Characterisation of the Rheotaxis and Thigmotaxis Responses of Stallion Sperm

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Degree: MSc Biological Science (Research)

Abstract

To navigate their way towards the site of fertilisation in the oviducts, sperm orientate and swim against a flow of mucus (rheotaxis) and while doing so, are guided by the sensation of touch along the epithelial lining of the reproductive tract (thigmotaxis). The aim of this study was to characterise the rheotactic and thigmotactic response of stallion sperm within a microfluidic channel (800 μm wide, 20 μm deep and 58.5 mm in length). Stallion sperm rheotaxis was assessed within the microfluidic channel in response to (i) A range of flow velocities (ii) Varying media viscosity (iii) Sperm distribution across the microfluidic channel and (iv) Sperm hyperactivation and compared to human and ram sperm. Stallion sperm progressed furthest at a velocity range of 10-30 μm/s, with an optimum velocity of 20 μm/s, and a viscosity of 2.5 cP or greater reduced sperm rheotaxis ($P<0.05$). Stallion sperm that were hyperactivated were unable to exhibit rheotaxis within the microfluidic channel, whereas, both hyperactivated human and ram sperm did exhibit positive rheotaxis. Both hyperactivated and non-hyperactivated human and ram sperm reorietated and swam against the flow, and while non-hyperactivated stallion sperm reoriented and swam against the flow, hyperactivated stallion sperm did not and were swept downstream with the flow. The number of sperm swimming near the microfluidic channel walls was higher than in the centre ($P<0.05$). This is the first study to illustrate that stallion sperm are rheotactically responsive and increasing viscosity reduces this response. We also demonstrated that sperm are inclined to swim along a surface and uniquely, hyperactivated stallion sperm are non-progressive and do not exhibit a rheotactic response unlike other species. The swimming pattern of hyperactivated stallion sperm against a fluid flow is uncoordinated and non-progressive

similar to the erratic pattern traditionally observed in a static environment. This study provides a novel insight into species differences in sperm rheotatic response.

Declaration

I hereby declare that this research is entirely my own work. It has not been submitted for any other academic award, or part thereof, at this or any other educational institution.

Acknowledgments

Firstly, I would like to thank my supervisor Dr. Seán Fair for giving me the opportunity to join his group and use his facilities. His guidance and expertise was invaluable throughout the course of this project. I would also like to acknowledge Dr. David Newport for his assistance and direction. Additionally, I would like to thank all the people who helped and supported me while completing this research project, in particular the members of the Fair research lab group and all the staff in the Department of Biological Science at the University of Limerick. I would like to express a special thanks to Sinead Cronin, Jon Romero and Laura Abril for all their advice and support during this project and my time in the lab. Finally, I would like to acknowledge all of my friends and family for their encouragement and support throughout my masters.

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List of Abbreviations

ADAM A Disintegrin and Metalloprotease

AI Artificial Insemination

ALH Amplitude of Lateral Head-displacement

ART's Assisted Reproduction Techniques

BCF Beat Cross Frequency

Ca^{2+} Calcium Ion

cAMP Cyclic Adenosine Monophosphate

CASA Computer Assisted Sperm Analysis

CatSper Cation channels of Sperm

CL Corpus Luteum

cP Cente Poise

dH2O Distilled Water

DNA Deoxyribonucleic Acid

ECM Extracellular Matrix

FSH Follicle Stimulating Hormone

GnRH Gonadotropin Releasing Hormone

HEPES 4-(2-Hydroxyethyl)-1-Piperazine Ethane Sulfonic acid

HSI Horse Sport Ireland

ICSI Intra Cytoplasmic Sperm Injection

ISH Irish Sport Horse

IVF *In vitro* Fertilisation

KWPN Dutch Warm Blood

LH Leutinizing Hormone

LIN Linearity

MUC4 Mucin 4

MUC5B Mucin 5B

OCC Oocyte Cumulus Complex

OPU Ovum Pick-Up

P4 Progesterone

PBS Phosphate Buffered Saline

PGF2α Prostaglandin

pH - Log H^+ Concentration

PR Pregnancy Rate

ROS Reactive Oxygen Species

s.e.m. Standard Error of the Mean

ST Seminal Tubule

STR Straightness

tACE Testis-Specific Angiotensin Converting Enzyme

TALP Tyrodes Albumin Lactate Pyruvate

UTJ Utero-tubal Junction

VAP Average Path Velocity

VCL Curvilinear Velocity

VSL Straight Line Velocity

Chapter 1

Literature Review

1.1 Introduction

Sperm have to navigate their way from the site of deposition in the lower reproductive tract to the site of fertilisation in the ampulla of the oviduct. While the location of semen deposition is species dependent, as well as varying between natural mating and artificial insemination, sperm have to make the tortuous journey of in excess of 1000-fold their length to the ampulla (Kantsler et al., 2014a, Kantsler et al., 2014b). Sperm undergo capacitation which leads them to exhibit hyperactive motility. Both of these events are necessary for fertilisation and provide sperm with the ability to recognize, bind and penetrate the zona pellucida on interaction with the oocyte. The spermatozoa's journey is facilitated by the increased contractility of the reproductive tract during the follicular phase of the mare's oestrous cycle, primarily under the control of oestrogen from the dominant follicle (Kunz et al., 1996). This contractility also stimulates mucus secretion which flows out through the cervix and results in the retrograde flow of sperm (Miki and Clapham, 2013a). In addition to this, the flow is also stimulated by the beating of epithelial cilia lining the reproductive tract which assume a more synchronised beating pattern at the time of ovulation, further increasing the flow (Hunter, 2012).

More recently, a number of sensing mechanisms have been proposed which guide sperm towards the oviducts (Eisenbach and Giojalas, 2006, Pérez-Cerezales et al., 2015), namely; chemotaxis (Zhang et al., 2015, Kaupp, 2012), thermotaxis (Hunter and Nichol, 1986, Boryshpolets et al., 2015), rheotaxis (Miki and Clapham, 2013a, Kantsler et al., 2014a) and thigmotaxis (Winet et al., 1984a, Denissenko et al., 2012).

Currently, the influence of hyperactivation on stallion sperm rheotaxis and thigmotaxis has not yet been reported. However, it has been shown in hamsters and mice that hyperactivated motility increased the straightness and allowed the spermatozoa to move more efficiently through a viscous medium similar to that of the oviduct (Suarez and Dai, 1992, Suarez et al., 1993). There is a dearth of published studies on how stallion sperm acquire the ability to fertilise and how they are hyperactivated within the microenvironment of the oviduct, as presently, the *in vivo* capacitating triggers for equine sperm have yet to be identified. The use of microfluidics can be used to mimic the *in vivo* microenvironments of the mammalian reproductive tract (Huang et al., 2014, Lopez-Garcia et al., 2008, Wheeler et al., 2007, Knowlton et al., 2015) and thus can be used to increase our understanding of how sperm interact with the female reproductive tract so as to develop strategies to improve assisted reproduction in horses. In this thesis microfluidics were used as a tool to study the behaviour of stallion sperm in both hyperactivated and non-hyperactivated states under the influence of differing flow velocities and media viscosities and to compare this with human and ovine sperm.

1.2 Equine Breeding

The Irish Sport Horse (ISH) breed has developed with a natural ability for the equestrian sports of showjumping and eventing. The unique composition of the breed gives the ISH it's much sought after, characteristics of athleticism, jumping ability, courage, intelligence and soundness. Irish Sport Horses compete successfully at the highest level of most equestrian disciplines and have dominated eventing competitions for decades. The ISH eventing studbook has been the leading studbook in the World Breeding Federation for Sport Horses (WBFSH) studbook rankings every year since 1994 with the exception of 2010 and 2011 when they finished in 2nd and 3rd place, respectively (HorseSportIreland, 2013).

Equine fertility and subsequent breeding success is multifactorial and some issues affecting it include; age, breed, health status, body condition and type of breeding method used. In order to achieve a specific breeding goal a dam and sire must be chosen and matched for the particular traits desired, for example, competition, showing or leisure horses. Informed choice of breeding stock and good management techniques will improve the quality and consistency of foals and young stock, and subsequently improve the productivity and profit of the ISH industry. To increase the efficiency of the ISH industry, Horse Sport Ireland (HSI) has compiled estimated breeding values (EBVs) for jumping, conformation, movement, temperament and athleticism (HorseSportIreland, 2013). Estimated breeding values are predictions of the traits that a stallion or mare will pass onto its offspring and estimate how much that improvement can be expected to be inherited by the offspring. They are expressed as an index with an average value of 100 and the higher the EBV figure, the better. A horse with an EBV of 140 for jumping will have a better chance of breeding jumpers than one with a value of 120. On the other hand, horses with values below 100 are unlikely to improve that quality in their offspring (Teagasc, 2016). An EBV with a reliability value of 0.7 or greater is considered accurate and the reliability increases as more information is provided on a particular horse, for example as the number of progeny competing increases, therefore, providing more results. Once elite horses have been identified, assisted reproductive techniques (ARTs) facilitate the widespread dissemination of genes.

1.2.1 Assisted Reproductive Techniques

There are a number of assisted reproductive techniques (ART's) available to the equine industry which can be used to increase the dissemination of genes from elite stallions and mares. Stallions can sire hundreds to thousands of offspring through artificial insemination (AI), whereby semen is collected from a stallion using an artificial vagina, diluted, processed and during AI sperm are deposited transcervically directly into the mare's uterus eliminating the need for natural covering. Artificial insemination in most cases is an effective, convenient and safe method of breeding. It can be carried out using fresh, cooled or frozen-thawed semen but the timing of insemination needs to be modified for each semen type (Table 1.1). This is due to the shortened fertile lifespan of processed semen within the mares reproductive tract.

Table 1.1 Optimum timing for insemination with fresh, cooled and frozen stallion semen (Teagasc, 2016).

Optimum timing of insemination	
Fresh semen	24-48 hours before ovulation
Chilled semen	12-18 hours before ovulation
Frozen semen	6 hours before to 6 hours after ovulation

Artificial insemination is by far the most common ART, however, rates in Ireland are very low in comparison to our European peers. For example, 100% of breeders in the Belgian warm blood stud book used AI and 90% of breeders in the Hannoverian and KWPN (Dutch warmblood) (HorseSportIreland, 2013), in contrast to this only 42% of ISH breeders used AI in 2016. Of these only 17% used frozen-thawed semen an increase from 12% in 2011 (HorseSportIreland, 2017), however, this indicates low levels of semen importation and limited use of internationally proven stallions. There is considerable scope

for expansion of this area of the industry but the sub-standard pregnancy rates following AI with frozen-thawed semen (<50%) (Sielhorst et al., 2016) is inhibiting this. This is a consequence of cryopreservation which causes irreversible damage to stallion sperm. According to Darr et al., (2016) there are three ways in which stallion sperm are affected these are: cold shock, osmotic shock and oxidative stress through the production of reactive oxygen species (ROS). This cryo-damage causes a decrease in sperm functions such as viability, mitochondrial membrane potential as well as motility, which decreases by approximately 50% after a single freeze-thaw cycle (Darr et al., 2016, Sielhorst et al., 2016).

The genetics of elite mares can be exploited through a number of ARTs. The most common, embryo transfer, whereby a donor mare is inseminated with fresh/cooled or frozen-thawed semen. Superovulation does not work well in mares as it does in other species (such as cows) due to a very large dominant follicle, the medulla covering the ovary and the presence of an ovulation fossa. A single embryo is normally recovered via uterine flushing on day 7-8 post insemination, the embryo transfer to a recipient mare is then carried out trans-cervically with an approximate pregnancy rate of 75% (Aurich, 2011).

Oocyte transfer is an ART, whereby, a mature oocyte of a donor mare is transferred via flank laparotomy to the oviduct of an inseminated recipient mare (Hinrichs, 2012). Both mare's cycles are synchronised and the oocyte from the recipient mare is aspirated to insure it is not available for fertilisation. This particular method was first developed to overcome the failure of equine *in vitro* fertilisation (IVF) and has a pregnancy rate of approximately 70% when *in vivo* matured oocytes are used, in comparison to pregnancy rates of 9-18% when *in vitro* matured oocytes are used (Aurich, 2011).

In vitro fertilisation (IVF) does not work in horses as stallion sperm are unable to complete the acrosome reaction *in vitro* and are thus unable to penetrate the zona pellucida of the oocyte (Hinrichs and Loux, 2012). Mare immature oocytes can be collected by transvaginal ultrasound guided ovum pick-up (OPU), matured for approximately 24h and fertilized by intra cytoplasmic sperm injection (ICSI). This involves the direct injection of a single spermatozoon into a matured oocyte *in vitro,* following which they are cultured until blastocyst stage and transferred to the uterus of the recipient mare (Aurich, 2011).

The use of OPU and/or oocyte/embryo transfer are advantageous in that they allow a mare to remain in competition while exploiting her genetic potential and make it possible to breed from sub-fertile mares and also reduces the spread of venereal diseases.

1.2.2 The Mares Reproductive System

The mare's reproductive system includes the ovaries, oviducts, uterus, cervix, vagina and the external genitalia (Figure 1.1. Senger, 2005). The walls of each region of the tract consists of four distinct layers; an outer serosal layer that covers the surface of the reproductive tract, a muscularis layer that consists of two layers of smooth muscle, this layer facilitate the contractility of the reproductive tract. Directly beneath this is the sub-mucosal layer which is rich in blood vessels, nerves and lymphatics, the final and inner most layer is the mucosa, this layer lines the lumen of the whole reproductive tract and secretions are specific to each region of the tract (Senger, 2005).

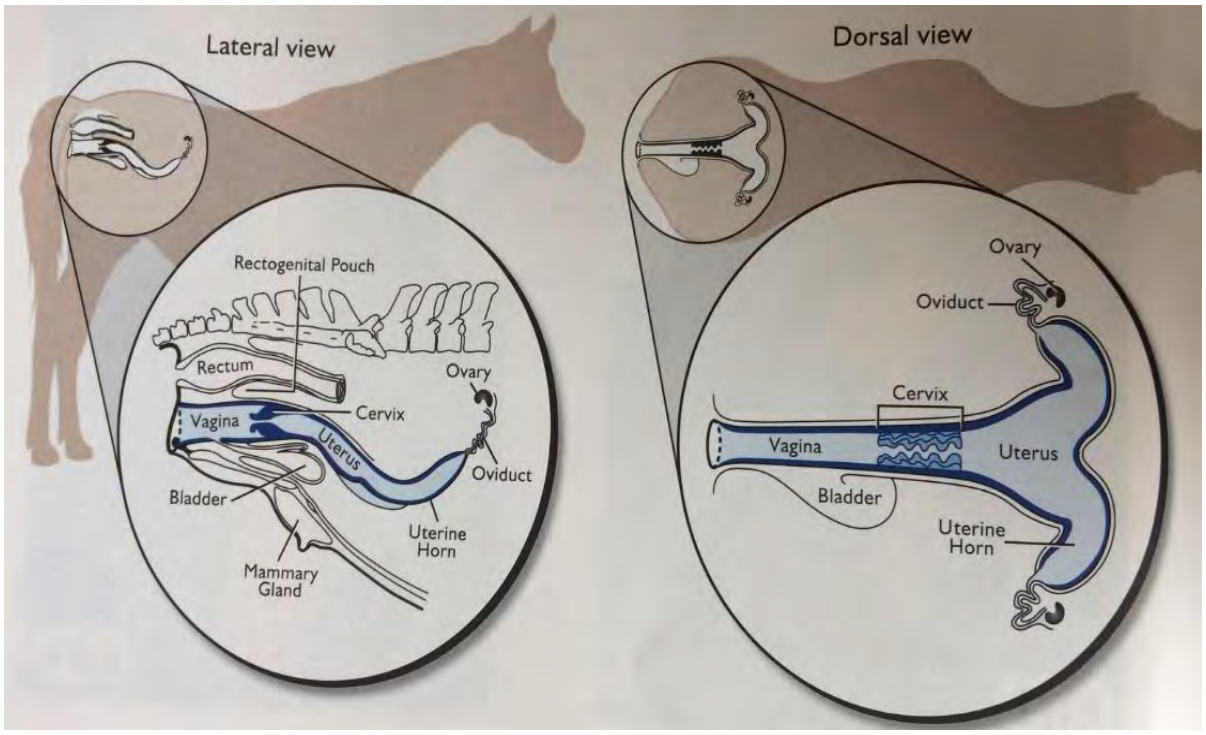


Figure 1.1 Lateral and dorsal view of the reproductive anatomy of the mare (source: Senger, 2005)

1.3 The Mares Reproductive Cycle

The mare is seasonally polyoestrus meaning that she exhibits multiple oestrous cycles during a specific season of the year. The oestrous cycle is the length of the interval between two successive ovulations, it is approximately 21 days and consists of 2 phases. The follicular phase which last approx. 4-7 days and the dioestrus stage luteal phase which last approx. 14-15 days (Aurich, 2011). Mares are classified as long-day breeders because they initiate cyclicity as the day length increases during spring (Aurich, 2011). The oestrous cycle is under the control of the endocrine system and as the day length increases there is a decrease in the release of melatonin from the pineal gland. This has a positive effect on the release of gonadotropin releasing hormone (GnRH) from the hypothalamus. Gonadotropin releasing hormone stimulates the production and release of the gonadotropins; follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. Follicle stimulating hormone concentrations peak in the mid luteal phase and this stimulates the initial cohort of follicular growth. Luteinizing hormone levels rise early in the follicular phase and peak just after ovulation leading to follicular and oocyte maturation and luteinisation of the granulosa cells of the pre-ovulatory follicle (Aurich, 2011). Ovulation which occurs 1-2 days before the end of follicular phase is triggered by the rise in circulating levels of LH. Post ovulation the follicular cells develop into a corpus haemorrhagicum and later into a corpus luteum which secretes progesterone (P4). If a viable embryo is not detected in the uterus (the signal from the embryo for maternal recognition of pregnancy is not known in horses), the corpus luteum is regressed by endogenous prostaglandin (PGF2α) that is released from the endometrium 13-15 days after ovulation (Aurich, 2011).

During the follicular phase the mare is receptive to the stallion, and the reproductive tract is prepared to transport sperm it to the site of fertilisation in the oviducts. A rise in

oestrogen levels prompts changes to the cervical mucus and oestrogen regulated secretions in the oviduct increase 2-3 fold during the follicular phase, and this in turn reduces the mucus viscosity and creates an outward flow of mucus, against which sperm must swim (Miki and Clapham, 2013b). In mice these changes are also instigated by natural mating which causes an increase in fluid secretions as a result of the release of prolactin from the anterior pituitary in response to coitus (Miki and Clapham, 2013b). It is a combination of these factors that makes the secretions less viscous and this in turn enables selective sperm penetration while still ensuring the sperm to egg ratio remains low to prevent polyspermy (Suarez, 2016). In addition to sperm swimming, sperm transport is also aided by uterine contractions. Increased muscular tone is characteristic of the follicular phase due to high circulating levels of oestrogen (Hunter, 2012). Hunter et al., (2011) reported that these contractions are enhanced in response to seminal plasma which causes the secretion of prostaglandins from the uterus.

The dominant hormone during the luteal phase is P4 and its high levels results in muscular relaxation which is characteristic of this stage (Hunter, 2012). The mare is not receptive to the stallion and the reproductive tract is prepared for pregnancy. This preparation includes the increase in viscosity of the mucus secretions to prevent sperm entering and also to protect the upper reproductive tract from pathogens as neither is capable of penetrating the thick mucus secretions.

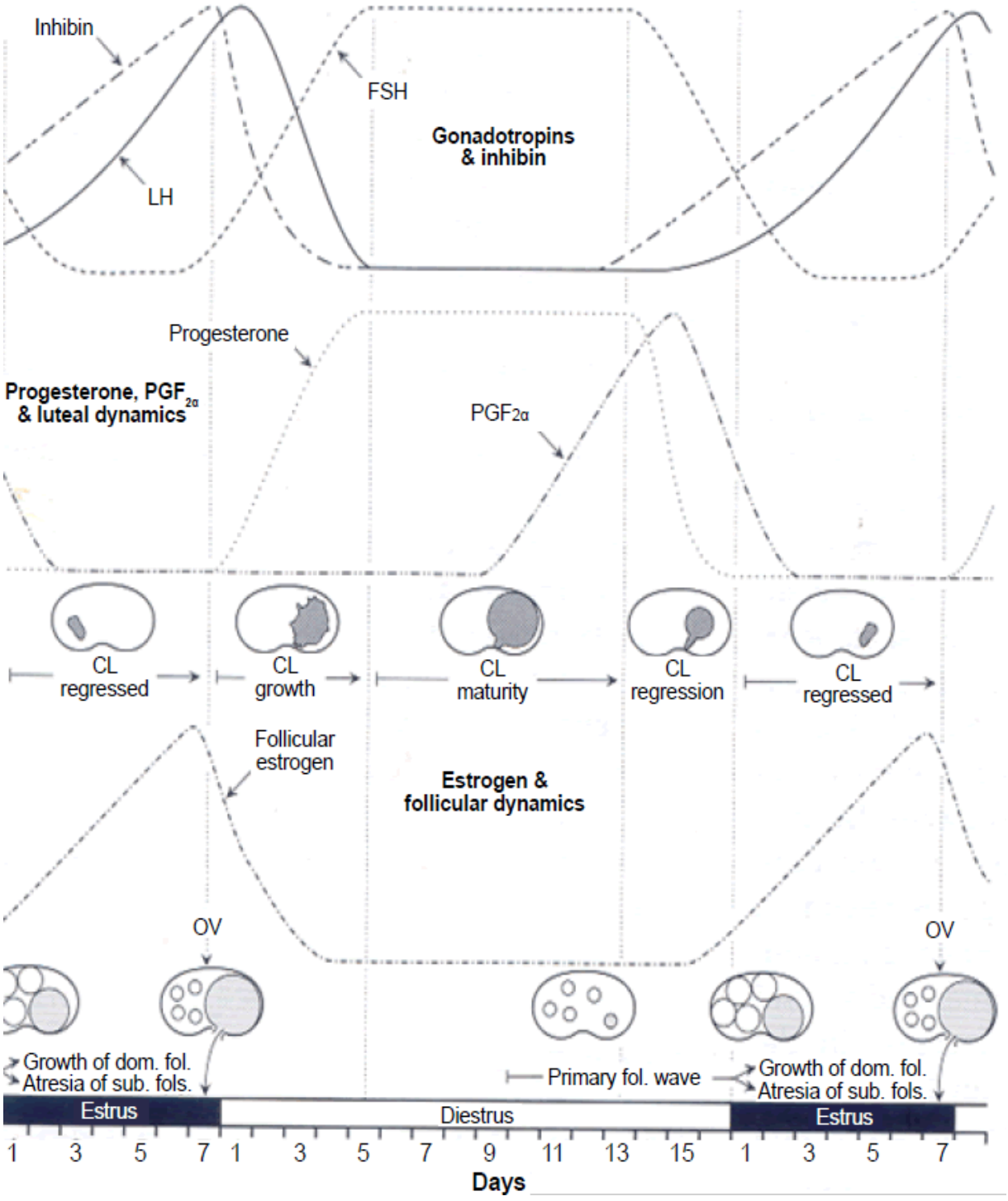


Figure 1.2 Relative blood hormone concentrations and relationship to follicular and luteal development during the oestrus cycle in the mare (source: Satue, 2013)

1.3.1 Mucus Composition

Mucus of the reproductive tract is composed of water, lipids, cholesterols, carbohydrates, inorganic ions, and proteins. These proteins include immunoglobulins, plasma proteins, enzymatic proteins such as sialidases and mucins which are large glycoproteins that provide structure to the mucus (Huang et al., 2014), MUC4 and MUC5B have been identified as the major gel-forming mucins in human cervical mucus (Figure 1.3).

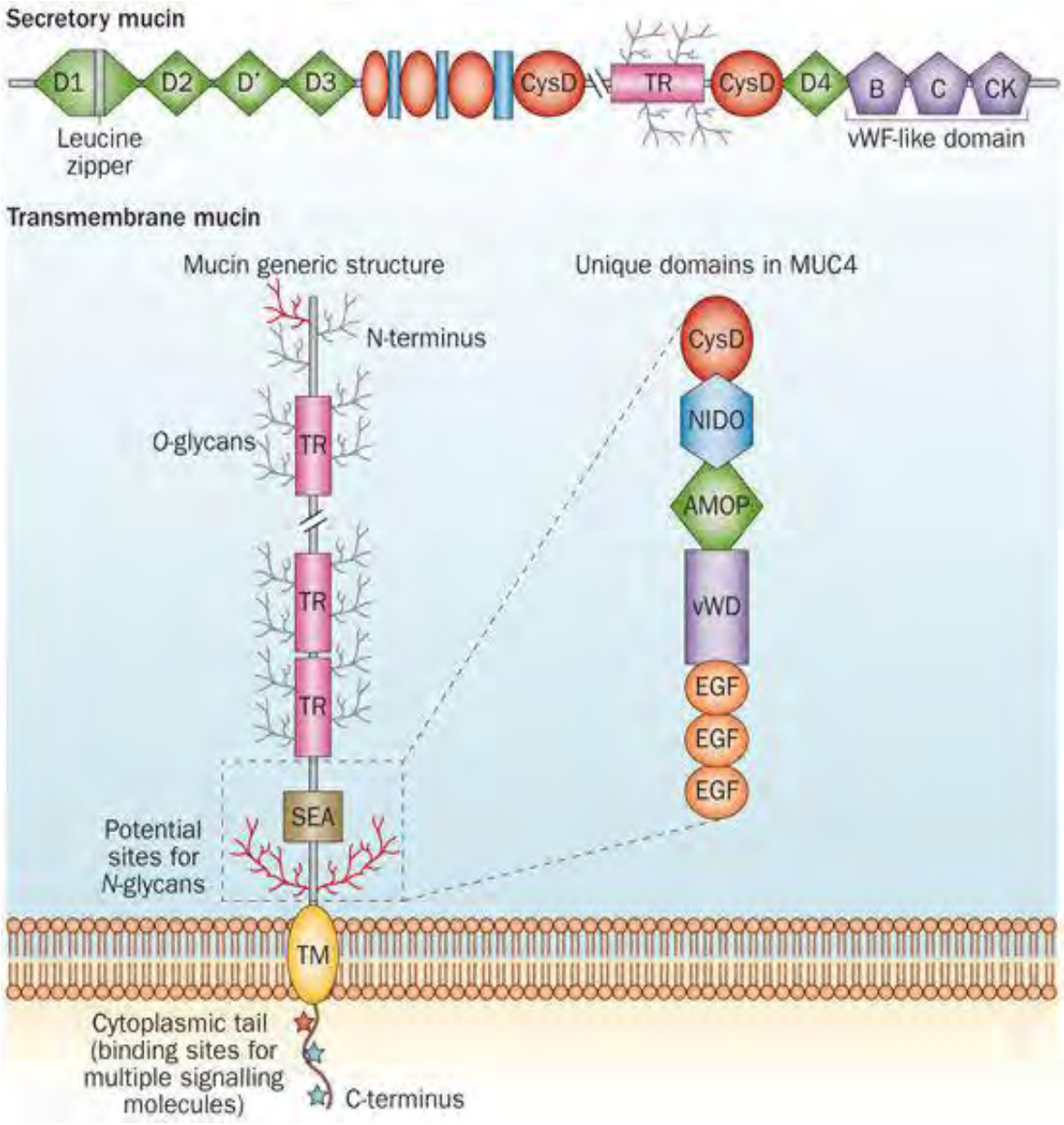


Figure 1.3 Structure of a secretory (MUC5B) and transmembrane (MUC4) mucin (source: Aguilar and Reyley, 2005)

The composition and secretion of the mucus present in the reproductive tract of the mare is largely influenced by cyclical endocrine changes (Hunter, 2012), and it has been suggested by Aguilar and Reyley (2005) that the rate of secretion of uterine tubal fluid in the mare is almost doubled from 2.82 mL/24h during dioestrus to 5.08 mL/24h during oestrus. It is this increase in secretions during oestrus that causes the polysaccharide component of the glycoproteins to absorb water resulting in hydration of the mucus, thus reducing the viscosity (Knowlton et al., 2015).

Sperm must swim through the viscous secretion of the reproductive tract en route to the oviduct, it is recognised that the viscosity plays a role in sperm selection and decreases the sperm to oocyte ratio (Hunter, 2012, Suarez, 2016). Mucus viscosity is at its lowest during oestrus to allow some sperm to penetrate. The ovary functioning as an endocrine organ secretes a range of steroid hormones such as oestrogen and progesterone that help coordinate oviduct activity (Hunter, 2012). Characteristics of the oviduct that are influenced by these hormones include surface morphology, muscular function and the volume and composition of the fluids present within the lumen of the oviduct. Viscous glycoprotein fluid accumulates in the isthmus and is especially prominent prior to ovulation (Hunter, 2012). The composition of the fluid within the oviduct and uterus is also important for providing a suitable environment for the early development of the embryo as it remains in the oviduct for approximately 4-5 days before being transported to the uterus where it remains free floating until fixation around Day 16 (Franciolli et al., 2011) and subsequent implantation at approximately Day 40.

In the cow it has been shown that there are two types of mucus secreted. The first is sialomucin, a mucus with a low viscosity secreted from the basal portion of the cervical crypts and the second type is sulfomucin, a mucus of high viscosity secreted from the apical portion of the cervical folds. Senger (2005) and Mullins and Saacke (1989) showed

in the cow it is these areas of low viscosity in the cervical crypts that allow the sperm to pass through thus creating 'privileged pathways'.

Fluid viscosity within the reproductive tract varies, depending on location and species. It has been suggested that the cervical mucus has the highest viscosity of all the regions of reproductive tract, with human cervical mucus reported to have a viscosity of 200-680 cP (Miki and Clapham, 2013b). Mullins and Saacke (1989) reported the viscosity of the human cervical mucus to vary from 100-1000 cP, however, it is unclear as to what stage of the cycle these measurements were taken.

In the mare a high proportion of sperm is deposited into the uterus during natural mating or AI, therefore, the cervix does not have a major role in sperm transport. Miki and Clapham (2013b) measured the viscosity of uterine fluid of mice and found it to be 81 ± 73 cP, further highlighting the variability in fluid viscosity. Importantly, it must be pointed out that the fluid of the reproductive tract is thixotropic meaning the viscosity decreases with shear (Hyakutake et al., 2015, Mullins and Saacke, 1989, Miki and Clapham, 2013b) and this could help explain why viscosity decreases as flow increases during oestrus. There are regional differences in mucus composition within the distinct microenvironments of the oviduct (Hunter, 2012). This is supported by the finding of Leemans et al., (2016) who showed that when spermatozoa were introduced directly into the ampulla of the oviduct via the infundibulum, the pregnancy rates are the same as AI into the uterine body. This suggests, that neither the uterine body nor the isthmus are essential for sperm to gain fertilising ability.

1.3.2 Effect of Mucus Viscosity on Swimming Pattern

The change in mucus viscosity influences both the swimming pattern and progression of sperm in particular the curvilinear velocity (VCL; the velocity of the sperm along its actual path), the amplitude of the lateral head displacement (ALH; the width of the lateral movement of the sperm head about its average path) and the average path velocity (VAP; the average velocity of a sperm along its average path) (Hunter et al., 2011). In addition, to this hyper-viscosity in human semen is associated with reduced motility and infertility (Hunter et al., 2011). Kirkman-Brown and Smith (2011) reported that a more accurate prediction of motility is the concentration of sperm migrating a set distance through viscous media compared to the commonly used vanguard distance which measures the distance travelled by the fastest sperm. The study also found that increasing viscosity reduces the side to side movement across the directional axis or 'head yaw'. Furthermore, the study reported that hyperactivation is a low-viscosity sperm behaviour that is expressed by an increased side to side head movement, and in a physiological viscosity hyperactivated sperm would swim in a more forward direction (Figure 1.4). This is further supported by Perez-Cerezales et al., (2016) who showed that mouse sperm treated with the hyperactivation agonist progesterone displayed erratic and non-progressive swimming patterns in low viscous media compared to a high viscous media whereby, the sperm displayed a more linear and progressive swimming pattern. In addition to this, artificial viscoelastic fluids have been shown to straighten the trajectories of hyperactivated mouse (Suarez and Dai, 1992) and hamster sperm (Suarez et al., 1993). Kantsler et al., (2014b) assessed the effect of different shear rates (0.2-9 s^{-1}) and viscosities (1-20 cP) on sperm rheotactic behaviour in microfluidic channels and found that spermatozoa upstream velocity decreased more strongly with higher viscosities for bull than for human sperm. The study reported that the difference in swimming behaviour could be due to differences

in head shape; bull sperm have a flatter head than human sperm which likely suppresses the rotational motion of the cell at high viscosities, thus leading to a smaller vertical beat amplitude.

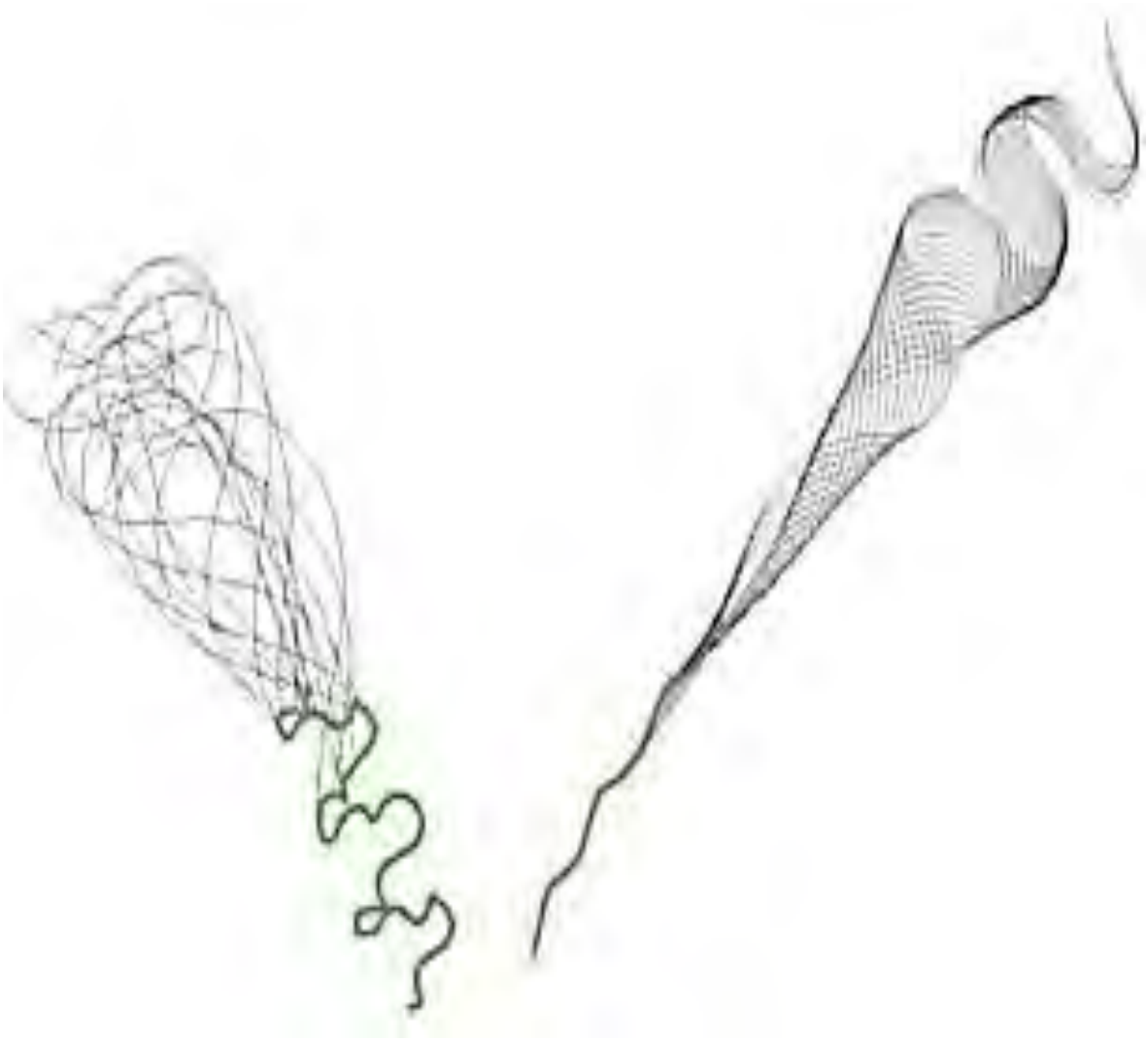


Figure 1.4 Plots of sperm in standard low-viscosity medium (left) and ovulatory physiological viscosity medium (right), showing the swimming trajectory of the sperm (green lines)(source; Kirkman-Brown and Smith, 2011).

1.4 Sperm Migration in the Female Reproductive Tract

1.4.1 Capacitation

Throughout the journey to the site of fertilisation, sperm undergo changes in the form of capacitation. Capacitation is a complex biochemical alteration of the sperm surface and is necessary for normal fertilisation. Some of the changes that occur include; the loss, rearranging and unmasking of various surface molecules. These changes subsequently cause an efflux of cholesterol, membrane permeablilization, hyperactivation and unbinding from the oviductal sperm reservoirs as it reduces the spermatozoa's binding affinity (Suarez, 2016). In both cattle and horses only non-capacitated spermatozoa bind to the oviduct epithelum, with sperm unbinding occurring in response to capacitation and or hyperactivation (Leemans et al., 2016). Capacitation allows fusion with the oocyte leading to the acrosome reaction (Rodriguez-Martinez, 2007). The epididymis and seminal plasma both contain molecules called de-capacitation factors that prevent the sperm from capacitating too early, therefore, preserving the fertility of the sperm. Capacitating triggers are present in the follicular and or the oviductal fluid and it is the absence of these triggers in current IVF media that prevents completion of capacitation of stallion sperm *in vitro* and subsequent *in vitro* fertilisation (Hinrichs and Loux, 2012). Leemans et al., (2016) hypothesized that in order for equine sperm to capacitate it is vital that sperm are exposed to oviductal secretions. The study emphasized the importance of the interaction of sperm with the ampulla in particular, as in cases where the sperm are introduced to the oviduct via the infundibulum they obtained similar pregnancy rates to those where the sperm were delivered into the uterus. This suggests that exposure to the uterus or the isthmus is not necessary for stallion sperm to gain the ability to fertilise.

1.4.2 Hyperactivation

Hyperactivation is an integral component of the capacitation process and in stallion sperm is characterised by a high amplitude, asymmetrical beating pattern of the sperm flagellum, manifested by a star shaped frenzied swimming pattern when viewed microscopically under a coverslip in aqueous media (Suarez and Ho, 2003, Hinrichs and Loux, 2012). *In vivo,* hyperactivation is necessary for successful fertilisation to occur, and it has been proposed as a mechanism, which assists the sperm in detaching from the oviductal epithelium (Curtis et al., 2012, Suarez, 2008). Although, the specific role it plays during this process remains unclear, it is thought that hyperactivity enables sperm to escape mucosal folds in the oviduct and break bonds between the sperm head and the oviductal epithelium (Simons et al., 2014). In order to hyperactivate, sperm require CatSper proteins which form Ca^{2+} channels. In support of this, in CatSper null mice alkalinisation inhibits calcium influx into the sperm and they are unable to hyperactivate, or detach from the oviductal epithelial cells (Ho et al., 2009), and are subsequently not capable of fertilising an oocyte (Hinrichs and Loux, 2012). There are a number of studies which indicate that hyperactivation is mediated by Ca^{2+} signaling pathways (Carlson et al., 2009) and it has been hypothesized that the asymmetric bending of the flagellum could be due to calcium binding to calmodulin receptors (Suarez, 2008). However, the exact mechanism for CatSper-mediated induction of hyperactivated motility is unclear; it may involve environmental calcium influx as well as the release of calcium from an internal store near the neck of the sperm, possibly the redundant nuclear envelope (Hinrichs and Loux, 2012). In mice, the CatSper channels are localised to the principal piece of the sperm and open in response to increased intracellular pH, which is typically associated with sperm capacitation. In addition to aiding sperm detachment from the reservoirs it is also understood that hyperactive motility is advantageous for sperm penetration through the

viscous oviductal fluid and the viscoelastic cumulus cells surrounding the oocyte as well as the zona pellucida (Suarez et al., 1991).

The local anaesthetic, procaine is commonly used as a hyperactivation agonist for stallion sperm (Hinrichs and Loux, 2012) due to its action as a sodium channel blocker. It also has effects on internal calcium stores, inhibiting calcium release from the endoplasmic reticulum through its inhibitory effect on ryanodine receptors, and is a weak base, thus causing intracellular alkalinization. This increase in intracellular calcium appears to trigger changes in the flagellar function leading to hyperactivation (Hinrichs and Loux, 2012). The influence of hyperactivation on the ability of stallion sperm to navigate mucus of varying viscosities have not been reported.

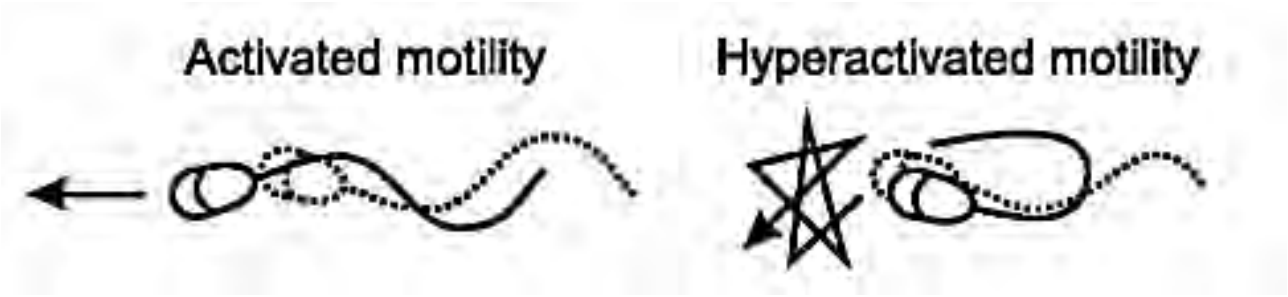


Figure 1.5 A diagrammatic representation of the swimming pattern of non-hyperactivated (left) and hyperactivated sperm (right)(source: Darszon et al., 2011)

1.4.3 Sperm Interactions with the Oviduct

The oviduct can be divided into four sections, the uterotubal junction (UTJ), the isthmus, the ampulla and the infundibulum (Hunter, 2012). The UTJ acts as a barrier to microbes and also regulates the entry of sperm into the oviduct. It has been shown in mice that the sperm head must express certain proteins, in particular, a disintegrin and metalloprotease 2 (ADAM2), calmegin and testis-specific angiotensin converting enzyme (tACE) in order to pass through the UTJ (Suarez, 2008). Mice that are null mutants for these particular proteins either lack or show abnormal distribution of ADAM3, implicating this particular protein in the control of sperm through the UTJ (Suarez, 2008). These mice are subsequently infertile because they cannot pass through the UTJ and not only this but they cannot bind to the zona pellucida either. This suggests that the UTJ has a key role in filtering abnormal sperm (Suarez, 2008). In mares, the isthmus is the site of sperm storage in the form of a sperm reservoir and lastly the ampulla is the site of fertilisation (Hunter, 2012, Leemans et al., 2016). There are a number of interactions that take place between the sperm and the oviduct that facilitate the migration of sperm from the uterus to the site of fertilisation. These various interactions help to maintain the sperms motility and viability until ovulation and select the best quality spermatozoa for fertilisation. These interactions can be divided into physical and molecular (Suarez, 2016). The physical interactions are the swimming response of the sperm to the environment within the oviduct in response to fluid flow, mucus viscosity and the surface make-up of the oviduct. Molecular interactions (such as chemoattractants) include the communication between sperm surface molecules and receptors present on the epithelial cells of the oviduct and also between the sperm and the secretions of the oviduct as well as the recently ovulated oocyte. It is these molecular interactions that allow the sperm to pass through the UTJ (Suarez, 2016). Sperm that pass into the isthmus may bind to the epithelial cells creating sperm reservoirs a process which

prolongs the fertilising capacity of the sperm. This is especially important in species such as horses which are in oestrus for a number of days and the timing of ovulation may be asynchronous with the timing of breeding. The gradual release of sperm from these reservoirs means that at any one time only a small number of sperm progress to the oocyte ensuring a low sperm to oocyte ratio (Hunter, 2012, Suarez, 2008). It was shown in the pig that removing the area of the reservoir and reconnecting the remaining oviduct that sperm numbers were significantly increased at the site of fertilisation, and subsequently increased the rate of polyspermy. It has also been shown that the release from the oviductal epithelium is due to changes in the sperm and not the epithelial cells. Changes to the surface proteins of the sperm during capacitation are understood to reduce the binding affinity for epithelial receptors and therefore, aid in the detachment from the epithelial cells (Leemans et al., 2016). Capacitated hamster sperm did not bind to epithelium whereas uncapacitated sperm did bind, this also appeared to be the case for bull sperm where much lower numbers bound after the sperm had been capacitated. Stallion sperm that are bound maintain low levels of Ca^{2+} compared to free swimming sperm (Dobrinski et al., 1997). This suggests that binding impedes capacitation, reiterating the point that binding of sperm prolongs their viability and fertilising ability (Suarez, 2016). It is possible that the epithelia may indirectly aid this unbinding by secreting capacitating factors.

1.4.4 Sperm Interaction with the Oocyte and Fertilisation

After unbinding from the storage reservoirs in the isthmus, the sperm must make its way to the site of fertilisation in the ampulla. It has been suggested that chemotaxis plays a role in guiding the sperm to the oocyte as sperm orientated towards a gradient of follicular fluid containing unknown chemoattractants (Perez-Cerezales et al., 2015). In mare's the oocyte is released from the ovulation fossa of the dominant graafian follicle (>30mm diameter) at

ovulation with a surrounding network of granulosa cells called the cumulus oocyte complex (COC), it is also accompanied by the efflux of follicular fluid (Franciolli et al., 2011). Once sperm reach the COC they must navigate through the cumulus cells and bind to the zona pellucida. Following this they undergo the acrosome reaction (Figure 1.6.) to successfully penetrate the zona pellucida and to fertilise the oocyte. In order to penetrate the zona pellucida it is necessary that the sperm are hyperactivated, it has been shown in hamsters that sperm which were capacitated and acrosome reacted but unable to hyperactivate could not penetrate the zona pellucida. This was also the case with mice that were null mutants for CatSper and therefore could not hyperactivate (Stauss et al., 1995 and Quill et al., 2003). Kim and Kim (2013) characterised the zona pellucida as being viscoeleatic, emphasising the importance that sperm must be able to penetrate viscous secretions.

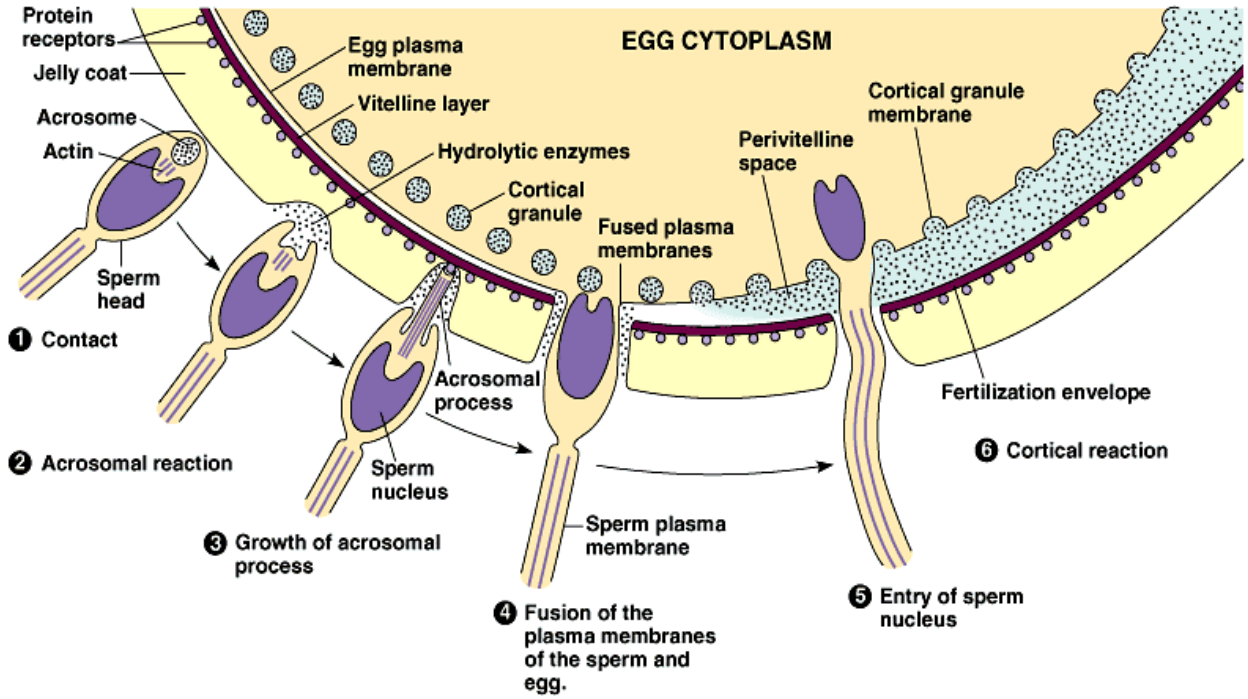


Figure 1.6 Sequential events occurring during the acrosome reaction (source: ID Biology, 2012)

1.5 Taxis Mechanisms for Sperm Guidance

Taxis refers to a number of sensing mechanisms used by sperm to guide them to the oocyte, these can be both physical (rheotaxis, thigmotaxis and thermotaxis) and chemical (chemotaxis). A possible sequence of events that lead to fertilisation is; Rheotaxis and thigmotaxis guide the sperm from the site of deposition in the uterus against the mucus flow created by muscular contractions and the beating of the epithelial cilia to the oviduct. Capacitated sperm are released from the sperm reservoir in the caudal isthmus and are then stimulated by thermotaxis towards the ampulla where they encounter chemoattractants secreted from the follicular fluid and or the COC (Perez-Cerezales et al., 2015).

1.5.1 Thermotaxis

Thermotaxis is the phenomenon whereby, the sperm swim up a temperature gradient as seen in humans and rabbits. The temperature difference between the sperm reservoir site in the isthmus and the warmer site of fertilisation in the ampulla is reported to be approx. +2°C (in rabbits, David et al., 1971), this appears to be significant enough to stimulate thermotactic behavior of the sperm. Thermotaxis has been reported to be mediated by calcium-permeable temperature-sensitive cation channels in human sperm (De Blas et al., 2009). However, Bahat et al., (2003) reported that only 3-5% of human sperm and 7-17% of rabbit sperm were able to sense this temperature difference *in vitro* and respond to it by thermotaxis. In addition, to this Miki and Clapham (2013b) suggested that thermotaxis is ovulation dependent in rabbits. This suggests that like chemotaxis, thermotaxis is a selective process, in that only a relatively small proportion of sperm respond and it is likely to be only effective in attracting sperm over short distances within the oviduct.

1.5.2 Chemotaxis

Chemotaxis is a phenomenon whereby sperm are attracted to and swim up a concentration gradient of chemoattractants (amino acids, peptides, lipids, sulfated steroids, Figure. 1.7) (Kaupp et al., 2008). Chemotaxis is a well established method by which marine invertebrate sperm are guided to the egg, and the detection of such chemoattractants has been shown to change the swimming direction of the sperm. In mammals these chemoattractants have been shown to be present in the follicular fluid (Cohen-Dayag et al., 1994, Gil et al., 2008) and secreted by the COC (Sun et al., 2005). Chemotaxis is triggered by the binding of chemoattractants to receptors present on the flagellum, this leads to a series of events that result in an influx of Ca^{2+}, this in turn causes the sperm to alter its swimming direction and orientate towards the source of chemoattractant (Alvarez et al., 2014). One such chemoattractant is progesterone, which has been shown by Perez-Cerezales et al., (2016) and Miki and Clapham (2013b) to be a physiological chemoattractant for mice and human sperm, respectively. Since only a fraction of capacitated human sperm (2-10 %) have been shown to be chemotactically responsive (Cohen-Dayag et al., 1994) it is likely that the main function of chemotaxis is the selective attraction of capacitated sperm within the ampulla of the mares reproductive tract (Cohen-Dayag et al., 1995, Eisenbach and Giojalas, 2006). Therefore, it could be considered a short range method for sperm guidance.

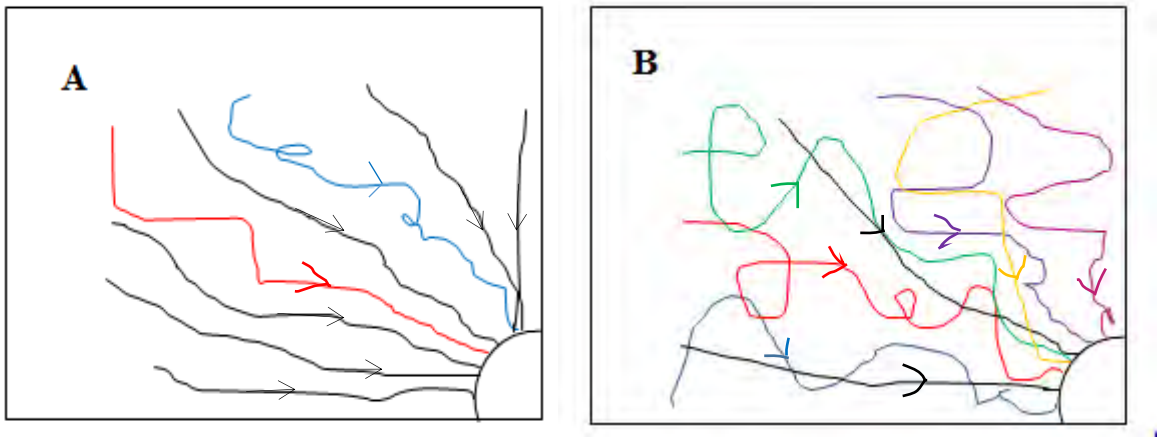


Figure 1.7 The swimming trajectories of human sperm in the presence of wells containing (A) Progesterone and (B) Follicular Fluid. The arrows indicate the direction of each trajectory, the sperm that exhibited directional changes towards the wells are coloured and those considered to swim straight are in black. The quarter circle in the lower right corner of each panel is part of the well (adapted from; Jaiswal et al., 1999).

1.5.3 Rheotaxis

rheotaxis is the ability of organisms to orientate and swim against a flow of fluid. It was proposed by Miki and Clapham (2013a) as a major determinant of sperm guidance over long distances in the mammalian female reproductive tract. The study observed it in both humans and mice and it also demonstrated rheotactic behaviour in both capacitated and uncapacitated sperm in low and high viscosity media. It is now well established that this flow is created by oviductal secretions, ciliary activity and smooth muscle contractions of the uterus. Coitus has also been shown to dramatically increase the secretion of oviductal fluid in mice (Miki and Clapham, 2013b), further, increasing the flow from the oviduct to the uterus. It has been suggested that the flow aids in clearance of the oviduct and reduces the viscosity of the reproduction secretions. Tung et al., (2014) studied the migration of bull sperm against fluid flow in a microfluidic device that recreated the biophysical environment of mammalian sperm with grooves embedded on a micro-channel surface. They reported that microgrooves allow sperm to swim faster and more efficiently in the

presence of the flow which suggests that the microgrooves present along the female reproductive tract have evolved, in part at least, to facilitate sperm swimming. Similarly, Kantsler et al., (2014b) reported that both human and bull sperm not only swim against the flow, but swim upstream in spiral-shaped trajectories along the walls of a micro-channel, and reported the ability of sperm to reverse their swimming direction upon flow reversal. El-Sherry et al., (2014) reported a rheotactic response in 80-84 % of bull sperm (except for very low flow velocities) and found shear stress, which is an indicator of velocity distribution, to play a critical role in regulating rheotactic behaviour of sperm. The velocity of the fluid flow within the reproductive tract of mares has not yet been quantified, however, the fluid flow in the oviduct of the mouse has been reported by Miki and Clapham (2013b) to be 18 ± 1.6 μm/s. In support of this, Tung et al., (2014) showed that bull sperm rheotaxis *in vitro* was stimulated by a flow of 15 μm/s.

Miki and Clapham (2013b) reported that without a flow sperm swam in uncoordinated random patterns. The study showed that 68% of mouse and 51% of human sperm (uncapacitated) exhibited rheotaxis. Interestingly, the same study showed that 82% of headless mouse sperm, which make up a tiny percentage (<0.1%), displayed rheotaxis. Indicating, the sperm head is not necessarily responsible for rheotactic behaviour. It did, however, suggest that CatSper channels are necessary for successful rheotaxis responses to take place. The same study demonstrated that sperm lacking CatSper subunits failed to create Ca^{2+} influx and, therefore, were unable to display rheotaxis. Finally, the study also showed the Ca^{2+} influx enhances sperm rotational swimming which is essential for rheotaxis.

1.5.4 Thigmotaxis

Thigmotaxis is the sperms apparent attraction to swimming near surfaces and it has been proposed that sperm prefer to swim along the epithelium of the uterus and oviducts where the mucus flow is slower and mucus is less viscous (Denissenko et al., 2012, Winet et al., 1984b). Sperm tend to follow microfluidic channel walls, and those moving along the wall move upstream faster than those swimming along the microfluidic channel centerline (El-Sherry et al., 2014). Sperm swimming along the walls tended to enter side pockets without any chemical binding. This tendency of motile cells to remain close to walls and swim along boundaries follows the principles of thigmotaxis (Denissenko et al., 2012). Miki and Clapham (2013b) reported that flow velocity is at its highest in the centre of the microfluidic channel and decreases considerably at the walls, which might in part explain why sperm have an affinity for swimming next to the wall. Furthermore, sperm swimming is influenced by the hydrodynamics of the fluid they are in (Alvarez et al., 2014). The same study reported that thigmotaxis occurs because the sperm flagellum is surrounded by a backward flow, and the sperm head is surrounded by a forward flow. The displaced fluid must be replaced by a flow from the side, when sperm are near a wall it hinders the fluid flow from that side and the fluid is mostly replaced from the side farthest from the wall, this in turn pushes the sperm towards the wall. In addition to this, the study showed that when sperm swim parallel to a wall they drift towards the wall with a set velocity dependent on the sperm size, strength of the flagellar beating and distance from the wall.

1.6 Research Objectives

It is now apparent that both rheotaxis and thigmotaxis are important long distance sperm transport mechanisms, however, there have been no published studies describing these in stallion sperm nor is there any evidence as to how they are affected by mucus viscosity.

The objectives of this research were to:

1. Establish if stallion sperm exhibit rheotaxis and thigmotaxis
2. Determine if there is an influence of flow velocity on stallion sperm rheotaxis
3. Assess the effect of viscosity on hyperactivated and non-hyperactivated stallion sperm kinematic parameters, stallion sperm rheotaxis and the thigmotactic behaviour of stallion sperm and their distribution across a microfluidic channel
4. Evaluate the effect of hyperactivation on stallion sperm rheotaxis and compare this to the effect of hyperactivation on both human and ram sperm rheotaxis.

Chapter 2

Material and Methods

2.1 Reagents

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

2.2 Microfluidic Channel

A microfluidic channel (Figure 2.1) manufactured from polymethyl methacrylate (PMMA), was purchased from the Microfluidic ChipShop (Jena, Germany). The microfluidic channel measured 800 μm wide, 20 μm deep and 58.5 mm in length. It was connected to a syringe pump (Figure 2.2; Standard Infuse/Withdraw PHD ULTRA™ Syringe Pump, Harvard Apparatus, Holliston, USA) by silicone tubing and a connector (Male Mini Luer fluid connector, Microfluidic ChipShop, Jena, Germany). The microfluidic channel was primed with media to remove air bubbles, following this, the flow was established at controlled rates of 0 – 100 μm/s. The sperm sample was loaded into the starting inlet of the microfluidic channel allowing sperm to orient against the oncoming flow and swim against it. The number of sperm that passed 15mm after 10 minutes was used as the criteria for the assessment of sperm rheotaxis. The 15mm point was measured using a Vernier calipers and sperm progression was assessed under X 200 magnification on an inverted microscope (CK40; Olympus, Centre Valley, PA, USA). The microfluidic channel was cleaned with dH_2O after use, and air was then pumped through to remove any excess fluid remaining in the channel.

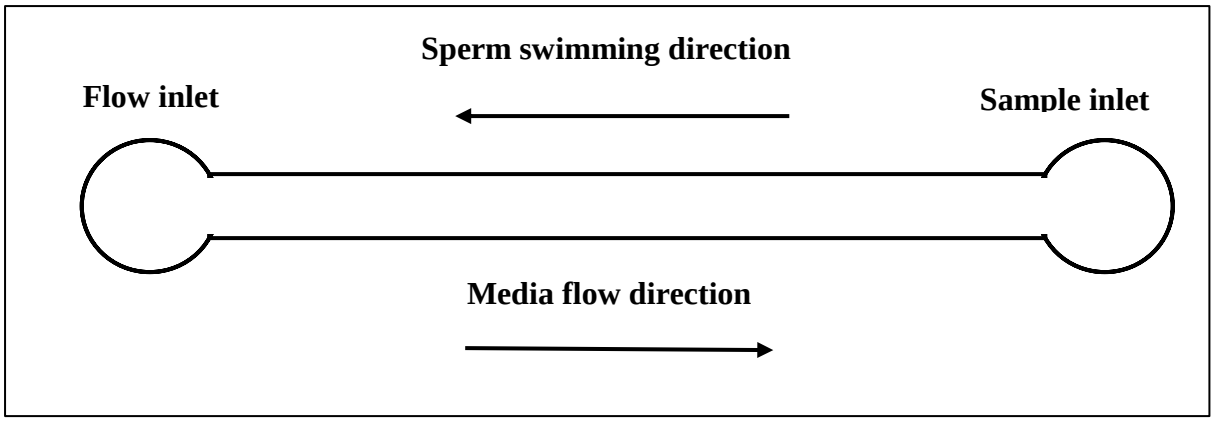


Figure 2.1 Schematic of the microfluidic channel**;** Depth 20 μm; Length 58.5 mm; Width 800 μm

2.3 Sample Preparation

Frozen-thawed sperm was donated from a commercial stud and thawed sperm from 2 ISH stallions was pooled to reduce inter-stallion variation in all experiments. Straws were thawed in a water bath at 37°C for 30 seconds. Post-thaw sperm motility of the pooled sample was assessed subjectively using a phase-contrast microscope (BX60; Olympus, Centre Valley, PA, USA) as a quality control check and only samples with greater than 30% total motility were used for experiments. Preliminary work demonstrated that a concentration of 20 x 10^6 sperm/mL was the optimum for use in the microfluidic channel (results not shown) and therefore this was used throughout all experiments. Sperm were diluted to this concentration using tyrosine, albumin, lactate and pyruvate (TALP, Lasko et al., 2012) media and a 10 μL sample was placed in the sample inlet. Media viscosity in all experiments was 0.9 cP and flow velocity was 20 μm/s, except the experiments in which different viscosities or flow velocities were assessed. The viscosity of TALP was assessed using a viscometer (Brookfield, DV2T, Lab Unlimited, Dublin, Ireland) and was determined to be 0.9 cP. Methyl cellulose (4000 cP) was added to TALP, at 0.1, 0.15 and 0.2% (w/v) in order to increase the viscosity of the media to 2.5, 4 and 6 cP respectively. All viscosities were confirmed using the viscometer.

2.4 Computer Assisted Sperm Analysis

Computer assisted sperm analysis (CASA) was used to assess motility and kinematic parameters. A 5 µL drop of diluted semen was placed in a pre-warmed leja chamber (10 µm in depth; IMV Technologies, LAigle, France) and analysed for sperm motion characteristics using factory CASA (stallion) settings. A minimum of five randomly selected microscopic fields with at least 200 sperm were analysed in each sample using a phase contrast microscope (BX60; Olympus, Centre Valley, PA, USA) with a fitted heated stage at 37°C. A manual correction was carried out to add or delete sperm or debris as required. The CASA derived motility and kinematic parameters assessed were motility (Mean velocity (VAP) greater than 10 µm/s), progressive motility (sperm which display a forward progressive linear movement), straight line velocity (VSL; µm/s; the time averaged velocity of a sperm along a straight line from its first position to its last position, average path velocity (VAP; µm/s; the time averaged velocity of a sperm along its average path), curvilinear velocity (VCL; µm/s; the time averaged velocity of the sperm along its actual path, amplitude of the lateral head displacement (ALH; µm; the width of the lateral movement of the sperm head about its average path, linearity (LIN; %; VSL/VCL x 100), straightness (STR; %; VSL/VAP x 100), and beat cross frequency (BCF; Hz; the number of times the sperm head crosses the direction of movement per second or the average rate at which the curvilinear path crosses the average path).

2.5 Experiment 1: Effects of flow velocity on rheotaxis

The aim of this experiment was to determine if stallion sperm exhibited rheotaxis and if so establish the optimum flow velocity for maximum rheotaxis. The sperm rheotactic response was assessed in seven different velocities, namely 0, 10, 20, 30, 50, 70 and 100 μm/s, as described above. Four replicates were completed.

2.6 Experiment 2: Effect of media viscosity on sperm swimming patterns, rheotaxis and thigmotaxis

This experiment tested the hypothesis that stallion sperm swimming patterns and rheotaxis response is affected by fluid viscosity, and that this is different in hyperactivated and non-hyperactivated stallion sperm. The first objective was to determine the effect of media viscosity on both hyperactivated and non-hyperactivated stallion sperm swimming patterns in a static droplet. Frozen-thawed stallion sperm were diluted to a concentration of 20 x 10^6 sperm/mL in different viscosities (0.9, 2.5, 4, 6 cP) and assessed for motility and kinematic parameters in a static environment within a pre-warmed leja chamber (10 μm in depth; IMV Technologies, LAigle, France), using CASA. Commonly used CASA derived hyperactivation parameters were used to classify sperm as being hyperactivated or not. These included an ALH > 7.0 μm/s, LIN<30% and a VCL >70 μm/s (Hinrichs and Loux, 2012, Olson et al., 2011). Stallion sperm were hyperactivated using 5mM procaine a known stallion sperm hyperactivation agonist (Hinrichs and Loux 2012). Five replicates were completed.

The second objective was to assess stallion sperm progression within the microfluidic channel by creating a flow with media of different viscosities, and to determine if the viscosity affected the optimum flow velocity for maximum sperm rheotaxis. The sperm rheotactic response was assessed in seven different velocities, namely 0, 10, 20, 30, 50, 70 and 100 μm/s, each in media of four different viscosities (0.9, 2.5, 4 and 6 cP) as described previously. Three replicates were completed.

The third objective was to assess the effect of viscosity on the stallion sperm distribution across the microfluidic channel in order to quantify sperm wall tracking behaviour (thigmotaxis). Sperm that were swimming touching the walls of the microfluidic channel were considered as sperm at the wall and the remainder of sperm that were not in contact with the wall were considered as sperm in the centre of the microfluidic channel. Three replicates were completed.

2.7 Experiment 3: Effect of hyperactivation on rheotaxis

The objective of this experiment was to assess if hyperactivated stallion sperm had a differential rheotactic response to non-hyperactivated sperm. To assess the effect of hyperactivation, stallion sperm were incubated either in the presence of 5mM procaine a hyperactivation agonist or not (control) for 10 min prior to loading the sample in the microfluidic channel. Following this, stallion sperm rheotactic response for both hyperactivated and non-hyperactivated samples was assessed when exposed to a flow velocity of 20 μm/s. In addition to this, the samples were also pre-loaded into the microfluidic channel and further assessed for a rheotactic response. Three replicates were completed.

Based on the results of this experiment, a further objective was to investigate if hyperactivated sperm from other species (human and ram) behaved in a similar way to hyperactivated stallion sperm. Based on the scientific literature, progesterone (10μM) (Sagare-Patil et al., 2012) and caffeine (10mM) (Colas et al., 2010) were used to hyperactivate human and ram sperm respectively. Subsequently, the rheotactic response of both human and ram sperm hyperactivated and non hyperactivated was assessed. Dose responses for all hyperactivation agonists (procaine, progesterone and caffeine) were carried out to confirm the ability of each agonist to induce hyperactivation. Three replicates were

carried out (results not shown). Finally, the swimming trajectories of hyperactivated stallion, human and ram sperm were assessed using CASA.

2.8 Statistical Analysis

Data were analysed using Statistical Package for the Social Sciences (SPSS) (version 22, IBM, Chicago, IL). Data were examined for normality of distribution using the Shapiro-Wilk test, transformed where appropriate and analysed using a univariate analysis of variance (ANOVA). Data from experiment 2b and 2c were transformed using a square root transformation. The transformed data were used to calculate the P values; however, the corresponding means and standard error of the non-transformed data are presented in the results. Post Hoc tests were conducted using the Bonferroni test and results were reported as the mean ± standard error of margin (s.e.m). $P<0.05$ was considered statistically significant.

Chapter 3

Results

3.1 Experiment 1: Effects of flow velocity on rheotaxis

After loading of the sample, the sperm had to find the microfluidic channel inlet and swim into it. During this initial period, the swimming pattern was uncoordinated, but once in the microfluidic channel, sperm quickly detected the flow and orientated against it (S1 Movie). When no flow was introduced at the inlet, sperm continued to swim in an uncoordinated pattern. There was an effect of flow velocity on sperm progression ($P < 0.05$; Figure 3.1) as stallion sperm required a mean flow velocity of at least 10 µm/s to initiate rheotaxis. The optimum flow velocity was between 10-30 µm/s ($P<0.05$) and peaked at 20 µm/s, and for this reason a velocity of 20 µm/s was chosen for all subsequent experiments. Sperm progression decreased when sperm were exposed to a flow of 50 µm/s or greater ($P<0.05$) as sperm found it more difficult to swim against a faster flow.

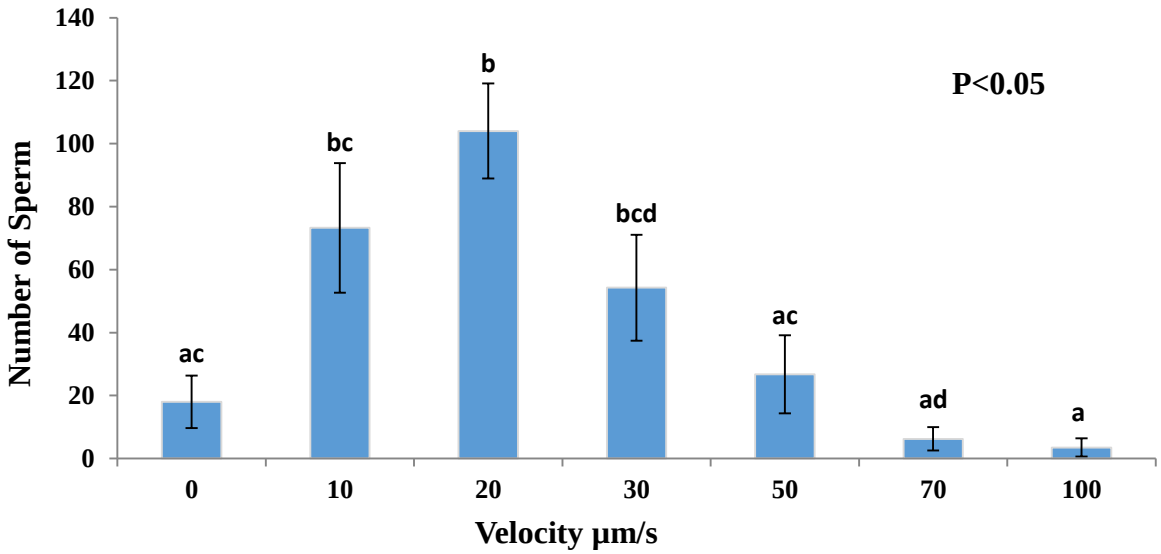


Figure 3.1 Stallion sperm progression in a microfluidic-channel at varying flow velocities in tyrosine, albumin, lactate and pyruvate (TALP) media (0.9cP). Vertical bars represent s.e.m. (n=4 replicates). ^{abcd} Differing superscripts differ significantly ($P<0.05$)

3.2 Experiment 2: Effect of media viscosity on sperm swimming patterns, rheotaxis and thigmotaxis

Stallion sperm swimming pattern in a static drop was not affected by viscosity in either the hyperactivated or non hyperactivated (control) treatment ($P>0.05$). However, when the two groups were compared there was a noticeable difference in the motility parameters between the hyperactivated and non hyperactivated treatment (Figure 3.2). Hyperactivated stallion sperm had an increased VCL (>90) and ALH (>4) and a decreased LIN (<20%) in all viscosities (0.9, 2.5, 4 and 6 cP). The results for both the VCL and LIN are consistent with hyperactivation parameters previously described in the literature (Hinrichs and Loux, 2012, Olson et al., 2011).

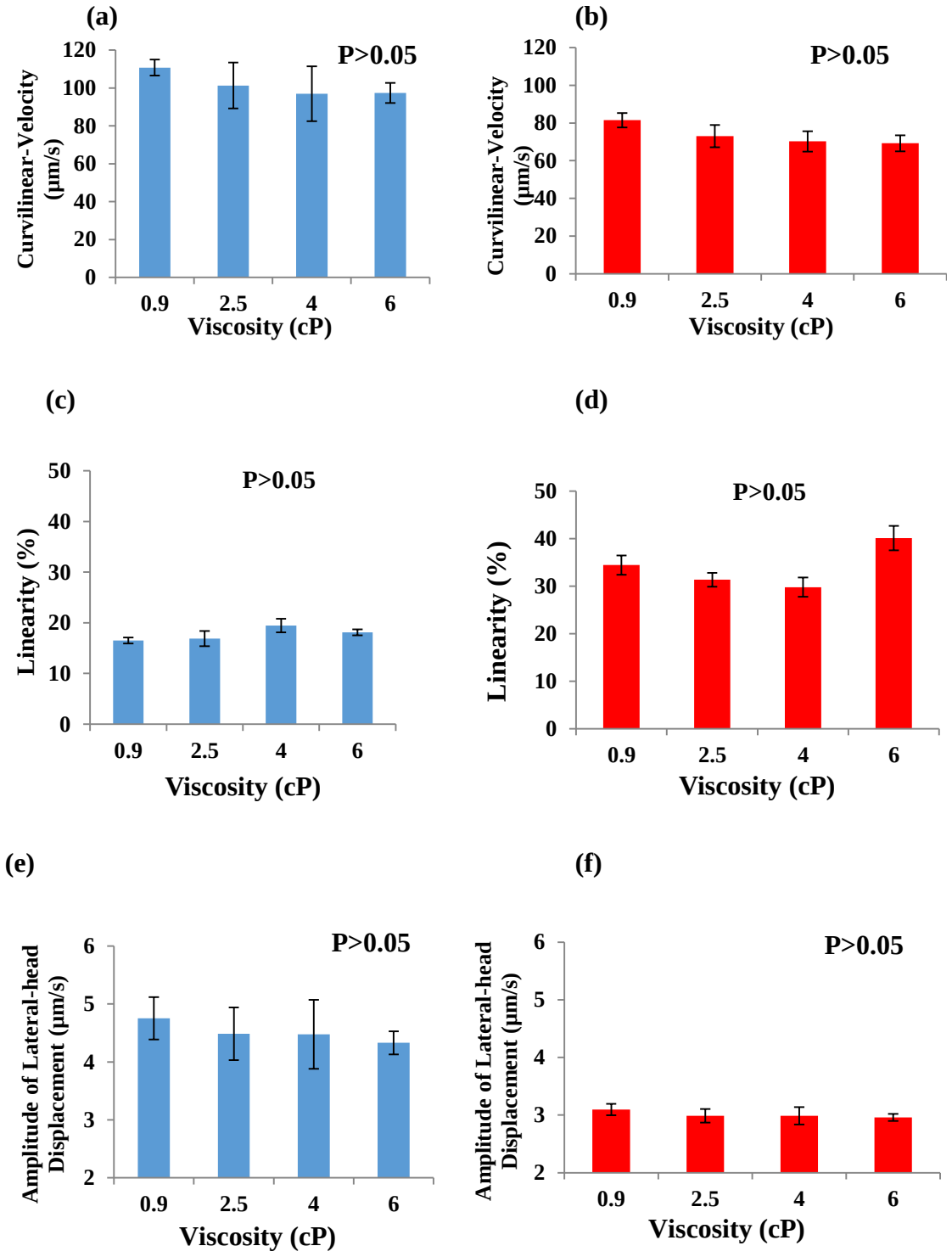


Figure 3.2 Motility parameters for hyperactivated (a, c and e) and non hyperactivated (b, d and f) sperm in a static chamber. Vertical bars represent s.e.m. (n=5 replicates).

Although the swimming pattern was not affected by the range of viscosities assessed in a static droplet, sperm progression in the microfluidic channel was affected by the change in media viscosity (P<0.05; Figure 3.3). Once exposed to a flow in the microfluidic channel sperm orientated and swam against the flow, their rate of progression was affected by the flow velocity and viscosity but there was no interaction. The optimum flow velocity for sperm progression remained the same as experiment 1 (20µm/s) for the range of viscosity tested (0.9 to 6 cP)**.** However, viscosity affected progression with the two highest viscosities (4 and 6 cP) resulting in the lowest number of sperm exhibiting a rheotactic response and the biggest decrease in rheotaxis occurred between 0.9-2.5 cP.

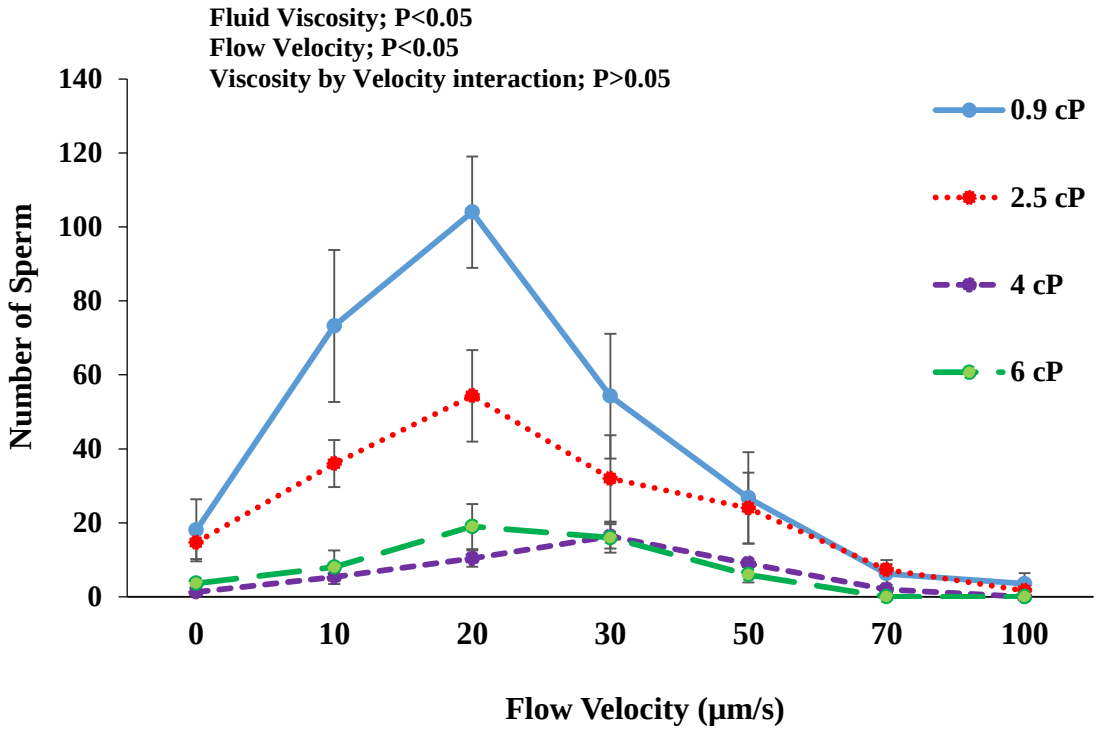


Figure 3.3 Number of sperm progressing passed 15mm in a microfluidic channel after 10 mins against differing media viscosities at varying flow velocities (0-100µm/s). Vertical bars represent s.e.m. (n=3 replicates)

Sperm thigmotactic behaviour was evident, with a higher percentage of sperm swimming next to the walls than in the microfluidic channel centre ($P < 0.05$; Figures 3.4; S1 Movie). There was no effect of media viscosity (0.9, 2.5, 4 and 6 cP) ($P>0.05$; Figure 3.4) or flow velocity (0-100 µm/s) ($P>0.05$; results not shown) on the percentage of sperm displaying wall tracking behaviour (thigmotaxis), within the microfluidic channel.

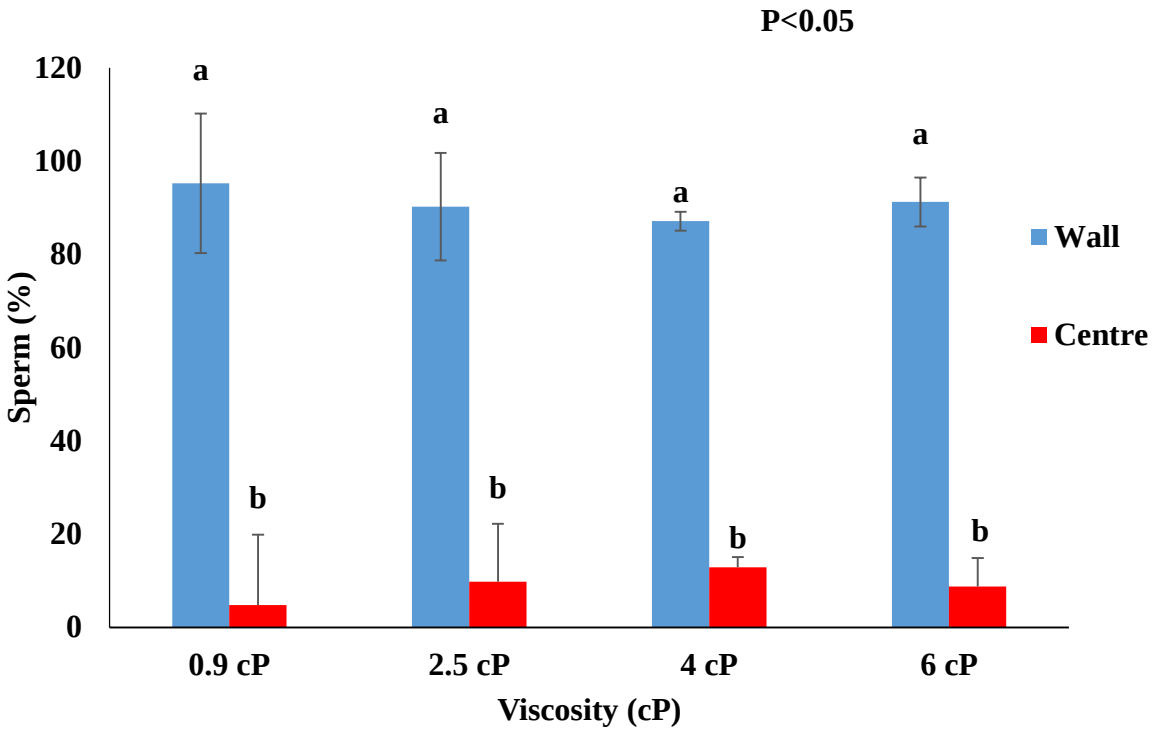


Figure 3.4 The percentage of sperm swimming at the microfluidic channel wall or in the centre position at 15mm in a range of viscosities (0.9-6 cP). Vertical bar represent the s.e.m. (n=3 replicates) ^{ab} differing subscripts differ significantly within viscosity ($P<0.05$)

3.3 Experiment 3: Effects of hyperactivation on rheotaxis

The number of stallion sperm that progressed to 15mm after 10 minutes in the microfluidic channel, in both hyperactivated and non-hyperactivated samples was assessed (Figure 3.8). Interestingly, when exposed to a fluid flow, stallion sperm which had been incubated in the presence of 5mM procaine, had reduced progression compared to the control treatment which had high levels of progression ($P < 0.05$; Figure 3.8). This result was confirmed by the observation of stallion sperm samples that were pre-loaded into the microfluidic channel, it was obvious from these that the hyperactivated stallion sperm did not exhibit rheotaxis (S4 Movie). Following this, the number of human and ram sperm that progressed to 15mm after 10 minutes in the microfluidic channel, in both hyperactivated and non-hyperactivated samples was also assessed (Figure 3.8). However, in both these species hyperactivated sperm exhibited rheotaxis ($P>0.05$), showing an evident difference between the species. Incubation of stallion, human and ram sperm with the hyperactivation agonists, procaine, progesterone and caffeine respectively increased the percentage of hyperactivated sperm compared to the control treatment ($P < 0.05$; Figure 3.7; S2 and S3 Movies, respectively), as characterised by high-amplitude flagellar waves and asymmetrical flagellar beating and assessed in a static non viscous droplet. There was a distinct difference in hyperactivated swimming patterns of the three species (Figure 3.9).

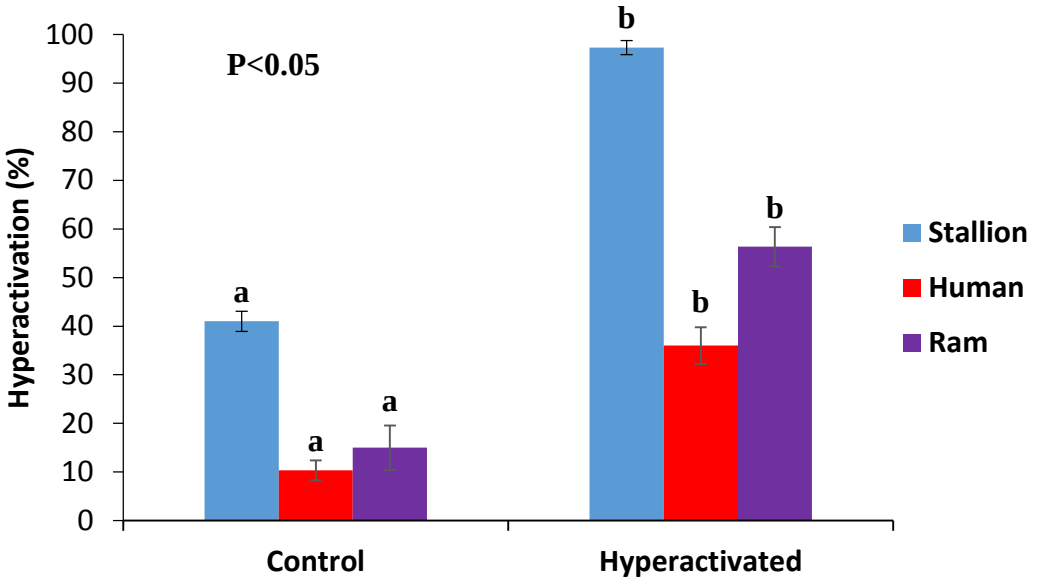


Figure 3.7. The percentage of hyperactivated sperm compared to the control treatment following incubation with the hyperactivation agonists, procaine (stallion), progesterone (human) and caffeine (ram) as characterised by high-amplitude flagellar waves and asymmetrical flagellar beating and assessed manually in a static saline droplet. Vertical bars represent the s.e.m. (n=3 replicates). ^{abcd} Differing superscripts differ significantly within control or hyperactivated groups.

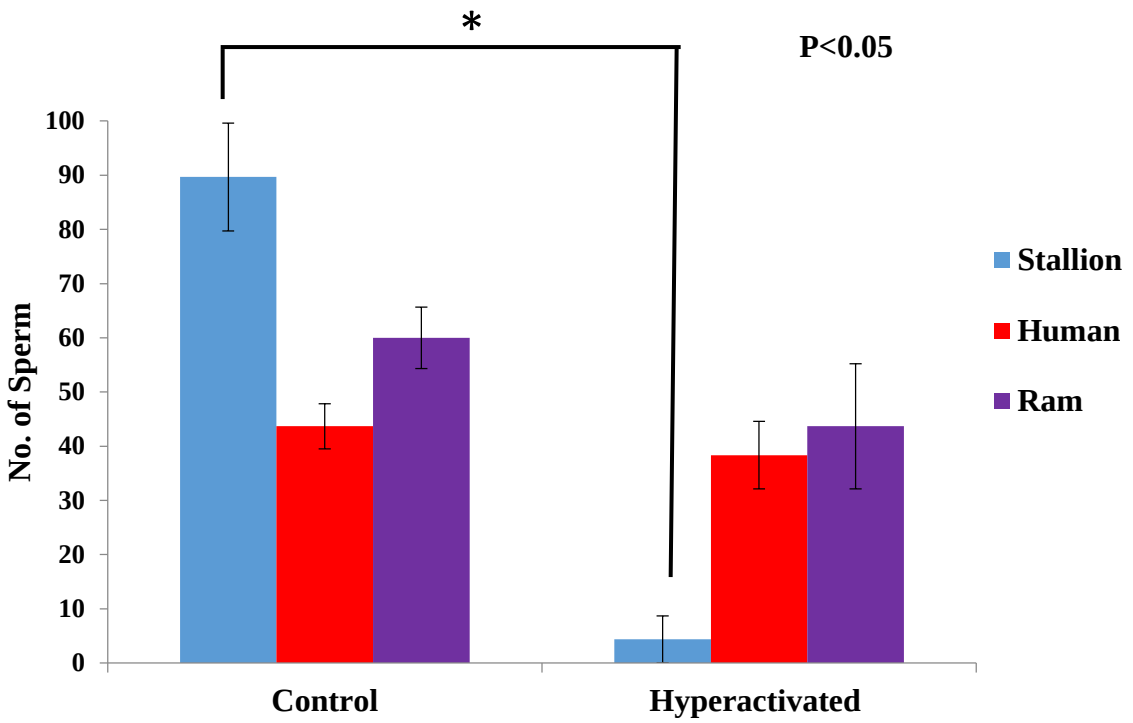
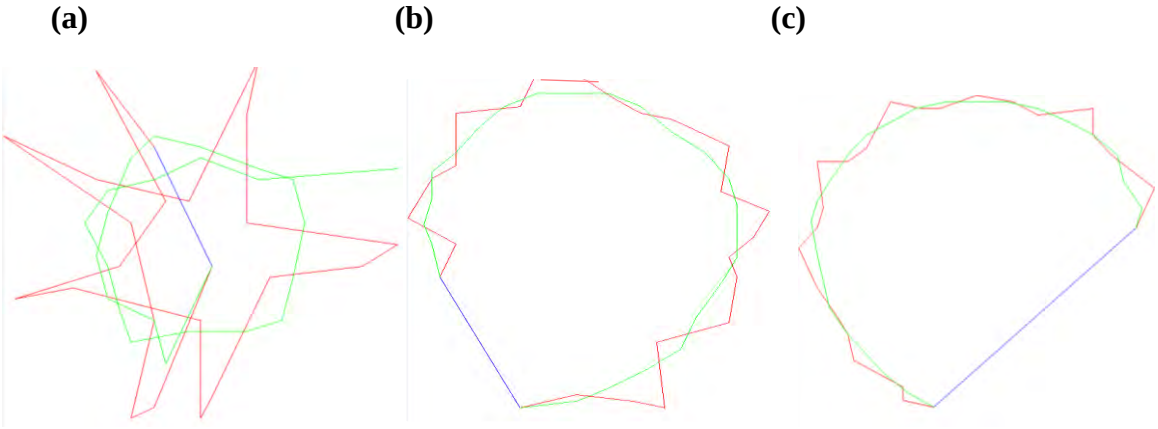


Figure 3.8 The number of stallion, human and ram sperm progressing passed 15mm in a microfluidic channel after 10 mins against a flow (20 µm/sec) following treatment with hyperactivation agonists or not (Control). Vertical bars represent s.e.m. (n=3 replicates). * represents statistical significance.



VCL-Curvilinear Velocity, **VAP**-Average Path Velocity, **VSL**-Straight line Velocity

Figure 3.9 Computer assisted sperm analysis showing the swimming patterns of hyperactivated stallion (a) human (b) and ram (c) sperm**.** The different coloured lines represent the curvilinear velocity (VCL, red), average path velocity (VAP, green) and the straight line velocity (VSL, blue)

Chapter 4

Discussion

To navigate their way towards the site of fertilisation in the oviducts, sperm orientate and swim against a flow of mucus, a phenomena known as rheotaxis (Miki and Clapham, 2013a, Perez-Cerezales et al., 2015) and while doing so, are guided by the sensation of touch along the epithelial lining of the reproductive tract (thigmotaxis) (Denissenko et al., 2012, Winet et al., 1984b). Both rheotaxis and thigmotaxis have been shown in a number of species, with evidence suggesting that rheotaxis in particular, is a major factor in long distance sperm guidance (Miki and Clapham, 2013a). However, the role of these responses in guiding stallion sperm towards the site of fertilisation and the effect of viscosity and flow velocity on them has not yet been investigated or characterised in stallion sperm.

This study offers a novel insight into stallion sperm rheotaxis and how it is influenced by viscosity and associated species differences in the ability of hyperactivated sperm to exhibit rheotaxis. It demonstrated (i) that stallion sperm respond to a fluid flow and also that their rheotactic response is affected by fluid viscosity (ii) it clearly illustrates for the first time that hyperactivated stallion sperm do not exhibit rheotaxis and that their swimming pattern against a fluid flow is uncoordinated and non-progressive, similar to the erratic figure of eight pattern traditionally observed in a static environment. In contrast, to this the study showed (iii) that both hyperactivated human and ram sperm do indeed display positive rheotaxis, however, hyperactivated motility did not affect their rate of progression as it does in bull sperm (Johnson et al., 2017).

This study, has shown that stallion sperm actively sense and adapt to fluid flow similar to human (Miki and Clapham, 2013a) and bull (Johnson et al., 2017) and is in agreement with reported differences in the percentage of sperm exhibiting rheotaxis at different flow velocities in a microfluidic channel (El-Sherry et al., 2014). The only known published data on the rate of fluid flow *in vivo* is that by Miki and Clapham (2013a) who found the

velocity of fluid flow within the oviduct of the mouse to be approximately 18 μm/s. This is similar to the current study which found the optimum flow velocity for maximum stallion sperm rheotaxis to be 20 μm/s. In addition to fluid flow sperm must also overcome mucus of varying viscosity en route to the site of fertilisation, and Coy et al., (2009) proposed that the viscosity of the reproductive secretions reduced the sperm to oocyte ratio thereby reducing polyspermy. The changes in oviductal fluid composition, under hormonal influences provide the optimum conditions for sperm transport, fertilisation and early embryo development (Špaleková et al., 2011), with oviductal fluid viscosity in the peri-ovulatory period estimated to be as low as 2-3 cP (Miki and Clapham (2013b). The results of the current study demonstrate that sperm progression was affected by the media viscosity within the range 0.9-6 cP. It was not possible to pump higher viscosities through the microfluidic channel, and although the viscosities were relatively low they were sufficient to demonstrate that the viscosity of the reproductive secretions does indeed have an effect on sperm rheotaxis**.** This also supports the hypothesis that fluctuations in the oviductal fluid viscosity during the oestrus cycle can contribute to reducing the number of sperm at the site of fertilisation (Coy et al., 2009).

A study by Kantsler et al., (2014a) reported changes in the flagellar beat pattern of human sperm in a static droplet, from a strong helical beat component in a low viscosity fluid (3 cP) to a more planar wave forms in a high viscosity fluid (20 cP). The same study reported differences in the beat component between human and bull sperm, which was explained by the differences in head shape, since bull sperm have a flatter head than human sperm. Therefore, the study suggested sperm alter their rheotactic swimming patterns but not their rate of progression in viscous fluid. This is in disagreement with the findings of this study, where there was a significant reduction in sperm progression with increasing viscosity.

Hyperactivation is a key step in the capacitation process and has long been proposed as a process by which sperm detach from the epithelial lining of the sperm reservoir in the isthmus, and as a means to penetrate the oocyte cumulus complex and zona pellucida (Ho et al., 2009, Simons et al., 2014). In support of this Ho et al., (2009) showed that mice sperm unable to hyperactivate were subsequently incapable of unbinding from the oviductal epithelium. Interestingly, this study found that not only were hyperactivated stallion sperm unable to progress within a microfluidic channel, they were also not affected by either flow rate or viscosity. This is in conflict with research by Hinrichs and Loux (2012) who stated that when exposed to a flow and viscous fluid hyperactivated sperm swim in a more linear progressive trajectory. This particular theory is also shared by Miki and Clapham (2013a) who proposed that as viscosity increases the swimming pattern of mouse sperm becomes more direct. In addition to this, Perez-Cerezales et al., (2015) reported that rheotaxis is dependant on hyperactivation. This is in contrast to the findings of this current study which showed that hyperactivation clearly inhibited stallion sperm rheotaxis. In contrast, to this human and ram sperm did display rheotaxis when hyperactivated, similar to results observed with bull sperm as described by Kantsler et al., (2014a) and Johnson et al., (2017). There was no difference observed between the hyperactivated and non hyperactivated treatments unlike the findings of Johnson et al., (2017) who reported greater progression of hyperactivated bull sperm. As all three species sperm displayed rheotaxis when not hyperactived, it suggests that stallion sperm hyperactivation is somehow different in function to the other species examined. It has also been reported that rheotaxis, thermotaxis and chemotaxis are only effective on capacitated sperm (human and mouse) (Perez-Cerezales et al., 2015), however, this was not the case in this current study, which showed that both human and stallion sperm displayed rheotaxis irrespective of being

capacitated. To rule out the possibility that the hyperactivated stallion were unable to find their way into the microfluidic channel due to the erratic swimming pattern, sperm were carefully pre-loaded into the microfluidic channel where they were exposed to a flow (S4 Movie). The same result was observed, whereby, the hyperactivated stallion sperm were non progressive, unable to display rheotaxis and were swept downstream with the flow.

The inability of hyperactivated stallion sperm to exhibit rheotaxis when exposed to a flow, or when preloaded into the microfluidic channel illustrates clear species difference in rheotaxis. This could possibly, be explained by the unique star shaped swimming pattern of the stallion sperm when hyeractivated, in comparison to the figure of eight swimming pattern of the human and ram sperm (Figure 3.9). The swimming pattern remained the same irrespective of whether the stallion sperm were hyperactivated with procaine or by using a more physiological strategy of increasing the extracellular pH. Commonly used motility parameters that define hyperactivation include but are not limited to, an increase in curvilinear velocity and amplitude of lateral head displacement and a decrease in linearity (Hinrichs and Loux, 2012). These changes were observed in the hyperactivated stallion sperm samples in this study and further support the finding that the hyperactived motility induced with procaine is representitve of physiological hyperactivation. It is likely that the variation in the swimming patterns is due to the different head shapes of the three species. This illustrates that, when viewed within the confines of a static low viscous saline fluid, hyperactivated stallion sperm have a vigorous, erratic and non-progressive movement pattern (Suarez et al., 1991), and this was not affected by exposure to a flow during rheotaxis within a microfluidic channel. However, hyperactivated human and ram sperm appear to develop a more progressive swimming pattern when exposed to a flow. This is also supported by previous work by our group in the bull (Johnson et al., 2017). These

species differences are particularly interesting as they raise questions over the function of hyperactivated motility in stallion sperm. In particular, the previous studies which suggest that the purpose of hyperactivated motility is to aid the sperm in detaching from the reservoirs and penetration of the viscous oviductal mucus (Ho et al., 2009, Simons et al., 2014). The non-progressive swimming pattern of the hyperactivated stallion sperm when exposed to a flow mean it is unlikely that the sperm would be able to swim to the site of fertilisation in the ampulla. One possible explanation is a model proposed by Armon and Eisenbach (2011), who suggested that sperm behavior was affected by a chemoattractant gradient and that capacitated sperm maintain their course of swimming, specifically, they swim straighter and repress hyperactivated motility (turn less) when swimming up a concentration gradient. The study suggested that in the presence of a chemoattractant sperm are continuously stimulated and this results in the sperm swimming straight without turns or hyperactivation events and a decrease in the concentration gradient increases the frequency of these hyperactivation events. Furthermore, it suggested that the function of hyperactivation is to re-orientate the sperm towards the concentration gradient through sharp turning. It is possible that chemotaxis and not rheotaxis would influence the hyperactivated stallion sperm to swim straighter and therefore, guide the sperm to the oocyte. Chemotaxis is thought to be a short range guidance mechanism occurring within in the order of millimeters within the oviduct, however, Bian et al., (2012) and Armon and Eisenbach (2011) reported that in mice a gradient of natriuretic peptide precursor A (NPPA; a known chemoattractant *in vitro*) was found as far as the utero tubule junction, raising the possibility that chemotaxis has a longer range of sperm guidance than previously believed. Therefore, we propose that rheotaxis is central to directing sperm to the oviducts in all mammalian species but there are species-specific mechanisms guiding hyperactivated

sperm towards the oocyte. In horses this is not rheotaxis, assuming of course that hyperactivation does actually occur *in vivo* in horses.

Sperm thigmotactic behaviour was evident in the non-hyperactivated stallion sperm, and in both hypercativated and non hyperactivated human and ram sperm. This suggests that non-hyperactivated stallion sperm and both hyperactivated and non-hyperactivated human and ram sperm have a preference to move against a flow (Tung et al., 2014) and along surfaces (Kantsler et al., 2013, Denissenko et al., 2012). This mimicks the privileged pathways of the cervix and uterus, perhaps in search of the epithelial lining of the utero-tubular junction and isthmus where they can bind and form a sperm reservoir. Indeed non-capacitated sperm have a greater affinity to bind to the oviductal epithelium but as sperm complete capacitation (Hinrichs, 2012), and hyperactive, this aids in them pulling away from the epithelium. Current methods of sperm analysis in media in which the conditions such as viscosity and temperature remain constant are useful, however, they fail to take into account the complex and varied conditions encountered by the sperm along the female reproductive tract (Hunter et al., 2011). This was also the sentiment of Kirkman-Brown and Smith (2011) who cautioned against making conclusions about sperm motility based on single factors without taking into consideration all the other elements that may be at play.

4.2 Conclusions

To the best of the author's knowledge, this is the first study to characterise stallion sperm rheotaxis. The results of this study clearly demonstrate that stallion sperm rheotaxis response varies with flow velocity and is reduced by increasing fluid viscosity. It also established that hyperactivated stallion sperm swim in an uncoordinated star shaped pattern and they are not rheotactically responsive when exposed to a flow or viscous media, unlike human and ram sperm. Thigmotaxis was confirmed by a clear preference of sperm to swim against the flow next to the walls of the microfluidic channel. In conclusion, the study of sperm in microfluidic channels is a useful tool to increase our understanding of how sperm behave in the female reproductive tract and facilitates the evaluation of sperm rheotactic and thigmotactic behaviour in response to varying physiological conditions

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Appendices

Appendix A.

Constituents of TALP media

Table 5.1 Composition of TALP media per 500mL

Reagent	Molecular Weight (g/mol)	Mass Required (g)	Final Concentration (Mm)
NaCl	58.44	2.922	100
KCl	74.55	0.115	3.1
$NaHCO_3$	84.01	1.05	25
NaH_2PO_4	119.98	0.018	0.3
Sodium Lactate	112.06	1.21	21.6
$CaCl_2$	110.98	0.165	3
$MgCl_2$	95.12	0.04	0.4
HEPES	238.3	1.19	10
Sodium Pyruvate	110	0.055	1
Glucose	180.16	0.45	5

Appendix B.

Preparation of Reagents

Procaine

Procaine hydrochloride (P9879 Sigma-Aldrich) with a molecular weight of 272.77 g/mol was reconstituted in dH_2O to make a stock concentration of 50 mM. This was further diluted in a final volume in order to get the desired concentration.

Caffeine

Caffeine (C0750 Sigma-Aldrich) with a molecular weight of 194.19 g/mol was reconstituted in dH_2O to make a stock concentration of 50 mM. This was further diluted in a final volume in order to get the desired concentration.

Progesterone

Progesterone (P0130 Sigma-Aldrich) with a molecular weight of 314.46 g/mol was reconstituted in dH_2O to make a stock concentration of 50 mM. This was further diluted in a final volume in order to get the desired concentration.

D-Penicillamine

D Penicillamine (P4875 Sigma-Aldrich) with a molecular weight of 149.21 g/mol was reconstituted in dH_2O to make a stock concentration of 50 mM. This was further diluted in a final volume in order to get the desired concentration.

Methylcellulose

Methylcellulose 4000 cP (M0512 Sigma Aldrich) was added to TALP to alter the viscosity. This was done by heating about 1/3 of the required volume of TALP to at least 80 °C on a heated magnetic block. The methylcellulose powder was added with agitation created with a magnetic stirrer, the solution was mixed until the particles are thoroughly wetted and evenly dispersed. The remainder of the media is then added cold or as ice to lower the temperature of the dispersion. It was then frozen at -18°C, this allows for complete solubilisation. Once the dispersion reaches the temperature, at which it becomes water-soluble, the powder begins to hydrate and the viscosity increases. Once thawed it was agitated for a further 30 minutes.

Supplementary information

S1 Movie: Non-hyperactivated stallion sperm in a microchannel exhibiting rheotaxis. Sperm detect the flow and swim against it during rheotaxis.

S2 Movie: Non-hyperactivated sperm on a slide. This video was recorded as a control in order to compare the movement pattern of hyperactivated and non-hyperactivated sperm.

S3 Movie: Hyperactivated stallion sperm in a static droplet. After incubation with procaine 5 mM sperm showed high-amplitude flagellar waves and asymmetrical flagellar beating when assessed in a static droplet.

S4 Movie: Hyperactivated stallion sperm in a microchannel. When hyperactivated, stallion sperm were non-progressive and unable to exhibit rheotaxis.