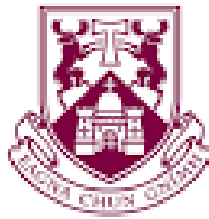


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The role of progesterone-induced hyperactivation in the detachment of bull sperm from the oviduct reservoir

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O L L S C O I L L U I M N I G H

The Role of Progesterone-Induced Hyperactivation in the Detachment of Bull Sperm from the Oviduct Reservoir.

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Submitted in accordance with academic requirements for the degree of Master of Science to the Department of Biological Sciences, School of Natural Sciences, Faculty of Science and Engineering, University of Limerick, Ireland.

September 2017

Declaration

I, the undersigned, hereby declare that I am the sole author of this work and it has not been submitted to any other University or higher education institution, or for any other academic award in this University. To identify the work of others, all sources have been fully acknowledged and referenced in both text and bibliography, in accordance with University of Limerick requirements.

Signature: _____

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Sinéad Cronin

Acknowledgements

I would like to express my gratitude to everyone who supported me throughout this thesis.

To Dr Seán Fair, I thank you sincerely for the advice and mentorship in my bad days and my good. Your feedback and support have been invaluable in the coordination of this learning experience. Thank you for the opportunity to work with your team.

To my remarkable parents, I thank you so much for the encouragement and love ye have given me throughout this masters. For the helping hand and the listening ear, the positivity and the reassurance and mostly for giving me the opportunity to make this thesis possible. I cannot thank you enough.

To my amazing boyfriend, you have been my rock throughout this masters. Thank you for being there for me through everything. Your patience and love have been incredible.

To my aunts, my siblings and my friends, thank you for the support, the cheer and the copious amounts of tea/coffee throughout the year. You have been the light to brighten my days and were always there when needed most.

To all in the Department of Biological Sciences past and present who truly enriched my time in the laboratory. I thank you for your excellent guidance and assistance. With a very special thanks to Shauna, Emer, Alan, Jon and Laura, this experience would not have been the same without each and everyone of you.

Abstract

For optimum fertility, sperm should reside in the bovine female reproductive tract for a number of hours before the onset of ovulation in order to ensure sperm have successfully acquired the ability to fertilise the oocyte. The formation of the sperm reservoir is aided by the complexity of the convoluted isthmus and the molecular recognition between the fucose receptor on the sperm plasma membrane and the carbohydrate residues on the oviduct epithelium. Sperm are then sequentially released from the reservoirs at a time that coincides with ovulation and these release mechanisms are not well understood in mammals, particularly in bovines. Therefore, the aim of this study was to elucidate the mechanisms that trigger the detachment of bull sperm from oviduct epithelial cells. Capacitation of frozen-thawed sperm *in vitro* resulted in a reduced binding affinity to bovine oviductal epithelial cell (BOEC) explants when treated with heparin, caffeine or heparin and caffeine in combination ($P < 0.001$). When non-capacitated sperm were co-incubated with BOEC explants, high binding affinity was apparent and the release of sperm was stimulated through capacitating factors (heparin) and hyperactivation of sperm through treatment with caffeine ($P < 0.001$). As the cumulus cells of the bovine oocyte are known to secrete progesterone (P4) it was hypothesised that P4 could induce hyperactivation and thus the release of sperm bound to the oviduct epithelium. Progesterone-treated sperm displayed a heightened level of hyperactivated motility ($P < 0.001$) which was suppressed when calcium (Ca^{2+}) influx was inhibited through Mibefradil (non-specific Ca^{2+} channel antagonist), NNC 550396 (CatSper specific Ca^{2+} channel antagonist), or when extracellular Ca^{2+} was chelated using Ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; $P < 0.001$). Hyperactivation induced by P4 stimulated the release of bound sperm from BOEC explants ($P < 0.01$), and this detachment required an influx of extracellular Ca^{2+} . Furthermore, we investigated the mode of action of P4 on bull sperm and the detachment of sperm from BOEC explants by P4, which was inhibited by Mifepristone (progesterone membrane receptor antagonist) and AG205 (progesterone membrane component 1 specific antagonist; PGRMC1, $P < 0.01$). These findings suggest the presence of a P4 membrane receptor on bull sperm and that P4 is capable of inducing the release of sperm from the bovine oviductal epithelium. This P4 induced release is mediated by extracellular Ca^{2+} . This study has increased our understanding of sperm interaction with the female reproductive tract and may aid in the development of fertility biomarkers in bovine reproduction.

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Abbreviations.

AF	Transcription activation factor
AI	Artificial Insemination
ANXA	Annexin protein
ATK/PKB	Protein kinase B
BOEC	Bovine oviductal epithelial cell
BSP	Bovine seminal plasma protein
Ca ²⁺	Calcium ion
Cav	Voltage-dependent L-type channel
cGMP	Cyclic guanosine monophosphate
Cl ⁻	Chloride Ion
CNG	Cyclic nucleotide-gated channel
DF	Decapacitation factors
diPUFA	phospholipids esterified with two PUFAs
DNA	Deoxyribonucleic acid
E ₂	Estrogen
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular receptor kinase
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GSH	Glutathione
HCN	Hyperpolarisation-activated cyclic nucleotide channel

HCO ₃ ⁻	Bicarbonate
Hv1	Voltage-gated proton channel
IATP	Adenosine triphosphate gated channel
IP ₃	Inositol triphosphate
IP ₃ R	IP ₃ receptor
K ⁺	Potassium Ion
MAPK	Mitogen-activated protein kinase
mGC	Membrane-bound guanylate cyclase
Na ⁺	Sodium Ion
NNC	NNC 55-0396; (1S,2S)-2-(2-(N-[(3-Benzimidazol-2-yl)propyl]-N-methylamino)ethyl)- 6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl cyclopropanecarboxylate dihydrochloride hydrate
NO	Nitric oxide
nPR	Nuclear progesterone receptor
P4	Progesterone
PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase
PEBP	Phosphatidylethanolamine-binding protein
PGRMC	Progesterone receptor membrane component
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLA	Phospholipase A
PLC	Phospholipase C

PLD	Phospholipase D
PMCA	Plasma membrane ATPase
PTK	Protein Tyrosine Kinase
PUFA	polyunsaturated fatty acid
RNE	Redundant nuclear envelope
RU-486	Mifepristone
ROS	Reactive oxygen species
s.e.m	Standard error of the mean
sAC	Soluble adenyl cyclase
sGC	Soluble guanylate cyclase
SOC	Store-operated calcium channel
STK	Serine/Threonine protein kinase
TALP	Tyrode's Albumin Lactate Pyruvate
TRPC	Transient receptor potential channel
UTJ	Utero-tubal junction
ZP	Zona Pelucida
β D126/	
DEF β 126	Beta-defensin 126

Chapter

1

Literature Review

Chapter One: Literature Review.

1.1 Introduction

In 2016, gross agricultural output in Ireland was valued at €6.92 billion, with the dairy industry accounting for 29.5% of this (DAFM, 2017). The dairy production system in Ireland predominantly operates on a seasonal grass-based system with 6,395 million litres of domestic milk produced in 2016 (Bord Bia, 2017; CSO, 2017). The utilisation of this cost-effective feed during a cows lactation is paramount to low-cost milk production and to achieve this, cows must calve in a compact time frame at the beginning of Spring to maximise milk output in accordance with grass availability (Teagasc, 2017). While the target calving interval is 365 days, currently the Irish national average calving interval stands at 391 days (ICBF, 2017). An extended calving interval costs an additional €2.20 per cow per each day outside of the 365 day target calving interval (Lenehan, 2017). Thus, fertility is a key driver of on-farm profitability in seasonal grass based production systems. With this in mind, the economic breeding index (EBI) was launched in 2001 which is a profit index that ranks animals on a combination of production, fertility and health traits. The fertility sub-index which is based on calving interval and survival rates accounts for 34.9% of the animals overall EBI. In the case of bulls, it ranks them on their daughter's predicted ability to yield a profit compared to a reference population (ICBF, 2017; Wickham, 2013).

While the advent of genomics in the dairy industry has increased the rate of genetic gain, it has also brought some challenges. Young bulls produce lower volumes of semen with less sperm per ejaculate (Mathevon et al., 1998; Baker et al., 1955). One way of increasing the number of progeny born to each of these sires is to lower the sperm number in each insemination dose. Currently, in Ireland, a 0.25 ml frozen-thawed straw contains 15-20 x 10⁶ sperm per dose (Al Naib et al., 2011). While acknowledging some bulls have compensable sperm traits, a reduction in the sperm concentration per straw without a concomitant reduction in fertility is highly desirable, as it would mean greater availability of straws of elite bulls. One way to achieve this is to better understand the interactions of sperm with the female reproductive tract. This thesis describes a series of experiments on how the propulsive force elicited by hyperactivation is a requirement for the detachment

of bull sperm from the sperm reservoirs in the oviduct and the role of progesterone (P4) and calcium (Ca²⁺) in this process.

1.2 Sperm Transport in the Oviduct

A critical step in the spermatozoa's journey to the female gamete is passing the utero-tubal junction (UTJ). The UTJ marks the distal end of the oviduct and presents anatomical, physiological and mucosal barriers to sperm transit to the oocyte (Hung and Suarez, 2010). The lumen of the UTJ in bovines is narrow and intricate with deep mucous folds in the lining which face back towards the uterus (Figure 1) (Hook and Hafez, 1968; Yaniz et al., 2006), thereby, creating a complex and branched passage for sperm (Suarez, 2015; Wrobel et al., 1993). It has been shown that knock-out mice for *ADAM1a* (Nishimura et al., 2004), *ADAM2* (Cho et al., 1998), *ADAM3* (Shamsadin et al., 1999), *Clgn* (Ikawa et al., 1997), *PDILT* (Tokuhiro et al., 2012), *Calr3* (Ikawa et al., 2011), *Ace* (Hagaman et al., 1998) and



Figure 1: Longitudinal section of the utero-tubal junction of a cow. Secondary folds forming dead ends. Crypts occupy the spaces between the folds X15 (Yaniz et al., 2000).

Tpst2 (Marcello et al., 2011) show difficulties in their sperm passing the UTJ. Interestingly, each of these genes encodes proteins which interact with *ADAM3* (a disintegrin and metalloprotease) (Nishimura et al., 2004; Tokuhiro et al., 2012). There is evidence that some molecular interaction must occur between the sperm cell surface and the UTJ (Nakanishi et al., 2004), yet the actual mechanism remains unknown.

The oviduct is divided into three anatomical regions composed of similar cell types but with differing proportions, while each region holds a distinct physiological function. The fimbriated infundibulum, the rostral portion of the oviduct, transports the

female gamete toward the ampulla segment post ovulation (Bosch and Wright, 2005). The ampulla is a dilated tubular region, with folds projecting toward the lumen where fertilisation occurs (Hess et al., 2006; Suarez, 2006). The proximal segment of the oviduct, the isthmus, is involved in male gamete storage and transport and thereafter embryo transport (Hunter, 1984; Suarez, 2002). The oviductal wall is comprised of distinct layers including an external serosal mesosalpinx, an intermediate double-layered mesosalpinx and an internal endosalpinx. This internal layer, also known as oviductal mucosa, contains folds of columnar ciliated epithelial cells (cilia length of ~10 μ m, diameter 0.25 μ m; Figure 2) and non-ciliated secretory cells (displaying surface microvilli; Figure 2) (Bosch and Wright, 2005; Satir, 1992).

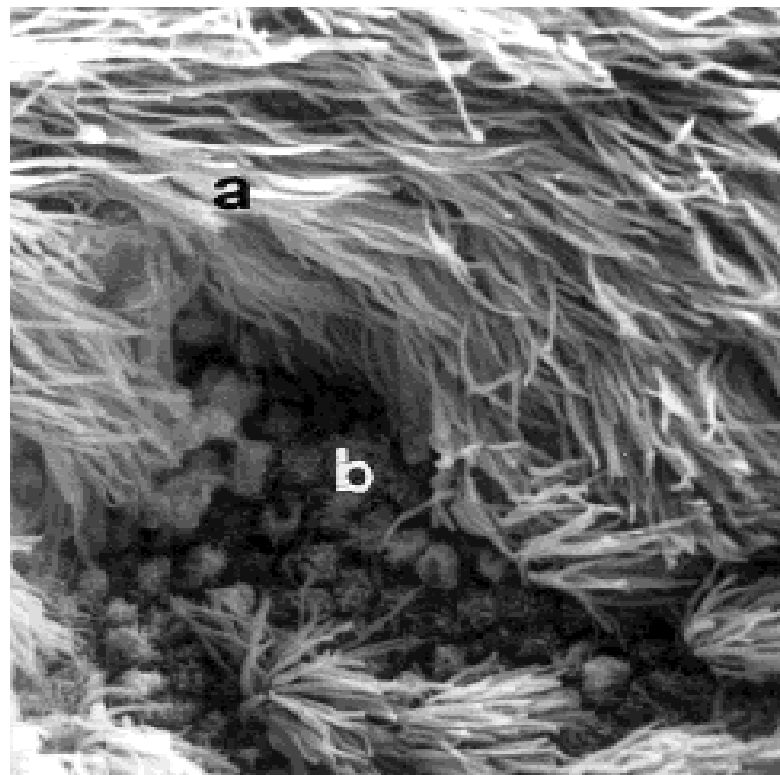


Figure 2: Cell components of the bovine isthmus region. (a) cells with beating cilia, which partially cover (b) the non-protruding secretory cells in basement pockets. X2000 (Yaniz et al., 2000).

Sperm that quickly migrate past the isthmus directly to the ampullary region have been shown to lack fertilisation capabilities, therefore, through interaction with the epithelium, sperm viability and fertilisation ability can be maintained for longer. (Pollard et al., 1991; Chian et al., 1995; Croxatto, 2002; Suarez, 2008). Moreover, sperm recovered from the isthmus of the rabbit have been observed as immotile or weakly motile (Overstreet and Cooper, 1975). This was interpreted as a transitory suppression or inhibition of flagellar movement, as a suppression of motility was seen when sperm were diluted with ampullary fluid or similar constituent media *in vitro* (Overstreet et al., 1980). Sperm that bind to epithelial cells have been shown to have intact acrosomes (Gualtieri and Talevi, 2000), uncapacitated status (Smith and Yanagimachi, 1991; Lefebvre and Suarez, 1996; Fazeli et al., 1999), low intracellular Ca²⁺ content, low tyrosine phosphorylation (Gualtieri et al., 2005; Petrunkina et al., 2001) and normal chromatin structure (Ellington et al., 1999). Moreover, it was previously thought that sperm bound equally well to the epithelium of the isthmus and the ampulla (Lefebvre et al., 1995), however, movement of fluorescently labelled sperm and co-culture bioassays of the oviduct determined that greater numbers of sperm associated with the isthmus compared the ampulla in mouse and human (Baillie et al., 1997; Chang and Suarez, 2012). Therefore, the isthmic portion of the oviduct has been the primary focus of the interaction of sperm-oviduct interactions *in vivo*.

1.3 Sperm Binding to the Oviductal Epithelium.

The isthmic portion of the oviduct is commonly known as the anatomical base of the sperm reservoir (Hunter, 1984; Suarez, 1987). The sperm reservoir is formed when many sperm accumulate together and bind by the apical region of the acrosome to the ciliated surface of the epithelial cells (Suarez, 1987; Yanagimachi and Chang, 1963). The isthmus features a narrow lumen with deep furrows formed by complex folds of mucosa which may contribute to sperm entrapment in this region (Hunter, 1995).

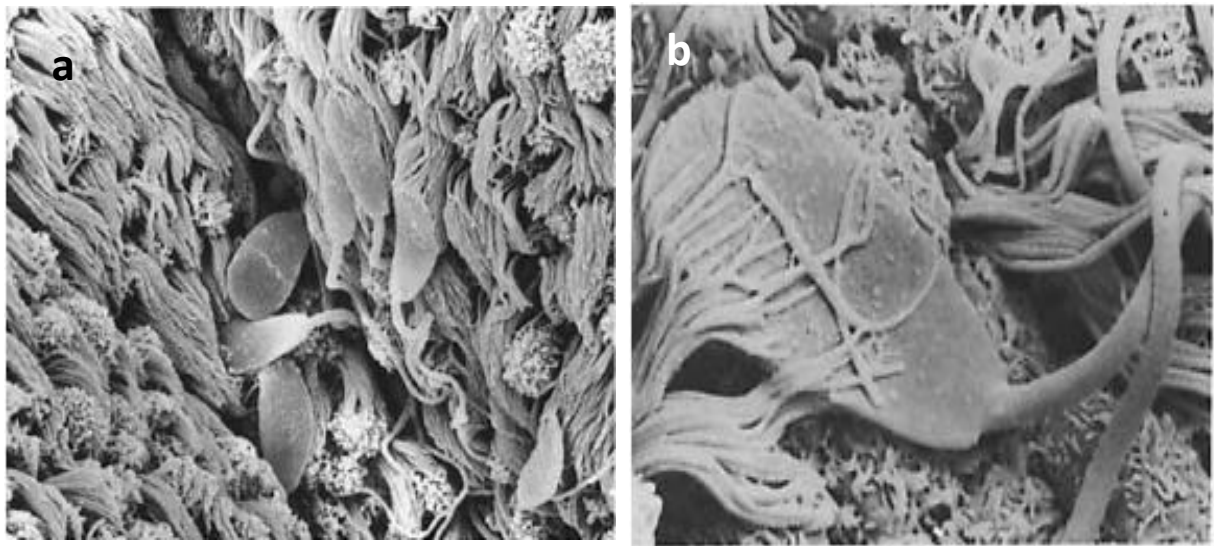


Figure 3: Bull sperm bound to cilia of epithelial cells of the isthmus. (a) in grooves of secondary folds. Scanning electron micrographs x1500. (b) apical region of sperm interacting with cilia of oviductal epithelial cells x5000 (Lefebvre et al., 1995).

The sperm-epithelial cell interaction is a reversible process and is mediated by lectin-like molecules on the sperm rostral surface, binding to oligosaccharide moieties on the oviductal cell membrane (DeMott et al., 1995; Dobrinski et al., 1996; Lefebvre et al., 1997; Ignatz et al., 2001; Green et al., 2001; Wagner et al., 2002; Cortes et al., 2004; Suarez et al., 1998). The sperm-epithelial cell association has long been hypothesised as ligand-receptor affiliation which, in studies to date, is different across species (DeMott et al., 1995). In hamsters, sialic acid and a sialoglycoprotein (fetuin) caused a reduction in the number of bound sperm on epithelial explants, thus proposing the role of such molecules in the sperm-epithelial cell interaction (DeMott et al., 1995). Dobrinski et al. (1996) demonstrated that the addition of galactose or glycoproteins (with the galactosyl residues exposed) to stallion sperm, prevented sperm attachment to epithelial cells. Formation of the sperm reservoir in pig sperm involve molecules on the sperm surface that interact with Lewis X trisaccharide and a mannosyl-oligosaccharide, lactosamine with 6-sialylated biantennary glycans on the apical surface of the oviductal epithelial cells (Töpfer-Petersen et al., 2002; Kadirvel et al., 2012)

In bull sperm, fucose in an alpha 1-4 linkage to N-acetylglucosamine which is found in the Lewis A trisaccharide suppressed bull sperm binding to epithelial cells (Lefebvre et al., 1997; Suarez et al., 1998). Lewis A, the significant carbohydrate moiety of the bull sperm receptor was used to determine a binding ligand for sperm, from which a 16.5 kDa sperm protein was detected (Ignotz et al., 2001). This protein, PDC-109, secreted by the seminal vesicles of the bull, associates with the sperm plasma membrane over the acrosomal region and recognises the fucosylated residues present on the epithelial cells (Figure 4) (Lefebvre et al., 1997; Suarez et al., 1998). PDC-109 (also known as BSP-A1/A2) is a member of the bovine seminal plasma (BSP) family of proteins (Srivastava et al., 2013). The absorption of PDC-109 on the surface of epididymal sperm is associated with volume regulatory ability of the cell (Sahin et al., 2009), which, coincidentally is a requirement for selection and preferential binding of high quality, fertile sperm to the oviductal epithelium (Khalil et al., 2006).

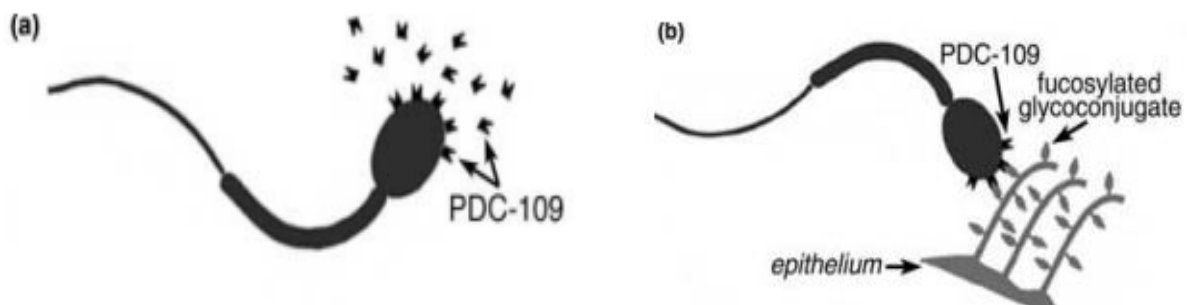


Figure 4: Bull sperm binding to oviductal epithelium. (a) PDC-109 addition to the sperm plasma membrane from seminal vesicles. (b) Sperm containing PDC-109 in the oviduct bind to fucosylated residues on the oviductal epithelium (Suarez, 2002).

Other BSP protein members such as BSP-30K (also known as BSP-5) and BSP-A3 have also been shown to enhance binding of sperm to the oviductal epithelium, although only expressed at a tenth of the level of PDC109 (Nauc and Manjunath, 2000; Gwathmey et al., 2006). Each of these BSP proteins consists of an N terminal domain, two FN2 domains with phospholipid and heparin binding sites (Fan et al., 2006). PDC109 is known to be

secreted by the seminal vesicles at 15-20 mg/mL and bind to sperm via the choline phospholipid domains at 9.05×10^6 molecules/cell (Manjunath et al., 1994; Calvete et al., 1994). Gwathmey et al. (2006), have reported that epididymal sperm have a low affinity to bind to oviduct epithelial cells, while incubation of epididymal sperm with purified BSP's significantly increases binding to the oviduct *in vitro* (Gwathmey et al., 2003; Gwathmey et al., 2006). Since members of the BSP family have been expressed in the bovine epididymis (Fan et al., 2006), this may explain the reduced binding ability of epididymal sperm in comparison to ejaculated sperm exposed to BSPs from seminal plasma (Gwathmey et al., 2006).

Bovine seminal plasma proteins were used to investigate oviductal ligands from the apical plasma membranes of the isthmus. From this, four proteins from the Ca^{2+} and lipid-binding protein family were immunolocalised. These included annexin 1, 2, 3 and 5 (ANXA1, 2, 3 and 5) (Ignotz et al., 2007). Through western blot analysis, it was found that ANXAs contain fucose and binding was inhibited by antibodies towards ANXAs (Ignotz et al., 2007). Similar to BSP proteins, ANXAs bind to Heparin, a glycosaminoglycan (GAG) in the oviduct (Parrish et al., 1989a), which may demonstrate its role, or a similar GAG, in the process of binding and release of sperm from the oviduct (Gualtieri and Talevi, 2000; Shao et al., 2006; Ishitsuka et al., 1998).

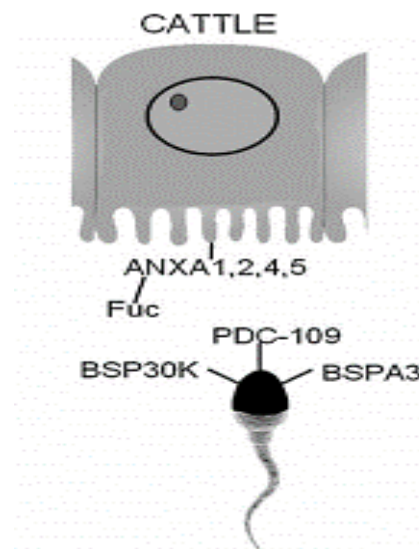


Figure 5: Annexin proteins and sperm bovine seminal plasma proteins (BSPs) involved in bull sperm-oviductal epithelial binding in cattle (Talevi and Gualtieri, 2010).

Another sperm protein, the cysteine-rich cationic polypeptide of beta-defensin (BD126 or DEFB126) has been shown to enhance binding of macaque sperm to the oviductal epithelium *in vitro* (Tollner et al., 2008). This protein is secreted in the caudal epididymis and adheres to the entire surface of the sperm in macaque (Yudin et al., 2003). Recent studies using immunohistochemistry and western blot analysis, show the BD126 protein exists as a dimer and was shown to be expressed by epithelial cells of the caudal epididymis and vas deferens in bulls and on the flagellum of sperm (Narciandi et al., 2016; Fernandez-Fuertes et al., 2016). The presence of this protein in bulls suggests a possible role in sperm adhesion to bovine epithelial cells. Lyons (2016) has recently demonstrated that bovine corpus sperm (i.e. sperm with no BD126) incubated with BD126 recombinant protein, had increased binding affinity to bovine oviductal explants *in vitro*. Moreover, the presence of similar proteins may also play a role in sperm-epithelial cell interactions and their removal or alteration may affect the sperm binding affinity for the epithelial cells.

1.4 Detachment of Sperm from the Oviductal Epithelium.

The mechanisms for sperm release from the oviductal reservoir are induced *in vivo* by oviductal microenvironments surrounding bound sperm. These can be modulated by alternative or synergic events including the preovulatory milieu or the presence of the cumulus cells surrounding the oocyte. There is evidence of two mechanisms which cause sperm to detach from the epithelial cells, namely 1) modification of sperm surface proteins during the process of capacitation and 2) hyperactivation (see section 1.4.2).

1.4.1 Capacitation and its Role in Sperm Detachment from the Oviductal Epithelium

Capacitation is the biochemical and biophysical priming of sperm which consists of a series of intracellular and surface transformations which results in acrosome exposure which is essential for fertilisation (Yanagimachi, 1994). It includes loss of cholesterol from the sperm plasma membrane, removal of the glycocalyx components (Saxena et al., 1986), increased protein tyrosine phosphorylation (Visconti et al., 1995; Visconti and Kopf, 1998), increased intracellular concentrations of Ca²⁺ and cyclic nucleotides such as cyclic

adenosine monophosphate (cAMP) and hyperpolarisation of sperm membrane potential (Visconti, 2009). In order to comprehend the process of sperm detachment from oviductal epithelial cells, it is important to elucidate membrane associated components and how external factors can influence sperm function.

1.4.1.1 Membrane Components Involved in Capacitation.

Capacitation involves significant rearrangement of membrane proteins and lipids of the sperm plasma membrane (Yanagimachi, 1994; Cross, 2004). The lipid fraction is made of approximately 50% phospholipids of which phosphatidylcholine, phosphatidylethanolamine and sphingomyelin are major components (Figure 6) (Flesch and Gadella, 2000). It also contains high amounts of polyunsaturated fatty acids (PUFA's) (Flesch and Gadella, 2000; Lenzi et al., 1996), along with diPUFAs (phospholipids esterified with two PUFAs), which are only found in sperm, retina and some brain tissues (Ladha, 1998; Wassall and Stillwell, 2009). PUFAs increase membrane fluidity and flexibility (Israelachvili et al., 1980; Meizel and Turner, 1983) and facilitate the bending and the flexing of the flagellum (Tavilani et al., 2006), which indicates a possible role in sperm motility. The lipid composition has been associated with specific functions, as it promotes the development of microdomains of different fluidity, fusogenicity and permeability characteristics (Wassall and Stillwell, 2009) which are necessary for sperm to reach and fertilise the oocyte.

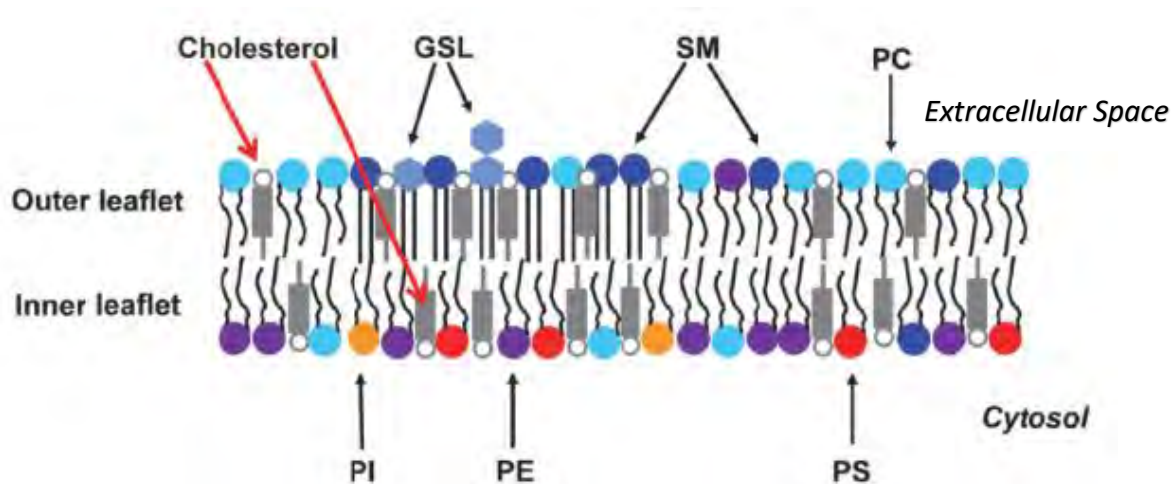


Figure 6: Asymmetric Lipid Bilayer of a cell.

Lipid components of the Lipid Bilayer: Sphingomyelin (SM), Glycosphingolipid (GSL), Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), Phosphatidylinositol (PI) with Cholesterol Adapted from (London, 2014)

Cholesterol is the second major component of the sperm plasma membrane and is known to regulate the order of the lipid chain thereby contributing to the rigidity of the plasma membrane (van Blitterswijk et al., 1987; Ohvo-Rekila et al., 2002). A subsequent decrease in the cholesterol/phospholipid ratio was found when sperm were exposed to serum albumin or β -cyclodextrins *in vitro* (Alberts et al., 2002; Visconti et al., 1999), thereby, altering the membrane fluidity, a change associated with the beginning of capacitation (Cross, 1998). The removal of cholesterol by BSA modulates the influx of bicarbonate and Ca^{2+} (Coy et al., 2012).

In vitro capacitation can be achieved by mimicking the oviductal milieu, therefore the media should contain bicarbonate, Ca^{2+} and serum albumin as the cholesterol-binding compound (Coy et al., 2012). Bicarbonate presence is associated with the regulation of the cyclic adenosine monophosphate (cAMP) pathway (Visconti et al., 2011) through stimulation of soluble adenylyl cyclase (sAC) (Buck and Levin, 2011). Functional analysis shows that sAC knockout mice are sterile due to the inability to capacitate or hyperactivate (Hess et al., 2005; Xie et al., 2006). The activation of protein kinase A (PKA) triggers the onset of tyrosine phosphorylation associated with sperm capacitation (Coy et al., 2012).

Proteins present on the membrane are absorbed to extracellular sites and originate from the testis, epididymis and seminal plasma. These can function as decapacitation factors and immune regulators to sperm (Dacheux et al., 1989; Rodriguez-Martinez et al., 2011). Decapacitation Factors (DF) are 40kDa proteins found in mice, human and bull (Arangasamy et al., 2011) which inhibits premature capacitation during the sperm transition through the female reproductive tract, therefore crucial to the maintenance of sperm viability (Fraser et al., 1990; Nixon et al., 2006). Their receptor, phosphatidylethanolamine binding protein 1 (PEBP1), is localised to the acrosomal, post-acrosomal and tail regions of sperm. This 23kD protein is bound to phosphatidylethanolamine on the inner leaflet of the sperm plasma membrane (Gibbons et al., 2005). In bovine species, the superfamily of BSPs bind at ejaculation, by interaction with choline phospholipids on the plasma membrane (Manjunath et al., 2009; Desnoyers and Manjunath, 1992). Three proteins BSP1, BSP3 and BSP5, play a dual role in bovine sperm capacitation; BSP proteins act as decapacitation factors through the stabilisation of the sperm plasma membrane, yet ultimately promote sperm capacitation through the induction of cholesterol and phospholipid efflux from sperm (Therien et al., 1998; Therien et al., 1999). This efflux can be stimulated by high concentrations of BSP proteins or through associations with high-density lipoproteins (HDL) and GAGs such as heparin sulphate from follicular fluid (Therien et al., 1997; Therien et al., 1995) BSP proteins in bovine species bind to various ligands such as heparin (Chandonnet et al., 1990), other GAG's (Therien et al., 2005), gelatine (Manjunath et al., 1987) and apolipoprotein A-I (Manjunath et al., 1989). Receptor proteins, like PEBPs, arise from integrated transmembrane proteins which hold the ability to function on both sides of the lipid bilayer. These receptors bind signal molecules from the extracellular space and generate intracellular signals (Alberts et al., 2002).

1.4.1.2. Sperm Detachment by Capacitation

The modification of the sperm surface through capacitation has been shown to result in the loss of oviduct binding proteins (e.g. PDC-109), thereby, reducing the affinity of sperm for the oviduct epithelium (Ignotz et al., 2001; Gwathmey et al., 2003). The incubation of capacitated sperm with PDC-109 restores the spermatozoa's binding ability to epithelial cells (Gwathmey et al., 2003). It has been found in bull sperm, that sulphated glycoconjugates (Talevi and Gualtieri, 2001) and disulphide-reductants (Talevi et al., 2007; Gualtieri et al., 2009) induce the release of ejaculated sperm bound from oviductal epithelium *in vitro*. These molecules are proposed to be a representation of oviductal components of heparin-like GAGs and reduced glutathione (GSH), respectively, each of which increases in concentration at the time of oestrus (Parrish et al., 1989a; Bergqvist and Rodriguez-Martinez, 2006; Lapointe and Bilodeau, 2003). Each BSP protein is endowed with a heparin binding site and four disulphide bridges (Gwathmey et al., 2006), therefore heparin and disulphide reductants eg Penicillamine, may directly affect the affinity of sperm for the oviductal epithelium. Epididymal sperm lacking PDC109, BSP-A3 or BSP-30kDa are released in response to heparin, fucoidan, dextran sulphate and penicillamine, indicating that other proteins on the plasma membrane surface of epididymal sperm can be altered by these signals and therefore lose their affinity for the oviductal epithelium (Gualtieri et al., 2010). Sperm adhesion to the oviductal epithelium is done selectively whereby the oviduct epithelium selects sperm endowed with surface proteins which expose low levels of sulfhydryl's (Gualtieri et al., 2009). Additionally, an increase in α -1-fucosidase activity in the oviduct was detected after ovulation which could be a cause of sperm release (Carrasco et al., 2008).

1.4.2 Sperm Detachment by Hyperactivation

Changes in motility from a symmetric, sinusoidal flagellar beat-form of a motile sperm to asymmetric hyperactivated motility, was first noted in mouse sperm as a vigorous flagellar beating, following incubation in follicular fluid (Yanagimachi, 1969; Gwatkin and Andersen, 1969; Yanagimachi, 1970). Since then, hyperactivation has been generally characterised as high amplitude asymmetric flagellar bending, a reduction in beat frequency and side to side yaw movement along with a star-spin movement of non-progressive motility of free swimming cells in fluids of low viscosity (Ooi et al., 2014; Simons et al., 2014). It has also been shown that mouse sperm display a hyperactivated motility pattern which accompanies frequent attachment and detachment from the oviduct *in vivo* (Chang and Suarez, 2012; Ho et al., 2009). Oviducts of mice were observed by transillumination where it was seen that only sperm that displayed hyperactivated motility detached from the oviductal epithelium (Demott and Suarez, 1992). Moreover, sperm were seen to use a rocking motion to depart from the epithelial cells (Chang and Suarez, 2012). Hyperactivated free-swimming sperm demonstrate an asymmetric waveform which results in a helical or curved trajectory (Yanagimachi, 1970), while a tethered sperm would generate a thrusting or ‘tugging’ forces to enable it to detach from a surface (Curtis et al., 2012; Demott and Suarez, 1992; Katz et al., 1989; Smith and Yanagimachi, 1991). Primate sperm in a hyperactivated state exhibit an increase in tangential forces (Ishijima, 2011). This motion is also essential to swim through viscoelastic substances of the oviduct and penetrate the cumulus layers that surround the oocyte (Suarez et al., 1991; Suarez and Dai, 1992; Quill et al., 2003; Alasmari et al., 2013). Mouse sperm that lack the sperm-specific cation channel of CatSper (described below) cannot hyperactivate and fail to make the transition beyond the oviductal sperm reservoir (Ho et al., 2009) as they appear to remain bound to oviductal epithelium *in vivo* (Ho et al., 2009; Simons et al., 2014). The attaching and detaching sequence has not been witnessed with symmetrical flagellar beating sperm (Chang and Suarez, 2012). Accumulating evidence suggests a greater role of sperm hyperactivation in the detachment of cells from the epithelium, however, it remains unclear as to what triggers sequential release through hyperactivation.

1.4.2.1 The Role of Ion Channels in Hyperactivation

Ion channels in sperm control sperm membrane potential, intracellular pH and cytoplasmic Ca^{2+} , which in turn regulates sperm motility, acrosome reaction and intracellular processes essential for fertilisation (Babcock et al., 1983; Yanagimachi and Usui, 1974). Membrane ion channels arise from integrated transmembrane proteins and are essential for the passage of ions such as Ca^{2+} (Flesch and Gadella, 2000). Ion channels in sperm physiology are essential for effective sperm function and processes of capacitation and hyperactivation, therefore, essential for the detachment of sperm from oviductal epithelial cells. Upon activation, ion channels can change the electric potential of the cell and the intracellular concentration of secondary messengers. The opening of ion channels can be triggered by small conformational changes induced by voltage, ligands, phosphorylation changes and membrane pressure, resulting in the influx of ions down their electrochemical gradient (Eaton, 1985). For example, Ca^{2+} ions act as central signalling molecules in sperm. An influx of Ca^{2+} exerts allosteric regulatory effects on many sperm proteins and on enzymes (Ren and Xia, 2010; Lishko et al., 2011; Strunker et al., 2011). The influx of Ca^{2+} involves the interaction of several channel proteins, directly or indirectly regulating Ca^{2+} entry (Rahman et al., 2014). Such channels include the voltage-gated proton channel (Hv1), located on the flagella (Lishko et al., 2011), several cyclic nucleotide-gated channels (CNG) (Esposito et al., 2004; Weyand et al., 1994) as well as, hyperpolarization-activated cyclic nucleotide-gated channels (HCN) (Wiesner et al., 1998). A summary of these ion channels is presented in Table 1.

Table 1: Ion channels involved in the regulation of Calcium influx in sperm.

Channel	Localisation on Sperm	Role in Sperm Physiology	Role in Ca²⁺ influx	Effect of absence	Reference
Hv1	Principal Piece	Proton extrusion, Alkalization	pH _i – alkalization; stimulation of Ca ²⁺ influx	Fertile	(Lishko et al., 2011; Lishko et al., 2010)
I_{ATP}	Midpiece	Alkalisiation, Ca ²⁺ influx	Selectively transports Ca ²⁺	Fertile	(Navarro et al., 2011)
CNG	Flagellum, Head	Ca ²⁺ influx	Triggers CatSper opening via cAMP/cGMP	Fertile	(Castellano et al., 2003; Biel and Michalakis, 2009)
TRPC	Midpiece, Principal Piece	Cell depolarisation, Ca ²⁺ influx	Triggers CatSper opening.	Fertile	(Gees et al., 2010; Castellano et al., 2003)
HCN	Flagellum	Ca ²⁺ influx	Depolarisation, Triggers opening of CatSper.	Fertile	(Wiesner et al., 1998)
SOC	Plasma Membrane	Sperm chemotactic response	Ca ²⁺ influx induced by ZP	Subfertile	(Yoshida et al., 2003)
CatSper (1,2,3,4, β,γ.)	Principal Piece	Ca ²⁺ influx	Primary Pathway	Sterile	(Barratt and Publicover, 2012; Qi et al., 2007)

Hv₁: Voltage Gated Channel; I_{ATP}:ATP Gated Channel; CNG: Cyclic Nucleotide-Gated Ion Channel; TRPC: Transient Receptor Potential Channels; HCN: Hyperpolarisation-activated cyclic nucleotide-gated channel SOC: Store-operated Ca²⁺ channel

1.4.2.1.1 CatSper: The Primary Calcium Channel in Sperm Hyperactivation

CatSper is a sperm-specific, heterotetrameric, voltage-gated and pH-sensitive Ca²⁺ channel composed of four main pore-forming subunit proteins; CatSper 1, 2, 3 and 4 (Ren et al., 2001; Quill et al., 2001; Lobley et al., 2003; Jin et al., 2007; Johnson et al., 2016), which have been localised to the plasma membrane of the principal piece (Quill et al., 2003; Ren et al., 2001; Xia et al., 2007). Accessory subunits of δ , β and γ have also been detected in murine and sea urchin sperm (Liu et al., 2007; Wang et al., 2009; Seifert et al., 2015). Each CatSper gene uniquely encodes a single, six-transmembrane spanning repeat, comparable to the voltage-dependent potassium channel, TRP and CNG channels (Lobley et al., 2003; Jin et al., 2005; Navarro et al., 2008; Qi et al., 2007; Quill et al., 2001; Ren et al., 2001). However, with its pore region, the CatSper channel is closer to the single domain of the larger four-repeat Ca_v, a voltage-dependent L-type Ca²⁺ channel, which both hold the signature T/S x E/D x W amino acid sequence in the ion selectivity filter region (Figure 7) (Ren et al., 2001; Ren and Xia, 2010).

	Pore region
CatSper	RFCQNI FTTLFTLFTMLILDDWSLIYED
I Ca _v 1.2	NFDNFAFAMLTVPQCITMEGWTDVLYN
I Ca _v 2.2	NFDNLFALLTVFQCITMEGWTDILYN
Ca _v 3.1	NFDNIGYAWIAIFQVITLEGWVDIMYE
II Ca _v 1.2	TFDNFPQSLLTVPQILLGEDWNSVMYD
II Ca _v 2.2	NFDTFPAALLTVFQILLGEDWNAVMYN
Ca _v 3.1	NFDSELLWAIIVTVFQILLQEDWNEKVLYN
III Ca _v 1.2	DFDNVLAAMMALFTVSTFEGWPELLYE
III Ca _v 2.2	HYDNVLWALLTLFTVSTGEGWPMYLYE
Ca _v 3.1	NFDNLGQALMSLFVLASKDGVVDIMYE
IV Ca _v 1.2	NFQTFPQAVLLLPRCATGEAWQDIMLA
IV Ca _v 2.2	NFRTFLOALMLLFRSATGEAWHBIMLS
Ca _v 3.1	IFRNFGMAPLTLFRVSTGEDNNGIMKI

Figure 7: Amino acid sequence homologies of the putative pore region of CatSper mouse, with the four domains (I-IV) from Ca_v1-3 human (Ren et al., 2001).

CatSper have been reported to be expressed in human (Lobley et al., 2003; Tamburrino et al., 2014), murine (Ren et al., 2001; Quill et al., 2001), bovine (Johnson et al., 2016), ovine (Vincente-Carrillo et al., 2015) and equine sperm (Loux et al., 2013). *In situ* hybridization studies have suggested that *CatSper* subtypes are differentially transcribed during spermatogenesis; *CatSper 1,3* and *4* transcribes to spermatids in the testes (Jin et al., 2005; Ren et al., 2001), while *CatSper 2* transcribes to sperm at the pachytene spermatocyte stage (Quill et al., 2001; Schultz et al., 2003).

All four members of the *CatSper* family are composed of cytoplasmic carboxyl and amino-termini six transmembrane segments. Similar to other voltage-gated channels, segments of *CatSper* are categorised into; Voltage sensory (segments 1-4) and the pore-forming (segment 5- P loop- segment 6) (Navarro et al., 2008). Segment 4 of the voltage sensory domain contains positively charged amino acids of Arginine (R) and Lysine (K) spaced at the helical turns and regulates the gating of the pore as a result of voltage changes (Navarro et al., 2008). While *CatSper 1* and *2* have 4 R/K residues in segment 4, *CatSper 3* and *4* contain 2, which may explain the reduced voltage dependency of the *CatSper* channel (Navarro et al., 2008).

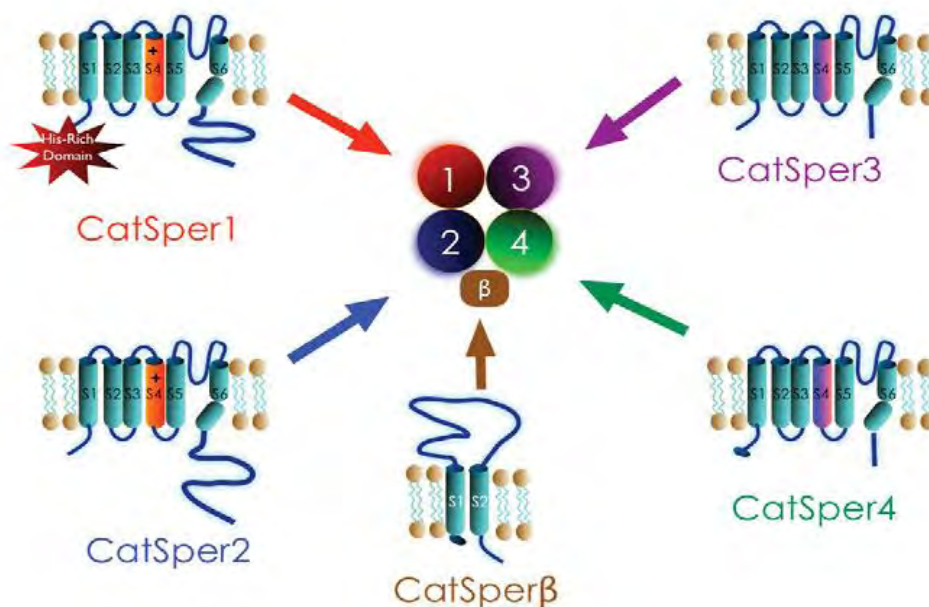


Figure 8: *CatSper* complex containing *CatSper 1-4* protein units and *CatSper β* subunit, *CatSper 1-4* proteins containing six transmembrane segments and a pore region (Navarro et al., 2008).

CatSper channels of human and mouse sperm have been shown to have weak voltage dependence (Lishko et al., 2010), however, they are pH sensitive, therefore rendering sperm sensitive to pH changes in the female reproductive tract. The CatSper 1 subunit possesses a large N terminus which is rich with histidine residues. This histidine-rich domain has been suggested to be involved in pH regulation of the CatSper channel currents (Ren et al., 2001; Navarro et al., 2008). Knock-out studies for CatSper genes carried out in mice indicate the necessity for sperm hyperactivation to facilitate sperm penetration into the oocyte (Qi et al., 2007; Hildebrand et al., 2010).

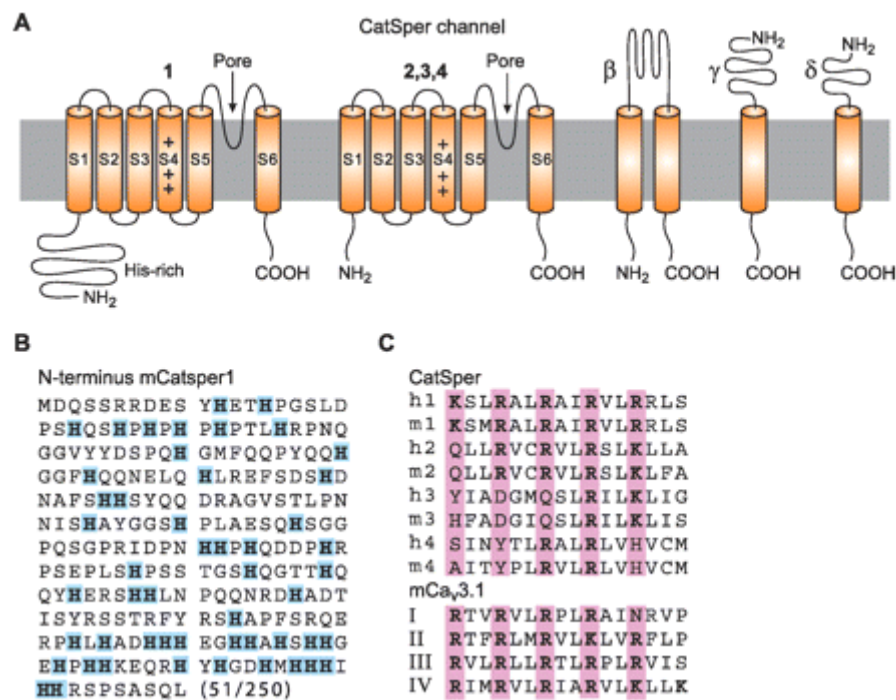


Figure 9: CatSper Structure and Protein Topology.

A: CatSper 1; six transmembrane segments including positively charged segment 4, pore loop, cytoplasmic COOH and the histidine-rich region of the NH₂ termini. CatSper 2,3,4; similar structure with short NH₂ and COOH cytoplasmic termini. CatSper β , γ , δ subunits; containing one, two and one transmembrane segments respectively. **B:** Mouse CatSper 1 NH₂ terminal contains 51 Histidines of 250 amino acids. **C:** Human (h) and mouse (m) segment 4 (voltage sensory) comparison of amino acid sequences (lower; comparison to mouse Ca_v3.1) (Darszon et al., 2011).

The mechanism of CatSper activation in different species has yet to be elucidated. In human sperm, CatSper can be activated through intracellular alkalization and P4 (Tamburrino et al., 2014; Lishko et al., 2011). Conversely, mouse spermatozoa have been shown to be insensitive to P4 (Lishko et al., 2011), while boar sperm exhibit acrosome exocytosis when capacitated *in vitro* and treated with supraphysiological doses of P4 (Yeste et al., 2015). It remains unclear as to the mechanism of activation of CatSper in bull and the role, if any, that P4 might play in inducing hyperactivation and the release of sperm from oviductal epithelial cells.

1.4.2.1.2 The Role of Calcium in Sperm Hyperactivation.

The influx of Ca^{2+} ions triggers multiple physiological events in sperm, including hyperactivated motility (Ho et al., 2002; Harper et al., 2004; Carlson et al., 2003), chemotaxis (Kaupp et al., 2006; Spehr et al., 2003), capacitation and the acrosome reaction (Breitbart, 2002; Kirkman-Brown et al., 2002b), each of which are essential for fertilisation.

Similar to somatic cells, Ca^{2+} is sourced either extracellularly, or released from intracellular stores (Benoff et al., 2007; Breitbart, 2002; Darszon et al., 2006; Florman et al., 2008; Publicover et al., 2007), however, the mechanisms by which intracellular Ca^{2+} is released from its stores remains to be elucidated. Unlike most somatic cells, mature sperm do not possess an endoplasmic reticulum (ER) which is the chief source for Ca^{2+} , hence, Ca^{2+} is thought to be held by a redundant nuclear envelope (RNE), which co-localises with the IP_3 receptor in the neck region, and tightly packed mitochondria in the midpiece (Costello et al., 2009; Ho and Suarez, 2001). Both of these structures contain calreticulin, a Ca^{2+} storage protein, and Ca^{2+} mobilisation from these stores involve IP_3 sensitive IP_3R and the Ca^{2+} induced Ca^{2+} release (CICR) of the ryanodine receptor (RyR) (Correia et al., 2015). A basal level of intracellular Ca^{2+} in sperm is sustained by Ca^{2+} absorption by mitochondria and Ca^{2+} extrusion pathways on the plasma membrane Ca^{2+} ATPase (PMCA) on the tail (Wennemuth et al., 2003). It has been shown that PMCA₄ is essential for normal sperm motility and leading to infertility (Okunade et al., 2004; Schuh et al., 2004).

Fluctuations of intracellular Ca^{2+} are fundamental transduction elements in cell signalling and the CatSper channel plays a preponderant role in the modulation of this divalent cation. It has been universally acknowledged that nuclei of sperm are transcriptionally inactive (Grunewald et al., 2005; Goodrich et al., 2013). Hence, sperm are dependent on activities of proteins gained through concentration changes of intracellular messengers of cyclic nucleotides (cAMP and cGMP), nitric oxide and Ca^{2+} . Parallel Ca^{2+} signalling pathways respond to unique stimuli, thereby mobilizing different sources of Ca^{2+} through various mechanisms throughout the cell with different intracellular Ca^{2+} kinematics (Jimenez-Gonzalez et al., 2006). Spatial overlap of pathways occur in sperm and specific targets are activated e.g. cAMP and protein kinases (Harper and Publicover, 2005).

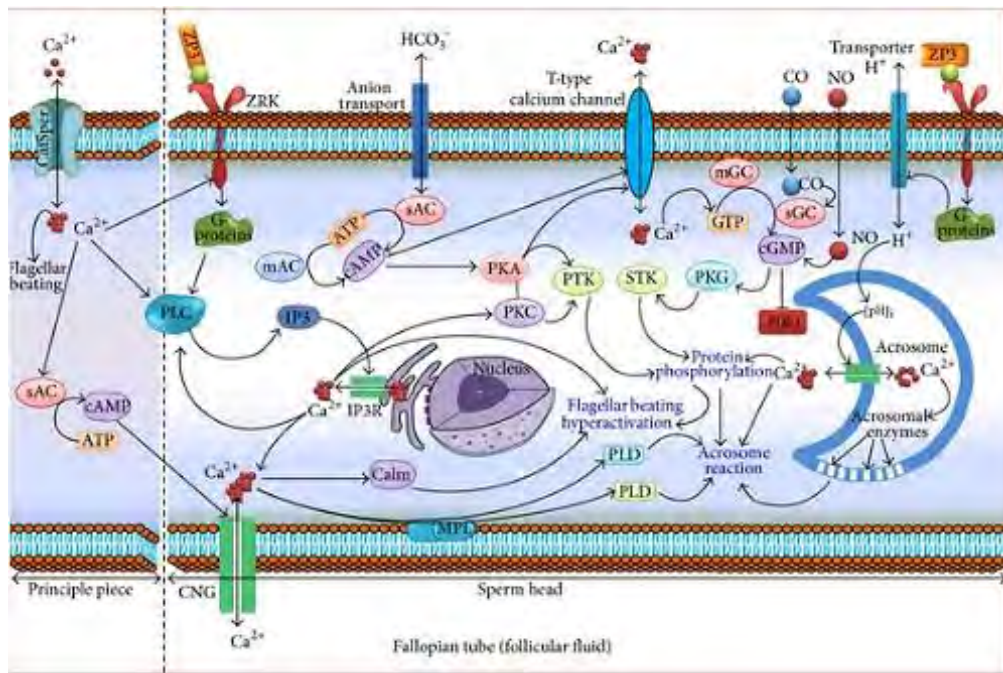


Figure 10: Schematic of calcium-regulated events of hyperactivation, capacitation and acrosome reaction (Rahman et al., 2014).

Bicarbonate ions and Ca^{2+} influx stimulate sAC and soluble guanylate cyclase (sGC) thereby, stimulating the production of cAMP and cGMP respectively from ATP through enzymatic catalysis in mature sperm. G proteins also activate sAC and phospholipase C (PLC) which results in the activation of tyrosine phosphorylation (Ho et al., 2002; Quill et al., 2003), a main signalling cascade involved in capacitation and the acrosomal reaction.

Diatomic messenger molecules of nitric oxide (NO) and carbon monoxide (CO) stimulate membrane-bound Guanylate cyclase (mGC) and sGC respectively. Cyclic GMP is synthesised in return. A rise in cGMP evokes a concomitant rise in cAMP through inhibition of phosphodiesterase activity (PDE_3) (Ravelli et al., 2002). Increased levels of intracellular Ca^{2+} can also directly catalyse cAMP (Luconi et al., 2006).

The result of sAC, sGC and PLC activation leads to the generation of inositol triphosphate (IP_3), another secondary messenger. The binding of IP_3 to the IP_3 receptor (IP_3R) triggers the release of Ca^{2+} ions from internal stores triggering hyperactivation (Kuroda et al., 1999). Protein kinases (PKA, PKG and PKC), activated by the secondary messengers, trigger various ion channels, while serine/threonine protein kinase (STK) and protein tyrosine kinases (PTK) also contribute to increased levels of protein phosphorylation in the cell, leading to hyperactivation (Ravelli et al., 2002; Luconi et al., 2006). PKA, in particular, is required for the activation of flagellar beating and asymmetrical waveform of hyperactivation (Nolan et al., 2004). Inhibitors including H89 and rpScAMP, disrupt PKA binding to proteins, thereby blocking sperm motility and *in vitro* fertilisation (Visconti et al., 1995; Vijayaraghavan et al., 1997).

Intracellular Ca^{2+} also plays a role in the activation of zona pellucida (ZP) receptors on the sperm plasma membrane which triggers G-proteins in the sperm head plasma membrane. Intracellular pH is increased due to activated G proteins stimulating the H^+ transporter and through catalysis of acrosomal enzymes, acrosome reaction and hyperactivation are triggered (Jeon et al., 2001). During the process of acrosome reaction, the influx of Ca^{2+}

through CatSper initiates Calmodulin; a Ca^{2+} modulated protein, phospholipase A (PLA), phospholipase D (PLD) with increased generation of other secondary messengers (Branham et al., 2006; Ickowicz et al., 2012).

The influence of oviductal microenvironments that surround sperm bound to the isthmus, undoubtedly trigger intracellular modifications and complex signalling processes in sperm leading to hyperactivation therefore, the release of sperm from oviductal cells. The stimulation of Ca^{2+} influx and thus hyperactivation in sperm has been demonstrated to be triggered by oviductal components such as hormones present in the oviduct (Lamy et al., 2016; Morales et al., 2000). Hormone action on any target cell involves the hormone binding to a receptor binding protein (Losel et al., 2003), thereby triggering a cell response.

1.4.2.2 Receptors in Sperm Physiology

Receptors for hormones have been reported to be present on the sperm plasma membrane in some species to date. These hormone receptors have been localised and identified on sperm through immunofluorescence and western blot techniques and play various roles in sperm guidance, motility and acrosome reaction. The main receptors are summarised in Table 2.

Table 2: Hormone receptors in sperm physiology

Receptor	Localisation in sperm	Localised by	Function	Species	Sperm Size (kDa)	Reference
Estrogen α	Equatorial segment	IF, CLS	Capacitation, exocytosis, Fertilising ability, motility	Human	66,45	(Solakidi et al., 2005) (Bai and Gust, 2009) (Guido et al., 2011)
Estrogen β	Mid-piece	IF, CLS	Metabolism modulation, Fertilising ability.	Human	64	(Solakidi et al., 2005) (Guido et al., 2011)
Androgen	Mid-piece	IF, CLS	Modulation of PI3K pathway	Human	110, 90, 85-87.	(Solakidi et al., 2005) (Aquila et al., 2007)
Progesterone	Acrosome Equatorial segment Post-acrosomal region	WB	Acrosome reaction, Motility, Chemotaxis	Human, Boar, Canine,	28, 54, 57, 65.	(Gadkar-Sable et al., 2005; Ambhaikar and Puri, 1998; Luconi et al., 2004)
Leptin	Tail, Acrosome	IF, PCR	Motility, Capacitation	Human, Boar,	145	(Jope et al., 2003; Aquila et al., 2008; Tartaglia et al., 1995)
Calcitonin	Tail, Acrosome, Mid-piece	IF	Regulation of Ca^{2+} , cAMP	Human, Equine	80	(Sjoberg et al., 1980; Adeoya-Osiguwa and Fraser, 2003)

IF: Immunofluorescence microscopy; CLS: Confocal laser scanning, WB: Western blot

1.4.2.2.1 Genomic Action of Steroid Hormones

Steroid hormones possess a minimum of four fused rings, which structurally coincides with their receptor proteins (Beato and Klug, 2000). A classic steroid nuclear receptor generally comprises of a condensed DNA binding domain of 66-68 amino acids (Conneely et al., 2003), a COOH terminus of ~220-250 amino acid (ligand binding domain) and a lengthy NH₂- terminal domain containing transcription activation domains (AF1 and AF3) (Beato, 1989; Gadkar-Sable et al., 2005). Three isoforms of nuclear P4 receptors (nPR) have been investigated in cells: PR-A (94kDa), PR-B (116kDa) and PR-C (60kDa) (Hanekamp et al., 2003). PR-A and PR-B are generated by alternate splicing of the same gene. PR-A has an inhibitory domain at the N terminal which suppresses its transcriptional abilities (Gellersen and Brosens, 2003). Studies in human and boar sperm have revealed that PR A and B proteins are localised to the sub-acrosome, equatorial region and midpiece (De Amicis et al., 2012; De Amicis et al., 2010; Gadkar et al., 2002; Plant et al., 1995). The accepted theory of genomic action of steroid hormones in cells involves the entry of steroid molecules into the cell passively by diffusion through the phospholipid membrane. Intracellular receptors in the nucleus or the cytosol undergo a conformational change as a result of steroid binding to the ligand domain, as reviewed by Losel et al. (2003). Proteins, which are affected by activity of the receptor, are dissociated and hold increased affinity for the DNA binding domain. Transcription of genes to mRNA, eventually trigger a cascade of biological actions (Beato et al., 1996; Beato and Klug, 2000; Losel et al., 2003). The activation of this pathway is a slow process, with hours to even days needed for protein synthesis (Losel et al., 2003).

1.4.2.2.2. Non-genomic Action of Steroid Hormones and Their Role in Sperm Motility.

It has been shown that steroid effects can occur in cells without a functional nucleus i.e. sperm, erythrocytes and platelets, therefore steroid signals can be transmitted by other means than through protein synthesis (Losel et al., 2003; Mitre-Aguilar et al., 2015). The highly-condensed DNA structure of mature sperm is not accessible for transcription, nor are ribosomes and other essential components available for translation (Imms et al., 2012; Luconi et al., 2004). Non-genomic steroid effects have been demonstrated in human sperm as a result of P4 and E2 stimulation (Luconi et al., 2004). Progesterone is a hormone

secreted by the cumulus cells of the oocyte *in vivo* and *in vitro* (Fatehi et al., 2002; Mingoti et al., 2002; Aardema et al., 2013; Lamy et al., 2016) and has been demonstrated to stimulate biological responses of human sperm including Ca^{2+} influx, tyrosine phosphorylation, extracellular signalling regulated kinases (ERK; also referred to as MAPK), chloride efflux and increases of cAMP levels (Losel et al., 2003; Blackmore et al., 1990; Falkenstein et al., 1999). In Jurkate T cells, immortalised T lymphocyte cells, it has been found that P4 causes a decrease in intracellular pH and an increase in intracellular Ca^{2+} . These biochemical changes are said to be transmitted by membrane receptors which are unrelated to the classical steroid receptors (Chien et al., 2009). Similarly, in human sperm, stimulation of rapid activities of P4, at nM- μ M concentrations, are proposed to originate from the cell surface and have been seen in many cells to be mediated through non-classical progestin membrane receptors (Falkenstein et al., 2000; Thomas et al., 2009; Harper et al., 2004).

The characterisation and action of putative membrane receptors in sperm is poorly understood, with various approaches such as fluorescent isothiocyanate (FITC)-labelling conjugated to BSA and P4, immunofluorescence microscopy, confocal laser scanning and western blot analysis used to depict a comprehensive representation of the model. As a result, many discrepancies and confusion in receptor nomenclature, molecular sizes and their role in sperm function have been reported. Two human homologues have been successfully isolated on sperm which coincides with membrane steroid receptors of porcine liver and are candidate P4 receptors on sperm (Meyer et al., 1996; Falkenstein et al., 1996). Progesterone membrane receptor component 1 (PGRMC1, MW~28kDa; also referred to as hmPR1/Hpr6.6) and the ortholog of P4 membrane receptor component 2 (PGRMC2; hmPR2/Dg6) are part of the membrane associated progesterone receptor (MAPR) family. PGRMC1 has been localised at the acrosome in human sperm and the inner plasma membrane of pig sperm (Losel et al., 2005; Thomas, 2008; Gerdes et al., 1998; Buddhikot et al., 1999). With shared homology with the prolactin/growth factor/cytokine receptor superfamily, the structure is composed of a proline rich sequence of box 1 motif for JAK2 for tyrosine kinase association, amino acid sequence analysis showed a single transmembrane unit close to the N terminus, no recognisable DNA

binding site or steroid binding pocket (Postel-Vinay and Finidori, 1995; Falkenstein et al., 1996; Williams and Sigler, 1998). Since work by Xu et al. (2011), PGRMC1 existence on sperm could potentially be a sigma receptor binding site on sperm, which act as a chaperone protein that modulate Ca^{2+} signalling and plays a role in metabolic regulation (Rousseaux and Greene, 2016). The PGRMC1 protein has proven various roles in cell types including providing cell insensitivity to chemotherapy (Peluso, 2011) and mediation of antiapoptotic action of P4 on granulosa cells (Peluso et al., 2008b), however, its role in rapid P4 responses in sperm still remains elusive.

Another membrane localised progestin receptor (mPR) has been found to induce oocyte maturation in multiple non-mammalian species including teleost fish (Zhu et al., 2003b; Thomas et al., 2002). Three gene isoforms of mPR α (PAQR VII), mPR β (PAQR VIII) and mPR γ (PAQR V), part of the P4 adiponectin Q receptor (PAQR) family, encodes a seven transmembrane protein, each ~40kDa (Thomas, 2008; Zhu et al., 2003a; Tang et al., 2005). The mPR α subunit has been identified in both human and mouse testes (Zhu et al., 2003a; Thomas, 2004). Through the use of RT-PCR and western blot, the presence of mPR α mRNA was elucidated with bands of ~40kDa and ~80kDa expressed in the plasma membrane of mature sperm (Thomas et al., 2009). It has been localised to the membranes of the mid piece and flagellum which are consistent with its proposed involvement in stimulation of hyperactivated motility in human sperm (Uhler et al., 1992). Evidence has accumulated that low mPR α expression is correlated with poor motility in human sperm samples (Thomas et al., 2009). The mechanism through which P4 exerts its effects is still unclear, however, it is proposed to activate membrane G proteins upon binding to mPR α . Various G-proteins of G_q, G_i, G_s and an olfactory G protein (G_{olf}) have been identified in human sperm (Glassner et al., 1991; Baxendale and Fraser, 2003) with progestin binding to mPR α , triggering the activation of one, or a combination of, these G proteins in human sperm (Thomas et al., 2007; Karteris et al., 2006; Dosiou et al., 2008; Thomas et al., 2009). G_q, G_s and G_{olf} activation are involved in rapid intracellular Ca^{2+} increase and elevation of cAMP concentrations i.e. factors that induce sperm hyperactivation (Thomas, 2003; Marquez and Suarez, 2004) and are therefore possibly involved in the progestin-mPR α effect on human sperm.

Studies suggest that the action of P4 on inducing hyperactivation and the acrosome reaction can occur independently of each other through different intracellular pathways (Marquez and Suarez, 2004; Ho and Suarez, 2001). Progesterone triggers an influx of Ca^{2+} in human sperm (Blackmore et al., 1990) within seconds of treatment and is known to be involved in sperm guidance in the female reproductive tract (Teves et al., 2009b) by means of chemotaxis and hyperactivation (Osman et al., 1989; Darszon et al., 2005). General tyrosine kinase and PKA inhibitors affect sperm chemotaxis response to inhibitors (Teves et al., 2009b). Tyrosine kinase and Ca^{2+} channels seem to be the primary effector of P4s action (Mendoza et al., 1995) with possible roles of protein kinase C (PKC) (Foresta et al., 1995) and MAPK/ERK (p42ERK) pathways (Lomax et al., 1991; Luconi et al., 1998). Interestingly, one intracellular cascade of the lipid signalling phosphatidylinositol 3-kinase (PI3K) pathway and its downstream effector of AKT (PKB) has been found to be triggered by P4 in many species including human (Sagare-Patil et al., 2013) and the Atlantic Croaker (Tan and Thomas, 2014). This pathway has been found to be a key regulator of hyperactivated motility and acrosome reaction in sperm, with pharmacological inhibition of PI3K leading to suppression of motility, hyperactivated motility and inhibition of acrosome reaction (Aparicio et al., 2005; Breitbart et al., 2010; Jungnickel et al., 2007; Koppers et al., 2011). Studies using a PI3K inhibitor on P4 treated sperm showed a reduction in hyperactivated sperm (Nauc et al., 2004). Thr308 and Ser473 phosphorylation at the tail region of AKT is upregulated by P4 treatment in capacitated sperm, thereby, preventing premature capacitation (Sagare-Patil et al., 2012; De Amicis et al., 2012; Breitbart et al., 2010). This phosphorylation of AKT however, is a Ca^{2+} dependent mechanism, yet has been shown to be crucial for P4 mediated tyrosine phosphorylation (Sagare-Patil et al., 2013). AKT phosphorylation has also been shown to prevent sperm from oxidative stress, DNA damage and spontaneous capacitation (Koppers et al., 2011). In bovine sperm, PIK3R1, the p85 α regulatory subunit of PI3K was localised to the post-acrosomal region of the head and the mid-piece (Etkovitz et al., 2007). This PI3K-AKT pathway is a possible mechanism of action of P4 to convey its biological effect.

Using the patch clamp technique Lishko et al. (2011) and Strunker et al. (2011), demonstrated that P4 induced currents match CatSper mediated currents in human sperm and CatSper-null mice are infertile. The electrophysiological patterns of P4 induced Ca^{2+} currents were demonstrated on the flagellum by Lishko et al. (2011), and have been shown to be enhanced in capacitating conditions (Strunker et al., 2011; Lishko et al., 2011). Progesterone stimulated an initial rapid transient influx of Ca^{2+} , followed by a slower, consistent flux of ions into the sperm cell (Strunker et al., 2011). Dose-dependent action of P4 was previously reported as biphasic with an EC_{50} in the nM range and a second phase in a μM range (Luconi et al., 1998). It has been suggested that the prolonged, slower, second phase is due to CICR from internal Ca^{2+} stores in sperm (Strunker et al., 2011). A point of note, P4 does not elicit the same CatSper response in mouse sperm (Lishko et al., 2011) in comparison to hyperactivated motility induced by 1-10nM P4 in human (Uhler et al., 1992; Gakamsky et al., 2009), and rhesus macaque sperm. (Sumigama et al., 2015).

It has been shown that incidences of polyspermic oocytes increased during the luteal phase of the oestrous cycle (Hunter, 1967). This formed the hypothesis that the release of sperm may be triggered by the presence of P4 in the oviductal milieu (Hunter, 2008). Progesterone, stimulates Ca^{2+} entry in human sperm (Blackmore et al., 1990; Falkenstein et al., 1999), by activation of the primary Ca^{2+} channel of CatSper (Lishko et al., 2011; Strunker et al., 2011) and these channels have been associated with stimulation of hyperactivated motility (Carlson et al., 2003). It has been demonstrated in pig sperm, that release from oviduct epithelial cells is triggered by P4 and involves the action of CatSper (Machado, 2013). This sperm release trigger was found to be P4 specific as the structurally related steroids of pregnenolone and $17\alpha\text{-OH-P4}$ did not affect sperm release from oviductal cell aggregates (Machado, 2013). Through fluorometric assessments, it was demonstrated that NNC 55-0396, a specific CatSper channel inhibitor, blocked the P4 induced Ca^{2+} influx in pig sperm, thus impeding the P4 induced release of sperm from oviductal cell aggregates (Machado, 2013). Moreover, the action of P4 was shown to be independent of a P4 receptor, with Mifepristone (RU-486), a P4 receptor antagonist, failing to suppress the P4 induced Ca^{2+} influx (Machado, 2013). To this authors knowledge at present, there have been no studies carried out in bovine species to demonstrate the release of sperm from bovine oviductal epithelial cells (BOEC) by hyperactivation.

Therefore, the objectives of this study were:

- To investigate the effect of capacitation on bull sperm binding to BOEC explants *in vitro*.
- To explore the effect of P4 on bull sperm hyperactivation as well as binding and unbinding to BOEC explants *in vitro*.
- To investigate the mechanism of action of P4 on sperm release from BOEC explants *in vitro*.

Chapter

2

Materials and Methods

Chapter Two: Materials and Methods.

2.1 Chemicals and Reagents.

All chemicals and reagents were purchased from Sigma Aldrich Chemical Co, (Arklow, Co. Wicklow, Ireland) unless otherwise stated.

2.2 Experiment 1: Effect of Capacitation Status on the Interaction of Sperm with Bovine Oviductal Epithelial Cell (BOEC) Explants.

The aim of this experiment was to assess the effect of capacitation on sperm interaction with oviductal epithelial cells. This was done by (i) Evaluating the effectiveness of Heparin and Caffeine in capacitating bull sperm, (ii) Assessing the ability of capacitated sperm to bind to BOEC explants, (iii) Assessing the effect of capacitation on the release of sperm from BOEC explants.

Experiment 1a: Evaluation of Capacitation Inducers in Vitro on Frozen-Thawed Bull Sperm using the Chlortetracycline (CTC) assay.

The aim of this experiment was to evaluate the effect of capacitation inducers, Caffeine and Heparin, on frozen-thawed bull sperm. Heparin is a naturally occurring glycosaminoglycan, which promote increases of intracellular Ca^{2+} and tyrosine phosphorylation in sperm (Galantino-Homer et al., 1997; Handrow et al., 1989; Parrish et al., 1994). Caffeine is a cyclic nucleotide phosphodiesterase inhibitor, which in turn, stimulates capacitation (Funahashi and Nagai, 2001) and at high concentrations (2-6 mM) can impact sperm motility (Levin et al., 1981). Frozen-thawed sperm from 3 different Holstein bulls was pooled following which motile sperm were selected using a 90:45% Percoll density gradient. The sperm pellet recovered was re-suspended in Tyrodes's Albumin Lactate Pyruvate (TALP) medium (NaCl 99 mM, KCl 3.1 mM, NaHCO_3 25 mM, NaH_2PO_4 400 μM , MgCl_2 1.1 mM, CaCl_2 2mM, HEPES 10 M, Sodium Pyruvate 1 mM, Sodium Lactate 25.4 mM, BSA 6 mg/ml; pH= 7.4, 38.5°C, 5% CO_2). Sperm suspensions (20×10^6 sperm/mL) were treated with (i) No treatment (Control), (ii) Caffeine (5 mM) (iii) Heparin (10 $\mu\text{g}/\text{mL}$) (iv) Heparin (10 $\mu\text{g}/\text{mL}$) and Caffeine (5 mM). Concentrations of heparin and caffeine were selected based on previous studies (Parrish et al., 1989a; Fukui et al., 1990; Coscioni et al., 2001). Aliquots of sperm (180 μL) were

incubated in heparin for 35 min (Ward and Storey, 1984; Fukui et al., 1990) followed by a 10 min incubation in the presence of caffeine at 38.5°C, in humidified air under 5% CO₂. A chlortetracycline (CTC) solution (750 µM) was prepared daily in a TRIS based buffer solution (NaCl 130 mM, 20 mM TRIS, Cysteine 5 µM; pH=7.8) and passed through a 0.22 µm filter. An aliquot (20 µL) of treated sperm was added to 20 µL of CTC solution and a 5 µL of 12.2% (w/v) paraformaldehyde in 0.5 M TRIS-HCl solution was added; pH= 7.8. Slides were prepared by spreading a 10 µL droplet of the fixed suspension on a clean slide. DABCO powder (1,4-diazabicyclo [2.2.2] octane; 0.22 M) was dissolved in glycerol: Phosphate Buffered Saline (PBS) (9:1) was mixed with the sample to prevent fading of fluorescence (Breininger et al., 2010). A coverslip (24x48mm) was added and excess fluid was removed by gently pressing down on the coverslip. Each slide was sealed using clear nail varnish and stored in the dark at 4°C. Samples were assessed using a fluorescent microscope (Olympus BX60; Centre Valley, PA, USA). Capacitation was evaluated through the pattern of fluorescence (Figure 11) expressed by 100 sperm. The assessor was blinded to all treatments and four replicates were completed.

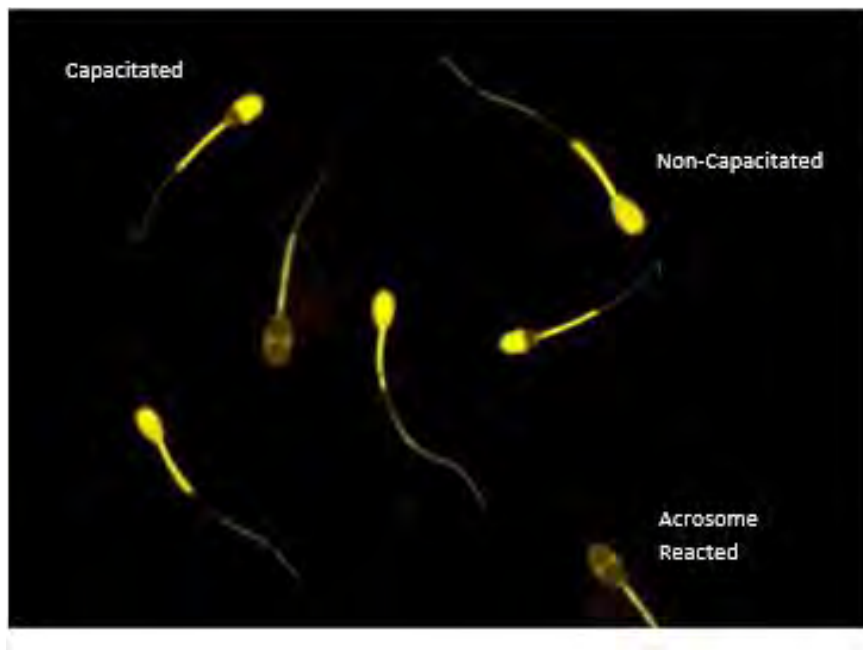


Figure 11: Indicative patterns of fluorescence from chlortetracycline staining of frozen-thawed bull sperm treated with both heparin and caffeine.

Sperm were classified in three categories as described by Fraser et al. (1995) namely non-capacitated - uniform fluorescence in the head; capacitated - fluorescence in the acrosome region; acrosome reacted - dull fluorescence over whole head except for a thin band of fluorescence in the equatorial segment.

Experiment 1b: Effect of Capacitating Treatments on Sperm Binding Density to Bovine Oviductal Epithelial Cells (BOEC) Explants.

The aim of this experiment was to assess the effect of capacitation on the ability of sperm to bind BOEC explants. The treatments were (i) No treatment (Control) (ii) Caffeine (5 mM) (iii) Heparin (10 µg/mL) and (iv) Heparin (10 µg/mL) + Caffeine (5 mM), to assess the binding ability of sperm to BOEC explants. Three replicates were completed.

Bovine Oviductal Epithelial Cell (BOEC) Binding Assay.

Non-pregnant heifer reproductive tracts were retrieved from a commercial abattoir immediately after slaughter. Reproductive tracts of different oestrus cycles stages were used as previous studies have shown that stage does not affect binding capabilities of sperm (Lefebvre et al., 1997; Gwathmey et al., 2003). The tracts were transported at 4-5°C to the laboratory in PBS supplemented with 0.5% Gentamycin. At the laboratory, the oviducts were trimmed free of surrounding connective tissue, ligated using sterile umbilical cord clamps and sterilised in 70% ethanol for 30 sec before being washed twice in PBS. Under the laminar flow, a vertical incision was made to separate the isthmic portion of the oviduct following which BOEC's were isolated from each oviduct through a mechanical scraping technique using a glass slide (Green et al., 2001). The BOECs were washed, recollected in 1 mL of PBS and were centrifuged at 200 g for 1 min. The supernatant was removed post centrifugation and 1 mL of M199 culture media was added to the BOEC's. The M199 medium was supplemented with Fetal Bovine Serum (FBS) and Gentamycin. The BOEC's were incubated in a 5% CO₂ incubator at 37.5°C for 1 h to allow the formation of everted vesicles with apical ciliated surfaces orientated outwards (Ignotz et al., 2007).

Straws from three different bulls were thawed and pooled to minimise bull variation. Using a Neubauer chamber, sperm concentration was assessed and samples were stained with 1% Hoechst 33342. Sperm were diluted to a concentration of 7.14×10^6 per mL using prewarmed TALP media and where appropriate were treated with caffeine, heparin or a combination of both, as per experiment 1(a). Post incubation, each treated sperm was added to 24-well plates in 140 µL aliquots.

PBS (5 mL) was added to the BOEC explants following which it was centrifuged at 200 g for 5 min and the supernatant discarded. BOEC explants (20 µL) were added to each aliquot of sperm. Sperm and BOEC's were co-incubated for 30 min at 37.5°C and 5% CO₂

following which loosely bound sperm were removed from the samples by gently pipetting through two 75 μ L droplets of TALP.

A 10 μ L droplet was then placed on a pre-warmed slide and covered with a coverslip. Slides were viewed on a heated stage (37°C) at 400x, under a half light, half fluorescence using a fluorescent microscope (Olympus BX60; Centre Valley, PA, USA). Ten fields of view were randomly assessed per slide. Sperm were classified as bound if the sperm head was in contact with the apical surface of the BOEC (Figure 12). A micrometre, placed in the eyepiece of the microscope, was used to measure the surface area of each explant. Bound sperm density was calculated by quantifying the number of sperm bound per 0.1mm² of the explant surface. The assessor was blinded to the treatments. One well was assessed per treatment per replicate, where one replicate (n) contained one heifer tract.

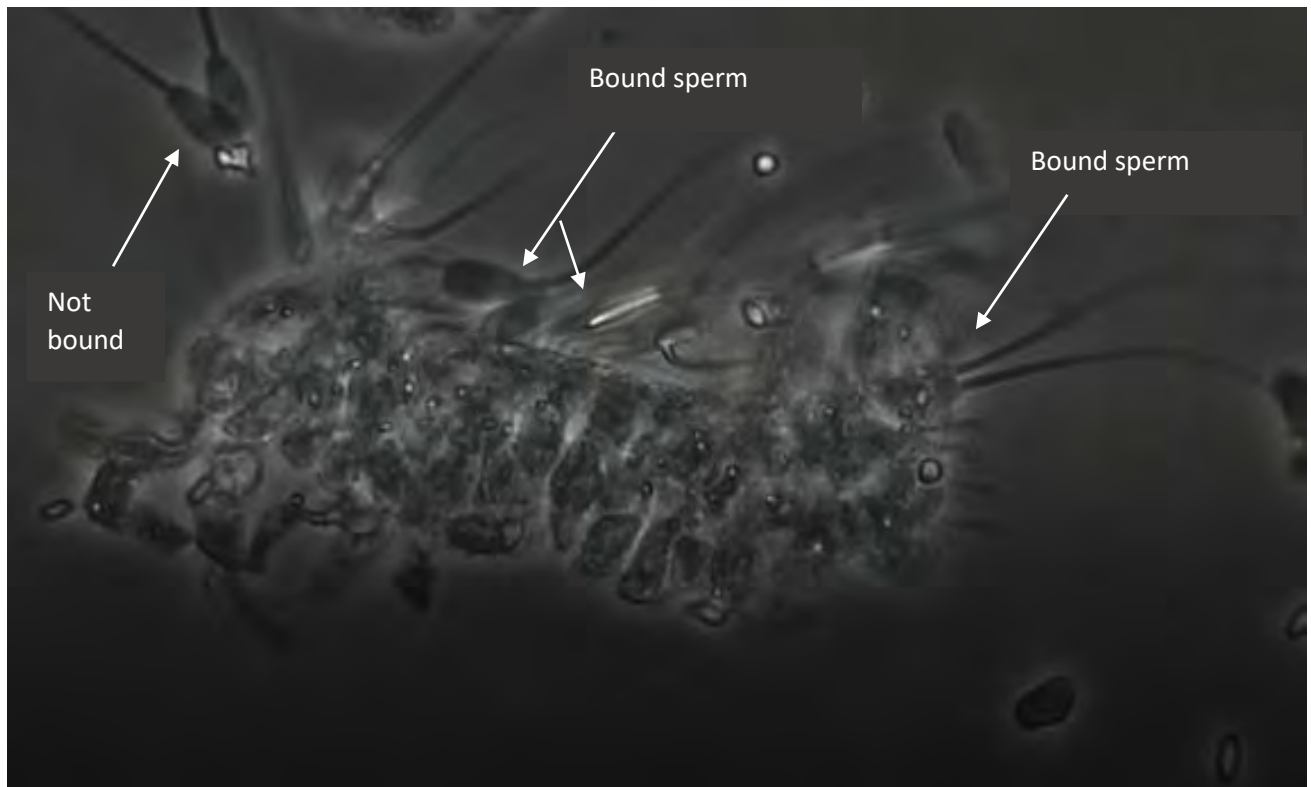


Figure 12: Sperm bound to bovine oviductal epithelial cell explants. Viewed under half-light, half-fluorescence at 400X.

Experiment 1c: Effect of Capacitation Treatments on the Release of Bound Sperm from Bovine Oviductal Epithelial Cell (BOEC) Explants.

The aim of this experiment was to assess the effect of capacitating treatments on the detachment of sperm bound to BOEC explants. Sperm were incubated with BOEC's following which they were treated with capacitating agents of (i) No treatment (Control), (ii) Caffeine, (iii) Heparin, (iv) Heparin and Caffeine before being assessed. One treatment per well, per replicate was assessed. Five replicates were completed.

Assessment of Bound Sperm Density- Post Incubation with Capacitating Agents.

Non-pregnant heifer tracts were prepared as per Experiment 1(b). Sperm diluted in TALP were diluted to 7.14×10^6 per mL and stained with 1% Hoechst 33342. Sperm were added to a 24-well plate in 140 μ L aliquots.

Post incubation with M199 culture media, the BOEC explants were washed and centrifuged with PBS (5 mL) as per Experiment 1(b) and the supernatant was removed. The BOEC explants (20 μ L) was added to each aliquot of sperm in the 24 well plate. Sperm and BOECs were co-incubated for 30 min at 37.5°C and 5% CO₂ following which loosely bound sperm were removed from the samples through gently pipetting through two 75 μ L droplets of TALP. Each sample was assessed for bound sperm density which confirmed equal binding in all wells (results not shown). Then sperm and BOECs were treated with (i) No treatment (Control) (ii) Caffeine (5mM), (iii) Heparin (10 μ g/mL) and (iv) Heparin (10 μ g/mL) + Caffeine (5 mM). Caffeine, Heparin and Heparin + Caffeine samples were incubated for 10, 35, 45 min respectively in 37.5°C and 5% CO₂. Post incubation, loosely bound and unbound sperm were removed by gently pipetting through two 75 μ L droplets of TALP.

Samples were placed on a glass slide and assessed as per Experiment 1(b) and the remaining bound sperm density was calculated by quantifying the number of sperm bound per 0.1mm² of the explant surface. The assessor was blinded to all treatments.

2.3 Experiment 2: Effect of Progesterone and Extracellular Calcium on Hyperactivation and Detachment from Bovine Oviductal Epithelial Cell (BOEC) explants.

The aim of this experiment was to investigate the effect of P4 on (i) Hyperactivated motility and (ii) Detachment from BOEC explants.

Experiment 2a: Role of Progesterone and Extracellular Calcium on Hyperactivation.

It was hypothesised that P4 would induce hyperactivation and would act through the influx of extracellular calcium, therefore, the aim of this experiment was to quantify the effect of calcium agonists and antagonists on sperm displaying hyperactivated motility. Experiments were carried out using P4 and calcium channel antagonists; Mibefradil and (1S,2S)-2-(2-(N-[(3-Benzimidazol-2-yl)propyl]-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthylcyclo-propane carboxylate dihydrochloride hydrate (NNC). Mibefradil is a calcium channel inhibitor which acts on both L-type and T-type channels (Mullins et al., 1998).

NNC 550396 is a derivative of Mibefradil and is a highly selective T-type calcium channel inhibitor (Li et al., 2005).

Initial dose response assessments were carried out to determine the optimum concentration of P4, Mibefradil and NNC on sperm hyperactivation. Vehicle controls were performed for treatments dissolved in ethanol (P4) and DMSO (NNC). Progesterone concentrations of (i) 0, 5, 8, 10, 50, 500 nM and 1, 10 and 50 μ M were assessed after 10 minutes. Hyperactivation was assessed as described below and three replicates were completed. Since it was hypothesised that P4 stimulates hyperactivation through extracellular Ca^{2+} influx, NNC concentrations were assessed by efficiency to antagonise T-type Ca^{2+} channels in the presence of P4 (10 nM). Treatments included: (i) No treatment (control) (ii) P4 (P4 control) (iii) P4 + 500 nM NNC (iv) P4 + 1 μ M NNC (v) P4 + 2 μ M NNC (vi) P4 + 10 μ M NNC (vii) P4 + 20 μ M NNC (viii) P4 + 30 μ M NNC. Hyperactivation was assessed as described below and three replicates were completed. Similarly, the concentration of Mibefradil was determined by its ability to suppress the proposed influx of Ca^{2+} . Treatments included (i) No Treatment (control) (ii) P4 (P4 control) (iii) P4 + 10 nM Mibefradil (iv) P4 + 100 nM Mibefradil (v) P4 + 500 nM Mibefradil (vi) P4 + 1 μ M Mibefradil (vii) P4 + 5 μ M Mibefradil (viii) P4 + 10 μ M

Mibefradil (ix) P4 + 20 μ M Mibefradil (x) P4 + 40 μ M Mibefradil. Hyperactivation was assessed as described below and three replicates were completed.

From each of the three dose response experiments above, the optimum concentration of each was chosen and assessed in one experiment to confirm their effects. These included: (i) No treatment (control) (ii) P4 (10 nM), (iii) NNC (2 μ M), (iv) Mibefradil (5 μ M), (v) P4 (10 nM) + Mibefradil (5 μ M), (vi) P4 (10 nM) + NNC (2 μ M), (vii) P4 (10 nM) + Mibefradil (5 μ M) + NNC (2 μ M).

Frozen-thawed straws from three different Holstein bulls were thawed at 37°C and pooled to minimise inter-bull variability. Only samples with a post-thaw motility of greater than 60% were used. Sperm concentration was determined using a Neubauer chamber and was diluted to 40 x 10⁶ sperm/ml in TALP media. Aliquots of treated sperm (200 μ l) were incubated for 10 min in 5% CO₂. Post incubation, a droplet of 10 μ l of a sample was placed on a pre-warmed slide with a coverslip.

Manual Assessment of Hyperactivation

Hyperactivation was subjectively assessed using a phase contrast microscope, fitted with a heated stage (Olympus: CX41, Hamberg, Germany) at x 400 magnification. Preliminary experiments deemed, subjective assessment of bull sperm hyperactivation to be more reliable, in comparison to Computer Assisted Sperm Analyser (CASA). The percentage of hyperactivated sperm in a sample was determined by recording the number of hyperactivated sperm from 100 motile sperm assessed. Hyperactivated motility of sperm was manifested in a characteristic figure of eight swimming trajectories with a helical pattern (Peedicayil et al., 1997; Alasmari et al., 2013; Ho et al., 2002), in comparison to the linear movement of non-hyperactivated sperm. Three technical replicates of each sample were completed (total of 300 sperm assessed per replicate) before getting an average percentage of hyperactivation per replicate. All assessments were carried out blind to the assessor.

Experiment 2b: Role of Progesterone and Extracellular Calcium on the Detachment of Bound Sperm from Bovine Oviductal Epithelial Cells (BOECs).

It was hypothesised that the incubation of bound sperm with P4 would stimulate a release of sperm from the BOEC explants due to an influx of extracellular calcium. Calcium channel antagonists of NNC and Mibefradil were used as antagonists of calcium channels. The calcium chelator, ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) was also used to chelate the calcium ions in the media. Therefore, the aim of this experiment was to investigate the action of P4 on the release of bound sperm from BOECs. Progesterone has been proposed to act upon CatSper channels in human sperm (Alasmari et al., 2013; Tamburrino et al., 2014; Strunker et al., 2011) and as CatSper is the only T-type calcium channel in sperm, NNC is an effective antagonist. The following treatments were assessed:

(i) No Treatment (control) (ii) P4 (10 nM) (iii) NNC (2 μ M) (iv) Mibefradil (5 μ M) (v) P4 (10 nM) + Mibefradil (5 μ M) (vi) P4 (10 nM) + NNC (2 μ M) (vii) P4 (10 nM) + Mibefradil (5 μ M) + NNC (2 μ M) and (viii) P4 (10 nM) + EGTA (2.5 mM). All treatments were assessed for bound sperm density post treatment as described above and three replicates were completed. All assessments were carried out blind to the assessor.

2.4 Experiment 3: Effect of Progesterone Receptor Antagonists on the Action of Progesterone.

The aim of this experiment was to investigate the mechanism of P4 action on bull sperm. It was hypothesised that P4 may act on sperm through non-genomic pathways to initiate the influx of Calcium. AG205 is a P4 Receptor Membrane Component 1 (PGRMC1) antagonist (Guo et al., 2016) and it has been used to investigate the involvement of PGRMC1 in numerous cell types, predominantly the liver, kidneys and cancerous cells (Peluso et al., 2008a; Mir et al., 2012). Mifepristone (RU-486) is an antiprogestin which exerts inhibitory effects on mPR mediated effects of P4, as seen in T cells (Chien et al., 2009). To examine the possible role of both membrane receptors on bull sperm the effect of P4 receptor blockers in the presence of P4 was assessed on (i) hyperactivated sperm and (ii) on the detachment of sperm from BOECs.

Experiment 3a: Effect of Progesterone Receptor Antagonists on Progesterone Induced Hyperactivation.

The aim of this experiment was to investigate if P4 induced hyperactivation is mediated through a membrane-bound P4 receptor. An initial dose response experiment was carried out for both Mifepristone and AG205 and the optimum concentration with the greatest effect on counteracting the P4 action on sperm hyperactivation was chosen. A vehicle control performed for Mifepristone as it was dissolved in DMSO.

Concentrations assessed included: Mifepristone: (i) No Treatment (control) (ii) P4 (10 nM), (iii) P4 (10 nM) + Mifepristone (40 nM), (iv) P4 (10 nM) + Mifepristone (400 nM), (v) P4 (10 nM) + Mifepristone (4 µM), (vi) P4 (10 nM) + Mifepristone (400 µM).

AG205: (i) No treatment (control) (ii) P4 (10 nM), (iii) P4 (10 nM) + AG205 (5 µM), (iv) P4 (10nM) + AG205 (10 µM), (v) P4 (10 nM) + AG205 (20 µM), (vi) P4 (10 nM) + AG205 (30 µM), (vii) P4 (10 nM) + AG205 (40 µM). Hyperactivation response of sperm were analysed using the above described methods.

From each of the three dose response experiments above, the optimum concentration of each was chosen and assessed in one experiment to confirm their effects. Treatments included (i) No treatment (control) (ii) P4 (10 nM) (iii) Mifepristone (400 nM; Mifepristone), (iv) AG205 (10 µM; AG205), (v) Mifepristone (400 nM) + AG205 (10 µM), (vi) P4 (10 nM) + Mifepristone (400 nM), (vii) P4 (10 nM) + AG205 (10 µM) (viii) P4 (10

nM) + Mifepristone (400 nM) + AG205 (10 μ M). All treatments were assessed blindly and three replicates were completed.

Experiment 3b: Effect of Progesterone and Progesterone Receptor Antagonists on the Detachment of Sperm from Bovine Oviductal Epithelial Cells (BOECs).

It was hypothesised that the action of P4 in the detachment of sperm from BOECs would be suppressed through the use of P4 receptor antagonists. Bound sperm were treated with; (i) No treatment (control) (ii) P4 (10 nM), (iii) Mifepristone (400 nM);, (iv) AG205 alone (10 μ M), (v) Mifepristone (400 nM) + AG205 (10 μ M), (vi) P4 (10 nM) + Mifepristone (400 nM), (vii) P4 (10 nM) + AG205 (10 μ M) (viii) P4 (10 nM) + Mifepristone (400 nM) + AG205 (10 μ M). The assessor was blind to the treatments being assessed and three replicates were completed.

2.5 Statistical Analysis

All data were examined for normality of distribution and were analysed using Statistical Package for the Social Sciences (IBM SPSS Software, Version 22, Chicago). All data were normally distributed and thus univariate Analysis of Variance (ANOVA) was used. Post hoc tests were carried out using the Bonferroni test whereby a P value <0.05 was considered statistically significant. All results are reported as the mean value \pm s.e.m (standard error of the mean).

Chapter

3

Results

Chapter Three: Results

3.1 Experiment 1: Effect of Capacitation Status on the Interaction of Sperm with Bovine Oviductal Epithelial Cell (BOEC) Explants.

Experiment 1a: Evaluation of Capacitation Inducers *in vitro* on Frozen-Thawed Bull Sperm using Chlortetracycline (CTC) Assay.

There was an effect of treatment on capacitated status ($P < 0.001$; Figure 13). While Caffeine alone induced a significantly higher capacitation in comparison to Heparin alone, the combination of Heparin and Caffeine was the most effective *in vitro* inducer of capacitation in frozen-thawed bull sperm.

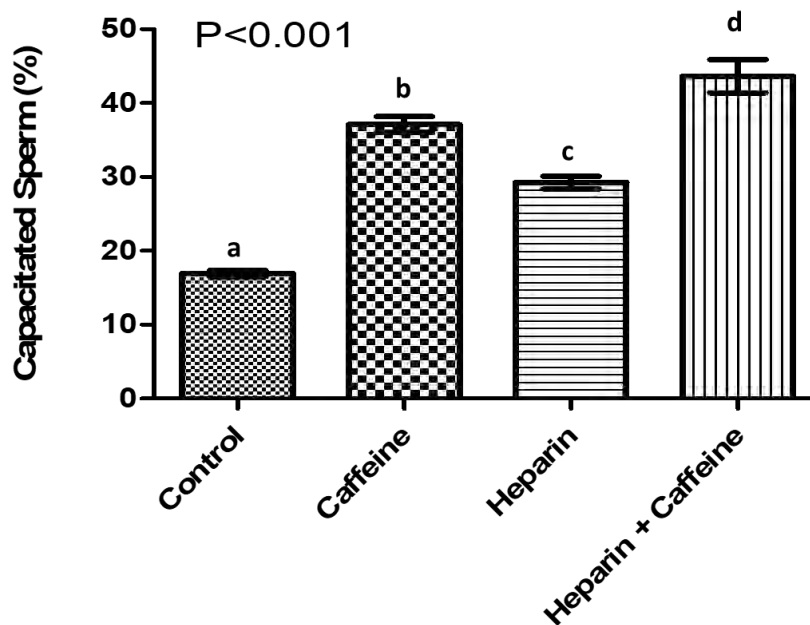


Figure 13: Capacitation status of frozen-thawed sperm assessed by Chlortetracycline staining. $n=4$ replicates. Vertical error bars represent s.e.m. ^{abcd} Differing superscripts differ significantly.

Experiment 1b: Effect of Capacitating Treatments on Sperm Binding Density to Bovine Oviductal Epithelial Cell (BOECs) explants.

There was an effect of capacitating treatment on the ability of frozen-thawed bull sperm to bind to BOEC explants ($P < 0.001$; Figure 14). Heparin and Caffeine treated sperm (i.e. capacitated sperm) had a significantly reduced binding density to BOEC than Caffeine alone ($P < 0.05$). The control treatment (i.e. non-capacitated sperm) had the highest binding ability.

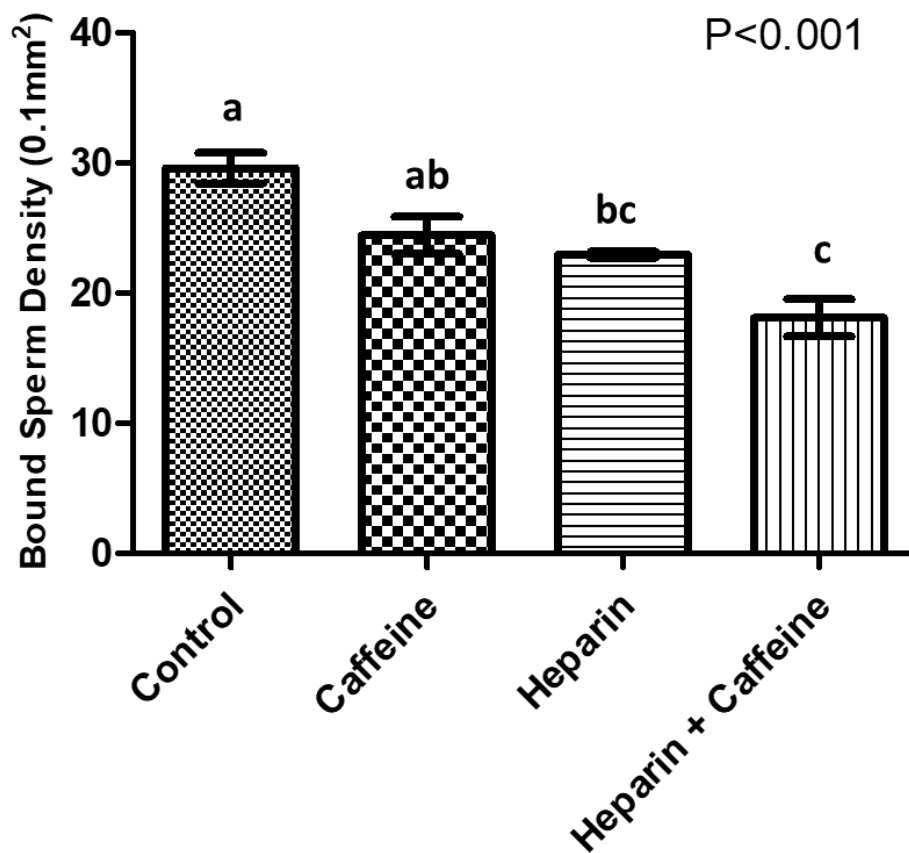


Figure 14: Sperm binding density of frozen-thawed sperm incubated with capacitating treatments to Bovine Oviductal Epithelial Cell explants. $n=3$ replicates. Vertical error bars represent s.e.m. ^{abc}Differing superscripts differ significantly.

Experiment 1c: Effect of Capacitation Treatments on the Release of Bound Sperm from Bovine Oviductal Epithelial Cell (BOEC) explants.

There was an effect of capacitation treatment on the release of sperm from BOEC explants ($P < 0.001$; Figure 15). The bound sperm density was reduced after incubation with caffeine alone heparin on its own as well as heparin and caffeine in combination ($P < 0.001$). There was no difference in the number of sperm remaining bound per 0.1 mm^2 after sperm-BOEC incubations were treated with caffeine, heparin and the combination of caffeine and heparin ($P > 0.05$).

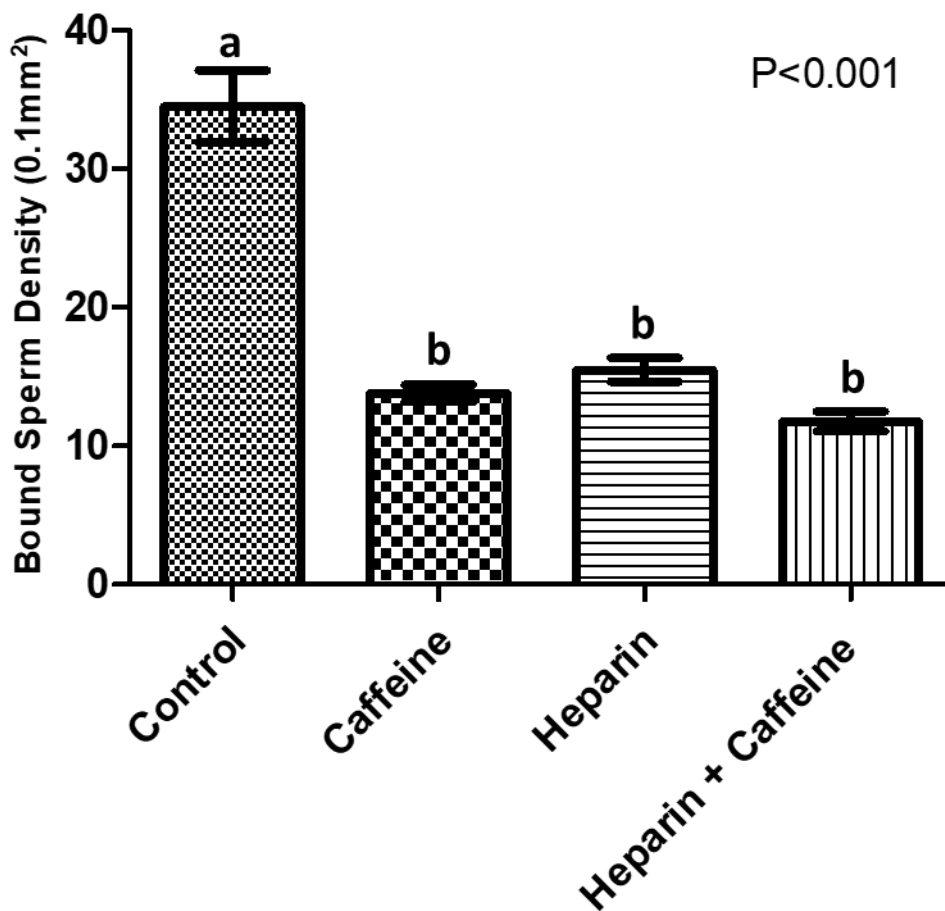


Figure 15: Remaining bound sperm density to bovine oviductal epithelial cell explants post treatment with hyperactivating (caffeine) and capacitating (heparin) inducing agents. $n = 5$ replicates; Vertical bars represent s.e.m. ^{ab}Differing superscripts differ significantly.

3.2 Experiment 2: Effect of Progesterone and Extracellular Calcium on Hyperactivation and Release of Bound Sperm from Bovine Oviductal Epithelial Cells (BOECs).

Experiment 2a: Role of Progesterone and Extracellular Calcium on Hyperactivation.

There was an effect of P4 treatments on hyperactivated motility of frozen-thawed bull sperm ($P < 0.001$; Figure 16). Increasing the concentration of P4 increased sperm hyperactivation with peak hyperactivation achieved at a concentration of 10 nM P4 (76.76 ± 2.963 ; $P < 0.001$). Hyperactivated motility rapidly developed when sperm were treated with P4. Calcium channel antagonists of NNC and Mibefradil successfully suppressed P4 induced hyperactivation, with concentrations of 2 μ M NNC ($P < 0.001$; Figure 17 (a)) and 5 μ M Mibefradil ($P < 0.001$; Figure 17(b)) having the greatest antagonistic effect and these concentrations were used for subsequent experiments.

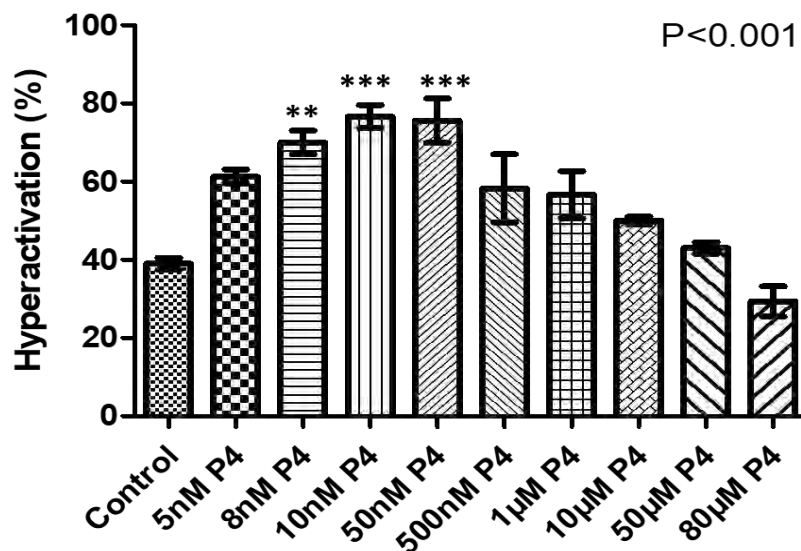


Figure 16: Sperm displaying hyperactivated motility following incubation with Progesterone. n=3 replicates per experiment. Vertical bars represent s.e.m. ^{abc} Differing superscripts differ significantly.

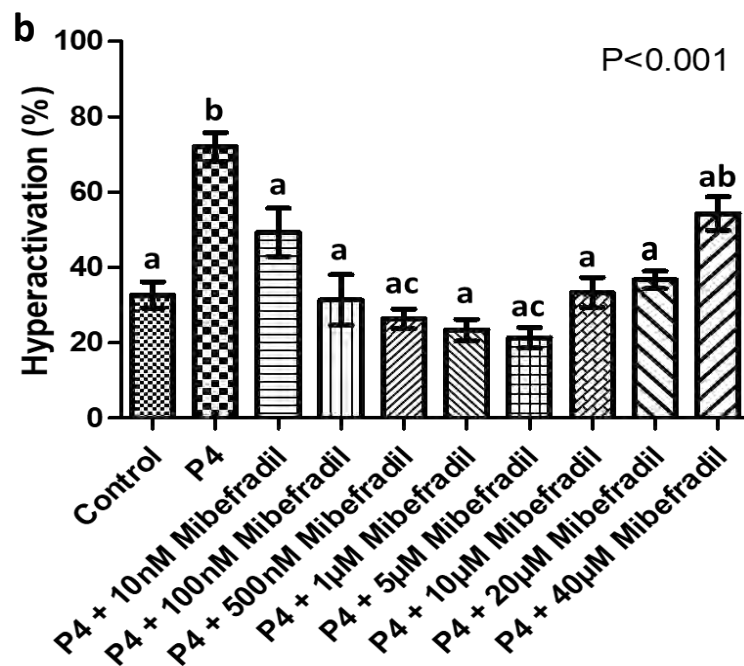
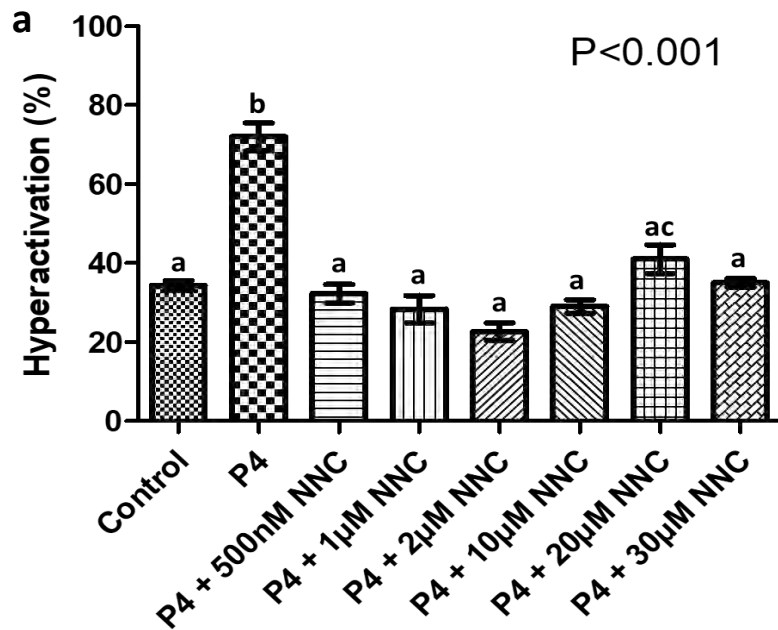


Figure 17: Hyperactivated sperm following incubation with (a) Progesterone with NNC and (b) Progesterone with Mibefradil. n=3 replicates per experiment. Vertical bars represent s.e.m. ^{abc} Differing superscripts differ significantly.

Experiment 2b: The Role of Progesterone and Extracellular Calcium on Hyperactivation and the Release of Bound Sperm from Bovine Oviductal Epithelial Cell (BOECs) Explants.

Progesterone increased sperm hyperactivation ($P < 0.001$; Figure 18) and stimulated the release of bound sperm from BOEC explants ($P < 0.001$; Figure 19). Incubation of sperm with calcium channel antagonists of NNC and Mibefradil alone had no effect on sperm hyperactivation ($P > 0.05$) in comparison to the control. However, with NNC and Mibefradil alone, there was an effect on the release of sperm from BOEC explants ($P < 0.01$). The action of NNC, Mibefradil and the calcium chelator, EGTA, inhibited P4 induced hyperactivation ($P < 0.001$) as well as the release of sperm from BOEC explants induced by P4 ($P < 0.001$).

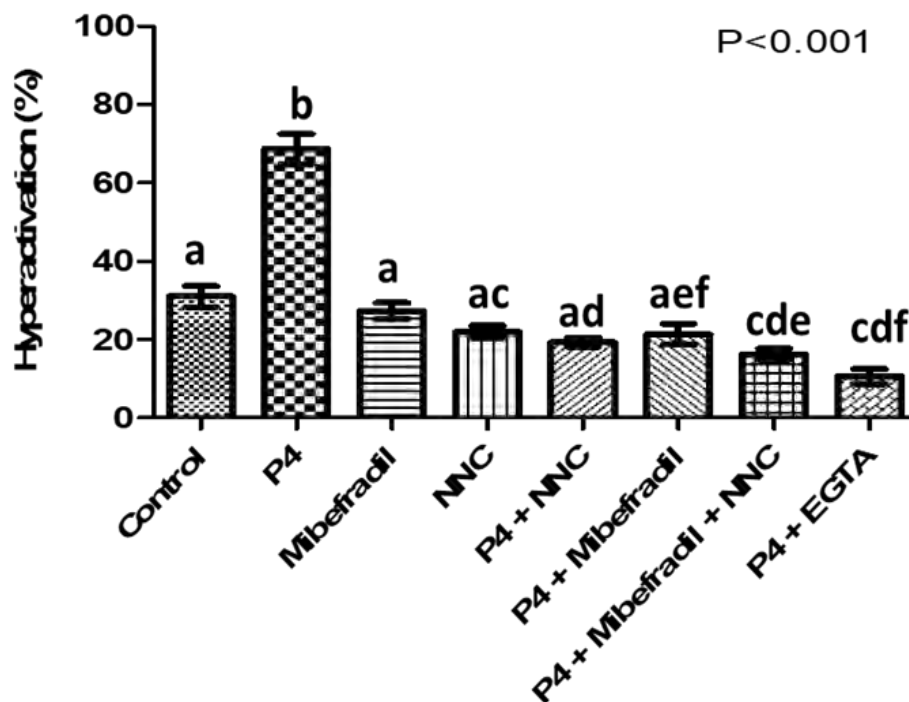


Figure 18: Hyperactivated motility of sperm treated with progesterone (P4), calcium channel antagonists (NNC and Mibefradil) and calcium chelator (EGTA). n=3 replicates per experiment; Vertical bars represent s.e.m. ^{abcdef}Differing superscripts differ significantly.

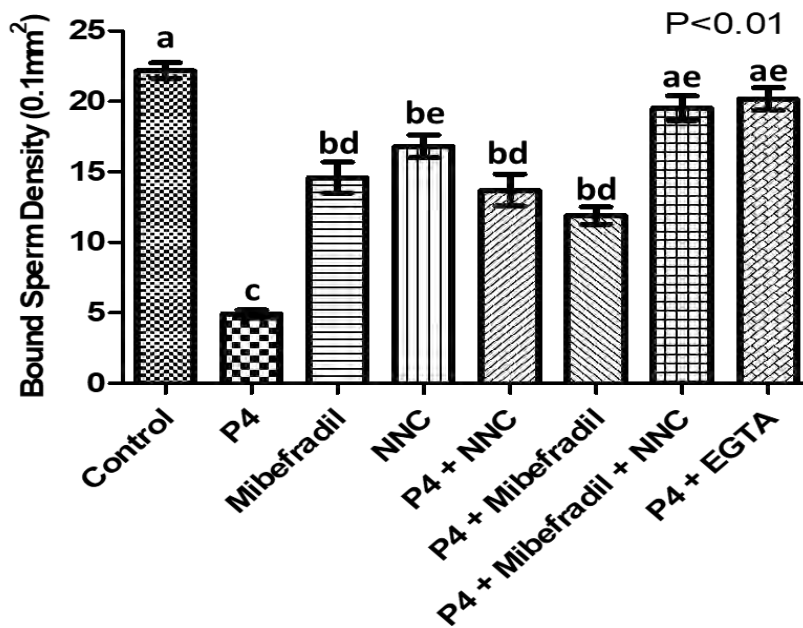


Figure 19: Release of sperm from BOEC explants of frozen-thawed bull sperm treated with progesterone (P4), calcium channel antagonists (NNC and Mibefradil) and calcium chelator (EGTA). n=3 replicates per experiment; Vertical bars represent s.e.m.

^{abcdef}Differing superscripts differ significantly.

3.3 Experiment 3: Effect of Progesterone Receptor Antagonists on the Action of Progesterone.

Experiment 3a: Effect of Progesterone Receptor antagonists on Progesterone induced Hyperactivation.

There was an effect of the P4 receptor antagonists of AG205 and Mifepristone on P4 induced hyperactivated motility ($P < 0.001$; Figure 18). All concentrations of AG205 inhibited effects on P4 induced hyperactivation ($P < 0.001$). Mifepristone (400 nM) had the greatest inhibitory effect against P4 ($P < 0.0001$). Therefore, the lowest, most effective concentrations of 10 μ M AG205 ($P < 0.0001$) and 400nM Mifepristone ($P < 0.0001$) were deemed optimum for subsequent experiments.

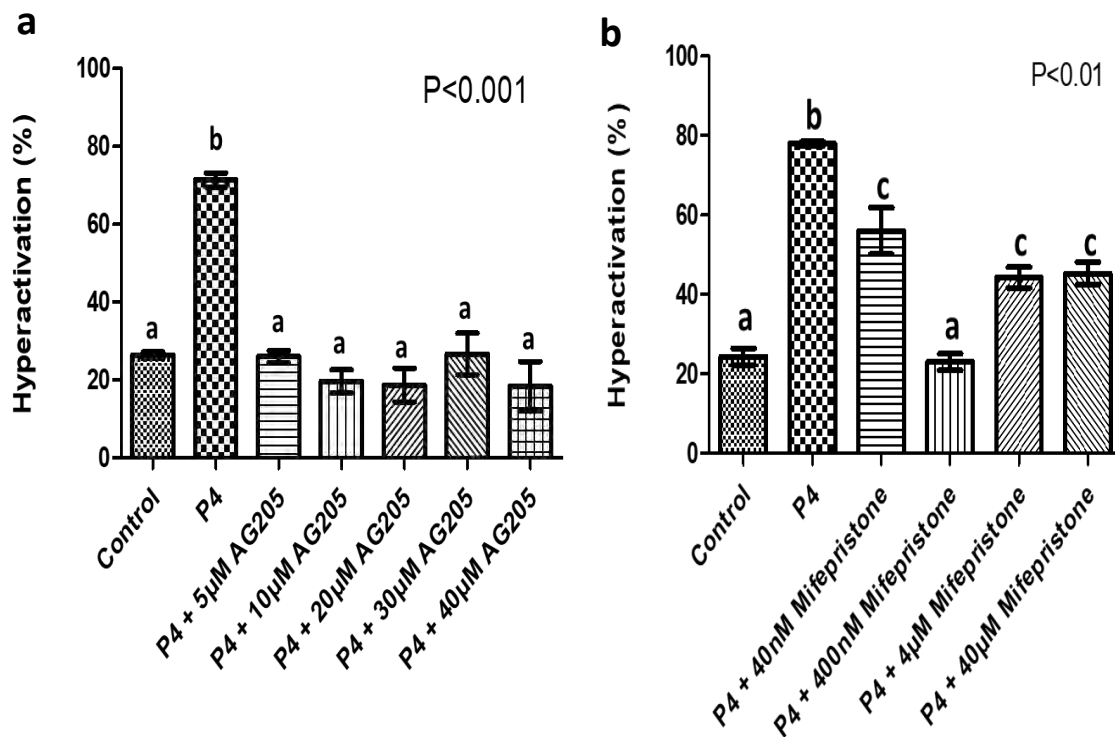


Figure 20: Sperm hyperactivation following incubation with progesterone and progesterone receptor antagonists of AG205 (a) and Mifepristone (b). n=3 replicates. Vertical bars show s.e.m. ^{abc}Differing superscripts differ significantly.

Experiment 3b: Effect of Progesterone and Progesterone Receptor Antagonists on the Detachment of Sperm from Bovine Oviductal Epithelial Cells (BOECs).

There was an inhibitory effect of P4 receptor antagonists on sperm hyperactivation ($P < 0.001$) and release of sperm from BOEC explants ($P < 0.01$; Figure 19). In the absence of P4, Mifepristone and AG205 alone or in combination did not affect hyperactivation ($P > 0.05$) or the release of sperm from BOECs ($P > 0.05$). Hyperactivation and the release of sperm from BOEC explants by P4 was inhibited in the presence of Mifepristone ($P < 0.0001$) and AG205 ($P < 0.01$). The combination of AG205 and Mifepristone had the highest significant effect on inhibiting the action of P4 in both hyperactivation and ability to release sperm from BOEC explants ($P < 0.001$). Bound sperm density was not affected by the P4 action when incubated with both AG205 and Mifepristone in comparison to the control treatment ($P > 0.05$).

a

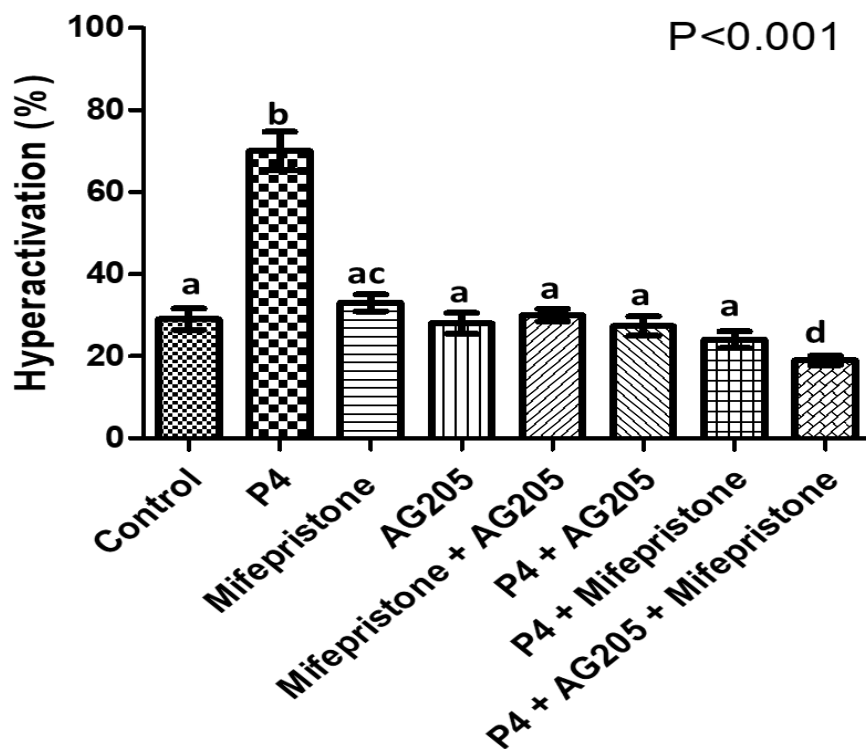


Figure 21: (a) Hyperactivated motility of sperm treated with Progesterone (P4) and Progesterone receptor antagonists (AG205 and Mifepristone). $n=3$ replicates; Vertical bars represent s.e.m. ^{abcd} Differing subscripts differ significantly.

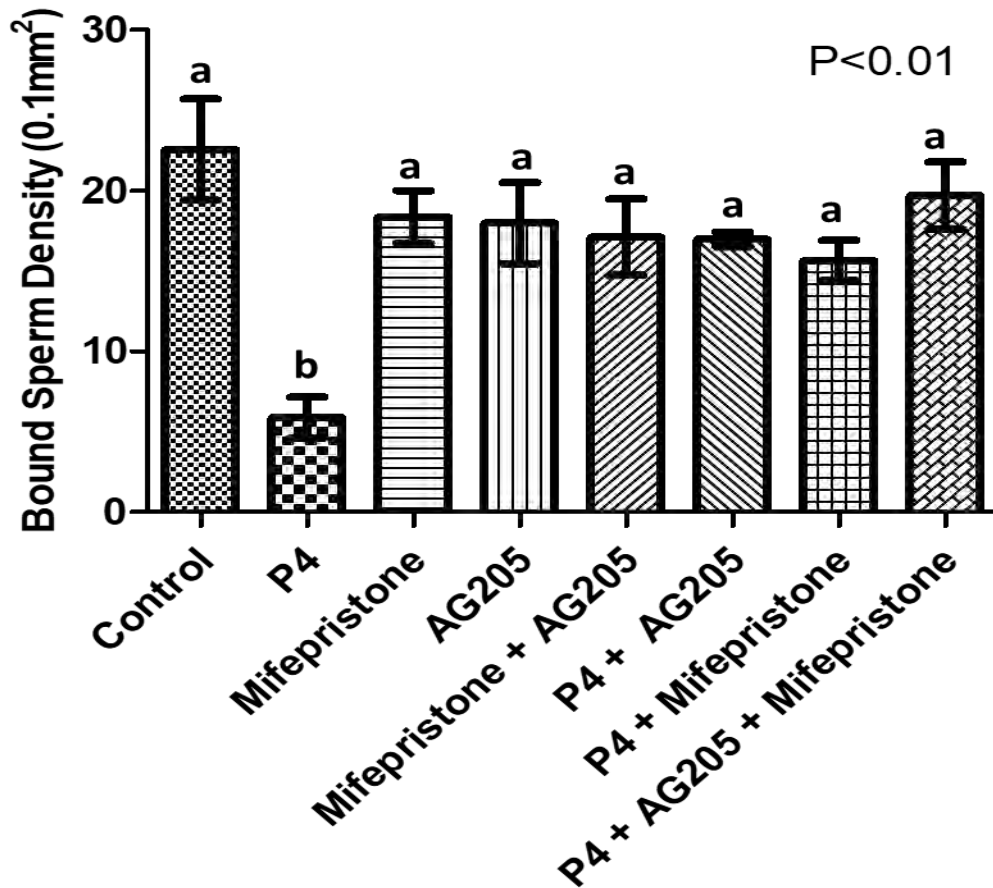


Figure 22: Release of sperm from BOEC explants of frozen-thawed bull sperm treated with Progesterone (P4) and Progesterone receptor antagonists (AG205 and Mifepristone). n=3 replicates; Vertical bars represent s.e.m. ^{abcd} Differing subscripts differ significantly.

Chapter

4

Discussion

Chapter Four: Discussion.

The sequential release of fertile sperm from the oviductal reservoir around the time of ovulation aids in delivering a supply of fertile sperm to the ampulla and increases the chance of successful fertilisation. The process that mediates their release has been the focus of a number of studies (Gervasi et al., 2016; Gualtieri et al., 2005; Hunter, 2008) and capacitation has been the focus of detachment in many of these studies (Bosch et al., 2001; Gualtieri et al., 2010; Talevi and Gualtieri, 2010). To the best of the author's knowledge, this is the first published study to assess the effect of P4 on bovine sperm hyperactivation and associated unbinding from the oviductal epithelium. The main findings of this study were; 1) Hyperactivation stimulates a significant release of sperm from BOEC explants, 2) Hyperactivation of bovine sperm can be induced by P4 by a rapid non-genomic action, 3) P4 elicits its effect by directly or indirectly acting on Ca^{2+} channels which facilitate the influx of extracellular Ca^{2+} into the cell and 4) The action of P4 on sperm which suppressed by P4 receptor antagonists in this data set, infer the possible presence of P4 membrane receptor.

In mammals, sperm must undergo the maturational process of capacitation, which is promoted by the interaction with the female reproductive tract secretions in order to gain the ability to successfully penetrate the zona pellucida of the oocyte. Through CTC staining, this study demonstrated that heparin and caffeine, alone or in combination induce capacitation of frozen-thawed bull sperm. The fluorescent antibiotic CTC moves into the cell, becomes negatively charged and binds to accumulations of intracellular free Ca^{2+} in cellular compartments (Gillan et al., 2005; Tsien and Pozzan, 1989). It should be noted that a rise in intracellular Ca^{2+} is just one of the parameters indicative of capacitation and it is unlikely that caffeine alone induces capacitation. Heparin, a sulphated glycosaminoglycan, has been found in bovine oviductal fluid (Hileman et al., 1998; Therien et al., 2005) from secretions by the cumulus and granulosa cells (Bergqvist et al., 2007). Heparin, bound to the sites on BSP proteins of the ejaculated sperm, in combination with caffeine affected the binding affinity sperm to the BOECs *in vitro* by preventing the association with the ANXA proteins on epithelial cells. The effect of GAGs, along with the influence of the phosphodiesterase inhibitor, to prevent the degradation of cAMP (Arnoult et al., 1997), likely resulted in maximised tyrosine phosphorylation levels

associated with capacitation (Hung and Suarez, 2012), thereby affecting the binding affinity and release of sperm from BOEC explants *in vitro* through capacitation. These results, where the BSP binding site is occupied by heparin, are in accordance with a previous study by Ignatz et al. (2001) who found that sperm that shed BSP proteins lack the capability to bind to oviduct epithelial cells. Additionally, Suarez (2008) described the binding ability of sperm is only functional in non-capacitated sperm.

The physiological factors that trigger the sequential release of sperm from oviductal reservoirs are not well understood. The attachment of sperm to the lower region of the isthmus maintains sperm viability and delays sperm capacitation *in vivo* (Pollard et al., 1991; Chian et al., 1995). It has been shown that the release mechanism does not affect the number of carbohydrate binding sites which are present in the oviductal epithelium (Lefebvre et al., 1995), therefore the key to release mechanisms is through an alteration to the sperm surface in response to tract components. The release of sperm from the isthmus epithelium has been reported to be dependent on sperm changes associated with capacitation (Bosch et al., 2001). In the current study, when bound sperm were treated with the capacitating agents heparin and caffeine *in vitro*, sperm were released. These results are consistent with other studies which demonstrate that sulphated glycoconjugates such as heparin, enhances disulphide reductants, and are powerful inducers of sperm release in hamster and bovine sperm (Hunter, 2008; Talevi and Gualtieri, 2001; Parrish et al., 1988; Parrish et al., 1989b), through the reduction of sperm surface protein disulphides to sulphhydryl's (Gualtieri et al., 2010). Moreover, heparin enhanced the percentage of bull sperm released from monolayer cultures (Bosch et al., 2001).

This study found that hyperactivation alone is sufficient for sperm detachment from the isthmus portion of the oviduct, indicating that hyperactivation is an important sperm release mechanism, in addition to capacitation. This supports the suggestion that sperm transport involves a cycle of detachment and attachment in the oviduct prior to capacitation but once capacitated, sperm do not rebind (Chang and Suarez, 2012). Studies of tethered sperm show that hyperactivated sperm can exert enough propulsive force to penetrate through viscoelastic fluids and detach from a surface (Curtis et al., 2012; Ishijima, 2011). Progesterone is known to be released by the cumulus cells of the bovine oocyte (Mingoti et al., 2002), therefore sperm are exposed to a P4 gradient with maximal

concentration in close proximity to the oocyte. In this current study, a concentration of 10nM P4 was used, which may be higher than physiological concentrations to which sperm are actually exposed to in the female reproductive tract. This, and similar concentrations have been used in functional studies of human sperm (Strunker et al., 2011; Sumigama et al., 2015; Luconi et al., 1998), with picomolar concentrations used to demonstrate chemotaxis (Villanueva-Diaz et al., 1995; Teves et al., 2009a), while the induction of the acrosome reaction in human sperm has been performed using micromolar concentrations (Blackmore et al., 1991; Baldi et al., 2009; Bronson et al., 1999; Kirkman-Brown et al., 2002a). To-date much of the focus of the effect of P4 on bull sperm has been on its ability to induce chemotaxis (Gil et al., 2007) yet, the effect of P4 in the induction of bovine sperm release from the oviductal reservoir has not been assessed.

This study has shown the positive effect of P4 in the induction of hyperactivation of bovine sperm, which enabled us to test our hypothesis that hyperactivated motility is responsible for sperm detachment from oviductal epithelial cells. It has been shown in human sperm that P4 activates a biphasic response, involving repeated intracellular Ca^{2+} oscillations of a rapid Ca^{2+} rise, followed by a sustained increase which is thought to be a result of intracellular Ca^{2+} store mobilisation (Harper et al., 2004; Kirkman-Brown et al., 2004). Intracellular Ca^{2+} is approximately 30-50 nM in a normal motile bull sperm which increases to 200-1000 nM in a hyperactivated sperm (Ho et al., 2002). We showed that hyperactivation induced by P4 occurs rapidly and is reliant on the function of the primary calcium channel in sperm, CatSper. The importance of Ca^{2+} influx via CatSper has been illustrated by CatSper null mice which are unable to make the transition beyond the sperm reservoir (Ho et al., 2009) and have been found to be infertile (Qi et al., 2007; Ren et al., 2001; Carlson et al., 2005). CatSper has been reported in human (Strunker et al., 2011), stallion (Loux et al., 2013) and bovine sperm (Johnson et al., 2016) and can be antagonised by Mibefradil (non-specific Ca^{2+} channel blocker) and more specifically by its derivative of NNC which is less cytotoxic to the sperm (Bui et al., 2008). Mibefradil acts through the reduction of P4 mediated AKT phosphorylation in sperm (Sagare-Patil et al., 2013). These antagonists suppress the effect of P4, showing that the effect of P4 acts through stimulating an influx of extracellular Ca^{2+} into the cell. When Ca^{2+} was removed from the media through the use of EGTA, the action of P4 was also nullified. Therefore, the presence of

extracellular Ca^{2+} is essential in order for P4 to elicit its effect on hyperactivation and its resultant release of sperm from BOEC explants.

Interestingly, analysis of hyperactivation and the release of sperm from BOEC explants was induced by P4 but was suppressed by P4 receptor antagonising agents of Mifepristone and AG205 when alone or in combination. Due to the rapid action of P4 on sperm, it depicts that P4 acts through a non-genomic mechanism on the transcriptional and translationally silent cell. The action of AG205 against a non-classical P4 receptor PGRMC1 in this study suggests the presence of this receptor in bovine sperm. This receptor, part of the MAPR family, has been located in human and porcine sperm with a molecular weight of ~ 28kDa (Thomas, 2008; Losel et al., 2005). Additionally, sperm treated with P4 and Mifepristone, blocked non genomic action in sperm. Mifepristone has been shown to antagonise both nPR and mPRs (Chien et al., 2009), therefore in this study, P4 conjugated to BSA was used. This renders the steroid impermeable to sperm, hence the suppression of P4 hyperactivation by Mifepristone must occur through the inhibition of a mPR on bovine sperm.

This study as well as previous work in human sperm (Lishko et al., 2011; Strunker et al., 2011), suggest P4 acts through non-genomic pathways by stimulating CatSper channels. The fast action of P4 on sperm hyperactivation and the demonstrated requirement of extracellular Ca^{2+} to elicit the response, therefore strongly suggests a direct extracellular effect of P4 on a membrane sperm protein. Interestingly, it has been found that P4 is more effective on the cell when it is present in the extracellular milieu in comparison to when P4 is microinjected into the cytoplasm (Maller and Krebs, 1977). It is therefore proposed that, the action of P4 on a surface receptor may trigger an influx of extracellular Ca^{2+} through CatSper (rapid rise), followed by a further CICR from intracellular stores (transient rise) and stimulation of an intracellular pathway e.g. PI3K/AKT, meaning phosphorylation of proteins in the sperm cell. Thus leading to hyperactivated motility and the development of the propulsive force to detach from the epithelial cells of the oviduct. Through this proposed mechanism, the sperm cell still holds the essential surface molecules to reattach to the oviduct cells on its journey to the oocyte.

In conclusion, this novel study has demonstrated that hyperactivation induced by P4 plays a role in the detachment of bull sperm from BOEC explants which is an indication of events occurring *in vivo*. These findings indicate that the presence of extracellular Ca^{2+} is essential for the sperm response to P4. In addition, we have presented evidence that the action of P4 is mediated by a membrane P4 receptor, however, the underlying mechanism that inflicts the action of P4 on intracellular pathways still remains to be elucidated. Dissecting the physiological components of the oviductal environment and their effect on sperm function will expand our understanding of sperm binding, sequential release and will ultimately enable the industry to improve assisted reproduction in cattle, such as reducing the sperm number in semen straws without affecting fertility.

Publications.

Johnson,G.P., English A.M., Cronin, S., Hoey, D.A., Meade, K.G., Fair, S 2017.
Genomic Identification, Expression Profiling and Functional Characterisation of CatSper
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Chapter

5

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Chapter

6

Appendices

Chapter Six: Appendices.

Tyrosdes Albumin Lactate Pyruvate (TALP)

Reagents	Concentration
NaCl	99 mM
KCl,	3.1 mM
NaHCO ₃	25 mM
NaH ₂ PO ₄	400 µM
MgCl ₂	1.1 mM
CaCl ₂	2mM
HEPES	10 M
Sodium Pyruvate	1 mM
Sodium Lactate	25.4 mM
BSA	6 mg/ml
pH	7.4
Temperature	38.5°c
Atmosphere	5% CO ₂

Phosphate Buffered Saline

Reagents	Concentration
NaCl	1.37 mM
KCl,	27 mM
NaH ₂ PO ₄	100 mM
pH	7.4

M199 Culture Media

Reagents	Volume
M199 media	3400 µL
Fetal Bovine Serum	400 µL
Gentamicin Sulphate Solution	200 µL