitation factors. These in turn result in the initiation of hyperactivated motility and the acquisition of fertilising ability.

Increase in $[Ca^{2+}]_i$ and activation of sperm motility

Following the completion of sperm maturation, sperm are stored in the cauda epididymis and maintained in a quiescent state (Verma, 2001; Das *et al.*, 2010). The activation of sperm motility commences immediately after sperm are released from the epididymis and come in contact with high concentrations of HCO^{3-} and Ca^{2+} present in

the seminal plasma (Salicioni *et al.*, 2007). This physiological change is modulated by Catsper - a sperm-specific, pH-sensitive, Ca^{2+} selective channel that controls the influx of calcium ions into sperm cells (Lishko and Kirichok, 2010; Strunker *et al.*, 2011). The transmembrane influx of HCO^{3-} into the sperm cell has been associated with the increase in intracellular pH observed during sperm during capacitation (Visconti, 2009). HCO^{3-} and $[Ca^{2+}]_i$ are also believed to be regulators of cAMP metabolism via the activation of a unique type of adenylyl cyclase (SACY) (Salicioni *et al.*, 2007; Visconti, 2009). Data from multiple studies reveal that SACY is the main HCO^{3-} target during sperm capacitation (Okamura *et al.*, 1985; Chen *et al.*, 2000; Visconti *et al.*, 2011). The activation of SACY causes an increase in intracellular cAMP levels which in turn activate protein kinase A (PKA) (Salicioni *et al.*, 2007). The activated PKA phosphorylates various target proteins which are presumed to initiate several signalling pathways that result in the activation of sperm motility (Visconti, 2009). See chapter 3 for a detailed overview of sperm calcium signalling and homeostasis.

Cholesterol Efflux

The loss of cholesterol from the sperm's cell membrane is a major physiological change associated with sperm capacitation, as was first described by Davis *et al.* (1979). Cholesterol functions to stabilise the sperm's plasma membrane during epididymal transport thus inhibiting the signalling interactions required to achieve a capacitated state (Visconti *et al.*, 1995b). During capacitation, cholesterol is removed by acceptor molecules in the sperm's extracellular environment such as albumin and high density lipoproteins. These molecules have been identified in follicular fluid (Revelli *et al.*, 2009), and are very important constituents of *in vitro* fertilisation culture media.

Tyrosine Phosphorylation

An increase in tyrosine phosphorylation has been shown to be a feature of mammalian sperm capacitation in all species that have been studied (Visconti *et al.*, 1995b; Aitken *et al.*, 1996a; Galantino-Homer *et al.*, 1997; Flesch *et al.*, 1999; Visconti *et al.*, 1999; Lewis and Aitken, 2001; Pommer *et al.*, 2003). This signalling event is localised to the sperm tail and is dependent on the presence of Ca^{2+}, HCO^{3-} and cholesterol acceptors present in follicular fluid e.g. serum albumin (Visconti, 2009). Cholesterol acceptors regulate tyrosine phosphorylation via the removal of plasma membrane cholesterol from the sperm cell. This efflux of cholesterol in turn induces the HCO^{3-} /SACY/cAMP/PKA signalling pathway (Figure 12) (Salicioni *et al.*, 2007; Visconti, 2009). Furthermore, *in vitro* studies have shown that the absence of Ca^{2+}, HCO^{3-} and bovine serum albumin (BSA) in capacitation media prevents both tyrosine phosphorylation and capacitation (Visconti *et al.*, 1995a; Visconti *et al.*, 1995b).

Loss of decapacitation factors

While it is established that mammalian sperm can achieve fertilisation *in vitro*, there are no regulatory mechanisms present in the culture media that control the rate of capacitation in order to ensure that the spermatozoa arrive the surface of the oocyte in a fully primed state. These regulatory mechanisms are present *in vivo* and involve the dynamic interaction of spermatozoa with decapacitation factors that prevent them from capacitating too early. These factors include cholesterol (Davis, 1981), phosphatidylethanolamine binding protein I (Nixon *et al.*, 2006), platelet-activating factor acetylhydrolase (Zhu *et al.*, 2006), protease inhibitors such as the serine protease inhibitor Kasal-type-like protein (SPINKL) (Lin *et al.*, 2008) and serine protein inhibitor

(Lu *et al.*, 2011), HongrESI (Ni *et al.*, 2009) and NYD-SP27, a phospholipase C Zeta 1 isoform localized to the sperm acrosome (Bi *et al.*, 2009). The dissolution of these factors from the sperm surface and intracellular environment are critical to the initiation of sperm capacitation.

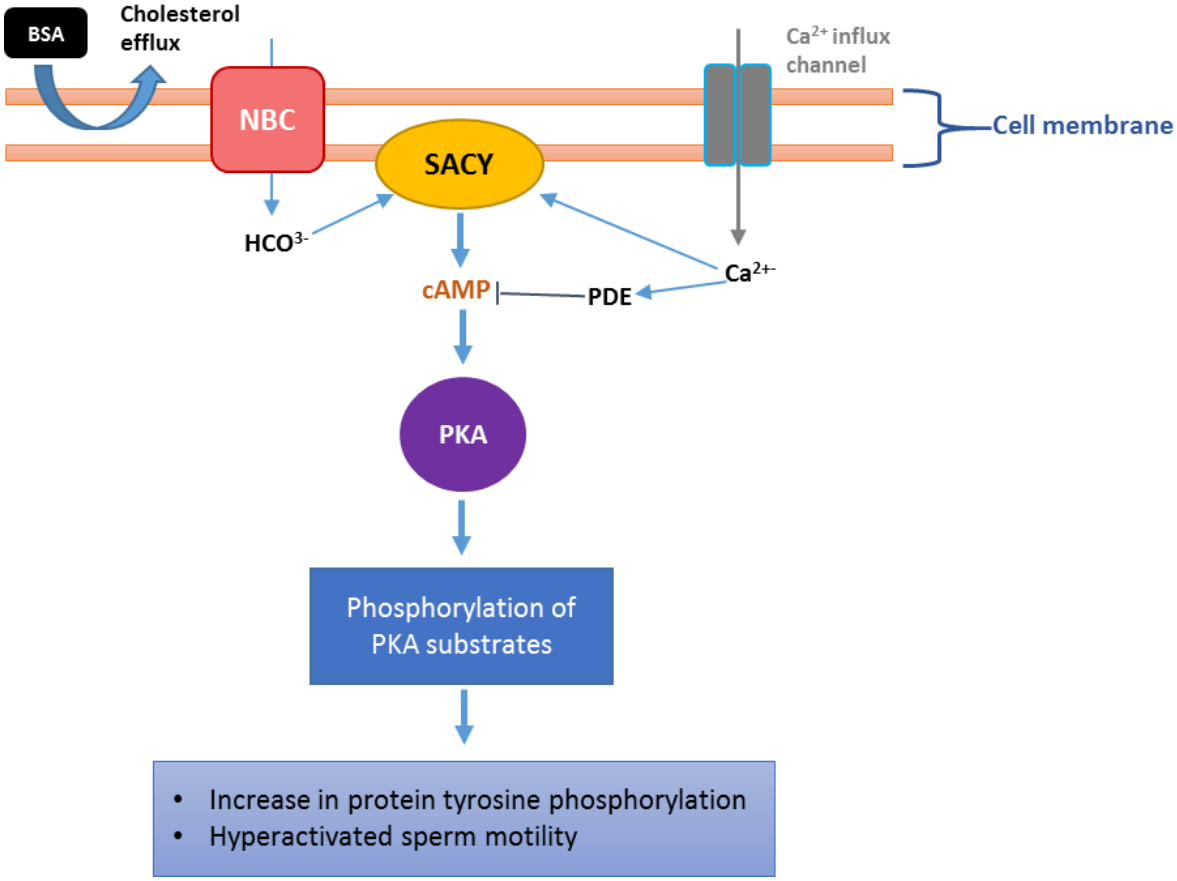


Figure 12 The HCO3-/SACY/cAMP/PKA signalling pathway. The acquisition of fertilising capacity by mammalian sperm is preceded by a switch in the sperm's motility pattern to a state of hyperactivated motility. At the molecular level, these changes are dependent on the increase in protein kinase A (PKA) activity, and are mediated by a Ca^{2+} and HCO^{3-} coordinated stimulation of the soluble adenylyl cyclase (SACY). This in turn results in an increase in tyrosine phosphorylation. This increase is downstream of PKA stimulation and is dependent on the presence of cholesterol acceptors in the extracellular environment. Adapted from Visconti (2009). [NBC=sodium bicarbonate transporter, PDE=phosphodiesterase].

1.4 THE FINAL STAGES OF A SPERM'S JOURNEY TO THE OOCYTE & FERTILISATION

Most animal species give rise to offspring through the process known as fertilization, which is the fusion of a male gamete (sperm) with a female gamete (oocyte) to give rise to a genetically unique individual. This multistep process has three functions: (1) transformation of genes from parents to offspring, (2) restoration of the chromosome number reduced during gamete formation (meiosis), and (3) the initiation of embryonic development.

Amongst capacitated spermatozoa, only sperm cells with a functionally-intact membrane have the ability to fertilise (Rath *et al.*, 2008). Once a few capacitated, fertilising spermatozoa make it to the fertilisation environment in the ampullary-isthmic region of the oviduct, they come in contact with the ovulated cumulus-oocyte complex (COC) and a much higher concentration of follicular fluid. Follicular fluid in addition to secretions from the COC e.g. progesterone further modulate the spermatozoa to initiate the process of sperm-oocyte interaction which includes: (a) penetration of the cumulus oophorus (b) zona pellucida binding (c) acrosome reaction (AR), and (d) the completion of fertilisation. With regards to mammalian fertilisation *in vivo*, the AR is an absolute requirement and is a highlight of sperm-oocyte interaction (Patrat *et al.*, 2000).

1.4.1 Sperm penetration of the cumulus-oophorus

The series of sperm-oocyte interactions associated with the AR begins with the sperm's contact with the cells that constitute the cumulus-oophorus cell mass surrounding the oocyte. Once spermatozoa come in contact with the cumulus-oophorus, the enzyme hyaluronidase present on the surface of the sperm head dissolves hyaluronic acid which

is the major cementing factor between the cells that make up the cumulus-oophorus cell mass (Lin *et al.*, 1994; Rath *et al.*, 2008). This enzymatic dissolution of hyaluronic acid allows the sperm to make its way through the cumulus-oophorus cell mass to the oocyte's *zona pellucida*.

1.4.2 Sperm-zona binding

Once the sperm cell makes its way to the *zona pellucida*, it comes in contact with ZP glycoproteins, ZP1, ZP2, and ZP3 (Gupta and Bhandari, 2011). The expression of a fourth ZP glycoprotein - ZP4 has been confirmed in the human (Lefievre *et al.*, 2004), rat (Hoodbhoy *et al.*, 2005) and hamster (Izquierdo-Rico *et al.*, 2009) however, its function is still being studied across species. In the conventional physiological models based on the 3 glycoprotein system, mammalian sperm have been reported to bind with ZP3 and ZP2 via receptors present on the sperm plasma membrane (Bleil and Wassarman, 1990; Storey, 1995; Reid *et al.*, 2011). Under physiological conditions in mammals, this binding is species-specific depending on the relevant carbohydrates associated with the ZP2 glycoprotein (Bleil and Wassarman, 1990; Wassarman, 1990).

Using mouse and human models, Yauger *et al.* (2011) and Baibakov *et al.* (2012) further investigated the roles of ZP2 and ZP3 in human sperm-zona binding. Transgenic mice were created in which the endogenous ZP proteins were replaced with human ZP1, ZP2, ZP3 and ZP4. Human sperm bound to the human-mouse chimeric ZP only in the presence of human ZP2, but not in the presence of human ZP1, ZP3 or ZP4 (Yauger *et al.*, 2011; Baibakov *et al.*, 2012). This sperm-zona binding was further characterised by the penetration of the zona however, acrosome-reacted human sperm accumulated in the perivitelline space unable to fuse or fertilise the mouse eggs (Baibakov *et al.*, 2012).

Taken together, these findings suggest that ZP2 rather than ZP3 is the ZP ligand to which human sperm bind.

Sperm-zona binding via the ZP glycoproteins is a key step in the process of fertilisation as it triggers an intracellular calcium influx which in turn initiates the AR (Breitbart, 2002). However, this primary binding is by itself not sufficient for the penetration of the zona pellucida and therefore requires the completion of the acrosome reaction (Bleil and Wassarman, 1990; Wassarman, 1990).

1.4.3 Acrosome Reaction (AR)

The acrosome reaction is an exocytotic process essential for the sperm's penetration of the *zona pellucida*, as well as sperm-oocyte binding and fusion. Subsequent to sperm-zona binding, the acrosome reaction takes place and is characterised by the fusion of the sperm plasma membrane with its outer acrosomal membrane (Figure 13). This results in the release of hydrolytic and proteolytic enzymes mainly hyaluronidase and acrosin which are essential for sperm penetration through the *zona pellucida* (Yanagimachi, 1994).

With regards to the onset of mammalian sperm acrosome reaction, the accepted dogma over the years points towards the occurrence of the acrosome reaction either very close to or on the surface of the *zona pellucida* (Austin, 1960; Yanagimachi, 1994; Cummins, 1995). Interestingly, a study by Jin *et al.* (2011) demonstrated that fertilising mouse sperm undergo the acrosome reaction before arriving at the *zona pellucida* of cumulus-enclosed oocytes. Additional data from this study showed that the *in vitro* fertilisation rates of cumulus-stripped oocytes increased when incubated with cumulus cells, suggesting an important physiological role for the cumulus matrix. The *in vivo* effects of

cumulus cells on mammalian spermatozoa remain largely unknown however, the secretion of micromolar progesterone by cumulus cells (de los Santos *et al.*, 2012), sufficient to trigger AR in capacitated human sperm (De Jonge *et al.*, 1988; Osman *et al.*, 1989) could support the hypothesis that cumulus cells function as an inducer of AR *in vivo* based on the findings of Jin *et al.* (2011).

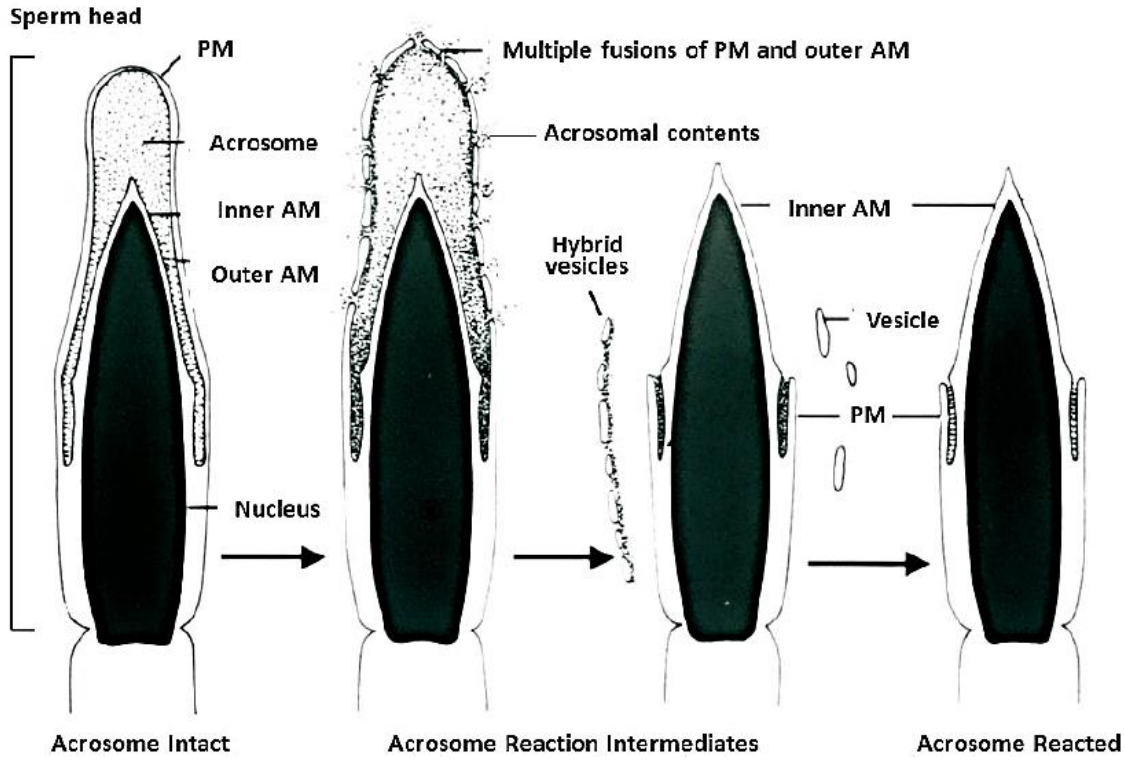


Figure 13 Schematic representation of sperm AR - showing a sperm acrosome before AR (acrosome intact), during AR (acrosome reaction intermediates) and after AR (acrosome reacted). AM – acrosomal membrane. PM – plasma membrane. (Adapted from Wassarman and Litscher, 2008)

1.4.4 sperm-oocyte fusion and the completion of fertilisation

Once the sperm reaches the perivitelline space following the degradation of the *zona pellucida*, it proceeds to fuse with the oocyte's plasma membrane in order to facilitate its entry into the oocyte. As soon as the sperm cell fuses with the oocyte, a process known as oocyte activation is initiated which is accompanied by physical and metabolic

changes in the oocyte (see Tosti and Ménézo (2016); Yeste *et al.* (2016); Swann and Lai (2016) for comprehensive reviews on oocyte activation and early fertilisation events). These changes include a rise in $[Ca^{2+}]_i$ concentration (Stricker, 1999), completion of the second meiotic division (Runft *et al.*, 2002), and the cortical reaction which is responsible for the exocytosis of cortical granules seen within the oocyte's plasma membrane shortly after sperm-oocyte fusion (Wessel *et al.*, 2001). Cortical granules are made up of different groups of enzymes such as glycosidases and proteinases which diffuse into the zona pellucida during exocytosis from the oocyte (Barros and Yanagimachi, 1971; Ghetler *et al.*, 1998; Wessel *et al.*, 2001; Burkart *et al.*, 2012). This alters the structure of the *zona pellucida* thereby triggering a zona reaction which acts as a block to polyspermy (Hoodbhoy and Talbot, 1994; Tsaadon *et al.*, 2006).

An overview of the numerous events that take place during the sperm's journey to the oocyte has been presented. Of all the earlier-mentioned sperm modulators encountered by a sperm cell during its spatio-temporal journey of the female reproductive tract, ovarian follicular fluid stands out for its vast array of biochemical constituents, many of which have been shown to exert physiological changes in human sperm. These include proteins, steroid hormones, prostaglandins, nitric oxide etc. (see Table 1). The research presented in the following chapters of this thesis focuses on the steroid hormone constituents of human follicular fluid and their modulation of human sperm in the female reproductive tract.

CHAPTER 2

FOLLICULAR FLUID STEROID HORMONES AND HUMAN SPERM I – CHARACTERISATION OF INTRACELLULAR CALCIUM RESPONSES AND ACROSOME REACTION

2.1 INTRODUCTION

2.1.1 Ovarian follicular fluid – A key modulator of sperm physiology

Follicular fluid consists of secretions from ovarian follicular cells and blood plasma exudates (Edwards, 1974). This mixture includes: steroid hormones; gonadotropins; growth factors; proteins; reactive oxygen species; sugars; prostanoids and anti-apoptotic factors (see Table 1).

Following its release from the mature ovarian follicle during ovulation, follicular fluid travels via the oviduct down the female reproductive tract, ahead of the oocyte, where it comes in contact with spermatozoa as they make their journey toward the vicinity of the oocyte.

The physiological changes resulting from the interaction between spermatozoa and follicular fluid have been studied by a number of researchers prior to the development of clinical IVF (Gwatkin and Andersen, 1969; Yanagimachi, 1969; Hicks *et al.*, 1972). This is from early recognition of the role of follicular fluid in the natural fertilisation environment. The most visible of changes is the stimulation of sperm motility and enhancement of sperm movement characteristics. This was first reported in hamster sperm, with a significant change in motility patterns observed following incubation in follicular fluid (Gwatkin and Andersen, 1969; Yanagimachi, 1969). *In vitro* studies on human sperm have also shown that exposure to follicular fluid significantly enhances sperm motility and sperm movement characteristics such as curvilinear velocity and lateral head movement (Mendoza and Tesarik, 1990; Falcone *et al.*, 1991; Chao *et al.*, 1992; Fabbri *et al.*, 1998; Jeon *et al.*, 2001; Getpook and Wirotkarun, 2007). Follicular fluid has also been suggested to be a sperm attractant in humans with the evidence

suggesting a chemotactic effect. (Villanueva-Diaz *et al.*, 1990; Ralt *et al.*, 1991; Ralt *et al.*, 1994; Jeon *et al.*, 2001; Wang *et al.*, 2001). This phenomenon has also been observed in mouse (Oliveira *et al.*, 1999) and rabbit (Fabro *et al.*, 2002). Interestingly, follicular fluid has also been recently reported to play a role in the preservation of sperm chromatin quality. Spermatozoa incubated in follicular fluid contained a higher proportion of cells with normal histone and protamine integrity when compared with spermatozoa incubated in conventional culture media (Bahmanpour *et al.*, 2012).

Ovarian follicular fluid and sperm capacitation

Follicular fluid has been shown to be a modulator of sperm capacitation – the functional maturation of sperm cells during which they acquire the ability to fertilise an oocyte. The majority of the studies published on this effect of follicular fluid assessed sperm capacitation based on the following criteria: (i) a switch to hyperactivated motility and (ii) *in vitro* fertilisation of oocytes post-exposure to follicular fluid. Using either or both of the above-mentioned physiological criteria, a number of studies have provided evidence of the stimulatory effect of follicular fluid on mammalian sperm capacitation (Gwatkin and Andersen, 1969; Yanagimachi, 1969; Mbizvo *et al.*, 1990; Ravnik *et al.*, 1993; Kulin *et al.*, 1994; McNutt *et al.*, 1994; Fabbri *et al.*, 1998; Hamdi *et al.*, 2010). Follicular fluid modulates sperm capacitation in part by inducing the efflux of cholesterol from the plasma membrane to acceptors such as albumin, high-density lipoproteins or lipid transfer protein-I all present in follicular fluid (Langlais *et al.*, 1988; Ravnik *et al.*, 1993; Hamamah *et al.*, 1995). Other active follicular fluid constituents, steroid hormones, and their role in sperm capacitation will be reviewed later in this chapter.

Ovarian follicular fluid and sperm acrosome reaction

With the induction of the acrosome reaction being a crucial physiological event preceding fertilisation, the importance and the effects of follicular fluid on this process have been widely investigated. Prolonged or continuous exposure to follicular fluid is reported to induce acrosome reaction in human sperm (Tesarik, 1985; Mortimer and Camenzind, 1989; Stock *et al.*, 1989; Siegel *et al.*, 1990; De Jonge *et al.*, 1993; Fabbri *et al.*, 1998; Burrello *et al.*, 2004). These studies also proposed several hypotheses on the induction of human sperm acrosome reaction by follicular fluid. These include a concentration-dependent action of follicular fluid (Mortimer and Camenzind, 1989), the activation of sperm proteinase systems (Siegel *et al.*, 1990), the activation of multiple signal transduction pathways in sperm (De Jonge *et al.*, 1993), biochemical action of granulosa cell secretions (Fabbri *et al.*, 1998), as well as follicular fluid interaction with γ-aminobutyric acid receptors - $GABA_A$ and $GABA_B$ (Burrello *et al.*, 2004). All of these studies however only provide a partial picture on the underlying mechanisms responsible for the induction of acrosome reaction which are still yet to be fully understood.

Ovarian follicular fluid and sperm-zona binding

In contrast to the previously discussed stimulatory effects of follicular fluid on human sperm physiology, an inhibitory effect on the zona-binding capacity of sperm cells was observed with human follicular fluid from both gonadotrophin-stimulated and natural reproductive cycles (Yao *et al.*, 1996; Qiao *et al.*, 1998). This creates an empirical paradox with follicular fluid inhibiting sperm-zona binding but facilitating the fertilisation of oocytes. Despite the presence of a high concentration of progesterone in

follicular fluid-which is known to stimulate sperm-zona binding (Sueldo *et al.*, 1993), this inhibitory effect was observed in all human follicular samples tested (Yao *et al.*, 1996). Yao *et al.* (1996) also reported that this observed inhibitory effect was not mediated via an observable effect on sperm viability, motility or acrosome reaction. Further studies carried out by Yao *et al.* (1998) led to the identification of two glycoproteins - zona-binding inhibitory factor 1 and zona-binding inhibitory factor 2 (ZIF-1 and ZIF-2), with molecular weights of 32kDa and 192kDa respectively. Further characterisation studies showed ZIF-1 to be a differentially glycosylated isoform of Glycodelin-A, and was found to be the main molecule responsible for the inhibitory activity of follicular fluid (Chiu *et al.*, 2003). Due to its presence in follicular fluid, ZIF-1 was renamed Glycodelin-F in line with the nomenclature of other glycodelins (Dell *et al.*, 1995; Chiu *et al.*, 2003). Furthermore, the sperm-zona binding inhibitory effect of follicular fluid was found to be significantly reduced by cumulus cells (Hong *et al.*, 2003), suggesting a finely tuned modulation of sperm function at the site of fertilisation.

Ovarian follicular fluid and sperm intracellular signalling

When studying the modulation of human sperm by follicular fluid at the single cell level, the next logical questions may be what active factors in follicular fluid are responsible, as well as the intracellular signalling mechanisms through which these active factors modulate sperm physiology in the female reproductive tract.

The signalling effects of follicular fluid on human sperm have been studied at the intracellular level. Using fluorescence spectroscopy, human follicular fluid was found to elicit a rapid $[Ca^{2+}]_i$ increase in human sperm (Fukui *et al.*, 1995; Minami *et al.*, 1995; Shiomi *et al.*, 1996; Frettsome, 2012). The observed rise in $[Ca^{2+}]_i$ was reduced by 50%

when spermatozoa were treated with lipid-stripped follicular fluid, and was restored when the lipid-stripped FF was supplemented with progesterone (Fukui *et al.*, 1995). However, the restoration of $[Ca^{2+}]_i$ levels was not observed when estradiol was used as a supplement thus suggesting a major role for progesterone in the lipid fraction of follicular fluid to mediate Ca^{2+} entry into human spermatozoa. Evidence from the above studies therefore suggests that follicular fluid steroid hormones are key to the modulation of the signalling events.

2.1.2 Steroid Hormones in Ovarian Follicular Fluid

Early studies on a range of mammalian species including human, led to the identification of a wide variety of sex steroid hormones (progestogens, estrogens and androgens) in follicular fluid (as reviewed by Edwards, 1974). Based on previously published studies on the effects of glucocorticoid hormones on ovarian physiology (reviewed by Michael *et al.*, 2003; Whirledge and Cidlowski, 2010), further characterisation studies on mammalian follicular fluid resulted in the identification of glucocorticoids in the follicular fluid milieu (Fateh *et al.*, 1989; Jimena *et al.*, 1992b; Harlow *et al.*, 1997; Lewicka *et al.*, 2003).

Early studies by Calzada *et al.* (1988) using isolated steroid hormones showed that exposure to progesterone, estrogens and testosterone increased the membrane potential of human spermatozoa as measured by the uptake of lipophilic cation radiolabelled triphenylmethylphosphonium ($TPMP^+$). Extensive research has since been carried out on the effects of steroid hormones on mammalian sperm physiology. An overview of current knowledge on the effects of follicular fluid steroid hormones on mammalian sperm is given below.

Progestogens

The effects of progestogens on mammalian female reproductive physiology have been widely studied. In addition to their role in the regulation of the menstrual cycle and early pregnancy, progestogens present in follicular fluid have been shown to have a significant impact on spermatozoa in the female reproductive tract as they travel towards the site of fertilisation. Studies have suggested that the physiological concentrations of progestogens in the female reproductive tract are at micromolar levels (de los Santos *et al.*, 2012).

Progesterone evokes sperm $[Ca^{2+}]_i$ signalling and enhances sperm capacitation

Data from a ground-breaking study by Blackmore *et al.* (1990) showed that progesterone and 17α-hydroxyprogesterone induced a rapid influx of Ca^{2+} in human sperm from the extracellular environment. Using 3.2μM progesterone, Kirkman-Brown *et al.* (2000) demonstrated that the observed influx of Ca^{2+} in human spermatozoa is biphasic in nature, characterised by a transient rise in $[Ca^{2+}]_i$ immediately followed by a sustained $[Ca^{2+}]_i$ increase. This response was shown to be dose-dependent, with nanomolar progesterone found to be sufficient to stimulate a Ca^{2+} influx (Harper *et al.*, 2003). Furthermore, a maximal $[Ca^{2+}]_i$ response was observed in response to 3μM progesterone. This $[Ca^{2+}]_i$ signalling event is believed to play a significant role in key physiological changes such as capacitation, which occur in spermatozoa upon exposure to follicular fluid progesterone.

Progesterone has been reported by several studies to have a stimulatory effect on mammalian sperm capacitation (Foresta *et al.*, 1992; Uhler *et al.*, 1992; Kay *et al.*, 1994; Yang *et al.*, 1994; de Lamirande *et al.*, 1998; Contreras and Llanos, 2001; Thundathil *et*

al., 2002; Yamano *et al.*, 2004; Sumigama *et al.*, 2015). However, the absence of a universally accepted empirical marker for sperm capacitation makes the comparative analysis of these studies difficult due to the use of different measures/markers of capacitation in different studies (Baldi *et al.*, 2009). Nevertheless, the direct induction of hyperactivated motility is a capacitation marker common to most of these studies.

Mammalian sperm Ca^{2+} influx and $[Ca^{2+}]_i$ signalling are believed to be key to the execution of progesterone-induced physiological responses in mammalian sperm. Based on studies using human sperm, a generally accepted mechanism of Ca^{2+} influx is the progesterone-induced activation of the principal flagellar Ca^{2+} channel, CatSper (Lishko *et al.*, 2011) (See chapter 3 for a detailed review on the CatSper channel). Using recordings of CatSper currents from epididymal and testicular sperm, Smith *et al.* (2013) showed that the sensitivity of the CatSper channel to progesterone presents itself early in sperm development and gradually increases until it reaches a peak when the spermatozoa are ejaculated. The expression and function of the CatSper channel has been directly associated with progressive motility, and may be involved in the pathogenesis of asthenozoospermia (Tamburrino *et al.*, 2014). Interestingly, the progesterone-induced activation of the CatSper channel does not occur in mice (Lishko *et al.*, 2011) but has been demonstrated in the rhesus monkey (Sumigama *et al.*, 2015). Nevertheless, an absence of the CatSper channel in mouse spermatozoa has been shown to be responsible for male infertility, based on the observed inability of the spermatozoa to undergo hyperactivation (Carlson *et al.*, 2003; Carlson *et al.*, 2005). Similar suggestions have also been made from studies carried out on human sperm carrying CatSper gene mutations or defects in CatSper function (Avenarius *et al.*, 2009;

Hildebrand *et al.*, 2010; Smith *et al.*, 2013; Williams *et al.*, 2015). In view of the evidence supporting the progesterone-induced activation of the CatSper channel, this brings into question the precise mechanism(s) of action of progesterone on transcriptionally inactive spermatozoa.

In addition to the published evidence supporting the role of CatSper as the primary Ca^{2+} influx channel in mammalian sperm (Lishko *et al.*, 2011; Strunker *et al.*, 2011), a number of studies have suggested that store operated channels (SOCs) also play a role in $[Ca^{2+}]_i$ signalling of human sperm (Blackmore, 1993; Costello *et al.*, 2009; Park *et al.*, 2011; Lefievre *et al.*, 2012; Morris *et al.*, 2015). The sperm Ca^{2+} stores with a suggested role in this proposed $[Ca^{2+}]_i$ signalling event are located both within the acrosome and at the sperm neck region, and are crucial to the completion of the acrosome reaction and the regulation of motility respectively (Costello *et al.*, 2009; Alasmari *et al.*, 2013b). With reference to the generally accepted $[Ca^{2+}]_i$ signalling models across species, this store-operated calcium entry (SOCE) or capacitative calcium entry (CCE) is believed to be mediated via the activation of SOCs by interaction with stromal interaction molecule (STIM), a sensor molecule localised to the membrane of the $[Ca^{2+}]_i$ store that detects depletion of stored calcium (Cahalan, 2009). Following the activation of the store, STIM repositions itself to interact with the SOC proteins (Orai and members of the transient receptor potential canonical [TRPC] family), causing the channels to open (Cahalan, 2009). Evidence in support of the existence of this signalling pathway in mammalian sperm can be obtained from localisation studies which have identified STIM I, Orai proteins and TRPC proteins in human sperm (Castellano *et al.*, 2003; Darszon *et al.*, 2012; Lefievre *et al.*, 2012). With the biphasic $[Ca^{2+}]_i$ response of human sperm to

progesterone already established (Kirkman-Brown *et al.*, 2000; Harper *et al.*, 2004), some studies suggest that the sustained phase of the $[Ca^{2+}]_i$ response in a sub-population of responding cells is characterised by a series of $[Ca^{2+}]_i$ oscillations (Harper *et al.*, 2004; Kirkman-Brown *et al.*, 2004; Aitken and McLaughlin, 2007; Sánchez-Cárdenas *et al.*, 2014) consistent with calcium-induced calcium release (CICR) from the store located at the sperm neck (Harper *et al.*, 2004). Using single-cell Ca^{2+} imaging, a recent attempt was carried out by Morris *et al.* (2015) to further investigate the role of CICR in progesterone-induced sperm calcium signalling. Based on their findings, it was proposed that SOCs contribute to the progesterone-induced transient $[Ca^{2+}]_i$ response in human sperm. It was also suggested that pharmacological interference with SOC regulatory mechanisms delays the closure of the channels thus causing a prolonged $[Ca^{2+}]_i$ transient response (Morris *et al.*, 2015).

Progesterone as a possible sperm chemoattractant

Progesterone has been reported to be a mammalian sperm chemoattractant and a likely modulator of sperm chemotaxis in the female reproductive tract (Villanueva-Diaz *et al.*, 1995; Jaiswal *et al.*, 1999; Wang *et al.*, 2001; Teves *et al.*, 2006; Teves *et al.*, 2009; Blengini *et al.*, 2011; Gatica *et al.*, 2013). Teves *et al.* (2006) demonstrated that picomolar progesterone is a chemoattractant for mammalian sperm. Further studies also showed that gradients of picomolar progesterone chemotactically selected functional human spermatozoa from sub-fertile sperm populations (Gatica *et al.*, 2013). As is seen with studies on progesterone-induced sperm capacitation, there is a conflict in the data from the above-mentioned studies on sperm chemotaxis, rooted in the varying observations on the simultaneous effects of progesterone on mammalian sperm

hyperactivation as well as the hypothesised involvement of sperm hyperactivated motility in the mediation of sperm chemotaxis. Armon and Eisenbach (2011) suggested that sperm hyperactivation is part of the progesterone-induced chemotactic response. This was based on their observations on the response of human spermatozoa to the spatial and temporal application of chemoattractants. A reduction of hyperactivated motility was observed in chemotactically-responding spermatozoa populations in the proximity of a spatial progesterone gradient, thus ensuring that the sperm stay on course as they swim towards the gradient. When a temporal increase in concentration of progesterone was applied via photorelease from its caged form, a delayed response was observed characterised by an initiation of hyperactivated motility (Armon and Eisenbach, 2011). There however remains an absence of data on the underlying mechanisms by which progesterone induces sperm chemotaxis and hyperactivated motility. This is confounded when one takes into account the practicalities of measuring and extrapolating such data whilst mimicking the viscous properties of the female tract environment *in vitro* (Smith *et al.*, 2009; Kirkman-Brown and Smith, 2011; Perez-Cerezales *et al.*, 2016)

Progesterone significantly induces sperm acrosome reaction

With sperm acrosome reaction being a key physiological prerequisite for mammalian fertilisation, the direct induction of mammalian sperm acrosome reaction by progesterone has been established (Osman *et al.*, 1989; Foresta *et al.*, 1992; Parinaud *et al.*, 1992; Bronson *et al.*, 1999; Kirkman-Brown *et al.*, 2000; Harper *et al.*, 2006). This is in accordance with data from studies demonstrating a significant correlation between progesterone-induced responses and fertilising ability during IVF (Krausz *et al.*, 1996;

Jacob *et al.*, 1998). As with most exocytotic processes, progesterone-induced acrosome reaction is believed to be mediated via the action of $[Ca^{2+}]_i$ (Thomas and Meizel, 1989). This informed an increased research focus on the identification of the precise $[Ca^{2+}]_i$ signalling mechanisms, and the involvement of $[Ca^{2+}]_i$ stores and membrane channels in the modulation of progesterone-induced sperm acrosome reaction. A number of signalling pathways have since been suggested by different studies with some controversy surrounding their validity. One of such pathways is the PLC/IP3/DAG/PKC signalling pathway (Thomas and Meizel, 1989; O'Toole *et al.*, 1996). However, Harper *et al.* (2004) reported that phospholipase C (PLC) blockers do not appear to affect $[Ca^{2+}]_i$ responses in progesterone-treated sperm, thus creating some doubt as to the validity of this model. Another proposed pathway is the cAMP/PKA pathway (Harrison *et al.*, 2000) however, Moseley *et al.* (2005) reported that progesterone-induced acrosome reaction is not associated with an increase in the activation of protein kinase A. Using human sperm, Harper *et al.* (2006) reported that maximal acrosome reaction occurred at the transient phase (rather than the sustained phase) of the $[Ca^{2+}]_i$ response triggered by exposure to progesterone. They also suggested that the amplitude of the sperm $[Ca^{2+}]_i$ response is not a critical determinant of the acrosome reaction. Varano *et al.* (2008) provided some evidence for the involvement of the tyrosine kinase Src in progesterone-induced acrosome reaction. A significant reduction in protein tyrosine phosphorylation and hence acrosome reaction was observed following the pharmacological inhibition of Src activity (Varano *et al.*, 2008). Furthermore, the phosphatidylinositol 3-Kinase (PI3K) pathway was suggested to be a modulator of progesterone-induced mammalian sperm hyperactivation and acrosome reaction, with the inhibition of PI3K shown to result in the suppression of hyperactivation and

acrosome reaction (Nauc *et al.*, 2004; Aparicio *et al.*, 2005; Breitbart *et al.*, 2010). Recently, in line with previous evidence supporting progesterone-induced CICR in sperm (Harper *et al.*, 2004; Bedu-Addo *et al.*, 2007), Sosa *et al.* (2016) reported that acrosomal swelling, an important step in mammalian sperm acrosome reaction is triggered by the activation of an adenylyl cyclase functioning downstream of the Ca^{2+} release from store-operated calcium channels.

Receptor-mediated action of progesterone

Due to the absence of a nuclear progesterone receptor in mammalian spermatozoa, coupled with the extremely rapid nature of progesterone-induced sperm $[Ca^{2+}]_i$ mobilisation, it is presumed that non-genomic membrane receptor(s) mediate this process (Revelli *et al.*, 1998; Losel and Wehling, 2003; Correia *et al.*, 2007). The presence of a progesterone receptor in human sperm has been studied by several research groups using different technical approaches (reviewed in Aquila and De Amicis, 2014). This supposed sperm membrane progesterone receptor was shown to have a specificity for progesterone in the presence of progesterone, testosterone and androstane analogues (Blackmore *et al.*, 1996). The identity of this non-genomic progesterone receptor and the precise signalling mechanisms through which it initiates Ca^{2+} influx remained unknown until very recently.

Using human sperm, Miller *et al.* (2016) provided evidence for an endocannabinoid signalling model that governs the activation of the CatSper channel via progesterone. In this study, the highly expressed orphan enzyme alpha/beta hydrolase domain containing protein 2 (ABHD2) was shown to function as the sperm progesterone receptor. In this proposed signalling model, progesterone binds and activates ABDH2 which in turn acts

as a progesterone-dependent lipid hydrolase by depleting endogenous endocannabinoid 2-arachidonoylglycerol (2AG) via hydrolysis into arachidonic acid (AA) and glycerol. 2AG inhibits CatSper and the depletion of 2AG ultimately leads to Ca^{2+} influx via the CatSper channel (Miller *et al.*, 2016). Evidence in support of the ABHD2 endocannabinoid signalling model was demonstrated via the treatment of spermatozoa with an irreversible metabolic serine hydrolase inhibitor- methyl arachidonyl fluorophosphanate (MAFP). MAFP inhibited ABHD2 activity resulting in the complete inhibition of progesterone-dependent CatSper activation (Miller *et al.*, 2016). This study also found that brief applications of AA transiently activate CatSper whilst a prolonged incubation of spermatozoa in 3μM AA resulted in CatSper inactivation and the loss of progesterone sensitivity, suggesting that ABHD2 activity may be regulated via a negative feedback mechanism whereby continuous application of progesterone will eventually result in an overabundance of AA in the plasma membrane ultimately resulting in the desensitisation of the CatSper channel. The identification of ABHD2 as the likely candidate for the non-genomic sperm membrane progesterone receptor has paved the way for further research into the modulation of sperm physiology via follicular fluid steroid hormones.

Estrogens

The effects of estrogens on the structure and function of cells and tissues within the mammalian female reproductive system have been widely studied. However, fewer studies have been carried out on the direct effects of estrogens on mammalian sperm physiology. The presence of estrogen receptors α and β (ERα and ERβ) in ejaculated spermatozoa and immature spermatogenic cells suggest a considerable role for estrogens

in the regulation of spermatogenesis, sperm maturation and capacitation (reviewed in Hess, 2014). Using murine species, studies revealed that the absence of estrogen receptors adversely affected male reproductive function and fertility (Hess *et al.*, 2000). Estrogen receptor α knock out mice (ERKO) possessed dilated epididymi as well as blind-ended efferent ductules with lesions. In addition to the classic estrogen receptors α and β, a G protein-coupled estrogen receptor (GPER) was recently identified in mature human and pig spermatozoa (Rago *et al.*, 2014). Whilst the GPER was detected in the acrosomal region, equatorial segment and mid-piece of pig spermatozoa, localisation studies found the GPER confined to the mid-piece region of human sperm (Rago *et al.*, 2014). This may suggest a role for both the GPER and the ERs in mediating the rapid non-genomic effects of estrogens on spermatozoa. In the context of spermatozoa traversing the steroid hormone milieu in the mammalian female reproductive tract, estrogens have been suggested to have a possible role in sperm capacitation. Estrone, estradiol and estriol have been shown to stimulate boar sperm capacitation *in vitro* (Ded *et al.*, 2010). In human sperm, estradiol has been shown to have a dose-dependent stimulatory effect on cholesterol efflux thus promoting an increase in Ca^{2+} influx and protein tyrosine phosphorylation (Luconi *et al.*, 1999; Guido *et al.*, 2011). Baldi *et al.* (2000) reported that estradiol evoked a biphasic $[Ca^{2+}]_i$ response in human spermatozoa about half the size of a typical progesterone-induced response. However, pre-exposure of human spermatozoa to estradiol was found to inhibit progesterone-induced Ca^{2+} influx (Baldi *et al.*, 2000; Kirkman-Brown, 2000). Similarly in hamster sperm, dual exposure to progesterone and estradiol or pre-exposure to estradiol supressed progesterone-induced hyperactivation (Fujinoki, 2010). Some studies suggest that estradiol has no direct effect on mammalian sperm acrosome reaction (Francavilla *et al.*,

2003; Vigil *et al.*, 2008) however, estradiol has been shown to negatively regulate progesterone-induced acrosome reaction (Luconi *et al.*, 1999; Baldi *et al.*, 2000; Kirkman-Brown, 2000; Vigil *et al.*, 2008). With estrogens shown to evoke mammalian sperm Ca^{2+} influx and capacitation as well as inhibit progesterone-induced physiological responses in mammalian sperm, it is likely that a signalling pathway connecting the progesterone receptor, estrogen receptor(s) and the CatSper channel is in effect. Interestingly, using the whole-cell patch clamp technique, Espinosa *et al.* (2000) and Lu *et al.* (2008) reported that estradiol has a dose-dependent inhibitory effect on T-type calcium channels in mouse spermatogenic cells. This might suggest that the precise mechanism by which estrogens modulate sperm Ca^{2+} signalling is reliant on a synergistic and/or antagonistic effect of other follicular fluid steroid hormones namely progesterone.

Androgens

Elevated androgen levels (testosterone) in FF have been associated with lower quality oocytes, particularly oocytes with lower cleavage rates following fertilisation (Uehara *et al.*, 1985). The ratio of estradiol to testosterone has also been reported to be higher in follicles containing pregnancy-associated oocytes (Andersen, 1993; Xia and Younglai, 2000). Taken together, the data from the above-mentioned studies suggest that a low estrogen/androgen ratio in FF may be associated with early follicular atresia, which in turn has a negative effect on the viability of the enclosed oocyte and this reduces the chances of fertilisation and pregnancy (Revelli *et al.*, 2009). Whilst the importance of androgens in male physiology has been widely studied (reviewed in Walker *et al.*, 2010), very few studies have been carried out on the effects of FF androgens on human sperm physiology in the female reproductive tract. Early studies by Chan *et al.* (1983) showed

that treatment with testosterone or dihydrotestosterone inhibited the fertilising capacity of human sperm *in vitro*. Blackmore *et al.* (1990) reported that testosterone stimulates only about 10% of the rise in sperm $[Ca^{2+}]_i$ levels when compared to progesterone induced $[Ca^{2+}]_i$ responses. There is however a lack of published data on the direct effects of androgens on sperm capacitation and hyperactivated motility. With regards to the possible effect of androgens on human sperm acrosome reaction, testosterone has been reported to exert no stimulatory effect (Vigil *et al.*, 2012).

Glucocorticoids

The presence of glucocorticoids in FF is believed to be important for oocyte maturation and subsequent embryo implantation, hence their production in FF has been highlighted as a further marker for fertilisation success (Keay *et al.*, 2002; Lewicka *et al.*, 2003). The enzyme 11β hydroxysteroid dehydrogenase (11βHSD) is responsible for catalysing the biochemical conversion of corticoids – the reversible generation of 'active' cortisol from 'inert' cortisone (see Figures 9 and 10). A reduction in the activity of this enzyme and/or the presence of increased cortisol: cortisone ratios in FF have been associated with improved pregnancy outcomes in patients undergoing ART treatments (Michael and Papageorghiou, 2008). However, a lot remains unknown about the effects of glucocorticoids on mammalian sperm in the female reproductive tracts resulting from the absence of published studies.

2.1.3 Combined effects of follicular fluid steroids hormones

Studies have shown that higher levels of certain steroid hormones in FF are associated with increased fertilisation rates in patients undergoing ART treatments (Mendoza *et al.*, 1999; Lamb *et al.*, 2010). Many of these studies however tend to focus on oocyte quality without giving much consideration to the potential effects these hormones could simultaneously have on sperm function and fertilising ability.

Based on the knowledge of the steroid hormones present in the ovarian steroidogenic pathway, Blackmore *et al.* (1990) studied the effects of a wide range of steroid hormones on sperm $[Ca^{2+}]_i$ (Table 2). However, the stimulatory effects of these steroid hormones were studied individually and not as a combined steroid hormone complement.

For several years, the lack of published studies on the simultaneous effects of follicular fluid steroids on human sperm has been largely due to the absence of published data on the full steroid hormone complement of FF thus making it difficult to create experimental analogues to model the *in vivo* interaction between sperm and all the steroid hormone constituents of FF. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS), Kushnir *et al.* (2009) made the first attempt to establish a steroid hormone concentration profile for human follicular fluid. This was carried out using follicular fluid samples obtained from regular menstruating women and women undergoing ovarian stimulation for IVF (see Table 3). Progestogens, estrogens, androgens and corticosteroids were identified however this study did not provide any data on the identification and concentration of progesterone. Due to its evident significance in human sperm physiology, the absence of progesterone data from this

follicular fluid steroid hormone profile makes it less than ideal for use in investigating the effects of the follicular fluid steroid hormone milieu on sperm. To that effect, a recent attempt has been made by Professor W. Arlt's research group at the Institute of Metabolism and Systems Research, University of Birmingham to profile extensively the steroid hormone constituents of human follicular fluid (hFF) via LC-MS/MS. This was carried out using FF samples taken with informed consent from 16 patients undergoing ovarian stimulation for IVF. Unpublished data from this hormonal assay (shown in Table 4) was made available to Dr. Jackson Kirkman-Brown's group at the School of Clinical and Experimental Medicine, University of Birmingham (personal communication).

Table 2 Effects of various ovarian steroid hormones on human sperm $[Ca^{2+}]_i$ elevation. The results for the effect of each steroid were expressed as a percentage of the maximum progesterone effect on human sperm (Blackmore *et al.*, 1990).

Steroid Hormone	Steroid Hormone Concentration (nM)	Progesterone Effect (%)
Progesterone	31.8	100.0
17α-hydroxyprogesterone	30.3	57.3
17β-hydroxyprogesterone	30.3	44.0
5α-pregnane-3,20-dione	31.6	37.5
Androstenedione	34.9	15.6
Pregnenolone	31.4	14.6
Corticosterone	28.9	10.4
20α-hydroxypregnen-3-one	31.6	10.4
β-estradiol	36	10.4
Testosterone	34.7	10.4
Estrone	37.0	7.3
Dehydroepiandrosterone	34.7	7.3

Table 3 Steroid hormone concentration profile as measured by LC-MS/MS, of follicular fluid from regular menstruating women and women undergoing ovarian stimulation. Data presented as the median and central 90% of the distribution. Concentration ranges are displayed in parentheses (Kushnir *et al.*, 2009).

Steroid Hormone	Regularly menstruating women (μg/L) n=21	Women with ovarian stimulation for IVF (μg/L) n=5
Pregnenolone	52 (16-89)	110 (74-460)
17-hydroxypregnenolone	32 (4.4-6.0)	3.0 (1.5-6.9)
17α-hydroxyprogesterone	180 (65-310)	520 (380-1400)
11-deoxycortisol	4.1 (1.8-6.6)	1.5 (0.6-3.2)
Cortisol	17 (3.9-38)	53 (38-64)
Cortisone	32 (19-47)	12 (7.7-27)
Dehydroepiandrosterone	86 (34-190)	2.7 (1.7-4.3)
Androstenedione	420 (200-830)	6.8 (2.9-67)
Testosterone	18 (6.2-43)	0.3 (0.01-2.7)
Androstanedione	2.0 (0.6-6.2)	0.5 (0.3-2.5)
Estrone	34 (3.3-140)	24 (12-36)
Estradiol	31 (2.6-302)	235 (119-389)
Estriol	0.47 (0.1-2.3)	0.86 (0.8-2.0)

Table 4 Steroid hormone profile of human follicular fluid showing the concentrations of its constituent steroid hormones. Steroid hormone concentrations (nM) presented as mean values from 16 women undergoing ART treatments. Concentration data ranges are also displayed for each steroid hormone. Data from Prof. W. Arlt's research group; Frettsome (2012).

Steroid Hormone	Concentration (nM)	Concentration ranges
Progesterone	13500	800nM-28μM
17α-hydroxyprogesterone (17OHP)	5600	1.9μM-8.9μM
Estradiol (E2)	800	433nM-1.7μM
Pregnenolone	700	234nM-1.7μM
Androstenedione	370	45.5nM-2.0μM
Estrone (E1)	180	77nM-397nM
Cortisol	50	24.3nM-83.3nM
17-hydroxypregnenolone (17OHPreg)	15	675pM-28.8nM
Cortisone	13	4.5nM-28.7nM
Dehydroepiandrosterone (DHEA)	11	9.5nM-13.6nM
Testosterone	4	7.0pM-17.9nM
11-deoxycortisol	3	795pM-6.9pM
Dihydrotestosterone (DHT)	2	0-5nM
Corticosterone	1	305pM-2.0nM

2.1.4 Aim of the study

The aim of this study was to investigate the effects of the physiological concentration mix of steroid hormones present in hFF on human sperm calcium signalling dynamics and acrosome reaction. A synthetic follicular fluid (shFF) was prepared according to the Table 4 above (see section 2.2.2 for preparation methods). The research objectives of this study were to (i) characterise the effect(s) of shFF on the $[Ca^{2+}]_i$ response dynamics in human spermatozoa via live cell calcium imaging and fluorimetry (ii) compare the effects of physiological and standard laboratory temperatures on the above responses (iii) observe the effects of shFF on human sperm acrosome reaction.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Bovine Serum Albumin, (Millipore, USA). Supplemented Earle's Balanced Salt Solution, (REF-06-2010-03-1B; Biological Industries, Israel). Calcium Green-1, AM, (Life Technologies, UK). 5ml Falcon tube - REF 352054, (Corning, USA). 50x4x0.4mm glass capillary tube (CM Scientific, UK). Perfusible imaging chamber (Harvard Apparatus, UK). BEEM® capsule (Biopoint, UK). Poly-D-lysine, (BD, USA). Fluoromount, (BDH Chemicals, UK). Multichannel pipette, (Eppendorf, UK). 1.5ml microcentrifuge tube, (Eppendorf, UK). Cristaseal, (Hawksley, UK). IVOS 10.9 Computer Assisted Sperm Analysis (CASA) system, (Hamilton Thorne Research, USA). Pluronic F-17. 20% solution in DMSO, (Life Technologies, UK). Fura-2 acetoxymethyl ester (fura-2/AM), (Life Technologies, UK). Ionomycin, (Life Technologies, UK).

All of the other materials listed below were obtained from Sigma-Aldrich, UK. Phosphate Buffered Saline (PBS). Dimethyl Sulfoxide. Sodium Citrate. Fructose. FITC-conjugated *Pisum sativum* agglutinin (FITC-PSA). Progesterone. 17α-hydroxyprogesterone. 17β-Estradiol. Pregnenolone. Androstenedione. Estrone. Cortisol. 17-hydroxypregnenolone. Cortisone. Dehydroepiandrosterone. Testosterone. 11-deoxycortisol. Dihydrotestosterone. Corticosterone. Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA). Methylcellulose.

2.2.2 Preparation of a synthetic human follicular fluid (shFF)

All the steroid hormones identified in the hFF assay were mixed in concentrations and proportions identical to the average concentrations from the LC-MS/MS data (see Table 4). All of the 14 steroid hormones used in the preparation of shFF were of ≥98% purity. Sterile cell culture grade Dimethyl Sulfoxide (DMSO) was used as a solvent to prepare solutions of the individual steroid hormones, as well as the shFF stock solution (1000x). Briefly, 2mg/ml solutions of each steroid hormone were prepared except for progesterone and 17α-Hydroxyprogesterone. These solutions were then mixed together in proportions required to make up the desired volume of shFF stock solution. The masses of progesterone and 17α-Hydroxyprogesterone required to make up the desired volume of the shFF stock solution were weighed out and added to the steroid hormone mixture to complete the 1000x shFF stock solution. The final concentrations of shFF used in this study were in 1:1000 dilution of DMSO or lower. This is based on observations made from previous studies by Dr. Kirkman-Brown (personal communication) where DMSO dilutions higher than 1:1000 had a negative effect on sperm survival.

2.2.3 Preparation of semen samples

All the sperm donors used in this study were recruited at the Birmingham Women's Hospital (HFEA Centre 0209; Ethics Committee Reference Number 13/EM/0272). Ejaculated sperm cells were obtained from healthy donors of proven fertility by masturbation. Following liquefaction of the semen sample (approximately 30 minutes), motile sperm cells were harvested via the direct swim-up technique. Briefly, the semen sample was divided into aliquots of 300µl and each aliquot was layered beneath 1ml of supplemented Earle's Balanced Salt Solution (sEBSS) + 0.3% BSA in a 5ml Falcon tube. The tubes were then incubated at an angle of 45° for 1 hour at 37°C and 6% CO_2. After 1 hour of incubation, the upper 0.7 ml of sEBSS medium (containing the motile fraction of sperm cells) was carefully removed from each tube using a sterile pipette. All the motile sperm cell aspirates were then pooled together and the total concentration was assessed using a Neubauer haemocytometer in accordance with WHO semen analysis protocols (WHO, 2010) - The concentration of the motile sperm preparation was adjusted to 6 million sperm cells per ml to allow for optimal live cell imaging (Kirkman-Brown *et al.*, 2000). 200µl aliquots of prepared sperm cells were then capacitated via incubation for 4 hours at 37ºC and 6% CO_2 for use in live cell Ca^{2+} imaging experiments.

2.2.4 Investigation of human sperm $[Ca^{2+}]_i$

The effects of shFF on human sperm $[Ca^{2+}]_i$ were investigated using two complementary experimental techniques – live cell fluorescence Ca^{2+} imaging and population fluorimetry. The live cell Ca^{2+} imaging technique made it possible to study sperm $[Ca^{2+}]_i$ dynamics at the single cell level, as well as the responses in the different sub populations of responding cells in a heterogeneous sperm sample. The ratiometric fluophore used in the population fluorimetry studies made it possible to calibrate and quantify the actual sperm $[Ca^{2+}]_i$ levels for the duration of an experiment. Population fluorimetry was preferred for the dose dependence studies which were longer in duration, as it reduces the overall exposure of each cell to the excitation wavelengths when compared to live cell Ca^{2+} imaging.

Live cell Ca^{2+} imaging

200µl aliquots of capacitated sperm (6 million sperm cells/ml) were then labelled with 7µM Calcium Green-1, AM dye and incubated at 37°C, 6% CO_2 for 30 minutes. 100µl of the labelled sperm cells were introduced into a purpose built, perfusible imaging chamber with a sample capacity of 100µl (CAIRN Research Ltd, UK). A coverslip previously coated with 0.001% poly-D-lysine and air-dried, was attached to the lower surface of the chamber. The chamber was incubated for a further 30 minutes to allow for further labelling of the sperm cells and for adhesion of the sperm cells to the coverslip.

Following the incubation period, the chamber was perfused with 10ml of sEBSS medium via a gravity driven perfusion system (Figure 14) at a perfusion rate of 0.4ml/min (Kirkman-Brown *et al.*, 2000) to remove traces of debris, non-adherent cells and

extracellular Calcium Green dye. Fluorescence images of the labelled spermatozoa were visualised whilst perfusing, using a Nikon Eclipse TE 300 inverted microscope (Nikon, Japan) illuminated with a blue LED – 490nm (CAIRN Research Ltd, UK) coupled to a Quantem 512SC CCD camera (Figure 14). Emitted fluorescence was filtered at 530nm.

In all experiments, there was an initial control period of 2 minutes during which the sperm cells were perfused with sEBSS before exposing the cells to 100% shFF in sEBSS. The shFF perfusion lasted for 5 minutes after which the sperm cells were further exposed to a two-minute sEBSS perfusion to wash off the shFF from the extracellular environment. Fluorescence images were acquired at intervals of 5 seconds using Metamorph software version 7.8.2.0 (Molecular Devices LLC, USA) with pre-set time points and perfusion controls.

Figure 14 Live cell fluorescence imaging setup with automated perfusion controls.

The imaging and perfusion environment was maintained at either 37°C (physiological temperature) or 26°C (standard laboratory temperature as employed in previously published live cell Ca^{2+} imaging studies e.g. Kirkman-Brown *et al.*, 2000; 2003) depending on the experiment. The fluorescence intensities of the Calcium Green -1 AM dye in each of the labelled sperm cells were recorded at 470nm throughout all time points in the imaging experiments undertaken. All captured fluorescence images were processed and analysed using Image-Pro Analyser 7.0 software package (Media Cybernetics, UK). Statistical analyses were carried out using Microsoft Excel 2010 software package (Microsoft, USA). Comparison of data was via two-tailed paired t-tests. A p-value of <0.05 was taken as significant.

Fluorimetric studies

Population fluorimetric studies were carried out using 2ml aliquots of capacitated spermatozoa (6 million sperm cells/ml) labelled with 5μM fura-2 acetoxymethyl ester (fura-2/AM) dye for 30 minutes at 37°C and 6% CO_2, centrifuged at 300g for 5 minutes, resuspended in 2ml of sEBSS and incubated for a further 20 minutes. A Perkin-Elmer LS-50B fluorescence spectrofluorimeter running FL WinLab™ version 4.0 was used for experiments (Figure 15). Excitation wavelengths were alternated between 340 and 380nm via a fast-filter setup, and emission was held at 510nm whilst the temperature of the cuvette was maintained at 37°C. Slit width was set at 15nm with a sampling rate of 12.5 Hz. Dose dependence of the shFF or progesterone-induced $[Ca^{2+}]_i$ response was assessed by measuring the amplitude of the initial $[Ca^{2+}]_i$ transient after stimulation. Sequential concentrations of shFF or Progesterone were added to the cuvette once a level baseline in resting $[Ca^{2+}]_i$ had been maintained for at least 200s. Calibration of

$[Ca^{2+}]_i$ was determined using 50µM ionomycin to generate a maximal response and subsequent addition of 50mM EGTA as a minimal response. Observations from trial experiments established that addition of further aliquots of EGTA caused no further reduction in fluorescence ratio. A Kd value of 224nM was assumed, as in other relevant studies (Luconi *et al.*, 1998; Blackmore and Eisoldt, 1999; Harper *et al.*, 2003), and calibration was performed according to the equation of Grynkiewicz *et al.* (1985).

Figure 15 A Perkin Elmer LS-50B spectrofluorimeter running FL WinLab™ version 4.0, connected to a temperature-controlled water bath.

2.2.5 Acrosome Reaction assay

After capacitation, as described in section 2.2.3, 100µl aliquots of spermatozoa were treated with either shFF (0.01%, 0.1%, 1%, 10% and 100% shFF) or progesterone (13.5 µM); ionomycin (10µM) or solvent control (0.1% DMSO). All working concentrations of the treatments used made use of DMSO as the carrier solvent at a final dilution of 0.1% or lower. After an incubation of 1 hour at 37C and 6% CO_2, spermatozoa were centrifuged briefly at 300g for 5 minutes. This was immediately followed by the hypo-osmotic swelling (HOS) test for the selection of viable spermatozoa. Briefly, the supernatant was removed, and the spermatozoa were resuspended in 0.5 ml of HOS medium (0.74% sodium citrate, 1.35% fructose in double-distilled H_2O). After a 45 minute incubation in HOS medium, the spermatozoa were centrifuged (300g for 5 minutes) and resuspended in a minimal volume of HOS medium (≤50 µl), smeared on microscope slides (duplicate slides, previously coated with 0.001% poly-D-lysine solution), and air-dried.

Following cell permeabilisation by immersion of smeared slides in 100% methanol for 1 minute, the sperm acrosome was labelled by incubation with 50µg/ml FITC-PSA in PBS for 1 hour in a moist chamber at 37°C. Slides were then washed in PBS for 15 minutes before air-drying and mounting with Fluoromount. Slides were stored in the dark at room temperature. Fluorescence microscopy was used to assess acrosomal status (Cross *et al.*, 1988). Slides were subjected to blind scoring and only viable (curly tailed) sperm cells were scored (Aitken *et al.*, 1993). The assessment of acrosomal status was carried out as described by Mendoza *et al.* (1992). A total of 200 sperm cells were scored per treatment (100 per slide). The Percentage stimulation of acrosome reaction was calculated using the following formula: % stimulation of AR=(([T]AR-

[DMSO]AR)/([Ionomycin]AR-[DMSO]AR)) X 100; where [T]AR represents the % of acrosome reacted spermatozoa in the treatment of interest, [DMSO]AR represents % of acrosome reacted spermatozoa in DMSO treatment, and [Ionomycin]AR represents % of acrosome reacted spermatozoa in ionomycin treatment. This derivation is based on the use of DMSO and ionomycin as the respective negative and positive experimental controls in this study. All calculations and statistical analyses were performed using Microsoft Excel 2010 software package. An arcsine transformation of the data was carried out prior to testing for statistical significance. Paired *t* tests (two tailed) were carried out to test for significance. Statistical significance was set at $P<0.05$.

2.3 RESULTS

2.3.1 Live cell imaging data - Effects of shFF on sperm $[Ca^{2+}]_i$

Initial (transient) response to shFF

Following exposure to 100% shFF at 37˚C, the majority of spermatozoa showed a rapid, significant increase in $[Ca^{2+}]_i$ in the head and the midpiece. This rise begins within seconds of shFF application, peaks within 20-30 seconds, and then decreases with a similar time course (Figure 16). This was observed in all experiments. The amplitude of the transient $[Ca^{2+}]_i$ response was typically a 10-20% increase in fluorescence intensity over baseline levels. However, a "tail" of larger responses up to 50% was present and in a very small number of cells the transient was greater in amplitude (Figure 17). In the 9 experiments chosen for detailed analysis, the proportion of cells showing this response was 56.1±3.5% (n=9; Table 5).

At 26˚C, a similar response kinetic was also observed. The transient $[Ca^{2+}]_i$ response began within seconds of shFF application, peaked within 30-40 seconds, and then decreased over a longer time course (70-90 seconds) (Figure 18). This was observed in all experiments and the amplitude of the transient $[Ca^{2+}]_i$ response was typically a 10-25% increase in fluorescence intensity over baseline levels. A "tail" of larger responses up to 80% was also present (Figure 19). In the 7 experiments chosen for detailed analysis, the proportion of cells showing this response was 77.1±3.3% (n=7; Table 6).

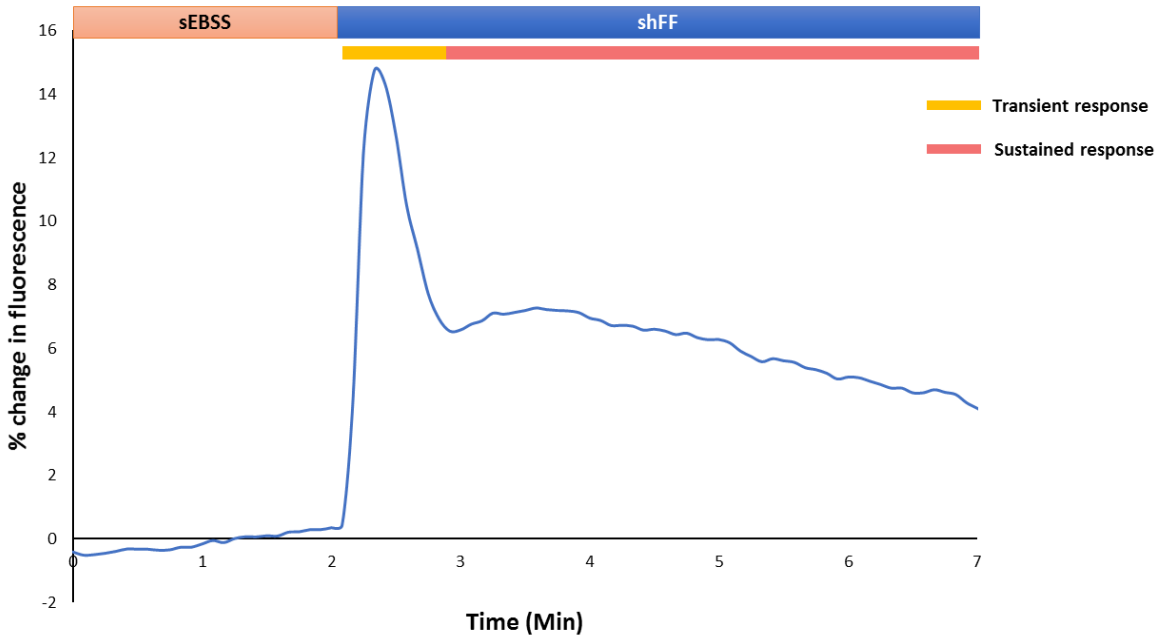


Figure 16 Mean $[Ca^{2+}]_i$ response (R_{tot}) at 37°C in 557 responding sperm cells from 9 experiments continuously perfused with 100% shFF for 5 minutes. shFF was introduced after the initial two minute control period. The response of spermatozoa to shFF is characterised by an initial transient response (T) peak, immediately followed by a second sustained response (S). Transient responses are typically a 10-20% increase in fluorescence whilst sustained responses are characterised by a rise in fluorescence intensity of about 5-15% after 5 minutes.

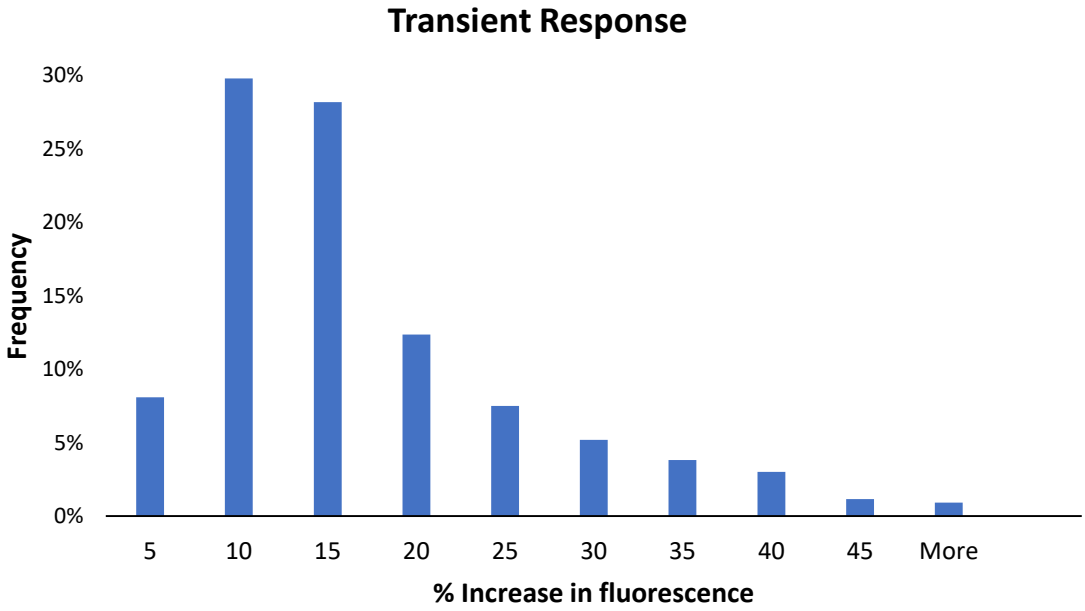


Figure 17 Summary amplitude distribution for transient responses (37°C) at time of peak for R_{tot} from 9 experiments. Data obtained from sub-population of cells significantly showing a transient response (T only; T&S).

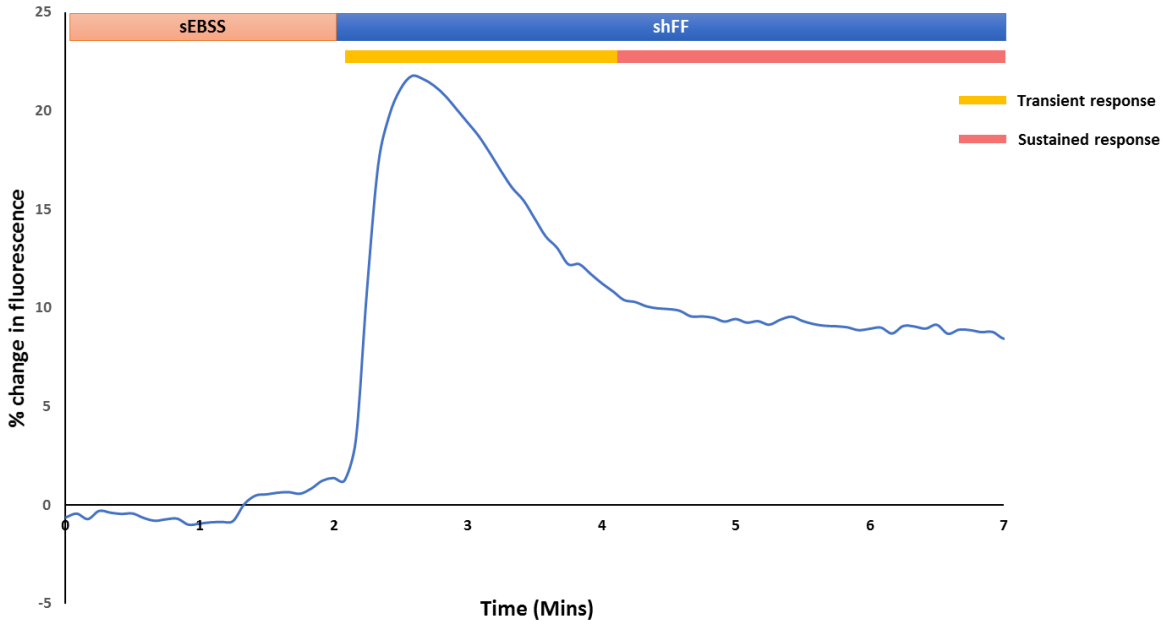


Figure 18 Mean $[Ca^{2+}]_i$ response (R_{tot}) at 26°C in 1099 responding sperm cells from 7 experiments continuously perfused with 100% shFF for 5 minutes. shFF was introduced after the initial two minute control period. The response of spermatozoa to shFF is characterised by an initial transient response (T) peak, immediately followed by a second sustained response (S). Transient responses are typically a 10-25% increase in fluorescence whilst sustained responses are characterised by a rise in fluorescence intensity of about 10-20% after 5 minutes.

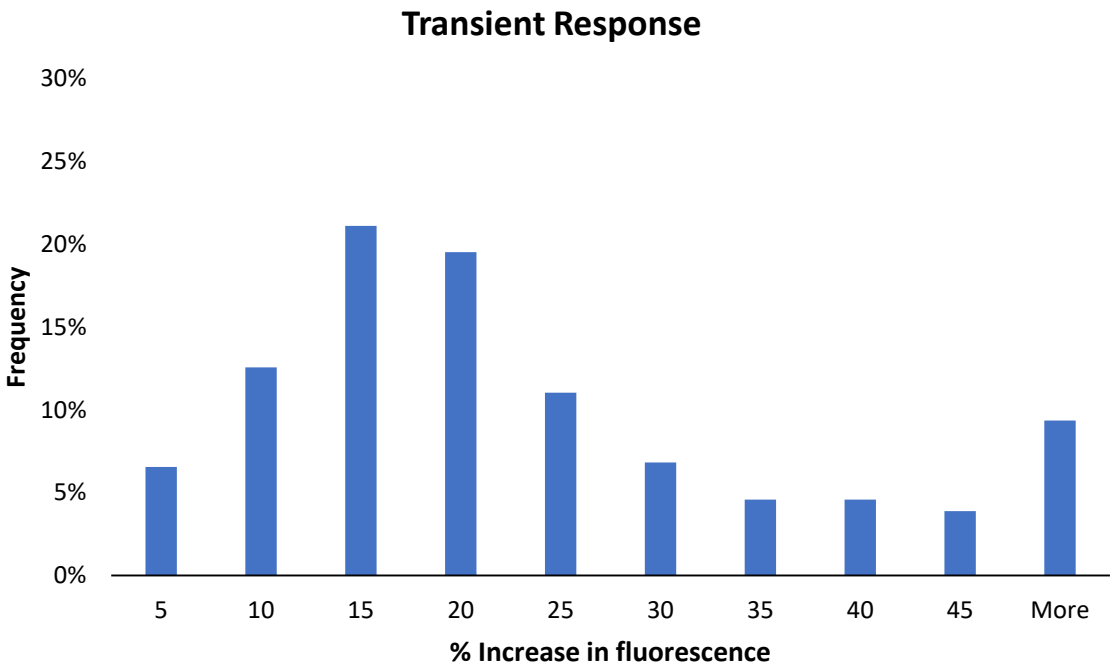


Figure 19 Summary amplitude distribution for transient responses (26°C) at time of peak for R_{tot} from 7 experiments. Data obtained from sub-population of cells significantly showing a transient response (T only; T&S).

Second (sustained) response to shFF

At 37°C, a proportion of the spermatozoa (mean 40.2±3.9%; n=9) showed a significant sustained rise in $[Ca^{2+}]_i$ (Figures 16 and 22; Table 5). This sustained response began immediately following the transient response decay, and lasted until shFF was removed from the perfusion medium and replaced with sEBSS. The amplitude of the sustained $[Ca^{2+}]_i$ response was typically a 5-15% increase in fluorescence intensity over levels before application of shFF (Figure 20).

At 26°C, a sub-population of the spermatozoa (mean 47.9±4.1%; n=7) also showed a significant sustained rise in $[Ca^{2+}]_i$ (Figures 18 and 23; Table 6). The sustained response was characterised by a much later onset resulting from the prolonged nature of the transient $[Ca^{2+}]_i$ response peak and subsequent decay (Figures 18 and 24). This response was maintained until shFF was removed from the perfusion medium and replaced with sEBSS. The amplitude of the sustained $[Ca^{2+}]_i$ response was typically a 10-20% increase in fluorescence intensity over levels before application of shFF (Figure 21).

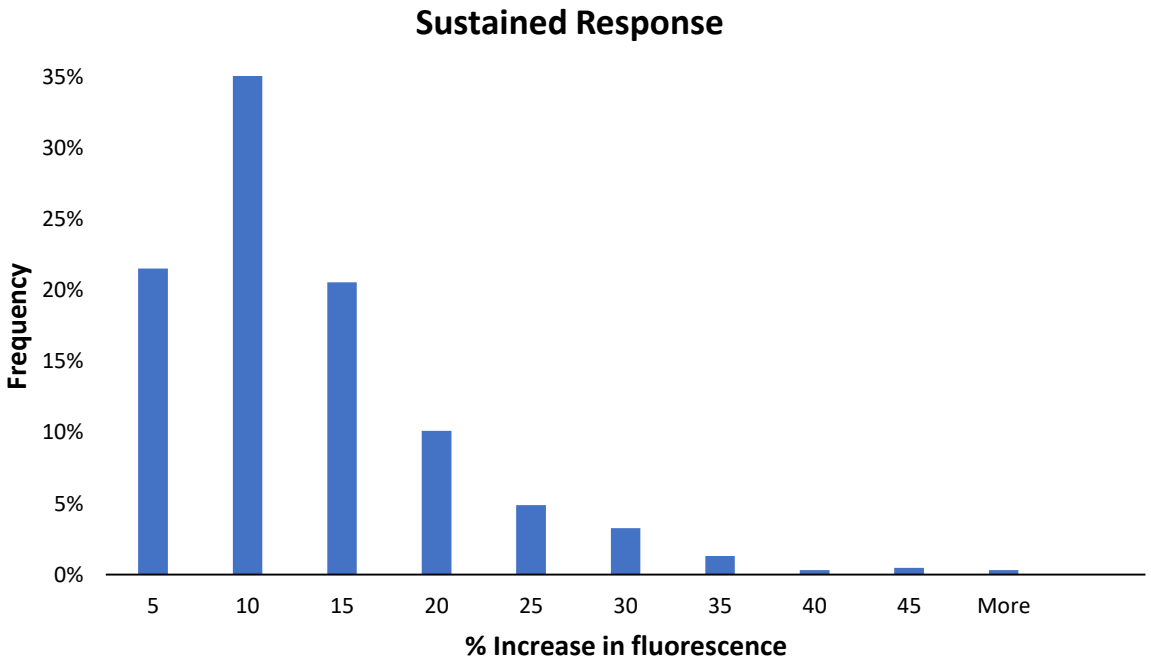


Figure 20 Summary amplitude distribution for sustained responses (37°C) from 9 experiments-normalised amplitude after 3-7 minutes. Data obtained from sub-population of cells significantly showing a sustained response (T&S; S only).

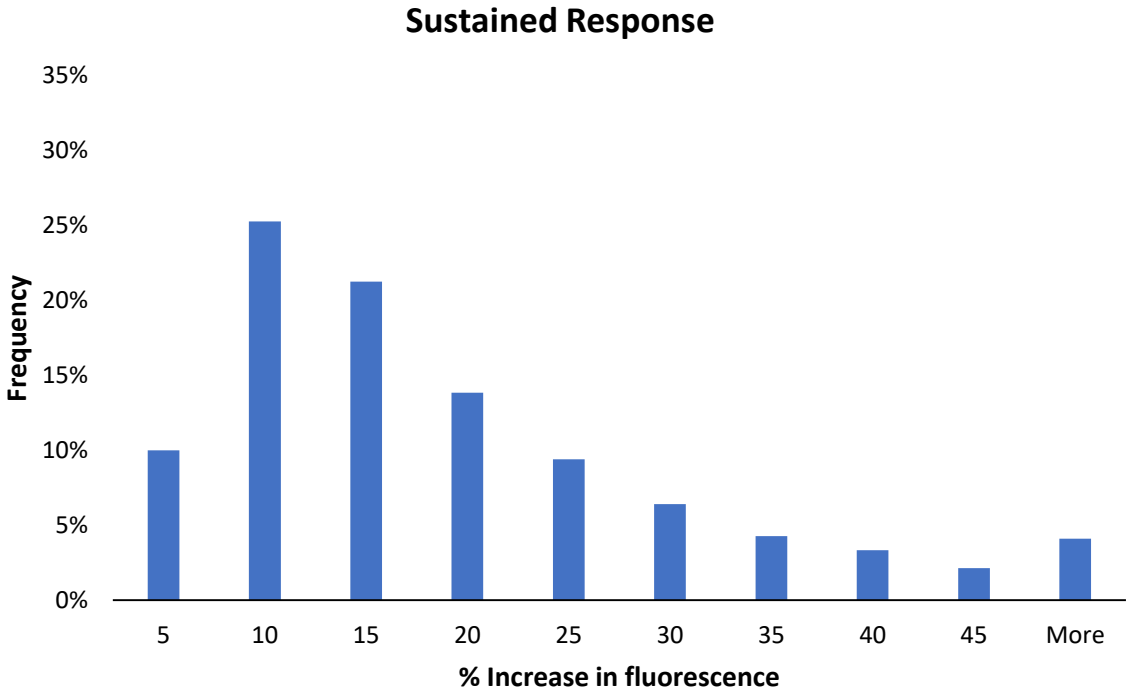


Figure 21 Summary amplitude distribution for sustained responses (26°C) from 7 experiments-normalised amplitude after 3-7 minutes. Data obtained from sub-population of cells significantly showing a sustained response (T&S; S only).

Table 5 Summary of data obtained at 37°C in the nine experiments used for detailed analysis. In these experiments, the sperm cells were continuously perfused with shFF for 5 minutes before being washed off with sEBSS.

No of Cells (% of total)						No of Cells (% of total)	
Experiment	Transient Response only (T)	T and S	Sustained Response only (S)	No response	Total	Total T	Total S
1	62 (27)	80 (35)	8 (4)	78 (34)	228	142 (62)	88 (39)
2	24 (11)	130 (60)	8 (4)	54 (25)	216	154 (71)	138 (64)
3	6 (12)	17 (35)	0 (0)	26 (53)	49	23 (47)	17 (35)
4	28 (20)	60 (44)	4 (3)	45 (33)	137	88 (64)	64 (47)
5	12 (16)	19 (26)	3 (4)	40 (54)	74	31 (42)	22 (30)
6	27 (16)	74 (45)	11 (7)	54 (33)	166	101 (61)	85 (51)
7	27 (24)	19 (17)	11 (10)	57 (50)	114	46 (40)	30 (26)
8	76 (25)	99 (33)	7 (2)	122 (40)	304	175 (58)	106 (35)
9	47 (27)	59 (33)	4 (2)	67 (38)	177	106 (60)	63 (36)
Total Cells	309	557	56	543	1465	866	613
Mean %	19.9±2.0	36.3±4.1	3.9±0.9	40.0±3.4		56.1±3.5	40.2±3.9

Table 6 Summary of data obtained at 26°C in the seven experiments used for detailed analysis. In these experiments, the sperm cells were continuously perfused with shFF for 5 minutes before being washed off with sEBSS.

No of Cells (% of total)						No of Cells (% of total)	
Experiment	Transient Response only (T)	T and S	Sustained Response only (S)	No response	Total	Total T	Total S
1	53(13)	227(55)	41(10)	90(22)	411	280(68)	268(65)
2	83(30)	154(55)	6(2)	38(14)	281	237(84)	160(57)
3	145(36)	189(46)	6(2)	68(17)	408	334(82)	195(48)
4	124(49)	80(32)	1(0)	48(19)	253	204(81)	81(32)
5	64(30)	111(52)	3(1)	36(17)	214	175(82)	114 (53)
6	162(36)	217(49)	1(0)	67(15)	447	379(85)	218(49)
7	112(31)	97(27)	16(5)	133(37)	358	209(58)	113 (32)
Total Cells	743	1075	74	480	2372	1818	1149
Mean %	32.1±3.6	45.1±3.8	2.9±1.1	20.0±2.7		77.1±3.3	47.9±4.1

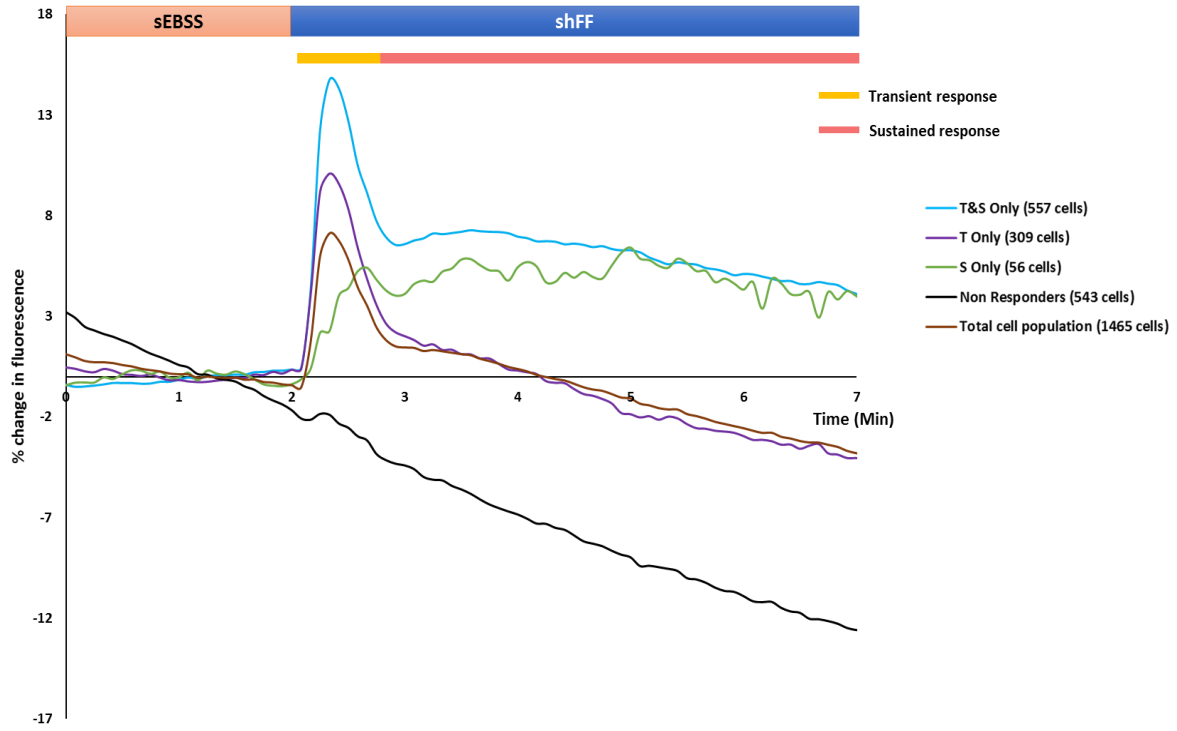


Figure 22 Mean $[Ca^{2+}]_i$ response (R_{tot}) at 37°C in all response sub categories.

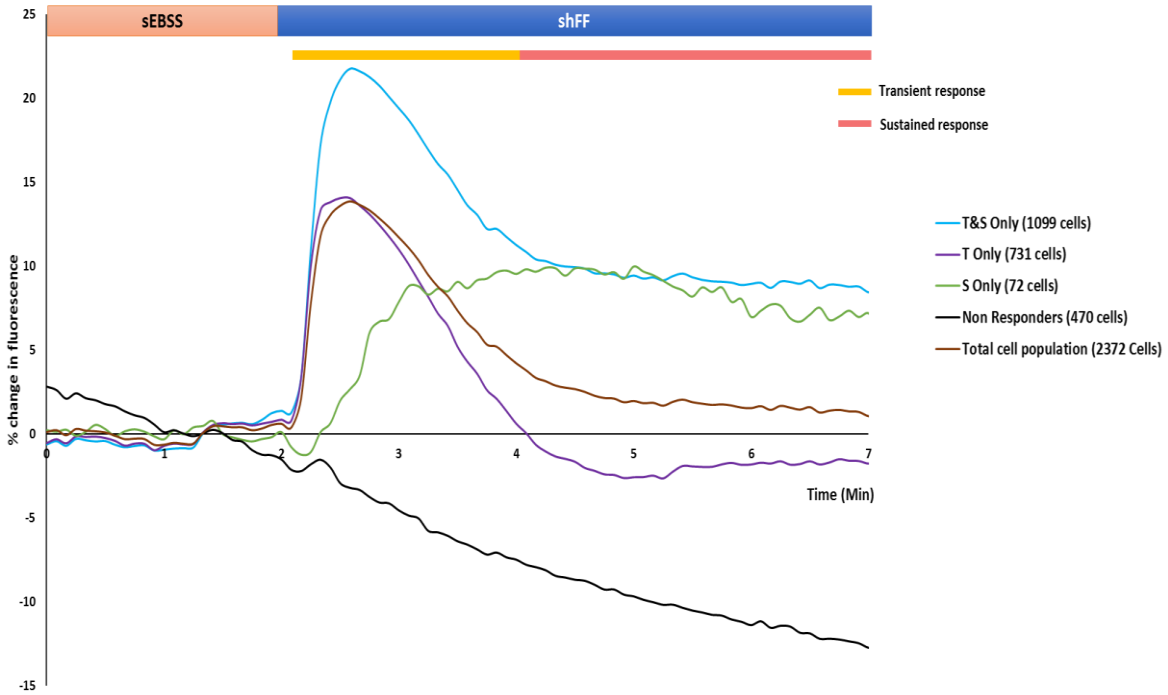


Figure 23 Mean $[Ca^{2+}]_i$ response (Rtot) at 26°C in all response sub categories..

Relationship between transient and sustained responses

At 37°C, the proportion of shFF responsive cells varied between experiments (Table 5), however there was a correlation between the proportion of cells giving a significant transient response and the proportion in which a sustained $[Ca^{2+}]_i$ response was observed (R=0.84). Detailed analysis of the occurrence of transient and sustained responses in a total of 1465 cells from 9 experiments showed that the two responses were significantly linked ($P<10^{-90}$; χ^2), with the occurrence of the sustained response being greatly biased towards those cells in which a transient response was observed (Figure 16; Table 5). In all of the 9 experiments used for analysis, a greater transient response was observed in the sub-population of cells that showed both transient and sustained responses (Table 7), with a small proportion of cells (mean 3.9±0.9%) showing a sustained increase in fluorescence which was not preceded by a significant transient response.

At 26°C, the proportion of shFF responsive cells also varied between experiments (Table 6) and a correlation was observed between the proportion of cells giving a significant transient response and the proportion in which a sustained $[Ca^{2+}]_i$ response was observed (R=0.25). Similarly, detailed analysis of the occurrence of transient and sustained responses in a total of 2372 cells from 7 experiments showed that the two responses were significantly linked ($P<10^{-90}$; χ^2), with the occurrence of the sustained response also being greatly biased towards a transient response (Figure 18; Table 6). In all of the 7 experiments used for analysis, a greater transient response was observed in the sub-population of cells that showed both transient and sustained responses (Table 8), with a small proportion of cells (2.9±1.1%) showing a sustained increase in fluorescence which was not preceded by a significant transient response.

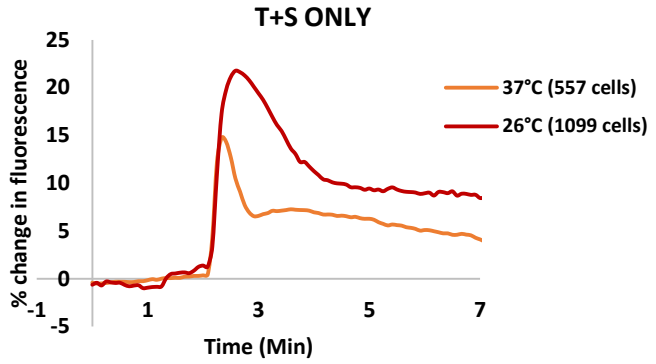
Table 7 Relationship between occurrence of the sustained response and amplitude of the transient response (37°C). In each of the nine experiments used for detailed analysis, the mean amplitude (±SEM) of the transient response (as percentage increase over baseline fluorescence) was determined for those cells that showed a transient response only (T only; column 2) and for those that gave a transient response which was followed by a sustained response (T+S; column 3). Numbers in parentheses show the number of cells in each group. P shows the level of significance for the difference between mean values for each experiment (t-test).

Experiment	T Only	T+S	P
1	6.1±0.32 (62)	7.2±0.3 (80)	0.014
2	10.0±0.8 (24)	13.5±0.4 (130)	0.0006
3	20.6±2.6 (6)	21.6±1.0 (17)	0.6624
4	21.5±2.5 (28)	28.5±1.4 (60)	0.0102
5	17.2±1.5 (12)	22.8±1.7 (19)	0.0301
6	22.6±2.5 (27)	28.3±1.0 (74)	0.0123
7	9.6±0.4 (27)	12.8±0.5 (19)	<0.0001
8	8.3±0.5 (76)	10.8±0.3 (99)	<0.0001
9	10.8±0.9 (47)	13.8±0.7 (59)	0.0087

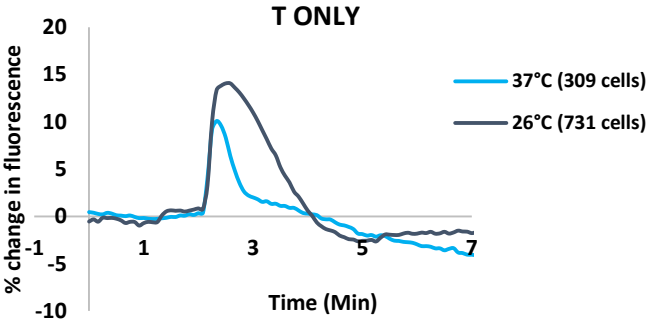
Table 8 Relationship between occurrence of the sustained response and amplitude of the transient response (26°C). In each of the seven experiments used for detailed analysis, the mean amplitude (±SEM) of the transient response (as percentage increase over baseline fluorescence) was determined for those cells that showed a transient response only (T only; column 2) and for those that gave a transient response which was followed by a sustained response (T+S; column 3). Numbers in parentheses show the number of cells in each group. P shows the level of significance for the difference between mean values for each experiment (t-test).

Experiment	T Only	T+S	P
1	21.8±1.5 (53)	28.0±0.9 (227)	0.0021
2	10.0±0.6 (83)	13.4±0.4 (154)	<0.0001
3	14.6±0.5 (145)	19.3±0.5 (189)	<0.0001
4	13.6±0.6 (124)	16.2±1.0 (80)	0.0187
5	11.1±0.8 (64)	17.1±0.6 (111)	<0.0001
6	30.4±1.4 (162)	46.8±1.3 (217)	<0.0001
7	12.3±0.6 (112)	17.9±0.7 (97)	<0.0001

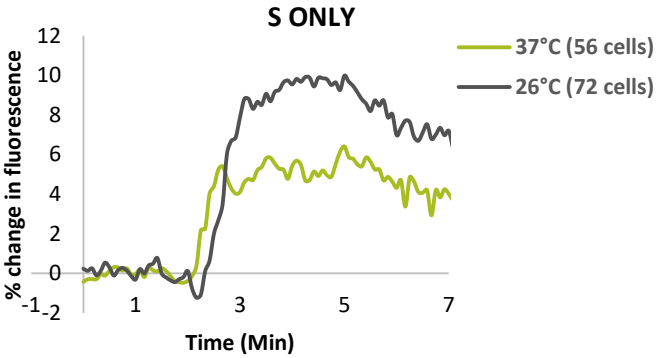
A



B



C



D

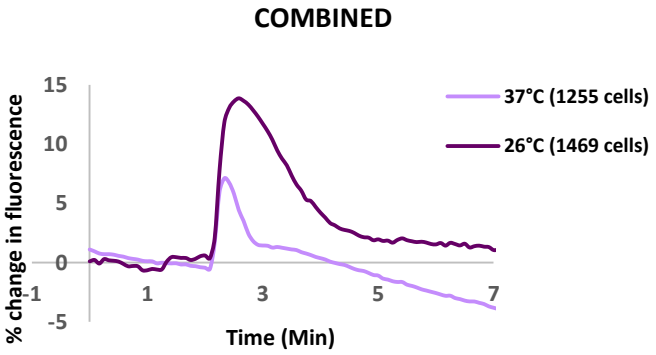


Figure 24 Comparison of Mean $[Ca^{2+}]_i$ responses at 26°C and 37°C. (a) T+S only (b) T only (c) S only (d) Combined cell population of T+S only, T only, S only and non-responders.

2.3.2 Fluorimetric studies - Effects of shFF on sperm $[Ca^{2+}]_i$

The mean calculated pre-treatment (resting) sperm $[Ca^{2+}]_i$ was 132±4nM (n=6 experiments). Treatment of spermatozoa with 100% shFF induced a biphasic elevation of $[Ca^{2+}]_i$ as observed in the live cell imaging data (Figure 25). The transient phase had a peak amplitude of 455±11nM and a time to peak of 13±1s. The transient then decayed for a period of 50-60s to a point of inflexion 47±1nM above control resting $[Ca^{2+}]_i$ after which there was a secondary sustained phase that took the form of a plateau (Figure 25). The amplitude of the sustained $[Ca^{2+}]_i$ after stimulation with 100% shFF was 136±1nM above resting $[Ca^{2+}]_i$ (Figure 25).

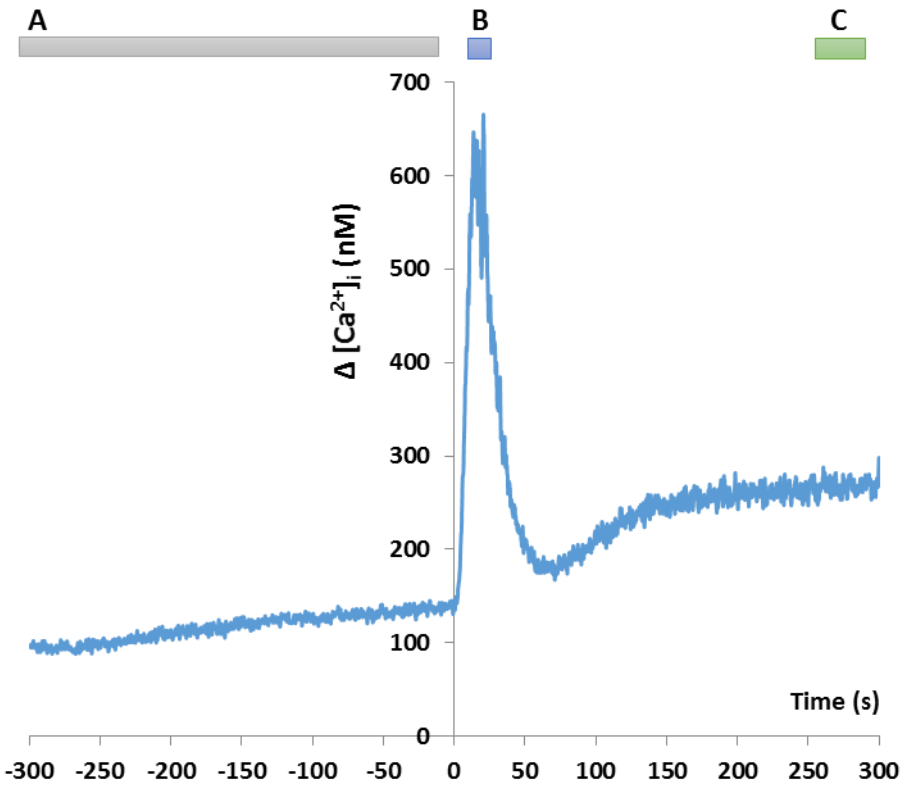


Figure 25 Fluorimetric (population) $[Ca^{2+}]_i$ response to 100% shFF (added at time 0). Moving average data from 6 experiments. Line A shows the period used for measurement of the initial resting $[Ca^{2+}]_i$; B shows the period used for measurement of the $[Ca^{2+}]_i$ transient; C shows the period used for measurement of the amplitude of the sustained response.

Some experiments were carried out (n=4) in which sperm cells were stimulated with a progesterone treatment corresponding to the concentration present in shFF (13.5μM). A similar biphasic response was observed with a peak amplitude of 477±12nM. The transient decay followed a similar time course, with the amplitude of the sustained response after stimulation with 13.5μM progesterone 119±3nM above control resting $[Ca^{2+}]_i$ (Figure 26).

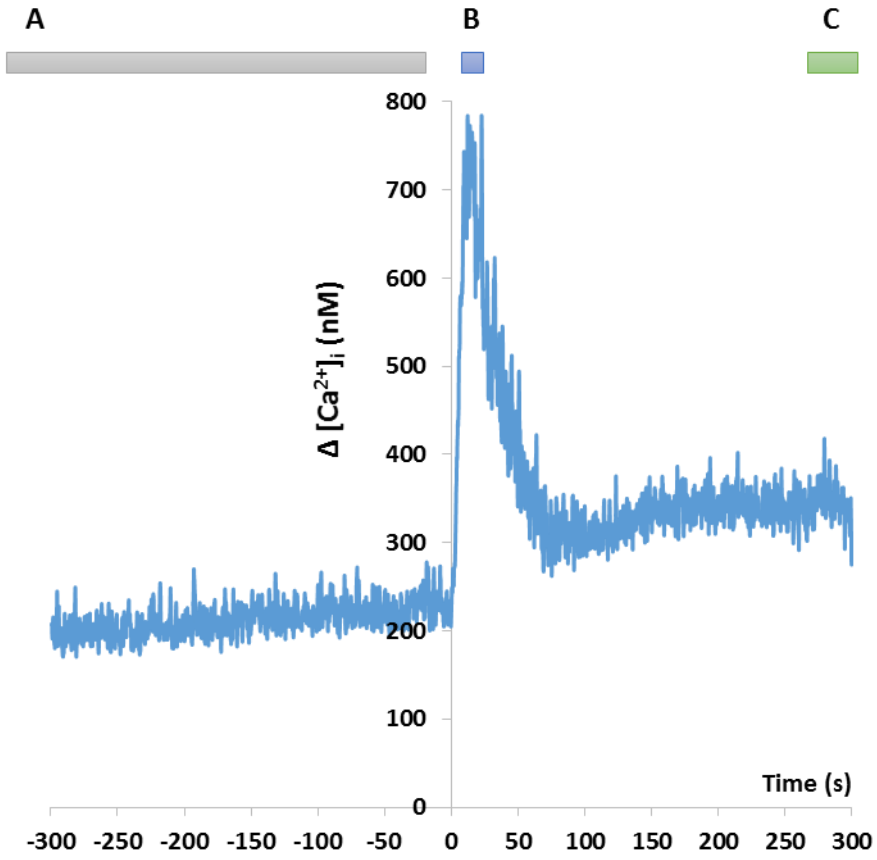


Figure 26 Fluorimetric (population) $[Ca^{2+}]_i$ response to 13.5μM progesterone (added at time 0). Moving average data from 4 experiments. Line A shows the period used for measurement of the initial resting $[Ca^{2+}]_i$; B shows the period used for measurement of the $[Ca^{2+}]_i$ transient; C shows the period used for measurement of the amplitude of the sustained response.

Dependence of the transient $[Ca^{2+}]_i$ response amplitude on shFF concentration was assessed by measuring the response to shFF doses over the range 0.001%-111.111% (Figure 27; 28). The first visible response peak was observed following the addition of 1% shFF in the majority of experiments carried out. Interestingly in some repeats, this was observed following the addition of 0.1% shFF (Figure 28). A dose-dependent response was observed characterised by a continuous increase in $[Ca^{2+}]_i$ in response to increasing concentrations of shFF. The maximum $[Ca^{2+}]_i$ was seen at a shFF concentration of 11.111% (following the addition of 10% shFF) across all experiments (Figure 27). This was then followed by a much lower $[Ca^{2+}]_i$ response following the addition of 100% shFF.

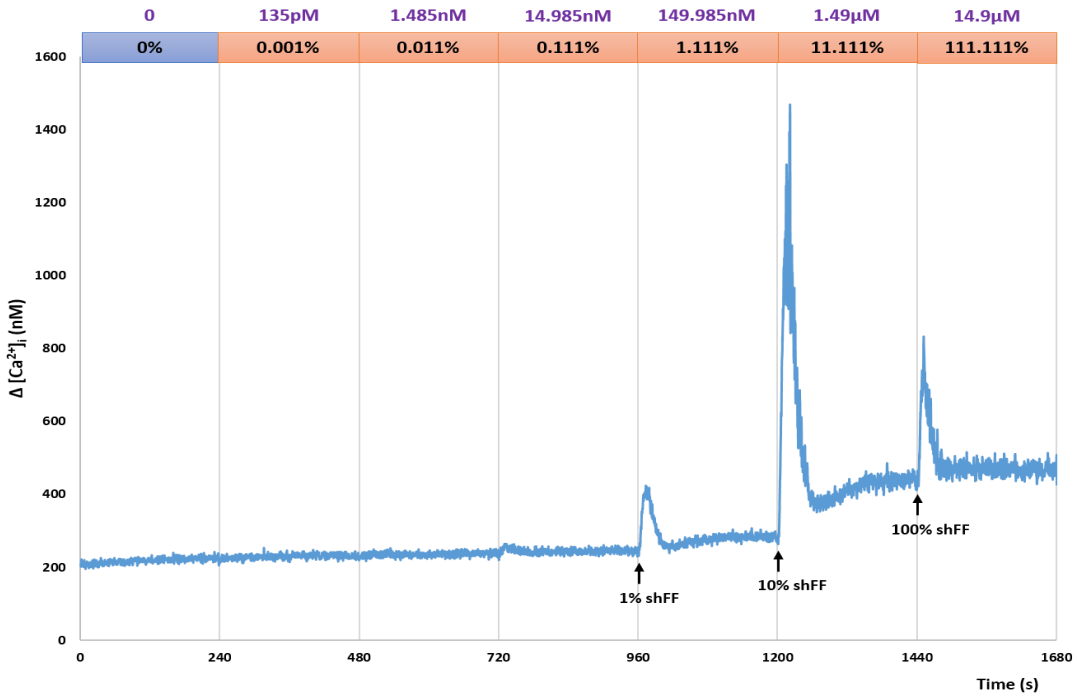
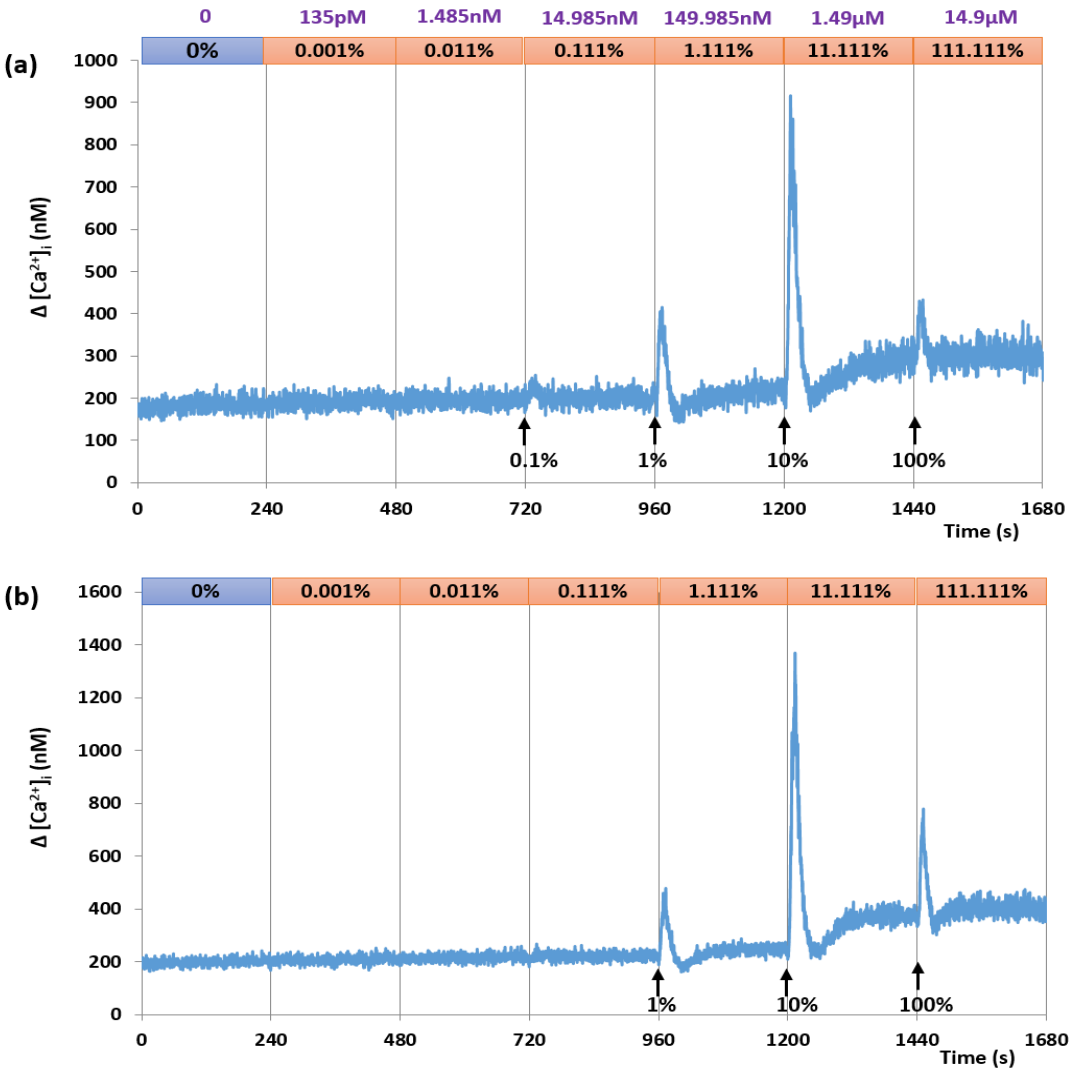


Figure 27 Fluorimetric (population) $[Ca^{2+}]_i$ response to sequential doses of shFF (0.001%, 0.01%, 0.1%, 1%, 10% and 100% shFF). Average data from 5 experiments. 240s between each addition. Rectangular bars indicate the concentration of shFF present in the cuvette. Purple text indicates the corresponding progesterone concentrations. Arrows indicate $[Ca^{2+}]_i$ responses to shFF dose treatments. Note that 100% shFF contains 13.5μM progesterone. Data is expressed as moving average due to the vast nature of the fluorimetric data readings (12 readings per second).



Progesterone	135pM	1.35nM	13.5nM	135nM	1.35µM	13.5µM
shFF concentration	**0.001%**	**0.01%**	**0.1%**	**1%**	**10%**	**100%**
Average Peak Amplitude - Progesterone (nM)	11±2	96±3	170±2	274±6	161±4	95±4
Average Peak Amplitude - shFF (nM)	0.1±2	0.4±2	20±2	124±4	682±19	231±9

Figure 28 Fluorimetric (population) $[Ca^{2+}]_i$ response to sequential doses of shFF (0.001%, 0.01%, 0.1%, 1%, 10% and 100% shFF). (a) Experimental trace from 1 experiment showing 4 response peaks in which a minimum $[Ca^{2+}]_i$ is observed in response to a 0.1% shFF treatment (b) Experimental trace from 1 experiment showing 3 response peaks with a minimum $[Ca^{2+}]_i$ only observed at 1% shFF. Purple text indicates the corresponding progesterone concentrations. Rectangular bars indicate the concentration of shFF present in the cuvette. Black arrows indicate $[Ca^{2+}]_i$ responses to shFF dose treatments. Data is expressed as moving average due to the vast nature of the fluorimetric data readings (12 readings per second). Descriptive table shows average peak amplitude of transient $[Ca^{2+}]_i$ responses to sequential doses of shFF and progesterone (n=5 experiments each).

The dose dependence study was furthered by measuring responses to progesterone concentrations that are present in the previously used shFF doses, over the range 135pM – 13.5μM (Figure 29, n=5). A minimum response was observed following the addition of 1.35nM progesterone across all experiments. A dose-dependent response was observed characterised by a continuous increase in $[Ca^{2+}]_i$ in response to increasing concentrations of progesterone. Maximum $[Ca^{2+}]_i$ was seen at a progesterone concentration of 149.985nM, following the addition of 135nM progesterone (Figure 29). This was then followed by a continuous decrease in $[Ca^{2+}]_i$ peak amplitude following the addition of 1.35μM and 13.5μM respectively.

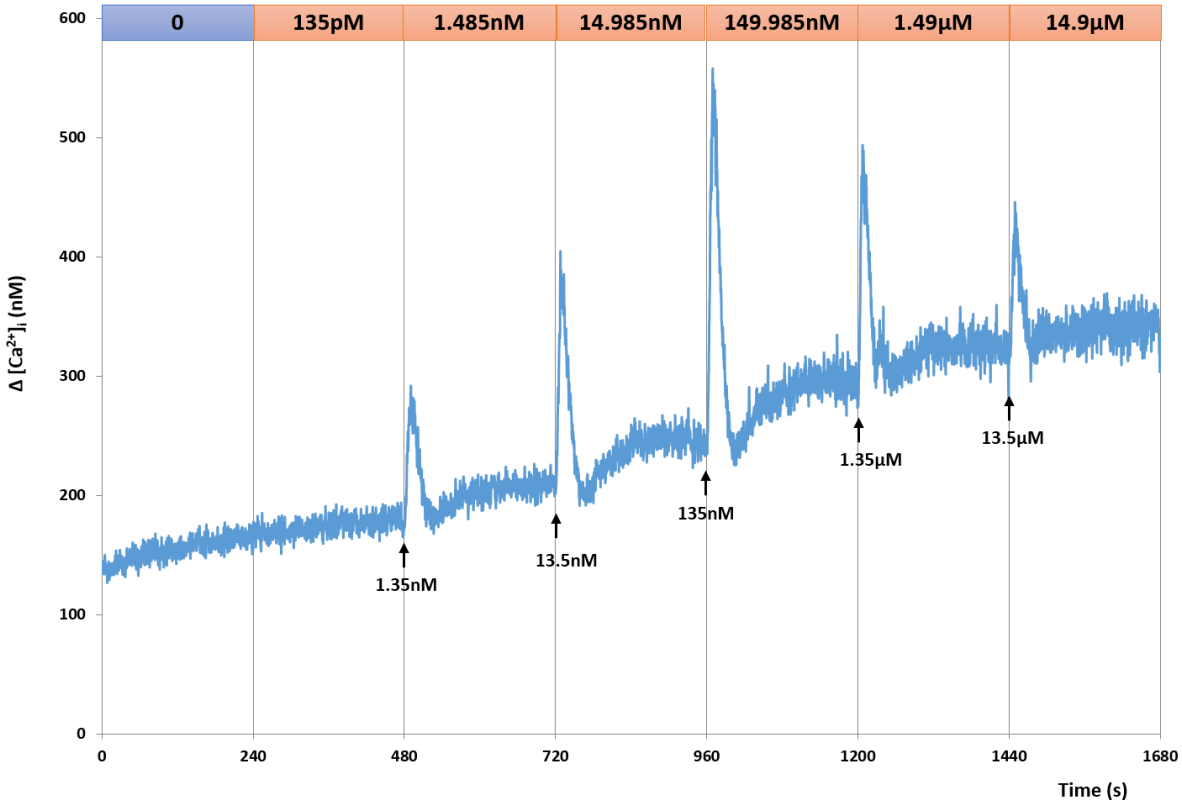


Figure 29 Fluorimetric (population) $[Ca^{2+}]_i$ response to sequential doses of progesterone corresponding to the respective shFF doses (135pM, 1.35Nm, 13.5nM, 135nM, 1.35μM, 13.5μM). Data average from 5 experiments. 240s between each addition. Rectangular bars indicate the concentration of progesterone present in the cuvette. Arrows indicate $[Ca^{2+}]_i$ responses to progesterone dose treatments. Data is expressed as moving average due to the vast nature of the fluorimetric data readings (12 readings per second).

2.3.3 Effects of shFF on sperm acrosome reaction

All sperm cells were labelled with FITC-PSA following incubation with either DMSO (solvent control) or Ionomycin or progesterone or shFF (see Table 9; Figure 30). Data obtained from the AR analysis of control experiments (sperm cells incubated with DMSO only) showed that AR occurred spontaneously in 8.1±0.01% of spermatozoa (mean ± SEM; n=6). Treatment of the cells with 13.5μM progesterone increased the percentage of acrosome-reacted spermatozoa to 20.3±0.01% (Figure 30; n=6; $P<0.05$). Sperm cells responded to Ionomycin treatment (positive control) with a further increase in the percentage of acrosome-reacted spermatozoa (58.4±0.03%; $P<0.05$). Samples treated with less than 1% shFF did not show a significant effect on the percentage of acrosome-reacted spermatozoa when compared with the solvent control. Higher shFF concentrations (1%, 10% and 100% shFF) evoked a significant increase in the percentage of acrosome-reacted spermatozoa when compared with the solvent control (10.5%±0.01%, 13.7±0.01% and 16.7±0.01% respectively; $P<0.05$, $P<0.01$, $P<0.001$; see Figure 30) however, these responses were all significantly lower than those from 13.5μM progesterone-treated spermatozoa ($P<0.01$). Furthermore, a statistical comparison between the populations of acrosome-reacted spermatozoa from 10% and 100% shFF treatments did not yield any significance. Progesterone-treated samples showed a 24.4±1.5% (n=6) stimulation of AR which was higher than that of the samples treated with 100% shFF (17.0±0.9%; see Table 9).

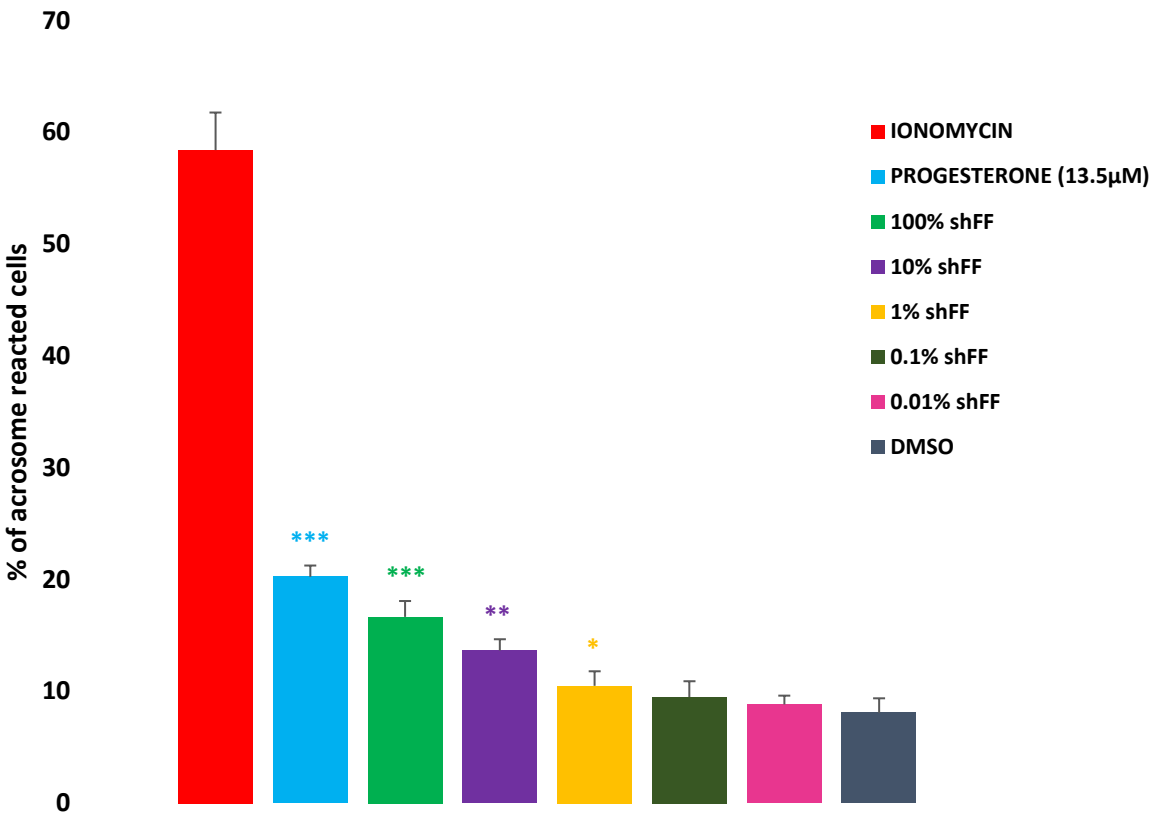


Figure 30 Acrosome reaction analysis data from 6 experiments showing the percentage of acrosome-reacted spermatozoa in different treatment populations (mean ± SEM). Sperm cells were treated with either DMSO (solvent control) or ionomycin (positive control) or progesterone or varying shFF concentrations (0.01%, 0.1%, 1%, 10% and 100%). Samples treated with progesterone and shFF concentrations (1% shFF or higher) showed a significant increase in the percentage of acrosome-reacted spermatozoa. However, the percentage of acrosome reacted sperm in the shFF treatments was significantly lower when compared to progesterone-treated spermatozoa. [*=P<0.05; **=P<0.01; ***=P<0.001].

Table 9 Percentage stimulation data derived from the percentage of acrosome-reacted spermatozoa in the each of the six experiments used for data analysis. Refer to section 2.2.5 for formula used. [DMSO=0%; Ionomycin=100%].

Treatments	% stimulation of acrosome reaction						
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Expt 6	Mean±SEM
DMSO	0	0	0	0	0	0	0±0.0
Ionomycin	100	100	100	100	100	100	100±0.0
Progesterone	24.0	21.9	26.4	31.0	22.0	21.3	24.4±1.5
100% shFF	17.6	17.1	17.2	19.0	18.4	12.8	17.0±0.9
10% shFF	12.0	1.0	13.8	20.2	12.8	8.5	11.4±2.6
1% shFF	8.0	1.0	10.3	3.6	3.7	2.1	4.8±1.5
0.1% shFF	5.6	1.9	0.0	-1.2	4.6	4.3	2.5±1.1
0.01% shFF	3.2	-1.9	2.3	5.6	-1.8	2.1	1.6±1.2

2.4 DISCUSSION

The data obtained from a recent steroid hormone profile of human follicular fluid has paved the way for this study in an attempt to further understand the dynamics associated with human sperm signalling in the female reproductive tract. This steroid hormone profile data was obtained from 16 human follicular fluid samples. Using this data, a novel attempt was made to study the combined effect of the physiological steroid hormone complement present in human follicular fluid on human sperm $[Ca^{2+}]_i$ signalling and acrosome reaction.

2.4.1 shFF and sperm $[Ca^{2+}]_i$ signalling

Using live cell calcium imaging and fluorimetry, the effects of shFF on human sperm calcium signalling were investigated at two working temperatures – 37°C and 26°C representative of physiological and standard laboratory temperatures respectively. At 37°C, 100% shFF evoked a rapid biphasic $[Ca^{2+}]_i$ response (influx) in human sperm characterised by an initial transient response peak immediately followed by a sustained response plateau (see Figures 16 and 25). This is in agreement with the findings of Kirkman-Brown *et al.* (2000) and Harper *et al.* (2003) who reported that the $[Ca^{2+}]_i$ response of human sperm to 3μM progesterone is biphasic in nature.

However, a significantly higher progesterone concentration (13.5μM) is present in 100% shFF and further data from fluorimetric studies using progesterone alone also showed a similar biphasic $[Ca^{2+}]_i$ response following the treatment of human sperm with 13.5μM progesterone (see Figure 26). Interestingly, the amplitude of the transient response to 100% shFF typically observed in the live cell imaging data is considerably lower than what was observed with 3.2μM progesterone by Kirkman-Brown *et al.* (2000) (10-25%

and 20-40% increase in fluorescence intensity above baseline levels for 100% shFF and 3.2μM progesterone respectively). This may be as a result of an antagonistic effect of the other steroid hormones present in the shFF mixture. The considerable amount of estradiol (800nM) in the shFF mixture makes it a potential candidate in concordance with the findings of Baldi *et al.* (2000) and Fujinoki *et al.* (2010) who reported that estradiol exhibits an inhibitory effect on progesterone-induced $[Ca^{2+}]_i$ responses and hyperactivation in mammalian sperm. This points in the direction of a hypothesis suggesting the possible existence of a signalling pathway that connects the sperm progesterone receptor, estrogen receptor(s) and the CatSper channel. Studies on CatSper have so far only provided individual response data for progesterone and estradiol alone, with progesterone shown to potentiate the CatSper channel (Lishko *et al.*, 2011; Strunker *et al.*, 2011) and estradiol not having any visible stimulatory effect on CatSper (Lishko *et al.*, 2011). To further explore this, more experiments could be carried out using the patch-clamp technique in an attempt to facilitate a direct comparative analysis of CatSper activity in response to 100% shFF, 100% shFF minus estrogens, 100% shFF minus progestogens and 13.5μM progesterone respectively. This could give a clearer picture of the combined effects of follicular fluid steroid hormones on sperm Ca^{2+} influx and CatSper activity.

At 37°C, the percentage of cells in which a significant transient response was observed in response to 100% shFF ranged from 42-71%. Since sperm capacitation and *in vitro* incubation conditions were maintained as constant as possible, this variability may reflect differences in responsiveness between donor samples. The mean percentage of cells responding to 100% shFF at 37°C (56.1±3.5%) was much lower than has

previously been reported from studies using progesterone (90-93%, Plant *et al.*, 1995; Aitken *et al.*, 1996 and 71.8±7.3%, Kirkman-Brown *et al.*, 2000). Whilst the effects of shFF on sperm responsiveness are open to speculation, part of the observed difference in responding populations to 100% shFF and progesterone may also be attributed to capacitation status (Baldi *et al.*, 1991; Mendoza and Tesarik, 1993; Garcia and Meizel, 1999). Following the perfusion of the spermatozoa with 100% shFF, the time period from the initiation to the peak of the transient response ranged from 10-30 seconds in individual cells. This variation between individual cells may be partly attributed to the latency of arrival of shFF at the sperm's cell membrane.

As the $[Ca^{2+}]_i$ transient decays, a sub-population (Mean 40.2%; see Table 5) of spermatozoa show a sustained ramp or plateau which persists till the end of shFF perfusion (up to 4 minutes). Further validation of the response kinetics was achieved when the pooling together of large amounts of single cell imaging data across 9 experiments (557 sperm cells) resulted in a trace very similar to what was observed in the fluorimetry experiments (see Figures 16 and 25). There appears to be a strong relationship between the transient and sustained response however the 'sustained only' response category occurs in a small number of spermatozoa which appear to show either a non-significant or non-discernible transient. As was also observed with progesterone by Kirkman-Brown *et al.* (2000), this shows that the transient response is not an absolute prerequisite for the sustained phase. Interestingly, the transient response in 'Transient and sustained only' spermatozoa was significantly higher than that of the 'transient only' cells. This suggests a correlation between the responsiveness of spermatozoa to shFF and the amplitude of the initial transient. With this trend absent

in previous studies using progesterone only, the presence of other progestogens such as 17α-hydroxyprogesterone in the shFF mixture may contribute to this.

The initiation of the transient and sustained $[Ca^{2+}]_i$ responses is the result of the activation of the CatSper channel by shFF according to current literature (Lishko *et al.*, 2011; Strunker *et al.*, 2011). This is likely to be modulated via the recently discovered endocannabinoid signalling pathway in which ABHD2 functions as the sperm progesterone receptor (Miller *et al.*, 2016). However, in the presence of a physiological steroid hormone complement as mimicked by shFF, the following scenarios are a possibility – (i) the presence of other signalling pathways activated by one or more of the other steroid hormones present in the shFF mixture, occurring synchronously with the earlier mentioned progesterone CatSper signalling pathway (ii) the presence of a much larger and complex signalling pathway, possibly involving other steroid hormones in which the progesterone CatSper pathway plays a significant part (Correia *et al.*, 2015; Morris *et al.*, 2015).

At a standard laboratory temperature of 26°C as in previous studies, 100% shFF also stimulated a biphasic $[Ca^{2+}]_i$ response (influx) in human sperm. This shows that the occurrence of the transient and sustained $[Ca^{2+}]_i$ responses in spermatozoa is not reliant on physiological temperature. Further comparison also showed that the time taken for the initiation of the transient response is similar at both 26 and 37°C however, the transient response at 26°C was characterised by a much slower peak as well as a much slower transient decay (see Figure 24). The different rates of sperm Ca^{2+} influx at 26 and 37°C respectively suggest that the endocannabinoid pathway responsible for the activation of the CatSper channel via the ABHD2 progesterone receptor is temperature-

sensitive. This in turn implies that extracellular temperature, as well as the speed of sperm Ca^{2+} influx are physiologically relevant in the sperm's journey through the follicular fluid milieu in the female reproductive tract. Furthermore, with regards to the experimental setup, photo bleaching of the calcium reporter dye used may account for a small part of the observed differences in transient response magnitude. However, with optical, image acquisition and perfusion settings kept constant at both working temperatures, photo bleaching could not have exerted a significant effect on the observed response kinetics.

Studies on other eukaryotic cell types have demonstrated a significant effect of temperature on $[Ca^{2+}]_i$ store pumps such as SERCAs and PMCAs (Thomas and Karon, 1994; Mackiewicz and Lewartowski, 2006). It may be suggested that the slower rate of decay of the shFF-induced transient $[Ca^{2+}]_i$ response at 26°C (see Figure 24) may partly be the result of a temperature interference with the regulation of sperm $[Ca^{2+}]_i$ store-operated channels likely to be responsible for the secondary portion of the transient Ca^{2+} influx mediated by CICR. A similar form of interference has been demonstrated using pharmacological agents (Kirkman-Brown *et al.*, 2003; Lefievre *et al.*, 2012; Morris *et al.*, 2015). It may therefore be possible for the function of $[Ca^{2+}]_i$ stores implicated in human sperm CICR to be affected by changes in temperature which in turn may lead to changes in transient $[Ca^{2+}]_i$ response decay rates.

Analysis of the response sub-categories revealed a higher proportion of 'Transient and sustained only' spermatozoa at 26°C. Whilst this difference in responsiveness may be a temperature effect, the significantly higher number of spermatozoa analysed in the 26°C experiments must also be taken into account. This difference in sample size between the

two temperature groups results from the adhesion of a larger population of spermatozoa when introduced into the perfusion chamber at 26°C, suggesting that a standard laboratory temperature of 26°C may be more conducive than physiological temperature (37°C) for the interaction of sperm with poly-D-lysine. Spermatozoa at 26°C showed a considerably higher amplitude for the transient and sustained $[Ca^{2+}]_i$ responses to 100% shFF (Figure 24). This suggests that the amount of shFF-induced Ca^{2+} influx into the sperm cell may be temperature-susceptible. Furthermore, the effects of temperature on the dissociation constant (Kd) of the Calcium Green dye may have a role to play as a reduction in experimental temperature has been shown to prolong fluorescence lifetimes thus affecting the reported $[Ca^{2+}]_i$ concentrations (Oliver *et al.*, 2000). This in turn may account for some of the observed difference in the sperm transient $[Ca^{2+}]_i$ response amplitudes.

Dose response

Using different doses of shFF (0.001%-100% shFF) and progesterone alone (135pM-13.5μM), analysis of fluorimetry data showed a complex relationship between shFF doses and the amplitude of the transient and sustained responses (Figures 27, 28 and 29). This was also observed using progesterone in previous studies (Harper *et al.*, 2003). However, no visible dose-dependence was observed in the response kinetics. The $[Ca^{2+}]_i$ peak amplitude of the shFF-induced transient responses was at its maximum following the addition of 10% shFF to the sample cuvette. In progesterone-only experiments, 135nM progesterone evoked a maximum peak amplitude. With 1% shFF containing 135nM progesterone, it is interesting that a much smaller response peak amplitude is observed in response to 1% shFF than to 135nM. This may also suggest the presence of

an antagonistic effect of the other steroid hormones present in the shFF mix on progesterone-induced sperm calcium influx. The considerably reduced transients observed following the addition of 100% shFF and 1.35μM progesterone are consistent with the hypothesis by Miller *et al.* (2016), suggesting that a continuous application of progesterone will eventually result in an overabundance of arachidonic acid (AA) in the plasma membrane ultimately resulting in the desensitisation of the CatSper channel.

Possible future research

Following the attempt to study the effects of shFF on sperm Ca^{2+} signalling and the data herein, more research could be carried out to further investigate the $[Ca^{2+}]_i$ response trends observed in the data from this study. Firstly, fluorimetry studies could be also carried out at a working temperature of 26°C. This will serve to confirm the presence of a temperature effect as suggested by the live cell imaging data. This will also take into account the possible effects of a reduction in temperature on the KD of fura-2/AM reporter dye. The dose dependence studies could also be improved by altering the experimental setup with the use of a microplate reader to record fluorescence intensity data. Briefly, equal volumes of prepared sperm cells labelled with FURA-2 will be aliquoted into 6 different wells in the microplate reader at 37°C and treated with 0.001%, 0.01%, 0.1%, 1%, 10% and 100% shFF respectively. The fluorescence readings obtained from this setup will provide $[Ca^{2+}]_i$ response data in the absence of pre-exposure as is present in a cuvette being read by a fluorimeter. Furthermore, the role and importance of the estrogens present in the shFF mixture could be investigated by creating a mixture of shFF minus estradiol and estrone-the 2 estrogens present in the LC-MS/MS profile of follicular fluid presented in this study (Table 4). This alteration of shFF

could be used in live cell imaging and fluorimetry experiments in an attempt to establish the effect(s) of estrogens on progesterone-mediated $[Ca^{2+}]_i$ responses. With regards to the role of the CatSper channel, the $[Ca^{2+}]_i$ kinetics during exposure to 100% shFF may be further characterised using patch clamp studies, with particular focus on the activity of sperm ion channels during the transient and sustained phase of the $[Ca^{2+}]_i$ response. Finally, the live cell imaging experiments carried out in this study may be repeated in the presence of a CatSper blocker in an attempt to investigate the presence of unconventional signalling pathways involving the action of other steroid hormones in the shFF mixture besides progesterone.

2.4.2 shFF and sperm acrosome reaction

The effects of some steroid hormone constituents of FF on sperm acrosome reaction have been investigated (Baldi *et al.*, 2000; Kirkman-Brown *et al.*, 2000; Harper *et al.*, 2006). Studies have established the effects of progesterone on human sperm as characterised in part by a significant induction of acrosome reaction (Kirkman-Brown *et al.*, 2000; Harper *et al.*, 2004). The data obtained from this study (n=6) showed a similar pattern, with sperm acrosome reaction significantly induced following exposure to 13.5μM progesterone. This phenomenon is thought to be mediated by a Ca^{2+} influx resulting from the same exposure to progesterone (Son *et al.*, 2000; Jensen and Publicover, 2012). Recently, it has been reported that progesterone via the CatSper channel activates the AKT-phosphatidylinositol 3-kinase (PI3K-AKT) signalling pathway which modulates human sperm motility and hyperactivation. However, the biochemical inhibition of this signalling pathway did not have an effect on acrosome reaction (Sagare-Patil *et al.*, 2013). This is in agreement with the findings from a study carried out using a mouse model, where it was shown that sperm acrosome reaction was not

diminished in CatSper knockout mice (Ren and Xia, 2010). These suggest that progesterone may potentially initiate sperm Ca^{2+} influx and acrosome reaction via discrete signalling mechanisms.

The effect of the varying shFF concentrations on sperm acrosome reaction as observed in the data from this study was found to be significantly reduced when compared to the AR response of progesterone-treated sperm ($P<0.05$). This brings into question the possible underlying mechanisms at work given that shFF contains a significant amount of progesterone (13.5μM). This reduced effect of shFF on sperm AR may be as a result of the antagonistic effect of one or more of the other 13 steroid hormones present in the shFF mixture. In support of this hypothesis, data from the studies carried out by Baldi *et al.* (2000) and Kirkman-Brown (2000) showed that pre-treatment of sperm with estradiol inhibited progesterone-stimulated $[Ca^{2+}]_i$ increase and acrosome reaction. A functional non-genomic estradiol receptor localised to the sperm membrane was also identified (Baldi *et al.*, 2000), however its role in molecular signalling pathways is still to be understood. Furthermore, the observed effect of shFF on AR may not be limited to the antagonistic effects of estrogen and/or any of the other steroid components. The presence of another progestogen, 17α-hydroxyprogesterone in shFF might have a role to play as the sperm's non-genomic progesterone receptor (ABHD2) may also be activated by this progesterone derivative (Blackmore *et al.*, 1990). This in turn lends focus to the sensitivity of the sperm progesterone receptor, which needs to be studied in more detail.

With 0.1% and 0.01% shFF inducing minimal $[Ca^{2+}]_i$ responses in human sperm as shown in the fluorimetry data, the absence of a significant AR in these treatment groups may be due to a much lower concentration of progesterone present in each dose – 13.5nM and 1.35nM respectively. This is in agreement with the findings of Sagare-Patil *et al.* (2012) who observed that low concentrations of progesterone (10-100nM) induced sperm motility and tyrosine kinase activation, whilst higher concentrations of progesterone were required to induce AR. Despite the low shFF doses, the possible antagonistic effect of the estrogens present in these shFF dilutions is worth considering.

The data obtained from the experiments carried out in this study reveal a significant dynamic as per the modulation of AR by follicular fluid steroid hormones. To further understand this phenomenon, the real time monitoring of the sperm acrosome as shown in previous studies (Harper *et al.*, 2006; Sánchez-Cárdenas *et al.*, 2014) may be used to potentially distinguish the sub-population of spermatozoa undergoing AR based on their physiological activity.

2.4.3 Overall conclusions

The significant stimulation of a biphasic Ca^{2+} influx by shFF coupled with its reduced effect on human sperm AR (in comparison to progesterone alone) as observed in this study suggests that human follicular fluid modulates Ca^{2+} influx and acrosome reaction via signalling pathways dependent on other steroid hormones besides progesterone. The AR data also implies that human follicular fluid in the female reproductive tract may not induce sperm AR in levels as high as was previously suggested based on studies using progesterone. Physiologically, this may suggest that a larger population of spermatozoa are likely to arrive at the vicinity of the oocyte with an intact acrosomal

membrane. The relationship between shFF-induced sperm Ca^{2+} influx and AR can be further studied and whilst the resulting data may reveal no apparent relationship between shFF-induced $[Ca^{2+}]_i$ response amplitude and AR as was previously reported to be the case in progesterone-treated sperm (Harper *et al.*, 2006), it may be possible to characterise the $[Ca^{2+}]_i$ response patterns associated with shFF-induced AR. This has been demonstrated using progesterone alone on human sperm (Sánchez-Cárdenas *et al.*, 2014) and mouse sperm (Romarowski *et al.*, 2016). In conclusion, the characterisation data presented in this study brings us a step closer in our understanding of the nature and complexity of the $[Ca^{2+}]_i$ signalling mechanisms that regulate pre-fertilisation events in human sperm, which are still a long way from being fully understood.

CHAPTER 3

FOLLICULAR FLUID STEROID HORMONES AND HUMAN SPERM II – CHARACTERISATION OF $[Ca^{2+}]_i$ KINETICS FOLLOWING REMOVAL OF THE EXTRACELLULAR STEROID MILIEU

3.1 INTRODUCTION

When the migratory journey through the female reproductive tract begins, human spermatozoa are exposed to a changing environment characterised by discrete regions that possess quite different conditions of viscosity (Gaffney *et al.*, 2011) and biochemical milieu (Revelli *et al.*, 2009).

The effect of the changing tract environment on human sperm $[Ca^{2+}]_i$ kinetics and homeostasis constitutes a key question in human reproductive physiology. The biochemical constituents of follicular fluid contribute significantly to the external chemical milieu of the female reproductive tract however, one question of relevance is how the exposure and removal of follicular fluid or any of its biochemical constituents may affect spermatozoa, particularly with relevance to the potential for a sperm cell to swim in and out of the plume of follicular fluid as it approaches the ovulated oocyte. The temporal and spatial dynamic surrounding this form of interaction with follicular fluid is likely to be crucial to the modulation of sperm chemotaxis as a possible means of sperm guidance towards the vicinity of the oocyte (Kaupp *et al.*, 2008; Perez-Cerezales *et al.*, 2015b),

The $[Ca^{2+}]_i$ signalling effects of the addition of follicular fluid steroid hormones to human sperm were previously examined (see Chapter 2). Steroid hormones are a key biochemical constituent of follicular fluid known to contribute significantly to the above-mentioned intracellular calcium responses in sperm (Blackmore *et al.*, 1990; Kirkman-Brown *et al.*, 2000; Harper *et al.*, 2003; Chapter 2). The sperm's calcium signalling system functions as a metabolomic 'control tower' through which the biochemical signalling pathways responsible for the initiation of physiological responses are

processed (see Figure 55). The However prior to this study, there has been an absence of published data on the effects of the full steroid hormone complement present in follicular fluid on human sperm, or their removal. Using previously obtained data on the steroid hormone complement of human follicular fluid (see Chapter 2), the effects of the addition and removal of follicular fluid steroid hormones on sperm intracellular calcium signalling were therefore examined.

3.1.1 Calcium homeostasis in mammalian sperm

The calcium ion (Ca^{2+}) is a ubiquitous intracellular messenger carrying biological information that ultimately controls an extensive range of cellular processes from the origin of cell life at fertilisation to the end of cell life during programmed cell death. Some of these processes include gene transcription, cell motility and cell proliferation (See Clapham (2007), Ren and Xia (2010) and Darszon *et al.* (2011) for comprehensive reviews on calcium signalling). In mammalian cells including spermatozoa, a variety of cellular functions are driven by changes in $[Ca^{2+}]_i$ levels regulated by a balance between the mechanisms responsible for $[Ca^{2+}]_i$ entry and clearance. The ambivalent nature of the Ca^{2+} signal makes this homeostatic balance essential and this is maintained either via the transport of Ca^{2+} across membrane boundaries, or the formation of reversible complexes in the cytoplasm or in the lumen of organelles.

Sperm $[Ca^{2+}]_i$ entry via ion channels

Studies have shown that the primary source of Ca^{2+} for the mammalian sperm cell is its extracellular milieu – the female reproductive tract (*in vivo*) and culture media (*in vitro*) (Foresta and Rossato, 1997). This brings into question, the mechanism(s) responsible for sperm $[Ca^{2+}]_i$ entry via plasma membrane boundaries. In eukaryotic cells, Ca^{2+} entry is

mediated by specific plasma membrane ion channel proteins that are permeable to Ca^{2+} (Pietrobon *et al.*, 1990; Berridge *et al.*, 2003). The literature reviewed shows the identification of sperm ion channels in different mammalian species (Jimenez-Gonzalez *et al.*, 2006). Recently a set of criteria was proposed to identify ion channel proteins of functional significance in sperm physiology (Ren and Xia, 2010). These criteria include (i) protein detectability in sperm, preferably using knockout sperm as a negative control (ii) production of ion channel current detectable by patch clamp recording (iii) impairment of normal sperm function in response to channel blockage (iv) sperm malfunctions resulting from ion channel protein gene mutations.

CatSper – The primary sperm Ca^{2+} entry channel

The cation channel of sperm (Catsper) is a family of sperm-specific, Ca^{2+} selective, pH sensitive, voltage-gated channels that modulates the influx of $[Ca^{2+}]_i$ from the extracellular environment (Kirichok *et al.*, 2006). In addition to being the only family of sperm ion channels that clearly meet most of the above criteria (Ren and Xia, 2010), Catsper is exclusively expressed in the testis and is essential for sperm hyperactivation and male fertility (Lishko *et al.*, 2011; Shukla *et al.*, 2012). Studies have revealed the presence of CatSper orthologs in all of the mammalian species examined so far (e.g. rat, dog, chimpanzee and human), however their identities may differ between species (Cai and Clapham, 2008; Navarro *et al.*, 2008). Catsper consists of 4 separate pore-forming α subunits (CatSper 1-4) and three additional auxiliary subunits – CatSper β, CatSper γ and CatSper δ (Figure 31). This heterotetrameric channel complex is localised to the sperm flagellum and has been found to be sensitive to changes in intracellular pH, progesterone, prostaglandins and odorants (Darszon *et al.*, 2011; Lishko *et al.*, 2012).

All of the CatSper subunits are functionally expressed in coordination with each other as knockout studies show that CatSper 2-4, CatSper β, CatSper γ and CatSper δ are all undetectable in CatSper 1 null sperm plasma membranes (Carlson *et al.*, 2005; Liu *et al.*, 2007; Qi *et al.*, 2007; Wang *et al.*, 2009; Chung *et al.*, 2011). Furthermore, targeted knockout or disruption of any the CatSper 1-4 genes results in complete loss of the CatSper channel and consequent male infertility indicating that all pore-forming α subunits are indispensable to the functionality of the CatSper channel (Carlson *et al.*, 2005; Qi *et al.*, 2007). The physiological role of the CatSper channel in mammalian sperm has been widely studied using murine species with evidence pointing to its role in the induction and maintenance of sperm hyperactivated motility (Carlson *et al.*, 2003). The significance of CatSper to the process of fertilisation in this species is also apparent as CatSper 1 and 2 null spermatozoa were found to be incapable of fertilising intact oocytes (Ren *et al.*, 2001; Quill *et al.*, 2003). In humans however, the physiological role played by the CatSper channel is not fully understood. The induction and maintenance of human sperm hyperactivated motility is largely dependent on Ca^{2+} influx via CatSper and Ca^{2+} release from intracellular calcium stores (Harper *et al.*, 2004; Kirkman-Brown *et al.*, 2004; Alasmari *et al.*, 2013b). CatSper in humans is also known to be critical for progesterone-induced sperm hyperactivation (Lishko *et al.*, 2011; Strunker *et al.*, 2011; Smith *et al.*, 2013), and appears to be essential for sperm to travel through viscous media (Alasmari *et al.*, 2013a). The apparent positive correlation between IVF success rates and progesterone-induced $[Ca^{2+}]_i$ increase (Alasmari *et al.*, 2013b) would prove useful in further understanding the clinical role of CatSper, which in turn may contribute to advancements in male fertility diagnosis.

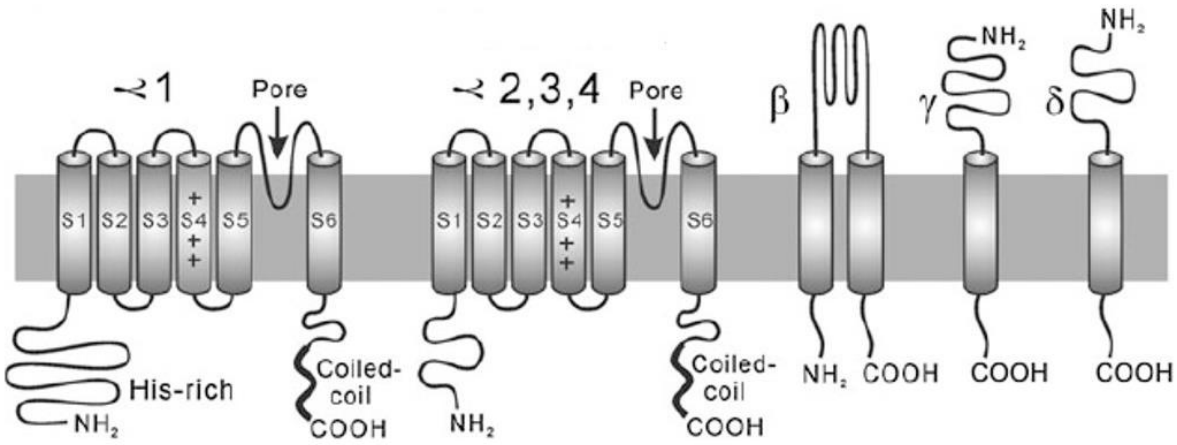


Figure 31 Structure of the CatSper channel protein showing the topology of the α pore-forming subunits (CatSper1-4) and auxiliary units (CatSper β, γ and δ). The pore-forming subunits each possess 6 transmembrane segments. CatSper β has 2 transmembrane segments whilst CatSper γ and δ each possess a single transmembrane segment. The presence of positively charged residues in the fourth segment of each pore-forming subunit is consistent with CatSper's function as a voltage-gated channel. Adapted from Nishigaki *et al.* (2014).

Maintaining Calcium Homeostasis in Mammalian Cells & Sperm

Calcium clearance is essential for the maintenance of $[Ca^{2+}]_i$ homeostasis in eukaryotic cells as it serves to maintain resting $[Ca^{2+}]_i$ levels or to bring $[Ca^{2+}]_i$ concentrations down to resting levels following a response to physiological stimuli (Berridge *et al.*, 2000). This ATP-dependent process is largely mediated either by Ca^{2+} ATPases (pumps) or Na^+-Ca^{2+} exchangers (NCXs) (Michelangeli *et al.*, 2005).

Na^+- Ca^{2+} exchangers (NCXs)

NCXs are present in the plasma membrane of most animal cells, and function by using the energy of the electrochemical Na^+ gradient present in the extracellular space to allow the flow of Na^+ across the plasma membrane into the cytoplasm in exchange for the export of Ca^{2+} at a stoichiometric ratio of $3Na^+$ for $1Ca^{2+}$ (Brini *et al.*, 2013). The NCX is present in mammalian spermatozoa (Babcock and Pfeiffer, 1987), and has been reported to contribute significantly to sperm calcium clearance mechanisms and homeostasis in mammalian species such as the mouse (Wennemuth *et al.*, 2003) and

human (Krasznai *et al.*, 2006). There is however a need for further characterisation studies in order for some correlation to be drawn between the functional expression of NCXs and male fertility.

Ca^{2+} ATPases (pumps)

Animal cells express three Ca^{2+} ATPases namely (i) plasma membrane Ca^{2+} ATPase (PMCA), located in the plasma membrane (ii) sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), located in the endoplasmic reticulum/sarcoplasmic reticulum (iii) secretory pathway Ca^{2+} ATPase (SPCA), located in the Golgi apparatus. They all belong to the family of P-Type ATPases, which are known to become transiently phosphorylated during the reaction cycle to aid the transport of ions across membrane boundaries (Pedersen and Carafoli, 1987). These three named Ca^{2+} pumps facilitate calcium clearance either by exporting $[Ca^{2+}]_i$ to the extracellular medium (PMCA), or to the lumen of the reticulum (SERCA) and of the Golgi apparatus (SPCA).

Pumping calcium to the sperm's extracellular environment

PMCAs are high affinity Ca^{2+} pumps that exist in 4 isoforms (PMCA 1-4), and are present in the plasma membrane of most mammalian cells including spermatozoa. Structurally, all 4 isoforms are laid out into 10 transmembrane segments, grouped into four main units that serve different functions (Triphan *et al.*, 2007). The distinctive functional roles of each of the 4 isoforms contribute significantly to the maintenance of resting $[Ca^{2+}]_i$ levels as well as the regulation of $[Ca^{2+}]_i$ signals with spatial and temporal resolution (Strehler and Treiman, 2004). Studies have shown that PMCA4 is the most abundant isoform present in mammalian testis and sperm (Okunade *et al.*, 2004; Schuh *et al.*, 2004). Consequently, PMCA4 null mice were found to be infertile characterised by a

failure of sperm hyperactivated motility (Okunade *et al.*, 2004; Prasad *et al.*, 2004; Schuh *et al.*, 2004). In human sperm, PMCA4 was localised to the acrosome, inner acrosomal membrane, posterior head, neck, midpiece and proximal piece (Andrews *et al.*, 2015).

Pumping calcium to intracellular stores / making a store

The transport of $[Ca^{2+}]_i$ via Ca^{2+} pumps into intracellular stores is an important feature of Ca^{2+} homeostasis regulation in all eukaryotic cells. SERCAs and SPCAs located on the endoplasmic reticulum and Golgi apparatus respectively, contribute significantly to the existing Ca^{2+} clearance mechanisms in mammalian cells (Brini *et al.*, 2013). With the endoplasmic reticulum constituting key Ca^{2+} stores in most cells, SERCA serves as a major conduit through which $[Ca^{2+}]_i$ is pumped back into these stores. This functions to terminate $[Ca^{2+}]_i$ signals as well as restore resting cytosolic Ca^{2+} levels (Michelangeli *et al.*, 2005; Michelangeli and East, 2011). SERCA exists in three isoforms (SERCA 1-3) with variations in size, localisation and function (Michelangeli and East, 2011). There is very little evidence to support the role of SERCAs in mammalian sperm Ca^{2+} clearance however observations from characterisation and localisation studies have led to a hypothesis suggesting that the functionality of SERCA is 'switched off' following the completion of spermatogenesis (Jimenez-Gonzalez *et al.*, 2006). Another Ca^{2+} pump localised to the Golgi apparatus of most eukaryotic cells is the SPCA. Two isoforms of SPCAs have been found to exist namely SPCA 1 and SPCA 2 (Gunteski-Hamblin *et al.*, 1992). Immunolocalisation studies provide evidence for the presence of SPCA 1 in the spermatids and spermatozoa of mammalian species such as the rat (Wootton *et al.*, 2004) and human (Harper *et al.*, 2005). Evidence for the expression of SPCA 2 in

mammalian sperm has also been revealed (Lawson *et al.*, 2007). Questions into the possible physiological roles PMCA and SPCA play either individually or synergistically in mammalian sperm may be further answered using knockout studies.

Sperm Ca^{2+} mobilisation from intracellular Stores

In most somatic cells, the most prevalent and widely studied Ca^{2+} stores that exist are the endoplasmic reticulum, sarcoplasmic reticulum, mitochondria and nuclear envelope. In mammalian spermatozoa, the underlying mechanisms controlling Ca^{2+} release from $[Ca^{2+}]_i$ stores remain unclear and this is attributed to the absence of an endoplasmic reticulum which typically constitutes a major $[Ca^{2+}]_i$ store. Ca^{2+} stores that have been identified in mammalian sperm include the acrosome in the head, a redundant nuclear envelope (RNE) in the neck region and mitochondria present in the midpiece (Ho and Suarez, 2001a; Ho and Suarez, 2003; Costello *et al.*, 2009). These stores function in the presence or absence of physiological stimuli by releasing or sequestering Ca^{2+} in order to elevate or restore basal $[Ca^{2+}]_i$ levels as required by the cell. The mobilisation of these sperm Ca^{2+} stores are believed to contribute significantly to the regulation of physiological responses such as hyperactivated motility and acrosome reaction (Correia *et al.*, 2015). As is widely present in somatic cells, two intracellular channels capable of mobilising stored Ca^{2+} have been identified in mammalian sperm namely the inositol 1,4,5-trisphosphate receptor (IP_3R) and the ryanodine receptor (RyR) (Jimenez-Gonzalez *et al.*, 2006).

The IP_3R is a receptor protein that is activated upon binding to IP3, which is a universal intracellular second messenger that communicates cell signaling information from the plasma membrane to intracellular organelles (Berridge *et al.*, 2003). The activation of the IP_3R confers on it the ability to release Ca^{2+} from intracellular stores (usually the ER) (Lencesova and Krizanova, 2012). In mammalian cells, the IP_3R exists in 3 isoforms (IP_3R 1-3) which are all similar in structure and function however, the distinctive Ca^{2+} signals associated with each isoform are due to differences in organelle localisation, agonist affinity and associated protein interactions (Taylor *et al.*, 1999; Parys and De Smedt, 2012). The published data reviewed by Jimenez-Gonzalez *et al.* (2006) shows the detection of IP_3R isoforms in mammalian sperm at different stages of spermatogenesis. Immunolocalisation studies on human sperm revealed the expression of IP_3R 1 in the acrosomal region while IP_3R 3 was localised to the posterior head/neck (PHN) and mid-piece region (Walensky and Snyder, 1995; Kuroda *et al.*, 1999; Naaby-Hansen *et al.*, 2001). Harper *et al.* (2004) demonstrated that progesterone stimulation resulted in the apparent mobilization of Ca^{2+} stores located in the PHN region of human spermatozoa. Similar results were obtained using thimerosal - a known IP_3R activator, resulting in sustained sperm $[Ca^{2+}]_i$ increase and hyperactivated motility in the presence of a CatSper inhibitor (Alasmari *et al.*, 2013b). A physiological role for IP_3R in the acrosome reaction was also demonstrated using thimerosal (Herrick *et al.*, 2005).

The RyR is known to be a calcium-induced calcium release (CICR) channel localised to the ER/SR membrane (Lanner, 2012). Three RyR isoforms exist in mammalian cells (RyR 1-3) with ~70% sequence similarity (Brini, 2004). In somatic cells, the degree of CICR by RyRs located on intracellular Ca^{2+} stores is dependent on the RyR isoform as well as

accessory proteins and secondary messenger binding. In concordance, Ikemoto *et al.* (1995) demonstrated that calmodulin inhibits both RYR 1 and 2 at high $[Ca^{2+}]_i$ levels however at low $[Ca^{2+}]_i$ concentrations, it inhibits RYR 2 only with RYR 1 remaining in an activated state. Studies on murine species have shown that both RyR1 and RyR2 are present in spermatocytes and spermatids (Giannini *et al.*, 1995; Trevino *et al.*, 1998). The RYR 3 isoform however could only be detected in the acrosomal region of mature spermatozoa using RT-PCR and immunolocalisation techniques (Trevino *et al.*, 1998). The expression of RYR in human sperm was first studied by Harper *et al.* (2004) who reported the co-localisation of RyR and SPCA 1 predominantly to the PHN region of human sperm, with some expression in the acrosome. This study also demonstrated the importance of RyRs in sperm calcium signalling as the cessation of progesterone-induced Ca^{2+} oscillations was observed upon the application of tetracaine – a RyR inhibitor. Furthermore, recent studies showed that RYR 2 depleted sperm is characterised by reduced sperm motility and consequently, low fertilisation success (Park *et al.*, 2011).

In summary, as a mature sperm cell traverses the female reproductive tract, it comes in contact with a wide range of physiological agonists which in most cases call for the deployment of the sperm's calcium signalling 'toolkit' (Figure 32). Physiological stimuli such as steroid hormones present in follicular fluid, or ZP proteins in the vicinity of the oocyte have been shown to modulate $[Ca^{2+}]_i$ responses via the synergistic action of the components of this 'toolkit'. Sperm Ca^{2+} entry via CatSper channel, sperm $[Ca^{2+}]_i$ store mobilisation, and calcium clearance via PMCA are all essential to the physiologically-induced changes that occur in the female tract such as hyperactivated motility and acrosome reaction. This chapter focuses on the characterisation of sperm calcium

clearance following the removal of physiological steroid hormone stimuli as is present in human follicular fluid.

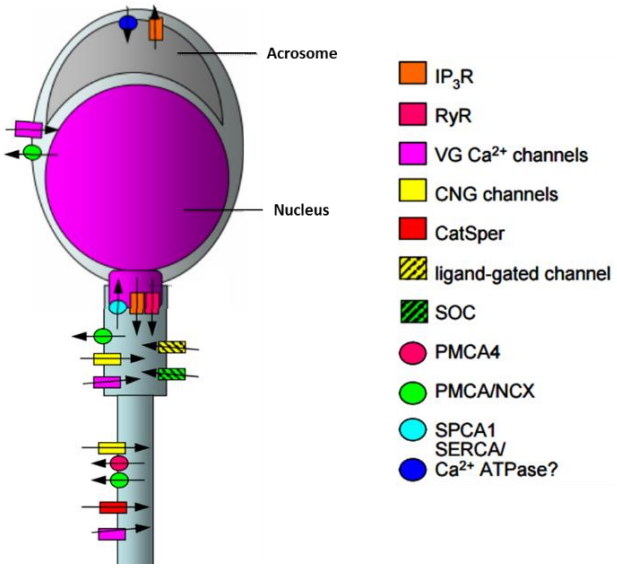


Figure 32 Schematic summary of the sperm cell Ca^{2+} signalling 'toolkit'. Refer to text for details. Adapted from Bedu-Addo *et al.* (2008).

3.1.2 Aim of the study

The aim of this study was to investigate the effects of the removal of a follicular fluid steroid hormone milieu in the form of shFF (see Chapter 2) from the sperm's extracellular environment immediately following short exposure to this milieu as may occur if a sperm cell swam in and out of follicular fluid in the female reproductive tract, or experienced a dynamic steroid hormone concentration change flowing over it whilst attached to the epithelia of the female reproductive tract. The research objectives of this study were to (i) characterise the effect(s) of shFF removal on the $[Ca^{2+}]_i$ response dynamics in human spermatozoa via live cell calcium imaging (ii) compare the effects of physiological and standard laboratory temperatures on the above responses.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Please refer to chapter 2, section 2.2.1 for a detailed list of research materials used in this study. The preparation of the shFF and semen samples used in this study was carried out as described in chapter 2.

3.2.2 Live cell Ca^{2+} imaging

See chapter 2, section 2.2.4 for a concise description of the live cell Ca^{2+} imaging protocol used in this study.

3.3 RESULTS

3.3.1 Live cell imaging data – shFF gradient removal and sperm $[Ca^{2+}]_i$ kinetics

Depletion of shFF causes a rapid reduction in $[Ca^{2+}]_i$ levels

An $[Ca^{2+}]_i$ response was observed following exposure of spermatozoa to 100% shFF at 37°C. This was characterised by a transient $[Ca^{2+}]_i$ increase which was then followed by a $[Ca^{2+}]_i$ plateau sustained for the duration of exposure to shFF (see chapter 2). A rapid decrease in $[Ca^{2+}]_i$ was observed when shFF was removed from the perfusing medium (Figures 33 and 34). At 37°C, this response is characterised by a sharp decrease in fluorescence intensity up to 2% below baseline levels prior to the addition of shFF.

This decrease in $[Ca^{2+}]_i$ brought about by the removal of shFF was found to have similar kinetic attributes to the $[Ca^{2+}]_i$ decrease that immediately follows the initial transient peak (Figure 35). Analyses of the above-mentioned decrease in sperm $[Ca^{2+}]_i$ immediately following initial transient, as well as immediately following shFF removal were carried out by comparing the average amplitudes of the corresponding reductions in fluorescence intensity across all of the 557 responding sperm cells. This revealed a positive correlation ($R=0.98$) between the $[Ca^{2+}]_i$ decrease immediately following the initial transient response and the rapid $[Ca^{2+}]_i$ decrease immediately after shFF removal (Figure 35).

Detailed analysis of the occurrence of a transient response and $[Ca^{2+}]_i$ depletion after shFF wash off from 9 experiments (557 sperm cells) revealed a relationship between both responses ($P<0.01$; χ^2), suggesting a significant link between the transient $[Ca^{2+}]_i$ increase following exposure to 100% shFF and the subsequent rapid decrease upon removal of extracellular shFF. A significant linkage was also found between the

occurrence of a sustained $[Ca^{2+}]_i$ response and subsequent rapid depletion upon removal of the shFF stimulus ($P<0.01$; χ^2).

Scatter plot analyses of the response data from each responding cell were carried out to further investigate the relationship between the biphasic $[Ca^{2+}]_i$ response to shFF and the subsequent $[Ca^{2+}]_i$ decrease following extracellular shFF removal. Average amplitudes of the percentage change in normalised fluorescence intensities corresponding to selected points within the transient peak, sustained plateau, and $[Ca^{2+}]_i$ decrease following shFF removal were plotted for each responding cell (See Figures 36 and 37). This revealed a weak negative correlation between the transient $[Ca^{2+}]_i$ response phase and the rapid $[Ca^{2+}]_i$ decrease following shFF removal (Figure 36). In contrast, a positive correlation was observed between the sustained $[Ca^{2+}]_i$ response phase and the rapid $[Ca^{2+}]_i$ decrease upon shFF removal. (Figure 37).

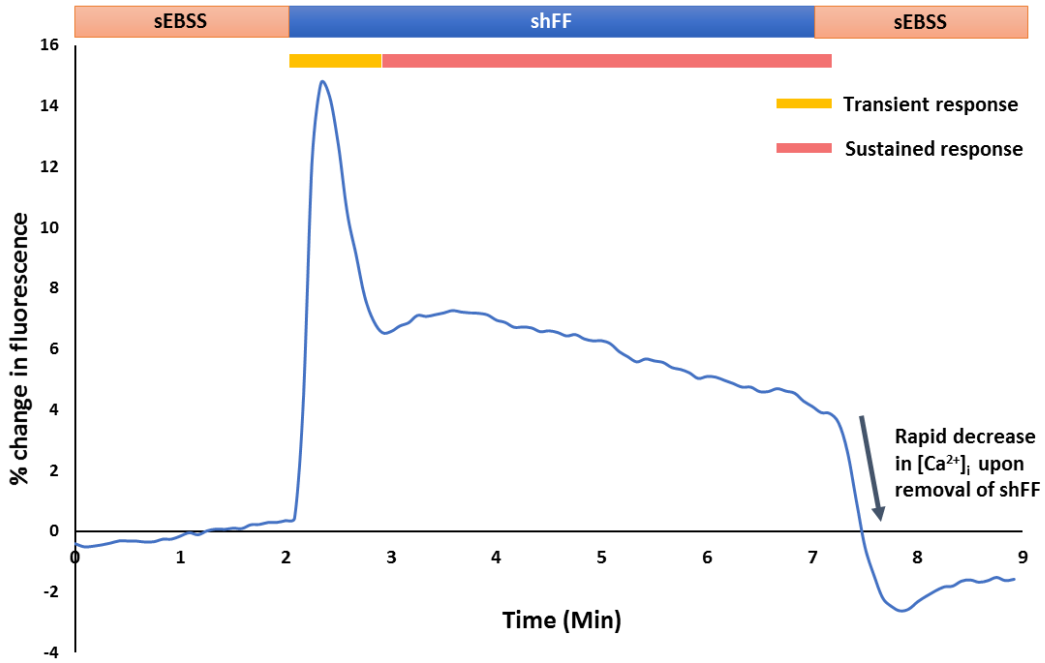


Figure 33 A rapid depletion in $[Ca^{2+}]_i$ is observed in 557 responding cells at 37°C following the removal of shFF from the perfusing medium (wash off). This is characterised by a decrease in fluorescence intensity by up to 2% below initial baseline levels prior to shFF stimulation, followed by the re-establishment of a new $[Ca^{2+}]_i$ baseline.

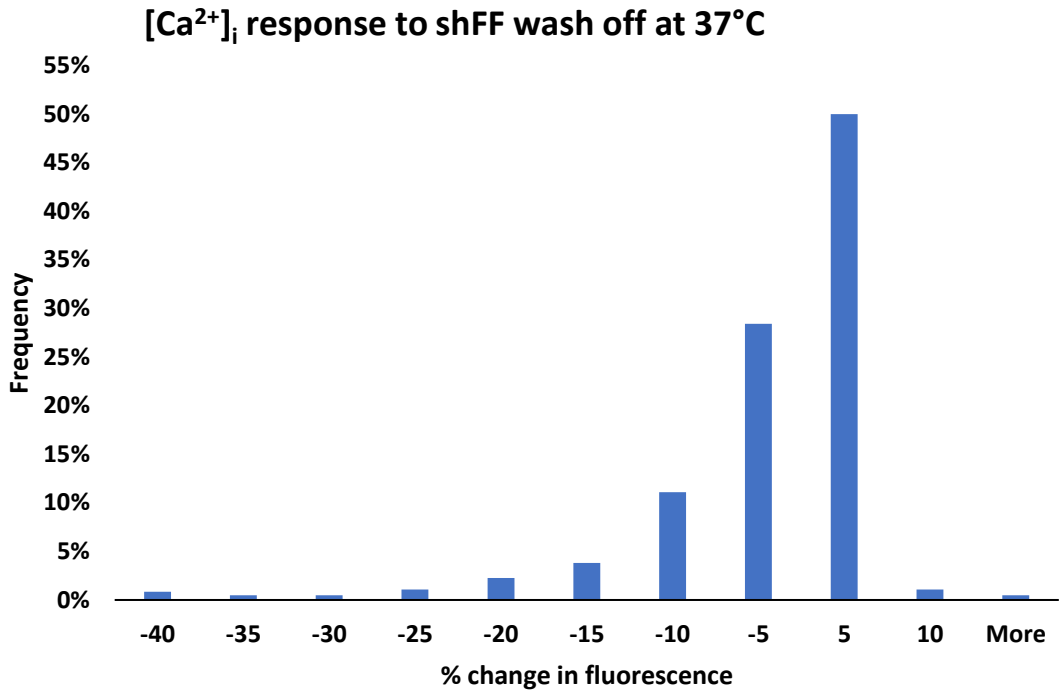


Figure 34 Summary amplitude distribution for $[Ca^{2+}]_i$ responses (37°C) at time of shFF wash off for R_{tot} from 9 experiments. Data obtained from sub-population of cells significantly showing a response to the removal of extracellular shFF.

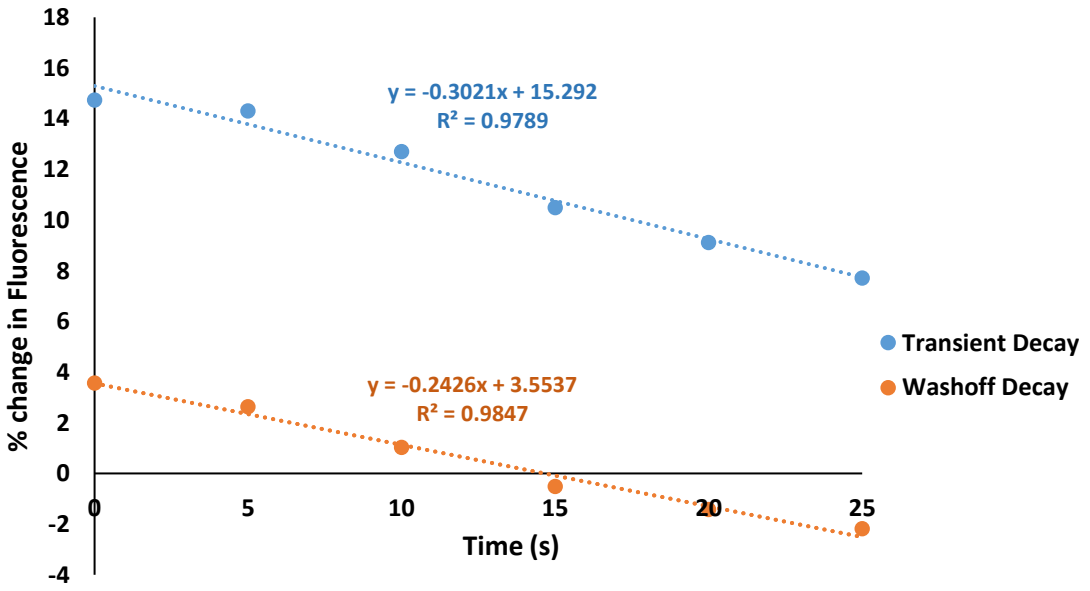


Figure 35 Comparison of the rapid $[Ca^{2+}]_i$ decrease that immediately follows the initial transient response to shFF and shFF wash off respectively at 37°C. Correlation analysis of 557 responding cells from 9 experiments revealed a positive correlation (R=0.98) between the $[Ca^{2+}]_i$ decrease immediately following the initial transient response and the rapid $[Ca^{2+}]_i$ decrease immediately after shFF removal (wash off).

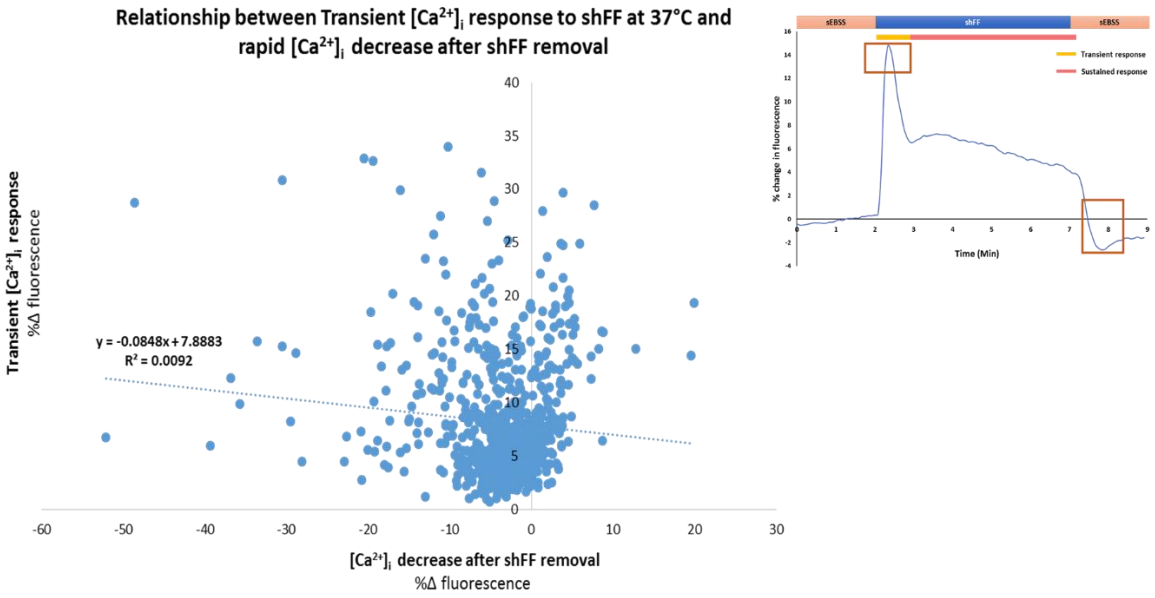


Figure 36 Relationship between transient $[Ca^{2+}]_i$ increase and rapid $[Ca^{2+}]_i$ decrease following the treatment of spermatozoa with shFF at 37°C and shFF wash off respectively. Analysis was carried out using data from all cells in 9 experiments (788 cells) that showed both a transient response and a rapid $[Ca^{2+}]_i$ decrease in response to shFF wash off. Highlighted sections on the response curve (upper right) shows the areas within each response phase for each cell that were selected and averaged for scatter plot analysis.

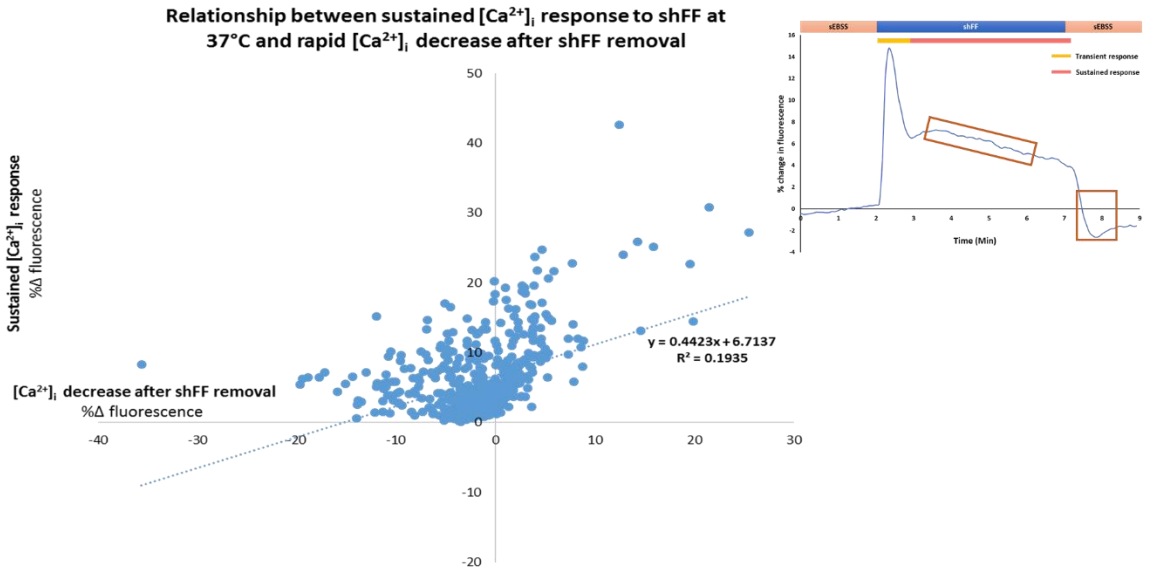


Figure 37 Relationship between sustained $[Ca^{2+}]_i$ responses and rapid $[Ca^{2+}]_i$ decrease following the treatment of spermatozoa with shFF at 37°C and shFF wash off respectively. Analysis was carried out using data from all cells in 9 experiments (569 cells) that showed both a sustained response and a rapid $[Ca^{2+}]_i$ decrease in response to shFF wash off. Highlighted sections on the response curve (upper right) shows the areas within each response phase for each cell that were selected and averaged for scatter plot analysis.

A rapid decrease in $[Ca^{2+}]_i$ was also observed at 26°C when shFF was removed from the perfusing medium (Figures 38 and 39). This response is characterised by a rapid decrease in fluorescence intensity up to 2% below baseline levels prior to the addition of shFF. As observed at 37°C, this decrease in $[Ca^{2+}]_i$ brought about by the removal of shFF was also found to have similar kinetic attributes to the $[Ca^{2+}]_i$ decrease that immediately follows the initial transient response (Figure 40). This rapid decrease however appeared to occur at a slower rate at 26°C when compared to the $[Ca^{2+}]_i$ response to shFF removal at 37°C (Figure 43). Detailed analysis of the occurrence of a transient response and $[Ca^{2+}]_i$ depletion after shFF wash off from 7 experiments (1099 cells) revealed a relationship between both responses ($P<0.01$; χ^2), suggesting a significant link between the transient $[Ca^{2+}]_i$ increase following exposure to 100% shFF and the subsequent rapid decrease upon removal of shFF from the cell's extracellular environment. A significant linkage was also found between the occurrence of a sustained $[Ca^{2+}]_i$ response and subsequent rapid depletion upon removal of shFF from the cell's extracellular environment ($P<0.01$; χ^2).

Scatter plot analyses of the above response data from each responding cell were also carried out. Average amplitudes of the percentage change in normalised fluorescence intensities corresponding to selected points within the transient peak, sustained plateau, and $[Ca^{2+}]_i$ decrease following shFF removal were plotted for each responding cell (See Figures 41 and 42). This revealed positive correlation between the transient $[Ca^{2+}]_i$ response phase and the rapid $[Ca^{2+}]_i$ decrease following shFF removal (Figure 41). Similarly, a positive correlation was also observed between the sustained $[Ca^{2+}]_i$ response phase and the rapid $[Ca^{2+}]_i$ decrease upon shFF removal. (Figure 42).

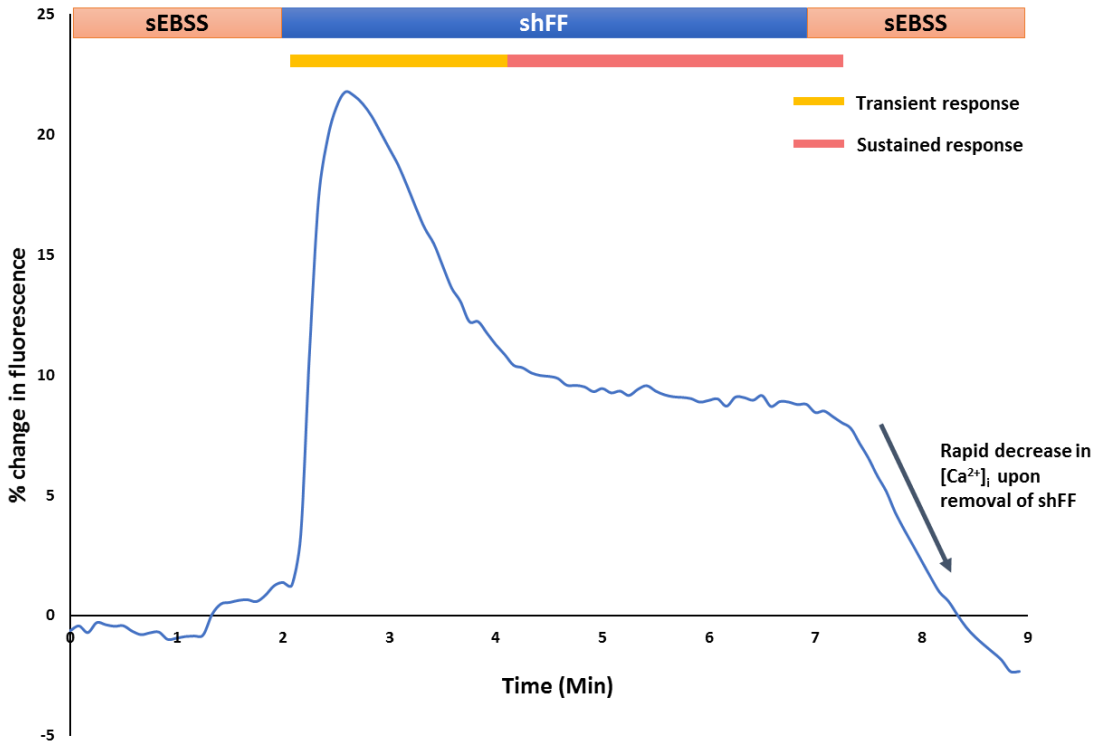


Figure 38 A rapid depletion in $[Ca^{2+}]_i$ is observed in 1099 responding cells at 26°C following the removal of shFF from the perfusing medium (wash off). This is characterised by a decrease in fluorescence intensity by up to 2% below initial baseline levels prior to shFF stimulation.

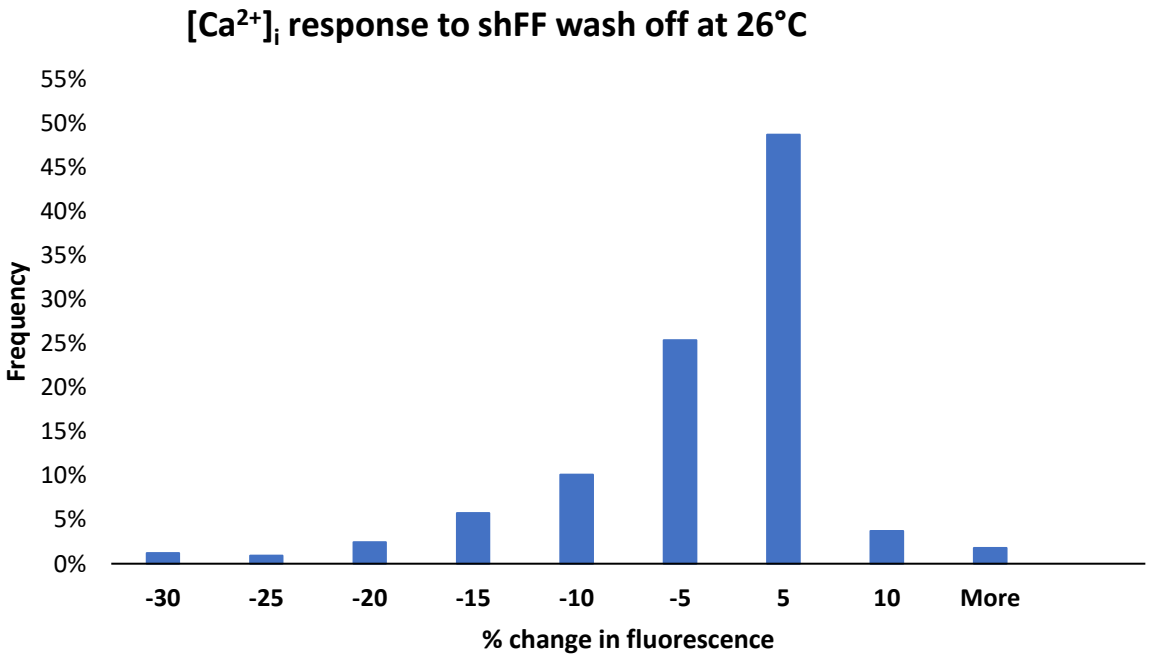


Figure 39 Summary amplitude distribution for $[Ca^{2+}]_i$ responses (26°C) at time of shFF wash off for R_{tot} from 7 experiments. Data obtained from sub-population of cells significantly showing a response to the removal of extracellular shFF.

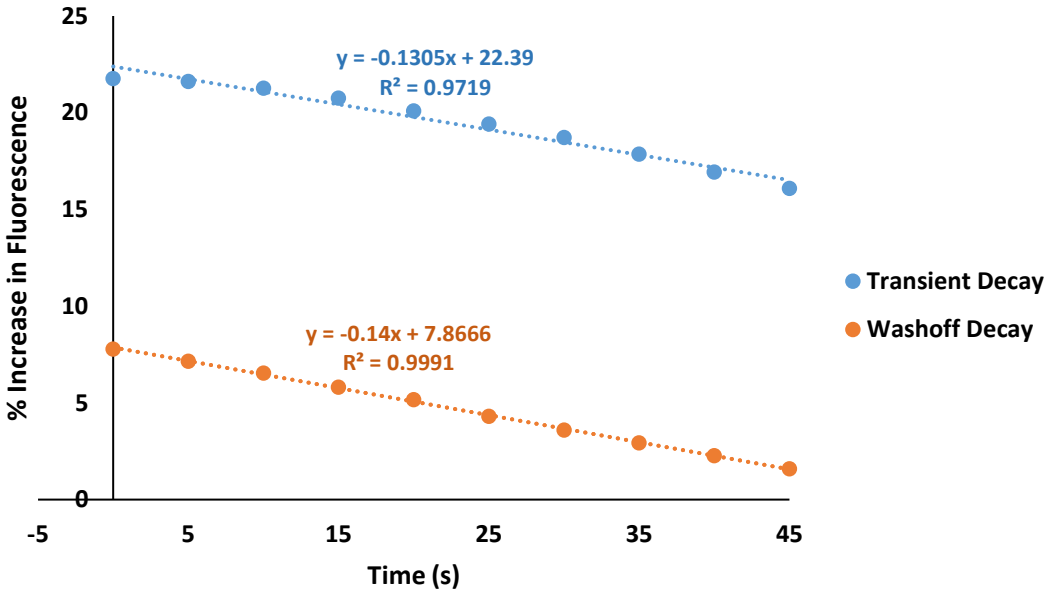


Figure 40 Comparison of the rapid $[Ca^{2+}]_i$ decrease that immediately follows the initial transient response to shFF and shFF wash off respectively at 26°C. Correlation analysis of 1099 responding cells from 7 experiments revealed a positive correlation (R=0.98) between the $[Ca^{2+}]_i$ decrease immediately following the initial transient response and the rapid $[Ca^{2+}]_i$ decrease immediately after shFF removal (wash off).

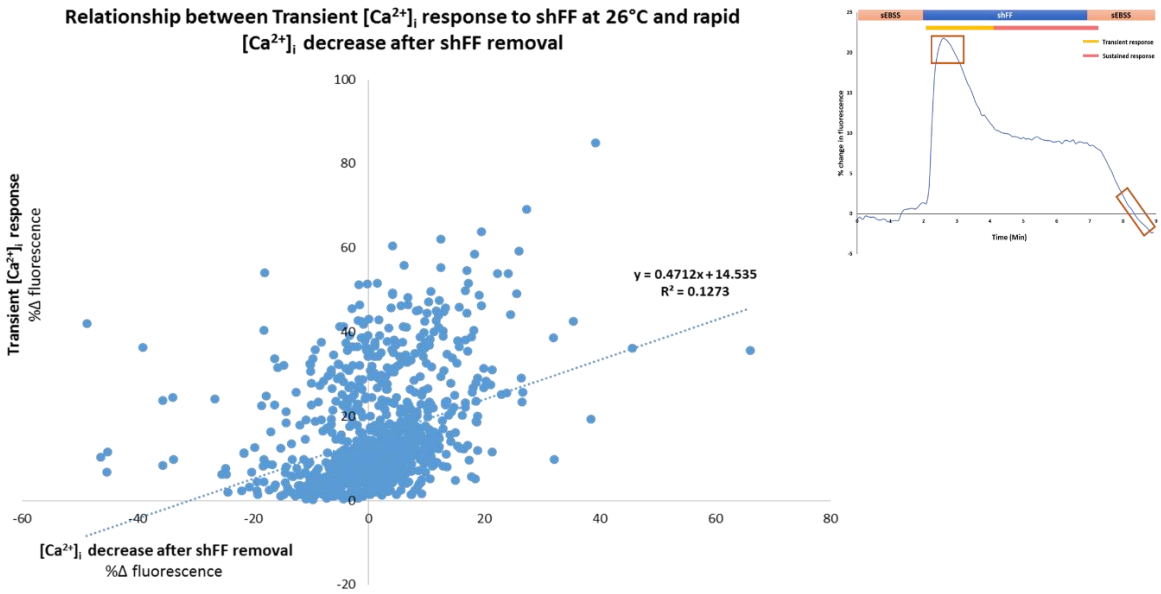


Figure 41 Relationship between transient $[Ca^{2+}]_i$ increase and rapid $[Ca^{2+}]_i$ decrease following the treatment of spermatozoa with shFF at 26°C and shFF wash off respectively. Analysis was carried out using data from all cells in 7 experiments (1077 cells) that showed both a transient response and a rapid $[Ca^{2+}]_i$ decrease in response to shFF wash off. Highlighted sections on the response curve (upper right) shows the areas within each response phase for each cell that were selected and averaged for scatter plot analysis.

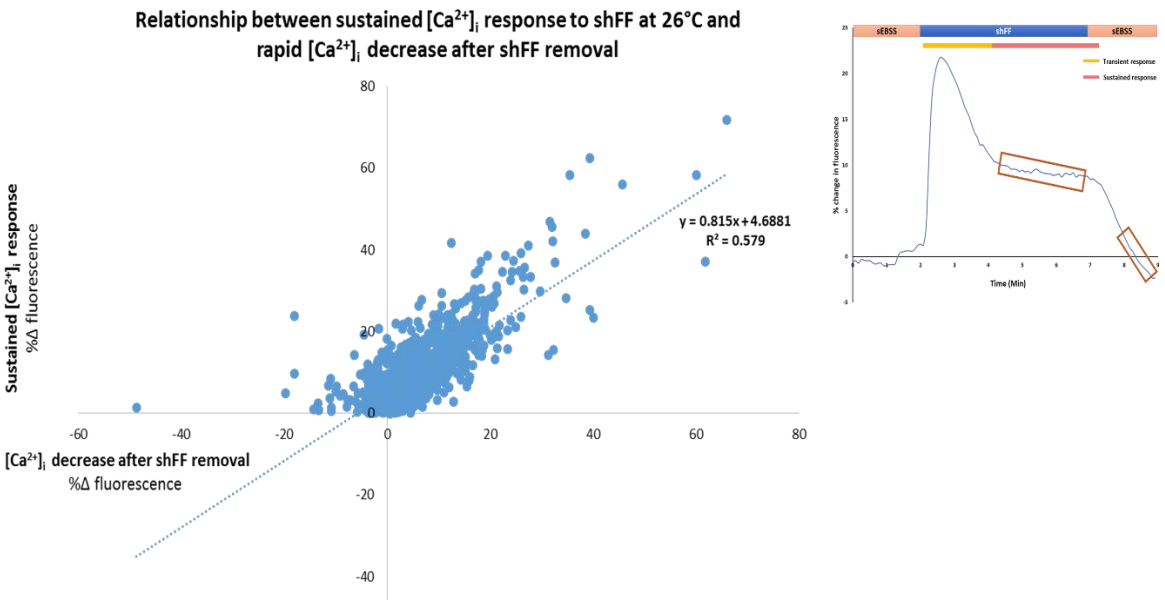
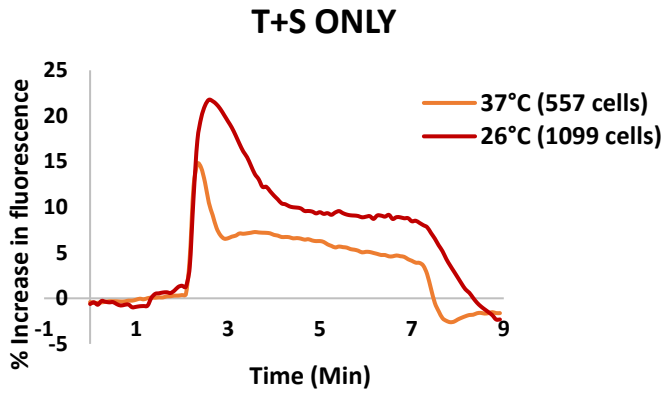
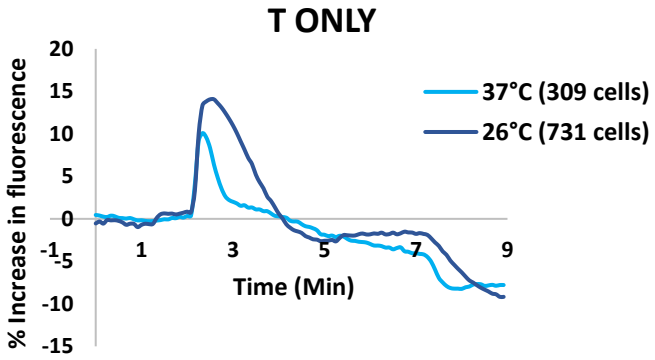


Figure 42 Relationship between sustained $[Ca^{2+}]_i$ responses and rapid $[Ca^{2+}]_i$ decrease following the treatment of spermatozoa with shFF at 26°C and shFF wash off respectively. Analysis was carried out using data from all cells in 7 experiments (1171 cells) that showed both a sustained response and a rapid $[Ca^{2+}]_i$ decrease in response to shFF wash off. Highlighted sections on the response curve (upper right) shows the areas within each response phase for each cell that were selected and averaged for scatter plot analysis.

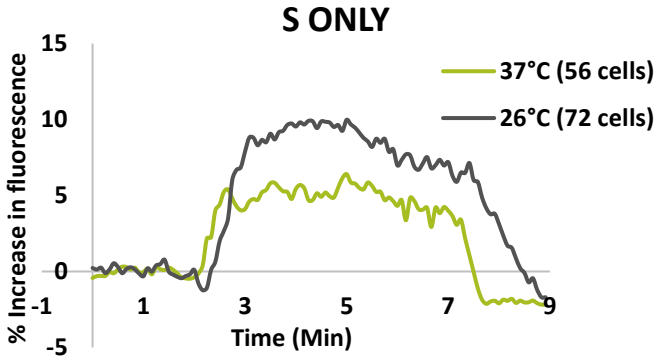
A



B



C



D

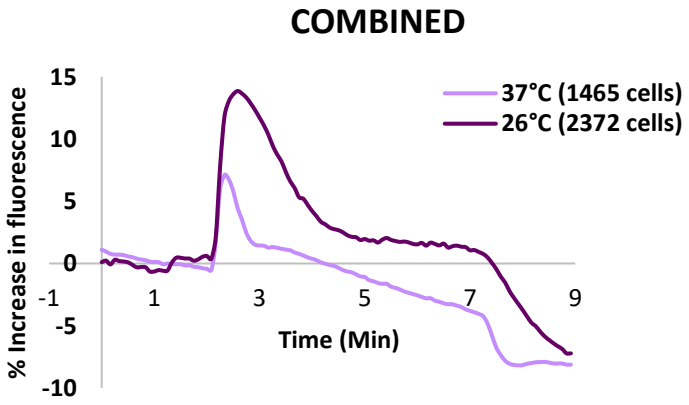


Figure 43 Comparison of Mean [Ca2+]i responses at 26°C and 37°C. (a) T+S only (b) T only (c) S only (d) Combined cell population of T+S only, T only, S only and non-responders.

3.3.2 Sperm $[Ca^{2+}]_i$ response to shFF following pre-exposure

Having studied the effects of shFF on sperm $[Ca^{2+}]_i$, an interesting question is the nature of change in $[Ca^{2+}]_i$ kinetics as the sperm continuously travel in and out of follicular fluid gradients as they traverse the female reproductive tract. Some live cell calcium imaging experiments were carried out in an attempt to study this. Preliminary data (541 sperm cells, n=2 experiments) showed a quicker onset of the second transient $[Ca^{2+}]_i$ response to 100% shFF following a prior exposure to the same concentration of shFF, when compared to a similar transient without pre-exposure (Figure 44). The second transient was also lower in amplitude by approximately 5% and shorter in duration by 25-30 seconds. Further preliminary data comparing 3.2μM progesterone transients with and without prior exposure (325 sperm cells, n=2 experiments) also showed a quicker second transient. However upon comparison, the amplitude and duration of both transients appeared to be similar (Figure 45).

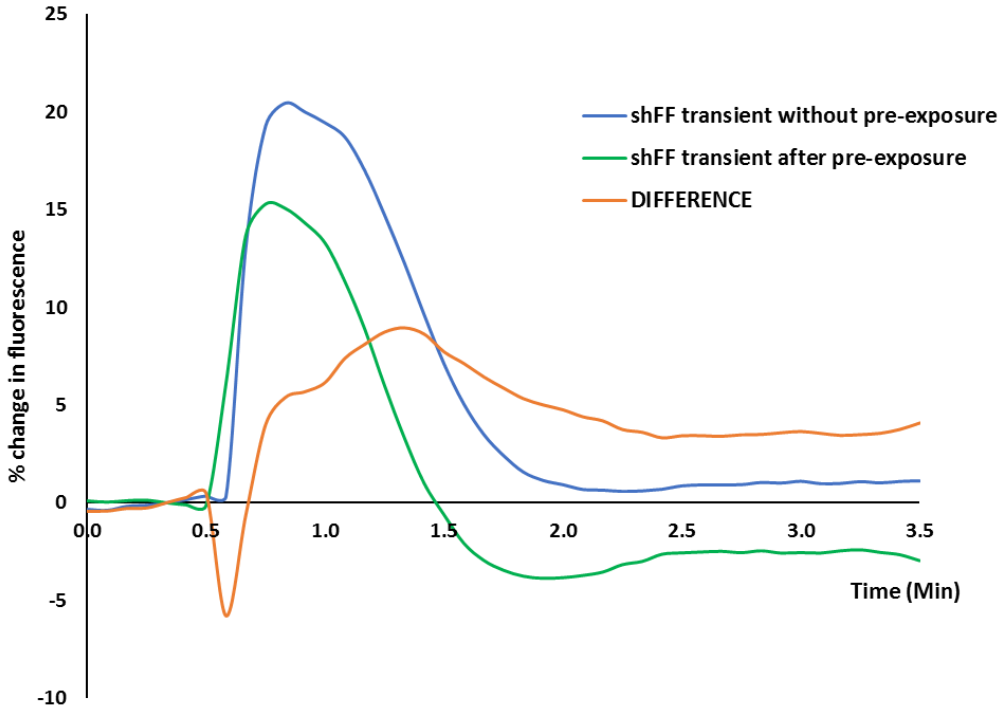


Figure 44 Comparison of two transient $[Ca^{2+}]_i$ responses (R_{tot}) each resulting from a 15s stimulation of spermatozoa with 100% shFF at 37°C, both of which were 2min 45s apart.

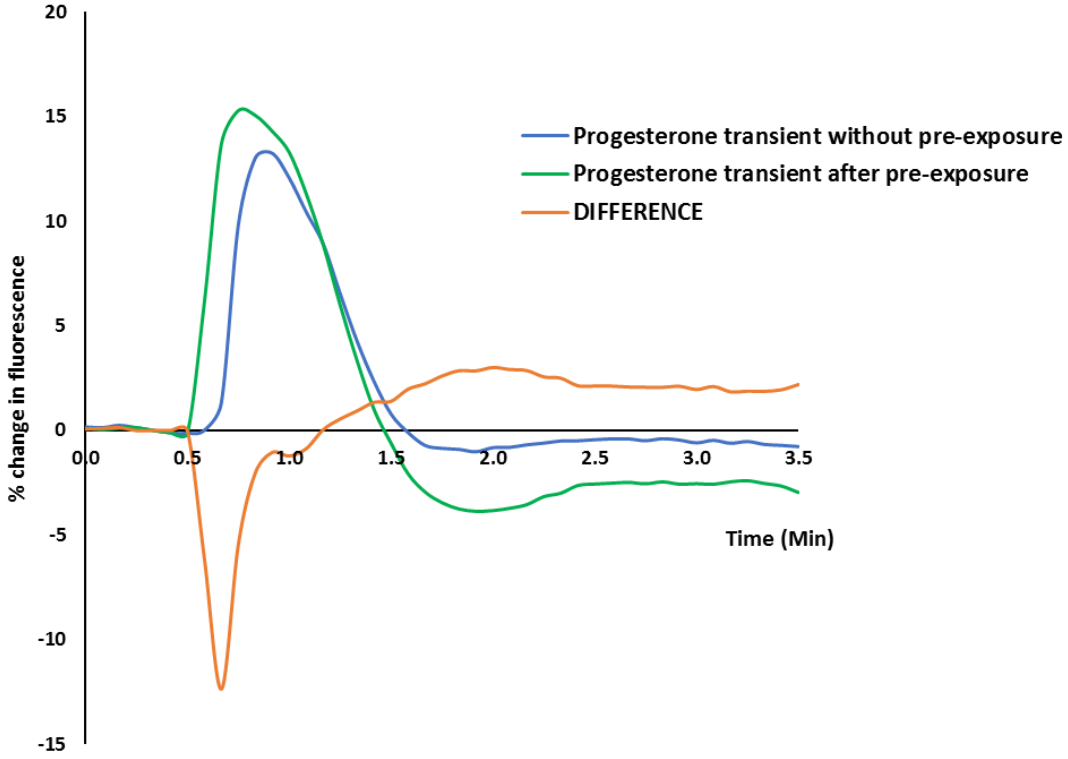


Figure 45 Comparison of two transient $[Ca^{2+}]_i$ responses (Rtot) each resulting from a 15s stimulation of spermatozoa with 3.2μM progesterone at 37°C, both of which were 2min 45s apart.

3.4 DISCUSSION

The elevation of human sperm $[Ca^{2+}]_i$ in response to the complex follicular fluid steroid hormone mix was characterised in chapter 2. This was demonstrated at two different working temperatures – 37°C and 26°C representative of physiological and standard laboratory temperatures respectively. Using the same experimental setup, this chapter presents further characterisation data showing human sperm $[Ca^{2+}]_i$ kinetics corresponding to the depletion and removal of shFF from the sperm's extracellular environment following a short exposure to shFF. This provides a clearer picture of the underlying sperm $[Ca^{2+}]_i$ signalling events associated with dynamic changes in follicular fluid steroid hormone concentrations which may be encountered by sperm in the female reproductive tract.

3.4.1 Effects of shFF depletion on sperm $[Ca^{2+}]_i$ kinetics

With the biphasic elevation of human sperm $[Ca^{2+}]_i$ in response to shFF stimulation already demonstrated at 37°C (see chapter 2), the sustained phase of this $[Ca^{2+}]_i$ response was followed by the depletion of shFF from the sperm's extracellular medium via perfusion with standard media. This was a novel attempt at understanding the sperm $[Ca^{2+}]_i$ signalling events associated with dynamic changes in extracellular steroid hormone concentrations which may be encountered by sperm as they swim through differing follicular fluid mixes in the female reproductive tract. As expected, removal of shFF resulted in a rapid decrease in $[Ca^{2+}]_i$ characterised by a sharp decrease in fluorescence intensity up to 2% below baseline levels (from prior to the addition of shFF to the sperm's extracellular medium). The observed decrease in sperm $[Ca^{2+}]_i$ is likely to comprise a number of factors. Firstly, the deactivation of the CatSper channel

brought about by the extracellular depletion of shFF containing progesterone and possibly other CatSper-activating steroids. This is in agreement with the findings of Lishko *et al.* (2011) and Smith *et al.* (2013), who demonstrated using the patch-clamp technique that the activation of the CatSper channel by progesterone is fully reversible. Secondly, downstream of CatSper deactivation, the observed drop in $[Ca^{2+}]_i$ levels as shown in Figure 33 will directly result from the Ca^{2+} clearing activity of the spermatozoa. According to Miller *et al.* (2016), the inactivation of the CatSper channel is brought about by the replenishment of endogenous 2AG levels following the removal of extracellular stimulus, thereby bringing sperm Ca^{2+} influx to a halt. Using patch-clamp studies, the rate of inactivation of the CatSper channel following the removal of extracellular shFF could be determined and compared with the rate of inhibition of CatSper by 2AG in order to establish the physiological significance of this mechanism. This could also be done at both working temperatures of 37°C and 26°C to investigate the presence of a temperature effect on the rate of CatSper inactivation.

The proposed sequestration of sperm Ca^{2+} is also likely to be based upon the pumping of cytosolic Ca^{2+} to the extracellular environment and into intracellular stores with PMCAs and SPCAs being implicated (Correia *et al.*, 2015). The localisation of PMCAs (Andrews *et al.*, 2015) and SPCAs (Harper *et al.*, 2005) in human sperm suggest possible roles in calcium clearance however, further studies need to be carried out in order to extensively characterise the Ca^{2+} homeostatic mechanisms modulated by these channels. In all eukaryotic cells, the transport of Ca^{2+} across the plasmalemma and intracellular store membranes is an energy-dependent process that requires the use of ATP (Burdakov *et al.*, 2005; Petersen *et al.*, 2005). Therefore, characterising the consumption of ATP

during shFF-induced sperm calcium signalling may help to further understand the relationship between sperm cytosolic Ca^{2+} concentrations, Ca^{2+} ATPase activity and ATP turnover. However in this case, sperm ATP levels may be monitored during the separate signalling events of shFF-induced $[Ca^{2+}]_i$ response and response decay upon shFF removal. This would in turn provide a clearer picture as to the difference between both signalling events (i.e. biphasic $[Ca^{2+}]_i$ response and subsequent response decay) with regards to Ca^{2+} ATPase activity. To that effect, further experiments may be carried out using a bioluminescence assay based on the luciferin-luciferase reaction as described by Gottlieb *et al.* (1987), modified to investigate ATP utilisation in sperm at closely marked time points following shFF treatment and shFF removal.

Previous studies using other eukaryotic cell types have characterised the Ca^{2+} turnover/clearance rates in the different P-Type Ca^{2+} ATPases under different experimental conditions (Lytton *et al.*, 1992; Missiaen *et al.*, 2002; Dode *et al.*, 2005; Duman *et al.*, 2008; Lamboley *et al.*, 2014). Similar data from human sperm could prove useful in further identifying and characterising the Ca^{2+} ATPases likely to be involved in shFF-induced CICR, as well as Ca^{2+} clearance in response to shFF removal. Further studies may be carried out with an experimental design centred on monitoring overall sperm Ca^{2+} turnover rates in the presence of Ca^{2+} ATPase inhibitors. Briefly, the rates of sperm Ca^{2+} turnover corresponding to the presence and absence of extracellular shFF stimulus are first of all characterised. This will then be followed by measuring the changes in overall sperm Ca^{2+} turnover following the application of Ca^{2+} ATPase inhibitors with selective affinity for SERCAs and PMCAs such as thapsigargin (Harper *et al.*, 2005) and caloxin (Pande *et al.*, 2008) respectively. The data obtained may help

characterise Ca^{2+} clearance and Ca^{2+} ATPase activity in the presence of these inhibitors, thus bringing us closer towards fully understanding the identity and roles of certain sperm Ca^{2+} ATPases in Ca^{2+} clearance.

Physiologically, the drop in fluorescence intensity below initial baseline levels may be as a result of a faster rate of $[Ca^{2+}]_i$ clearance downstream of CatSper deactivation possibly triggered by the previous exposure to shFF and the resulting high $[Ca^{2+}]_i$ levels. The absence of significant dye leakage at the population level also suggests that the rapid decrease in $[Ca^{2+}]_i$ is not due to the loss of acrosomal calcium, characteristic of the acrosome reaction. With a shift in focus to the experimental setup in which the labelling of spermatozoa is consistent as possible across samples, the photo bleaching of the fluophore (Calcium Green 1-AM) during exposure to excitation wavelengths may contribute to a steady decline in fluorescence as observed in non-responders. However, this does not explain the rapid decrease in fluorescence intensity observed across all experiments.

The sperm $[Ca^{2+}]_i$ kinetics associated with extracellular shFF depletion following pre-exposure was also characterised at a standard laboratory temperature of 26°C. As observed at 37°C, the removal of extracellular shFF resulted in a rapid decline in $[Ca^{2+}]_i$ up to 2% below initial baseline levels. However in comparison to the $[Ca^{2+}]_i$ kinetics observed at 37°C, the sperm populations in the experiments conducted at 26°C exhibited a slower rate of $[Ca^{2+}]_i$ decrease requiring the entire duration of the shFF wash off (2 mins) to reach the lowest level of $[Ca^{2+}]_i$. The observed rates of $[Ca^{2+}]_i$ depletion at 37°C and 26°C are indicative of the different speeds at which sperm Ca^{2+} clearance occurs at these temperatures as reflected in the speed of $[Ca^{2+}]_i$ signal decay immediately

following the transient $[Ca^{2+}]_i$ response to shFF (see Chapter 2). A direct physiological effect of temperature on the shFF-induced sperm Ca^{2+} responses presents itself in the signalling patterns observed at the end of the shFF wash off period. In particular, the spermatozoa in the experiments conducted at 37°C appeared to re-establish a new $[Ca^{2+}]_i$ baseline within the duration of the shFF wash off (2 mins) while this was not observed at 26°C as the $[Ca^{2+}]_i$ decrease still appeared to be ongoing. In support of this hypothesis, studies on other eukaryotic cell types suggest that changes in temperature can induce significant functional changes in Ca^{2+} ATPase activity (Masuda and de Meis, 1977; Rubtsov *et al.*, 1986; Thomas and Karon, 1994; Mackiewicz and Lewartowski, 2006). It is therefore likely that a temperature effect is observed in human sperm PMCAs and SPCAs influencing the modulation of Ca^{2+} active transport and passive leakage pathways.

With spermatozoa possibly swimming in and out of follicular fluid in the female reproductive tract, the distance covered during the rapid decrease in $[Ca^{2+}]_i$ associated with extracellular shFF depletion is of significant interest. The average time taken for the spermatozoa to reach minimal $[Ca^{2+}]_i$ levels following extracellular shFF depletion were 45s and 120s at 37°C and 26°C respectively. With the rapid swimming speed of human spermatozoa at about 5mm/min (Mortimer and Swan, 1995), this suggests that a sperm cell could cover a distance of approximately 4mm under physiological conditions in the female reproductive tract. However with sperm $[Ca^{2+}]_i$ signalling key to the modulation of sperm motility and hyperactivation, it may be that such a rapid decline in $[Ca^{2+}]_i$ levels consequently induces a change in sperm motility parameters with swimming speed likely to be altered.

At both working temperatures (37°C and 26°C), a statistically significant linkage was established between the occurrence of the shFF-induced biphasic sperm Ca^{2+} influx and the rapid $[Ca^{2+}]_i$ decrease following the removal of shFF. This suggests that the activation of the Ca^{2+} clearance mechanism responsible for the rapid decline in sperm $[Ca^{2+}]_i$ is reliant on the prior initiation of sperm Ca^{2+} influx. At 37°C, the negative correlation observed between the amplitudes of the transient $[Ca^{2+}]_i$ influx and the rapid $[Ca^{2+}]_i$ decline following shFF removal suggests that many of the sperm cells with larger transient $[Ca^{2+}]_i$ responses exhibited the tendency for a subsequent $[Ca^{2+}]_i$ decrease below baseline levels whilst the cells with much smaller $[Ca^{2+}]_i$ transients managed to maintain $[Ca^{2+}]_i$ at or above baseline levels following shFF removal. Interestingly at 26°C, a positive correlation between the amplitudes of the transient Ca^{2+} influx and the rapid $[Ca^{2+}]_i$ decline following shFF removal was observed. Whilst the change in temperature may have a role to play in this observed difference in correlation, it may be useful to carry out some live cell imaging experiments using fura-2/AM reporter dye to obtain ratiometric sperm $[Ca^{2+}]_i$ data. Using this data, a more detailed correlation analysis could be carried out taking into account the relationship between the response amplitudes and the sperm's absolute resting $[Ca^{2+}]_i$.

Preliminary data showing a quicker onset of sperm $[Ca^{2+}]_i$ responses to shFF or progesterone following pre-exposure to the same may be as a result of the latter $[Ca^{2+}]_i$ response occurring with the CatSper channel not completely deactivated from the initial exposure. However due to the very small sample size in each set of experiments (n=2), further experiments would be required to validate this trend and more importantly, fully investigate the effects of pre-exposure on sperm $[Ca^{2+}]_i$ amplitude and kinetics. In

addition to studying the effects of pre-exposure, it may also be useful to characterise the effects of spatio-temporal exposure to changing concentrations of extracellular shFF as likely encountered in the female reproductive tract where spermatozoa experience a possible stochastic drift from higher to lower concentrations (and vice versa) of follicular fluid steroid hormones likely influenced by the different diffusion rates of follicular fluid gradients that exist in different regions of viscosity (Kirkman-Brown and Smith, 2011). Similar to the study carried out by Harper *et al.* (2004), human sperm could be stimulated with shFF concentration gradients in an attempt to simulate the earlier described exposure to changing concentrations of follicular fluid steroid hormones in the female tract. The resulting implications on sperm Ca^{2+} influx Ca^{2+} clearance would in turn be characterised. Furthermore, the effects of decreasing shFF concentration gradients could be studied and the data compared with the sperm $[Ca^{2+}]_i$ data presented in this chapter.

Possible future research

The data obtained from this study provides a glimpse of the possible physiological changes that occur in human sperm as they are sequentially exposed to extracellular environments with and without follicular fluid steroid hormones. Follow up studies could be carried out using the present shFF wash off model to further characterise the intracellular responses that occur during the depletion of extracellular shFF. The 'temperature effect' hypothesis may be further investigated by characterising sperm $[Ca^{2+}]_i$ during shFF wash off with incremental changes in temperature, starting from 26°C all the way up to 37°C. This may provide a possible correlation between increase in temperature and sperm $[Ca^{2+}]_i$ decrease during shFF wash off. In addition, the wash

off phase of the experiments conducted at 26°C may be prolonged in order to see whether new $[Ca^{2+}]_i$ baseline levels will be established. The $[Ca^{2+}]_i$ kinetics during shFF wash off may be further characterised using patch clamp studies, with particular focus on the activity of sperm ion channels during the rapid $[Ca^{2+}]_i$ decrease. Furthermore, a closer look may be taken at the acrosomal dynamics of the sperm population in real time during the shFF wash off period. Using the FM4-64 fluorescent dye, the real time monitoring of acrosomal status/reaction could be carried out in combination with Ca^{2+} imaging as was demonstrated by Sánchez-Cárdenas *et al.* (2014). In addition to the real time acrosomal data obtained, this proposed study may also answer questions on the possible contribution of acrosomal Ca^{2+} leakage to the $[Ca^{2+}]_i$ decrease observed during the depletion of extracellular shFF. It will also be of interest to explore the $[Ca^{2+}]_i$ responses that may be induced by re-introducing shFF to the sperm's extracellular environment immediately following the wash off period. The data obtained from this characterisation study will provide a fuller picture of the $[Ca^{2+}]_i$ signalling that occurs *in vivo* when spermatozoa swim in and out of the follicular fluid steroid environment.

In conclusion, maintaining sperm calcium homeostasis in the presence of agonist-induced Ca^{2+} influx involves a complex dynamic characterised by Ca^{2+} transport and clearance mechanisms which are yet to be fully characterised. With regards to follicular fluid steroid agonists, the effects of progesterone on human sperm $[Ca^{2+}]_i$ signalling have been widely studied. There however remains little or no characterisation data from other steroid hormone groups present in follicular fluid. Below is a proposed model for sperm calcium signalling as modulated by the addition and removal of shFF (Figure 46). In this model, shFF is portrayed as a progesterone-containing stimulus that modulates sperm

calcium signalling via the progesterone-activated endocannabinoid signalling pathway (Miller *et al.*, 2016). This portrayal is due to the absence of data on the signalling pathways through which other steroid hormone groups possibly contribute to sperm Ca^{2+} influx. The removal of the shFF stimulus results in the initiation of rapid Ca^{2+} clearance pathways which are yet to be fully understood.

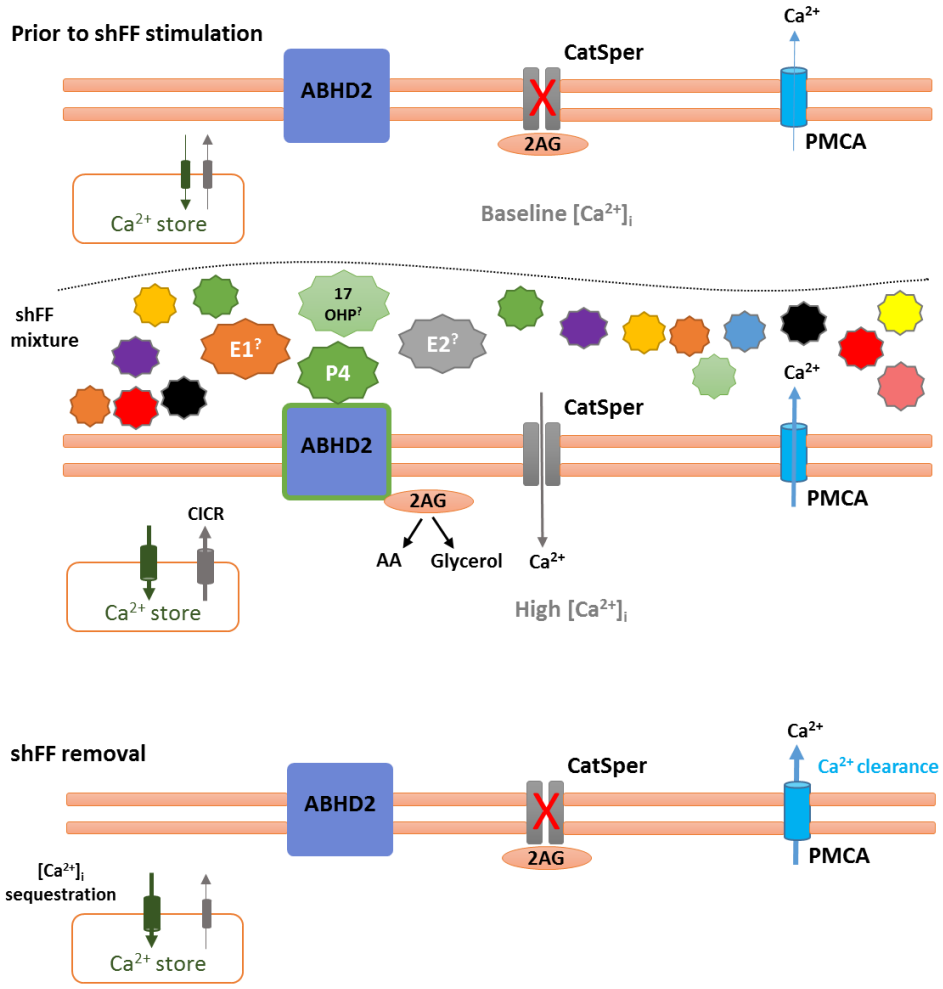


Figure 46 Illustrated summary of proposed model for human sperm Ca^{2+} signalling in the presence and absence of shFF stimulus. Progesterone (P4) present in shFF activates ABHD2 which in turn hydrolyses the endogenous CatSper inhibitor-2AG, resulting in Ca^{2+} influx via CatSper and subsequent CICR from intracellular store(s). Upon removal of shFF, PMCA rapidly pumps Ca^{2+} to the extracellular space, complemented by the sequestration of $[Ca^{2+}]_i$ to the intracellular store(s).

CHAPTER 4

FOLLICULAR FLUID STEROID HORMONES AND HUMAN SPERM III – EFFECTS ON HUMAN SPERM KINESIS

4.1 INTRODUCTION

4.1.1 An overview of sperm kinetic interactions in the female tract

In mammalian reproduction, studies have revealed the existence of dynamic interaction mechanisms between sperm and the female reproductive tract which to varying extents facilitate the sperm's journey to the oocyte. So far three of such mechanisms have been proposed namely chemotaxis - swimming up a chemoattractant's concentration gradient, rheotaxis- swimming against a current of fluid, and thermotaxis- swimming up a temperature gradient. Despite the fact that there is little or no linkage between these three mechanisms due to the specificity of the different stimuli involved, sperm kinesis being an essential feature of all three tactile movements is a shared similarity (Perez-Cerezales *et al.*, 2015b).

Sperm chemotaxis

Chemotaxis is a dynamic feature of cell physiology characterised by the migration of a cell along a shallow gradient of biochemical stimuli that is less than 5% difference between the anterior and posterior of the cell (Wu, 2005). Cell motility and directionality are the two distinct components of chemotaxis, both of which are independent but interconnected and are regulated by external stimuli otherwise known as chemoattractants. In reproductive physiology, sperm chemotaxis is known to play a significant role in the migration of spermatozoa through the female tract towards the vicinity of the oocyte (Kaupp *et al.*, 2008). Despite the key role of chemotaxis in the process of fertilisation, a lot is yet to be known about the chemotaxis of mammalian sperm with published literature only dating back to the last two and a half decades. Studies by Villanueva-Diaz *et al.* (1990) and Ralt *et al.* (1991) provided initial evidence for mammalian sperm chemotaxis. They reported that follicular fluid contains substances

that stimulate human sperm chemotaxis *in vitro*. Studies have since been carried out in order to further understand this physiological phenomenon in mammals (Ralt *et al.*, 1994; Eisenbach, 1999; Oliveira *et al.*, 1999; Fabro *et al.*, 2002; Sun *et al.*, 2005; Eisenbach and Giojalas, 2006; Teves *et al.*, 2009; Armon and Eisenbach, 2011; Armon *et al.*, 2014; Caballero-Campo *et al.*, 2014). In order for fertilisation to occur in the mammalian female tract, the role of chemotaxis implies that a swimming sperm cell is guided upwards a gradient of chemoattractant(s) secreted by the oocyte and its surrounding cumulus cells (Sun *et al.*, 2005). This ultimately relies on the availability of chemotactically responsive spermatozoa in the female reproductive tract. Evidence from studies on human and rabbit spermatozoa led to the conclusion that only capacitated spermatozoa are chemotactically responsive (Cohen-Dayag *et al.*, 1994; Cohen-Dayag *et al.*, 1995; Eisenbach, 1999; Fabro *et al.*, 2002; Giojalas *et al.*, 2004). This is based on the similarities observed between the fractions of the sperm population that are capacitated and chemotactically responsive such as the size, similarly short lifespan and continuous replenishment of both sub-populations. The continuous replenishment of these sub-populations in the female tract is dependent on the fact that different spermatozoa become capacitated and chemotactically responsive at different times (Cohen-Dayag *et al.*, 1994). In mammals that ovulate periodically (e.g. human), this ensures that there are capacitated and chemotactically competent sperm cells present in the female tract for extended periods (Cohen-Dayag *et al.*, 1994; Giojalas *et al.*, 2004). Physiological changes in sperm such as capacitation and hyperactivated motility make the study of sperm chemotaxis quite challenging due to the following reasons: (i) the small sub-population of capacitated cells (i) the disruption of forward progressive movement by vigorous whiplash flagellar movement, characteristic of hyperactivated

sperm. The latter results in the 'trapping' of spermatozoa in a small area, a phenomenon that may be mistaken for sperm accumulation resulting from a true chemotactic response (Eisenbach and Giojalas, 2006; Kaupp *et al.*, 2008).

Attempts have been made to study the behavioural and molecular mechanisms of mammalian sperm chemotaxis. Although these remain unclear, a plethora of chemoattractants and chemokinetic receptors have been implicated (Eisenbach and Giojalas, 2006; Naz and Sellamuthu, 2006; Wehling *et al.*, 2007). In line with previously published evidence for the role of follicular fluid in mammalian sperm chemotaxis, further *in vitro* studies proposed that progesterone-a key follicular fluid constituent, is a chemoattractant of major significance (Sliwa, 1995; Villanueva-Diaz *et al.*, 1995; Wang *et al.*, 2001). Progesterone at micromolar concentrations is secreted by cumulus cells and is present in follicular fluid forming a downstream chemoattractant gradient (Correia *et al.*, 2007; see Table 4).

Conversely, evidence from an extensive study by Teves *et al.* (2006) suggests that progesterone at picomolar concentrations functions as a chemoattractant for rabbit and human spermatozoa. Whilst there is evidence for a chemotactic response of spermatozoa to picomolar progesterone, it is unlikely that the chemoattractant gradient present in the vicinity of the oocyte, responsible for guiding spermatozoa toward the oocyte is made up of picomolar progesterone. The data obtained from the steroid hormone profiling of human follicular fluid further supports this hypothesis with progesterone found to be present at micromolar concentrations (see Table 4).

The precise signalling pathway(s) through which progesterone evokes a chemotactic response in mammalian sperm remains unknown however the occurrence of sperm

chemotaxis is believed to coincide with a rise in sperm $[Ca^{2+}]_i$ (Yoshida and Yoshida, 2011). This brings about the question as to whether sperm $[Ca^{2+}]_i$ dynamics mediate chemotactic responses in response to progesterone. Progesterone has been shown to stimulate a biphasic elevation of human sperm $[Ca^{2+}]_i$ (Kirkman-Brown *et al.*, 2000; Harper *et al.*, 2003), thus suggesting a possible relationship between progesterone-induced calcium signalling and chemotactic signalling pathway(s) in mammalian spermatozoa (Harper *et al.*, 2004; Publicover *et al.*, 2008; Teves *et al.*, 2009). It is however important to note that the sub-population of spermatozoa that exhibit an $[Ca^{2+}]_i$ response to progesterone far outnumber the fraction of spermatozoa considered to be chemotactically active. In consideration of these proposed signalling relationships, the recently characterised candidate for the non-genomic progesterone receptor is once again implicated (Miller *et al.*, 2016). The progesterone-induced activation of ABHD2 in the sperm's plasma membrane results in the depletion of endogenous CatSper inhibitors thus resulting in the activation of the CatSper channel and consequently the influx of Ca^{2+}.

In addition to steroid hormones, odorants are another category of chemoattractants that have been examined. This is due to the presence of olfactory receptors that have been identified in mammalian testis and sperm (Walensky *et al.*, 1995; Vanderhaeghen *et al.*, 1997; Spehr *et al.*, 2003; Spehr *et al.*, 2004; Fukuda and Touhara, 2006). Two non-physiological odorants-lyral and burgeonal were suggested to be chemoattractants for mouse and human spermatozoa respectively (Spehr *et al.*, 2003; Fukuda *et al.*, 2004). In these studies, lyral and burgeonal were reported to be agonists of MOR23 (mouse testicular olfactory receptor) and hOR17-4 (human testicular olfactory receptor)

respectively. Furthermore, the induction of sperm Ca^{2+} entry and motility by these odorants was proposed to be via G protein-coupled receptor (GCPR) and cAMP signalling pathways (Spehr *et al.*, 2004). However, a later study by Brenker *et al.* (2012) provided evidence to the contrary by demonstrating that CatSper functions as a polymodal chemosensor in human sperm. A wide range of odorants including burgeonal were found to activate CatSper without the involvement of GCPRs and cAMP (Brenker *et al.*, 2012).

The non-physiological nature of the above-mentioned chemotactic agonists suggests that there are substances present in the female reproductive tract and potentially in follicular fluid that may also function as physiological chemoattractants. The biochemical complexity of follicular fluid in the mammalian female reproductive suggests that there may be other chemoattractants present which are yet to be identified. Follicular fluid constituents such as proteins, amino acids and nitric oxide may be of physiological significance in mammalian sperm chemotaxis (Kaupp *et al.*, 2008).

Sperm rheotaxis and thermotaxis

Rheotaxis in mammalian spermatozoa is a physiological response characterised by the ability to sense a flow of fluid in the extracellular environment and respond with a change of directional path to swim against the fluid flow (Miki and Clapham, 2013). This has so far been demonstrated in capacitated and non-capacitated sperm from mouse and humans. Recently, Miki and Clapham (2013) characterised this response by demonstrating significant differences between capacitated and non-capacitated sperm in response to fluid velocity changes. When the extracellular fluid viscosity was raised to a level that mimics that of the oviductal lumen milieu, non-capacitated sperm were

observed to move in a more planar path which in the physiological context increases their chances of attachment to the oviductal epithelium. Capacitated sperm on the other hand responded by rotating around their longitudinal axis, faster than the non-capacitated sub-population. This was proposed to facilitate the detachment of capacitated spermatozoa from the oviductal surface, enabling them to swim into the main fluid current in the oviduct. Further data from this study also suggests that rheotaxis occurs in at least 50% of the sperm population. This differs from thermotactic and chemotactic responses which occur in a much smaller fraction of the sperm population. In an attempt to further understand the precise mechanisms behind this tactic response, studies need to be carried out in an attempt to identify the receptors that function in a rheosensory role.

The mammalian female reproductive tract consists of different areas with marginal differences in temperature which in turn may evoke thermotactic responses. Studies carried out on some mammalian species have shown that the fertilisation site in the oviduct is 1 to 2°C warmer than the site of the sperm reservoir (David *et al.*, 1971; Hunter and Nichol, 1986). The response of spermatozoa to changes in the extracellular temperature gradient has been characterised *in vitro* (Bahat *et al.*, 2003; Bahat *et al.*, 2012; Boryshpolets *et al.*, 2015). This was found to occur in a small fraction of the sperm population (~10%) and was accompanied by a change in the velocity and linearity of sperm movement. A recent study by Perez-Cerezales *et al.* (2015a) implicated opsins (photoreceptors) as having a thermosensory role in mammalian sperm thermotaxis however, the precise signalling mechanism(s) responsible for mediating thermotactic responses remain unknown. In view of the evidence available to support sperm

thermotaxis, there currently appears to be a lack of reproducibility in the published evidence supporting the involvement of thermotaxis as a mammalian sperm interaction mechanism in the female reproductive tract. This is based on the fact that nearly all published studies to date on mammalian sperm thermotaxis spanning that last one and a half decades come from a single research group led by Prof. Michael Eisenbach at the Weizmann Institute of Science, Israel (Bahat *et al.*, 2003; Eisenbach and Giojalas, 2006; Bahat *et al.*, 2012; Boryshpolets *et al.*, 2015; Perez-Cerezales *et al.*, 2015a; Perez-Cerezales *et al.*, 2015b). Consequently, this currently raises significant doubt as to the physiological importance of thermotaxis in human sperm guidance as is currently being suggested by the above-mentioned studies.

In addition to the above-mentioned interaction mechanisms, some studies have demonstrated the existence of a boundary-following navigation as a sperm guidance mechanism (Denissenko *et al.*, 2012; Eamer *et al.*, 2016; Nosrati *et al.*, 2016). This is characterised by the ability of sperm to turn corners in response to surface boundaries in a dynamic *in vitro* microenvironment. This further supports the idea that sperm migration near female tract surfaces plays a key role in fertilisation *in vivo*. A recent study by Eamer *et al.* (2016) also demonstrated a strong correlation between high sperm DNA integrity and boundary-following navigation. Human sperm with preference to follow boundaries on the left or right-hand sides were shown to possess higher (>51%) DNA integrity than straight swimming sperm, as well as a significantly higher (>67%) DNA integrity than sperm in raw semen (Eamer *et al.*, 2016). Using a microfluidic chip with a surface-modified microchannel, Huang *et al.* (2014) recently demonstrated the sorting of motile/non-motile and live/dead spermatozoa with high efficiency. This

sperm selection technique utilizes boundary-navigation and rheosensory capabilities of human spermatozoa but does not incorporate any physiological response to agonists. Finally, with regards to clinical ART treatments, these advancements in characterization and application of sperm-boundary navigation may prove to be useful tools for the selection of sperm with the highest DNA integrity and fertilizing capability which in turn would help improve success rates

In summary, the sperm guidance mechanisms employed in the journey to the oocyte are accompanied by essential physiological changes as spermatozoa traverse a dynamic female reproductive tract characterised by variations in temperature, viscosity and fluid biochemical composition. From the point of ejaculation, the ability of spermatozoa to penetrate the cervical mucus and viscous fluid milieu, as well as respond to biochemical and temperature gradients ultimately relies on the efficiency of the signalling mechanisms responsible for sperm motility, sperm hyperactivation and sperm capacitation. In addition to the study of sperm chemotaxis and other tactile responses, this also makes the exclusive study of sperm motility and migration very significant.

4.1.2 Aim of the study

The aim of this study was to investigate the effects of the steroid hormone milieu as present in follicular fluid on the different forms of sperm kinesis that bear physiological significance in human reproduction. The research objectives of this study were to characterise the effect(s) of extracellular shFF on (i) human sperm chemotaxis (ii) human sperm motility (iii) human sperm migration through a mucus analogue.

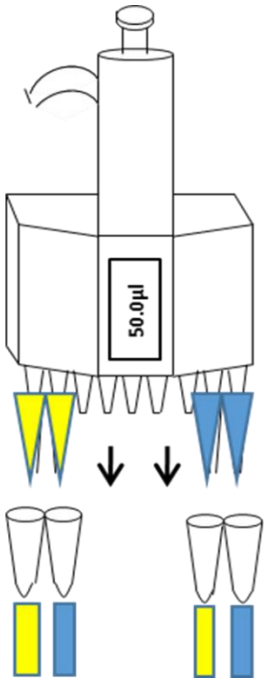
4.2 MATERIALS AND METHODS

4.2.1 Materials

Please refer to chapter 2, section 2.2.1 for a detailed list of research materials used in this study. The preparation of shFF and the semen samples used in this study was carried out as described in chapter 2.

4.2.2 Chemotaxis assay

The chemotactic effects of shFF on human sperm were studied using a multichannel pipette system (Fig 5.5) devised by a previous member of the Kirkman-Brown research group at the University of Birmingham (Dr. João Correia, personal communication). Briefly, 50µl of supplemented Earle's Balanced Salt Solution (sEBSS) medium with or without shFF was aspirated into the pipette tips which were then inserted into Eppendorf tubes containing prepared sperm of known concentrations in either sEBSS medium or shFF (as per Figure 47). The experimental setup was incubated at 37°C and 6% CO_2 for 1 hour. Following the incubation, the contents of the tips were ejected and analysed on the IVOS 10.9 CASA system. The percentage migration of sperm cells into the pipette tip was determined from the concentrations of sperm (millions/ml) recorded in each pipette tip and the corresponding microcentrifuge tube.



Colour tag	Pipette tip	Eppendorf tube
	sEBSS + shFF	-
	sEBSS - shFF	-
	-	Sperm + shFF
	-	Sperm + sEBSS

Figure 47 Experimental setup for the study of shFF-induced chemotaxis. shFF is present in the yellow pipette tip and Eppendorf tube containing sEBSS and sperm respectively while shFF is absent in the blue pipette tip and tube.

4.2.3 Motility studies - Computer assisted sperm analysis (CASA)

Quantitative analysis of sperm motility parameters was carried using an IVOS 10.9 CASA system (as per ESHRE, 1998). Briefly, 45µl of prepared sperm was mixed with 5µl of the required amount of shFF to give the correct final concentration (100% or 10% or 1% or 0.1% or 0.01% shFF or 13.5μM progesterone). 1:1000 DMSO-treated sperm was used as control. 3µl of the sperm treatment mixture was then loaded into 20μm pre-warmed CellVision® CASA slides. Loaded chambers were sealed with vacuum grease to reduce evaporation and incubated for 30 minutes at 37°C and 6% CO_2. Motility parameters were assessed from the CASA slides at 0, 5, 15 and 30 minutes post-incubation. 10 fields per slide chamber were acquired by the CASA system for analysis. The quantitative sperm motility parameters analysed by the CASA system across all experiments include curvilinear velocity (VCL) - the total distance travelled by a sperm

cell divided by the time elapsed, straight line velocity (VSL) - the straight line distance from the beginning to the end of a sperm track divided by the time taken, and linearity (LIN) – the measure of cell track departure from a straight line determined by the percentage ratio of VSL/VCL. Spermatozoa were considered to exhibit hyperactivated motility if their curvilinear velocity (VCL) was greater than 150μm/s, their linearity (LIN) was less than 50%, and their lateral head displacement was greater than 7μm (classed as 'sort 7' algorithm on the CASA system).

4.2.4 Kremer migration assay

The effect of shFF on sperm cervical mucus migration was studied using the Kremer test, making use of a cervical mucus analogue - methylcellulose (Kremer, 1965; Ivic *et al.*, 2002; Figure 48). Briefly, capillary glass tubes (50x4x0.4mm) were filled by capillary action with 1% methylcellulose (4000cp viscosity on a 2% solution at 20°C – Sigma-Aldrich, UK) in sEBSS, treated with either 100% shFF or 0.001% DMSO. The tubes were then sealed at one end with cristaseal and equilibrated in a humidified chamber at 37°C and 6% CO_2. Liquefied fresh semen samples were divided into 50µl aliquots in BEEM® capsules treated in duplicate with either 100% shFF in sEBSS or 0.1% DMSO in sEBSS. The open ends of the capillary tubes were then inserted into each BEEM® capsule and incubated for 1 hour at 37°C and 6% CO_2. The degree of sperm migration into the methylcellulose column was then assessed at 1, 2, 3 and 4cm migration distances (4 fields of view per distance) using a x20 microscope objective.

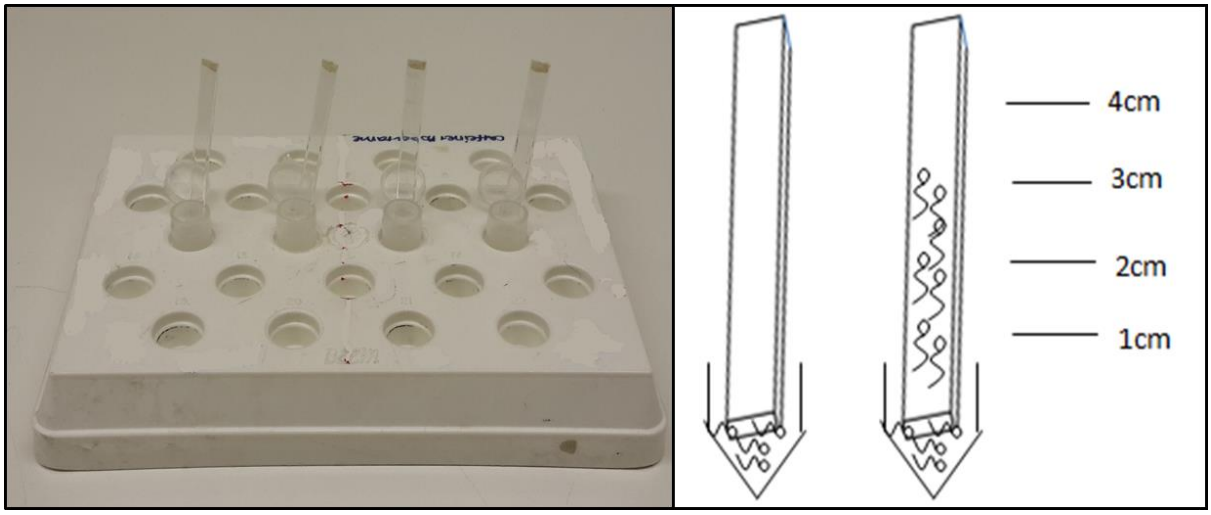


Figure 48 Kremer migration assay setup. Sperm cells migrate from the BEEM capsules upstream of the viscous methylcellulose column.

4.3 RESULTS

4.3.1 Effects of shFF gradient on sperm chemotaxis

Sperm migration from the Eppendorf tube into the pipette tip was observed in all 4 experimental setups, with percentage migration ranging from 0.5 to 10%. Sperm migration into the pipette tip was significantly inhibited in the setup where shFF is present only in the pipette tip versus all other experimental conditions ($P<0.05$) (see Table 10 below).

Table 10 Effects of shFF on sperm chemotaxis. See bottom left column for the calculated mean % sperm migration from Eppendorf tube into pipette tip in each of the 4 setups (n=14). Paired T-tests were carried out to determine any significant differences in sperm migration between the different experimental setups. A P-value of <0.05 was taken as statistically significant. Yellow Eppendorf tube or tip indicates the presence of shFF. Blue indicates no shFF addition.

	Mean percentage migration from tube to pipette tip		
	**P = 0.03*** **(3.1% vs 1.7%)**	P = 0.66 (3.1% vs 3.5%)	P = 0.46 (3.1% vs 2.7%)
	N/A	**P = 0.02*** **(1.7% vs 3.5%)**	**P = 0.02*** **(1.7% vs 2.7%)**
	N/A	N/A	P = 0.29 (3.5% vs 2.7%)
			

4.3.2 Effects of shFF on sperm motility

The very low number of cells counted by the sort 7 algorithm proved to be less than ideal for a detailed analysis of shFF-induced hyperactivation across experiments. A visible reduction in the sperm VCL and VSL was observed over time in the 100%, 10% and 1% shFF treatments across all experiments however significant changes in sperm VCL and VSL were only observed between 0 and 5 minutes post-treatment with shFF (see Figures 49 and 50). At 5min post-exposure to shFF, a significant reduction in LIN was observed in sperm treated with 100% shFF and 13.5μM progesterone when compared to the control ($P<0.05$; $n=5$) (Figure 51). At 15min post-exposure to shFF, a significant reduction in LIN was observed in sperm treated with 10% and 100% shFF when compared to the control ($P<0.05$; $n=5$) (see Figure 51). A similar trend was also observed 30min post-exposure but was found not to be statistically significant. In all of the CASA slides incubated for analysis, some degree of sample evaporation was observed.

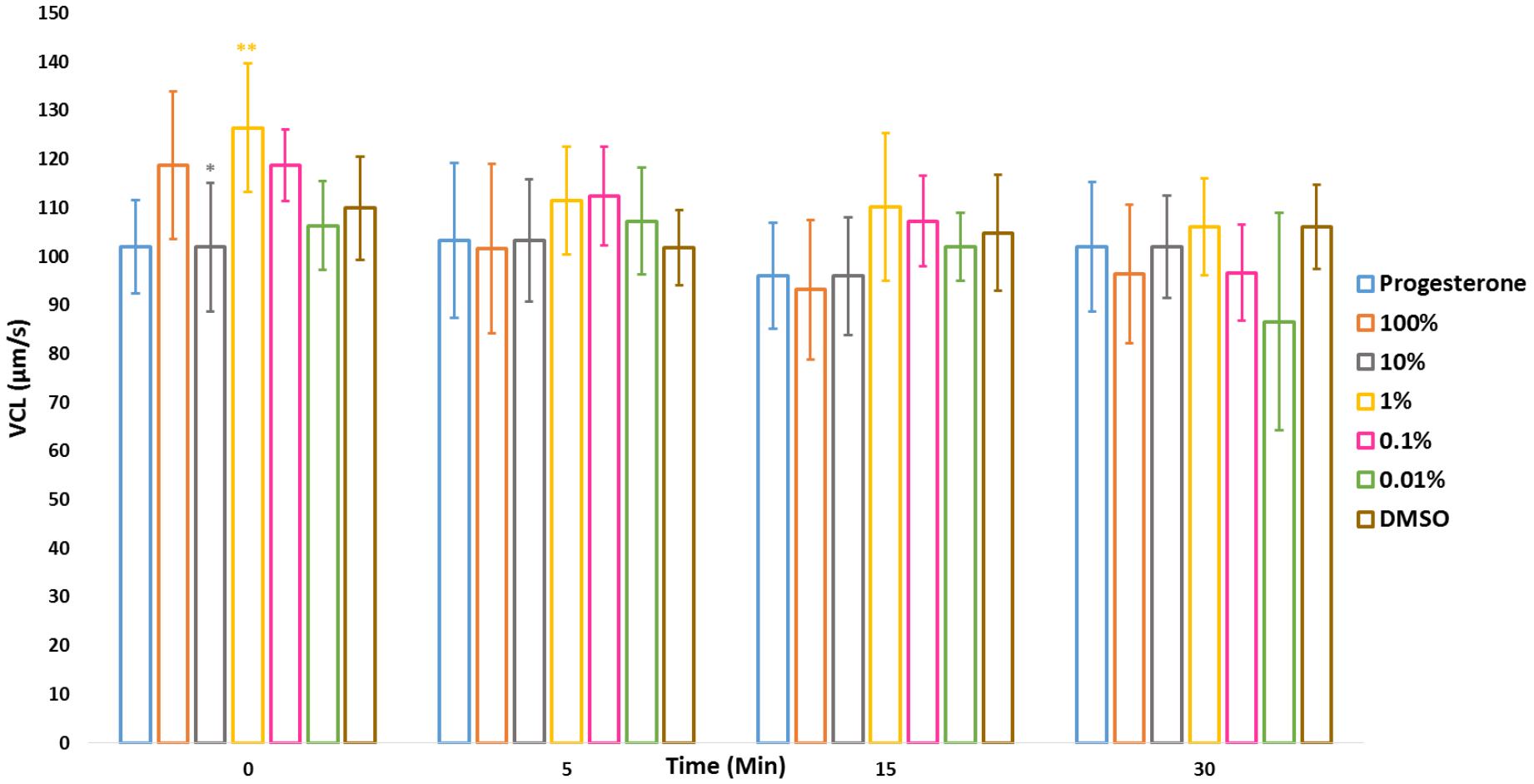


Figure 49 Effects of shFF on sperm curvilinear velocity (VCL). Data expressed as Mean ± SEM from 5 experiments. At each time point, paired T-tests were carried out between data from the different treatments and the control to determine any significant effects of shFF on sperm curvilinear velocity over time (*=P<0.05; **=P<0.01).

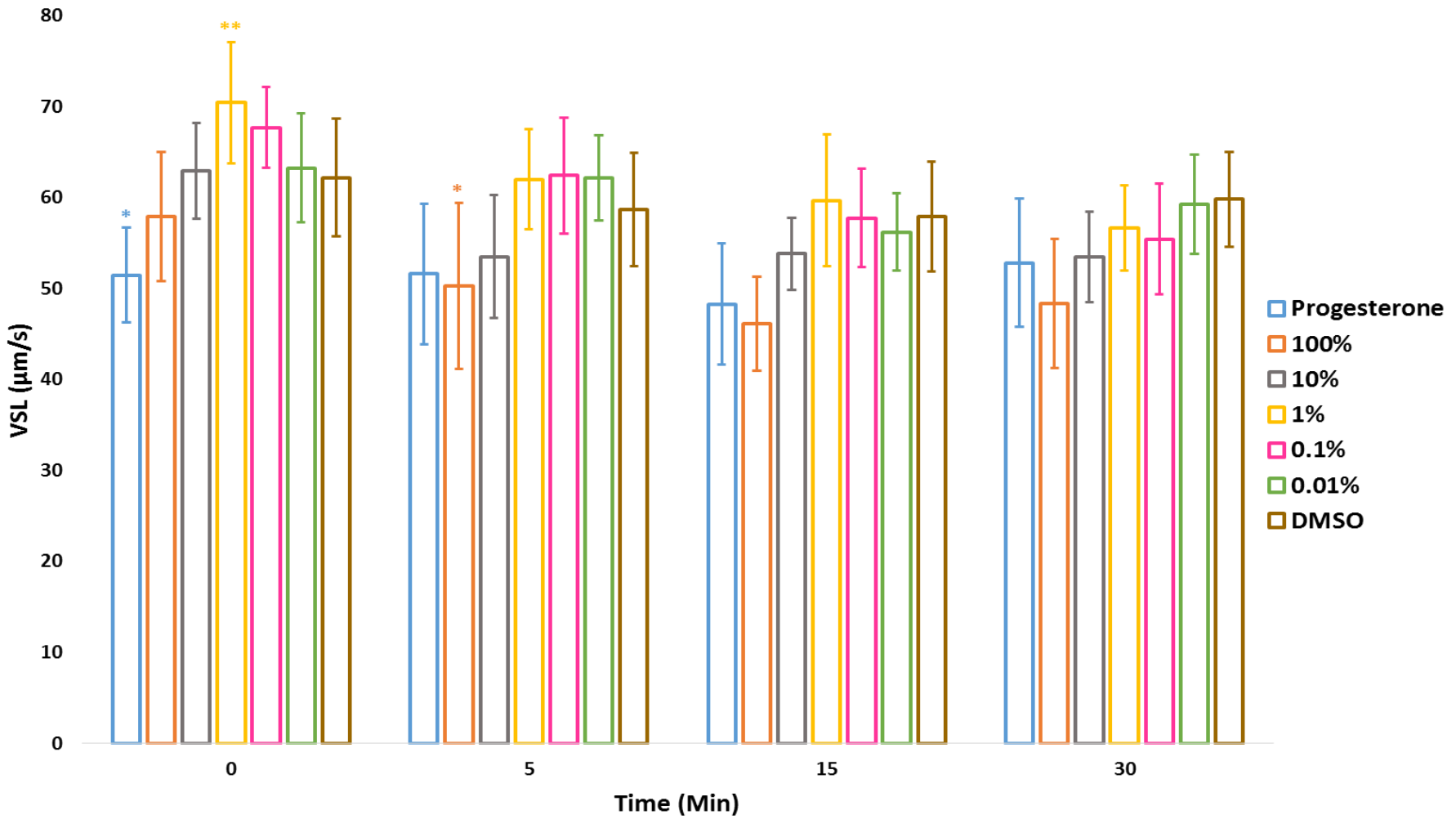


Figure 50 Effects of shFF on sperm straight line velocity (VSL). Data expressed as Mean ± SEM from 5 experiments. At each time point, paired T-tests were carried out between data from the different treatments and the control to determine any significant effects of shFF on sperm straight line velocity over time. (*=P<0.05; **=P<0.01).

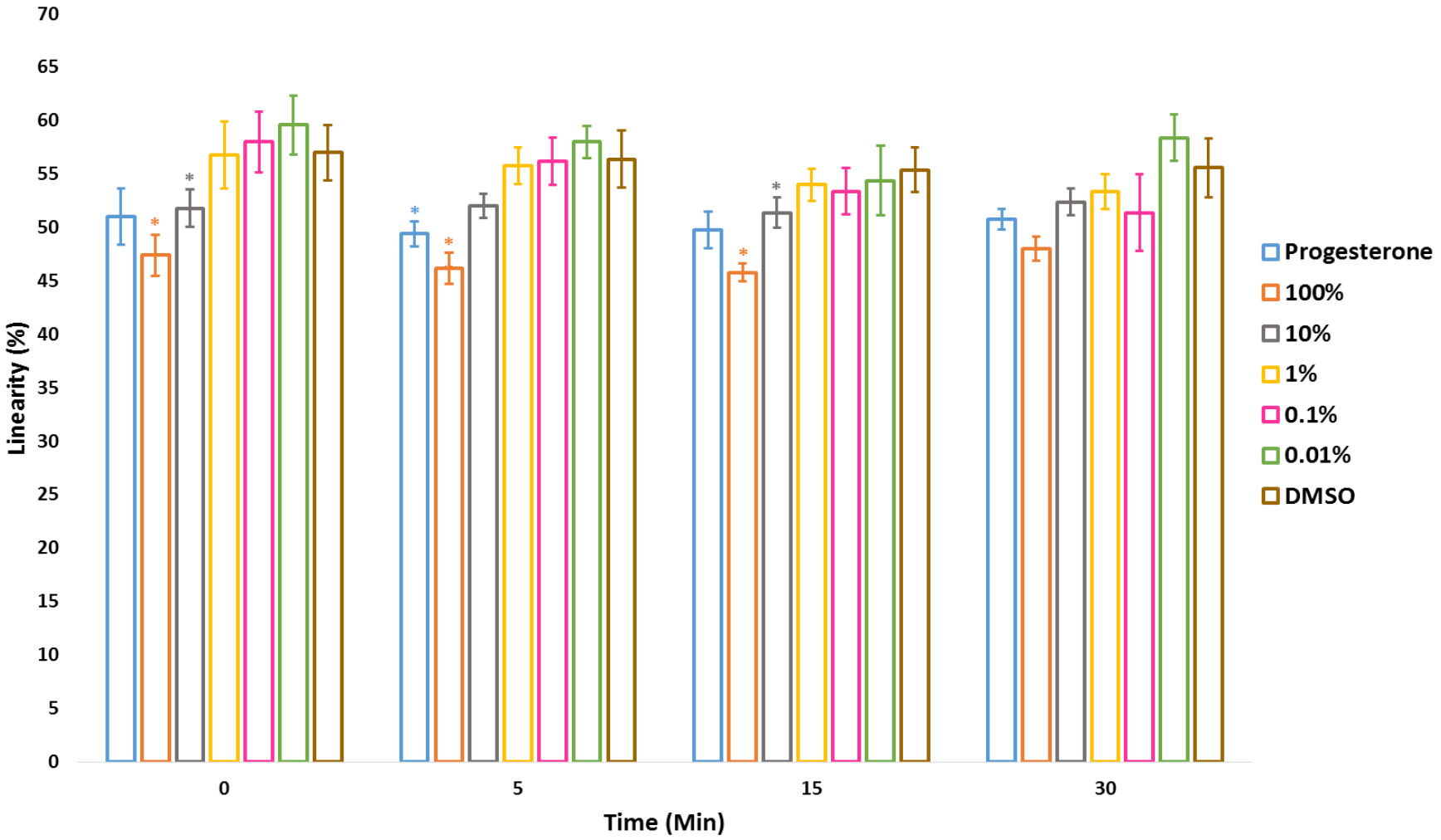


Figure 51 Effects of shFF on sperm linearity (LIN). Data expressed as Mean ± SEM from 5 experiments. At each time point, Paired T-tests were carried out between data from the different treatments and the control to determine any significant effects of shFF on sperm linearity over time. (*=P<0.05; **=P<0.01).

4.3.3 Effects of shFF on sperm migration through cervical mucus

The effect of shFF on sperm migration was studied using 4 different experimental conditions. These were: shFF-treated methylcellulose (MC) columns inserted in shFF-treated semen, shFF-treated MC columns inserted in null-treated semen, null-treated MC columns inserted in shFF-treated semen, and control (null-treated MC columns inserted in null-treated semen). A visible reduction in sperm migration versus control was observed at 3 and 4 cm migration distances in both of the experimental setups containing shFF-treated MC columns (Figures 52 and 53). However, this apparent reduction in sperm migration did not reach statistical significance (Paired T-test. At 3cm, P=0.06; 0.15. At 4cm, P=0.18; 0.15). At the 3cm migration distance, the spermatozoa from both of the experimental setups containing shFF-treated MC columns had experienced a reduction in migration by more than 70% (Figure 52). A similar trend was also observed at 4cm with a further reduction in migration by more than 80% of migrated cells at 1cm.

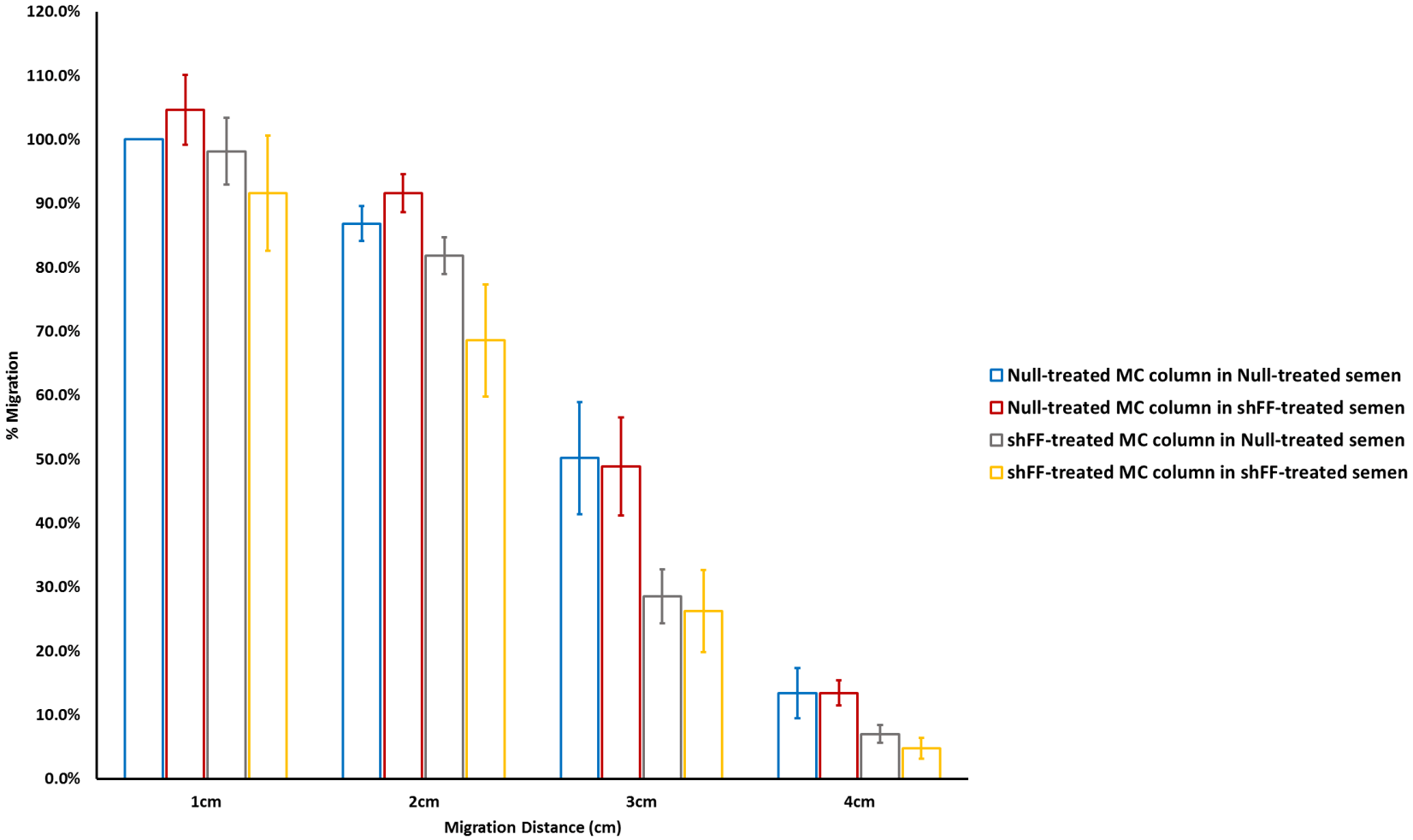


Figure 52 Effects of 100% shFF on sperm migration. Data presented as Mean ± SEM from 5 experiments. Sperm migration for each of the 4 Kremer setups at every migration distance was expressed as a % of the control setup (Null-treated MC column in Null-treated semen) migration at 1cm migration distance (blue bar at 1cm).

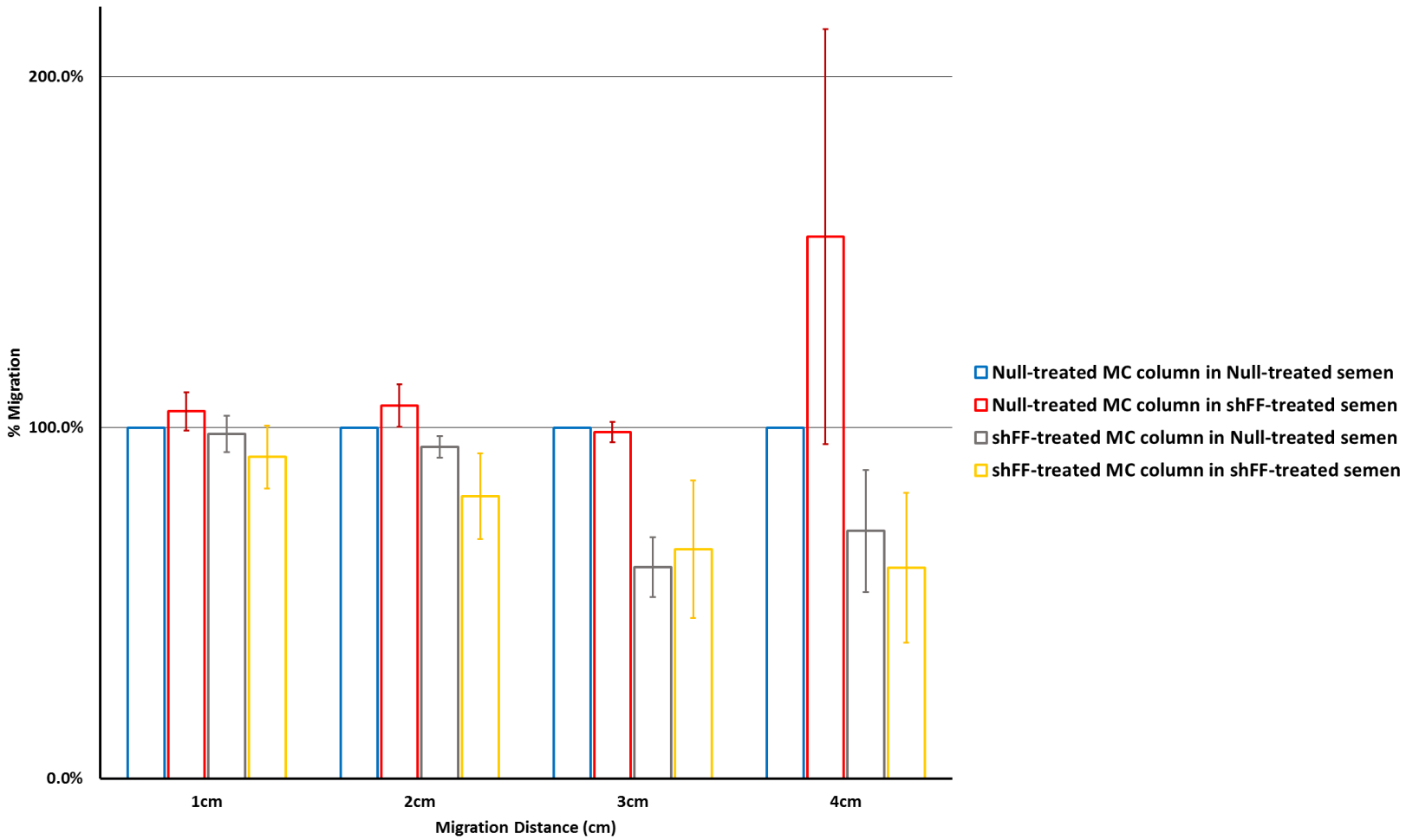


Figure 53 Effects of 100% shFF on sperm migration. Data presented as Mean ± SEM from 5 experiments. Sperm migration for each of the 4 Kremer setups at each migration distance was expressed as a % of the control setup (Null-treated MC column in Null-treated semen) migration at the corresponding migration distances (blue bars). At each migration distance, paired T-tests were carried out between data from the different setups and the control to determine any significant differences in sperm migration. Tests revealed no statistical significance.

4.4 DISCUSSION

4.4.1 shFF and sperm chemotaxis

Chemotaxis has been proposed by many studies as a potential candidate mechanism used by sperm in locating the oocyte for fertilisation however, the mechanism employed in mammals still remains unclear (Kirkman-Brown and Smith, 2011). Data from the chemotaxis experiments revealed an apparent chemotactic effect in which there was a significant reduction in the percentage migration of spermatozoa swimming up the shFF gradient into the pipette tip (see Table 10). This observed phenomenon may be a result of 'hyperactivated trapping' (Eisenbach and Giojalas, 2006; illustrated in Figure 54) where spermatozoa responding to the shFF gradient in the Eppendorf tube switch to a state of hyperactivated motility and as a result become non-progressive. This would affirm the findings of Ralt *et al.* (1994) who reported that sperm acquired hyperactivated motility upon approach to a follicular fluid gradient.

The results obtained may be further characterised via the live monitoring of shFF-induced chemotaxis. Briefly, human spermatozoa could be introduced into a purpose-built imaging chamber containing shFF gradients with spatial proximity to the cells via micro channels. This combination of qualitative and quantitative data can then be used in experiments to determine the direct effects of the shFF gradient concentration on sperm characteristics such as progressive motility and hyperactivation.

The occurrence of 'hyperactivated trapping' may be further studied by making use of a microfluidic chip as described by Huang *et al.* (2014), modified to accommodate shFF gradients. This will make it possible for the microscopic observation of sperm hyperactivated trapping along micro surfaces in response to shFF gradients. Finally,

studies investigating the effects of calcium channel blockers on sperm chemotaxis should be carried out in order to ascertain the Catsper dependency of follicular fluid-induced chemotaxis. These studies would however be complicated as exposure to these inhibitors may already affect baseline motility (Campbell *et al.*, 2013).

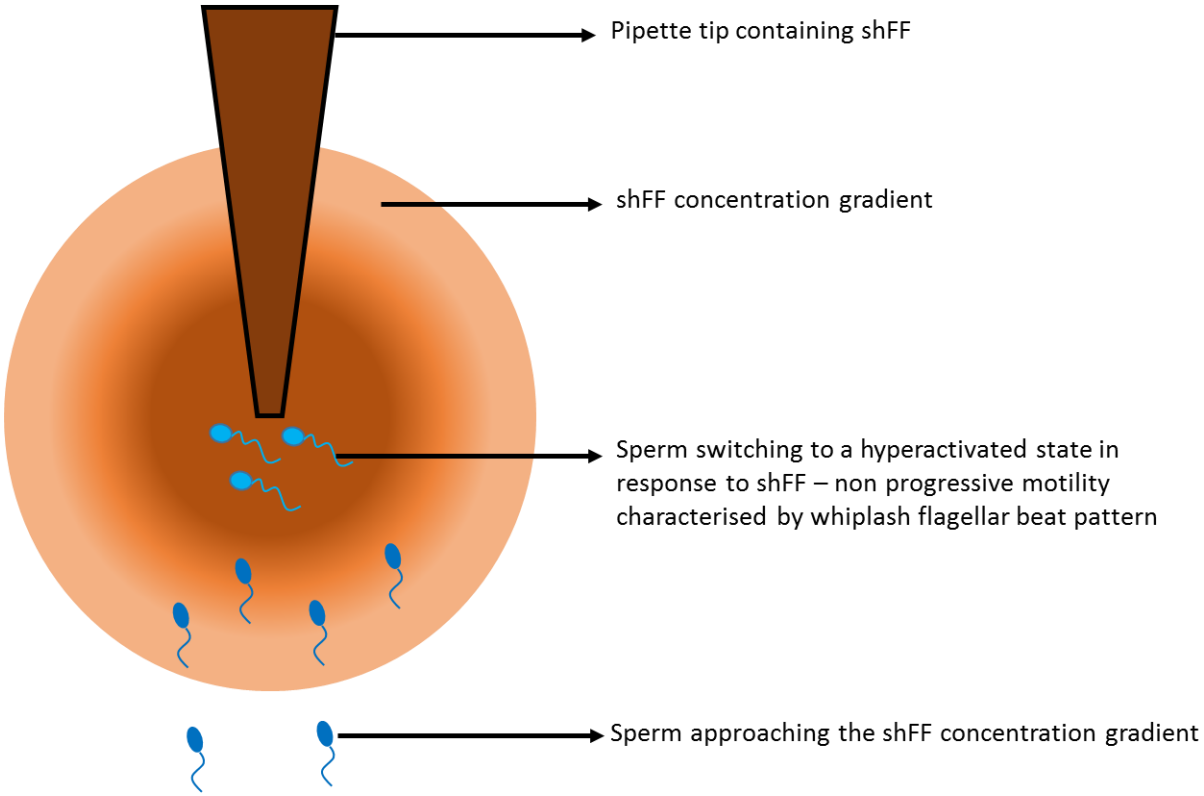


Figure 54 Illustration of the proposed sperm 'hyperactivated trapping' as a chemokinetic response to 100% shFF. Spermatozoa (dark blue) swim towards shFF resulting in the initiation of hyperactivated motility (light blue). This renders them non-progressive and less likely to enter into the narrow opening of the pipette tip.

4.4.2 shFF and sperm motility

Follicular fluid steroid hormones have been shown to modulate sperm hyperactivation (Uhler *et al.*, 1992; Yang *et al.*, 1994; Jaiswal *et al.*, 1999; Contreras and Llanos, 2001; Fujinoki, 2010; Fujinoki, 2014). However, the effects of shFF on sperm hyperactivated motility appeared unchanged in this study with a low number of cells detected by the sort 7 algorithm on the CASA system.

The low number of sort 7 cells across all experiments at later time points where they may be expected, could be due to the sample evaporation observed in the CASA slide chambers during the 30 minute incubation period. This could change the tonicity of the sperm's extracellular medium within the CASA slide chambers, possibly altering sperm motility parameters. However, the sort 7 count may also be influenced by other factors such as the depth of the slide chamber.

The significant changes in the VCL, VSL and LIN of shFF-treated sperm observed at 0 mins (Figures 49, 50 and 51) is evidence of a rapid stimulatory effect which is also seen in the intracellular calcium response to shFF (see chapter 2). With the time period between loading of CASA chambers and motility analysis spanning between 0 and 90s, a rapid increase in $[Ca^{2+}]_i$ is likely to have taken place within that time. This in turn triggers the cascade of signalling events responsible for the induction of rapid changes in sperm motility. The significantly reduced LIN from 0 to 15 minutes post exposure to 100% shFF may be indicative of sperm hyperactivation as the mean linearity values from the 5 experiments carried out are less than 50%. Despite the direct relationship between LIN, VSL and VCL (LIN=VSL/VCL x 100), 100% shFF only appeared to significantly reduce sperm VSL at 5 minutes. This may be attributed to the relatively

small sample size used for analysis (n=5), thus requiring further experimental repeats to ascertain the validity of this trend.

The reduced effect of 100% shFF on sperm motility parameters over time when compared to progesterone may be due to a possible inhibitory effect of the other steroid hormones present in the shFF mix. This effect was also observed in acrosome reaction studies (see Chapter 2), and is in agreement with data from previous studies suggesting that exposure of spermatozoa to estrogens have an inhibitory effect on the modulation of human sperm by progesterone (Luconi *et al.*, 1999; Baldi *et al.*, 2000; Vigil *et al.*, 2008; Fujinoki, 2010). This reduced effect may also result from the prolonged incubation of sperm with shFF (30 minutes), possibly leading to excessive $[Ca^{2+}]_i$ mobilisation and clearance, which in turn may result in signalling fatigue. This in turn would have implications for sperm calcium homeostasis and the regulation of sperm motility by $[Ca^{2+}]_i$. The absence of a significant shFF effect on sperm motility after 30 minutes might have clinical implications as to the importance of time in the response of sperm to follicular fluid steroids. This may be used as a basis for clinical studies that could attempt to establish a relationship between motility responses to shFF, subsequent fertilisation and IVF pregnancy rates.

In consideration of the relatively small sample size (n=5), the absence of hyperactivation data, and the sample evaporation may affect the degree of data accuracy, but this may also reflect the depth of the CASA slide chamber (20μm) or other factors. In addition to the study of an extended donor group making for a larger sample size, the experimental setup could be changed to replace the 20μm CASA slides with capillary glass tubes (50x4x0.4mm). The tubes would then be filled with the treated

sperm samples via capillary action, and sealed at both ends with cristaseal. This setup should allow some improvement in the study of sperm motility modulated by shFF over time, due to its observed efficacy in minimising sample evaporation when used in the Kremer migration assay. It does however have its limitations. At present, the capillary glass tubes cannot be imaged in the existing IVOS 10.9 CASA system; and large numbers of cells in a sealed environment may have effects on media pH not seen at lower cell density as in the Kremer assay.

4.4.3 shFF and sperm migration through cervical mucus

The reduction in sperm migration observed in the shFF-treated MC columns appears to be in agreement with the findings from the chemotaxis and motility data presented in this study, all suggesting that the presence of other steroid hormones in the shFF mixture creates a modulating effect on progesterone-induced sperm migration. However, for some understanding of its physiological significance, the Kremer test data may be interpreted as follows. Firstly, the steroid hormone concentrations as present in 100% shFF are unlikely to be found in the cervix region of the female reproductive tract, suggesting the need for more Kremer tests using lower shFF concentrations (10%, 1%, 0.1% and 0.01% shFF). Barring the above-mentioned disparity in steroid hormone concentrations, the setup containing shFF-treated MC column in null-treated semen is the closest analogue to migration of spermatozoa through the cervical mucus during the ovulatory phase of the female menstrual cycle, with a very low dose gradient of human follicular fluid likely to be physiologically present. The lack of statistical significance in the observed data despite visible changes in sperm migration may be attributed to a relatively small sample size (n=5). More experimental repeats need to be carried out in an attempt to statistically validate the observed trend.

CHAPTER 5

GENERAL DISCUSSION

5.1 GENERAL DISCUSSION

The series of studies presented focus upon a more detailed understanding of the role of the follicular fluid steroid hormone mix in the modulation of human sperm. These follicular fluid steroid hormones are encountered by sperm *en route* the site of natural fertilisation. The key fixture present in every experimental setup is synthetic human follicular fluid (shFF), the first formulated complex steroid hormone analogue of human follicular fluid (see Chapter 2).

shFF was utilised in the study of the effects of isolated follicular fluid steroid hormone mix on different aspects of human sperm physiology as will be reviewed below.

5.1.1 shFF-induced sperm $[Ca^{2+}]_i$ signalling and acrosome reaction

The $[Ca^{2+}]_i$ kinetics observed in shFF-treated spermatozoa, were consistent with those of progesterone-treated sperm as previously characterised by Kirkman-Brown *et al.* (2000) and others. The very high concentrations of progesterone and 17α-hydroxyprogesterone (13.5μM and 5.6μM respectively) present in shFF are likely to be largely responsible for the characteristic transient and sustained $[Ca^{2+}]_i$ response. The large sample size of spermatozoa in these single-cell imaging experiments (>2000 sperm cells in total) gives a high degree of statistical power to the observed data.

The induction of a biphasic Ca^{2+} influx at both physiological and standard laboratory temperatures of 37°C and 26°C respectively suggests that the characteristic kinetics of human sperm Ca^{2+} influx, as induced by follicular steroid hormones present in the female reproductive tract, is a cellular response occurring in capacitated spermatozoa across a broad temperature range of surrounding medium. The consistency of the biphasic $[Ca^{2+}]_i$ response (as demonstrated previously with progesterone alone) in the presence of a

complete follicular fluid steroid complement (shFF), may also suggest that follicular fluid steroid hormones act in synergy via a central signalling pathway to evoke $[Ca^{2+}]_i$ responses in human sperm when in contact with follicular fluid in the female reproductive tract.

However, the amplitude and duration of either phase of the Ca^{2+} influx may be affected by changes in extracellular temperature as observed in the different rates of transient Ca^{2+} influx and decay at 37°C and 26°C respectively. The speed of the shFF-induced transient Ca^{2+} influx at the physiological temperature of 37°C may be representative of the typical $[Ca^{2+}]_i$ response speeds of human sperm to follicular fluid in the female reproductive tract. The apparent temperature-induced differences in sperm Ca^{2+} influx speeds may also suggest that maintaining well-regulated physiological temperatures within the female tract environment is essential for successful sperm interaction with the various sperm modulators present in the female tract.

The rapid nature of sperm $[Ca^{2+}]_i$ decrease in response to shFF removal in turn reveals a rapid rate of $[Ca^{2+}]_i$ sequestration and clearance. However, the rapid rates of sperm Ca^{2+} influx and clearance in response to steroid hormones bring into question the speed at which spermatozoa maintain $[Ca^{2+}]_i$ homeostasis as they continuously swim in and out of the plume of follicular fluid in the female reproductive tract. It is expected that the viscous nature of the female tract environment has a role to play in this signalling dynamic, particularly with regards to the effect of viscosity on the swimming speed of spermatozoa and diffusion rates of any chemicals.

The induction of sperm acrosome reaction by shFF supports the hypothesis that follicular fluid stimulates human sperm acrosome reaction. Nevertheless, the significantly lower

rate of shFF-induced AR when compared to progesterone alone suggests the presence of an antagonistic effect from the other steroid hormones on progesterone-induced AR. Physiologically, this could lead one to hypothesise that the full steroid hormone complement of human follicular fluid creates a stabilising medium for spermatozoa through its journey across the female tract, which to an extent inhibits the mass induction of AR until the sperm cells arrive the vicinity of the oocyte where other factors within the cumulus-oocyte complex may then come into play.

The characterisation data presented in chapters 2 and 3 on the effects of a complex follicular fluid steroid hormone analogue, shFF on human sperm $[Ca^{2+}]_i$ signalling and acrosome reaction is the first of its kind and brings us a step closer towards fully understanding the role follicular fluid steroid hormones have to play in the modulation of human sperm *en route* the site of fertilisation in the female reproductive tract. This in turn paves the way for further studies, some of which are proposed below.

Use of the patch-clamp technique is at the core of many of the recent advancements in progesterone-induced sperm Ca^{2+} signalling (Lishko *et al.*, 2011; Strunker *et al.*, 2011; Smith *et al.*, 2013; Williams *et al.*, 2015; Miller *et al.*, 2016). The introduction of this technique into the further study of shFF-induced sperm Ca^{2+} signalling may prove valuable. Useful data that could be obtained from this setup includes CatSper channel current recordings from sperm treated with shFF and 13.5μM progesterone, to better understand how the complex mix affected channel gating.

Use of CatSper channel blockers such as NNC 55-0396 and mibefradil, in both patch-clamp and live cell imaging experiments may also reveal more about the role of CatSper in the signalling occurring. However, the rate of shFF-induced AR is likely to be significantly reduced in the presence of the above-mentioned CatSper blockers in accordance with the findings of Tamburrino *et al.* (2014) using progesterone alone.

In line with the physiological reality that spermatozoa come in contact with different concentration gradients of follicular fluid at different points in the female reproductive tract, a steroid hormone dose dependence may also be investigated via live cell Ca^{2+} imaging. Briefly, human spermatozoa could be exposed to a concentration gradient of shFF (0.01-100%shFF) to simulate approach to the oocyte. As demonstrated by Harper *et al.* (2004) using progesterone alone, exposure to gradual increases in shFF gradient concentration may result in a slowly developing $[Ca^{2+}]_i$ increase further characterised by $[Ca^{2+}]_i$ oscillations in a subpopulation of cells. Furthermore, CatSper channel recordings could be carried out using the patch-clamp technique on human sperm treated with gradually increasing shFF concentration gradients. The disadvantage of patch-clamp is the present inability to perform recordings simultaneously on many spermatozoa in the same experiment. Therefore, the data generated possesses a much lower degree of information about attributes in a heterogeneous population when compared to the live cell imaging data presented in this study.

The very recent identification and characterisation of an unconventional sperm progesterone receptor by Miller *et al.* (2016) will pave the way for further research investigating the extent to which human sperm physiological responses are modulated by the ABHD2 endocannabinoid signalling pathway. To that effect, shFF should be used

in further studies in an attempt to elucidate the possible signalling roles of the combined physiological steroid hormone mix. This may be done by characterising other interdependent signalling pathways involving other steroid hormones. Importantly, Estrogens have been shown to have a dose-dependent inhibitory effect on T-type calcium channels in mouse spermatogenic cells (Espinosa *et al.*, 2000; Lu *et al.*, 2008); as well as having no visible stimulatory effect on human CatSper (Lishko *et al.*, 2011). Using the patch-clamp technique, it may however be useful to study the effects of estrogens as present in shFF (800nM estradiol; 180nM estrone) on CatSper activity. This could be further expanded into dose-dependence studies, and comparative studies alongside shFF. This data obtained from these proposed experiments could represent a significant update of our knowledge of follicular fluid steroid-induced sperm $[Ca^{2+}]_i$ signalling

Characterisation of the physiological significance of shFF-induced calcium signalling may also be carried out via a comparative study analysing the effects of ovarian follicular fluid alongside shFF on sperm $[Ca^{2+}]_i$ signalling. The similarities and differences in the Ca^{2+} influx kinetics that may be observed between both treatment categories will provide a good measure of the role steroid hormones play in sperm $[Ca^{2+}]_i$ signalling, in the presence of other follicular fluid constituents.

The AR study presented in chapter 2 could be followed up with further characterisation studies investigating shFF-induced acrosomal changes in real time. Harper *et al.* (2006) attempted the real time monitoring of sperm acrosomal dispersal via staining with organelle probes such as LysoTracker DND-99 and dapoxyl (2-aminoethyl) sulphonamide (DAES) with subsequent observation via phase contrast and fluorescence

microscopy. This technique could be employed in the further study of shFF-induced acrosome reaction. However, the main challenge encountered in the application of this technique is reported to be the heterogeneous labelling of the sperm population (Harper *et al.*, 2006). The real time monitoring of shFF-induced AR could also be carried using the protocol described by Sánchez-Cárdenas *et al.* (2014). This involves the use of FM4-64 reporter dye which labels the sperm's plasma membrane and acrosome, with the ability to report morphological changes before, during and after the AR.

5.1.2 shFF-induced sperm kinesis

With directionality and motility the key features of agonist-induced sperm kinesis, the data obtained from each of the different studies presented in chapter 4 tell a story about shFF-induced kinesis. However when brought together, a joint analysis of the data could be utilised to postulate a simple hypothesis – human sperm exposed to shFF experience a reduced rate of motility and chemokinetic migration over time when compared to untreated sperm. With further reference to directionality, the reduced rate of chemokinetic sperm migration towards shFF observed in the Kremer and chemotaxis experiments may suggest that the other steroid hormones present in shFF may have an antagonistic effect on progesterone-induced sperm chemokinesis. This hypothesis is in line with the presented data on shFF-induced acrosome reaction.

Whilst this has physiological implications for sperm-follicular fluid interactions in the female reproductive tract, it is important to note that additional factors present in the female tract such as viscosity and follicular fluid constituents e.g. proteins, prostaglandins also play a joint role in the modulation of steroid hormone-induced chemokinesis. The current data obtained from the study of shFF-induced chemotaxis could be enhanced

by further studies using different experimental setups as employed by Dr. Maria Teves and colleagues (Teves *et al.*, 2006; Publicover *et al.*, 2008; Blengini *et al.*, 2011; Unates *et al.*, 2014). However, the chemotaxis setup used in this study could also be used to study the chemotactic effects of ovarian follicular fluid on human sperm. Unlike shFF, this may reveal a visible chemotactic effect. Further studies also need to be carried out on the microscopic evaluation of shFF or progesterone-induced 'hyperactivated trapping'.

A more detailed study of the effects of shFF on human sperm motility using short-burst time-lapse recordings during incubation of human sperm with different concentrations of shFF would provide a vast source of raw data that could be analysed thoroughly after the experimental incubations and recordings have been concluded. An example of additional data that could be analysed qualitatively and quantitatively are the beat patterns associated with sperm flagellar movement. The data from the Kremer tests may be supported with further experimental repeats using lower shFF concentrations. In addition to providing a higher degree of statistical power to the data obtained, this proposed study will contain a more accurate recreation physiological conditions as the cervix region of the female reproductive tract is expected to contain a follicular fluid gradient of a much lower steroid concentration than present in shFF.

All of the research presented in this thesis on the effects of shFF on human sperm $[Ca^{2+}]_i$ signalling, acrosome reaction and kinesis constitutes a novel study on the modulation of human sperm by a complete follicular fluid steroid hormone complement. Using follicular fluid steroid hormones as extracellular stimuli, a proposed $[Ca^{2+}]_i$ signalling model for the modulation of certain physiological responses in human sperm is hereby

illustrated (Figure 55). Despite the physiological significance of follicular fluid steroid hormones which is this basis of this entire study, studies have also identified other biochemical constituents of follicular fluid that have been characterised as modulators of sperm in the female reproductive tract. These include prostaglandins (Aitken and Kelly, 1985; Shimizu *et al.*, 1998; Strunker *et al.*, 2011; Rios *et al.*, 2016), glycodelins (Chiu *et al.*, 2007; Yeung *et al.*, 2009), and nitric oxide (Herrero *et al.*, 2003; Machado-Oliveira *et al.*, 2008; de Lamirande *et al.*, 2009). To that effect, a comprehensive concentration profile could be obtained via LC-MS/MS for some of the above-mentioned biochemical constituents as physiologically present in ovarian follicular fluid. This data will in turn facilitate further research on the effects of these follicular fluid constituents on the modulation of human sperm $[Ca^{2+}]_i$ signalling, acrosome reaction and kinesis.

In conclusion, the formulation of a novel follicular fluid steroid hormone analogue has resulted in significant data on the modulation of human sperm by follicular fluid steroid hormones. With previously published studies mostly focused on progesterone alone, the data presented in this thesis represents current knowledge on the effects on follicular fluid steroid hormones on human sperm. This in turn could be applied to the improvement of diagnostic tests that rely on progesterone such as motility change (Mortimer, 2000) and acrosome reaction (De Jonge and Barratt, 2013). With a synthetic human follicular fluid formulation now tried and tested, it will be important going forwards to examine how more complete conditions such as this can be used in diagnosis and the assessment of sperm function.

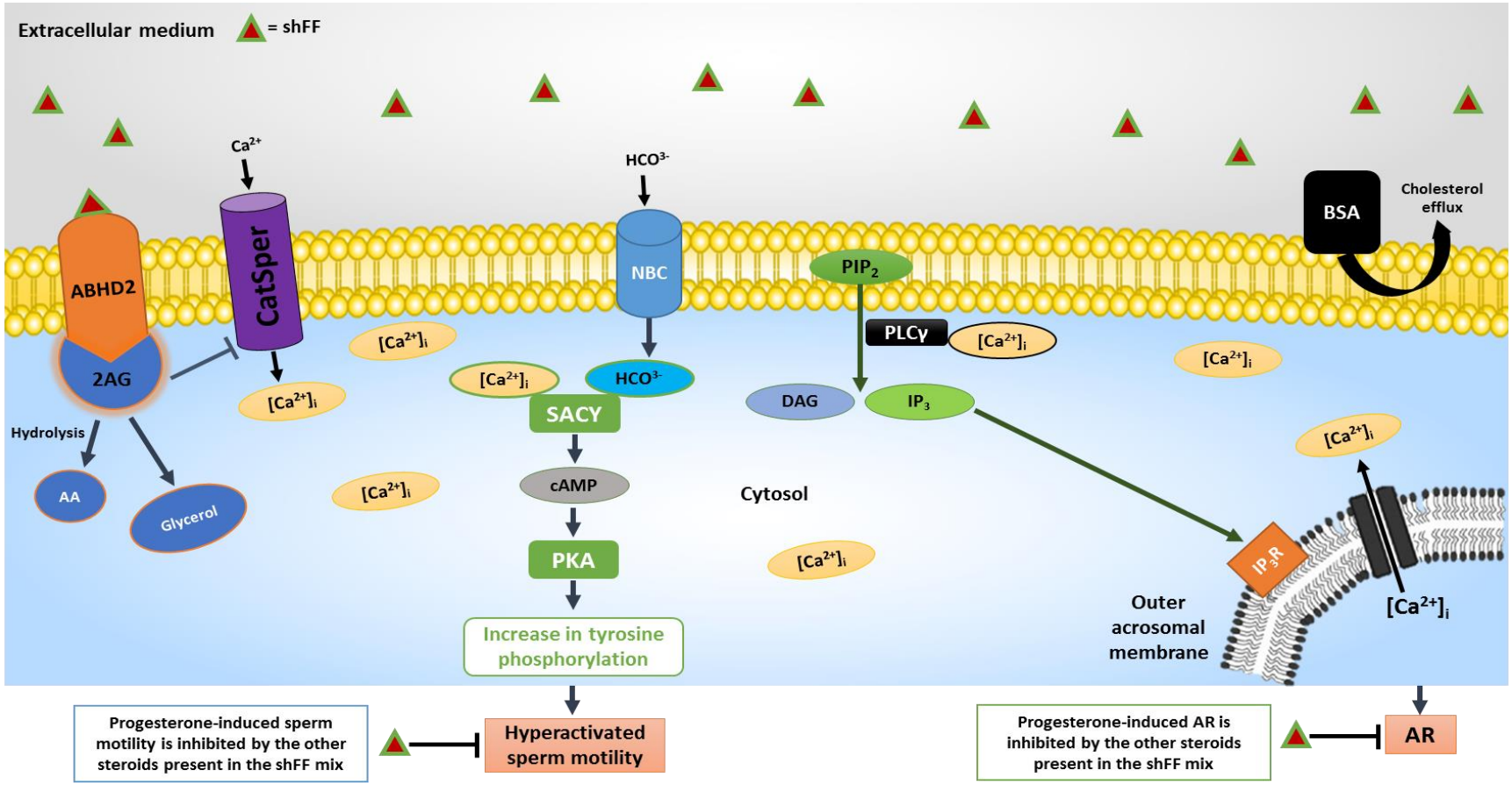


Figure 55 Proposed signalling model for the modulation of human sperm $[Ca^{2+}]_i$ influx, kinesis and acrosome reaction by shFF. These physiological changes are dependent on a characteristic rise in $[Ca^{2+}]_i$. shFF activates ABHD2 which in turn hydrolyses 2AG, an endogenous CatSper inhibitor. This results in Ca^{2+} influx via CatSper which is crucial for the activation of the HCO^{3-}/SACY/cAMP/PKA signalling pathway, responsible for the modulation of sperm capacitation and hyperactivated motility. The rise in $[Ca^{2+}]_i$ is also responsible for the activation of PLCγ which in turn hydrolyses PIP_2 into IP_3 and DAG. During the acrosome reaction, IP_3 activates the IP_3 receptor resulting in $[Ca^{2+}]_i$ release characteristic of acrosomal exocytosis. The other steroid hormones present in shFF have an inhibitory effect on the signalling pathway responsible for progesterone-induced AR.

APPENDIX

MEDIA

Supplemented Earle's Balanced Salts Solution

Components	Molecular weight	Concentration (g/L)	Molarity (mM)
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	146.98	0.264	1.80
Potassium chloride (KCl)	74.55	0.40	5.37
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	246.50	0.20	0.81
Sodium bicarbonate ($NaHCO_3$)	84.01	2.20	26.19
Sodium phosphate ($NaH_2PO_4 \cdot 2H_2O$)	156.00	0.158	1.01
Sodium chloride (NaCl)	58.44	6.80	116.36
D-Glucose	180.20	1.00	5.55
Sodium pyruvate ($C_3H_3O_3Na$)	110.00	0.30	2.73
Sodium lactate ($C_3H_3O_5Na$)	112.10	4.68	41.75

285 <mOsm< 295; 7.15 <pH< 7.45; 0.3% BSA added before use.

REFERENCES

Abou-Haila, A. & Tulsiani, D. R. (2000). Mammalian sperm acrosome: formation, contents, and function. *Arch Biochem Biophys,* 379(2), pp 173-82.

Abou-haila, A. & Tulsiani, D. R. (2009). Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. *Arch Biochem Biophys,* 485(1), pp 72-81.

Agarwal, A., Saleh, R. A. & Bedaiwy, M. A. (2003). Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril,* 79(4), pp 829-43.

Aitken, R. J., Buckingham, D. W. & Fang, H. G. (1993). Analysis of the responses of human spermatozoa to A23187 employing a novel technique for assessing the acrosome reaction. *J Androl,* 14(2), pp 132-41.

Aitken, R. J., Buckingham, D. W., Harkiss, D., Paterson, M., Fisher, H. & Irvine, D. S. (1996a). The extragenomic action of progesterone on human spermatozoa is influenced by redox regulated changes in tyrosine phosphorylation during capacitation. *Mol Cell Endocrinol,* 117(1), pp 83-93.

Aitken, R. J., Buckingham, D. W. & Irvine, D. S. (1996b). The extragenomic action of progesterone on human spermatozoa: evidence for a ubiquitous response that is rapidly down-regulated. *Endocrinology,* 137(9), pp 3999-4009.

Aitken, R. J. & Kelly, R. W. (1985). Analysis of the direct effects of prostaglandins on human sperm function. *J Reprod Fertil,* 73(1), pp 139-46.

Aitken, R. J. & McLaughlin, E. A. (2007). Molecular mechanisms of sperm capacitation: progesterone-induced secondary calcium oscillations reflect the attainment of a capacitated state. *Soc Reprod Fertil Suppl,* 63(273-93.

Alasmari, W., Barratt, C. L., Publicover, S. J., Whalley, K. M., Foster, E., Kay, V., Martins da Silva, S. & Oxenham, S. K. (2013a). The clinical significance of calcium-signalling pathways mediating human sperm hyperactivation. *Hum Reprod,* 28(4), pp 866-76.

Alasmari, W., Costello, S., Correia, J., Oxenham, S. K., Morris, J., Fernandes, L., Ramalho-Santos, J., Kirkman-Brown, J., Michelangeli, F., Publicover, S., *et al.* (2013b). Ca2+ signals generated by CatSper and Ca2+ stores regulate different behaviors in human sperm. *J Biol Chem,* 288(9), pp 6248-58.

Albertini, D. F. 2015. The Mammalian Oocyte. *In:* Plant, T. M. & Zeleznik, A. J. (eds.) *Knobil and Neill's Physiology of Reproduction.* 4th ed. New York: Elsevier.

Albertini, D. F., Fawcett, D. W. & Olds, P. J. (1975). Morphological variations in gap junctions of ovarian granulosa cells. *Tissue Cell,* 7(2), pp 389-405.

Ambekar, A. S., Nirujogi, R. S., Srikanth, S. M., Chavan, S., Kelkar, D. S., Hinduja, I., Zaveri, K., Prasad, T. S., Harsha, H. C., Pandey, A., *et al.* (2013). Proteomic analysis of human follicular fluid: a new perspective towards understanding folliculogenesis. *J Proteomics,* 87(68-77.

Andersen, C. (2002). Possible new mechanism of cortisol action in female reproductive organs: physiological implications of the free hormone hypothesis. *Journal of Endocrinology,* 173(2), pp 211-217.

Andersen, C. Y. (1993). Characteristics of human follicular fluid associated with successful conception after in vitro fertilization. *J Clin Endocrinol Metab,* 77(5), pp 1227-34.

Anderson, E. & Albertini, D. F. (1976). Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. *J Cell Biol,* 71(2), pp 680-6.

Andrews, R. E., Galileo, D. S. & Martin-DeLeon, P. A. (2015). Plasma membrane Ca2+-ATPase 4: interaction with constitutive nitric oxide synthases in human sperm and prostasomes which carry Ca2+/CaM-dependent serine kinase. *Mol Hum Reprod,* 21(11), pp 832-43.

Angelucci, S., Ciavardelli, D., Di Giuseppe, F., Eleuterio, E., Sulpizio, M., Tiboni, G. M., Giampietro, F., Palumbo, P. & Di Ilio, C. (2006). Proteome analysis of human follicular fluid. *Biochim Biophys Acta,* 1764(11), pp 1775-85.

Aparicio, I. M., Gil, M. C., Garcia-Herreros, M., Peña, F. J. & Garcia-Marin, L. J. (2005). Inhibition of phosphatidylinositol 3-kinase modifies boar sperm motion parameters. *Reproduction,* 129(3), pp 283-289.

Armon, L., Ben-Ami, I., Ron-El, R. & Eisenbach, M. (2014). Human oocyte-derived sperm chemoattractant is a hydrophobic molecule associated with a carrier protein. *Fertility and Sterility,* 102(3), pp 885-890.

Armon, L. & Eisenbach, M. (2011). Behavioral Mechanism during Human Sperm Chemotaxis: Involvement of Hyperactivation. *PLoS ONE,* 6(12), pp e28359.

Attaran, M., Pasqualotto, E., Falcone, T., Goldberg, J., Miller, K., Agarwal, A. & Sharma, R. (2000). The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. *Int J Fertil Womens Med,* 45(5), pp 314 - 320.

Austin, C. (1960). Capacitation and the release of hyaluronidase from spermatozoa. *J Reprod Fertil,* 3(310–11.

Austin, C. R. (1951). Observations on the penetration of the sperm in the mammalian egg. *Aust J Sci Res B,* 4(4), pp 581-96.

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

Austin, C. R. (1985). *Sperm Maturation in the Male and Female Gential Tracts*, Florida: Academic Press.

Avenarius, M. R., Hildebrand, M. S., Zhang, Y., Meyer, N. C., Smith, L. L., Kahrizi, K., Najmabadi, H. & Smith, R. J. (2009). Human male infertility caused by mutations in the CATSPER1 channel protein. *Am J Hum Genet,* 84(4), pp 505-10.

Babcock, D. F. & Pfeiffer, D. R. (1987). Independent elevation of cytosolic [Ca2+] and pH of mammalian sperm by voltage-dependent and pH-sensitive mechanisms. *J Biol Chem,* 262(31), pp 15041-7.

Baccetti, B. & Afzelius, B. A. (1976). The biology of the sperm cell. *Monogr Dev Biol,* 10), pp 1-254.

Bahat, A., Caplan, S. R. & Eisenbach, M. (2012). Thermotaxis of human sperm cells in extraordinarily shallow temperature gradients over a wide range. *PLoS One,* 7(7), pp e41915.

Bahat, A., Tur-Kaspa, I., Gakamsky, A., Giojalas, L. C., Breitbart, H. & Eisenbach, M. (2003). Thermotaxis of mammalian sperm cells: A potential navigation mechanism in the female genital tract. *Nat Med,* 9(2), pp 149-150.

Bahmanpour, S., Namavar, M. R., Talaei-Khozani, T. & Mazaheri, Z. (2012). The effect of the follicular fluid on sperm chromatin quality in comparison with conventional media. *Eur Rev Med Pharmacol Sci,* 16(13), pp 1840-6.

Baibakov, B., Boggs, N. A., Yauger, B., Baibakov, G. & Dean, J. (2012). Human sperm bind to the N-terminal domain of ZP2 in humanized zonae pellucidae in transgenic mice. *J Cell Biol,* 197(7), pp 897-905.

Baillie, H. S., Pacey, A. A., Warren, M. A., Scudamore, I. W. & Barratt, C. L. (1997). Greater numbers of human spermatozoa associate with endosalpingeal cells derived from the isthmus compared with those from the ampulla. *Hum Reprod,* 12(9), pp 1985-92.

Baker, T. G. (1963). A QUANTITATIVE AND CYTOLOGICAL STUDY OF GERM CELLS IN HUMAN OVARIES. *Proc R Soc Lond B Biol Sci,* 158(417-33.

Baldi, E., Casano, R., Falsetti, C., Krausz, C., Maggi, M. & Forti, G. (1991). Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. *J Androl,* 12(5), pp 323-30.

Baldi, E., Luconi, M., Muratori, M. & Forti, G. (2000). A novel functional estrogen receptor on human sperm membrane interferes with progesterone effects. *Mol Cell Endocrinol,* 161(1-2), pp 31-5.

Baldi, E., Luconi, M., Muratori, M., Marchiani, S., Tamburrino, L. & Forti, G. (2009). Nongenomic activation of spermatozoa by steroid hormones: facts and fictions. *Mol Cell Endocrinol,* 308(1-2), pp 39-46.

Barrionuevo, M. J., Schwandt, R. A., Rao, P. S., Graham, L. B., Maisel, L. P. & Yeko, T. R. (2000). Nitric oxide (NO) and interleukin-1beta (IL-1beta) in follicular fluid and their correlation with fertilization and embryo cleavage. *Am J Reprod Immunol,* 44(6), pp 359-64.

Barros, C. & Yanagimachi, R. (1971). Induction of zona reaction in golden hamster eggs by cortical granule material. *Nature,* 233(5317), pp 268-9.

Bedu-Addo, K., Barratt, C. L., Kirkman-Brown, J. C. & Publicover, S. J. (2007). Patterns of [Ca2+](i) mobilization and cell response in human spermatozoa exposed to progesterone. *Dev Biol,* 302(1), pp 324-32.

Bedu-Addo, K., Costello, S., Harper, C., Machado-Oliveira, G., Lefievre, L., Ford, C., Barratt, C. & Publicover, S. (2008). Mobilisation of stored calcium in the neck region of human sperm--a mechanism for regulation of flagellar activity. *Int J Dev Biol,* 52(5-6), pp 615-26.

Benoit, J. (1926). Researches anatomiques, cytologiques et histophysiologiques sur les voies excretrices du testicule chez les mammiferes. *Arch Anat Histol Embryol (Strasb),* 5(175-412.

Berridge, M. J., Bootman, M. D. & Roderick, H. L. (2003). Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol,* 4(7), pp 517-529.

Berridge, M. J., Lipp, P. & Bootman, M. D. (2000). The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol,* 1(1), pp 11-21.

Bi, Y., Xu, W. M., Wong, H. Y., Zhu, H., Zhou, Z. M., Chan, H. C. & Sha, J. H. (2009). NYD-SP27, a novel intrinsic decapacitation factor in sperm. *Asian J Androl,* 11(2), pp 229-39.

Bili, H., Tarlatzis, B. C., Daniilidis, M., Fleva, A., Bontis, J., Tourkantonis, A. & Mantalenakis, S. (1998). Cytokines in the human ovary: presence in follicular fluid and correlation with leukotriene B4. *J Assist Reprod Genet,* 15(2), pp 93-8.

Blackmore, P. F. (1993). Rapid non-genomic actions of progesterone stimulate Ca2+ influx and the acrosome reaction in human sperm. *Cell Signal,* 5(5), pp 531-8.

Blackmore, P. F., Beebe, S. J., Danforth, D. R. & Alexander, N. (1990). Progesterone and 17 alpha-hydroxyprogesterone. Novel stimulators of calcium influx in human sperm. *J Biol Chem,* 265(3), pp 1376-80.

Blackmore, P. F. & Eisoldt, S. (1999). The neoglycoprotein mannose-bovine serum albumin, but not progesterone, activates T-type calcium channels in human spermatozoa. *Mol Hum Reprod,* 5(6), pp 498-506.

Blackmore, P. F., Fisher, J. F., Spilman, C. H. & Bleasdale, J. E. (1996). Unusual steroid specificity of the cell surface progesterone receptor on human sperm. *Mol Pharmacol,* 49(4), pp 727-39.

Bleil, J. D. & Wassarman, P. M. (1990). Identification of a ZP3-binding protein on acrosome-intact mouse sperm by photoaffinity crosslinking. *Proceedings of the National Academy of Sciences of the United States of America,* 87(14), pp 5563-5567.

Blengini, C. S., Teves, M. E., Unates, D. R., Guidobaldi, H. A., Gatica, L. V. & Giojalas, L. C. (2011). Human sperm pattern of movement during chemotactic re-orientation towards a progesterone source. *Asian J Androl,* 13(5), pp 769-73.

Boryshpolets, S., Perez-Cerezales, S. & Eisenbach, M. (2015). Behavioral mechanism of human sperm in thermotaxis: a role for hyperactivation. *Hum Reprod,* 30(4), pp 884-92.

Boundless. (2014). *Gametogenesis (Spermatogenesis and Oogenesis)* [Online]. Available: https://www.boundless.com/biology/textbooks/boundless-biology-textbook/animal-reproduction-and-development-43/human-reproductive-anatomy-and-gametogenesis-239/gametogenesis-spermatogenesis-and-oogenesis-891-12142/ [Accessed 13 Apr 2014].

Braun, R. E., Behringer, R. R., Peschon, J. J., Brinster, R. L. & Palmiter, R. D. (1989). Genetically haploid spermatids are phenotypically diploid. *Nature,* 337(6205), pp 373-6.

Breitbart, H. (2002). Intracellular calcium regulation in sperm capacitation and acrosomal reaction. *Mol Cell Endocrinol,* 187(1-2), pp 139-44.

Breitbart, H., Rotman, T., Rubinstein, S. & Etkovitz, N. (2010). Role and regulation of PI3K in sperm capacitation and the acrosome reaction. *Mol Cell Endocrinol,* 314(2), pp 234-8.

Brenker, C., Goodwin, N., Weyand, I., Kashikar, N. D., Naruse, M., Krahling, M., Muller, A., Kaupp, U. B. & Strunker, T. (2012). The CatSper channel: a polymodal chemosensor in human sperm. *Embo j,* 31(7), pp 1654-65.

Brini, M. (2004). Ryanodine receptor defects in muscle genetic diseases. *Biochemical and Biophysical Research Communications,* 322(4), pp 1245-1255.

Brini, M., Cali, T., Ottolini, D. & Carafoli, E. 2013. Intracellular Calcium Homeostasis and Signalling. *In:* Banci, L. (ed.) *Metallomics and the Cell.* New York: Springer.

Britannica, E. (2010). *Spermatogenesis* [Online]. Available: http://www.britannica.com/EBchecked/topic/559418/spermatogenesis [Accessed 03 March, 2015.

Bronson, R. A., Peresleni, T. & Golightly, M. (1999). Progesterone promotes the acrosome reaction in capacitated human spermatozoa as judged by flow cytometry and CD46 staining. *Mol Hum Reprod,* 5(6), pp 507-12.

Burdakov, D., Petersen, O. H. & Verkhratsky, A. (2005). Intraluminal calcium as a primary regulator of endoplasmic reticulum function. *Cell Calcium,* 38(3-4), pp 303-10.

Burkart, A. D., Xiong, B., Baibakov, B., Jimenez-Movilla, M. & Dean, J. (2012). Ovastacin, a cortical granule protease, cleaves ZP2 in the zona pellucida to prevent polyspermy. *J Cell Biol,* 197(1), pp 37-44.

Burrello, N., Vicari, E., D'Amico, L., Satta, A., D'Agata, R. & Calogero, A. E. (2004). Human follicular fluid stimulates the sperm acrosome reaction by interacting with the gamma-aminobutyric acid receptors. *Fertil Steril,* 82 Suppl 3(1086-90.

Byskov, A. G. & Andersen, C. Y. 2013. Ontogeny of the Mammalian Ovary. *In:* Trounson, A., Gosden, R. & Eichenlaub-Ritter, U. (eds.) *Biology and Pathology of the Oocyte: Role in Fertility, Medicine and Nuclear Reprograming.* New York: Cambridge University Press.

Caballero-Campo, P., Buffone, M. G., Benencia, F., Conejo-García, J. R., Rinaudo, P. F. & Gerton, G. L. (2014). A Role for the Chemokine Receptor CCR6 in Mammalian Sperm Motility and Chemotaxis. *Journal of Cellular Physiology,* 229(1), pp 68-78.

Cahalan, M. D. (2009). STIMulating store-operated Ca(2+) entry. *Nat Cell Biol,* 11(6), pp 669-77.

Cai, X. & Clapham, D. E. (2008). Evolutionary Genomics Reveals Lineage-Specific Gene Loss and Rapid Evolution of a Sperm-Specific Ion Channel Complex: CatSpers and CatSper? *PLoS ONE,* 3(10), pp e3569.

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

Calzada, L., Bernal, A. & Loustaunau, E. (1988). Effect of steroid hormones and capacitation on membrane potential of human spermatozoa. *Arch Androl,* 21(2), pp 121-8.

Campbell, J., Savage, A., Madamilola, O., Tamhane, K., Soriano, R., Adya, A. & Brown, S. (2013). Progesterone significantly enhances the mobility of boar spermatozoa. *BioDiscovery,* 9(5), pp.

Carlson, A. E., Quill, T. A., Westenbroek, R. E., Schuh, S. M., Hille, B. & Babcock, D. F. (2005). Identical Phenotypes of CatSper1 and CatSper2 Null Sperm. *Journal of Biological Chemistry,* 280(37), pp 32238-32244.

Carlson, A. E., Westenbroek, R. E., Quill, T., Ren, D., Clapham, D. E., Hille, B., Garbers, D. L. & Babcock, D. F. (2003). CatSper1 required for evoked Ca2+ entry and control of flagellar function in sperm. *Proc Natl Acad Sci U S A,* 100(25), pp 14864-8.

Carlsson, I. B. (2008). *Regulation of Human Ovarian Folliculogenesis In Vitro.* PhD, Karolinska Institutet.

Carpintero, N. L., Suarez, O. A., Mangas, C. C., Varea, C. G. & Rioja, R. G. (2014). Follicular steroid hormones as markers of oocyte quality and oocyte development potential. *J Hum Reprod Sci,* 7(3), pp 187-93.

Castellano, L. E., Trevino, C. L., Rodriguez, D., Serrano, C. J., Pacheco, J., Tsutsumi, V., Felix, R. & Darszon, A. (2003). Transient receptor potential (TRPC) channels in human sperm: expression, cellular localization and involvement in the regulation of flagellar motility. *FEBS Lett,* 541(1-3), pp 69-74.

Cha, K. Y., Barnes, R. B., Marrs, R. P. & Lobo, R. A. (1986). Correlation of the bioactivity of luteinizing hormone in follicular fluid with oocyte maturity in the spontaneous cycle. *Fertil Steril,* 45(3), pp 338-41.

Chan, S. Y., Tang, L. C., Tang, G. W. & Chan, P. H. (1983). Effects of androgens on fertilizing capacity of human spermatozoa. *Contraception,* 28(5), pp 481-8.

Chang, M. C. (1951). Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature,* 168(4277), pp 697-8.

Chao, H. T., Ng, H. T., Tsai, K. L., Hong, C. Y. & Wei, Y. H. (1992). Human follicular fluid stimulates motility and velocity of washed human sperm in vitro. *Andrologia,* 24(1), pp 47-51.

Chen, Y., Cann, M. J., Litvin, T. N., Iourgenko, V., Sinclair, M. L., Levin, L. R. & Buck, J. (2000). Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science,* 289(5479), pp 625-8.

Chiu, P. C., Chung, M. K., Koistinen, R., Koistinen, H., Seppala, M., Ho, P. C., Ng, E. H., Lee, K. F. & Yeung, W. S. (2007). Cumulus oophorus-associated glycodelin-C displaces sperm-bound glycodelin-A and -F and stimulates spermatozoa-zona pellucida binding. *J Biol Chem,* 282(8), pp 5378-88.

Chiu, P. C., Koistinen, R., Koistinen, H., Seppala, M., Lee, K. F. & Yeung, W. S. (2003). Zona-binding inhibitory factor-1 from human follicular fluid is an isoform of glycodelin. *Biol Reprod,* 69(1), pp 365-72.

Chiu, T. T., Rogers, M. S., Law, E. L., Briton-Jones, C. M., Cheung, L. P. & Haines, C. J. (2002). Follicular fluid and serum concentrations of myo-inositol in patients undergoing IVF: relationship with oocyte quality. *Hum Reprod,* 17(6), pp 1591-6.

Cho, W. K., Stern, S. & Biggers, J. D. (1974). Inhibitory effect of dibutyryl cAMP on mouse oocyte maturation in vitro. *Journal of Experimental Zoology,* 187(3), pp 383-386.

Chung, J.-J., Navarro, B., Krapivinsky, G., Krapivinsky, L. & Clapham, D. E. (2011). A novel gene required for male fertility and functional CATSPER channel formation in spermatozoa. *Nat Commun,* 2(153.

Clapham, D. E. (2007). Calcium Signaling. *Cell,* 131(6), pp 1047-1058.

Clarke, H. G., Hope, S. A., Byers, S. & Rodgers, R. J. (2006). Formation of ovarian follicular fluid may be due to the osmotic potential of large glycosaminoglycans and proteoglycans. *Reproduction,* 132(1), pp 119-31.

Clermont, Y. (1963). The cycle of the seminiferous epithelium in man. *Am J Anat,* 112(35-51.

Cohen-Dayag, A., Ralt, D., Tur-Kaspa, I., Manor, M., Makler, A., Dor, J., Mashiach, S. & Eisenbach, M. (1994). Sequential acquisition of chemotactic responsiveness by human spermatozoa. *Biol Reprod,* 50(4), pp 786-90.

Cohen-Dayag, A., Tur-Kaspa, I., Dor, J., Mashiach, S. & Eisenbach, M. (1995). Sperm capacitation in humans is transient and correlates with chemotactic responsiveness to follicular factors. *Proc Natl Acad Sci U S A,* 92(24), pp 11039-43.

Connolly, T. (2011). *Calcium signalling during human sperm interaction with cells of the female reproductive tract.* PhD, University of Birmingham.

Conti, M. 2013. Hormones and growth factors in the regulation of oocyte maturation. *In:* Trounson, A., Gosden, R. & Eichenlaub-Ritter, U. (eds.) *Biology and Pathology of the Oocyte.* 2nd ed. Cambridge: Cambridge University Press.

Contreras, H. R. & Llanos, M. N. (2001). Detection of progesterone receptors in human spermatozoa and their correlation with morphological and functional properties. *Int J Androl,* 24(4), pp 246-52.

Cooper, T. G. (2007). Sperm maturation in the epididymis: a new look at an old problem. *Asian J Androl,* 9(4), pp 533-9.

Cooper, T. G., Noonan, E., von Eckardstein, S., Auger, J., Baker, H. W., Behre, H. M., Haugen, T. B., Kruger, T., Wang, C., Mbizvo, M. T., *et al.* (2010). World Health Organization reference values for human semen characteristics. *Hum Reprod Update,* 16(3), pp 231-45.

Correia, J., Michelangeli, F. & Publicover, S. (2015). Regulation and roles of Ca(2+) stores in human sperm. *Reproduction (Cambridge, England),* 150(2), pp R65-R76.

Correia, J. N., Conner, S. J. & Kirkman-Brown, J. C. (2007). Non-genomic steroid actions in human spermatozoa. "Persistent tickling from a laden environment". *Semin Reprod Med,* 25(3), pp 208-19.

Costello, S., Michelangeli, F., Nash, K., Lefievre, L., Morris, J., Machado-Oliveira, G., Barratt, C., Kirkman-Brown, J. & Publicover, S. (2009). Ca2+-stores in sperm: their identities and functions. *Reproduction,* 138(3), pp 425-37.

Cross, N. L., Morales, P., Overstreet, J. W. & Hanson, F. W. (1988). Induction of acrosome reactions by the human zona pellucida. *Biol Reprod,* 38(1), pp 235-44.

Croxatto, H. 1995. Gamete Transport. *In:* Adashi, J. & Rosenwaks, Z. (eds.) *Reproductive Endocrinology, Surgery and Technology.* New York: Raven.

Cummins, J. 1995. Tests of sperm funtion. *In:* Grudzinskas, J. & Yovich, J. (eds.) *In Gametes - The Spermatozoon.* Cambridge University Press.

Cupisti, S., Dittrich, R., Mueller, A., Strick, R., Stiegler, E., Binder, H., Beckmann, M. W. & Strissel, P. (2007). Correlations between anti-mullerian hormone, inhibin B, and activin A in follicular fluid in IVF/ICSI patients for assessing the maturation and developmental potential of oocytes. *Eur J Med Res,* 12(12), pp 604-8.

Curlin, M. & Bursac, D. (2013). Cervical mucus: from biochemical structure to clinical implications. *Front Biosci (Schol Ed),* 5(507-15.

Dacheux, J. L., Castella, S., Gatti, J. L. & Dacheux, F. (2005). Epididymal cell secretory activities and the role of proteins in boar sperm maturation. *Theriogenology,* 63(2), pp 319-41.

Darszon, A., Nishigaki, T., Beltran, C. & Treviño, C. L. (2011). Calcium Channels in the Development, Maturation, and Function of Spermatozoa. *Physiological Reviews,* 91(4), pp 1305-1355.

Darszon, A., Sanchez-Cardenas, C., Orta, G., Sanchez-Tusie, A. A., Beltran, C., Lopez-Gonzalez, I., Granados-Gonzalez, G. & Trevino, C. L. (2012). Are TRP channels involved in sperm development and function? *Cell Tissue Res,* 349(3), pp 749-64.

Das, S., Saha, S., Majumder, G. C. & Dungdung, S. R. (2010). Purification and Characterization of a Sperm Motility Inhibiting Factor from Caprine Epididymal Plasma. *PLoS ONE,* 5(8), pp e12039.

David, A., Vilensky, A. & Nathan, H. (1971). [Temperature changes in different parts of the rabbit oviduct. Preliminary report]. *Harefuah,* 80(4), pp 180-2.

Davis, B. K. (1981). Timing of fertilization in mammals: sperm cholesterol/phospholipid ratio as a determinant of the capacitation interval. *Proc Natl Acad Sci U S A,* 78(12), pp 7560-4.

Davis, B. K., Byrne, R. & Hungund, B. (1979). Studies on the mechanism of capacitation. II. Evidence for lipid transfer between plasma membrane of rat sperm and serum albumin during capacitation in vitro. *Biochim Biophys Acta,* 558(3), pp 257-66.

De Jonge, C. J. & Barratt, C. L. (2013). Methods for the assessment of sperm capacitation and acrosome reaction excluding the sperm penetration assay. *Methods Mol Biol,* 927(113-8.

De Jonge, C. J., Barratt, C. L., Radwanska, E. & Cooke, I. D. (1993). The acrosome reaction-inducing effect of human follicular and oviductal fluid. *J Androl,* 14(5), pp 359-65.

De Jonge, C. J., Rawlins, R. G. & Zaneveld, L. J. (1988). Induction of the human sperm acrosome reaction by human oocytes. *Fertil Steril,* 50(6), pp 949-53.

de Lamirande, E., Harakat, A. & Gagnon, C. (1998). Human sperm capacitation induced by biological fluids and progesterone, but not by NADH or NADPH, is associated with the production of superoxide anion. *J Androl,* 19(2), pp 215-25.

de Lamirande, E., Lamothe, G. & Villemure, M. (2009). Control of superoxide and nitric oxide formation during human sperm capacitation. *Free Radic Biol Med,* 46(10), pp 1420-7.

de los Santos, M. J., Garcia-Laez, V., Beltran-Torregrosa, D., Horcajadas, J. A., Martinez-Conejero, J. A., Esteban, F. J., Pellicer, A. & Labarta, E. (2012). Hormonal and molecular characterization of follicular fluid, cumulus cells and oocytes from pre-ovulatory follicles in stimulated and unstimulated cycles. *Hum Reprod,* 27(6), pp 1596-605.

de Rooij, D. G. & Russell, L. D. (2000). All you wanted to know about spermatogonia but were afraid to ask. *J Androl,* 21(6), pp 776-98.

Ded, L., Dostalova, P., Dorosh, A., Dvorakova-Hortova, K. & Peknicova, J. (2010). Effect of estrogens on boar sperm capacitation in vitro. *Reproductive Biology and Endocrinology,* 8(1), pp 1-11.

Dell, A., Morris, H. R., Easton, R. L., Panico, M., Patankar, M., Oehniger, S., Koistinen, R., Koistinen, H., Seppala, M. & Clark, G. F. (1995). Structural analysis of the oligosaccharides derived from glycodelin, a human glycoprotein with potent immunosuppressive and contraceptive activities. *J Biol Chem,* 270(41), pp 24116-26.

Denissenko, P., Kantsler, V., Smith, D. J. & Kirkman-Brown, J. (2012). Human spermatozoa migration in microchannels reveals boundary-following navigation. *Proc Natl Acad Sci U S A,* 109(21), pp 8007-10.

Dode, L., Andersen, J. P., Raeymaekers, L., Missiaen, L., Vilsen, B. & Wuytack, F. (2005). Functional Comparison between Secretory Pathway Ca2+/Mn2+-ATPase (SPCA) 1 and Sarcoplasmic Reticulum Ca2+-ATPase (SERCA) 1 Isoforms by Steady-state and Transient Kinetic Analyses. *Journal of Biological Chemistry,* 280(47), pp 39124-39134.

Dogan, S., Vargovic, P., Oliveira, R., Belser, L. E., Kaya, A., Moura, A., Sutovsky, P., Parrish, J., Topper, E. & Memili, E. (2015). Sperm Protamine-Status Correlates to the Fertility of Breeding Bulls. *Biol Reprod.*

Duman, J. G., Chen, L. & Hille, B. (2008). Calcium Transport Mechanisms of PC12 Cells. *The Journal of General Physiology,* 131(4), pp 307-323.

Dym, M. (1994). Spermatogonial stem cells of the testis. *Proc Natl Acad Sci U S A,* 91(24), pp 11287-9.

Dym, M. & Fawcett, D. W. (1971). Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. *Biol Reprod,* 4(2), pp 195-215.

Dym, M., Kokkinaki, M. & He, Z. (2009). Spermatogonial stem cells: mouse and human comparisons. *Birth Defects Res C Embryo Today,* 87(1), pp 27-34.

Eamer, L., Vollmer, M., Nosrati, R., San Gabriel, M. C., Zeidan, K., Zini, A. & Sinton, D. (2016). Turning the corner in fertility: high DNA integrity of boundary-following sperm. *Lab Chip,* 16(13), pp 2418-22.

Eddy, E. M. (1998). Regulation of gene expression during spermatogenesis. *Semin Cell Dev Biol,* 9(4), pp 451-7.

Edström Hägerwall, A. M. L., Rydengård, V., Fernlund, P., Mörgelin, M., Baumgarten, M., Cole, A. M., Malmsten, M., Kragelund, B. B. & Sørensen, O. E. (2012). β-Microseminoprotein Endows Post Coital Seminal Plasma with Potent Candidacidal Activity by a Calcium- and pH-Dependent Mechanism. *PLoS Pathog,* 8(4), pp e1002625.

Edwards, R. G. (1974). Follicular Fluid. *Journal of Reproduction and Fertility,* 37(1), pp 189-219.

Eichenlaub-Ritter, U. & Plancha, C. 2013. Structural basis for oocyte–granulosa cell interactions. *In:* Trounson, A., Gosden, R. & Eichenlaub-Ritter, U. (eds.) *Biology and Pathology of the Oocyte.* 2nd edn ed. Cambridge: Cambridge University Press.

Eisenbach, M. (1999). Mammalian sperm chemotaxis and its association with capacitation. *Dev Genet,* 25(2), pp 87-94.

Eisenbach, M. & Giojalas, L. C. (2006). Sperm guidance in mammals - an unpaved road to the egg. *Nat Rev Mol Cell Biol,* 7(4), pp 276-85.

Enien, W., el Sahwy, S., Harris, C., Seif, M. & Elstein, M. (1995). Human chorionic gonadotrophin and steroid concentrations in follicular fluid: the relationship to oocyte maturity and fertilization rates in stimulated and natural in-vitro fertilization c. *Hum Reprod,* 10(2840 - 2844.

Eppig, J. J. (1991). Intercommunication between mammalian oocytes and companion somatic cells. *Bioessays,* 13(11), pp 569-74.

Eppig, J. J., Viveiros, M. M., Bivens, C. M. & De La Fuente, R. 2004. Regulation of Mammalian Oocyte Maturation. *In:* Leung, P. C. K. & Adashi, E. Y. (eds.) *The Ovary.* Amsterdam: Elsevier Academic Press.

Erickson, G. 2000. The Graafian Follicle: A Functional Definition. *In:* Adashi, E. (ed.) *Ovulation.* Springer New York.

Erickson, G. F. 2004. Follicle Growth and Development. *In:* Sciarra, J. J. (ed.) *Gynecology and Obstetrics.* Philadelphia: Lippincott Williams & Wilkins.

Espinosa, F., Lopez-Gonzalez, I., Munoz-Garay, C., Felix, R., De la Vega-Beltran, J. L., Kopf, G. S., Visconti, P. E. & Darszon, A. (2000). Dual regulation of the T-type Ca(2+) current by serum albumin and beta-estradiol in mammalian spermatogenic cells. *FEBS Lett,* 475(3), pp 251-6.

Fabbri, R., Porcu, E., Lenzi, A., Gandini, L., Marsella, T. & Flamigni, C. (1998). Follicular fluid and human granulosa cell cultures: influence on sperm kinetic parameters, hyperactivation, and acrosome reaction. *Fertil Steril,* 69(1), pp 112-7.

Fabro, G., Rovasio, R. A., Civalero, S., Frenkel, A., Caplan, S. R., Eisenbach, M. & Giojalas, L. C. (2002). Chemotaxis of capacitated rabbit spermatozoa to follicular fluid revealed by a novel directionality-based assay. *Biol Reprod,* 67(5), pp 1565-71.

Fahiminiya, S. & Gerard, N. (2010). [Follicular fluid in mammals]. *Gynecol Obstet Fertil,* 38(6), pp 402-4.

Falcone, L., Gianni, S., Piffaretti-Yanez, A., Marchini, M., Eppenberger, U. & Balerna, M. (1991). Follicular fluid enhances sperm motility and velocity in vitro. *Fertil Steril,* 55(3), pp 619-23.

Fateh, M., Ben-Rafael, Z., Benadiva, C. A., Mastroianni, L., Jr. & Flickinger, G. L. (1989). Cortisol levels in human follicular fluid. *Fertil Steril,* 51(3), pp 538-41.

Fawcett, D. W. (1975). The mammalian spermatozoon. *Dev Biol,* 44(2), pp 394-436.

Findlay, J. K. & Drummond, A. E. (1999). Regulation of the FSH Receptor in the Ovary. *Trends in Endocrinology & Metabolism,* 10(5), pp 183-188.

Flesch, F. M., Colenbrander, B., van Golde, L. M. & Gadella, B. M. (1999). Capacitation induces tyrosine phosphorylation of proteins in the boar sperm plasma membrane. *Biochem Biophys Res Commun,* 262(3), pp 787-92.

Florman, H. M., Jungnickel, M. K. & Sutton, K. A. (2007). What can we learn about fertilization from cystic fibrosis? *Proc Natl Acad Sci U S A,* 104(27), pp 11123-4.

Forabosco, A., Sforza, C., De Pol, A., Vizzotto, L., Marzona, L. & Ferrario, V. F. (1991). Morphometric study of the human neonatal ovary. *Anat Rec,* 231(2), pp 201-8.

Foresta, C. & Rossato, M. (1997). Calcium influx pathways in human spermatozoa. *Mol Hum Reprod,* 3(1), pp 1-4.

Foresta, C., Rossato, M., Mioni, R. & Zorzi, M. (1992). Progesterone induces capacitation in human spermatozoa. *Andrologia,* 24(1), pp 33-5.

Francavilla, F., Romano, R., Pandolfi, C., Macerola, B., Santucci, R., Necozione, S. & Francavilla, S. (2003). Evaluation of the effect of 17alphaOH-progesterone and 17beta-oestradiol on human sperm ability to fuse with oocytes: comparison and possible interference with the effect of progesterone. *Int J Androl,* 26(6), pp 342-7.

Frettsome, R. L. (2012). *The Effects of the Human Oocyte Vestments and Follicular Fluid on Spermatozoa.* PhD, University of Birmingham.

Fujinoki, M. (2010). Suppression of progesterone-enhanced hyperactivation in hamster spermatozoa by estrogen. *Reproduction,* 140(3), pp 453-64.

Fujinoki, M. (2014). Regulation and disruption of hamster sperm hyperactivation by progesterone, 17β-estradiol and diethylstilbestrol. *Reproductive Medicine and Biology,* 13(3), pp 143-152.

Fukuda, N. & Touhara, K. (2006). Developmental expression patterns of testicular olfactory receptor genes during mouse spermatogenesis. *Genes Cells,* 11(1), pp 71-81.

Fukuda, N., Yomogida, K., Okabe, M. & Touhara, K. (2004). Functional characterization of a mouse testicular olfactory receptor and its role in chemosensing and in regulation of sperm motility. *J Cell Sci,* 117(Pt 24), pp 5835-45.

Fukui, K., Takeda, Y., Yano, J., Kaura, R., Kitagawa, H., Suginami, H., Matsuura, S. & Oka, K. (1995). The effect of follicular fluid on intracellular free calcium levels in human spermatozoa. *J Obstet Gynaecol (Tokyo 1995),* 21(1), pp 43-9.

Gaffney, E. A., Gadêlha, H., Smith, D. J., Blake, J. R. & Kirkman-Brown, J. C. (2011). Mammalian Sperm Motility: Observation and Theory. *Annual Review of Fluid Mechanics,* 43(1), pp 501-528.

Galantino-Homer, H. L., Visconti, P. E. & Kopf, G. S. (1997). Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3'5'-monophosphate-dependent pathway. *Biol Reprod,* 56(3), pp 707-19.

Garcia, M. A. & Meizel, S. (1999). Progesterone-mediated calcium influx and acrosome reaction of human spermatozoa: pharmacological investigation of T-type calcium channels. *Biol Reprod,* 60(1), pp 102-9.

Gatica, L. V., Guidobaldi, H. A., Montesinos, M. M., Teves, M. E., Moreno, A. I., Unates, D. R., Molina, R. I. & Giojalas, L. C. (2013). Picomolar gradients of progesterone select functional human sperm even in subfertile samples. *Mol Hum Reprod,* 19(9), pp 559-69.

Getpook, C. & Wirotkarun, S. (2007). Sperm motility stimulation and preservation with various concentrations of follicular fluid. *Journal of Assisted Reproduction and Genetics,* 24(9), pp 425-428.

Ghetler, Y., Raz, T., Ben Nun, I. & Shalgi, R. (1998). Cortical granules reaction after intracytoplasmic sperm injection. *Mol Hum Reprod,* 4(3), pp 289-94.

Giannini, G., Conti, A., Mammarella, S., Scrobogna, M. & Sorrentino, V. (1995). The ryanodine receptor/calcium channel genes are widely and differentially expressed in murine brain and peripheral tissues. *J Cell Biol,* 128(5), pp 893-904.

Gilbert, S. F. (2014). *Developmental Biology*, 10th Edition, Massachussets, USA: Sinauer Associates, Inc.

Gilks, C. B., Reid, P. E., Clement, P. B. & Owen, D. A. (1989). Histochemical changes in cervical mucus-secreting epithelium during the normal menstrual cycle. *Fertil Steril,* 51(2), pp 286-91.

Gill-Sharma, M. K., Choudhuri, J. & D'Souza, S. (2011). Sperm chromatin protamination: an endocrine perspective. *Protein Pept Lett,* 18(8), pp 786-801.

Giojalas, L. C., Rovasio, R. A., Fabro, G., Gakamsky, A. & Eisenbach, M. (2004). Timing of sperm capacitation appears to be programmed according to egg availability in the female genital tract. *Fertil Steril,* 82(1), pp 247-9.

Goldenberg, R. L., Powell, R. D., Rosen, S. W., Marshall, J. R. & Ross, G. T. (1976). Ovarian morphology in women with anosmia and hypogonadotropic hypogonadism. *Am J Obstet Gynecol,* 126(1), pp 91-4.

Gondos, B. & Zamboni, L. (1969). Ovarian development: the functional importance of germ cell interconnections. *Fertil Steril,* 20(1), pp 176-89.

Gook, D. A., Edgar, D. H., Borg, J. & Martic, M. (2008). Detection of zona pellucida proteins during human folliculogenesis. *Human Reproduction,* 23(2), pp 394-402.

Gosden, R., Krapez, J. & Briggs, D. (1997). Growth and development of the mammalian oocyte. *BioEssays,* 19(10), pp 875-882.

Gosden, R. G., Mullan, J., Picton, H. M., Yin, H. & Tan, S.-L. (2002). Current perspective on primordial follicle cryopreservation and culture for reproductive medicine. *Human Reproduction Update,* 8(2), pp 105-110.

Gottlieb, C., Svanborg, K., Eneroth, P. & Bygdeman, M. (1987). Adenosine triphosphate in human semen: a study on conditions for a bioluminescence assay. *Fertil Steril,* 47(6), pp 992-9.

Gougeon, A. (1986). Dynamics of follicular growth in the human: a model from preliminary results. *Hum Reprod,* 1(2), pp 81-7.

Gougeon, A. (1996). Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr Rev,* 17(2), pp 121-55.

Gougeon, A. 2004. Dynamics of Human Follicular Growth: Morphologic, Dynamic, and Functional Aspects. *In:* Leung, P. C. K. & Adashi, E. Y. (eds.) *The Ovary.* 2nd ed.: Elsevier.

Gougeon, A., Ecochard, R. & Thalabard, J. C. (1994). Age-related changes of the population of human ovarian follicles: increase in the disappearance rate of non-growing and early-growing follicles in aging women. *Biol Reprod,* 50(3), pp 653-63.

Govin, J., Caron, C., Lestrat, C., Rousseaux, S. & Khochbin, S. (2004). The role of histones in chromatin remodelling during mammalian spermiogenesis. *Eur J Biochem,* 271(17), pp 3459-69.

Griswold, M. D. (1998). The central role of Sertoli cells in spermatogenesis. *Semin Cell Dev Biol,* 9(4), pp 411-6.

Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985). A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem,* 260(6), pp 3440-50.

Guido, C., Perrotta, I., Panza, S., Middea, E., Avena, P., Santoro, M., Marsico, S., Imbrogno, P., Ando, S. & Aquila, S. (2011). Human sperm physiology: estrogen receptor alpha (ERalpha) and estrogen receptor beta (ERbeta) influence sperm metabolism and may be involved in the pathophysiology of varicocele-associated male infertility. *J Cell Physiol,* 226(12), pp 3403-12.

Gunteski-Hamblin, A. M., Clarke, D. M. & Shull, G. E. (1992). Molecular cloning and tissue distribution of alternatively spliced mRNAs encoding possible mammalian homologues of the yeast secretory pathway calcium pump. *Biochemistry,* 31(33), pp 7600-8.

Gupta, S. K. & Bhandari, B. (2011). Acrosome reaction: relevance of zona pellucida glycoproteins. *Asian J Androl,* 13(1), pp 97-105.

Guraya, S. S. (1987). *Biology of Spermatogenesis and Spermatozoa in Mammals*: Springer Berlin Heidelberg.

Gwatkin, R. B. & Andersen, O. F. (1969). Capacitation of hamster spermatozoa by bovine follicular fluid. *Nature,* 224(5224), pp 1111-2.

Hamamah, S., Lanson, M., Barthelemy, C., Garrigue, M. A., Muh, J. P., Royere, D. & Lansac, J. (1995). Analysis of the lipid content and the motility of human sperm after follicular fluid treatment. *Andrologia,* 27(2), pp 91-7.

Hamdi, S. M., Vieitez, G., Jaspard, B., Barbaras, R., Perret, B., Mieusset, R., Parinaud, J. & Collet, X. (2010). Effects of human follicular fluid and high-density lipoproteins on early spermatozoa hyperactivation and cholesterol efflux. *J Lipid Res,* 51(6), pp 1363-9.

Han, S. J. & Conti, M. (2006). New Pathways from PKA to the Cdc2/cyclin B Complex in Oocytes: Wee1B as a Potential PKA Substrate. *Cell Cycle,* 5(3), pp 227-231.

Hanrieder, J., Nyakas, A., Naessen, T. & Bergquist, J. (2008). Proteomic analysis of human follicular fluid using an alternative bottom-up approach. *J Proteome Res,* 7(1), pp 443-9.

Harlow, C. R., Jenkins, J. M. & Winston, R. M. (1997). Increased follicular fluid total and free cortisol levels during the luteinizing hormone surge. *Fertil Steril,* 68(1), pp 48-53.

Harper, C., Wootton, L., Michelangeli, F., Lefievre, L., Barratt, C. & Publicover, S. (2005). Secretory pathway Ca(2+)-ATPase (SPCA1) Ca(2)+ pumps, not SERCAs, regulate complex [Ca(2+)](i) signals in human spermatozoa. *J Cell Sci,* 118(Pt 8), pp 1673-85.

Harper, C. V., Barratt, C. L. & Publicover, S. J. (2004). Stimulation of human spermatozoa with progesterone gradients to simulate approach to the oocyte. Induction of [Ca(2+)](i) oscillations and cyclical transitions in flagellar beating. *J Biol Chem,* 279(44), pp 46315-25.

Harper, C. V., Barratt, C. L., Publicover, S. J. & Kirkman-Brown, J. C. (2006). Kinetics of the progesterone-induced acrosome reaction and its relation to intracellular calcium responses in individual human spermatozoa. *Biol Reprod,* 75(6), pp 933-9.

Harper, C. V., Kirkman-Brown, J. C., Barratt, C. L. & Publicover, S. J. (2003). Encoding of progesterone stimulus intensity by intracellular [Ca2+] ([Ca2+]i) in human spermatozoa. *Biochem J,* 372(Pt 2), pp 407-17.

Harrison, D. A., Carr, D. W. & Meizel, S. (2000). Involvement of protein kinase A and A kinase anchoring protein in the progesterone-initiated human sperm acrosome reaction. *Biol Reprod,* 62(3), pp 811-20.

Heller, C. G. & Clermont, Y. (1963). Spermatogenesis in man: an estimate of its duration. *Science,* 140(3563), pp 184-6.

Hennet, M. L. & Combelles, C. M. (2012). The antral follicle: a microenvironment for oocyte differentiation. *Int J Dev Biol,* 56(10-12), pp 819-31.

Herrero, M. B., de Lamirande, E. & Gagnon, C. (2003). Nitric oxide is a signaling molecule in spermatozoa. *Curr Pharm Des,* 9(5), pp 419-25.

Herrick, S. B., Schweissinger, D. L., Kim, S. W., Bayan, K. R., Mann, S. & Cardullo, R. A. (2005). The acrosomal vesicle of mouse sperm is a calcium store. *J Cell Physiol,* 202(3), pp 663-71.

Hess, R. A. (2014). Disruption of estrogen receptor signaling and similar pathways in the efferent ductules and initial segment of the epididymis. *Spermatogenesis,* 4(2), pp e979103.

Hess, R. A., Bunick, D., Lubahn, D. B., Zhou, Q. & Bouma, J. (2000). Morphologic changes in efferent ductules and epididymis in estrogen receptor-alpha knockout mice. *J Androl,* 21(1), pp 107-21.

Hess, R. A. (2003). Estrogen in the adult male reproductive tract: A review. *Reproductive biology and endocrinology : RB&E,* 1(52-52.

Hicks, J. J., Pedron, N. & Rosado, A. (1972). Modifications of human spermatozoa glycolysis by cyclic adenosine monophosphate (cAMP), estrogens, and follicular fluid. *Fertil Steril,* 23(12), pp 886-93.

Hildebrand, M. S., Avenarius, M. R., Fellous, M., Zhang, Y., Meyer, N. C., Auer, J., Serres, C., Kahrizi, K., Najmabadi, H., Beckmann, J. S., *et al.* (2010). Genetic male infertility and mutation of CATSPER ion channels. *Eur J Hum Genet,* 18(11), pp 1178-84.

Hinton, B. T. 2010. What does the epididymis do and how does it do it? *In:* Robaire, B. & Chan, P. (eds.) *Handbook of Andrology.* 2nd ed. Kansas, USA: Allen Press.

Ho, H.-C. & Suarez, S. S. (2001a). An Inositol 1,4,5-Trisphosphate Receptor-Gated Intracellular Ca2+ Store Is Involved in Regulating Sperm Hyperactivated Motility. *Biology of Reproduction,* 65(5), pp 1606-1615.

Ho, H. C. & Suarez, S. S. (2001b). Hyperactivation of mammalian spermatozoa: function and regulation. *Reproduction,* 122(4), pp 519-26.

Ho, H. C. & Suarez, S. S. (2003). Characterization of the intracellular calcium store at the base of the sperm flagellum that regulates hyperactivated motility. *Biol Reprod,* 68(5), pp 1590-6.

Holdcraft, R. W. & Braun, R. E. (2004). Hormonal regulation of spermatogenesis. *Int J Androl,* 27(6), pp 335-42.

Holstein, A.-F., Schulze, W. & Davidoff, M. (2003). Understanding spermatogenesis is a prerequisite for treatment. *Reproductive Biology and Endocrinology,* 1(1), pp 107.

Homer, H. 2013. Getting into and out of oocyte maturation. *In:* Trounson, A., Gosden, R. & Eichenlaub-Ritter, U. (eds.) *Biology and Pathology of the Oocyte.* 2nd ed. Cambridge: Cambridge University Press.

Hong, S. J., Tse, J. Y., Ho, P. C. & Yeung, W. S. (2003). Cumulus cells reduce the spermatozoa-zona binding inhibitory activity of human follicular fluid. *Fertil Steril,* 79 Suppl 1(802-7.

Hoodbhoy, T., Joshi, S., Boja, E. S., Williams, S. A., Stanley, P. & Dean, J. (2005). Human sperm do not bind to rat zonae pellucidae despite the presence of four homologous glycoproteins. *J Biol Chem,* 280(13), pp 12721-31.

Hoodbhoy, T. & Talbot, P. (1994). Mammalian cortical granules: contents, fate, and function. *Mol Reprod Dev,* 39(4), pp 439-48.

Hu, J., Zhang, Z., Shen, W. J. & Azhar, S. (2010). Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. *Nutr Metab (Lond),* 7(47.

Huang, H. Y., Wu, T. L., Huang, H. R., Li, C. J., Fu, H. T., Soong, Y. K., Lee, M. Y. & Yao, D. J. (2014). Isolation of motile spermatozoa with a microfluidic chip having a surface-modified microchannel. *J Lab Autom,* 19(1), pp 91-9.

Hunter, R. H. (1981). Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation. *J Reprod Fertil,* 63(1), pp 109-17.

Hunter, R. H. & Nichol, R. (1983). Transport of spermatozoa in the sheep oviduct: preovulatory sequestering of cells in the caudal isthmus. *J Exp Zool,* 228(1), pp 121-8.

Hunter, R. H. & Nichol, R. (1986). A preovulatory temperature gradient between the isthmus and ampulla of pig oviducts during the phase of sperm storage. *J Reprod Fertil,* 77(2), pp 599-606.

Ikemoto, T., Iino, M. & Endo, M. (1995). Enhancing effect of calmodulin on Ca(2+)-induced Ca2+ release in the sarcoplasmic reticulum of rabbit skeletal muscle fibres. *The Journal of Physiology,* 487(3), pp 573-582.

Ivic, A., Onyeaka, H., Girling, A., Brewis, I. A., Ola, B., Hammadieh, N., Papaioannou, S. & Barratt, C. L. (2002). Critical evaluation of methylcellulose as an alternative medium in sperm migration tests. *Hum Reprod,* 17(1), pp 143-9.

Izquierdo-Rico, M. J., Jimenez-Movilla, M., Llop, E., Perez-Oliva, A. B., Ballesta, J., Gutierrez-Gallego, R., Jimenez-Cervantes, C. & Aviles, M. (2009). Hamster zona pellucida is formed by four glycoproteins: ZP1, ZP2, ZP3, and ZP4. *J Proteome Res,* 8(2), pp 926-41.

Jacob, A., Hurley, I., Mandel, F. S., Hershlag, A., Cooper, G. W. & Benoff, S. (1998). Human sperm non-nuclear progesterone receptor expression is a novel marker for fertilization outcome. *Mol Hum Reprod,* 4(6), pp 533-42.

Jaiswal, B. S., Tur-Kaspa, I., Dor, J., Mashiach, S. & Eisenbach, M. (1999). Human sperm chemotaxis: is progesterone a chemoattractant? *Biol Reprod,* 60(6), pp 1314-9.

Jarkovska, K., Martinkova, J., Liskova, L., Halada, P., Moos, J., Rezabek, K., Gadher, S. J. & Kovarova, H. (2010). Proteome Mining of Human Follicular Fluid Reveals a Crucial Role of Complement Cascade and Key Biological Pathways in Women Undergoing in Vitro Fertilization. *Journal of Proteome Research,* 9(3), pp 1289-1301.

Jensen, M. B. & Publicover, S. J. (2012). Progesterone and CatSper dependency. *Int J Androl,* 35(5), pp 631-2.

Jeon, B. G., Moon, J. S., Kim, K. C., Lee, H. J., Choe, S. Y. & Rho, G. J. (2001). Follicular fluid enhances sperm attraction and its motility in human. *J Assist Reprod Genet,* 18(8), pp 407-12.

Jeremy, J. Y., Okonofua, F. E., Thomas, M., Wojdyla, J., Smith, W., Craft, I. L. & Dandona, P. (1987). Oocyte maturity and human follicular fluid prostanoids, gonadotropins, and prolactin after administration of clomiphene and pergonal. *J Clin Endocrinol Metab,* 65(3), pp 402-6.

Jia, X. C., Kalmijn, J. & Hsueh, A. J. (1986). Growth hormone enhances follicle-stimulating hormone-induced differentiation of cultured rat granulosa cells. *Endocrinology,* 118(4), pp 1401-9.

Jimena, P., Castilla, J. A., Peran, F., Molina, R., Ramirez, J. P., Acebal, M., Vergara, F. & Herruzo, A. (1992a). Insulin and insulin-like growth factor I in follicular fluid after induction of ovulation in women undergoing in vitro fertilization. *J Reprod Fertil,* 96(2), pp 641-7.

Jimena, P., Castilla, J. A., Peran, F., Ramirez, J. P., Vergara, F., Jr., Molina, R., Vergara, F. & Herruzo, A. (1992b). Adrenal hormones in human follicular fluid. *Acta Endocrinol (Copenh),* 127(5), pp 403-6.

Jimenez-Gonzalez, C., Michelangeli, F., Harper, C. V., Barratt, C. L. R. & Publicover, S. J. (2006). Calcium signalling in human spermatozoa: a specialized 'toolkit' of channels, transporters and stores. *Human Reproduction Update*, 12(3), pp 253-267.

Jin, M., Fujiwara, E., Kakiuchi, Y., Okabe, M., Satouh, Y., Baba, S. A., Chiba, K. & Hirohashi, N. (2011). Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. *Proc Natl Acad Sci U S A*, 108(12), pp 4892-6.

Johnson, M. H. (2013). *Essential Reproduction*, 7th ed., UK: Wiley-Blackwell.

Jones, R. E. & Lopez, K. H. (2014). *Human Reproductive Biology*, Fourth: Academic Press.

Juyena, N. S. & Stelletta, C. (2012). Seminal plasma: an essential attribute to spermatozoa. *J Androl*, 33(4), pp 536-51.

Karasu, Y., Dilbaz, B., Demir, B., Dilbaz, S., Secilmis Kerimoglu, O., Ercan, C. M., Keskin, U., Korkmaz, C., Duru, N. K., Ergün, A., *et al.* (2012). REPRODUCTIVE ENDOCRINOLOGY. *Human Reproduction*, 27(suppl 2), pp ii302-ii337.

Kaupp, U. B., Kashikar, N. D. & Weyand, I. (2008). Mechanisms of sperm chemotaxis. *Annu Rev Physiol*, 70(93-117.

Kay, V. J., Coutts, J. R. & Robertson, L. (1994). Effects of pentoxifylline and progesterone on human sperm capacitation and acrosome reaction. *Hum Reprod*, 9(12), pp 2318-23.

Keay, S. D., Harlow, C. R., Wood, P. J., Jenkins, J. M. & Cahill, D. J. (2002). Higher cortisol:cortisone ratios in the preovulatory follicle of completely unstimulated IVF cycles indicate oocytes with increased pregnancy potential. *Hum Reprod*, 17(9), pp 2410-4.

Kervancioglu, M. E., Djahanbakhch, O. & Aitken, R. J. (1994). Epithelial cell coculture and the induction of sperm capacitation. *Fertil Steril*, 61(6), pp 1103-8.

Kierszenbaum, A. L., Rivkin, E. & Tres, L. L. (2003). Acroplaxome, an F-Actin–Keratin-containing Plate, Anchors the Acrosome to the Nucleus during Shaping of the Spermatid Head. *Molecular Biology of the Cell*, 14(11), pp 4628-4640.

Kirichok, Y., Navarro, B. & Clapham, D. E. (2006). Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca2+ channel. *Nature,* 439(7077), pp 737-740.

Kirkman-Brown, J. C. (2000). *Second Messenger Systems in the Human Spermatozoon.* PhD, University of Birmingham.

Kirkman-Brown, J. C., Barratt, C. L. & Publicover, S. J. (2003). Nifedipine reveals the existence of two discrete components of the progesterone-induced [Ca2+]i transient in human spermatozoa. *Dev Biol,* 259(1), pp 71-82.

Kirkman-Brown, J. C., Barratt, C. L. & Publicover, S. J. (2004). Slow calcium oscillations in human spermatozoa. *Biochem J,* 378(Pt 3), pp 827-32.

Kirkman-Brown, J. C., Bray, C., Stewart, P. M., Barratt, C. L. & Publicover, S. J. (2000). Biphasic elevation of [Ca(2+)](i) in individual human spermatozoa exposed to progesterone. *Dev Biol,* 222(2), pp 326-35.

Kirkman-Brown, J. C. & Smith, D. J. (2011). Sperm motility: is viscosity fundamental to progress? *Mol Hum Reprod,* 17(8), pp 539-44.

Kopito, L. E., Kosasky, H. J., Sturgis, S. H., Lieberman, B. L. & Shwachman, H. (1973). Water and electrolytes in human cervical mucus. *Fertil Steril,* 24(7), pp 499-506.

Kotaja, N. (2014). MicroRNAs and spermatogenesis. *Fertil Steril,* 101(6), pp 1552-62.

Krasznai, Z., Krasznai, Z. T., Morisawa, M., Bazsáné, Z. K., Hernádi, Z., Fazekas, Z., Trón, L., Goda, K. & Márián, T. (2006). Role of the Na+/Ca2+ exchanger in calcium homeostasis and human sperm motility regulation. *Cell Motility and the Cytoskeleton,* 63(2), pp 66-76.

Krausz, C., Bonaccorsi, L., Maggio, P., Luconi, M., Criscuoli, L., Fuzzi, B., Pellegrini, S., Forti, G. & Baldi, E. (1996). Andrology: Two functional assays of sperm responsiveness to progesterone and their predictive values in in-vitro fertilization. *Human Reproduction,* 11(8), pp 1661-1667.

Kremer, J. (1965). A simple sperm penetration test. *Int J Fertil,* 10(3), pp 209-15.

Kulin, S., Bastiaans, B. A., Hollanders, H. M., Janssen, H. J. & Goverde, H. J. (1994). Human serum and follicular fluid stimulate hyperactivation of human spermatozoa after preincubation. *Fertil Steril,* 62(6), pp 1234-7.

Kunz, G., Beil, D., Deininger, H., Wildt, L. & Leyendecker, G. (1996). The dynamics of rapid sperm transport through the female genital tract: evidence from vaginal sonography of uterine peristalsis and hysterosalpingoscintigraphy. *Hum Reprod,* 11(3), pp 627-32.

Kunz, G., Beil, D., Huppert, P. & Leyendecker, G. (2007). Oxytocin--a stimulator of directed sperm transport in humans. *Reprod Biomed Online,* 14(1), pp 32-9.

Kuroda, Y., Kaneko, S., Yoshimura, Y., Nozawa, S. & Mikoshiba, K. (1999). Are there inositol 1,4,5-triphosphate (IP3) receptors in human sperm? *Life Sci,* 65(2), pp 135-43.

Kushnir, M. M., Naessen, T., Kirilovas, D., Chaika, A., Nosenko, J., Mogilevkina, I., Rockwood, A. L., Carlstrom, K. & Bergquist, J. (2009). Steroid profiles in ovarian follicular fluid from regularly menstruating women and women after ovarian stimulation. *Clin Chem,* 55(3), pp 519-26.

Lamb, J. D., Zamah, A. M., Shen, S., McCulloch, C., Cedars, M. I. & Rosen, M. P. (2010). Follicular fluid steroid hormone levels are associated with fertilization outcome after intracytoplasmic sperm injection. *Fertil Steril,* 94(3), pp 952-7.

Lamboley, C. R., Murphy, R. M., McKenna, M. J. & Lamb, G. D. (2014). Sarcoplasmic reticulum Ca(2+) uptake and leak properties, and SERCA isoform expression, in type I and type II fibres of human skeletal muscle. *The Journal of Physiology,* 592(Pt 6), pp 1381-1395.

Langlais, J., Kan, F. W., Granger, L., Raymond, L., Bleau, G. & Roberts, K. D. (1988). Identification of sterol acceptors that stimulate cholesterol efflux from human spermatozoa during in vitro capacitation. *Gamete Res,* 20(2), pp 185-201.

Lanner, J. T. (2012). Ryanodine receptor physiology and its role in disease. *Adv Exp Med Biol,* 740(217-34.

Lanuza, G. M., Fischman, M. a. L. & Barañao, J. L. (1998). Growth Promoting Activity of Oocytes on Granulosa Cells Is Decreased upon Meiotic Maturation. *Developmental Biology,* 197(1), pp 129-139.

Lanzone, A., Fortini, A., Fulghesu, A. M., Soranna, L., Caruso, A. & Mancuso, S. (1996). Growth hormone enhances estradiol production follicle-stimulating hormone-induced in the early stage of the follicular maturation. *Fertil Steril,* 66(6), pp 948-53.

Lasserre, A., Barrozo, R., Tezon, J. G., Miranda, P. V. & Vazquez-Levin, M. H. (2001). Human epididymal proteins and sperm function during fertilization: un update. *Biol Res,* 34(3-4), pp 165-78.

Lau, C. P., Ledger, W. L., Groome, N. P., Barlow, D. H. & Muttukrishna, S. (1999). Dimeric inhibins and activin A in human follicular fluid and oocyte-cumulus culture medium. *Hum Reprod,* 14(10), pp 2525-30.

Laufer, N., Botero-Ruiz, W., DeCherney, A. H., Haseltine, F., Polan, M. L. & Behrman, H. R. (1984). Gonadotropin and prolactin levels in follicular fluid of human ova successfully fertilized in vitro. *J Clin Endocrinol Metab,* 58(3), pp 430-4.

Lawson, C., Dorval, V., Goupil, S. & Leclerc, P. (2007). Identification and localisation of SERCA 2 isoforms in mammalian sperm. *Mol Hum Reprod,* 13(5), pp 307-16.

Ledee, N., Lombroso, R., Lombardelli, L., Selva, J., Dubanchet, S., Chaouat, G., Frankenne, F., Foidart, J. M., Maggi, E., Romagnani, S., *et al.* (2008). Cytokines and chemokines in follicular fluids and potential of the corresponding embryo: the role of granulocyte colony-stimulating factor. *Hum Reprod,* 23(9), pp 2001-9.

Lee, M. S., Ben-Rafael, Z., Meloni, F., Mastroianni, L., Jr. & Flickinger, G. L. (1987). Relationship of human oocyte maturity, fertilization, and cleavage to follicular fluid prolactin and steroids. *J In Vitro Fert Embryo Transf,* 4(3), pp 168-72.

Lefievre, L., Conner, S. J., Salpekar, A., Olufowobi, O., Ashton, P., Pavlovic, B., Lenton, W., Afnan, M., Brewis, I. A., Monk, M., *et al.* (2004). Four zona pellucida glycoproteins are expressed in the human. *Hum Reprod,* 19(7), pp 1580-6.

Lefievre, L., Nash, K., Mansell, S., Costello, S., Punt, E., Correia, J., Morris, J., Kirkman-Brown, J., Wilson, S. M., Barratt, C. L., *et al.* (2012). 2-APB-potentiated channels amplify CatSper-induced Ca2+ signals in human sperm. *Biochem J.*

Lei, Z. M., Mishra, S., Zou, W., Xu, B., Foltz, M., Li, X. & Rao, C. V. (2001). Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. *Mol Endocrinol,* 15(1), pp 184-200.

Lencesova, L. & Krizanova, O. (2012). IP(3) receptors, stress and apoptosis. *Gen Physiol Biophys,* 31(2), pp 119-30.

Lewicka, S., von Hagens, C., Hettinger, U., Grunwald, K., Vecsei, P., Runnebaum, B. & Rabe, T. (2003). Cortisol and cortisone in human follicular fluid and serum and the outcome of IVF treatment. *Hum Reprod,* 18(8), pp 1613-7.

Lewis, B. & Aitken, R. J. (2001). A redox-regulated tyrosine phosphorylation cascade in rat spermatozoa. *J Androl,* 22(4), pp 611-22.

Lewis, J. D., Abbott, D. W. & Ausió, J. (2003). A haploid affair: core histone transitions during spermatogenesis. *Biochemistry and Cell Biology,* 81(3), pp 131-140.

Li, R., Norman, R. J., Armstrong, D. T. & Gilchrist, R. B. (2000). Oocyte-Secreted Factor(s) Determine Functional Differences Between Bovine Mural Granulosa Cells and Cumulus Cells. *Biology of Reproduction,* 63(3), pp 839-845.

Lin, M. H., Lee, R. K., Hwu, Y. M., Lu, C. H., Chu, S. L., Chen, Y. J., Chang, W. C. & Li, S. H. (2008). SPINKL, a Kazal-type serine protease inhibitor-like protein purified from mouse seminal vesicle fluid, is able to inhibit sperm capacitation. *Reproduction,* 136(5), pp 559-71.

Lin, Y., Mahan, K., Lathrop, W. F., Myles, D. G. & Primakoff, P. (1994). A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. *J Cell Biol,* 125(5), pp 1157-63.

Lindner, C., Lichtenberg, V., Westhof, G., Braendle, W. & Bettendorf, G. (1988). Endocrine parameters of human follicular fluid and fertilization capacity of oocytes. *Horm Metab Res,* 20(4), pp 243-6.

Lishko, P. V., Botchkina, I. L. & Kirichok, Y. (2011). Progesterone activates the principal Ca2+ channel of human sperm. *Nature,* 471(7338), pp 387-91.

Lishko, P. V. & Kirichok, Y. (2010). The role of Hv1 and CatSper channels in sperm activation. *J Physiol,* 588(Pt 23), pp 4667-72.

Lishko, P. V., Kirichok, Y., Ren, D., Navarro, B., Chung, J. J. & Clapham, D. E. (2012). The control of male fertility by spermatozoan ion channels. *Annu Rev Physiol,* 74(453-75.

Liu, J., Xia, J., Cho, K. H., Clapham, D. E. & Ren, D. (2007). CatSperbeta, a novel transmembrane protein in the CatSper channel complex. *J Biol Chem,* 282(26), pp 18945-52.

Liu, L., Kong, N., Xia, G. & Zhang, M. (2013). Molecular control of oocyte meiotic arrest and resumption. *Reprod Fertil Dev,* 25(3), pp 463-71.

Losel, R. & Wehling, M. (2003). Nongenomic actions of steroid hormones. *Nat Rev Mol Cell Biol,* 4(1), pp 46-56.

Lu, C. H., Lee, R. K., Hwu, Y. M., Chu, S. L., Chen, Y. J., Chang, W. C., Lin, S. P. & Li, S. H. (2011). SERPINE2, a serine protease inhibitor extensively expressed in adult male mouse reproductive tissues, may serve as a murine sperm decapacitation factor. *Biol Reprod,* 84(3), pp 514-25.

Lu, L., Wang, C. S., Gao, X. H., Wang, J., Cheng, J., Gao, R. & Xiao, H. (2008). [17 beta-estradiol inhibits T-type calcium channels in mouse spermatogenic cells]. *Zhonghua Nan Ke Xue,* 14(6), pp 483-8.

Luconi, M., Bonaccorsi, L., Maggi, M., Pecchioli, P., Krausz, C., Forti, G. & Baldi, E. (1998). Identification and characterization of functional nongenomic progesterone receptors on human sperm membrane. *J Clin Endocrinol Metab,* 83(3), pp 877-85.

Luconi, M., Muratori, M., Forti, G. & Baldi, E. (1999). Identification and characterization of a novel functional estrogen receptor on human sperm membrane that interferes with progesterone effects. *J Clin Endocrinol Metab,* 84(5), pp 1670-8.

Lyons, R. A., Saridogan, E. & Djahanbakhch, O. (2006). The reproductive significance of human Fallopian tube cilia. *Human Reproduction Update,* 12(4), pp 363-372.

Lytton, J., Westlin, M., Burk, S. E., Shull, G. E. & MacLennan, D. H. (1992). Functional comparisons between isoforms of the sarcoplasmic or endoplasmic reticulum family of calcium pumps. *J Biol Chem,* 267(20), pp 14483-9.

Machado-Oliveira, G., Lefievre, L., Ford, C., Herrero, M. B., Barratt, C., Connolly, T. J., Nash, K., Morales-Garcia, A., Kirkman-Brown, J. & Publicover, S. (2008). Mobilisation of Ca2+ stores and flagellar regulation in human sperm by S-nitrosylation: a role for NO synthesised in the female reproductive tract. *Development,* 135(22), pp 3677-86.

Mackiewicz, U. & Lewartowski, B. (2006). Temperature dependent contribution of Ca2+ transporters to relaxation in cardiac myocytes: important role of sarcolemmal Ca2+-ATPase. *J Physiol Pharmacol,* 57(1), pp 3-15.

Malamitsi-Puchner, A., Sarandakou, A., Baka, S., Vrachnis, N., Kouskouni, E. & Hassiakos, D. (2004). Soluble Fas concentrations in the follicular fluid and oocyte-cumulus complex culture medium from women undergoing in vitro fertilization: association with oocyte maturity, fertilization, and embryo quality. *J Soc Gynecol Investig,* 11(8), pp 566-9.

Malcuit, C., Kurokawa, M. & Fissore, R. A. (2006). Calcium oscillations and mammalian egg activation. *J Cell Physiol,* 206(3), pp 565-73.

Manau, D., Balasch, J., Jimenez, W., Fabregues, F., Civico, S., Casamitjana, R., Creus, M. & Vanrell, J. A. (2000). Follicular fluid concentrations of adrenomedullin, vascular endothelial growth factor and nitric oxide in IVF cycles: relationship to ovarian response. *Hum Reprod,* 15(6), pp 1295-9.

Marquez, B. & Suarez, S. S. (2004). Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. *Biol Reprod,* 70(6), pp 1626-33.

Masuda, H. & de Meis, L. (1977). Effect of temperature on the Ca2+ transport ATPase of sarcoplasmic reticulum. *J Biol Chem,* 252(23), pp 8567-71.

Matson, C. K., Murphy, M. W., Griswold, M. D., Yoshida, S., Bardwell, V. J. & Zarkower, D. (2010). The mammalian doublesex homolog DMRT1 is a transcriptional gatekeeper that controls the mitosis versus meiosis decision in male germ cells. *Dev Cell,* 19(4), pp 612-24.

Matzuk, M. M., Burns, K. H., Viveiros, M. M. & Eppig, J. J. (2002). Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science,* 296(5576), pp 2178-80.

Mbizvo, M. T., Burkman, L. J. & Alexander, N. J. (1990). Human follicular fluid stimulates hyperactivated motility in human sperm. *Fertil Steril,* 54(4), pp 708-12.

McGee, E., Spears, N., Minami, S., Hsu, S. Y., Chun, S. Y., Billig, H. & Hsueh, A. J. (1997). Preantral ovarian follicles in serum-free culture: suppression of apoptosis after activation of the cyclic guanosine 3',5'-monophosphate pathway and stimulation of growth and differentiation by follicle-stimulating hormone. *Endocrinology,* 138(6), pp 2417-24.

McLachlan, R. I., O'Donnell, L., Meachem, S. J., Stanton, P. G., de Kretser, D. M., Pratis, K. & Robertson, D. M. (2002). Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. *Recent Prog Horm Res,* 57(149-79.

McLaughlin, E. A. & McIver, S. C. (2009). Awakening the oocyte: controlling primordial follicle development. *Reproduction,* 137(1), pp 1-11.

McNatty, K., P., Moore Smith, D., Osathanondh, R. & Ryan, K., J. (1979). The human antral follicle : Functional correlates of growth and atresia. *Ann. Biol. anim. Bioch. Biophys.,* 19(5), pp 1547-1558.

McNutt, T. L., Olds-Clarke, P., Way, A. L., Suarez, S. S. & Killian, G. J. (1994). Effect of follicular or oviductal fluids on movement characteristics of bovine sperm during capacitation in vitro. *J Androl,* 15(4), pp 328-36.

Meduri, G., Charnaux, N., Driancourt, M. A., Combettes, L., Granet, P., Vannier, B., Loosfelt, H. & Milgrom, E. (2002). Follicle-stimulating hormone receptors in oocytes? *J Clin Endocrinol Metab,* 87(5), pp 2266-76.

Mehlmann, L. M. (2005). Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction,* 130(6), pp 791-9.

Mendoza, C., Carreras, A., Moos, J. & Tesarik, J. (1992). Distinction between true acrosome reaction and degenerative acrosome loss by a one-step staining method using Pisum sativum agglutinin. *J Reprod Fertil,* 95(3), pp 755-63.

Mendoza, C., Cremades, N., Ruiz-Requena, E., Martinez, F., Ortega, E., Bernabeu, S. & Tesarik, J. (1999). Relationship between fertilization results after intracytoplasmic sperm injection, and intrafollicular steroid, pituitary hormone and cytokine concentrations. *Hum Reprod,* 14(3), pp 628-35.

Mendoza, C. & Tesarik, J. (1990). Effect of follicular fluid on sperm movement characteristics. *Fertil Steril,* 54(6), pp 1135-9.

Mendoza, C. & Tesarik, J. (1993). A plasma-membrane progesterone receptor in human sperm is switched on by increasing intracellular free calcium. *FEBS Lett,* 330(1), pp 57-60.

Messinis, I. E. & Templeton, A. A. (1987). Relationship between intrafollicular levels of prolactin and sex steroids and in-vitro fertilization of human oocytes. *Hum Reprod,* 2(7), pp 607-9.

Michael, A. E. & Papageorghiou, A. T. (2008). Potential significance of physiological and pharmacological glucocorticoids in early pregnancy. *Hum Reprod Update,* 14(5), pp 497-517.

Michael, A. E., Thurston, L. M. & Rae, M. T. (2003). Glucocorticoid metabolism and reproduction: a tale of two enzymes. *Reproduction,* 126(4), pp 425-41.

Michelangeli, F. & East, J. M. (2011). A diversity of SERCA Ca2+ pump inhibitors. *Biochem Soc Trans,* 39(3), pp 789-97.

Michelangeli, F., Ogunbayo, O. A. & Wootton, L. L. (2005). A plethora of interacting organellar Ca2+ stores. *Curr Opin Cell Biol,* 17(2), pp 135-40.

Miki, K. & Clapham, D. E. (2013). Rheotaxis guides mammalian sperm. *Curr Biol,* 23(6), pp 443-52.

Miller, M. R., Mannowetz, N., Iavarone, A. T., Safavi, R., Gracheva, E. O., Smith, J. F., Hill, R. Z., Bautista, D. M., Kirichok, Y. & Lishko, P. V. (2016). Unconventional endocannabinoid signaling governs sperm activation via the sex hormone progesterone. *Science,* 352(6285), pp 555-9.

Miller, W. L. (1988). Molecular biology of steroid hormone synthesis. *Endocr Rev,* 9(3), pp 295-318.

Minami, S., Yamano, S., Ishikawa, H. & Aono, T. (1995). Increase of intracellular free [Ca2+] in single human motile spermatozoa treated with human follicular fluid. *Arch Androl,* 34(3), pp 115-23.

Missiaen, L., Vanoevelen, J., Parys, J. B., Raeymaekers, L., De Smedt, H., Callewaert, G., Erneux, C. & Wuytack, F. (2002). Ca2+ uptake and release properties of a thapsigargin-insensitive nonmitochondrial Ca2+ store in A7r5 and 16HBE14o- cells. *J Biol Chem,* 277(9), pp 6898-902.

Monteleone, P., Giovanni Artini, P., Simi, G., Casarosa, E., Cela, V. & Genazzani, A. R. (2008). Follicular fluid VEGF levels directly correlate with perifollicular blood flow in normoresponder patients undergoing IVF. *J Assist Reprod Genet,* 25(5), pp 183-6.

Morris, J., Jones, S., Howl, J., Lukanowska, M., Lefievre, L. & Publicover, S. (2015). Cell-penetrating peptides, targeting the regulation of store-operated channels, slow decay of the progesterone-induced [Ca2+]i signal in human sperm. *Mol Hum Reprod,* 21(7), pp 563-70.

Mortimer, D. (1994). *Practical Laboratory Andrology*, Oxford: Oxford University Press.

Mortimer, D. & Camenzind, A. R. (1989). The role of follicular fluid in inducing the acrosome reaction of human spermatozoa incubated in vitro. *Hum Reprod,* 4(2), pp 169-74.

Mortimer, S. T. (2000). CASA--practical aspects. *J Androl,* 21(4), pp 515-24.

Mortimer, S. T. & Swan, M. A. (1995). Variable kinematics of capacitating human spermatozoa. *Hum Reprod,* 10(12), pp 3178-82.

Naaby-Hansen, S., Wolkowicz, M. J., Klotz, K., Bush, L. A., Westbrook, V. A., Shibahara, H., Shetty, J., Coonrod, S. A., Reddi, P. P., Shannon, J., *et al.* (2001). Co-localization of the inositol 1,4,5-trisphosphate receptor and calreticulin in the equatorial segment and in membrane bounded vesicles in the cytoplasmic droplet of human spermatozoa. *Molecular Human Reproduction,* 7(10), pp 923-933.

Nauc, V., de Lamirande, E., Leclerc, P. & Gagnon, C. (2004). Inhibitors of Phosphoinositide 3-Kinase, LY294002 and Wortmannin, Affect Sperm Capacitation and Associated Phosphorylation of Proteins Differently: Ca2+-Dependent Divergences. *Journal of Andrology,* 25(4), pp 573-585.

Navarro, B., Kirichok, Y., Chung, J. J. & Clapham, D. E. (2008). Ion channels that control fertility in mammalian spermatozoa. *Int J Dev Biol,* 52(5-6), pp 607-13.

Naz, R. K. & Sellamuthu, R. (2006). Receptors in spermatozoa: are they real? *J Androl,* 27(5), pp 627-36.

Neal, P. & Baker, T. G. (1975). RESPONSE OF MOUSE GRAAFIAN FOLLICLES IN ORGAN CULTURE TO VARYING DOSES OF FOLLICLE-STIMULATING HORMONE AND LUTEINIZING HORMONE. *Journal of Endocrinology,* 65(1), pp 27-32.

Newton, S. C., Blaschuk, O. W. & Millette, C. F. (1993). N-cadherin mediates Sertoli cell-spermatogenic cell adhesion. *Dev Dyn,* 197(1), pp 1-13.

Ni, Y., Zhou, Y., Chen, W. Y., Zheng, M., Yu, J., Li, C., Zhang, Y. & Shi, Q. X. (2009). HongrES1, a cauda epididymis-specific protein, is involved in capacitation of guinea pig sperm. *Mol Reprod Dev,* 76(10), pp 984-93.

Nishigaki, T., Gonzalez-Cota, A. L. & Orta Salazar, G. J. 2014. CatSper in Male Infertility. *In:* Weiss, N. & Koschak, J. (eds.) *Pathologies of Calcium Channels.* Berlin: Springer-Verlag.

Niswender, G. D., Juengel, J. L., Silva, P. J., Rollyson, M. K. & McIntush, E. W. (2000). Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev,* 80(1), pp 1-29.

Nixon, B., MacIntyre, D. A., Mitchell, L. A., Gibbs, G. M., O'Bryan, M. & Aitken, R. J. (2006). The identification of mouse sperm-surface-associated proteins and characterization of their ability to act as decapacitation factors. *Biol Reprod,* 74(2), pp 275-87.

Nosrati, R., Graham, P. J., Liu, Q. & Sinton, D. (2016). Predominance of sperm motion in corners. *Scientific Reports,* 6(26669.

O'Toole, C. M., Roldan, E. R. & Fraser, L. R. (1996). Protein kinase C activation during progesterone-stimulated acrosomal exocytosis in human spermatozoa. *Mol Hum Reprod,* 2(12), pp 921-7.

Okamura, N., Tajima, Y., Soejima, A., Masuda, H. & Sugita, Y. (1985). Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. *J Biol Chem,* 260(17), pp 9699-705.

Oktay, K., Briggs, D. & Gosden, R. G. (1997). Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. *J Clin Endocrinol Metab,* 82(11), pp 3748-51.

Oktay, K., Newton, H., Mullan, J. & Gosden, R. G. (1998). Development of human primordial follicles to antral stages in SCID/hpg mice stimulated with follicle stimulating hormone. *Hum Reprod,* 13(5), pp 1133-8.

Oktay, K., Schenken, R. S. & Nelson, J. F. (1995). Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat. *Biol Reprod,* 53(2), pp 295-301.

Oktem, O. & Oktay, K. (2008). The ovary: anatomy and function throughout human life. *Ann N Y Acad Sci*, 1127(1-9.

Okunade, G. W., Miller, M. L., Pyne, G. J., Sutliff, R. L., O'Connor, K. T., Neumann, J. C., Andringa, A., Miller, D. A., Prasad, V., Doetschman, T., *et al.* (2004). Targeted ablation of plasma membrane Ca2+-ATPase (PMCA) 1 and 4 indicates a major housekeeping function for PMCA1 and a critical role in hyperactivated sperm motility and male fertility for PMCA4. *J Biol Chem,* 279(32), pp 33742-50.

Oliva, R. (2006). Protamines and male infertility. *Human Reproduction Update,* 12(4), pp 417-435.

Oliva, R. & Castillo, J. (2011). Proteomics and the genetics of sperm chromatin condensation. *Asian J Androl,* 13(1), pp 24-30.

Oliveira, R. G., Tomasi, L., Rovasio, R. A. & Giojalas, L. C. (1999). Increased velocity and induction of chemotactic response in mouse spermatozoa by follicular and oviductal fluids. *J Reprod Fertil,* 115(1), pp 23-7.

Oliver, A. E., Baker, G. A., Fugate, R. D., Tablin, F. & Crowe, J. H. (2000). Effects of temperature on calcium-sensitive fluorescent probes. *Biophysical Journal,* 78(4), pp 2116-2126.

Omura, T. & Morohashi, K.-i. (1995). Gene regulation of steroidogenesis. *The Journal of Steroid Biochemistry and Molecular Biology,* 53(1–6), pp 19-25.

Oosterhuis, G. J., Vermes, I., Lambalk, C. B., Michgelsen, H. W. & Schoemaker, J. (1998). Insulin-like growth factor (IGF)-I and IGF binding protein-3 concentrations in fluid from human stimulated follicles. *Hum Reprod,* 13(2), pp 285-9.

Osman, R. A., Andria, M. L., Jones, A. D. & Meizel, S. (1989). Steroid induced exocytosis: the human sperm acrosome reaction. *Biochem Biophys Res Commun,* 160(2), pp 828-33.

Owen, D. H. & Katz, D. F. (2005). A review of the physical and chemical properties of human semen and the formulation of a semen simulant. *J Androl,* 26(4), pp 459-69.

Pande, J., Szewczyk, M. M., Kuszczak, I., Grover, S., Escher, E. & Grover, A. K. (2008). Functional effects of caloxin 1c2, a novel engineered selective inhibitor of plasma membrane Ca(2+)-pump isoform 4, on coronary artery. *Journal of Cellular and Molecular Medicine,* 12(3), pp 1049-1060.

Pangas, S. A. & Rajkovic, A. 2015. Follicular Development: Mouse, Sheep and Human Models. *In:* Plant, T. M. & Zeleznik, A. J. (eds.) *Knobill and Neil's Physiology of Reproduction.* USA: Academic Press.

Parinaud, J., Labal, B. & Vieitez, G. (1992). High progesterone concentrations induce acrosome reaction with a low cytotoxic effect. *Fertil Steril,* 58(3), pp 599-602.

Park, K. H., Kim, B. J., Kang, J., Nam, T. S., Lim, J. M., Kim, H. T., Park, J. K., Kim, Y. G., Chae, S. W. & Kim, U. H. (2011). Ca2+ signaling tools acquired from prostasomes are required for progesterone-induced sperm motility. *Sci Signal,* 4(173), pp ra31.

Parys, J. B. & De Smedt, H. (2012). Inositol 1,4,5-trisphosphate and its receptors. *Adv Exp Med Biol,* 740(255-79.

Patrat, C. & Serres, C. (2009). [Role of the female environment in sperm capacitation]. *Gynecol Obstet Fertil,* 37(6), pp 536-9.

Patrat, C., Serres, C. & Jouannet, P. (2000). The acrosome reaction in human spermatozoa. *Biol Cell,* 92(3-4), pp 255-66.

Patrizio, P., Tucker, M. J. & Guelman, V. (2003). *A Color Atlas for Human Assisted Reproduction: Laboratory and Clinical Insights*, Philadelphia: Lippincott Williams & Wilkins.

Payne, A. H. & Hales, D. B. (2004). Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr Rev,* 25(6), pp 947-70.

Pedersen, P. L. & Carafoli, E. (1987). Ion motive ATPases. I. Ubiquity, properties, and significance to cell function. *Trends in Biochemical Sciences,* 12(146-150.

Pepling, M. E. & Spradling, A. C. (1998). Female mouse germ cells form synchronously dividing cysts. *Development,* 125(17), pp 3323-8.

Perez-Cerezales, S., Boryshpolets, S., Afanzar, O., Brandis, A., Nevo, R., Kiss, V. & Eisenbach, M. (2015a). Involvement of opsins in mammalian sperm thermotaxis. *Sci Rep,* 5(16146.

Perez-Cerezales, S., Boryshpolets, S. & Eisenbach, M. (2015b). Behavioral mechanisms of mammalian sperm guidance. *Asian J Androl,* 17(4), pp 628-32.

Perez-Cerezales, S., Lopez-Cardona, A. P. & Gutierrez-Adan, A. (2016). Progesterone effects on mouse sperm kinetics in conditions of viscosity. *Reproduction,* 151(5), pp 501-7.

Petersen, O. H., Michalak, M. & Verkhratsky, A. (2005). Calcium signalling: past, present and future. *Cell Calcium,* 38(3-4), pp 161-9.

Pietrobon, D., Di Virgilio, F. & Pozzan, T. (1990). Structural and functional aspects of calcium homeostasis in eukaryotic cells. *Eur J Biochem,* 193(3), pp 599-622.

Plant, A., McLaughlin, E. A. & Ford, W. C. (1995). Intracellular calcium measurements in individual human sperm demonstrate that the majority can respond to progesterone. *Fertil Steril,* 64(6), pp 1213-5.

Pommer, A. C., Rutllant, J. & Meyers, S. A. (2003). Phosphorylation of protein tyrosine residues in fresh and cryopreserved stallion spermatozoa under capacitating conditions. *Biol Reprod,* 68(4), pp 1208-14.

Prasad, V., Okunade, G. W., Miller, M. L. & Shull, G. E. (2004). Phenotypes of SERCA and PMCA knockout mice. *Biochem Biophys Res Commun,* 322(4), pp 1192-203.

Publicover, S. J., Giojalas, L. C., Teves, M. E., de Oliveira, G. S., Garcia, A. A., Barratt, C. L. & Harper, C. V. (2008). Ca2+ signalling in the control of motility and guidance in mammalian sperm. *Front Biosci,* 13(5623-37.

Qi, H., Moran, M. M., Navarro, B., Chong, J. A., Krapivinsky, G., Krapivinsky, L., Kirichok, Y., Ramsey, I. S., Quill, T. A. & Clapham, D. E. (2007). All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. *Proceedings of the National Academy of Sciences,* 104(4), pp 1219-1223.

Qiao, J., Yeung, W. S., Yao, Y. Q. & Ho, P. C. (1998). The effects of follicular fluid from patients with different indications for IVF treatment on the binding of human spermatozoa to the zona pellucida. *Hum Reprod,* 13(1), pp 128-31.

Quill, T. A., Sugden, S. A., Rossi, K. L., Doolittle, L. K., Hammer, R. E. & Garbers, D. L. (2003). Hyperactivated sperm motility driven by CatSper2 is required for fertilization. *Proc Natl Acad Sci U S A,* 100(25), pp 14869-74.

Rago, V., Giordano, F., Brunelli, E., Zito, D., Aquila, S. & Carpino, A. (2014). Identification of G protein-coupled estrogen receptor in human and pig spermatozoa. *Journal of Anatomy,* 224(6), pp 732-736.

Ralt, D., Goldenberg, M., Fetterolf, P., Thompson, D., Dor, J., Mashiach, S., Garbers, D. L. & Eisenbach, M. (1991). Sperm attraction to a follicular factor(s) correlates with human egg fertilizability. *Proceedings of the National Academy of Sciences of the United States of America,* 88(7), pp 2840-2844.

Ralt, D., Manor, M., Cohen-Dayag, A., Tur-Kaspa, I., Ben-Shlomo, I., Makler, A., Yuli, I., Dor, J., Blumberg, S., Mashiach, S., *et al.* (1994). Chemotaxis and chemokinesis of human spermatozoa to follicular factors. *Biol Reprod,* 50(4), pp 774-85.

Rath, D., Schuberth, H. J., Coy, P. & Taylor, U. (2008). Sperm interactions from insemination to fertilization. *Reprod Domest Anim,* 43 Suppl 5(2-11.

Rathke, C., Baarends, W. M., Awe, S. & Renkawitz-Pohl, R. (2014). Chromatin dynamics during spermiogenesis. *Biochim Biophys Acta,* 1839(3), pp 155-68.

Ravnik, S. E., Albers, J. J. & Muller, C. H. (1993). A novel view of albumin-supported sperm capacitation: role of lipid transfer protein-I. *Fertil Steril,* 59(3), pp 629-38.

Reid, A. T., Redgrove, K., Aitken, R. J. & Nixon, B. (2011). Cellular mechanisms regulating sperm-zona pellucida interaction. *Asian J Androl,* 13(1), pp 88-96.

Ren, D., Navarro, B., Perez, G., Jackson, A. C., Hsu, S., Shi, Q., Tilly, J. L. & Clapham, D. E. (2001). A sperm ion channel required for sperm motility and male fertility. *Nature,* 413(6856), pp 603-9.

Ren, D. & Xia, J. (2010). Calcium signaling through CatSper channels in mammalian fertilization. *Physiology (Bethesda),* 25(3), pp 165-75.

Revelli, A., Delle Piane, L., Casano, S., Molinari, E., Massobrio, M. & Rinaudo, P. (2009). Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reprod Biol Endocrinol,* 7(40.

Revelli, A., Massobrio, M. & Tesarik, J. (1998). Nongenomic actions of steroid hormones in reproductive tissues. *Endocr Rev*, 19(1), pp 3-17.

Rios, M., Carreno, D. V., Oses, C., Barrera, N., Kerr, B. & Villalon, M. (2016). Low physiological levels of prostaglandins E2 and F2alpha improve human sperm functions. *Reprod Fertil Dev*, 28(4), pp 434-9.

Robaire, B. & Hinton, B. T. 2015. The Epididymis. *Knobil and Neill's Physiology of Reproduction.* 4th ed. USA: Elsevier.

Robinson, D. N. & Cooley, L. (1996). Stable intercellular bridges in development: the cytoskeleton lining the tunnel. *Trends Cell Biol*, 6(12), pp 474-9.

Romarowski, A., Sanchez-Cardenas, C., Ramirez-Gomez, H. V., Puga Molina Ldel, C., Trevino, C. L., Hernandez-Cruz, A., Darszon, A. & Buffone, M. G. (2016). A Specific Transitory Increase in Intracellular Calcium Induced by Progesterone Promotes Acrosomal Exocytosis in Mouse Sperm. *Biol Reprod*, 94(3), pp 63.

Rubtsov, A. M., Sentjurc, M. & Schara, M. (1986). Effect of temperature on Ca-ATPase from sarcoplasmic reticulum membranes: ESR studies. *Gen Physiol Biophys*, 5(5), pp 551-61.

Runft, L. L., Jaffe, L. A. & Mehlmann, L. M. (2002). Egg activation at fertilization: where it all begins. *Dev Biol*, 245(2), pp 237-54.

Sagare-Patil, V., Vernekar, M., Galvankar, M. & Modi, D. (2013). Progesterone utilizes the PI3K-AKT pathway in human spermatozoa to regulate motility and hyperactivation but not acrosome reaction. *Mol Cell Endocrinol*, 374(1-2), pp 82-91.

Salicioni, A. M., Platt, M. D., Wertheimer, E. V., Arcelay, E., Allaire, A., Sosnik, J. & Visconti, P. E. (2007). Signalling pathways involved in sperm capacitation. *Soc Reprod Fertil Suppl*, 65(245-59.

Saling, P. M., Sowinski, J. & Storey, B. T. (1979). An ultrastructural study of epididymal mouse spermatozoa binding to zonae pellucidae in vitro: sequential relationship to the acrosome reaction. *J Exp Zool*, 209(2), pp 229-38.

Sánchez-Cárdenas, C., Servín-Vences, M. R., José, O., Treviño, C. L., Hernández-Cruz, A. & Darszon, A. (2014). Acrosome Reaction and Ca2+ Imaging in Single Human Spermatozoa: New Regulatory Roles of [Ca2+]i. *Biology of Reproduction,* 91(3), pp 67, 1-13.

Sanderson, J. T. (2006). The Steroid Hormone Biosynthesis Pathway as a Target for Endocrine-Disrupting Chemicals. *Toxicological Sciences,* 94(1), pp 3-21.

Sarandakou, A., Malamitsi-Puchner, A., Baka, S., Rizos, D., Hassiakos, D. & Creatsas, G. (2003). Apoptosis and proliferation factors in serum and follicular fluid from women undergoing in vitro fertilization. *Fertil Steril,* 79(3), pp 634-6.

Schuh, K., Cartwright, E. J., Jankevics, E., Bundschu, K., Liebermann, J., Williams, J. C., Armesilla, A. L., Emerson, M., Oceandy, D., Knobeloch, K. P., *et al.* (2004). Plasma membrane Ca2+ ATPase 4 is required for sperm motility and male fertility. *J Biol Chem,* 279(27), pp 28220-6.

Sheng, K. & Liang, X. (2014). The role of histone ubiquitination during spermatogenesis. 2014(870695.

Shimizu, Y., Yorimitsu, A., Maruyama, Y., Kubota, T., Aso, T. & Bronson, R. A. (1998). Prostaglandins induce calcium influx in human spermatozoa. *Mol Hum Reprod,* 4(6), pp 555-61.

Shiomi, H., Yamano, S., Shono, M. & Aono, T. (1996). Characteristics of calcium ion influx induced by human follicular fluid in individual human sperm. *Arch Androl,* 37(2), pp 79-86.

Shukla, K. K., Mahdi, A. A. & Rajender, S. (2012). Ion channels in sperm physiology and male fertility and infertility. *J Androl,* 33(5), pp 777-88.

Siegel, M. S., Paulson, R. J. & Graczykowski, J. W. (1990). The influence of human follicular fluid on the acrosome reaction, fertilizing capacity and proteinase activity of human spermatozoa. *Hum Reprod,* 5(8), pp 975-80.

Sirard, M. A. (2001). Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. *Theriogenology,* 55(6), pp 1241-54.

Sliwa, L. (1995). Effect of some sex steroid hormones on human spermatozoa migration in vitro. *Eur J Obstet Gynecol Reprod Biol,* 58(2), pp 173-5.

Smith, D. J., Gaffney, E. A., Gadelha, H., Kapur, N. & Kirkman-Brown, J. C. (2009). Bend propagation in the flagella of migrating human sperm, and its modulation by viscosity. *Cell Motil Cytoskeleton,* 66(4), pp 220-36.

Smith, J. F., Syritsyna, O., Fellous, M., Serres, C., Mannowetz, N., Kirichok, Y. & Lishko, P. V. (2013). Disruption of the principal, progesterone-activated sperm Ca2+ channel in a CatSper2-deficient infertile patient. *Proc Natl Acad Sci U S A,* 110(17), pp 6823-8.

Sobinoff, A. P., Mahony, M., Nixon, B., Roman, S. D. & McLaughlin, E. A. (2011). Understanding the Villain: DMBA-induced preantral ovotoxicity involves selective follicular destruction and primordial follicle activation through PI3K/Akt and mTOR signaling. *Toxicol Sci,* 123(2), pp 563-75.

Son, W. Y., Lee, J. H., Lee, J. H. & Han, C. T. (2000). Acrosome reaction of human spermatozoa is mainly mediated by alpha1H T-type calcium channels. *Mol Hum Reprod,* 6(10), pp 893-7.

Sosa, C. M., Zanetti, M. N., Pocognoni, C. A. & Mayorga, L. S. (2016). Acrosomal Swelling Is Triggered by cAMP Downstream of the Opening of Store-Operated Calcium Channels During Acrosomal Exocytosis in Human Sperm. *Biol Reprod,* 94(3), pp 57.

Spehr, M., Gisselmann, G., Poplawski, A., Riffell, J. A., Wetzel, C. H., Zimmer, R. K. & Hatt, H. (2003). Identification of a testicular odorant receptor mediating human sperm chemotaxis. *Science,* 299(5615), pp 2054-8.

Spehr, M., Schwane, K., Riffell, J. A., Barbour, J., Zimmer, R. K., Neuhaus, E. M. & Hatt, H. (2004). Particulate adenylate cyclase plays a key role in human sperm olfactory receptor-mediated chemotaxis. *J Biol Chem,* 279(38), pp 40194-203.

Stock, C. E., Bates, R., Lindsay, K. S., Edmonds, D. K. & Fraser, L. R. (1989). Extended exposure to follicular fluid is required for significant stimulation of the acrosome reaction in human spermatozoa. *J Reprod Fertil,* 86(1), pp 401-11.

Storey, B. T. (1995). Interactions between gametes leading to fertilization: the sperm's eye view. *Reprod Fertil Dev,* 7(4), pp 927-42.

Strehler, E. E. & Treiman, M. (2004). Calcium pumps of plasma membrane and cell interior. *Curr Mol Med,* 4(3), pp 323-35.

Stricker, S. A. (1999). Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev Biol,* 211(2), pp 157-76.

Strunker, T., Goodwin, N., Brenker, C., Kashikar, N. D., Weyand, I., Seifert, R. & Kaupp, U. B. (2011). The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. *Nature,* 471(7338), pp 382-6.

Suarez, S. S. 2002. Gamete transport. *In:* Hardy, D. (ed.) *Fertilization.* New York: Academic Press.

Suarez, S. S. (2016). Mammalian sperm interactions with the female reproductive tract. *Cell Tissue Res,* 363(1), pp 185-94.

Suarez, S. S. & Pacey, A. A. (2006). Sperm transport in the female reproductive tract. *Human Reproduction Update,* 12(1), pp 23-37.

Suchanek, E., Simunic, V., Juretic, D. & Grizelj, V. (1994). Follicular fluid contents of hyaluronic acid, follicle-stimulating hormone and steroids relative to the success of in vitro fertilization of human oocytes. *Fertil Steril,* 62(2), pp 347-52.

Sueldo, C. E., Oehninger, S., Subias, E., Mahony, M., Alexander, N. J., Burkman, L. J. & Acosta, A. A. (1993). Effect of progesterone on human zona pellucida sperm binding and oocyte penetrating capacity. *Fertil Steril,* 60(1), pp 137-40.

Sumigama, S., Mansell, S., Miller, M., Lishko, P. V., Cherr, G. N., Meyers, S. A. & Tollner, T. (2015). Progesterone Accelerates the Completion of Sperm Capacitation and Activates CatSper Channel in Spermatozoa from the Rhesus Macaque. *Biol Reprod,* 93(6), pp 130.

Sun, F., Bahat, A., Gakamsky, A., Girsh, E., Katz, N., Giojalas, L. C., Tur-Kaspa, I. & Eisenbach, M. (2005). Human sperm chemotaxis: both the oocyte and its surrounding cumulus cells secrete sperm chemoattractants. *Hum Reprod,* 20(3), pp 761-7.

Sun, X. & Yang, W.-X. (2010). Mitochondria: transportation, distribution and function during spermiogenesis. *Advances in Bioscience and Biotechnology,* 1(2), pp 97-109.

Swann, K. & Lai, F. A. (2016). Egg Activation at Fertilization by a Soluble Sperm Protein. *Physiol Rev,* 96(1), pp 127-49.

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

Tarlatzis, B. C., Pazaitou, K., Bili, H., Bontis, J., Papadimas, J., Lagos, S., Spanos, E. & Mantalenakis, S. (1993). Growth hormone, oestradiol, progesterone and testosterone concentrations in follicular fluid after ovarian stimulation with various regimes for assisted reproduction. *Hum Reprod,* 8(

Taylor, C. W., Genazzani, A. A. & Morris, S. A. (1999). Expression of inositol trisphosphate receptors. *Cell Calcium,* 26(6), pp 237-51.

Tesarik, J. (1985). Comparison of acrosome reaction-inducing activities of human cumulus oophorus, follicular fluid and ionophore A23187 in human sperm populations of proven fertilizing ability in vitro. *J Reprod Fertil,* 74(2), pp 383-8.

Teves, M. E., Barbano, F., Guidobaldi, H. A., Sanchez, R., Miska, W. & Giojalas, L. C. (2006). Progesterone at the picomolar range is a chemoattractant for mammalian spermatozoa. *Fertil Steril,* 86(3), pp 745-9.

Teves, M. E., Guidobaldi, H. A., Unates, D. R., Sanchez, R., Miska, W., Publicover, S. J., Morales Garcia, A. A. & Giojalas, L. C. (2009). Molecular mechanism for human sperm chemotaxis mediated by progesterone. *PLoS One,* 4(12), pp e8211.

Thomas, D. D. & Karon, B. S. (1994). Temperature dependence of molecular dynamics and calcium-ATPase activity in sarcoplasmic reticulum. *The Temperature Adaptation of Biological Membranes, ed Cossins AR (Portland, London, UK),* 1-12.

Thomas, P. & Meizel, S. (1989). Phosphatidylinositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca2+ influx. *Biochem J,* 264(2), pp 539-46.

Thundathil, J., de Lamirande, E. & Gagnon, C. (2002). Different signal transduction pathways are involved during human sperm capacitation induced by biological and pharmacological agents. *Mol Hum Reprod,* 8(9), pp 811-6.

Tisdall, D. J., Watanabe, K., Hudson, N. L., Smith, P. & McNatty, K. P. (1995). FSH receptor gene expression during ovarian follicle development in sheep. *J Mol Endocrinol,* 15(3), pp 273-81.

Toshimori, K. (2009). *Dynamics of the Mammalian Sperm Head*, Berlin: Springer.

Toshimori, K. & Eddy, E. M. 2015. The Spermatozoon. *In:* Plant, T. M. & Zeleznik, A. J. (eds.) *Knobil and Neill's Physiology of Reproduction* 4th ed. USA: Elsevier.

Tosti, E. & Ménézo, Y. (2016). Gamete activation: basic knowledge and clinical applications. *Human Reproduction Update,* 22(4), pp 420-439.

Trevino, C. L., Santi, C. M., Beltran, C., Hernandez-Cruz, A., Darszon, A. & Lomeli, H. (1998). Localisation of inositol trisphosphate and ryanodine receptors during mouse spermatogenesis: possible functional implications. *Zygote,* 6(2), pp 159-72.

Tripathi, A., Kumar, K. V. P. & Chaube, S. K. (2010). Meiotic cell cycle arrest in mammalian oocytes. *Journal of Cellular Physiology,* 223(3), pp 592-600.

Triphan, J., Aumüller, G., Brandenburger, T. & Wilhelm, B. (2007). Localization and regulation of plasma membrane Ca2+-ATPase in bovine spermatozoa. *European Journal of Cell Biology,* 86(5), pp 265-273.

Tsaadon, A., Eliyahu, E., Shtraizent, N. & Shalgi, R. (2006). When a sperm meets an egg: block to polyspermy. *Mol Cell Endocrinol,* 252(1-2), pp 107-14.

Tulsiani, D. R., Abou-Haila, A., Loeser, C. R. & Pereira, B. M. (1998). The biological and functional significance of the sperm acrosome and acrosomal enzymes in mammalian fertilization. *Exp Cell Res,* 240(2), pp 151-64.

Tunquist, B. J. & Maller, J. L. (2003). Under arrest: cytostatic factor (CSF)-mediated metaphase arrest in vertebrate eggs. *Genes & Development,* 17(6), pp 683-710.

Turnbull, K. E., Braden, A. W. & Mattner, P. E. (1977). The pattern of follicular growth and atresia in the ovine ovary. *Aust J Biol Sci,* 30(3), pp 229-41.

Turner, R. M. (2006). Moving to the beat: a review of mammalian sperm motility regulation. *Reprod Fertil Dev,* 18(1-2), pp 25-38.

Uehara, S., Naganuma, T., Tsuiki, A., Kyono, K., Hoshiai, H. & Suzuki, M. (1985). Relationship between follicular fluid steroid concentrations and in vitro fertilization. *Obstet Gynecol,* 66(1), pp 19-23.

Uhler, M. L., Leung, A., Chan, S. Y. & Wang, C. (1992). Direct effects of progesterone and antiprogesterone on human sperm hyperactivated motility and acrosome reaction. *Fertil Steril,* 58(6), pp 1191-8.

Unates, D. R., Guidobaldi, H. A., Gatica, L. V., Cubilla, M. A., Teves, M. E., Moreno, A. & Giojalas, L. C. (2014). Versatile action of picomolar gradients of progesterone on different sperm subpopulations. *PLoS One,* 9(3), pp e91181.

Valdimarsson, G., De Sousa, P. A. & Kidder, G. M. (1993). Coexpression of gap junction proteins in the cumulus-oocyte complex. *Molecular Reproduction and Development,* 36(1), pp 7-15.

van Wezel, I. L. & Rodgers, R. J. (1996). Morphological characterization of bovine primordial follicles and their environment in vivo. *Biol Reprod,* 55(5), pp 1003-11.

Vanderhaeghen, P., Schurmans, S., Vassart, G. & Parmentier, M. (1997). Specific repertoire of olfactory receptor genes in the male germ cells of several mammalian species. *Genomics,* 39(3), pp 239-46.

Vanderhyden, B. C., Telfer, E. E. & Eppig, J. J. (1992). Mouse oocytes promote proliferation of granulosa cells from preantral and antral follicles in vitro. *Biol Reprod,* 46(6), pp 1196-204.

Varano, G., Lombardi, A., Cantini, G., Forti, G., Baldi, E. & Luconi, M. (2008). Src activation triggers capacitation and acrosome reaction but not motility in human spermatozoa. *Hum Reprod,* 23(12), pp 2652-62.

Ventela, S. 2006. Cytoplasmic Bridges as Cell-Cell Channels of Germ Cells. *In:* Baluska, F., Volkmann, D. & Barlow, P. W. (eds.) *Cell-Cell Channels.* New York: Springer.

Verma, R. J. (2001). Sperm quiescence in cauda epididymis: a mini-review. *Asian J Androl,* 3(3), pp 181-3.

Vigil, P., Barrientos, V. M., Vargas, G. G., Machuca, D. A. & Cortes, M. E. (2012). Assessment of the effect of testosterone on the acrosome reaction of human spermatozoa. *Andrologia,* 44 Suppl 1(627-33.

Vigil, P., Toro, A. & Godoy, A. (2008). Physiological action of oestradiol on the acrosome reaction in human spermatozoa. *Andrologia,* 40(3), pp 146-51.

Villanueva-Diaz, C., Arias-Martinez, J., Bermejo-Martinez, L. & Vadillo-Ortega, F. (1995). Progesterone induces human sperm chemotaxis. *Fertil Steril,* 64(6), pp 1183-8.

Villanueva-Diaz, C., Vadillo-Ortega, F., Kably-Ambe, A., Diaz-Perez, M. A. & Krivitzky, S. K. (1990). Evidence that human follicular fluid contains a chemoattractant for spermatozoa. *Fertil Steril,* 54(6), pp 1180-2.

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

Visconti, P. E., Bailey, J. L., Moore, G. D., Pan, D., Olds-Clarke, P. & Kopf, G. S. (1995a). Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development,* 121(4), pp 1129-37.

Visconti, P. E., Krapf, D., de la Vega-Beltran, J. L., Acevedo, J. J. & Darszon, A. (2011). Ion channels, phosphorylation and mammalian sperm capacitation. *Asian J Androl,* 13(3), pp 395-405.

Visconti, P. E., Moore, G. D., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, D., Olds-Clarke, P. & Kopf, G. S. (1995b). Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development,* 121(4), pp 1139-50.

Visconti, P. E., Stewart-Savage, J., Blasco, A., Battaglia, L., Miranda, P., Kopf, G. S. & Tezon, J. G. (1999). Roles of bicarbonate, cAMP, and protein tyrosine phosphorylation on capacitation and the spontaneous acrosome reaction of hamster sperm. *Biol Reprod,* 61(1), pp 76-84.

Walensky, L. D., Roskams, A. J., Lefkowitz, R. J., Snyder, S. H. & Ronnett, G. V. (1995). Odorant receptors and desensitization proteins colocalize in mammalian sperm. *Mol Med,* 1(2), pp 130-41.

Walensky, L. D. & Snyder, S. H. (1995). Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. *J Cell Biol,* 130(4), pp 857-69.

Walker, W. H. (2010). Non-classical actions of testosterone and spermatogenesis. *Philos Trans R Soc Lond B Biol Sci,* 365(1546), pp 1557-69.

Wandji, S. A., Srsen, V., Voss, A. K., Eppig, J. J. & Fortune, J. E. (1996). Initiation in vitro of growth of bovine primordial follicles. *Biol Reprod,* 55(5), pp 942-8.

Wang, H., Liu, J., Cho, K.-H. & Ren, D. (2009). A Novel, Single, Transmembrane Protein CATSPERG Is Associated with CATSPER1 Channel Protein. *Biology of Reproduction,* 81(3), pp 539-544.

Wang, Q., Kim, J. Y., Xue, K., Liu, J. Y., Leader, A. & Tsang, B. K. (2012). Chemerin, a novel regulator of follicular steroidogenesis and its potential involvement in polycystic ovarian syndrome. *Endocrinology,* 153(11), pp 5600-11.

Wang, T. H., Chang, C. L., Wu, H. M., Chiu, Y. M., Chen, C. K. & Wang, H. S. (2006). Insulin-like growth factor-II (IGF-II), IGF-binding protein-3 (IGFBP-3), and IGFBP-4 in follicular fluid are associated with oocyte maturation and embryo development. *Fertil Steril,* 86(5), pp 1392-401.

Wang, Y., Storeng, R., Dale, P. O., Åbyholm, T. & Tanbo, T. (2001). Effects of follicular fluid and steroid hormones on chemotaxis and motility of human spermatozoa in vitro. *Gynecological Endocrinology,* 15(4), pp 286-292.

Wassarman, P. M. (1990). Profile of a mammalian sperm receptor. *Development,* 108(1), pp 1-17.

Wassarman, P. M. & Litscher, E. S. (2008). Mammalian fertilization: the egg's multifunctional zona pellucida. *Int J Dev Biol,* 52(5-6), pp 665-76.

Wassarman, P. M., Liu, C. & Litscher, E. S. (1996). Constructing the mammalian egg zona pellucida: some new pieces of an old puzzle. *Journal of Cell Science,* 109(2001-2004.

Wehling, M., Schultz, A. & Losel, R. (2007). To be or not to be (a receptor). *Steroids,* 72(2), pp 107-10.

Wen, X., Tozer, A. J., Butler, S. A., Bell, C. M., Docherty, S. M. & Iles, R. K. (2006). Follicular fluid levels of inhibin A, inhibin B, and activin A levels reflect changes in follicle size but are not independent markers of the oocyte's ability to fertilize. *Fertil Steril,* 85(6), pp 1723-9.

Wendler, A., Albrecht, C. & Wehling, M. (2012). Nongenomic actions of aldosterone and progesterone revisited. *Steroids*, 77(10), pp 1002-6.

Wennemuth, G., Babcock, D. F. & Hille, B. (2003). Calcium clearance mechanisms of mouse sperm. *J Gen Physiol*, 122(1), pp 115-28.

Wessel, G. M., Brooks, J. M., Green, E., Haley, S., Voronina, E., Wong, J., Zaydfudim, V. & Conner, S. (2001). The biology of cortical granules. *Int Rev Cytol*, 209(117-206.

Whirledge, S. & Cidlowski, J. A. (2010). Glucocorticoids, stress, and fertility. *Minerva Endocrinol*, 35(2), pp 109-25.

White-Cooper, H. (2004). Spermatogenesis: analysis of meiosis and morphogenesis. *Methods Mol Biol*, 247(45-75.

WHO. (2010). *WHO laboratory manual for the Examination and processing of human semen*, 5th Edition: WHO Press, Geneva.

Williams, H. L., Mansell, S., Alasmari, W., Brown, S. G., Wilson, S. M., Sutton, K. A., Miller, M. R., Lishko, P. V., Barratt, C. L., Publicover, S. J., *et al.* (2015). Specific loss of CatSper function is sufficient to compromise fertilizing capacity of human spermatozoa. *Hum Reprod*, 30(12), pp 2737-46.

Wootton, L. L., Argent, C. C., Wheatley, M. & Michelangeli, F. (2004). The expression, activity and localisation of the secretory pathway Ca2+ -ATPase (SPCA1) in different mammalian tissues. *Biochim Biophys Acta*, 1664(2), pp 189-97.

Wu, D. (2005). Signaling mechanisms for regulation of chemotaxis. *Cell Res*, 15(1), pp 52-56.

Wu, Y. T., Tang, L., Cai, J., Lu, X. E., Xu, J., Zhu, X. M., Luo, Q. & Huang, H. F. (2007). High bone morphogenetic protein-15 level in follicular fluid is associated with high quality oocyte and subsequent embryonic development. *Hum Reprod*, 22(6), pp 1526-31.

Xia, P. & Younglai, E. V. (2000). Relationship between steroid concentrations in ovarian follicular fluid and oocyte morphology in patients undergoing intracytoplasmic sperm injection (ICSI) treatment. *J Reprod Fertil*, 118(2), pp 229-33.

Yamano, S., Yamazaki, J., Irahara, M., Tokumura, A., Nakagawa, K. & Saito, H. (2004). Human spermatozoa capacitated with progesterone or a long incubation show accelerated internalization by an alkyl ether lysophospholipid. *Fertil Steril,* 81(3), pp 605-10.

Yanagimachi, H. 1994. Mammalian Fertilization. *In:* Knobil, E. & Neill, J. D. (eds.) *The Physiology of Reproduction.* New York: Raven Press.

Yanagimachi, R. (1969). In vitro capacitation of hamster spermatozoa by follicular fluid. *J Reprod Fertil,* 18(

Yanagimachi, R. 1981. Mechanisms of Fertilization in Mammals. *In:* Mastroianni, L., Jr. & Biggers, J. (eds.) *Fertilization and Embryonic Development In Vitro.* Springer US.

Yanagimachi, R. & Chang, M. C. (1963). Sperm ascent through the oviduct of the hamster and rabbit in relation to the time of ovulation. *J Reprod Fertil,* 6(413-20.

Yang, J., Serres, C., Philibert, D., Robel, P., Baulieu, E. E. & Jouannet, P. (1994). Progesterone and RU486: opposing effects on human sperm. *Proceedings of the National Academy of Sciences,* 91(2), pp 529-533.

Yao, Y. Q., Chiu, C. N., Ip, S. M., Ho, P. C. & Yeung, W. S. (1998). Glycoproteins present in human follicular fluid that inhibit the zona-binding capacity of spermatozoa. *Hum Reprod,* 13(9), pp 2541-7.

Yao, Y. Q., Yeung, W. S. & Ho, P. C. (1996). Human follicular fluid inhibits the binding of human spermatozoa to zona pellucida in vitro. *Hum Reprod,* 11(12), pp 2674-80.

Yauger, B., Boggs, N. A. & Dean, J. (2011). Human ZP4 is not sufficient for taxon-specific sperm recognition of the zona pellucida in transgenic mice. *Reproduction,* 141(3), pp 313-9.

Yeste, M., Jones, C., Amdani, S. N., Patel, S. & Coward, K. (2016). Oocyte activation deficiency: a role for an oocyte contribution? *Hum Reprod Update,* 22(1), pp 23-47.

Yeung, W. S., Lee, K. F., Koistinen, R., Koistinen, H., Seppala, M. & Chiu, P. C. (2009). Effects of glycodelins on functional competence of spermatozoa. *J Reprod Immunol,* 83(1-2), pp 26-30.

Yoshida, M. & Yoshida, K. (2011). Sperm chemotaxis and regulation of flagellar movement by Ca2+. *Molecular Human Reproduction*, 17(8), pp 457-465.

Young, J. M. & McNeilly, A. S. (2010). Theca: the forgotten cell of the ovarian follicle. *Reproduction*, 140(4), pp 489-504.

Zamah, A. M., Hassis, M. E., Albertolle, M. E. & Williams, K. E. (2015). Proteomic analysis of human follicular fluid from fertile women. *Clinical Proteomics*, 12(1), pp 5.

Zaneveld, L. J., De Jonge, C. J., Anderson, R. A. & Mack, S. R. (1991). Human sperm capacitation and the acrosome reaction. *Hum Reprod*, 6(9), pp 1265-74.

Zhang, S. 1999. Male Reproductive Syatem. *An Atlas of Histology.* New York: Springer-Verlag.

Zhu, J., Massey, J. B., Mitchell-Leef, D., Elsner, C. W., Kort, H. I. & Roudebush, W. E. (2006). Platelet-activating factor acetylhydrolase activity affects sperm motility and serves as a decapacitation factor. *Fertil Steril*, 85(2), pp 391-4.

Zollner, K. P., Hofmann, T. & Zollner, U. (2013). Good fertilization results associated with high IL-1beta concentrations in follicular fluid of IVF patients. *J Reprod Med*, 58(11-12), pp 485-90.