Characterisation of the immunomodulatory properties of spermatozoa from high and low field fertility bulls

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Characterisation of the immunomodulatory properties of spermatozoa from high and low field fertility bulls

The thesis is submitted to the University of Limerick in fulfilment of the academic requirements for the degree of Doctor of Philosophy (PhD) by

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Under the supervision of:

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Abstract

Despite passing all microscopy based quality control (QC) assessments at artificial insemination (AI) centres, bulls with apparently normal semen quality yield unacceptably low pregnancy rates. Based on a statistical animal adjusted model (AAM), bulls can vary in fertility rates by up to 25 % when used in the field. The overall aim of this thesis was to further our understanding of the biology underpinning the causes of sire sub-fertility and to pinpoint where along the pregnancy establishment axis variation occurs. Bulls with divergent fertilities were chosen from a population of 840 Holstein Friesian bulls with > 500 inseminations and with a minimum of 10 % between high fertility (HF) and low fertility (LF) treatment groups. *Ex vivo* assessments of sperm transport in the female reproductive tract identified a higher curvilinear velocity (VCL) as well as a higher number of spermatozoa bound to oviductal explants ($p<0.05$). As the uterus is normally the site of semen deposition during AI, using an *ex vivo* model with heifer follicular phase uterine explants, the inflammatory response to frozen-thawed spermatozoa from HF and LF bulls was characterised. Results identified a significant up-regulation of inflammatory gene expressions that were confirmed at both gene and protein level from frozen-thawed spermatozoa although, there was no difference between HF and LF treatments. Subsequently, sperm - polymorphonuclear neutrophils (PMN) binding was assessed with results reporting a higher number of spermatozoa from the LF group bound per PMN. An *in vivo* trial was carried out to profile the transcriptome of the uterus of heifer inseminated with HF or LF semen or no insemination (control group). RNA-sequencing revealed a more active transcriptomic response in the uterus of heifers inseminated with HF bulls (845 differentially expressed genes (DEGs)) compared to the control group with just four DEGs between the LF and control group. Uterine transcriptomic analysis showed a prominent role for the immune response with inflammatory marker Interleukin 1 alpha (*IL1A)* identified as the top DEG between HF and LF treatments. As well as delivering the paternal haploid genome, spermatozoa also deliver an abundance of miRNAs and mRNAs to the oocyte. Transcriptomic profiling of micro RNAs (miRNAs) and messenger RNAs (mRNA) from HF and LF bulls identified differentially expressed miRNAs related to embryonic development and an important involvement in protamine 1 (PRM1) which is a core element of sperm chromatin structure. Overall, this thesis has reported significant advances in the understanding of bull fertility variation and the molecular mechanisms underpinning sire sub-fertility. In particular, it has highlighted differences between semen from HF and LF bulls in relation to the inflammatory response in the uterus with consequences for sperm transport to the site of fertilisation and priming the endometrium for pregnancy as well as identifying differentially expressed miRNA and mRNAs that deliver transcripts to the oocyte that have roles in implantation and embryo development.

Declaration

I declare that the work within this thesis is original work that has not been submitted to any other university or higher education institution. The work contained within this thesis is entirely my own work, except from the assistance mentioned in the acknowledgments and the work stated below:

- ELISA assay by Margaret Murray, Teagasc

- Library preparation and RNA-sequencing performed by Macrogen, South Korea.

- RNA-sequencing data analysis (data quality assessment, mapping of reads to genome, generation of differentially expressed genes, gene ontology analysis and co-expression analysis) performed by Paul Cormican and Kate Keogh (Teagasc) and Jean Philippe-Perrier (UL)

- Histology analysis by Colm Reid (UCD)

- Western blotting analysis was carried out by Caitriona Collins (NUIG)

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Publications

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Presentations

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List of Abbreviations

AAM	Animal adjusted model
ABAM	Antibiotic-antimycotic
AF647-PNA	Alexa Fluor 647-peanut agglutin
AI	Artificial insemination
ALH	Amplitude of lateral head movement
AO	Acridine Orange
AP1	Activator protein 1
BSA	Bovine serum albumin
BSP	Binder of sperm proteins
BUEC	Bovine uterine epithelial cells
Ca^{2+}	Intracellular calcium
cAMP	Cyclic adenosine monophosphate
CES	Cauda epididymal sperm
CMA3	Chromomycin A3
CTRL	Control
DAG	Diacylglycerol
DAMPs	Damage associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DEG	Differentially expressed genes
EBI	Economic breeding index
ELISA	Enzyme-linked immunosorbent assay
FOV	Fields of view
HF	High fertility
ICBF	Irish cattle breeding federation
IL-8	Interleukin 8
IP3	Inositol 1,4,5-trisphosphate
IVF	*in vitro* fertilisation
LF	Low fertility
LIN	Linearity
LPS	Lipopolysaccharide
M540	Merocyanine 540
MAPK	Mitogen-activated protein kinase
Medium 199	Medium 199
MHC	Major histocompatibility complex
miRNA	Micro RNA
mRNA	Messenger RNA
NET	Neutrophil extracellular traps
NFκB	Nuclear factor kappa B
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCA	Principal component analysis
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$

piRNA	Piwi-interacting RNA
PKA	Protein kinase A
PLC	Phospholipase C
PMN	Polymorphonuclear neutrophils
PRM1	Protamine 1
PRR	Pathogen recognition receptors
QC	Quality control
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
rRNA	ribosomal RNA
SDF1	Stromal cell derived factor 1
SP	Seminal plasma
STR	Straightness
TALP	Tyrodes's Albumin Lactate Pyruvate
Treg	T regulatory
tRNA	transfer RNA
UTJ	Uterotubal junction
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight line velocity
ZP	zona pellucida

List of Genes

ADAM2	Metallopeptidase domain 2
ADIPOQ	Adiponectin, C1Q And Collagen Domain
AHSG	Alpha 2-HS glycoprotein
AIM1L	Absent in melanoma 1 like
AK1	Adenylate Kinase 1
ALOX12	Arachidonate 12-Lipoxygenase, 12S Type
AQP7	Aquaporin 7
AR1, AR2	Aldehyde reductase
BLA-DQB	MHC class II
BMP2	Bone Morphogenetic Protein 2
BoLA	MHC class I
CB1	Cannabinoid receptor
CCDC	Coiled-Coil Domain Containing
CCDC181	Coiled-coil domain containing 181
CCT8	Containing TCP1 Subunit 8
CDH1	Cadherin-1
CHMP5	Charged Multivesicular Body Protein 5
CLGN	Calmegin
COL9A3	Collagen type IX alpha 3 chain
CRISP2	Cysteine Rich Secretory Protein 2
CSF2	Colony stimulating factor 2
CXCL1	C-X-C chemokine ligand 1
CXCL8	Chemokines (C-X-C chemokine ligand 8
GABRA4	Gamma-aminobutyric acid type A receptor subunit alpha4
HMBG4	High mobility group box 4
IFI47	Interferon gamma inducible protein 47
IL10	Interleukin 10
IL11	Interleukin 11
IL11RA	Interleukin 11 Receptor Subunit Alpha
IL16	Interleukin 16
IL17A	Interleukin 17A
IL1A	Interleukin 1 alpha
IL1B	Interleukin 1 beta
IL6	Interleukin 6
INHBA	Inhibin Subunit Beta A
KIF5C	Kinesin family member 5C
KIT	Mast/stem cell growth factor receptor Kit
LDLR	Low-density lipoprotein receptor
LTA4H	Leukotriene A4 Hydrolase
OXT	Oxytocin
PCDH9	Protocadherin 9
PEBP1	Phosphatidylethanolamine Binding Protein 1

PGES	Prostaglandin E synthase
PGHS	Prostaglandin-endoperoxide synthase 2
PLA2	Phospholipase A2
PLCz1	Phospholipase C Zeta 1
PRM1	Protamine 1
PTGDS	Prostaglandin D2 Synthase *(PTGDS*
PTGS2	Prostaglandin endoperoxide synthase 2
PTPRC	Protein tyrosine phosphatase receptor type C
RBBP6	RB Binding Protein 6
SCARA5	Scavenger receptor class A, member 5
SCP2D1	SCP2 Sterol Binding Domain Containing 1
SH3D21	SH3 Domain Containing 21
SLC	Solute carrier
SLC24A1	solute carrier family 24 member 1
SLC7A3	Solute Carrier Family 7 Member 3
tACE	Testis-specific angiotensin convertin enzyme
TGFB	Transforming growth factor beta
TIMP2	Metallopeptidase Inhibitor 2
TNFA	Tumor necrosis factor alpha
TRADD	NFRSF1A Associated Via Death Domain
TRAPPC9	Trafficking protein particle complex 9
YWHAZ	Yrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

Chapter 1   Literature Review

1.1 Introduction - Artificial insemination industry

The most important driving force behind AI is its potential to increase the rate of genetic gain of livestock populations using elite sires of high genetic merit (Moore and Hasler, 2017). The importance of fertility is greater in seasonal systems of dairy production where a compact calving period in spring is essential to meet the demands of the cow through grass (Shalloo et al., 2014). Farmers require a fertile herd with a 365 day calving interval to maximise the efficiency and profitability of their enterprise. To achieve this, farmers rely heavily on genetic improvement programmes. In Irish dairy herds the economic breeding index (EBI) is used which is a profit index and ranks bulls across seven sub-indexes based on their daughter's predicted potential which includes milk production, fertility and health related traits. In 2009, Ireland was the second country in the world to include genomic selection in their evaluations, which allows AI centres to reliably identify genetically valuable sires within weeks of birth thereby reducing the generation interval (Cromie et al., 2011).

Using AI, a single ejaculate from a mature bull can be used to inseminate thousands of cows and therefore with the EBI index and genomic breeding programmes, farmers can increase the genetic gain of their herd more rapidly. However, despite stringent quality control checks at animal breeding centres worldwide, bulls with apparently normal semen quality which have passed microscopy based pre-freeze and post-thaw QC checks at the AI centre vary significantly in their field fertility. Animal breeding centres use a range of microscopy based QC checks including motility and morphological analysis, however, bulls that pass these can still vary in their fertility by up to 25%. Studies to-date have assessed a combination of computer assisted sperm analysis (CASA) and flow cytometry assessments to improve semen quality control and explain more of the variation in bull field fertility including motility and kinematic parameters (Kutchy et al., 2019), sperm morphology (Gillan et al., 2008), viability (Gliozzi et al., 2017), acrosome integrity, DNA fragmentation and reactive oxygen species (ROS) production (Kumaresan et al., 2017). More attributes associated with bull fertility include sperm capacitation status and cleavage rates post *in vitro* fertilisation (IVF) (Rahman et al., 2017, Ortega et al., 2018). A combination of parameters including oxidation, acrosomal integrity, DNA compaction, viability, velocity, mitochondrial activity and spermatozoa morphology have been shown to explain up to 40% of the variation in bull fertility (Sellem et al., 2015). A more recent

study by our group developed a linear model that explain 47% of the variation in bull fertility (Bernecic et al., 2021). However, a significant proportion of the variation in fertility remains unexplained. Although these studies are comprehensive and include a wide range of sperm attributes associated with subfertility, they do not identify where reproductive loss is occurring during the establishment of pregnancy i.e. sperm transport failure, fertilisation failure or embryo failure.

Therefore, the focus of this PhD thesis was to further understand the etiology of bull subfertility and to establish if this is due to differences between bulls in the ability of their spermatozoa to interact with the female reproductive tract and its secretions. A particular focus was on the interactions of HF and LF semen with the female reproductive tract, specifically the uterus, the site of semen deposition during AI.

1.2 Spermatozoa transport in the female reproductive tract

1.2.1 Spermatozoa guidance mechanisms

Regardless of species, during their journey in the female reproductive tract, spermatozoa face physical, biochemical and immunological barriers which determines the probability of them reaching the site of fertilisation in the ampulla of the oviduct (Rickard et al., 2019). In addition to sperm swimming ability, mechanisms that facilitate sperm transport to the oviduct include rheotaxis, thigmotaxis, chemotaxis, thermotaxis, as well as smooth muscle contractions.

1.2.2 Rheotaxis

Rheotaxis is the ability of cells and organisms to swim against a fluid flow and has been put forward as a long distance guidance mechanism (Miki and Clapham, 2013). In recent years there has been particular focus on rheotactic behaviour with Kantsler et al. (2014) among one of the first to characterise human and bull sperm behaviour in microchannels at different viscosities and flow rates. They found that spermatozoa swim upstream in particular along the walls of channels in a phenomena known as thigmotaxis and quantified the effects of flow rates (0.2 s^{-1} to 9 s^{-1}) and viscosities (1 mPa·s (that of water) to 20 mPa) on spermatozoa swimming patterns. The maximum upstream velocity decreased more strongly for bull spermatozoa compared to human which has been hypothesised to be due to differences in cell morphology with bull spermatozoa having a

flatter head which is likely to suppress the rotational motion of the cell at high viscosities (Kantsler et al., 2014). The majority (80-84%) of bull spermatozoa observed in a microfluidic device exhibited positive rheotaxis above a flow rate of 27 µm/s (El-Sherry et al., 2014). *In vivo* studies in mice have measured flow rates in the mouse oviduct, with a flow being generated 4 h post coitus and ampullar flow velocity estimated to be 18 ± 1.6 µm/s (Miki and Clapham, 2013). Microfluidic devices allow for the complete control of physical and chemical environments *in vitro* and have been used for sperm sorting, IVF and sperm diagnostics (El-Sherry et al., 2014).

As spermatozoa travel along the female reproductive tract the epithelial lining contains acid base receptors which control pH (Liu et al., 2012). In the uterus and oviduct pH becomes more alkaline due to increases in HCO_3 concentrations (Nishigaki et al., 2014). To optimise *in vitro* sperm sorting assays, the effects of pH on rheotaxis have been examined where bull spermatozoa have shown increased positive rheotaxis within pH 6.4 and 6.8 but above or below these values reduced positive rheotaxis significantly (El-Sherry et al., 2017). In recent years, the mechanisms underpinning the rheotactic response have been explored. Intracellular calcium (Ca^{2+}) control the flagellar beat and swimming patterns of mammalian spermatozoa (Kaupp, 2012, Fechner et al., 2015). Intracellular calcium is set by voltage gated Catsper Ca^{2+} channels which comprise of four subunits (Catsper 1-4) (Seifert et al., 2015). In mouse spermatozoa devoid of *Catsper1,* longitudinal rolling and rheotaxis were eradicated suggesting Ca^{2+} via Catsper is required for rheotaxis (Miki and Clapham, 2013). However, more recent literature has shown that Ca^{2+} via Catsper of both human and mouse spermatozoa is not required for rolling or rheotaxis and instead rheotaxis is a passive biochemical and hydrodynamic process resulting from hydrodynamic interactions between the flagellum of the spermatozoa and fluid flow (Schiffer et al., 2020). Low energy consumption for rheotaxis reserves the energy of spermatozoa for travelling the long journey to the oocyte (Zhang et al., 2016, Schiffer et al., 2020). No calcium influx was observed in human spermatozoa exhibiting positive rheotaxis, suggesting Catsper channels play a role through a different mechanism yet to be described (Zhang et al., 2016). In terms of hydrodynamics, spermatozoa have also been previously classified as pushers where the flagellum is located behind the head and propels the cell forward. The head is considered a passive load at the front (Alvarez et al., 2014). It is plausible, and likely, that males vary in the rheotactic response of their sperm but is unclear if this is related to field fertility. Currently, there are no published

bovine studies which have assessed the rheotactic response of bulls with varying field fertility data.

1.2.3 Thigmotaxis

Thigmotaxis is the tendency of a cell to swim along a surface irrespective of whether spermatozoa are swimming against a fluid flow or not (Denissenko et al., 2012). The movement of spermatozoa along channel walls has been widely reported (Miki and Clapham, 2013, Lopez-Garcia et al., 2008) with explanations for this wall effect seen as a consequence of hydrodynamics in tubes where flow is highest in the centre and falls next to the wall (Roberts, 1970). Bull spermatozoa have been shown to have a preference for swimming within grooves compared to a flat surface (Tung et al., 2014). El-Sherry et al. (2014) also found that bull sperm motility was significantly higher near the walls of the microchannel. Biologically, this addresses factors that facilitate spermatozoa swimming close to the epithelium of the female reproductive tract. There are a number of explanations for this including, in human sperm, where the amplitude in oscillations in the sperm head is less than the end of the tail and therefore the head is closer to the wall. The cone-shaped envelope of the flagellar wave aligns with the surface, resulting in the direction of spermatozoa being inclined towards the wall (Denissenko et al., 2012).

1.2.4 Chemotaxis

Chemotaxis is the process by which spermatozoa are guided by a chemical gradient towards the oocyte (Kaupp et al., 2008). Chemotaxis has been shown towards follicular fluid in human, mice and rabbit spermatozoa (Eisenbach and Giojalas, 2006) as well as low concentrations of progesterone in humans and rabbits (Teves et al., 2006). This mechanism along with thermotaxis is restricted to capacitated spermatozoa which is ~ 10% of sperm population in the oviduct of humans (Eisenbach and Giojalas, 2006). The role of sperm chemotaxis is to selectively recruit capacitated spermatozoa to fertilise the oocyte (Eisenbach and Giojalas, 2006). As chemotactic and capacitation responses are transient mechanisms with a 1-4 h life span, each individual spermatozoa undergoes the process at different stages, prolonging the time that spermatozoa are available in the female reproductive tract (Eisenbach, 1999, Giojalas et al., 2004). This is an essential mechanism particularly in species that are spontaneous ovulators such as bovine and ensures the disposal of capacitated and chemotactic competent cells throughout the female tract in the lead up to ovulation (Giojalas et al., 2004).

Sperm flagellar movement as well as chemoattractant stimuli control sperm swimming trajectories (Yoshida and Yoshida, 2011). Intracellular calcium has been well studied and is linked with regulating these flagellar motor responses (Shiba et al., 2008, Guerrero et al., 2010). Due to their simpler nature and homogenous sperm populations, spermatozoa from sea urchins have been used to study the process of chemotaxis (Kaupp, 2012). *In vitro* studies using human spermatozoa identified more potent chemoattractants from the oocyte compared to surrounding cumulus cells characterising chemotaxis as a short range mechanism (Sun et al., 2005). The chemoattractant response has been characterised as having two phases: a delay and a turn and this is best characterised in human spermatozoa by Armon and Eisenbach (2011) which show capacitated spermatozoa being continuously stimulated towards the chemoattractant gradient where they are swimming straight without turns or hyperactivation until they stop sensing the gradient. Spermatozoa then turn and experience hyperactivation episodes, which change their swimming pattern.

The mechanism behind this delay and turn phase is the transient increase in Ca^{2+} triggered by chemoattractants with each turning event resulting in a rapid increase in intracellular calcium (Alvarez et al., 2014). Bovine follicular fluid has been suggested to be involved in the increase of sperm straight line velocity (VSL) and VCL and in the flagellar bending of chemotactic spermatozoa (Mondal et al., 2017). Progesterone is a well known chemoattractant in follicular fluid as well as cumulus cells of the oocyte (Jaiswal et al., 1999, Mingoti et al., 2002) however, studies in humans suggest other potent spermatozoa chemoattractants including stromal cell derived factor 1 (SDF1) expressed in human oocytes has a chemotactic affinity for spermatozoa (Zuccarello et al., 2011). More recent literature has identified SDF1 as a chemotactic factor of bovine spermatozoa regulating spermatozoa migration to the cumulus oocyte complex in cattle *in vitro* (Umezu et al., 2020).

1.2.5 Thermotaxis

Thermotaxis is the cell response to a thermal gradient (Karbalaei and Cho, 2018). The mechanism suggests that a 2°C temperature difference between the isthmus, site of sperm storage, and the site of fertilisation in the ampulla, in pig and rabbit, is responsible for guiding spermatozoa to the ampulla (Bahat et al., 2003, Hunter, 1981). Three different mechanisms have been proposed for the temperature decrease at the spermatozoa storage site including; a localised release of acid mucus glycoprotein that undergoes extensive endothermic hydration (Luck et al., 2001), an ovulation dependent change where the

uterine artery is cooler after ovulation (Cicinelli et al., 2004) and blood from the ovarian vein cools the blood that enters the storage site by heat exchange (David et al., 1971). Unlike sperm chemotaxis, which is characterised as a short-range mechanism, thermotaxis is a long-range mechanism which guides capacitated spermatozoa to the site of fertilisation and is then followed by chemotaxis at close proximity to the oocyte (Bahat et al., 2003).

The opsin family which are present in the eye for light sensing have been identified as being at least partly responsible for mammalian sperm thermotaxis (Perez-Cerezales et al., 2015). They are a family of G-protein coupled receptors that act as thermosensors for spermatozoa and opsins have a heterogeneous distribution in human spermatozoa with different sub populations depending on the type of opsin present. Blocking out rhodopsin has been shown to significantly reduce thermotaxis in mouse spermatozoa (Perez-Cerezales et al., 2015). Rhodospin staining has been used as a marker of thermotactic spermatozoa in mouse and human *in vitro* and *in vivo* tracking of mouse spermatozoa in the fallopian tube showed that only spermatozoa with thermosensors (rhodopsin) correctly located on the spermatozoa are able to undergo thermotaxis (Perez-Cerezales et al., 2018). The pathways involved in sperm thermotaxis include the phospholipase C (PLC) pathway and the transduction/cyclic nucleotide pathway (Bahat and Eisenbach, 2010, Perez-Cerezales et al., 2015) where inhibition of both pathways completely stops sperm thermotaxis. Melanopsin (PLC pathway) has been identified in mouse spermatozoa where melanopsin knock-out mice caused a 50% decrease in sperm thermotaxis and rhodopsin (transduction nucleotide pathway) knock-out mice caused a 70% decrease in thermotaxis (Roy et al., 2020). The understanding of thermotaxis mechanisms has improved spermatozoa selection methods and more recently, a biomimetric microfluidic device has been developed for human spermatozoa which can enable testing of sperm thermotactic and chemotactic responses simultaneously (Yan et al., 2021).

1.3 Spermatozoa binding to the oviductal epithelium

After spermatozoa migrate through the uterus they arrive at the oviduct which plays an important role in successful fertilisation and has three distinct structures each with their own function; the uterotubal junction (UTJ), the isthmus and ampulla. The UTJ is considered the second major selective barrier beyond the cervix, the isthmus stores spermatozoa and fertilisation occurs in the ampulla (Suarez, 2008, Mahe et al., 2021). In the cow the morphology of the UTJ has been shown to have mucosal folds and lined by

a columnar epithelium containing ciliated and non-ciliated cells (Wrobel et al., 1993). Spermatozoa cannot readily pass through the UTJ unless key proteins are present on the head of their plasma membrane. Studies in mice showed how mice that lacked the genes such as metallopeptidase domain 2 (*ADAM2)*, calmegin (*CLGN*) and testis-specific angiotensin convertin enzyme (*tACE*) were infertile as their spermatozoa cannot pass through the UTJ despite normal sperm morphology and motility (Figure 1.1) (Yamaguchi et al., 2009, Nakanishi et al., 2004). It is also interesting to note that the oviduct must maintain an aseptic environment for successful fertilisation while also being able to control the response of the female to allogenic spermatozoa and embryos. The mucosal system in the oviduct produces a pro-inflammatory response in the oviduct to pathogens while it has also been shown that bovine spermatozoa in the oviduct produce anti-inflammatory Interleukin-10 (*IL10)* and Transforming growth factor beta (*TGFB)* which signifies spermatozoa induce their own protection from an immune perspective in the oviduct (Marey et al., 2019).
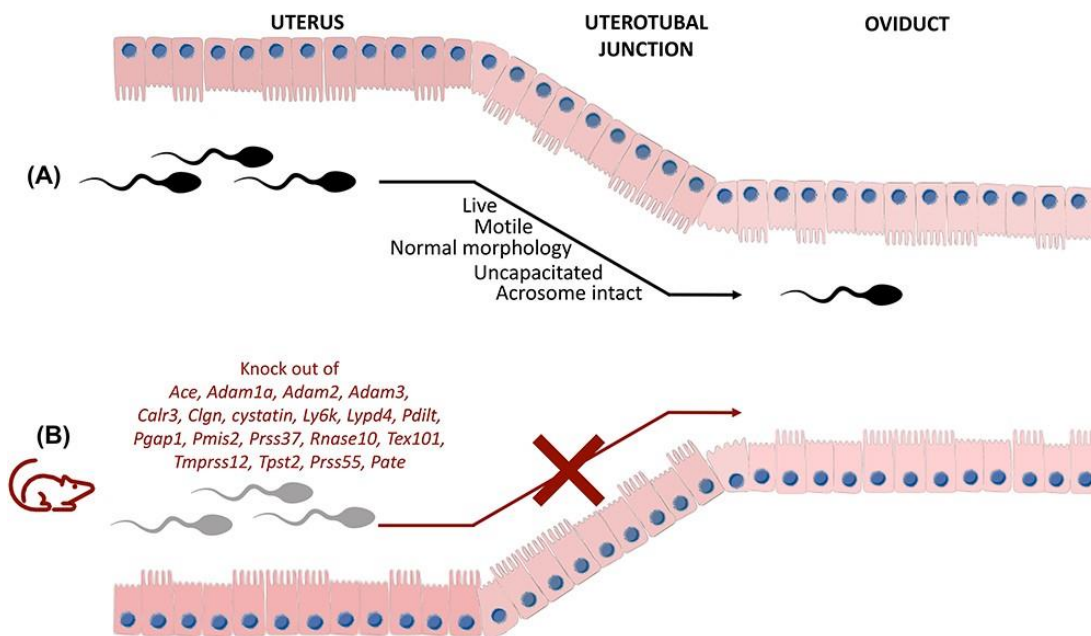
Figure 1.1 Sperm selection by the utero-tubal junction in mammals with a list of genes (red) required for sperm passage through to the oviduct as identified by knockout murine models (Mahe et al., 2021).

After passing through the UTJ, spermatozoa are stored in the isthmus and bind to the epithelium thereby prolonging the fertile lifespan of ruminant spermatozoa (Chian et al., 1995, Pollard et al., 1991). In cattle, it has been shown that sperm-oviductal epithelium interaction is mediated by carbohydrate recognition mechanisms, where fucose has been

identified as a key receptor for bull sperm binding (Suarez, 2016). The main binder of sperm (BSP) proteins identified were PDC109 (BSPA1/A2), BSPA3 and BSP30K (Gwathmey et al., 2006) as well as the integrin α5β1 (Osycka-Salut et al., 2017) and beta-defensin 126 (Lyons et al., 2018) that are also involved in sperm capacitation and detachment from the reservoir (Hung and Suarez, 2012) (Figure 1.2). Spermatozoa that bind to the epithelia have been shown to have low tyrosine phosphorylation and intracellular calcium (Gualtieri et al., 2005) as well as normal chromatin structure (Ellington et al., 1999). Bull spermatozoa can be stored in the isthmus for 18-20 h before they are released to ascend into the ampulla (Hunter and Wilmut, 1984) thereby, widening the window for when successful fertilisation can occur. Spermatozoa bind to the oviduct at different stages of the estrus cycle (Lefebvre et al., 1995) and therefore it is likely hormonal changes that are triggered around the time of ovulation trigger the release of spermatozoa (Hung and Suarez, 2010). The two main mechanisms by which spermatozoa are released from the reservoir are by shedding of sperm surface proteins during capacitation and by hyperactivation (Bosch et al., 2001, Curtis et al., 2012). During the process of capacitation, bull spermatozoa shed the BSP protein PDC109 and lose their affinity to bind to the epithelium (Gwathmey et al., 2003).

1.3.1   Capacitation

Sperm capacitation is a pre-requisite for fertilisation to occur and involves biochemical modifications that allow spermatozoa to acrosome react on exposure to the zona pellucida (ZP) (Florman and First, 1988). Capacitation is a lengthy process that begins in the uterus and is accelerated with arrival in the oviduct (Parrish et al., 1989). It involves the loss of decapacitating factors including cholesterol, an increase in Ca^{2+}, and an increase in sperm specific adenylyl cyclase, raising levels of cyclic adenosine monophosphate (cAMP) (Sinclair et al., 2000). This in turn activates protein kinase A (PKA) leading to increased tyrosine phosphorylation of sperm proteins such as calcium binding protein and testis specific serine protein (Platt et al., 2009). *In vitro,* bovine spermatozoa can be capacitated through incubation in a heparin media which is best described by Parrish (2014). Heparin binds to bull spermatozoa and a series of BSPs which interact with cholesterol and phospholipids in the plasma membrane leading to their loss, thereby, increasing membrane fluidity (Manjunath and Therien, 2002). Heparin also causes changes to intracellular pH, Ca^{2+} and cAMP levels while calcium is also important in capacitating media to lead to a rise in Ca^{2+} in the head of spermatozoa (Parrish et al., 1999). In sperm,

specific transmembrane Ca^{2+} channel family, known as Catsper channels play a significant role in capacitation and the acrosome reaction (Nguyen et al., 2016). These Catsper channels are weakly voltage dependent Ca^{2+} selective ions that control the entry of Ca^{2+} into the spermatozoa (Singh and Rajender, 2015). Disruption of these Catsper genes have been shown to lead to complete infertility in mice and humans (Ren et al., 2001, Li et al., 2006).

A bicarbonate-enriched media with a minimum of 10 mmol/L is also required which increases levels of cAMP and also induces a rapid disordering of the phospholipid packing of the sperm membrane, increasing membrane fluidity (Parrish, 2014). In parallel to this, but later than membrane fluidity changes and with the presence of bovine serum albumin (BSA) in the media, cholesterol is removed. Bicarbonate is also involved in the escape of the sperm-specific seminolipid from the apical membrane to the equatorial domain. This seminolipid stabilises the plasma membrane in low levels of bicarbonate and its relocation with bicarbonate exposure allows spermatozoa to complete the acrosome reaction (Gadella et al., 1995). It is difficult to fully characterise the time course and agents that capacitate spermatozoa *in vivo*. However, bovine oviductal fluid can capacitate spermatozoa in a similar time to heparin where glycosaminoglycans and other protein capacitating agents have been identified in the oviduct fluid (McNutt and Killian, 1991).
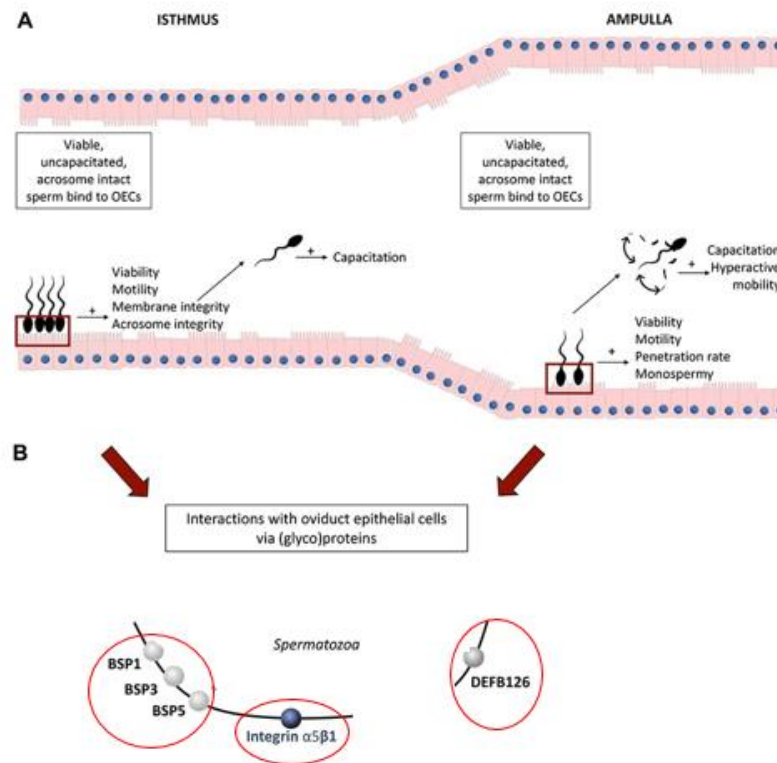
Figure 1.2 Mechanisms involved in sperm interactions with oviductal epithelial cells and their consequences on sperm physiology. After crossing the utero-tubal junction, a sub-population of spermatozoa are able to bind to the epithelial cells in the oviduct and isthmus maintaining viability, motility, membrane and acrosome integrity. After the release of spermatozoa from the epithelial cells, capacitation is induced followed by the acrosome reaction and hyperactivation. The proteins that have been identified as playing a role in bull sperm binding are circled in red. Image adjusted from Mahe et al. (2021).

1.3.2   Hyperactivation

After capacitation, spermatozoa become hyperactivated where they exhibit increased flagellar bend amplitudes which causes an asymmetrical beating of the flagellum (Suarez and Ho, 2003). Hyperactivation of bull spermatozoa is characterised by twisting and figure eight like movements in non-viscous media however; they swim progressively through viscous medium (Harayama, 2018). As mentioned previously, hyperactivation helps spermatozoa detach from the epithelium and it has been shown in cows that mucus which fills the UTJ extends to the isthmus (Suarez et al., 1997) therefore, spermatozoa that are hyperactivated are better able to penetrate these substances (Quill et al., 2003). Hyperactivation is triggered by an intracellular rise in pH and a Ca^{2+} influx that plays a key role in the regulation of hyperactivation (Ho and Suarez, 2001). The upper region of

the oviduct is more alkaline (~ pH 7.6) due to greater levels of bicarbonate and in turn opening pH gated Ca^{2+} channels (Olson et al., 2011). Catsper have been recently identified in bull spermatozoa and play a critical role in bull sperm hyperactivation (Johnson et al., 2017)

1.3.3 Fertilisation

The mechanisms described previously including capacitation, hyperactivation, thermotaxis and chemotaxis are all pre-requisites for sperm binding to the ZP (Suarez, 2007). The initial contact with the ZP begins a cascade of events leading to the acrosome reaction which again involves a Ca^{2+} influx whereby the plasma membrane fuses with the outer acrosomal membrane (Mattioli et al., 1996). The contents of the acrosome which consists mainly of hydrolytic enzymes (hyaluronidase and arcsin) disperses and digests the ZP (Breitbart, 2002). The oocyte becomes activated when a soluble sperm factor, PLCζ, released from the post acrosomal perinuclear theca, triggers calcium release from the oocyte endoplasmic reticulum and begins a cascade of events guiding oocyte meiosis, mobilisation and anti-polyspermy defence, pronuclear development and embryo cleavage (Kashir et al., 2010). Calcium oscillations are essential for oocyte activation and studies have identified PLCζ as a candidate for sperm-borne oocyte activation (Kashir et al., 2010, Amdani et al., 2016). PLCζ hydrolyses phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Yang et al., 2013). IP3 causes the release of Ca^{2+} from the endoplasmic reticulum and DAG reacts with Ca^{2+} to activate protein kinase C (Taylor et al., 2014). The bovine ZP contains three heavily glycosylated proteins, ZPA, ZPB and ZPC which are receptors for sperm binding to take place (Yi et al., 2007). The apical plasma membrane and the outer acrosomal membrane form vesicles and disperse. During this process, the inner acrosomal membrane becomes a part of the outer barrier of the cell and form a continuous membrane that looks like a hairpin structure.

Spermatozoa that have been hyperactivated and have reacted with the ZP enter the perivitelline space (between the ZP and egg membrane). Subsequently, the spermatozoon cell plasma and the oolemma fuse and the spermatozoon is incorporated into the oocyte (Flesch and Gadella, 2000). Bull spermatozoa found within the oocyte periviteline space have a nucleus covered with a reduced nuclear envelope and pernuclear theca (Thompson et al., 2003). Sperm DNA is mainly composed of protamines, mainly protamine 1 (Balhorn, 2007). Upon entry into the ooplasm the sperm nucleus is dismembranated and

protamines inserted in the nucleus at spermiogenesis are removed and replaced with histones (Sutovsky et al., 1997, Gao et al., 2004). At this time, the nuclear envelope is reconstructed by membrane vesicle fusion and nuclear lamina formation on both maternal and paternal chromatin (Sutovsky et al., 1998). As the pro-nuclei grow, maternal proteins re-enter the nuclear compartment to form nucleolus bodies that are formed into ribosomal RNA producing nucleoli at the time of zygote activation (Ogushi et al., 2008).

1.3.4 Embryogenesis and implantation

The formation of the zygote marks the end of fertilisation, and the beginning of embryo development. After fertilisation the embryo migrates from the oviduct to the uterus and as it does it undergoes cleavage leading to a rapid increase in the number of blastomeres (Imakawa et al., 2004). By Day 4 (16-cell stage) the embryo enters the uterus and develops into the morula (32-cell stage) forming cell to cell tight junctions in a process known as compaction (Van Soom et al., 1992). By Day 7 the embryo will have developed into a blastocyst with an inner cell mass which gives rise to the embryo and the outer layer (trophoblast) which forms the placenta (Holm et al., 1998). On Days 9-10, after hatching from the ZP, the blastocyst elongates and continues to grow. Conceptus elongation involves transition from a spherical blastocyst (Day 7) to an ovoid (Days 12-13), to a tubular (Days 14-15) and filamentous form, which is best described by Degrelle et al. (2005). By Day 19 the elongated conceptus begins implantation with attachment of the trophoblast to endometrial luminal epithelium (Guillomot, 1995).

1.3.5 Sire contribution to fertilisation, embryo development and implantation

Defects in sperm chromatin structure affect sperm function during fertilisation and first cleavage. An inadequate chromatin protamination and decreased DNA integrity were linked with reduced *in vivo* fertility in bull spermatozoa (Dogan et al., 2015). Numerous studies have demonstrated a relationship between DNA fragmentation levels in bull spermatozoa and field fertility (Kumaresan et al., 2017, Nagy et al., 2013, Karoui et al., 2012). However, it should be noted that not all bulls that have low field fertility, have high levels of DNA fragmentation (Rodriguez-Martinez and Barth, 2007).

IVF is a powerful tool that allows the assessment of sperm fertilising abilities. Previous studies have reported a clear relationship between the time of first cleavage of a bovine oocyte and its developmental ability (Lonergan et al., 1999, Dinnyes et al., 1999). The

timing of first cleavage is related to the polyadenylation of several mRNA transcripts in the oocyte (Lonergan et al., 1999). However, studies have shown that the time of first oocyte cleavage varies between bulls and late cleaving embryos develop slower and are usually poorer (Ward et al., 2001). In particular, embryos fertilised from HF bulls cleaved first and significantly more of these early cleaving zygotes were more capable of developing to the blastocyst stage than those that cleaved later (Ward et al., 2001). Ward et al. (2001) also reported a correlation between blastocyst yield and HF bulls while Al Naib et al. (2011) reported an increase in cleavage rate from HF bulls. Ortega et al. (2018) reported that bulls with a higher conception rate had advantages in terms of *in vivo* and *in vitro* productions of embryos however, there was no effect of sire fertility on conceptus elongation and development.

Recent advances in transcriptomics have revealed that mature spermatozoa are not only carriers of the paternal haploid genome. Spermatozoa also deliver an abundant cargo of various types of RNAs into the oocyte, including messenger mRNAs, as well as long and small non-coding RNAs, such as transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), Piwi-interacting RNAs (piRNAs), and miRNAs (Selvaraju et al., 2017, Sellem et al., 2020). The expression of certain mRNAs were reported to be associated with semen quality and sire fertility including Cysteine Rich Secretory Protein 2 (*CRISP2),* Chaperonin Containing TCP1 Subunit 8 (*CCT8)* and Phosphatidylethanolamine Binding Protein 1 *(PEBP1)* (Arangasamy et al., 2011), Adenylate Kinase 1 *(AK1), IB5,* Metallopeptidase Inhibitor 2 *(TIMP2)* and Phospholipase C Zeta 1 *(PLCz1)* (Kasimanickam et al., 2012) Protamine 1 (*PRM1)* (Ganguly et al., 2013), Aquaporin 7 (*AQP7)* (Kasimanickam et al., 2017), or cannabinoid receptor (*CB1)* and Fatty Acid Amide Hydrolase (*FAAH)* (Kumar et al., 2018). The transcription of mRNA is halted by the time germ cells reach the late spermatid stage but the accumulation of non-coding RNAs occurs during spermatogenesis and during epididymal transit through the inclusion of epididymosomes (Reilly et al., 2016). These miRNAs have been reported as having an integral role in regulating gene expression during early embryo development (Yuan et al., 2016).

1.4 Uterine innate immunity and the female inflammatory response defence mechanisms

Spermatozoa are deposited in the vagina during natural mating, whereas the site of sperm deposition during AI is normally in the uterine body, thereby bypassing the cervix. To focus on the potential effects of the immunological response to spermatozoa during AI, it is necessary to understand the immunology of the uterus. The uterus itself has a unique local immune system which can tolerate spermatozoa and semi-allogenic blastocysts while also being able to fight infections (Lee et al., 2015). The endometrium can identify pathogens and damages through pattern recognition receptors (PRRs) which recognise pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) (Sheldon et al., 2018). These innate immune responses to PAMPs and DAMPs are driven mostly by classical immune cells including macrophages, neutrophils and dentritic cells.

Toll-like receptors are the most common PRRs and are expressed on 'non-classical' immune stromal and epithelial cells on the endometrium (Turner et al., 2014). These cells are able to recognise DAMPs and PAMPs and in turn initiate several signalling cascades. The molecule MYD88 is essential for downstream signalling of all TLRs apart from TLR3 (Sheldon et al., 2014). MYD88 is activated and stimulates IL1R- associated kinases which in turn activate tumor necrosis factor receptor (TNFR) associated factor 6 that catalyses a number of members from the TAK1 binding protein family. These activate two important pathways of inflammation namely, the mitogen-activated protein kinase (MAPK) pathway and a nuclear factor kappa B (NFκB) subunit 2. The MAPkinase activates the activator protein 1 (AP1) transcription factor and NFκB and drives the transcription of genes including cytokine (Interleukin 1 beta (*IL1B),* Interleukin 1 alpha *(IL1A),* Interleukin 6 *(IL6)* and tumor necrosis factor alpha (*TNFA*)), chemokines (C-X-C chemokine ligand 8 (*CXCL8)* and C-X-C chemokine ligand 1 (*CXCL1)*) and lipids (phospholipase A2 (*PLA2),* prostaglandin-endoperoxide synthase 2 *(PGHS)* and prostaglandin E synthase (*PGES))* (Sheldon et al., 2014). Consequently, PMNs are recruited to the site of inflammation which ensures the phagocytosis of invading pathogens and spermatozoa to prepare the endometrium for pregnancy (Sheldon et al., 2019).
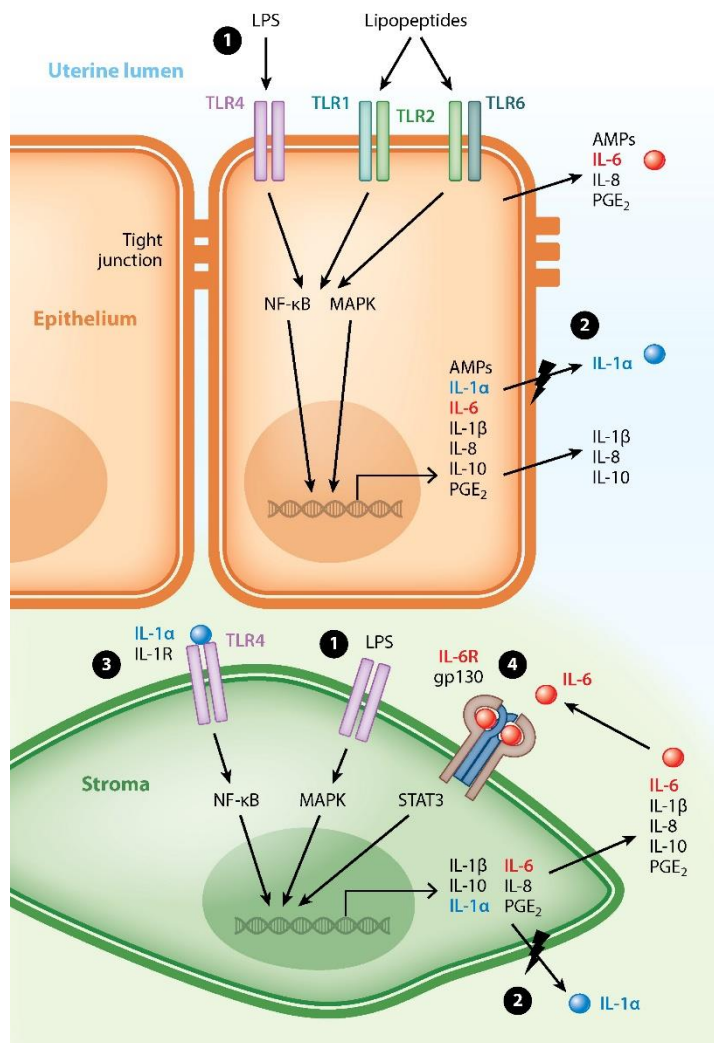
Figure 1.3 The innate immune response in the endometrium. Epithelial and stromal cells are represent by orange and green, respectively and express Toll-like receptor 4 (TLR4) and TLR1/TLR2 and TLR2/TLR6 heterodimers. Pathogen associated molecular patterns (PAMPs) bind to the TLRs which in turn activates signalling pathways and the transcription of genes which encode inflammatory mediators (Sheldon et al., 2019).

As illustrated in Figure 1.3 where PAMPs bind to TLRs, the NF-κB and MAPK signalling pathways are activated leading to the transcription of genes that encode several inflammatory mediators, including antimicrobial peptides, cytokines, chemokines, and prostaglandins. Damaged cells release the intracellular cytokine interleukin (IL)-1α in a paracrine manner. IL-1α can bind to the IL-1R of nearby cells, further activating the NF-κB and MAPK signalling pathways. Epithelial and stromal cells respond differently to IL-6, and in stromal cells IL-6 has a positive feedback through the IL-6R/gp130 receptor

heterodimer and signal transducer and activator of transcription-3) signalling to enhance the secretion of IL-6 and Interleukin 8 (IL-8) (Sheldon et al., 2019).

The production of chemokines and cytokines promote the extravasation of leukocytes (mainly composed of PMNs) in the bloodstream, into the infected tissue (Zlotnik and Yoshie, 2012). Endothelial cells which line the blood vessels are stimulated to express adhesion molecules such as selectins which allows the selective extravasation of PMNs and prevents the exit of erythrocytes (Pober and Sessa, 2007). Neutrophils are the first immune cells to reach the site of infections where they become activated either by direct contact with pathogens or through cytokines secreted by other resident cells (Medzhitov, 2008). Neutrophils eliminate pathogens through phagocytosis which is the production of reactive oxygen species and the release of potent bactericidal enzymes by degranulation or by the formation of neutrophil extracellular traps (NETs) which involves the extrusion of DNA to ensnare pathogens (Hahn et al., 2019). A successful acute inflammatory response results in the removal of pathogens followed by the resolution of tissue and the restoration of homeostasis. Resolution involves the release of anti-inflammatory molecules, IL-10 and lipid mediators, lipoxins (Serhan, 2014). Lipoxins and resolvins stop the recruitment of PMNs and instead stimulate the recruitment of monocytes which remove dead cells and promote tissue remodelling (Fullerton and Gilroy, 2016).

1.4.1 Uterine inflammatory response: Seminal plasma

Semen has been shown to induce a uterine immune response across several species including mice, pigs and horses (Katila, 2012, Robertson, 2007). Early studies on post breeding inflammation were focused on the role of seminal plasma (SP) in particular (Robertson, 2007).
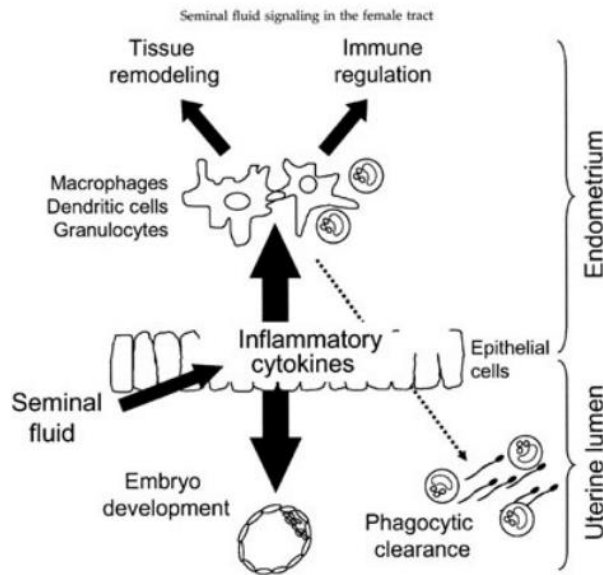
Figure 1.4 An illustration of the actions of seminal fluid in the female reproductive tract. Mice and pig studies have shown that active components of seminal fluid interact with the uterine epithelial cells and synthesis pro-inflammatory cytokines which cause the recruitment of macrophages, dendritic cells and PMNs (Robertson, 2007).

As demonstrated in Figure 1.4, seminal fluid initiates a pro-inflammatory response in the endometrium and with the recruitment of macrophages, dendritic cells and PMNs which are proposed to have roles in re modelling the endometrium and clearing seminal debris and maintaining uterine sterility (Robertson, 2007). *In vitro* studies in rodents and humans have identified TGFB to be the key modulatory factor in SP induced post insemination uterine inflammation (Gutsche et al., 2003, Sharkey et al., 2012). However, during natural mating semen is deposited directly into the bovine cervix in comparison to uterus in rodents and pigs (Hunter, 1981, Katila, 2012). In vaginal depositors of semen, such as cattle, it is not clear how much SP reaches the uterus following natural mating and during AI, semen is diluted considerably (0.8-12% the original ejaculate volume) (Nongbua et al., 2018). Recently, the effects of bovine SP in the endometrium has been assessed (Ibrahim et al., 2019). The presence of SP with or without spermatozoa has been shown to induce the inflammatory mediators colony stimulating factor 2 (*CSF2), IL1B, IL6,* Interleukin 17A *(IL17A), CXCL8,* prostaglandin endoperoxide synthase 2 *(PTGS2)* and *TGFB1* (Ibrahim et al., 2019). The increase in *TGFB1* suggests that a TGFB signal is amplified by bovine SP (Ibrahim et al., 2019).

1.4.2 Uterine inflammatory response – spermatozoa

More recent studies have focused specifically on the effects of spermatozoa in the post-insemination inflammatory response. Using an *in vitro* bovine model of bovine uterine epithelial cells (BUECs) (Elweza et al., 2018) demonstrated that frozen-thawed bull spermatozoa induced expression of pro-inflammatory genes, *IL1B, IL8, TNFA and NFkB*. Further investigation of this response with BUECs suggested the response is at least in part mediated via the TLR2/4 signalling pathway (Ezz et al., 2019) (Figure 1.5).
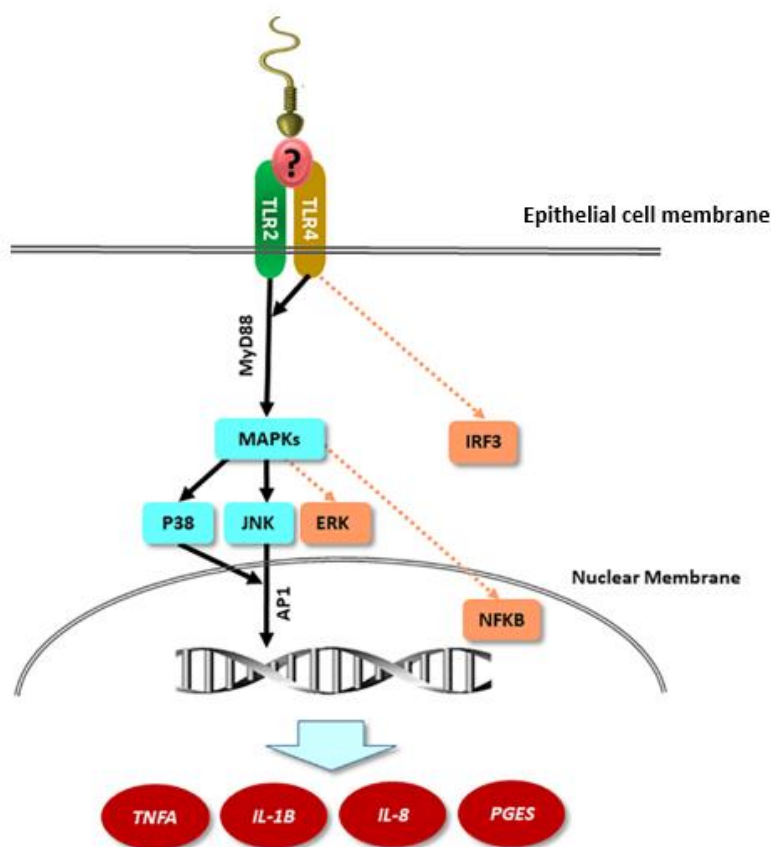
Figure 1.5 An illustration of the mechanism by which spermatozoa activate the toll-like receptor (TLR) TLR2/4 signalling pathway via an unknown endogenous ligand and activates the downstream mitogen-activated protein (MAPKs) which leads to the translocation of the AP1 protein and the transcription of pro-inflammatory mediators. (Ezz et al., 2019).

This work proposes that spermatozoa activate the MAPK component C-Jun N- terminal kinase and p38MAPK but do not have effects on other downstream TLR2/4 signalling pathways (Interferon regulatory factor 4 (IRF4) or NFKB or extracellular receptor kinase

(ERK)). The nuclear translocation of the AP-1 protein regulates the transcription of pro-inflammatory genes in BUECs. However, the endogenous ligand linking spermatozoa to this pathway remains unknown (Ezz et al., 2019). A subsequent study using a more physiological *ex vivo* uterine explant model demonstrated that during the preovulatory phase, bull spermatozoa enter uterine glands and interact with PMNs (Akthar et al., 2019). This study suggests that spermatozoa which enter uterine glands trigger an immune response and interact with PMNs present in these glands (Akthar et al., 2019).

1.4.3   Sperm-PMN interactions

PMNs are a key part of the innate immune system and inflammation where they provide the first line of defence and are the most abundant leukocytes in blood (Lumsden et al., 1980). It has been shown across a range of species, including cows, gilts, goats and mares that PMNs enter the uterine lumen in response to semen (Alghamdi et al., 2009) (Rozeboom et al., 1998, Mattner, 1968). PMNs have been detected in the uterus with a peak in numbers within 2 h in cattle (Alghamdi et al., 2009). PMNs have been shown to follow a chemoattractant gradient initiated in affected/injured tissue. Spermatozoa have been shown to activate the complement cascade in pigs and horses which facilitates the chemotaxis of PMNs (Troedsson et al., 2005). This complement system is made of a large number of distinct proteins that interact with pathogens and mark them for destruction by phagocytes (Charles A Janeway, 2001). The activation of this complement system is an important part of the inflammatory process where raised serum C3 and C4 are strong inflammatory markers (Liu et al., 2016). Spermatozoa have been shown to activate the complement system in BUECs with increased C3 mRNA expression (Elweza et al., 2018) and C3 plays an integral role in activating the entire inflammatory system (Kasperska-Zajac et al., 2013). Furthermore, the presence of inflammatory cytokines *IL1B* and *TNFA* upregulated bull spermatozoa phagocytosis by PMNs *in vitro* (Elweza et al., 2018). *IL8* has also been shown to be a powerful chemoattractant for PMNs (Keshari et al., 2012).

The binding of spermatozoa to PMNs is mediated by direct cell-to-cell attachment and sperm phagocytosis or by the formation of NETs (Hong et al., 2017, Alghamdi et al., 2009). The direct cell-to-cell attachment involves a receptor ligand or opsonisation (Alghamdi et al., 2009). The mechanism by which spermatozoa bind to PMNs is not fully known but it has been shown not to be lectin dependent (Taylor et al., 2008). When PMNs are activated PMNs extrude their DNA and histones to form NETs that engulf and kill both pathogens and spermatozoa (Alghamdi and Foster, 2005).

Sperm – PMN binding has been characterised in many species with a large inter-species variation. There are a number of factors that can have contrasting effects on sperm-PMN binding including the amount of SP, the cryopreservation process as well as the cryodiluent used. Ram sperm have been shown to undergo significant changes to their surface carbohydrates from the contact of SP as well as from cryopreservation (Pini et al., 2017). The same study demonstrated that cryopreserved ram sperm adhered less to PMNs compared to epididymal or ejaculated spermatozoa and concluded changes were due to cryodiluents (Pini et al., 2017). Bovine studies have shown an increased effect of SP on sperm-PMN binding (Alghamdi et al., 2009, Fichtner et al., 2020).

1.5   Consequences of the immune response for spermatozoa transport

Following on from the characterisation of the inflammatory response and NET formation with spermatozoa, its impact on fertility must be considered. Although this is not entirely clear across the literature and more studies are required, some *in vitro* studies have reported a link between sperm NET formation with decreased sperm motility. Human sperm studies have shown monocytes in contact with spermatozoa entrap the spermatozoa, forming small aggregates with motility significantly decreasing after 10 min of co-incubation (Schulz et al., 2019). This has been confirmed in other human studies where motility diminished after 1 h signifying that this interaction impedes sperm transport and decreases the probability of spermatozoa reaching the site of fertilisation (Zambrano et al., 2016). Similarly in cattle there is a strong correlation between increased percentage of adherent PMNs and a decrease in free spermatozoa (Alghamdi et al., 2009). The elimination of spermatozoa begins almost immediately with sperm transport (Katila, 2012). There are conflicting discussions around the appropriate extent of the inflammatory response and infiltration of PMNs (Chastant and Saint-Dizier, 2019, Katila, 2012) however, an *in vivo* bovine study examining PMN concentrations post insemination and its effects on pregnancy rates found a moderate influx of PMNs led to significantly higher conception rates (Kaufmann et al., 2009) suggesting a moderate inflammatory response is required to allow spermatozoa reach the site of fertilisation. Spermatozoa that fertilise the oocyte leave the uterus within the first 1-2 h (Hawk, 1987), therefore, perhaps this NET formation is also important in clearing the endometrium in preparation for pregnancy.

The inflammatory response of spermatozoa contributes to their rapid removal, preparing the uterus for the embryo but also has a role in preventing the female developing an acquired immune response against spermatozoa. However, as spermatozoa contain proteins that are foreign to the female it is possible that the female can develop antibodies to spermatozoa proteins and block the survival of spermatozoa thereby inhibiting their ability to reach the site of fertilisation (Wigby et al., 2019). Lower penetration of bull spermatozoa was recorded in cervical mucus of heifers with positive anti sperm antibodies (ASAs) suggesting a regulation of spermatozoa to reach the site of fertilisation (Zralý et al., 2003). Spermatozoa can also acquire ASAs through the breakdown of the blood epididymal barrier. Recent literature reported a high level of ASA present in serum and SP of bulls had a negative effect on their fertility through the prevention of capacitation (Ferrer et al., 2017).

1.5.1 Consequences of the seminal plasma induced immune response for priming of the endometrium for pregnancy

A large part of the literature focuses on SP signalling and its consequences for priming the endometrium in preparation for pregnancy. This section addresses the consequences of this SP immune response signalling. Female responses to SP including T regulatory (Treg) cell generation, macrophage and dendritic cell infiltration which are key factors in endometrial receptivity, which have been shown in humans, mice and pigs (Sharkey et al., 2012, Meuleman et al., 2015, Kim et al., 2015, O'Leary et al., 2004). Seminal plasma induces immune suppressive Treg cells that mediate adaptive tolerance necessary for pregnancy (Robertson et al., 2018). This happens when dendritic cells enter the draining lymph nodes where they present male SP to prime T-cells where the T cells then enter the uterine endometrium before and during implantation (Johansson et al., 2004). This constrains inflammation and mediates immune tolerance of the semi-allogenic embryo (Robertson et al., 2018). The capacity of seminal fluid to induce Treg cells is attributed to molecules including TGFB and prostaglandins that induce the presence of macrophages and dendritic cells and differentiate pro-tolergenic phenotypes that induce Treg cells (Remes Lenicov et al., 2012).

Despite the extensive body of evidence in mice and pigs, the effect of bovine SP on priming the endometrium and on embryo survival is less clear. Bovine SP has been shown to induce a pro-inflammatory response in the uterus (Ibrahim et al., 2019). A recent study has identified TGFB-1 and TGFB-2 in bovine SP, which have been identified as the active

moieties in SP in mice and humans, to be also present in bull SP with absolute values of 63 ng and 58 ng, respectively (Rizo et al., 2019). Comparing this to humans the values for TGFB-1 and TGFB-2 are 220 to 240 ng/ml and 5 to 18 ng/ml, respectively while mouse and swine have been estimated at 74 ng/ml and 150 ng/ml, respectively (Nocera and Chu, 1995, O'Leary et al., 2011). The use of reproductive technologies and studies which have primed the endometrium with SP prior to AI, demonstrate that SP is not required for pregnancy (Odhiambo et al., 2009, Lima et al., 2009). However, a recent study has shown that SP supplementation at the time of AI increased the weight of heifer calves (Ortiz et al., 2019). An *in vivo* study where heifers were mated to vasectomised bulls shows there was an increase in conceptus length in embryos from Day 7 to 14 compared to unmated heifers (Mateo-Otero et al., 2020). Despite this, recent *in vitro* work by Fernandez-Fuertes et al. (2019) showed a detrimental effect of bull SP on endometrial RNA suggesting SP alone doesn't play a significant role in modifying the endometrial environment in preparation for pregnancy.

1.5.2 Consequences of the spermatozoa plus seminal plasma induced immune response for priming of the endometrium for pregnancy

Further *in vivo* bovine experiments suggest a greater uterine immune response to spermatozoa that has come in contact with SP rather than SP alone, in altering and preparing the endometrium for pregnancy. Mating to intact bulls induces endometrial transcriptional changes compared to vasectomised bulls (SP alone) (Recuero et al., 2020). Spermatozoa from intact bulls demonstrated a differential regulation of genes that play a role in bovine fertility. Genes including scavenger receptor class A, member 5 (*SCARA5*) were upregulated from intact bulls (Recuero et al., 2020) and it has a central role in the innate immune system and also in regulating histotroph secretions and remodelling tissue (Vitorino Carvalho et al., 2019). The downregulation of oxytocin (*OXT*) was observed in the endometrium from mating with intact bulls (Recuero et al., 2020) where previously high levels reported a decrease in embryo survival due to the increase in prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) which induced luteolysis and a drop in progesterone (Lemaster et al., 1999).

In terms of the immune response, when directly compared (intact vs vasectomised bulls), there was an upregulation of interferon gamma inducible protein 47 (*IFI47*) in the endometrium of heifers mated with intact bulls which has previously been identified in

more HF heifers compared to those classed as sub fertile (Minten et al., 2013). Another immune upregulated gene reported is Interleukin 17F (*IL17F*) (Recuero et al., 2020). *IL17* was also upregulated *in vitro* by bovine spermatozoa action, not SP (Ibrahim et al., 2019). In bovine explants *IL17A* has been shown to be regulated by Day 15 conceptuses, not by interferon-tau, suggesting a role in the embryo-endometrium crosstalk during pregnancy in cattle (Sanchez et al., 2019). *IL17* is a cytokine produced by T helper 17 lymphocytes which participates in the establishment of this maternal tolerance towards paternal antigens (Song et al., 2016). A recent study in mice has also confirmed that spermatozoa play an active role in initiating the female immune response, inducing cytokines that assist in PMN recruitment and T cell activation (Schjenken et al., 2021).

Another process of maternal tolerance which is driven by the male is the regulation of major histocompatibility complex (MHC) genes which enable antigen presenting cells to recognise self and non-self molecules.The MHC class II gene *BLA-DQB* had increased expression in heifers inseminated with intact bulls (Recuero et al., 2020). In a genome wide association study, MHC class II genes were significantly associated with pregnancy success in beef cows (Melo et al., 2017). In mice, MHC disparity leads to a stronger immune response to pregnancy with more T regulatory cells in the uterus which has been shown to be essential in maintaining immune tolerance to the conceptus (Robertson et al., 2018) and this disparity has also been shown to increase fetal weight and more efficient placental function (Hemberger et al., 2011). It has been previously been shown that males and females chose MHC dissimilar partners which works as a non-self/self perception that the off spring will be immunogenetically viable as the more polymorphic the gene the higher probability of fighting infectious diseases (Ziegler et al., 2010). However, AI does not allow mate choice, therefore a dam could be inseminated with semen from a sire with similar MHC although further studies are required (Melo et al., 2017).

1.6   Artificial insemination and field fertility variation

Animal breeding centres worldwide typically use microscopy based sperm assessments pre freezing and post thawing to assess sperm motility, viability and morphology. Despite these stringent QC checks, bulls that have passed often have reduced fertility in the field (Fair and Lonergan, 2018). It is clear from numerous studies, bull fertility is a multifaceted phenotype and despite it being the most reliable male fertility model (due to

hundreds to thousands of inseminations per bull) there is 'noise' surrounding fertility datasets which are attributed to male and female factors, the AI procedure itself and the environment (Amann and DeJarnette, 2012, Sellem et al., 2015). Accurate predictions require both biological and statistical knowledge (Utt, 2016). Futhermore, 1000 inseminations are required to accurately detect differences, ± 4%, between high and LF bulls and a minimum of 500 insemination to detect differences ± 6% (Amann and DeJarnette, 2012). The sire fertility model used in Ireland is the animal AAM (Berry et al., 2011) and this is typical of many other countries. This is a multiple mixed regression model that adjusts the fertility values by a multitude of fixed effects such as successful pregnancies, number of inseminations, days in milk and cow parity; Non-additive effects such as heterosis, fresh, frozen or sexed semen and random effects such as cow breed, AI technician, herd and service bull (Berry et al., 2011). The AAM reports sire fertility around the mean of the population (zero). Sires can vary in fertility by up to 20-30%, albeit having passed all QC checks in the AI centre.

As outlined at the beginning of this review, there are numerous studies which have used *in vitro* assays to account for this variation in field fertility including sperm morphometric, motility and flow cytometry analysis (Gliozzi et al., 2017, Kumaresan et al., 2017). These resulted in a panel of assessments and a linear model that accounted for 47% of bull fertility variation (Bernecic et al., 2021). However, it is clear that there is a gap in the scientific literature in the understanding of the etiology and molecular mechanisms underpinning sire sub-fertility. Therefore, a more detailed analysis of sperm interactions with the female reproductive tract, in particular, the uterus is required. Given the aforementioned evidence outlined in this review, we hypothesised that HF and LF bulls differ in their interactions throughout the female reproductive tract. We hypothesised that in particular, spermatozoa may have a differential immunological response in the uterus from HF and LF bulls thereby affecting spermatozoa transport to the site of fertilisation and/or priming the endometrium for pregnancy. In addition, we aimed to profile both the miRNA and mRNA profiles of spermatozoa from HF and LF bulls further comprehend their role in fertilisation and early embryonic development.

1.7   Objectives

In order to address the above hypotheses, we used a reliable bull fertility animal adjusted model to identify and select HF and LF bulls used for AI based on > 500 inseminations per bull. The specific objectives of this thesis were:

To assess sperm transport in the female reproductive tract using spermatozoa from high and low field fertility bulls through a range of *in vitro* assessments.

To compare the uterine inflammatory response to frozen-thawed spermatozoa from high and low fertility bulls using an *ex vivo* model

To characterise the uterine transcriptomic response to spermatozoa from high and low fertility bulls using and *in vivo* model

To examine the disparity in sperm mRNA and miRNA fingerprints and associated biological pathways between high and low fertility bulls.

Chapter 2 An *ex-vivo* assessment of differential sperm transport in the female reproductive tract between high and low fertility bulls

2.1 Abstract:

Despite passing all quality control checks at animal breeding centres, bulls with apparently normal semen quality can yield unacceptably low field fertility rates. This study took an *ex-vivo* approach to assess if bulls of divergent field fertility differ in the ability of their spermatozoa to interact with the female reproductive tract and its secretions. Six HF and six LF Holstein Friesian bulls ($+4.0 \pm 0.2$ and -15.7 ± 3.13, respectively; adjusted mean fertility $\pm$ s.e.m. mean of the bull population was 0) were selected from a population of 840 bulls with >500 field inseminations per bull. Thawed spermatozoa from each bull were analysed across a range of *in vitro* assays to assess their ability to transverse the female reproductive tract including; motility and kinematic parameters using computer-assisted sperm analysis, viability, membrane fluidity and acrosomal integrity using flow cytometry as well as mucus penetration tests, rheotactic behaviour and sperm binding ability to the oviductal epithelium. While there was no significant difference between HF and LF bulls in most of the sperm motility, kinematic and sperm functional parameters (namely, motility, average path velocity (VAP), linearity (LIN), straightness (STR) , amplitude of lateral head movement (ALH)), viability, membrane fluidity or acrosome intactness, HF bulls had higher VCL compared to the LF group ($P<0.05$) and a higher straight-line velocity (VSL) was observed although it did not reach statistical significance ($P=0.08$). There was no difference between treatment groups in the ability of spermatozoa to penetrate periovulatory cervical mucus or in their rheotactic response ($P>0.05$). Interestingly, there was a positive correlation between the VSL of spermatozoa and their rheotactic response ($r=0.45$, $P<0.001$) and further linear regression analysis indicated 18.9% of the variance in sperm rheotaxis was accounted for by VSL. A higher number of spermatozoa from the HF group compared to the LF group bound to oviductal explants (15.1 ± 0.98 and 12.5 ± 0.76, respectively; mean $\pm$ s.e.m; $P<0.05$). In conclusion, the differences in the kinematics of sperm motility and ability to bind to oviductal explants between HF and LF bulls were modest and are unlikely to explain the inherent differences in fertility between these cohorts of bulls.

2.2 Introduction:

Artificial insemination centres internationally typically use microscopy-based sperm assessments pre- and post-thawing to assess sperm motility, viability and morphology. Despite these stringent QC checks, bulls that have passed, often display reduced fertility in the field (Fair and Lonergan, 2018). Bull fertility is a multifaceted phenotype and accurate predictions require both biological and statistical knowledge (Utt, 2016). The sire fertility model used in Ireland is the AAM (Berry et al., 2011), typical of the model used in many other countries. This is a multiple regression mixed model that takes into account a multitude of fixed effects such as number of inseminations, year and month of service, days since calving and cow parity, non-additive effects such as heterosis, semen type (fresh or frozen) and random effects such as cow breed, cow genotype, AI technician, herd and service bull (Berry et al., 2011). Data generated through this AAM highlight sires varying in fertility by up to 20-30%, despite the fact that their semen had passed all QC checks in the AI centre.

The underlying causes of differential fertility between service sires are unclear but are likely to be multifactorial, encompassing sperm transport and fertilizing ability, preimplantation embryonic development, and development of the embryo and placenta after conceptus elongation and pregnancy recognition. A variety of tools have been used to identify *in vitro* markers of fertility, which can be used to explain some of the variation in bull fertility. These include CASA that provides a large volume of data on kinematic parameters which have proved useful in identifying associations between sperm characteristics and functional aspects (Ramón and Martínez-Pastor, 2018). Other tools involve flow cytometry for the assessment of parameters such as mitochondrial activity, DNA integrity and acrosomal status (Holden et al., 2017a, Odhiambo et al., 2014, Sellem et al., 2015). Indeed, recently, our group used a linear model of combined data from *in vitro* assessments to explain 47% of the variation in bull field fertility (Bernecic et al., 2021). However, these studies do not directly inform us of the etiology underpinning sire subfertility and it is unclear if bulls differ in the ability of their spermatozoa to get to the site of fertilisation, interact with the oocyte, complete fertilisation or to sustain embryo and foetal development.

Measures of the ability of spermatozoa to progress through various media can be used as a proxy for their ability to transit from the site of semen deposition (uterine body in AI) to the site of fertilisation (oviduct). Our group (Al Naib et al., 2011) and others (Tas et

al., 2007) have previously demonstrated that spermatozoa from HF bulls were better able to swim through artificial mucus than spermatozoa of LF bulls; however, others found no differential effect (Verberckmoes et al., 2002). The ability of bovine spermatozoa to swim against an active fluid flow (referred to as rheotaxis) has been previously characterised (El-Sherry et al., 2014, Johnson et al., 2017). However, there are no published studies on the rheotactic response of spermatozoa from bulls differing in field fertility.

Sperm migration in the female reproductive tract is a selective process with only hundreds of spermatozoa reaching the oviduct (Mahe et al., 2021). Binding to the oviductal epithelium is critical in establishing a viable population of fertile spermatozoa in the oviduct where bovine sperm binding is thought to be mediated through fucose recognition (Suarez, 2016). β-defensin are host defence peptides that coat spermatozoa and have been identified as having a role in modulating the immune response to enhance spermatozoa survival in the oviduct (Meade and O'Farrelly, 2018). Previous work by our group has shown HF bulls with a specific β-defensin haplotype had higher sperm binding to the oviduct (Whiston et al., 2017).

Despite average fertilisation rates of >90% in cows and heifers being reported, birth rates to a single insemination are considerably lower due to the occurrence of embryonic mortality (Forde and Lonergan, 2017). Most losses occur during the peri-implantation stage of pregnancy due to poor oocyte quality and early embryo development as well as implantation and placentation failure (Wiltbank et al., 2016). While early pregnancy events in cattle are undoubtedly influenced by genetic contributions from the sire (Starbuck et al., 2004), the exact nature of these contributions and the underlying mechanisms are unclear. There is growing evidence that spermatozoa influence post fertilisation events through the cargo of non-coding RNAs that they bring with them into the oocyte at fertilisation (Jodar et al., 2013). O'Callaghan et al. (2021) recently assessed whether subfertility in AI bulls is due to issues of sperm transport to the site of fertilization, fertilisation failure, or failure of early embryo or conceptus development. They reported a higher accessory sperm number and embryos at more advanced stages of development by Day 7 in superovulated heifers inseminated with spermatozoa from HF bulls. A study by Ortega et al. (2018) reported a two-fold higher pregnancy loss between Days 19 and 33 following the insemination of superovulated heifers with frozen-thawed semen from LF sires. In addition, more unfertilised oocytes were recovered from heifers inseminated with LF spermatozoa compared to HF bulls. Taken together this suggests that processes

involved in sperm transport in the female tract resulted in decreased fertilisation with fertility from LF bulls (Ortega et al., 2018).

Despite the aforementioned studies, there is still a gap in our ability to explain the high proportion of inter-individual variation in field fertility between bulls and to locate exactly where along the developmental axis this variation occurs. In this study, a series of *ex vivo* experiments were designed to assess the ability of spermatozoa from HF and LF bulls to traverse the female reproductive tract and to mimic physical challenges that spermatozoa face *in vivo* to further understand sperm interactions with the female reproductive tract and its secretions.

2.3   Materials and Methods:

2.3.1   Reagents

Unless otherwise stated, all chemicals were sourced from Sigma-Aldrich (Dublin, Ireland).

Experimental Design:

2.3.2   Bull Selection:

All bulls used in this study were sourced from commercial AI centres in Ireland. These bulls were selected according to the AAM (Berry et al., 2011) and were selected from a population of bulls with > 500 inseminations (mean adjusted to 0%; Figure 2.1). Frozen-thawed spermatozoa from 12 Holstein Friesian bulls (six with high field fertility and six with low field fertility) were selected. The average AAM for LF bulls was -15.7% and for HF bulls was 4.0% with a minimum difference of 12.5% between the two treatment groups (Table 2.1).
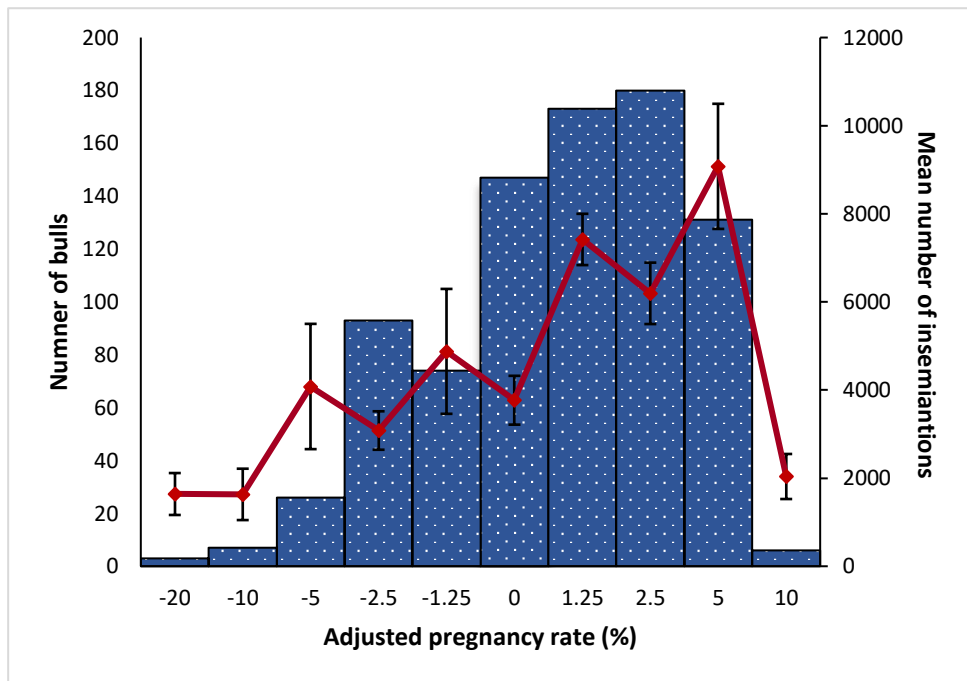
Figure 2.1 The population of Holstein Friesian bulls (n=840) with >500 inseminations from which six HF and six LF bulls were chosen for this study. The number of bulls (left y-axis) are shown in the histogram and the mean number of inseminations per bull (± s.e.m.) is represented by the line and read from the right y-axis.

Table 2.1 Fertility data of high and LF bulls with >500 inseminations based on the animal adjusted model (AAM) (Source: Irish Cattle Breeding Federation,www.icbf.ie). The mean of the population was zero.

Bull	AAM (%)	Number of inseminations	Fertility status
1	-28.73	777	Low
2	-20.23	1724	Low
3	-15.23	1429	Low
4	-12.13	519	Low
5	-8.93	510	Low
6	-8.93	1072	Low
7	+ 3.57	12417	High
8	+ 3.67	5121	High
9	+ 3.87	37856	High

10	+ 3.87	17323	High
11	+ 3.97	99953	High
12	+ 4.97	34859	High

2.3.3 Experiment 1: Computer assisted spermatozoa analysis and flow cytometry

The aim of this experiment was to assess *in vitro* sperm functional parameters including: CASA motility, as well as viability, membrane fluidity and acrosomal intactness of spermatozoa from bulls with high and low field fertility. Three ejaculates from each of six HF and six LF bulls were assessed. One straw per ejaculate was assessed for viability, membrane fluidity and acrosome intactness using flow cytometry while two straws per ejaculate were assessed for motility and kinematic parameters using CASA. For CASA and flow cytometry analysis, spermatozoa were diluted to a final concentration of 10 x 10^6 spermatozoa per ml and 2 x 10^6 spermatozoa per ml, respectively.

2.3.4 Experiment 2: Cervicovaginal mucus penetration

The aim of this experiment was to assess the ability of spermatozoa from HF and LF bulls to penetrate cervicovaginal mucus. Mucus was collected from heifers in spontaneous standing oestrus as described by Fernandez-Fuertes et al. (2016). Spermatozoa from one straw from each of three ejaculates from six HF and six LF bulls were assessed for their ability to penetrate cervicovaginal mucus *in vitro*.

2.3.5   Experiment 3: Rheotactic response
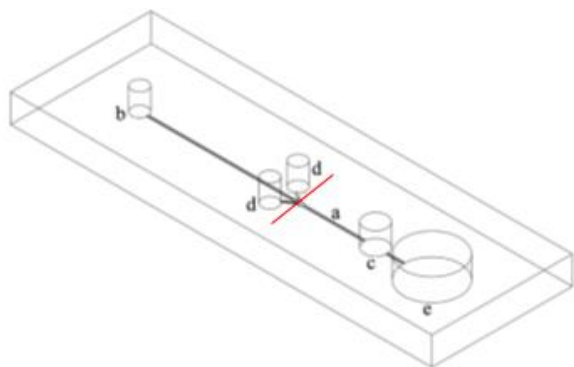
Figure 2.2 The microfluidic device used to assess sperm rheotaxis, measuring 300 µm in width, 100 µm in depth and 10 mm in length (a). The device was connected to a syringe pump and the device was primed with media before 20 µL of sperm sample was inserted (c). Spermatozoa were allowed to orient against a flow of 55.5 µm/s (positive rheotaxis) for 10 minutes before the number of spermatozoa at the 10 mm mark (as marked in red) were counted using an inverted microscope over a two minute period (Romero-Aguirregomezcorta et al., 2021).

The aim of this experiment was to assess the ability of spermatozoa from HF and LF bulls to swim against a flow i.e. the rheotactic response. A microfluidic device (Figure 2.2) was attached to a syringe pump and the rheotactic response of spermatozoa was assessed by assessing the number of spermatozoa over a 2 min period that passed the 10 mm point along the microchannel after a 10 min period. Two straws from each of three ejaculates were assessed from six HF and six LF bulls.

2.3.6   Experiment 4: Spermatozoa-oviduct binding

The aim of this experiment was to assess the binding ability of spermatozoa from HF and LF bulls to the oviductal epithelium. Reproductive tracts were collected from non-pregnant nulliparous heifers (n=18; heifer = replicate) at a commercial abattoir. The stage of the cycle was determined to be at the follicular phase as per Ireland et al. (1980). Explants were cultured from the isthmic portion of the oviduct and explants from both oviducts were pooled for each tract as described by Lyons et al. (2018). Three ejaculates from six HF and six LF bulls were assessed, with one ejaculate from each of one high and one LF bull assessed across each reproductive tract.

2.3.7 Spermatozoa preparation:

Straws were thawed at 37°C for 30s in a water bath. The sperm concentration from each was calculated using a haemocytometer and diluted 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 10M, Sodium Pyruvate 1 mM, Sodium Lactate 25.4 mM, bovine serum albumin (BSA) 6 mg/ml; pH= 7.4; 37°C). For Experiments 2, 3 and 4, spermatozoa were washed by centrifugation at 300 g for 5 min prior to sperm concentration assessment. For flow cytometry analysis (Experiment 1), TALP was modified without BSA, as BSA can produce a fluorescence signal in the presence of Merocyanine 540 (M540) (Banerjee et al., 2012).

2.3.8 Computer assisted spermatozoa analysis:

Sperm samples were assessed on a Sperm Class Analyzer® CASA system (SCA Microptic SL, Barcelona, Spain). The system included an Olympus®CX41 negative phase contrast microscope, a digital camera to capture images Basler A312 (Basler Inc., Vision-Technology, Ahrensburg, Germany), a Thermo® plate at 37°C and SCA® computer software. The sperm concentration was calculated using a haemocytometer and diluted to a final concentration of 10 x 10^6 spermatozoa per ml in TALP. A 3 µL sample was placed in a 20 micron Leja chamber (IMV Technologies, France) and was then analysed using the SCA® system. A minimum of 200 spermatozoa were analysed in six fields of view at 25 frames/second.

The kinematic parameters assessed included: total motility (%), defined as the % of cells with a velocity >20 µm/s; Progressive motility, defined as the number of motile cells swimming in a linear fashion; VCL (µm/s), defined as the velocity of a sperm head along the curvilinear trajectory; VSL (µm/s), the velocity of a sperm head along the straight line before the first and last detected position; VAP (µm/s), the velocity of a sperm head along a derived, smoothed trajectory path; STR(%) which is calculated as VSL/VAP and is defined as the linearity of the average path distance; LIN (%) which is calculated as the ratio of the of the straight line distance to the real path distance (VSL/VCL); and, the ALH (µm).

2.3.9   Flow cytometry assessments:

Sperm functional assessments were performed on a CytoFLEX flow cytometer from Beckman Coulter (Labplan, Dublin, Ireland). Analyses were carried out as described by Bernecic et al. (2021). Briefly, quality control fluorophores were used prior to each experiment to verify optical alignment and a sperm population gated following identification with the forward and side scatter. M540 was sourced from Sigma-Aldrich while Alexa Fluor 647-peanut agglutin (AF647-PNA) and 4',6-diamidino-2-phenylindole (DAPI) were sourced from Invitrogen (Biosciences, Dublin, Ireland). A final concentration of 0.5 µg/ml A7647-PNA, 0.8 µM M540 and 2 µM DAPI were used in combination to assess acrosome integrity, membrane fluidity and viability, respectively. Samples were incubated for 15 min at 37ºC prior to assessment. Compensation was performed to correct spectral overlap and 10,000 events were captured for each sample.

2.3.10   Mucus penetration test:

Mucus penetration tests were carried out as described by Al Naib et al. (2011) and (O'Hara et al., 2010) with some minor modifications. Briefly, cervical mucus was collected from heifers in standing oestrus. Semen was diluted to 10 x 10^6 spermatozoa per ml in TALP medium containing Hoechst 33342 stain (10 µg/ml). Flattened capillary tubes (0.3 mm × 3.0 mm × 100 mm; CM Scientific, UK) were marked at 10 mm intervals between 10 and 80 mm. These were then filled with mucus using an adapted 1 mL syringe. Two capillary tubes were placed vertically in a 1.5 mL eppendorf containing a 100 µL aliquot of the stained spermatozoa from one bull. In each replicate, each of the bulls were represented by two capillary tubes. The tubes were incubated for 45 min at 37 °C. After incubation, the tubes were placed on a hotplate at 45 °C for 1 min to immobilize the spermatozoa. Spermatozoa were counted from wall to wall across the tube, within the width of a single field of view, at each 10 mm interval using a fluorescent microscope (10x; Olympus BX 60).

2.3.11   Rheotaxis:

The microchannel (300 µm x 100 µm x 10 mm; width x depth x length) of a microfluidic device was primed with TALP media using a Hamilton 1 mL glass syringe and a Harvard PHD ULTRA syringe pump (Standard Infuse/Withdraw PHD ULTRA™ Syringe Pump, Harvard Apparatus, Holliston, USA) . The velocity was then set at 555 µm/sec for 20 sec

before 20 μL of a diluted sperm sample was inserted. The velocity was decreased stepwise over three minutes to 55.5 μm/s. The velocity of 55.5 μm/s was kept constant over five minutes and then the number of spermatozoa passing the 10 mm mark in the microchannel was counted over a 2 min period. Spermatozoa were assessed in the microchannel under $200 \times$ magnification on an inverted microscope (CK40; Olympus).

2.3.12 Preparation of oviductal explants and sperm binding assay:

Bovine oviductal explants were prepared and sperm binding was assessed as previously described by our group (Lyons et al., 2018). Reproductive tracts from the follicular phase of the oestrous cycle were collected from non-pregnant cross-bred beef heifers at a commercial abattoir immediately post mortem and transported to the laboratory within 1 h. Briefly, oviducts were trimmed free of connective tissue, washed twice with phosphate-buffered saline solution and the isthmic segment isolated at ambient temperature. The epithelial cells were extruded in sheets by squeezing the oviduct with a sterile glass slide, fragmented by pipetting, centrifuged for 1 min (200 g, at 38°C), transferred to medium 199 (M199) culture medium supplemented with fetal calf serum (10%) and gentamicin sulphate (0.25 mg/mL) and incubated for 1 h at 38°C in 5% CO_2 in air to form everted vesicles with apical ciliated surfaces oriented outwardly. Explants were used for binding assessments within 5 h of animal slaughter. M199 culture media was added (5 mL) to explants of each reproductive tract and centrifuged at 200 g for 5 min at 38°C. After centrifugation, the supernatant was removed and explants (20 μL) from each tract were added to sperm aliquots (140 μL) that were prestained for 30 min with 10 μg/ml Hoechst 33342 at 38°C for enhanced binding visualisation (Al Naib et al. 2011). The final concentration of spermatozoa in the binding assay was 5 x 10^6 spermatozoa per ml. After 30 min incubation at 38°C in 5% CO_2, loosely bound spermatozoa were removed from explants by gently pipetting through two 75 μL droplets of M199 media on a warmed 24-well culture plate (38°C). For each treatment 10 μL of the spermatozoa – explant mixture was placed on a slide, a coverslip was added and samples were viewed at 400x using a microscope fitted with a heated stage at 38°C (BX60; Olympus) under half light and half fluorescence. The number of spermatozoa bound were recorded and the relative surface area of each explant was determined using a micrometer. Ten explants of each treatment were assessed at random and sperm binding density was calculated by determining the number of spermatozoa bound per 0.1 mm^2 explant surface. The evaluator was blinded to treatment for all sperm binding assessments.

2.3.13 Statistical Analysis:

For all experiments, data were analysed using the Statistical Package for Social Sciences (IBM SPSS for Windows 25). Normality of distribution was assessed using the Shapiro-Wilko test and a Univariate analysis of variance (ANOVA) was performed (factor: treatment). For Experiments 1 and 4, Pearson correlation coefficients were calculated and a linear regression analysis was performed between kinematic parameters and the rheotactic response. The level of significance was set at $P<0.05$. All results are presented as mean ± s.e.m.

2.4   Results:

2.4.1   Experiment 1: Spermatozoa from HF bulls exhibit increased curvilinear velocity

There was no difference between HF and LF treatments in sperm kinematic parameters including motility (total and progressive), VAP, STR, LIN and ALH (Table 2.2; $P>0.05$). Spermatozoa from HF bulls exhibited a higher mean VCL than spermatozoa from LF bulls (88.6 µm/s ± 5.14 and 76.1 µm/s ± 4.25, respectively; Table 2.2; $P<0.05$). A trend was observed for the parameter VSL with a higher VSL from spermatozoa of HF bulls although it did not reach significance ($P=0.08$). There was no difference in the viability, membrane fluidity or acrosome intactness of spermatozoa from HF and LF bulls (Table 2.2; $P>0.05$). Figure 2.3 represents histograms and scatter plots obtained from viability, membrane fluidity and acrosome intactness assessments.

2.4.2   Experiment 2: Spermatozoa from HF and LF bulls do not differ in their ability to penetrate cervicovaginal mucus

There was no difference in the ability of spermatozoa from HF and LF bulls to penetrate cervicovaginal mucus (Table 2.2; $P>0.05$). Within treatment, the number of spermatozoa able to penetrate cervicovaginal mucus ranged from 178 to 301 and 160 to 303 for the HF and LF bulls, respectively.

Table 2.2 Mean ± s.e.m post-thaw sperm functional, morphological and intracellular variables assessed in Holstein Friesian bulls of high and LF (n = six bulls per phenotype; Experiment 1, 2 and 3).

Parameter	LF	HF	Difference in fertility (P value)
CASA			
Total motility (%)	56.6 ± 3.4	56.9 ± 3.4	NS
Progressive motility (%)	40.8 ± 2.9	43.9 ± 3.2	NS
Curvilinear velocity (VCL; μm/s)	76.1 ± 4.2	88.6 ± 2.6	P<0.05
Straight-line velocity (VSL; μm/s)	47.2 ± 2.6	55.9 ± 8.9	NS
Average path velocity (VAP; μm/s)	56.0 ± 3.4	64.6 ± 3.3	NS
Linearity (LIN; %)	55.6 ± 1.7	56.4 ± 2.8	NS
Straightness (STR; %)	74.2 ± 1.3	76.4 ± 2.0	NS
Amplitude of lateral head movement (ALH; μm)	3.3 ± 0.2	3.8 ± 0.1	NS
Wobble (WOB; %)	70.6 ± 1.5	69.7 ± 2.0	NS
Mucus penetration	235.2 ± 60.8	248.5 ± 66.4	NS
Rheotaxis	54.3 ± 5.4	84.8 ± 30.7	NS
Flow cytometry			
Viable (%)	53.0 ± 2.7	42.7 ± 4.3	NS
Acrosome intact (%)	76.4 ± 2.8	76.3 ± 1.8	NS
High membrane fluidity (%)	50.8 ± 2.2	59 ± 4.3	NS

Data for all variables were averaged across ejaculates within bull (3 ejaculates per bull), then across treatment (high or LF).

NS = non-significant.

Mucus penetration = number of spermatozoa traversing cervicovaginal mucus
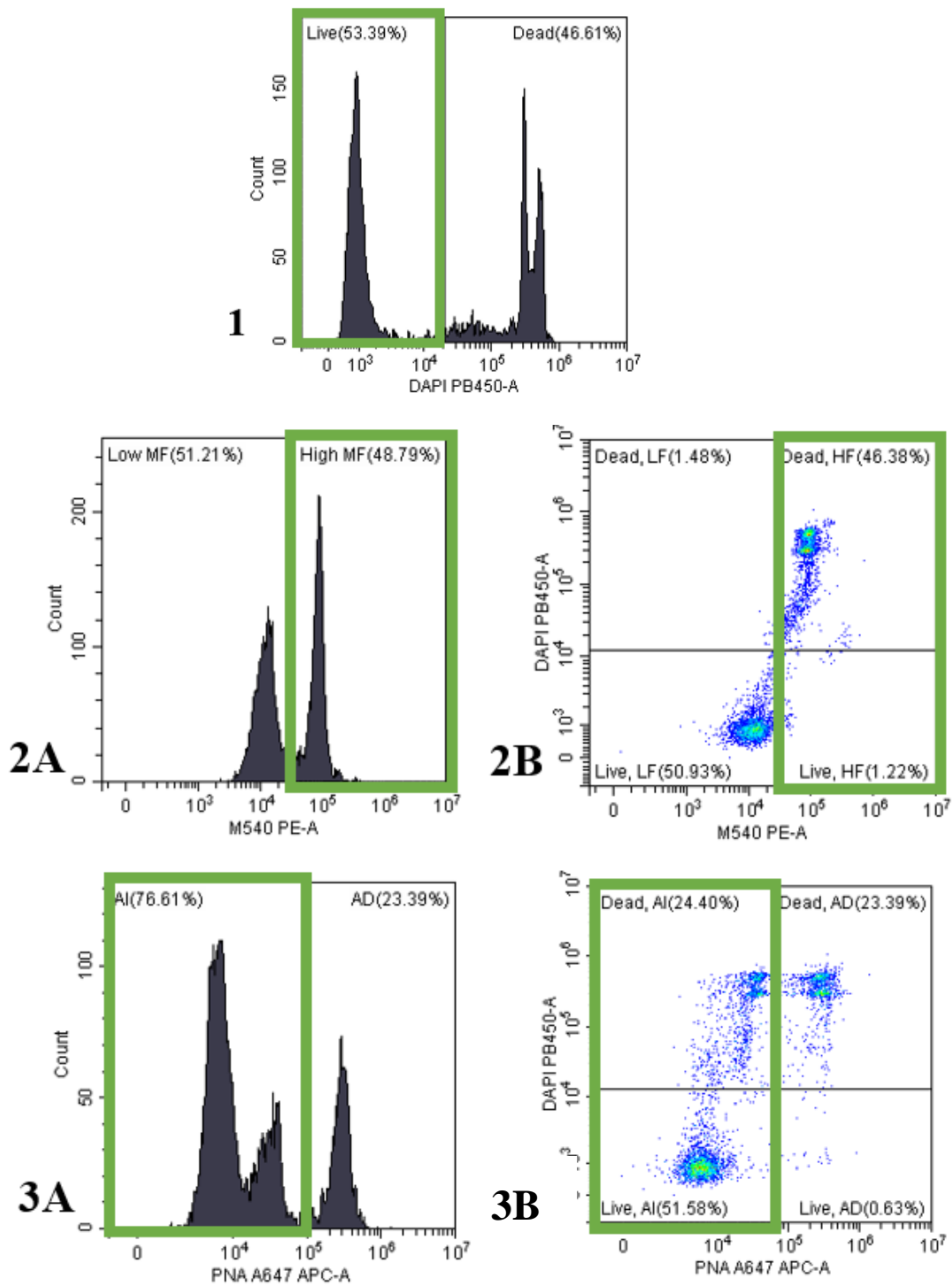
Figure 2.3 Representative histograms and scatter plots obtained following the simultaneous assessment of viability (1), membrane fluidity (2A and B) and acrosome intactness (3A and B) using DAPI, M540 and AF647 respectively. The areas of the histograms or plots highlighted by coloured boxes identify the populations analysed in this study. For membrane fluidity, the green coloured boxes highlight all spermatozoa

with high membrane fluidity, as observed by the scatter plot and histogram (2A and B). For acrosome intactness, the green coloured boxes highlights all spermatozoa that are acrosome intact, as shown by the scatter plot and histogram (3A and 3B).

2.4.3 Experiment 3: Straight line velocity is positively correlated to the rheotactic response

There was no difference in the rheotactic response of spermatozoa from HF and LF bulls (Table 2.2; P>0.05). Further analysis of the data illustrated a moderate positive correlation observed between the kinematic parameter VSL and the rheotactic response of spermatozoa (Figure 2.4; r = 0.45, P<0.001). Linear regression analysis demonstrated that 18.9% of variation in the rheotactic response was accounted for by VSL.
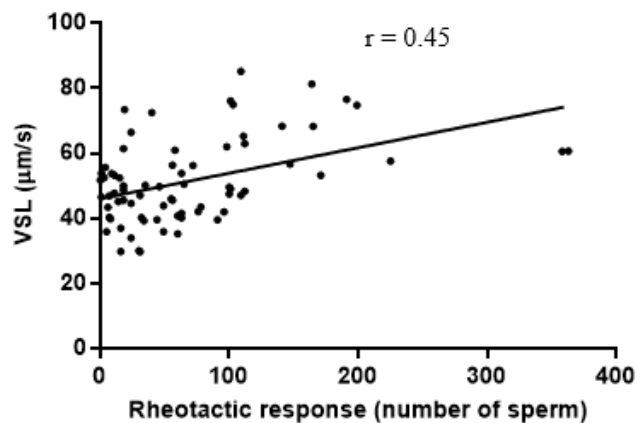
Figure 2.4 Correlation between rheotactic response and Straight Line Velocity (VSL µm/s). There was a moderate, positive Pearson correlation (r = 0.45, P<0.001) between the straightness of spermatozoa and their rheotactic response. Further linear regression yielded an adjusted $R^2 = 0.189$ (P <0.001), with 18.9% of the variance in the rheotactic response being accounted for by VSL.

2.4.4 Experiment 4: Spermatozoa from HF bulls have a greater ability to bind to the oviductal epithelium.

Spermatozoa from HF bulls exhibited a 21% higher binding affinity for oviductal epithelium compared to the those from LF bulls (15.1 per 0.1 mm^2 ± 0.98 and 12.5 per 0.1 mm^2 ± 0.76, respectively; Figure 2.5; P<0.05).
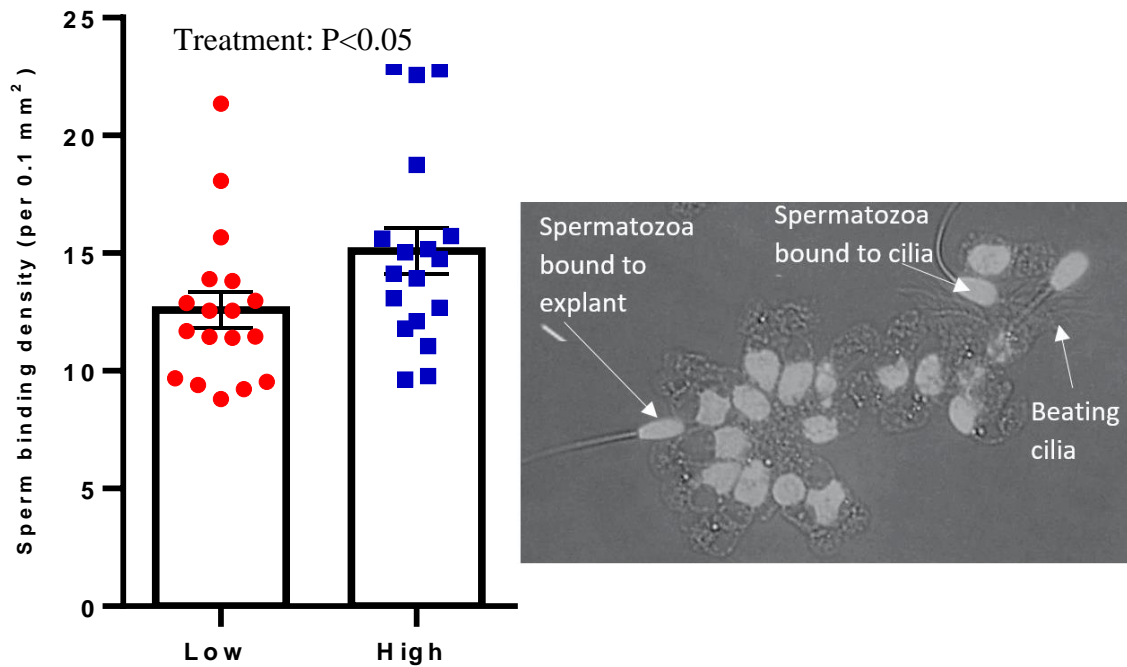
Figure 2.5 Binding density of spermatozoa from high and LF bulls to bovine oviduct epithelial cell explants. Binding density is expressed as the number of bound spermatozoa per 0.1 mm^2 explant. Each data point represents an individual bull (n=6 per fertility group) assessed across three separate ejaculates. Data are the mean ± s.e.m. (n = 18 replicates). Inserted image to the right shows three spermatozoa bound to the cilia of an explant.

2.5   Discussion

This study sought to establish and further the understanding of the etiology underlying the variation in bull fertility by assessing sperm functional parameters and using *ex vivo* assays that mimic the journey of spermatozoa along the female reproductive tract. Through our broad analysis of sperm functional attributes, including kinematic parameters, viability, acrosomal integrity, membrane fluidity, mucus penetration, rheotactic response and sperm binding ability to oviductal epithelium, we identified differences in VCL and in the ability of spermatozoa to bind to the oviductal epithelium which is a pre-requisite for fertilisation. Overall, these differences were subtle and are unlikley to explain the large variation in field fertility between the two cohorts of bulls studied.

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In terms of sperm transport to the site of fertilisation, spermatozoa from high and LF bulls had similar *ex vivo* functional attributes. CASA and flow cytometry assessments and their link with fertility have been widely studied (Sellem et al., 2015, Kutchy et al., 2019, Gliozzi et al., 2017, Bernecic et al., 2021). Gliozzi et al. (2017) reported differences in motility, STR, LIN and viability while Kutchy et al. (2019) reported a significant difference in VAP, ALH and STR between high and LF bulls. Results from the present study indicated an increase in VCL in the HF group which has been shown previously to have a strong positive correlation with non-return rates in Holstein bulls (Shojaei et al., 2012). Consistent with the observations of Kutchy et al. (2019), there were no differences in the overall motility between high and LF bulls. The acrosome integrity levels in the current study were in line with previous studies of bull spermatozoa (Gurler et al., 2015, Sellem et al., 2015). There was no difference in the ability of spermatozoa from high and LF bulls to penetrate cervicovaginal mucus which was in agreement with some studies (Verberckmoes et al., 2002) but not with others that used artificial mucus (Al Naib et al., 2011, Tas et al., 2007).

Sperm rheotaxis has been described as a passive physical process from hydrodynamic interactions between the flagellum of spermatozoa and fluid flow resulting in low energy consumption, thereby preserving the spermatozoa's energy for the journey to the site of fertilisation (Zhang et al., 2016, Schiffer et al., 2020). In this study, although there was no difference in sperm rheotaxis between spermatozoa from HF and LF bulls, an increase in VSL that had a positive correlation with the sperm rheotactic response was reported, with 18.9% of the variation in the rheotactic response accounted for by VSL. Although it did not reach statistical significance (P=0.08), a trend was observed with higher VSL from spermatozoa from HF bulls, and therefore should be pursued in future work.

This parameter could be used as an indicator of the ability of spermatozoa to reach the site of fertilisation and our results are in agreement with a previous study which demonstrated that bovine sperm samples with higher VSL as well as VCL and VAP had a superior rheotactic progression (El-Sherry et al., 2017). Regardless of differences between HF and LF bulls, the variation between individual bulls, even within the same fertility group, must also be noted and was observed across all sperm parameters assessed. Figure 2.5 gives a clear example of the variation between individual bulls and gives further justification that the variation between bulls is not due to one factor/assay. It emphasises the requirement to further our understanding of the etiology of sire subfertility as demonstrated in this study.

The differences in bull fertility were primarily manifested in differences in sperm binding to the oviduct epithelial cells. This observation is consistent with previous studies which linked sperm binding with fertility (De Pauw et al., 2002, Whiston et al., 2017). The oviductal receptors for spermatozoa indicate carbohydrates are part of the sperm binding site where bull spermatozoa favourably bind to the Lewis A motif (Suarez, 2016). Proteins that bind to spermatozoa during maturation in the epididymis and at ejaculation that are involved in forming the sperm resevoir include integrin $\alpha5\beta1$ (Osycka-Salut et al., 2017), BSPs 1,3 and 5 (Gwathmey et al., 2003, Gwathmey et al., 2006) and beta-defensin 126 (Lyons et al., 2018). As it has been previously reported, the beta-defensin haplotype present in HF bulls increased bull sperm binding to the oviduct (Whiston et al., 2017), however, further research is required to understand the role of BSPs and integrin $\alpha5\beta1$ on spermatozoa and their relationship to bull fertility. It is plausible LF spermatozoa are lacking or have modified versions of these sperm proteins impairing their ability to bind to the epithelial cells thus preventing the formation of an adequate sperm reservoir in the oviduct.

In conclusion, this study demonstrated differences between HF and LF bulls including VCL and a higher binding ability of HF spermatozoa to the oviductal epithelium. However, these differences are subtle and unlikely to explain the large variation in bull fertility.

Chapter 3   Comparison of the uterine inflammatory response to frozen-thawed spermatozoa from high and low fertility bulls

3.1 Abstract

Some bulls with apparently normal semen quality yield unacceptably low pregnancy rates. We hypothesised that a differential uterine immunological response to spermatozoa from HF and LF bulls may contribute to these differences. The experimental model used was heifer follicular phase uterine explants incubated with frozen-thawed spermatozoa from HF and LF bulls (3-5 replicates per experiment). Inflammatory gene expression of *IL1A, IL1B, IL6, TNFA* and *CXCL8* were assessed by qPCR and IL1-β and IL-8 were quantified in explant supernatants by ELISA. Neutrophil binding affinity to spermatozoa from high and LF bulls was also assessed. There was a significant up-regulation of *IL1A, IL1B* and *TNFA* from frozen-thawed sperm, irrespective of fertility status, compared to the unstimulated control. This response was confirmed at the protein level, with an increase of IL-1β and IL-8 protein concentrations by 5 and 2.7 fold, respectively ($P<0.05$). Although no significant differences in the inflammatory response at the gene or protein level were evident between HF and LF bulls, more spermatozoa from low compared to HF bulls bound to PMNs ($P<0.05$). Using bulls of unknown fertility, cauda epididymal sperm (CES) plus SP upregulated *IL6* ($P<0.05$) but there was no upregulation of any inflammatory gene expression for CES alone. Overall, this *ex vivo* study demonstrated an upregulation of inflammatory gene expression in the uterus in response to frozen-thawed bull spermatozoa. While there was no difference between spermatozoa from HF and LF bulls, there was a greater binding affinity of LF sperm by PMNs.

3.2 Introduction:

Through the use of AI semen from one bull can be used to inseminate tens of thousands of cows and which enables rapid genetic progress. The corollary however, is that the use of a sire with lower than expected fertility can result in widespread significant economic losses for farmers. To reduce this risk, cattle breeding centres use a range of microscopy-based quality control checks of sperm motility and morphology prior to releasing semen for use on farm. However, despite these stringent checks, some bulls with apparently normal spermatozoa still have significantly reduced fertility when used in the field, illustrating the recognised limitations in these *in vitro* checks as predictors of bull fertility (Amann and DeJarnette, 2012, Utt, 2016). Multiple previous studies have used flow cytometry based techniques to assess parameters that may account for the variation in bull field fertility including sperm capacitation status, mitochondrial activity, DNA integrity and sperm acrosome status to name but a few (Sellem et al., 2015, Dogan et al., 2013, Odhiambo et al., 2014). While a combination of these parameters can account for up to 40% of the variation in bull fertility (Sellem et al., 2015) there is still a significant proportion of the variation in bull fertility unexplained.

In contrast to the situation with natural mating where bull spermatozoa are deposited in conjunction with SP, a significantly reduced number of spermatozoa are used in AI (typically 10-20 million spermatozoa) with only marginal amounts of SP deposited, in order to achieve pregnancy rates equivalent to that of natural mating (as reviewed by (Miller, 2018)). Additionally, whereas spermatozoa are deposited in the vagina during natural mating, the site of sperm deposition during AI is in the uterine body, thereby bypassing the cervix. While depositing spermatozoa higher up the tract may expedite sperm migration, the consequences of sperm deposition in the uterine body, particularly in terms of interactions with epithelial and immune cells of the female tract remain poorly understood.

It is now clear from studies that semen (i.e. spermatozoa plus SP) induces an immune response in the uterus – and this response is common across several species including in mice, pigs, and horses (Katila, 2012, Robertson, 2007). Early work in rodent and pigs, showed a specific recruitment of immune cell including neutrophils, macrophages and dendritic cells into the uterine lumen in response to SP (Robertson, 2007). The authors proposed that this response promoted phagocytic clearance of spermatozoa but also uterine tissue remodelling in preparation for embryo development. A recent *in vivo* bovine

study showed that spermatozoa but not secretions from the accessory glands induce endometrial gene expression after natural mating (Recuero et al., 2020).

The role of this immune response from semen is not yet fully characterised and literature to date lacks clarity on how this response changes as a result of AI. Factors such as the timing of the recruitment of PMNs post-insemination and the effect of SP and diluents on sperm-PMN binding, vary significantly between equine, bovine and ovine species (Alghamdi et al., 2009, Pini et al., 2017, Troedsson et al., 2005). In cattle, after AI, PMNs migrate to the uterine lumen following a chemo-attractant gradient which has been proposed to peak in less than 2h (Alghamdi et al., 2009). Spermatozoa are phagocytosed by PMNs by cell to cell attachment or via the formation of neutrophil extracellular traps (NETs) (Alghamdi et al., 2009, Hong et al., 2017, Fichtner et al., 2020). Using an *in vitro* model of primary BUECs, a recent study by showed that washed frozen-thawed bull spermatozoa induced expression of pro-inflammatory genes - namely *IL1B, IL8, TNFA* and *NFkB2*. A follow on study by the same group suggested the response was mediated via the TLR2/4 signalling pathway (Ezz et al.). However, culturing BUECs *in vitro* necessitates disruption of the endometrial layer and the release of damage-associated DAMPs which could enhance inflammatory responses.

A subsequent study by this group (Akthar et al., 2019) using a more physiological *ex vivo* uterine explant model which demonstrated that during the preovulatory phase, bull sperm enter uterine glands and interact with PMNs to clear excess spermatozoa from the uterine environment in preparation for subsequent embryo implantation. Whether this immune cell infiltration is beneficial or detrimental to pregnancy success in cattle is unclear. An *in vivo* study examining PMN concentrations post-insemination and its effect on pregnancy rates in dairy cows, found a moderate influx of PMNs to the uterus (0-15%) led to significantly higher conception rates (Kaufmann et al., 2009). However, the aforementioned study did not assess where along the development axis reproductive wastage occurred. All the evidence to-date indicates that bull spermatozoa induce a uterine immune response but there are no published studies on whether this response differs between HF and LF bull spermatozoa. Differential responses could have knock-on consequences on sperm transport and/or differential priming of the uterus for subsequent embryo development. We hypothesised that spermatozoa from HF and LF bulls elicit a differential uterine immune response that at least partially explains the variation in pregnancy rates between bulls of varying field fertility.

3.3 Materials and Methods

All procedures described were conducted under ethical approval from the Teagasc Animal Ethics Committee and experimental license from the Irish Health Products Regulatory Authority (AE19132/P100) in accordance with the Cruelty to Animals Act 1876 and the European Communities (Amendments of the Cruelty to Animals Act 1876) Regulations, 1994.

3.3.1 Reagents

Unless otherwise stated, all chemicals were sourced from Sigma-Aldrich (Dublin, Ireland).

3.3.2 Experimental Design:

3.3.2.1 Experiment 1

The experimental model was based on previous bovine studies both *in vitro* and *in vivo* that showed spermatozoa inducing an immune response in the uterus (Elweza et al., 2018, Ezz et al., 2019, Akthar et al., 2019, Recuero et al., 2020). The aim of this experiment was to determine optimum time points and sperm concentrations for the pro-inflammatory response of uterine explants to frozen-thawed bull spermatozoa. Uteri were collected from non-pregnant nulliparous beef heifers (n=4; heifer = replicate) at a commercial abattoir. Heifer reproductive tracts were inspected and the appearance was consistent with normal bovine heifer reproductive tracts. The stage of the cycle was determined to be at the follicular phase as per Ireland et al. (1980). Endometrial explants (n=12 per animal) were recovered from the horn ipsilateral to the ovary containing the pre-ovulatory follicle and incubated for either 1, 3 or 6 h with 750 µl of (i) Roswell park memorial institute (RPMI) medium (control), (ii) RPMI medium + 5×10^6 sperm per ml (iii) RPMI + 10×10^6 sperm per ml or (iv) RPMI + Lipopolysaccharide (LPS), 2 µg/ml. Frozen-thawed semen from Holstein Friesian bulls was donated from commercial AI centres. Nine straws (three straws from three different ejaculates for each bull) were used for each replicate. A panel of pro-inflammatory gene expressions namely *IL6, IL1B, CXCL8, TNFA* and *IL1A* were assessed for each explant by qPCR.

3.3.2.2 Experiment 2A

The aim of this experiment was to characterise the inflammatory response of uterine explants to frozen-thawed spermatozoa from HF and LF bulls. Uteri at the follicular phase of the oestrous cycle were collected from non-pregnant nulliparous heifers (n=5; heifer = replicate) at a commercial abattoir. Endometrial explants (n=8 per animal) were recovered from the ipsilateral horn and incubated for 6 h with 750 µl (i) RPMI medium (control) (ii) RPMI+ HF sperm 5×10^6 sperm per ml (iii) RPMI+ LF sperm 5×10^6 sperm per ml and (iv) RPMI + LPS, 2 µg/ml. In the treatments where spermatozoa was used, one straw from each of three individual ejaculates was pooled per bull. Three explants were used for each replicate/heifer (1 per bull) and a concentration of 5×10^6 sperm per ml was used. These bulls were selected according to AAM (Berry et al., 2011). Three HF bulls were selected with an average fertility rate of + 4.27% ± 0.35 (mean ± s.e.m,) and three LF bulls with an average fertility rate of -12.2% ± 1.81 (mean ± s.e.m) from an average population fertility of 0% (Table 3.1, Bulls 1 to 3 and 5 to 7). A panel of pro-inflammatory genes namely *IL6, IL1B, CXCL8, TNFA* and *IL1A* were assessed for each explant by qPCR.

Table 3.1 In vivo fertility data of Holstein Friesian bulls of high and LF as determined by an adjusted animal model (AAM). Mean of the population in the AAM was 0%.

Bull	Number of inseminations	AAM (%)	Fertility status
1	1479	-15.53	Low
2	519	-12.13	Low
3	510	-8.93	Low
4	1024	-6.03	Low
5	37856	+3.87	High
6	99953	+3.97	High
7	34859	+4.97	High
8	5121	+3.67	High

3.3.2.3 Experiment 2B

The aim of this experiment was to quantify the concentrations of the pro-inflammatory IL-8 and IL-1β proteins using an ELISA (enzyme-linked immunosorbent assay) in explant supernatants from experiment 2A. IL-8 ELISA was performed as described by Cronin et al. (2015) and IL-1B ELISA used a bovine specific antibody (Thermo Fisher). IL-8 was chosen in correspondence with the mRNA *CXCL8* gene in experiment 2A as it is known to be associated with PMN recruitment (Proost et al., 1996) and IL-1B was chosen as it is a predominant inflammatory marker and corresponds to *IL1B* mRNA expression. This experiment had the same level of replication as experiment 2A.

3.3.2.4 Experiment 3

The aim of this experiment was to evaluate the number of PMNs with sperm bound from HF and LF bulls and to assess the number of sperm bound to a subsample of PMNs. Whole blood was collected from three heifers. One semen straw from each of three individual ejaculates was pooled per bull. Four LF bulls (Bulls 1 to 4; Table 1) with an average fertility rate of -10.65% ± 1.9 (mean ± s.e.m) and four HF bulls (Bulls 5 to 8; Table 1) were selected with an average fertility rate of + 4.12% ± 0.35 (mean ± s.e.m; Table 1). As serum levels have been previously reported to have an effect on sperm-PMN binding (Pini et al., 2017), we assessed three serum concentrations (1.5, 4 and 7.5%) as well as uterine fluid on sperm binding (Figure 3.1). There was no effect of treatment and therefore uterine fluid selected as it was considered a more physiological media for PMNs. A pool of uterine fluid was collected as described by Simintiras et al. (2019) from three heifers. It was centrifuged, bio banked and used across all bull replicates.
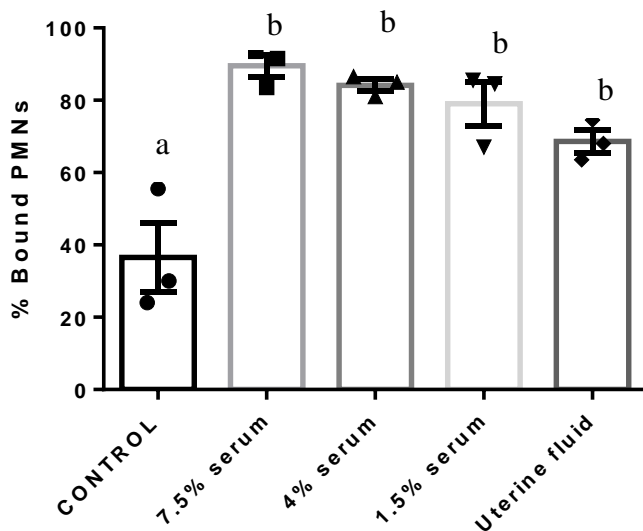
Figure 3.1 Percentage of frozen thawed spermatozoa bound to PMNs in four different treatments; 7.5% serum, 4% serum, 1.5% serum and uterine fluid. Data are presented as mean ± s.e.m. of three independent replicates. [abc]Different superscripts denote a significant difference between the treatments (p<0.05).

3.3.2.5   Experiment 4A

The aim of this experiment was to use a cauda epididymal sperm model to identify if the source of the uterine inflammatory response is of spermatozoa or SP origin. Uteri at the follicular phase of the oestrus cycle were collected from non-pregnant nulliparous heifers (n=3) at a commercial abattoir. Testes were collected from three bulls at a local abattoir post slaughter. Epididymal spermatozoa recovered from the three bulls were pooled and washed twice in 3 mL of TALP at 300 g for 5 min. Sperm concentration was assessed with a haemocytometer and adjusted to 5×10^6 sperm per ml. To obtain fresh SP, ejaculates from 3 Holstein Friesian bulls collected at a local AI centre were pooled. SP was obtained by centrifugation of the pooled ejaculates (9 min at 700 g). Endometrial explants (n=4 per animal) were recovered from the ipsilateral horn and incubated for 6 h with 750 µl (i) RPMI medium (control) (ii) RPMI + 5×10^6 CES sperm per ml (iii) RPMI + 5×10^6 CES sperm per ml + 5% SP or (iv) RPMI + LPS (2 µg/ml). A concentration of 5% SP was selected to represent the approximate concentration present in semen straws. A panel of pro-inflammatory genes namely *IL6, IL1B, CXCL8, TNFA* and *IL1A* were assessed for each explant by real time qPCR.

3.3.2.6   Experiment 4B

The aim of this experiment was to quantify the concentration of pro-inflammatory gene expression of IL-8 and IL-1B proteins using a bovine ELISA in uterine explant supernatants treated with CES and CES + 5% SP. IL-8 ELISA was performed as described by Cronin et al. (2015) and IL-1B ELISA used a bovine specific antibody (Thermo Fisher). IL-8 was chosen to correspond with the mRNA CXCL8 gene in experiment 4A as it has been associated with PMN recruitment while IL-1B was selected as it has been reported as a marker of inflammation. This experiment had the same level of replication as experiment 4A.

3.3.3   Spermatozoa and seminal plasma preparation:

For all experiments using frozen-thawed semen, straws were thawed in a water bath at 37° for 30s. For each bull a pool of three straws from three ejaculates were washed in 3 mL TALP (Romero-Aguirregomezcorta et al., 2019) and centrifuged at 300 g for 5 min. The supernatant was removed to remove excess diluent and sperm concentration was assessed using a haemocytometer and adjusted to 5×10^6 sperm per ml.

For experiments 4A and 4B testes were collected from a local abattoir and transported within 1 h to the laboratory at ambient temperature. A small incision was made in the cauda epididymis and the lumen of the deferent duct was cannulated with a blunted 22G needle. Spermatozoa were then gently flushed through the cauda with a 5 ml syringe loaded with phosphate buffered saline (PBS) at 37 °C, as described by Holden et al. (2017b).

3.3.4   Uterine explant culture:

As inflammation is modulated by multiple cell types, we postulated that an *ex vivo* uterine explant model would offer a more physiological model than a monolayer of endometrial cells to investigate the molecular mechanisms of immunity and inflammation (Borges et al., 2012). Heifer reproductive tracts were collected at the follicular stage of the cycle and uteri processed as described previously (Borges et al., 2012). Briefly, the uterine horn ipsilateral to the ovary containing the pre-ovulatory follicle was opened longitudinally with a sterile scissors. The exposed endometrium was washed with Dulbecco's PBS solution supplemented with 1% antibiotic-antimycotic (ABAM) (Gibco, ThermoFisher

Scientific, Dublin, Ireland), and tissue biopsy's were obtained from intercaruncular areas with the use of a sterile 8 mm biopsy punch (Stiefel Laboratories Ltd, High Wycome, UK). Sterile blades were used to dissect the endometrium away from the myometrium. Once dissected, the explants were washed twice in Hank's balanced salt solution (Gibco, ThermoFisher Scientific) containing 1% ABAM, and then transferred into a 24-well plate, so that each well contained one explant in 1 ml RPMI medium (Gibco, ThermoFisher Scientific) supplemented with 1% ABAM. Explants were cultured, endometrial side up, at 39 °C under an atmosphere of 5% CO_2 for 1 h prior to use. Following treatment stimulations, explants were snap frozen in liquid nitrogen and stored at -80°C for later RNA extraction while the supernatants were frozen and stored at -20° C.

3.3.5 RNA Extraction and cDNA synthesis:

Total RNA was extracted from ~50 mg of endometrial explants using Trizol reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. On-column DNase digestion and RNA clean-up was performed using the Qiagen mini kit (Qiagen, Hilden, Germany). The quantity of RNA was determined using the Nano Drop 1000 (Thermo Fisher Scientific). For experiment 1 and 2A, RNA quality was assessed by RNA gel electrophoresis as illustrated in Figure 3.2A. To prepare the gel, a 50 ml solution of RNA free water, 0.5X TBE, 1.5% agarose was boiled and left cool at ambient temperature. SYBR Safe (Thermo Fisher Scientific) at 0.5 μg/ml was added and the gel left harden for ~ 1h. Each sample (200 ng) was prepared with loading dye (New England Biolabs, United States) and RNA ladder prepared as per manufacturer's instructions (New England Biolabs). Samples were heated to 65°C for 5 min and subsequently kept on ice. The electrophoresis tank with the gel was filled with TBE 0.5X. Samples were loaded and the gel was run at 100v for ~30 min. Gels were viewed on a transilluminator. The analysis was performed on the observation of the 28S and 18S peaks. For experiment 3A, RNA quality was assessed with the Agilent Bioanalyzer (Agilent Technologies) and all samples had a RNA integrity number (RIN) >7.0 as illustrated in Figure 3.2B. For each sample, cDNA was prepared from 1 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA solutions were diluted 1:10 before qPCR analysis.
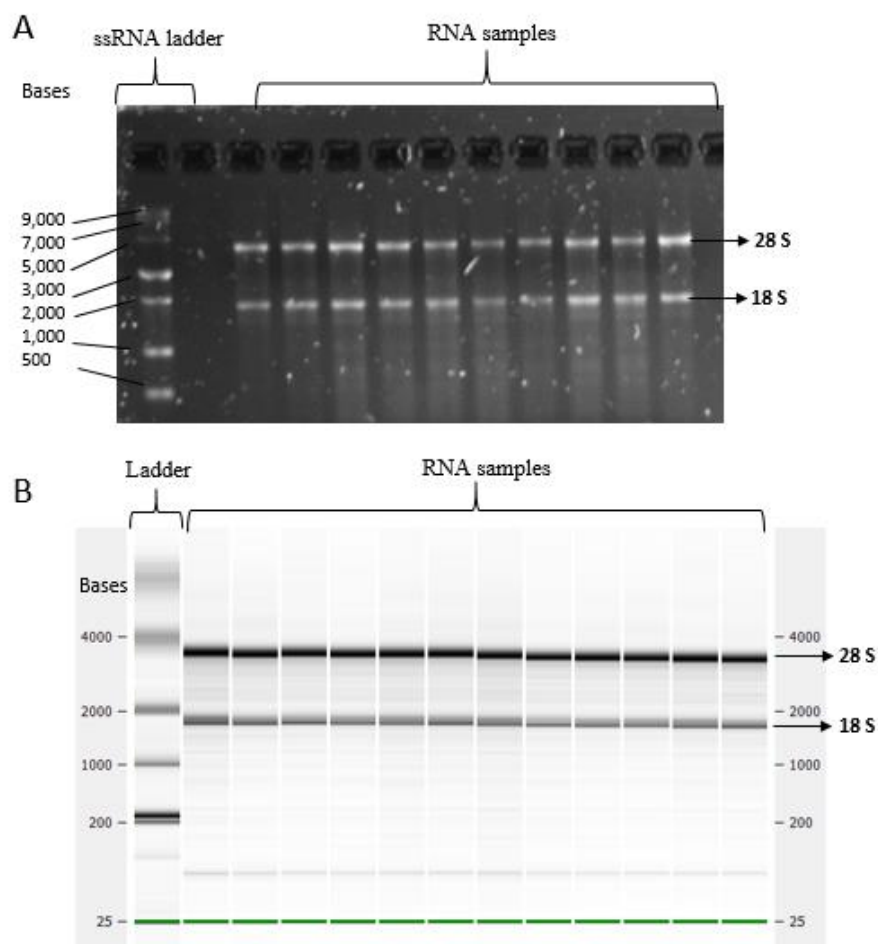
Figure 3.2 Representative images of RNA quality A) assessed by gel electrophoresis and B) assessed by the Agilent Bioanalyser with 28S and 18S bands visible and indicative of good quality RNA samples.

3.3.6 RT-qPCR:

All primers were designed using Primer Blast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) Table 3.2. Briefly, cDNA was prepared on a MicroAmp^{TM} Fast Optical 96-Well Reaction Plate (Applied Biosystems) with primers using Fast SYBR® Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. Controls without nucleic acid template and without reverse transcriptase were also included. The samples were run on a 7500 Fast Real-Time PCR System (Applied Biosystems). The following parameters were used: 95 °C for 20 s, 40 cycles of 95 °C for 3 s and 60 °C for 30 s. From a panel of reference genes (*GAPDH*, *ACTB*, *H3F3A, PPIA* and *RPS9)*, *H3F3A* was selected to use for normalisation based on the lowest M-value calculated by the geNorm tool in the GenEx software (v.5.2.7.44). For qPCR data, Ct values were imported into GenEx software. Each experiment had a no

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sperm control at each time point where relevant, and all stimulations were compared to the control explant. Ct values were adjusted for primer efficiency and replicate values were averaged. Data were normalized using the geometric mean of the reference gene by geNorm. Relative expression values were automatically calculated by the software using a modified version of delta-delta Ct method (ΔΔCq; also known asΔΔCT) as described (Livak and Schmittgen, 2001).

Table 3.2 Gene symbol, accession number, primers sequence and amplicon for all genes analysed by quantitative real-time PCR.

Gene symbol	Accession number	Primer sequence (5'-3')	Amplicon size
IL1A	NM_174092.1	GATGGCCAAAGTCCCTGACC GGTTTCCGAGGTATCCAGGG	178
IL1B	NM_174093.1	CCCTGCAGCTGGAGGAAGTA CTTCGATTTGAGAAGTGCTGATGT	150
IL6	NM_173923.2	ACCCCAGGCAGACTACTTCT CCCAGATTGGAAGCATCCGT	195
CXCL8	NM_173925.2	CATTCCACACCTTTCCACCC CCTTCTGCACCCACTTTTCC	145
TNFA	NM_173966.3	CCATCAACAGCCCTCTGGTT TCACACCGTTGGCCATGA	150
H3F3A	NM_001014389.2	CATGGCTCGTACAAAGCAGA ACCAGGCCTGTAACGATGAG	136

3.3.7 ELISA (Enzyme-linked immunosorbent assay)

The IL-8 ELISA was used to assess concentration in cell supernatants as described by Cronin et al. (2015) where the IL-8 values are based on an ovine antibody, which has been shown to cross-react with cattle. The IL-1β ELISA (Thermo Scientific) used a bovine specific antibody and was performed as per the manufacturer's instructions.

3.3.8 PMN magnetic labelling, separation and sperm-PMN binding assay

PMN magnetic labelling and separation was carried out as described in Stojkovic et al. (2016).Viability of cells were analysed using propidium iodide (Thermo Scientific) and cell purity and viability were >90%. Both positively and negatively selected cells were analysed by collecting 10,000 events in the granulocyte gate based on forward scatter (FSC) and side scatter (SSC). Cells were resuspended in 1 mL PBS 0.5% BSA.

Spermatozoa were diluted in PBS with 0.5% BSA and added at a 1:1 ratio to PMNs diluted in uterine fluid. A final concentration of 2×10^6 PMNs per ml in 100 µl and 20×10^6 sperm per ml in 100 µl were incubated for 1 h at 37° and 5% CO_2. After 1 h, samples were mixed to disassociate clumps and 10 µl was smeared on a slide and air-dried. Slides stained using Wright stain solution according to manufacturer's instructions. Slides were examined using brightfield and phase contrast microscopy (Olympus BX60) at 400x magnification. Slides were assessed as described by Pini et al. (2017), where 200 PMNs were counted per slide and classified as free or bound to ≥1 spermatozoa. For each slide, the number of sperm bound to a random subset of 50 PMNs were assessed to calculate the number of bound spermatozoa per PMN.

3.3.9 Statistical Analysis

Heifer was the experimental unit in all experiments. For all experiments, data were analyzed using the statistical package for the social sciences (IBM SPSS for Windows 25.0). One-way ANOVA and repeated measures ANOVA were performed on Delta Ct values (normalized data). Assumptions of normality and equality of variances were assessed prior to performing a one-way ANOVA. A test of sphericity was used prior to performing a repeated measures ANOVA using Shapiro-Wilko, Levene's and Maulachy's tests, respectively. In Experiment 1, data were analysed with a two-way repeated measures ANOVA (Within subject factor: time; Between subject factor: treatment and sperm concentration) with Bonferroni post-hoc tests. In experiments 2A,

2B, 3 as well as 4A and 4B a one-way ANOVA was used (factor: treatment) followed by Tukey-HSD post-hoc tests. The level of significance was set at P<0.05. All results are presented as mean ± s.e.m.

3.4 Results:

3.4.1 Experiment 1: Time and sperm concentration dependent effects of frozen-thawed spermatozoa on mRNA expression of pro-inflammatory genes in bovine uterine explants

There was no time by sperm concentration interaction for any of the inflammatory gene expression but there was an effect of time for *IL1A, IL1B, IL6* and *CXCL8* detected (P<0.05, Figure 3.3). Expression levels were lowest at the 1 h time point across all genes with maximal expression detected at 6 h. All three sperm concentrations assessed (5, 10 and 15 million per ml) resulted in an upregulation of both *IL1B* and *IL6* compared to the control with no spermatozoa (P<0.05). There was no difference between the three sperm concentrations and therefore a sperm concentration of 5 x 10^6 sperm per ml was chosen for subsequent experiments.
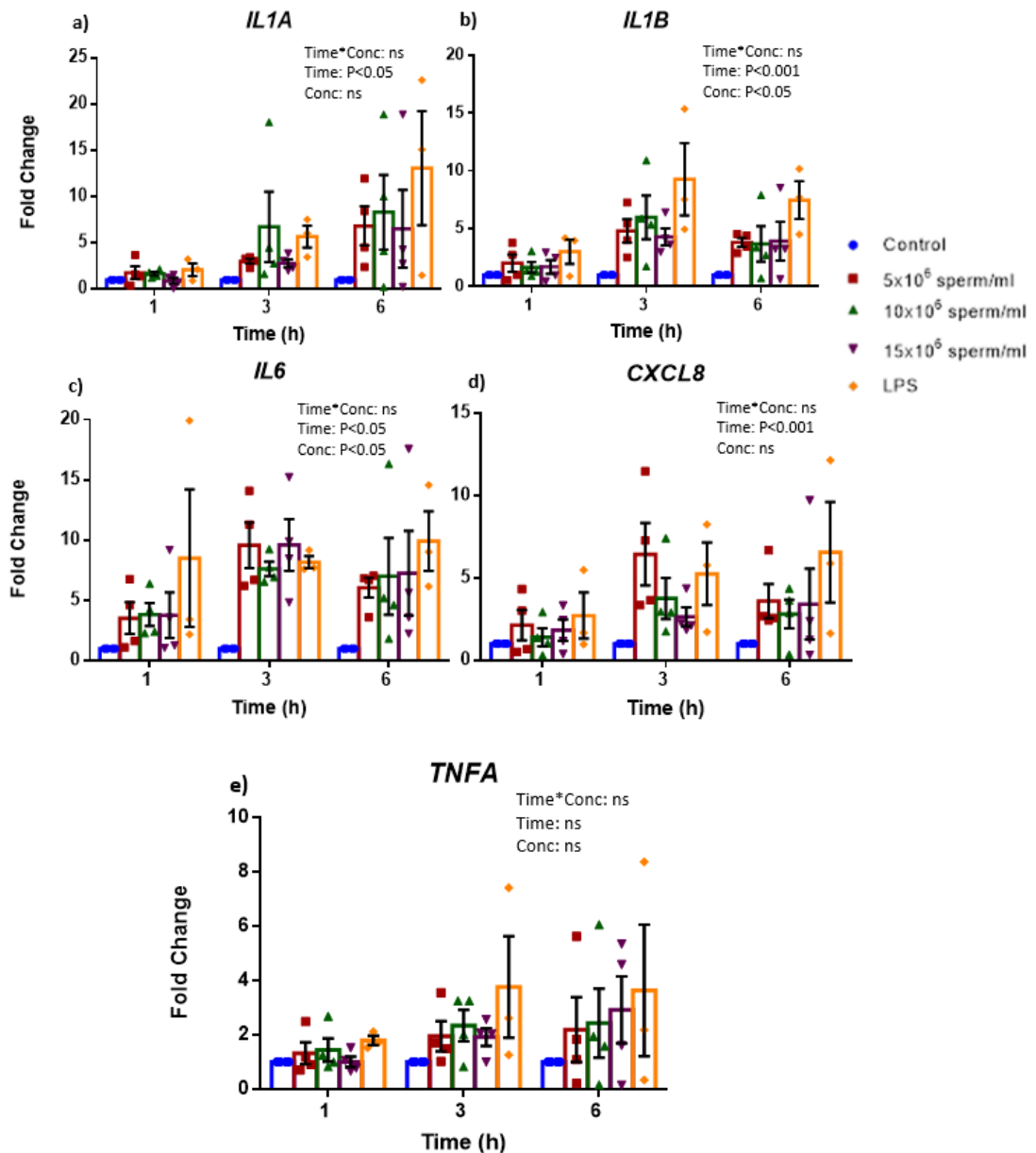
Figure 3.3 Time and sperm concentration (conc) dependent effects of frozen-thawed bovine spermatozoa on the mRNA expression of pro-inflammatory cytokines (a) *ILA* (b) *IL1B*, (c) *IL6*, (d) *CXCL8* and (e) *TNFA* from follicular phase uterine explants of nulliparous heifers. Uterine explants were co-cultured with three different concentrations of washed frozen-thawed spermatozoa (5, 10 and 15×10^6 sperm per ml) at three time points (1, 3 and 6 h). A pool of three straws (1 from each of 3 ejaculates) from three bulls was used for each replicate. Lipopolysaccharide (LPS) at 2 µg/ml was used as a positive control treatment. Data are presented as mean fold change ± s.e.m. relative to the reference gene *H3F3A* for four independent replicates (3 replicates for LPS). Each concentration is compared to a control (no spermatozoa) explant at each time point. ns = non-significant.

3.4.2 Experiment 2A: Frozen-thawed spermatozoa from both HF and LF bulls stimulates pro-inflammatory cytokine gene expression in bovine uterine explants

LPS stimulation was used as a positive control and it significantly induced pro-inflammatory gene expression in this model. The mRNA expression of pro-inflammatory markers *IL1A, IL1B* and *TNFA* was increased in uterine explants in response to frozen-thawed spermatozoa from both high and LF bulls relative to the no sperm control (Figure 3.4; $P<0.05$). Induced expression levels were highest for *IL1A* and *IL1B* but no significant difference between HF and LF sperm treatments were detected for any of the 5 genes assessed ($P>0.05$).
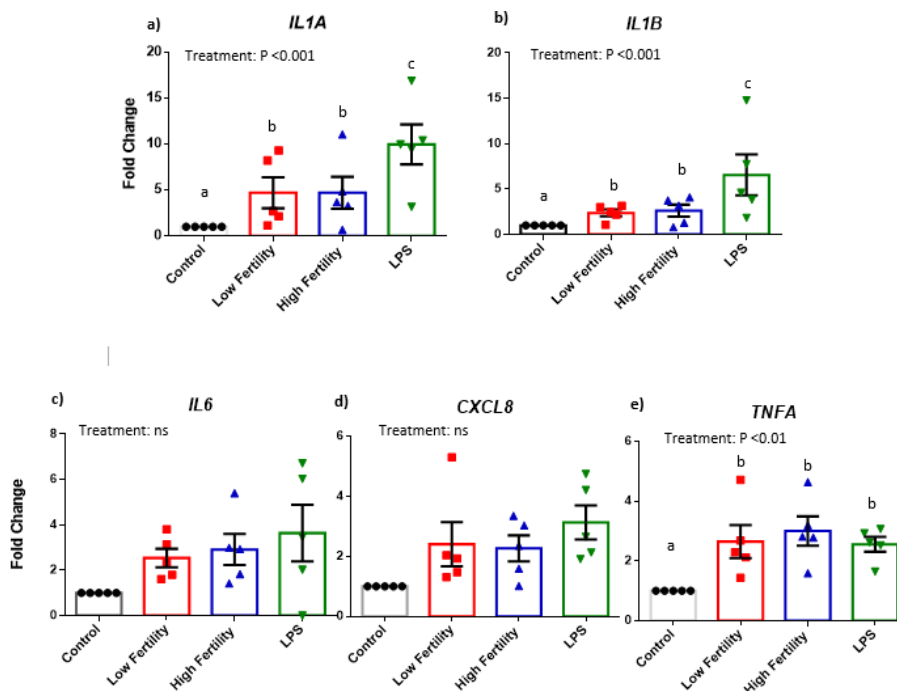
Figure 3.4 mRNA expression of pro-inflammatory cytokines (a) *ILA*, (b) *IL1B*, (c) *IL6*, (d) *CXCL8* and (e) *TNFA* from follicular phase uterine explants of nulliparous heifers in response to frozen-thawed spermatozoa from HF and LF bulls. Uterine explants were cultured with media (control), washed spermatozoa (5×10^6 sperm per ml) from three high and three LF bulls and lipopolysaccharide (LPS; 2 µg/ml) for 6 h. Each bull was represented on a single explant with a pool of three different ejaculates. Data are presented as mean fold change ± s.e.m. relative to the reference gene H3F3A and a control no sperm explant at 6 h for five independent replicates. [abc]Differing superscript denote a significant difference between the treatments within each gene ($p<0.05$). ns = non-significant.

3.4.3 Experiment 2B: Media incubated with uterine explants and frozen-thawed spermatozoa from high and LF bulls contain higher concentrations of IL-1B and IL-8 protein

Concentrations of IL-1β in explant conditioned media was elevated for both the HF and LF bulls compared to the no sperm control (P<0.01, Figure 3.5B) but there was no difference in the response to spermatozoa from HF and LF bulls. Concentrations of IL-8 in explant conditioned media was higher in response to spermatozoa from HF and LF bulls compared to the no sperm control (P<0.001; Figure 3.5B). However, there was no difference in IL-8 concentration between HF and LF bulls (P>0.05). The HF bulls had lower IL-8 concentrations compared to the LPS treatment (P<0.05) while there was no difference between the LF bulls and the LPS treatment (P>0.05).
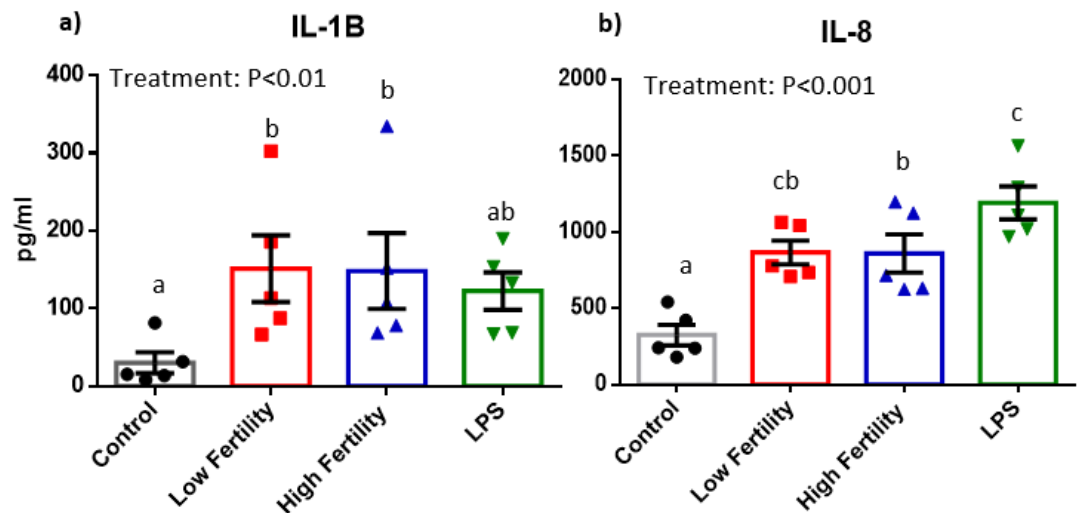
Figure 3.5 Concentration of (a) IL-1β and (b) IL-8 proteins in explant-conditioned media measured by enzyme linked immunosorbent assay after co culture with media (no sperm control), washed frozen-thawed spermatozoa from HF and LF bulls and lipopolysaccharide (LPS; 2 µg/ml) as a positive control. Follicular phase uterine explants from nulliparous heifers were cultured for 6 h with a sperm concentration of 5 x 10^6 sperm per ml. Data are presented as mean ± s.e.m. of five independent replicates. [abc]Different superscripts denote a significant difference between the treatments within each gene (p<0.05).

3.4.4   Experiment 3: PMNs bind more spermatozoa from LF bulls

The number of sperm bound per PMN was higher for LF compared to HF bulls (P<0.05; Figure 3.6) with an average of 2.25 ± 0.1 (mean ± s.e.m.) sperm bound per PMN in HF bulls compared to 2.46 ± 0.09 (mean ± s.e.m.) spermatozoa per PMN from LF spermatozoa (Figure 3.6b). There was no difference between HF and LF bulls in the overall percentage of PMNs which had at least one bound spermatozoon (P>0.05).
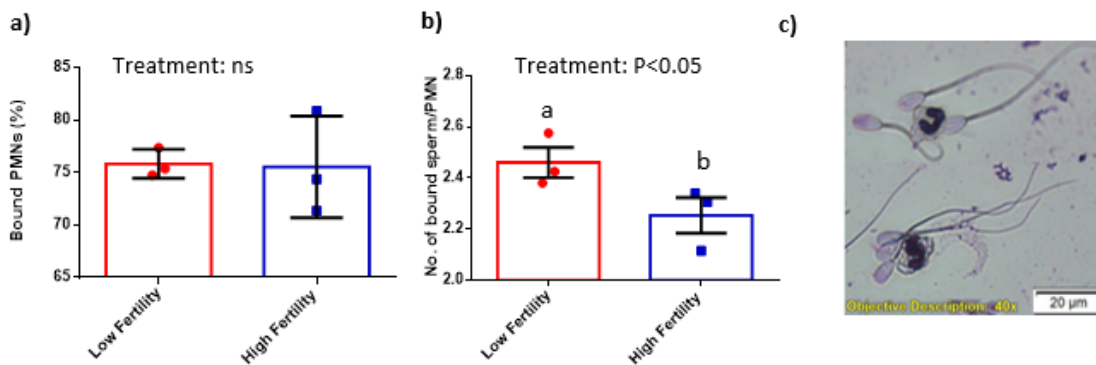
Figure 3.6 **a)** Percentage of bound PMNs (≥ 1 spermatozoon bound) from HF and LF bulls. PMNs were diluted to a concentration of 2 x 10^6 PMNs per ml and incubated with 20 x 10^6 sperm per ml for 1 h at 37 °C and 5% CO_2 **b)** Number of bound sperm per PMN from HF and LF bulls. **c)** Representative image showing a PMN with two sperm bound (top) and three sperm bound (bottom). A spermatozoon was characterised as bound to a PMN if the head or mid-piece region of the spermatozoon were attached to the PMN. Data are presented as mean ± s.e.m. of three independent replicates. [abc]Different superscripts denote a significant difference between the treatments (p<0.05).

3.4.5 Experiment 4A: Cauda epididymal spermatozoa in combination with seminal plasma upregulates the expression of the inflammatory gene marker, Interleukin 6.

There was no effect of treatment on mRNA expression of the pro-inflammatory cytokines *IL1A, IL1B, TNFA* or the chemokine *CXCL8* (P>0.05; Figure 3.7). In contrast, *IL6* expression was upregulated in response to CES + SP, but not CES, relative to the no sperm control (P<0.05; Figure 3.7). There was no difference between gene expression for CES or CES + SP treatments (P>0.05).
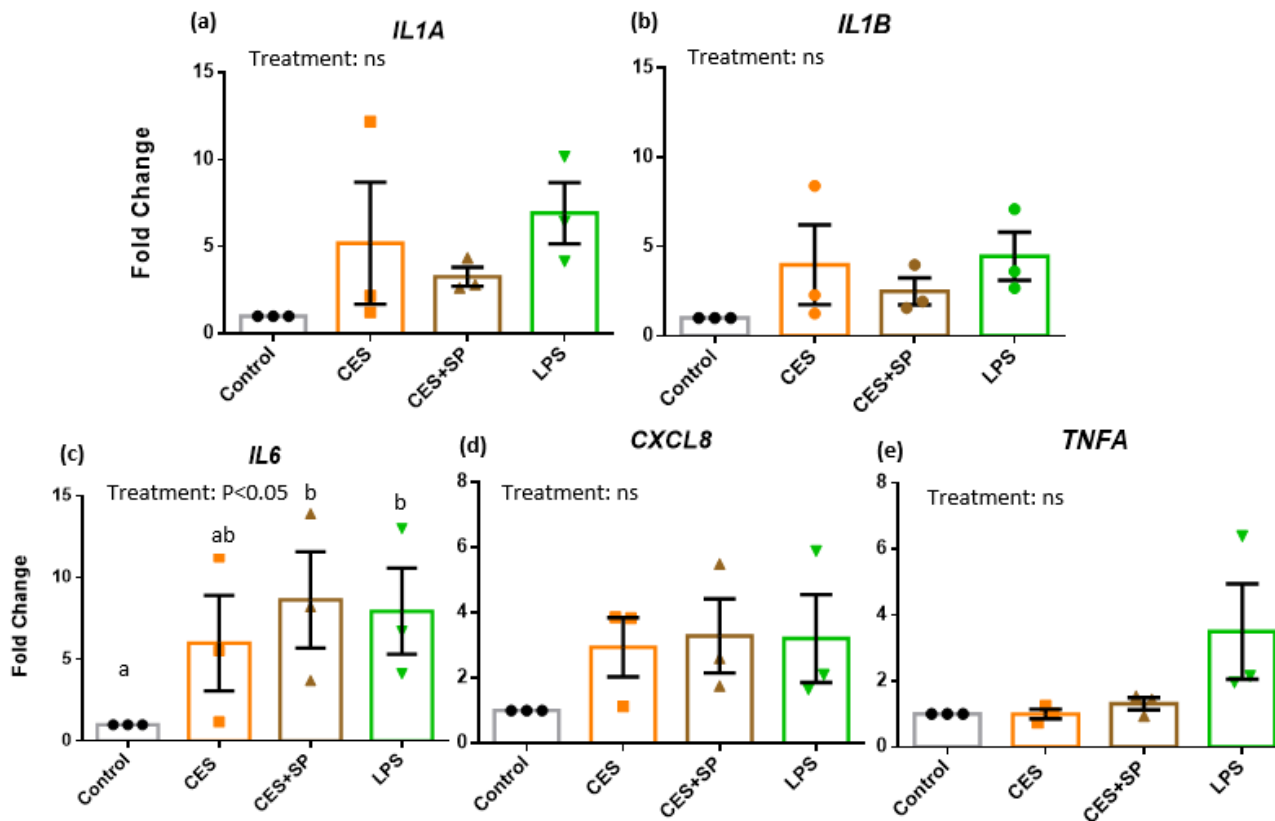
Figure 3.7 mRNA expression of pro-inflammatory cytokines a) ILA (b) IL1B, (c) IL6, (d) CXCL8, (e) TNFA from uterine explants in response to caudal epididymal spermatozoa (CES) and CES + 5% seminal plasma (SP). Follicular phase uterine explants were cultured with media (control), CES, CES + 5% SP (5 x 10^6 sperm per ml) and LPS (2 µg/ml) for 6 h. Data are presented as mean fold change ± s.e.m. relative to the reference gene H3F3A and a control no spermatozoa explant at 6 h for three independent replicates. [abc]Different superscripts denote a significant difference between the treatments (p<0.05), ns = non-significant

3.4.6 Experiment 4B: CES or CES in combination with SP do not alter cytokine concentrations

There was no effect of treatment on IL-1B or IL-8 protein concentrations (P>0.05; Figure 3.8). However, it is noteworthy that uterine explants secreted, on average, 8 fold higher protein concentrations of IL-8 compared to IL-1B across all treatments.
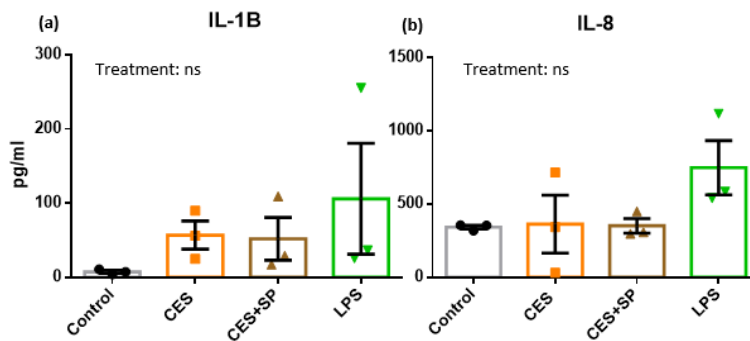
Figure 3.8 Concentration of (a) IL-1B and (b) IL-8 in explant-conditioned media measured by enzyme linked immunosorbent assay after co culture with media (no sperm control), caudal epididymal spermatozoa (CES), CES + seminal plasma (SP; 5%) and lipopolysaccharide (LPS; 2 µg/ml) as a positive control. Uterine explants were cultured for 6 h with a sperm concentration of 5 x 10^6 sperm per ml. Data are presented as mean ± s.e.m. of three independent replicates, ns = non-significant.

3.5 Discussion

We sought to ascertain if spermatozoa from HF and LF bulls induce a differential uterine inflammatory response using an *ex-vivo* uterine explant model which was selected as the tissue architecture is likely to be more physiologically relevant than a monoculture of epithelial cells used in previous studies (Borges et al., 2012). The main findings of this *ex vivo* study were i) frozen-thawed spermatozoa initiate a pro-inflammatory uterine response but there was no difference between HF and LF bulls ii) PMNs bind more spermatozoa from LF than HF bulls and iii) neither CES nor CES + SP elicit as potent a pro-inflammatory response as observed in frozen thawed bull spermatozoa and therefore the exact source of the spermatozoa induced uterine inflammatory response is yet to be determined.

The presence of frozen-thawed bull spermatozoa significantly upregulated *IL1A, IL1B* and *TNFA* gene expression in the current model but there was no difference in the magnitude of response to spermatozoa from HF and LF bulls. This was also confirmed by ELISA results from explant supernatants for IL-1B and IL-8. Interestingly, the concentration of IL-1B was similar in response to spermatozoa than it was to LPS, suggesting a potent response. Both *IL1A* and *IL1B* are potent inflammatory cytokines that activate the inflammatory process (Di Paolo and Shayakhmetov, 2016) while *TNFA* is another common pro-inflammatory mediator and has been previously shown to be linked with the inflammatory chemokine Interleukin-8 in a human endometriotic stromal cell *in vitro* model (Sakamoto et al., 2003). The results of this study are also in agreement with work using BUECs which detected significant induction of the pro-inflammatory markers *IL1B, IL8* and *TNFA* in response to frozen-thawed bull spermatozoa (Elweza et al., 2018, Ezz et al., 2019) although maximal expression occurred earlier – at 3 h post stimulation compared to 6 h in this study A related study using explants reported significant induction of *IL1B, IL8, TNFA* and *TLR2* at 2 h from washed fresh spermatozoa but did not assess other time points (Akthar et al., 2019). This innate immune response depends on PRRs to distinguish 'self' from 'non-self' molecules. Toll-like receptors are PRRs which can detect changes in a micro environment and trigger intracellular signalling resulting in the expression of pro-inflammatory molecules (Mogensen, 2009). This sperm uterine immune response has been shown to be mediated via the TLR2/4 signalling in a BUEC culture model using frozen thawed bull spermatozoa (Ezz et al., 2019) and later confirmed

in an explant model (Akthar et al., 2019) using fresh bull spermatozoa however, the exact molecular link between spermatozoa and the TLR2/4 pathway has yet to be determined. The TLR4 pathway has also been identified as the key mediator of the female uterine response to spermatozoa *in vivo* in mice and this same study identified a direct action of spermatozoa in signalling female uterine tissues, which subsequently affect female tract receptivity to spermatozoa, fertility, implantation and foetal development (Schjenken et al., 2021). The bovine sperm – uterine immune response has also been confirmed *in vivo* where changes in the uterine transcriptome were due to spermatozoa and not seminal fluid alone (Recuero et al., 2020).

The up regulation of mRNA expression for *CXCL8* in response to spermatozoa from both HF and LF bulls, which was significant at the protein level in explant supernatants, suggests that PMN responses to spermatozoa may shed additional light on the immune response. In an *in vivo* AI model, an influx of innate immune cells, composed predominantly of PMNs has been reported and the resulting moderate inflammatory response is thought to contribute to higher conception rates (Kaufmann et al., 2009). IL-8 is a powerful chemoattractant for PMNs (Alghamdi et al., 2009) and these PMNs have been shown in an *ex vivo* bovine model to react with spermatozoa via uterine glands (Akthar et al., 2019). Further characterisation of the PMN binding abilities from HF and LF bulls in this study showed a significant increase in the number of sperm bound per PMN from LF bulls, which could be preventing these spermatozoa from reaching the site of fertilisation in the ampulla. Human and equine studies have reported reduced sperm motility from the formation of neutrophil extracellular traps (Zambrano et al., 2016, Alghamdi and Foster, 2005). Similarly, in an *in vitro* study there was a strong correlation between an increased percentage of adherent PMNs and a decrease in free spermatozoa in the remaining sample (Alghamdi et al., 2009).

There are a number of factors that can have contrasting effects on sperm-PMN binding including the amount of SP, the cryopreservation process as well as the cryodiluent used. Ram spermatozoa have been shown to undergo significant changes to their surface carbohydrates from the contact of SP as well as from cryopreservation (Pini et al., 2017). The same study demonstrated that cryopreserved ram spermatozoa adhered less to PMNs compared to epididymal or ejaculated spermatozoa and concluded changes were due to cryodiluents (Pini et al., 2017). Bovine studies have shown an increased effect of SP on sperm-PMN binding (Alghamdi et al., 2009, Fichtner et al., 2020), while Alghamdi et al. (2009) reported egg-yolk to have a diminishing effect on sperm-PMN binding, Fichtner

et al. (2020) showed that this varied according to the cryodiluent used. The results of this study, which focused on differences between high and LF bulls and mirroring conditions during AI to characterise the stimulus of the uterine immune response, provides evidence that spermatozoa need to be incubated with SP to initiate an inflammatory response but additionally, the processing of frozen-thawed spermatozoa could be contributing to an inflammatory response and subsequently sperm-PMN interactions. The sperm membrane may be altered either by the addition of cryodiluent and/or by the cryopreservation process where further study is required.

In conclusion, this *ex vivo* study has clearly shown that frozen-thawed bull spermatozoa are immunogenic but the consequences of this remain to be elucidated. While PMNs trap spermatozoa from LF bulls more, no differences were detected in the immunological response of spermatozoa from HF and LF bulls. The preparation of explants from intact uteri inevitably release DAMPs, and these DAMP-associated inflammatory response may obscure more subtle differences in responses between spermatozoa from bulls with divergent field fertility. Thus, an *in vivo* study is warranted to further our understanding of how spermatozoa from bulls of varying fertility interact with the endometrium.

Chapter 4   The transcriptomic response of bovine uterine tissue is altered in response to spermatozoa from high and low fertility bulls

4.1 Abstract

Despite stringent quality control checks in animal breeding centres, some bulls with apparently normal semen quality yield lower than expected pregnancy rates. The hypothesis of this study was that this variation in fertility could be at least in part due to molecular differences in sperm-uterine interactions. The aim of this study was to profile the transcriptome and perform histological analysis of the bovine uterus in response to spermatozoa from HF and LF bulls. Uterine biopsies and uterine explants were collected post-mortem from nulliparous heifers 12 h after a fixed time AI to a synchronised oestrus with frozen-thawed semen from 5 HF (fertility rate $4.01\% \pm 0.25$, mean±s.e.m.) and 5 LF (fertility rate $-11.29\% \pm 1.11$, mean±s.e.m.) bulls. Semen from each bull was used to inseminate two heifers (10 heifers per treatment). Uterine biopsies were also collected from control (CTRL) heifers (n=8) which were not inseminated. High quality RNA extracted from uterine biopsies was analysed by RNA-seq and differential gene expression assessed. Uterine explants were collected for histological analysis and PMN quantification. In the HF treatment relative to CTRL heifers there were 845 genes significantly differentially expressed in the endometrium with just 4 genes differentially expressed in the LF treatment relative to CTRL heifers. Comparing the HF and LF treatments directly there were 67 significantly DEGs (FDR<0.1). Transcriptomic analysis shows a predominant role for the inflammatory marker Interleukin-1 and gene ontology analysis identified enriched pathways for cell adhesion, sperm cell movement, cell renewal as well as the inflammatory response. Further characterisation of the inflammatory response by the quantification of PMNs in the endometrium showed a significant effect of spermatozoa, however, there was no difference in PMN numbers between HF and LF groups. In conclusion, this novel study clearly shows a distinct inflammatory response to spermatozoa in the endometrium and a divergent transcriptomic response in the female reproductive tract to semen from high and low field fertility bulls, which could have important consequences for the outcome of AI.

4.2 Introduction:

Artificial insemination is used worldwide to improve the genetic merit of herds as one bull with desirable traits can be used to inseminate thousands of cows. Prior to the release of semen into the field animal breeding centres worldwide perform a number of microscopy based pre-freeze and post-thaw QC checks. Despite these stringent controls, some bulls with apparently normal semen quality, yield unacceptable low pregnancy rates which can have significant economic losses for farmers. The reason for this is largely unknown and most studies have focused on attempting to predict bull fertility using a range of *in vitro* sperm functional parameters combined with various statistical models (Utt, 2016, Bernecic et al., 2021). These *in vitro* assays range from microscopic evaluations to flow cytometry analysis of plasma membrane integrity, oxidation level, mitochondrial status and sperm chromatin structure, to name but a few (Odhiambo et al., 2014, Sellem et al., 2015, Holden et al., 2017a). These studies however do not attempt to assess where along the developmental pathway reproductive wastage occurs for some bulls.

During AI, semen is deposited in the uterus where spermatozoa interact with the uterine and oviductal epithelia as well as the mucus secretions before reaching the site of fertilisation in the ampulla (Miller, 2018). In many species to-date, it has been shown that semen (spermatozoa and seminal plasma) is capable of initiating an immune response in the uterus and has been best described in mice, pigs, horses and more recently cattle (Katila, 2012, Christoffersen and Troedsson, 2017, Recuero et al., 2020). Bovine *in vitro* studies have shown spermatozoa alone can induce a pro-inflammatory response in the uterus (Chapter 3) mediated by the TLR2/4 pathways (Elweza et al., 2018, Ezz et al., 2019). *In vivo* spermatozoa that have come in contact with SP but not SP alone, induce transcriptional changes in the uterus after natural mating in cattle (Recuero et al., 2020).

An *ex vivo* model showed spermatozoa enter these uterine glands where they interact with PMNs (Akthar et al., 2019). Post insemination, PMNs are known to rapidly enter the uterine lumen of cows. The route of this PMN migration to the uterus is via uterine glands where they phagocytise spermatozoa or form neutrophil extracellular traps (Alghamdi et al., 2009). The role of this immune response appears to clear the endometrium of excess spermatozoa and bacteria and to prepare the endometrium for pregnancy (Katila, 2012). The level of inflammation and PMN migration which is beneficial to priming the uterus for the subsequent pregnancy is a complex process and some studies suggest that

moderate influx of PMNs leads to significantly higher conception rates (Chastant and Saint-Dizier, 2019, Kaufmann et al., 2009).

We hypothesised that spermatozoa from bulls of differing fertility phenotypes elicit a differential immune response which can affect outcomes by altering sperm transport and/or by differential priming of the uterus which may affect embryo development once the blastocyst comes back down into the uterus approximately 5 days' post fertilisation. To address this, we used a molecular based approach to investigate the uterine biology of uterine interactions *in vivo* with spermatozoa from HF and LF AI bulls.

4.3   Materials and Methods

4.3.1   Ethical approval

Protocols were developed in accordance with the Cruelty to Animals Act (Ireland 1876, as amended by European Communities regulations 2002 and 2005) and the European Community Directive 86/609/EC. All animal procedures were conducted under experimental license from the Health Products Regulatory Authority and approved by the University of Limerick animal ethics committee.

4.3.2   Experimental Model

The oestrus cycles of thirty, cross-bred heifers were synchronised and inseminated using a fixed-time AI protocol. An 8-day intravaginal progesterone device (PRID® Delta) was used with gonadotrophin releasing hormone (Ovarelin; 2 ml) administered intra muscularly at the time of PRID insertion. All heifers were administered prostaglandin F2 alpha (Enzaprost®; 5 ml) intra muscularly to induce luteolysis 24 h prior to PRID removal. Heifers received a single fixed time insemination of frozen-thawed semen at 72 h post PRID removal or were not inseminated and left as part of a control group. Ovarelin (2 ml) was administered intramuscularly to all heifers at the time of AI.

Heifers were allocated to one of three treatments and were inseminated by a single trained technician with semen from 5 HF bulls (2 heifers/bull) and 5 LF bulls (2 heifers/bull) or

not inseminated (CTRL; n=8 heifers). Two semen straws (one from each of two ejaculates) from each bull were used. The study was carried out over two replicates.

4.3.3   Bull Selection

Data on the field fertility of a population of Holstein-Friesian bulls (n=840) used in Ireland were obtained from the Irish Cattle Breeding Federation (ICBF) database. Each bull had a minimum of 500 inseminations based on an adjusted sire fertility model (Berry et al., 2011). Adjusted bull fertility was defined as pregnancy to a given service identified retrospectively either from a calving event or where a repeat service (or a pregnancy scan) deemed the animal not to be pregnant to the said service. Cows and heifers that were culled or died on farm were omitted. These raw data were then adjusted for factors including semen type (frozen, fresh), cow parity, days in milk, month of service, day of the week when serviced, service number, cow genotype, herd, AI technician and bull breed and were weighted for number of service records resulting in an adjusted pregnancy rate centred at 0%. For this study, bulls classified as having HF had an average adjusted fertility score of +4.01±0.25% (n = 5; average no. inseminations per bull = 38,041: Table 4.1), whereas, those classified as LF had an average of -11.29±1.11% (n = 5; average no. inseminations = 978; Table 4.1).

Table 4.1 In vivo fertility data of Holstein Friesian bulls of high fertility and low fertility as determined by an adjusted animal model (AAM). Mean of the population in the AAM was 0%.

Bull	Number of inseminations	AAM (%)	Fertility status
1	1479	-15.53	Low
2	519	-12.13	Low
3	609	-10.33	Low
4	1772	-9.83	Low
5	510	-8.93	Low
6	12417	+3.57	High
7	5121	+3.67	High
8	37856	+3.87	High
9	99953	+3.97	High
10	34859	+4.97	High

4.3.4 Tissue collection

Twelve hours after insemination heifers were slaughtered at a commercial abattoir. Reproductive tracts were collected immediately post mortem and the uterine horn ipsilateral to the ovulated ovary was opened longitudinally with a sterile scissors. Tissue samples were obtained from the intercaruncular area of the uterine horn close to the side of the uterine body using a sterile 8 mm biopsy punch (Stiefel Laboratories Ltd, High Wycome, UK). Sterile blades were used to dissect the endometrium away from the myometrium. Samples were immediately stored in liquid nitrogen, transported to the laboratory, and stored at -80 °C.

4.3.5 RNA extraction, library prep and RNA sequencing

One uterine biopsy from each heifer was selected for RNA transcriptomic analysis. Samples were homogenised in Trizol, followed by RNA extraction using an RNeasy Mini Kit (Qiagen) as per manufacturer's instructions. The quantity of RNA was determined using the Nano Drop 1000 (Thermo Fisher Scientific) and quality with the Agilent Bioanalyzer (Agilent Technologies). The average RNA integrity number value of all samples was >7. TruSeq (Illumina TruSeq stranded mRNA library construction) RNA libraries were prepared for all 28 samples. All libraries were sequenced over Illumina NovaSeq sequencer; generating 100bp paired end reads (50 million reads/sample).

4.3.6 Quality control, mapping and differential read count quantification.

Raw sequence reads were downloaded in FASTQ format and these sequence reads were quality assessed using software fastqc (v.0.11.8) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequences from all samples were quality trimmed, and cleaned of adaptor sequences using BBDuk java package. On average 0.2% of the bases were trimmed per sample. Trimmed reads were mapped to the Bovine Reference Genome in UMD3.1. using STAR RNA-seq aligner v2.5.2 (Dobin et al., 2013) and uniquely mapped read counts per Ensemble annotated gene/ transcript were estimated using the STAR –quantMode option. Genes with zero read counts across all samples as well as non-protein coding genes were removed prior to subsequent analysis

Differential gene expression analysis and data transformations and visualization were carried out using DeSeq2 v1.30 (Love et al., 2014)in R 4.0.2. Sample clustering was

carried out on variance stabilizing transformed data and visualised using principal component analysis (PCA). DEGs lists were generated using a negative binomial generalized linear model and pairwise comparisons using each combination of the uterine biopsy groups. P values were adjusted for multiple comparisons using a Benjamini and Hochberg (B-H) method. Genes with an adjusted P value < 0.1 were considered differentially expressed and used for further data exploration and pathway analysis. Gene Ontology and KEGG pathway analysis of DEGs was carried out using clusterProfiler (v3.18.0) (Yu et al., 2012).

4.3.7 PMN assessments and quantification

The ability of PMNs to move from the mucosal region of the endometrium true the epithelium in response to the external environment was assessed as follows:

Endometrial samples (~1 cm^2), which included the surface epithelium, were harvested from three animals for each group (heifers exposed to LF semen, heifer exposed to HF and CTRL heifers- no semen exposure) within 30 minutes of slaughter and fixed in 10% neutral buffered formalin for 48 hrs. Following processing into paraffin, tissue blocks were sectioned at 5 µm thickness using a Leica microtome and stained with haematoxylin and eosin. The sections were examined using an Olympus BX43 microscope fitted with an image analyser (Image - Pro Premium; Media Cybernetics). Using a 63x oil immersion objective, between 20 and 40 Fields of View (FOV) were selected per sample. PMNs within the columnar epithelium region only were quantified. The count was expressed as the average number of PMNs per FOV per group. On average, 106 FOV were analysed per treatment group.

4.4 Results:
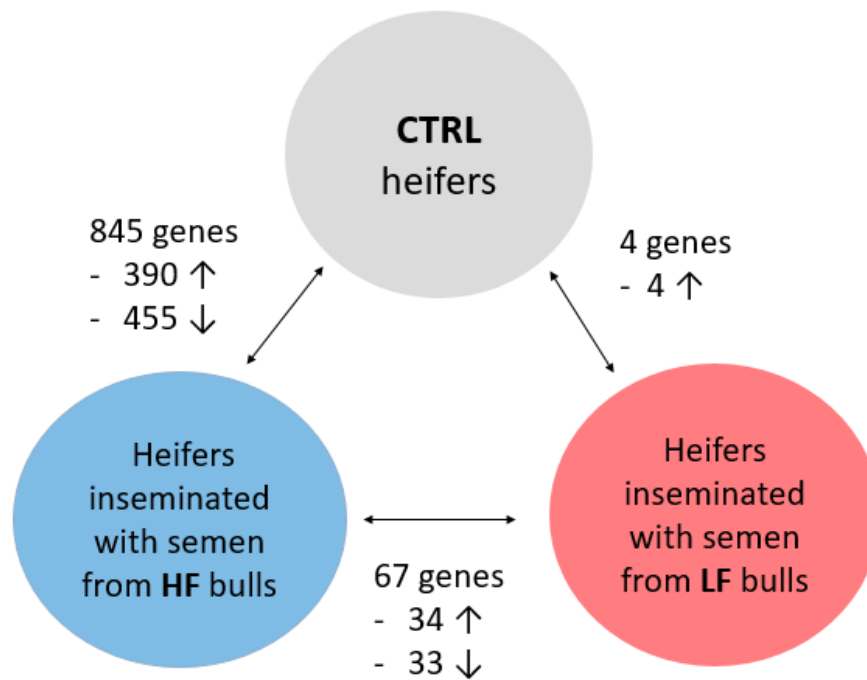
Figure 4.1 Number of differentially expressed genes in the endometrium of heifers where three comparisons were performed. (i) Heifers inseminated with frozen-thawed semen from HF bulls (n=10 heifers; 2 heifers/bull) relative to Control (CTRL; n=8 heifers) (ii) Heifers inseminated with semen from LF (LF) bulls (n=10 heifers; 2 heifers/bull) relative to CTRL(iii) Heifers inseminated with HF bulls relative to LF bulls. Gene expression was determined by RNA-seq using an adjusted p<0.1 and log2FC>1. Arrows represent the direction of fold change.

Comparison 1: Significant differential expression of genes in the endometrium of heifers in response to semen from HF bulls relative to CTRL heifers.

Using rigorous statistical filtering, 845 genes were identified as significantly differentially expressed (adjusted p value <0.1) in the endometrium of heifers inseminated with HF bulls relative to CTRL heifers (Figure 4.1). There is clear separation between samples in both groups as shown in the PCA plot (Figure 4.2). Of the 845 DEG's there were 390 genes with increased expression in the HF treatment and 455 with decreased expression in the HF treatment group and this relatively equal divide is also evident from the volcano plot (Figure 4.2). The fold change values which were increased in HF compared to CTRL treatments ranged from 1.14 to 48.05 fold. The top ten most significant DEGs are shown in Table 4.2. The gene with the largest fold change was a novel gene with the second largest fold change of 21.22 from Gamma-aminobutyric acid type A receptor subunit alpha4 (*GABRA4)* which is involved in transmembrane signalling receptor activity. The top genes with increased expression in HF treatments included

Prostaglandin D2 Synthase *(PTGDS),* Coiled-Coil Domain Containing (*CCDC*) 73 *(CCDC73)* and Solute Carrier Family 7 Member 3 *(SLC7A3).* Of the 390 upregulated genes there were multiple genes encoding coiled-coil domain containing proteins (*CCDC114, CCDC154, CCDC28B, CCDC62, CCDC73, CCDC80, CCDC88B*) with genes from this family involved in protein folding (Burkhard et al., 2001). In relation to immune genes, there was increased expression of Interleukin 11 Receptor Subunit Alpha (*IL11RA),* Interleukin 16 *(IL16)* and *IL1A*. The potent pro-inflammatory molecule *IL1A* was also significantly increased in the HF treatment. Of the 455 significantly downregulated genes in the endometrium of HF heifers relative to the CTRL treatment, the fold change ranged from -18.29 to -1.09. The most significantly decreased gene in the HF group was Arachidonate 12-Lipoxygenase, 12S Type (*ALOX12)* which is known to regulate platelet aggregation and cell migration as well as inflammation and apoptosis (Zheng et al., 2020). Inhibin Subunit Beta A (*INHBA)* was also downregulated in HF heifers which is a key regulator of follicle stimulating hormone (Namwanje and Brown, 2016). Multiple genes encoding solute carrier (SLC) proteins (*SLC12A2, SLC13A5, SLC17A9, SLC22A3, SLC25A15, SLC39A14, SLC4A4, SLC02A1, SLC04A1*) were decreased in HF treatment heifers. The top GO molecular function was cell adhesion molecule (Appendix I) and the top biological process identified by GO for this comparison was sterol biosynthesis process (Appendix I).
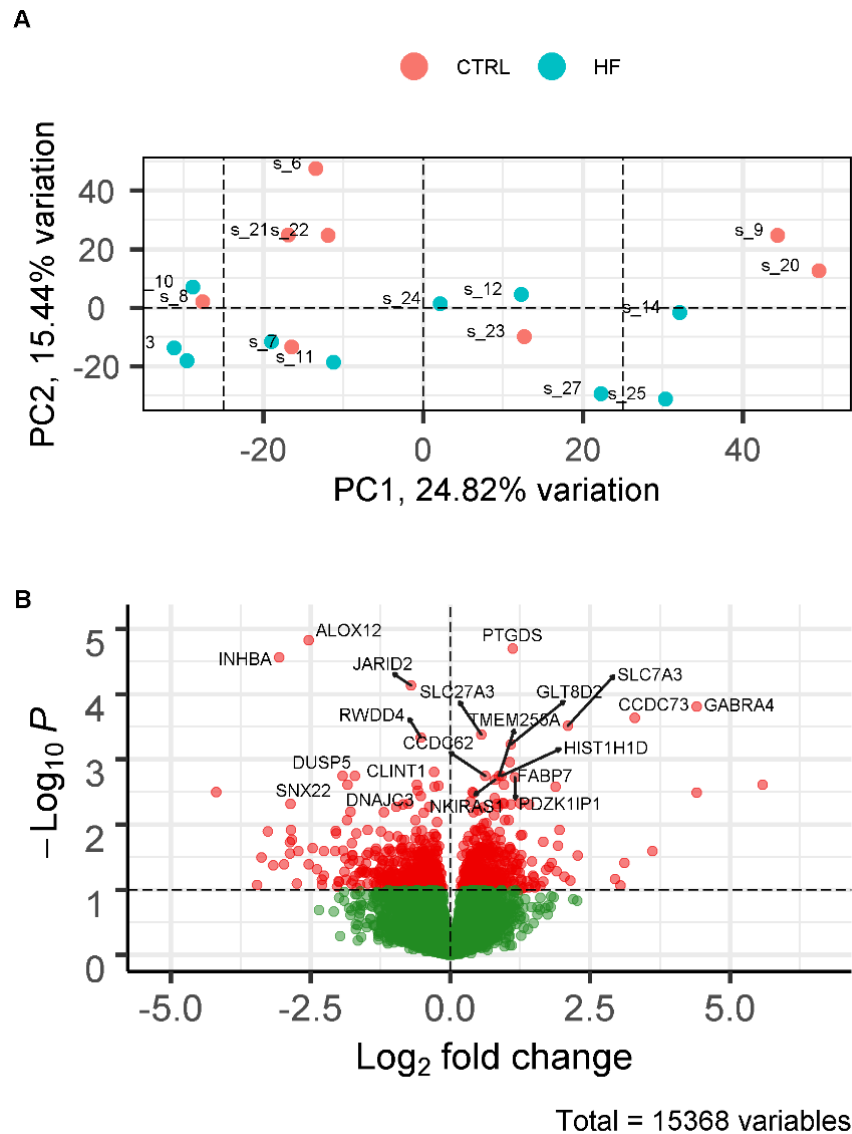
Figure 4.2 Differentially expressed genes in the endometrium of heifers inseminated with frozen-thawed semen from HF bulls (n=10 heifers) relative to Control (CTRL) heifers (n=8 heifers). A) PCA plot showing the distribution of RNA-seq samples, where colours indicate the two treatments and numbers refer to specific heifer IDs. B) Gene expression data are presented as a volcano plot using log values of the fold change and p value. Each data point represents a single gene, with those in black representing genes that survived the cut off thresholds of adjusted p<0.1. The most significant differentially expressed genes are labelled, where gene names are available.

Table 4.2 Top 10 differentially expressed genes (DEG's) in the endometrium of heifers inseminated with frozen-thawed semen from HF (HF) bulls relative to Control (CTRL)

heifers. Positive absFC values indicate higher expression in HF relative to CTRL; negative values indicate higher expression in CTRL relative to HF.

Gene symbol	Ensembl ID	Gene Name	Gene functions	absFC
ALOX12	ENSBTAG00000021933	Arachidonate 12-lipoxygenase	arachidonate 12(S)-lipoxygenase activity	-5.807319
PTGDS	ENSBTAG00000015074	Prostaglandin D2 synthase	Prostaglandin D synthase activity	2.164330
INHBA	ENSBTAG00000002912	Inhibin subunit beta A	To generate subunit of activin and inhibin complexes	-8.358133
JARID2	ENSBTAG00000012938	jumonji and AT-rich interaction domain containing 2	Chromatin/DNA binding	-1.629754
GABRA4	ENSBTAG00000016645	gamma-aminobutyric acid type A receptor alpha4 subunit	Transmembrane signaling receptor activity	21.22029
CCDC73	ENSBTAG00000032151	Coiled coil domain containing 73	Protein coding gene	9.835058793
SLC7A3	ENSBTAG00000007403	solute carrier family 7 member 3	Transmembrane transporter activity	4.287618253
SLC27A3	ENSBTAG00000021862	solute carrier family 27 member 3	Catalytic activity	1.468612493
RWDD4	ENSBTAG00000003081	RWD domain containing 4	Protein binding	-1.44336697
GLT8D2	ENSBTAG00000000925	glycosyltransferase 8 domain containing 2	Transferase activity	2.113567817

4.4.1 Comparison 2: Minor but significant differential expression of genes in the endometrium of heifers in response to semen from LF bulls relative to CTRL heifers.

In contrast to the relatively high number of DEGs in the HF compared to the CTRL heifers, LF heifers has just 4 DEG's relative to the CTRL heifers. There was a reduced amount of segregation within groups as illustrated in PCA plot (Figure 4.3). The low number of DEGs are evident in the volcano plot with all four DEGs skewed to the right signifying increased expression in the LF treatment (Figure 3). These four genes include Leukotriene A4 Hydrolase (*LTA4H),* SH3 Domain Containing 21 *(SH3D21),* Absent in melanoma 1 like *(AIM1L),* Kallikrein related peptidase 10 *(KLK10)* (Table 4.3) with a range of fold change from 1.38 to 2.02.
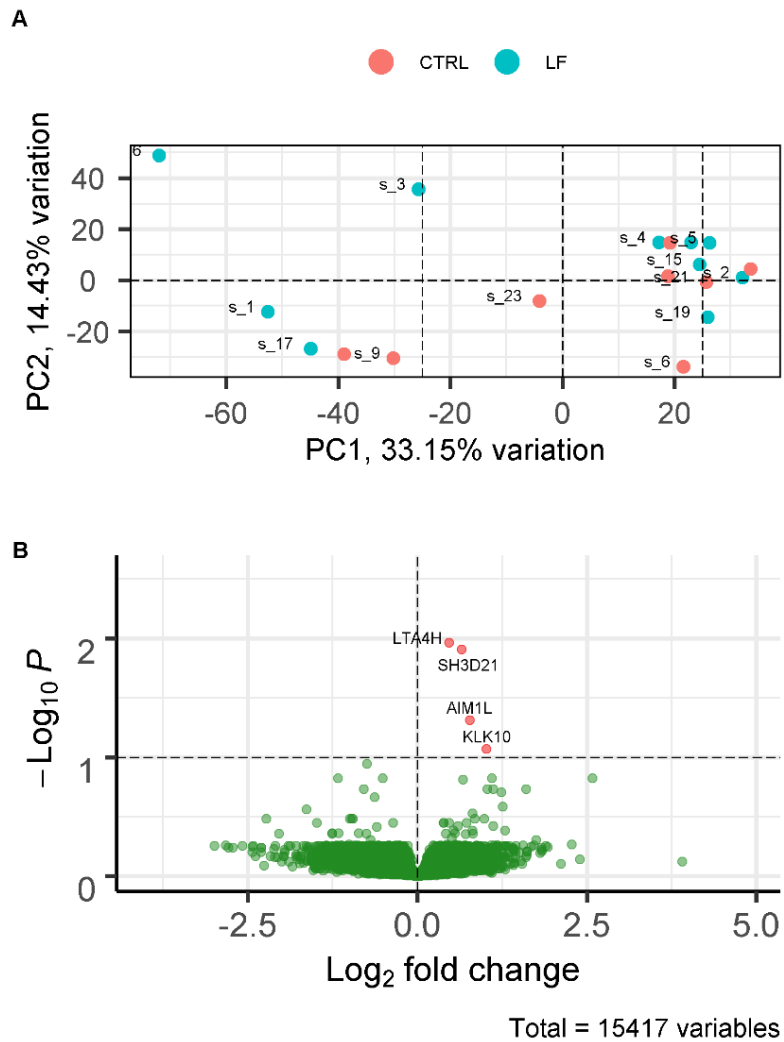
Figure 4.3: Differentially expressed genes in the endometrium of heifers inseminated with frozen-thawed semen from LF (LF) bulls relative to Control (CTRL). A) PCA plot showing the distribution of RNA-seq samples, where colours indicate the two groups and numbers refer to specific heifer IDs (as detailed in Supplementary File 1). B) Gene expression data are presented as a volcano plot using log values of the fold change and p value. Each data point represents a single gene, with those in red representing genes that survived the cut off thresholds of adjusted p<0.1. The four most significant differentially expressed genes are labelled, where gene names are available.

Table 4.3 Top 4 differentially expressed genes (DEG's) in the endometrium of heifers inseminated with frozen-thawed semen from LF (LF) bulls relative to Control (CTRL) heifers. Positive absFC values indicate higher expression in LF relative to CTRL; negative values indicate higher expression in CTRL relative to LF

Gene symbol	Ensembl ID	Gene Name	Gene functions	absFC
LTA4H	ENSBTAG00000016415	Leukotriene A4 hydrolase	Leukotriene A4 hydrolase protein coding	1.383163
SH3D21	ENSBTAG00000038617	SH3 Domain containing 21	protein coding	1.568792
AIM1L/CRYBG2	ENSBTAG00000005629	Absent in melanoma 1 like/ Crystallin beta-gamma domain containing 2	carbohydrate binding	1.706712
KLK10	ENSBTAG00000015129	Kallikrein related peptidase 10	proteolysis	2.029709

4.4.2 Comparison 3: Moderate but significant differential gene expression in the endometrium of heifers in response to semen from HF bulls relative to LF heifers.

When the HF and LF treatments were directly compared, there were 67 DEGs in the endometrium of heifers inseminated with HF bulls relative to LF bulls. From the PCA plot it is evident there is a mixed signal between the groups (Figure 4.4) with an almost equal distribution of genes with increased expression in the HF treatment (34 genes) as the LF treatment (33 genes; Figure 4.4). Fold changes which were increased in the HF relative to LF treatment varied from 1.15 to 40.108. The top 10 DEGs with increased expression in the HF treatment are shown in Table 4.4. The top DEG was pro-inflammatory *IL1A*. The top 10 genes with lower expression in the HF treatment compared to the HF are displayed in Table 4.4. The fold change of the 33 genes decreased in HF relative to LF ranged from -29.69 to – 1.23. Major histocompatibility class (MHC) I and II molecules (*BoLA* and *BOLA-DQB*) had lower expression in the HF treatment signifying a further role of the immune response. These are antigen presenting cells which allow the immune system to distinguish between self and non-self and so they only react to foreign antigens (Sordillo, 2016).
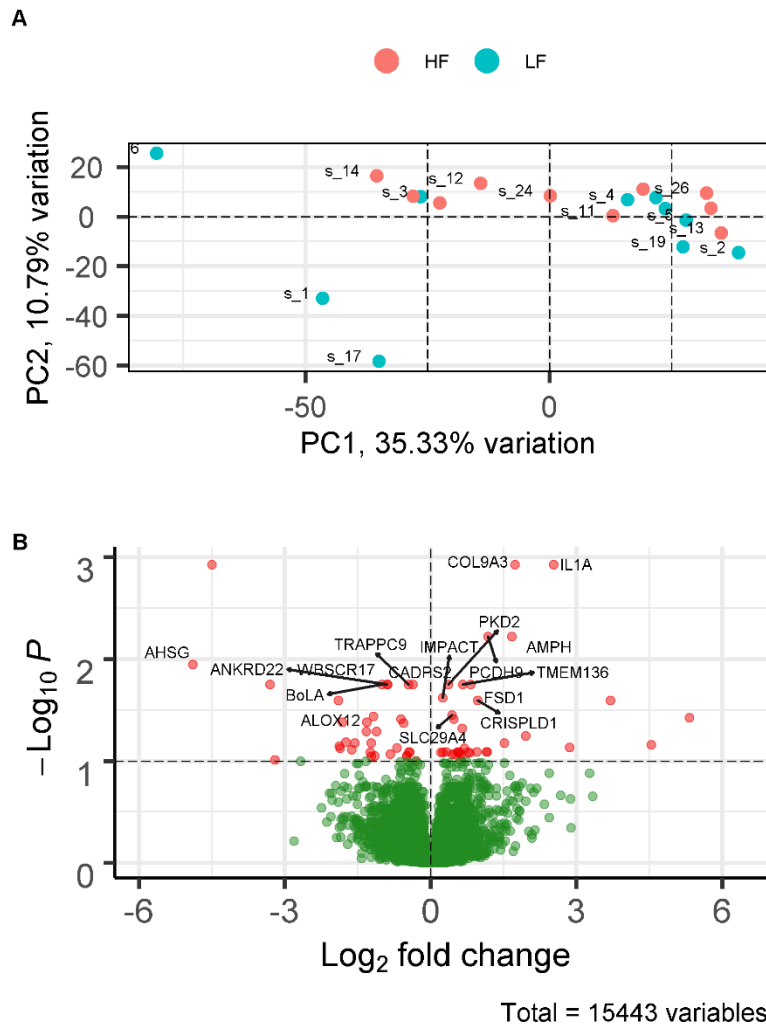
Figure 4.4 Differentially expressed genes (DEG's) in the endometrium of heifers inseminated with frozen-thawed semen from HF bulls relative to bulls LF (LF) bulls. A) PCA plot showing the distribution of RNA-seq samples, where colours indicate the two groups and numbers refer to specific heifer IDs (as detailed in Supplementary file 1. B) Gene expression data are presented as a volcano plot using log values of the fold change and p value. Each data point represents a single gene, with those in red representing genes that survived the cut off thresholds of adjusted p<0.1. The 20 most significant differentially expressed genes are labelled, where gene names are available.

Table 4.4 Differentially expressed genes in the endometrium of heifers inseminated with frozen-thawed semen from HF (HF) bulls relative to LF bulls. Top 10 genes with positive absFC values indicating higher expression in the HF treatment.

Gene symbol	Ensembl ID	Gene Name	Gene functions	absFC
IL1A	ENSBTAG00000010349	Interleukin 1 Alpha	Cytokine activity, immune response	5.768126

COL9A3	ENSBTAG00000015581	collagen type IX alpha 3 chain	extracellular matrix structural constituent	3.336228
PCDH9	ENSBTAG00000019340	protocadherin 9	Calcium ion binding/ cell adhesion	2.266951
AMPH	ENSBTAG00000031967	amphiphysin	Protein binding	3.181240
FSD1	ENSBTAG00000005999	fibronectin type III and SPRY domain containing 1	Protein binding	1.771381394
PKD2	ENSBTAG00000020031	polycystin 2, transient receptor potential cation channel	signaling receptor binding	1.291015573
TMEM136	ENSBTAG00000021342	TLC domain containing 5	integral component of membrane	1.57782319
IMPACT	ENSBTAG00000003035	impact RWD domain protein	actin binding	1.18981983
CRISPLD1	ENSBTAG00000004411	cysteine rich secretory protein LCCL domain containing 1	face morphogenesis	1.951912881
	ENSBTAG00000047700	Novel gene		12.9604735

Table 4.5 Differentially expressed genes in the endometrium of heifers inseminated with frozen-thawed semen from HF (HF) relative to LF bulls. Top 10 genes with negative absFC values indicating lower expression in the HF treatment.

Gene symbol	Ensembl ID	Gene Name	Gene functions	absFC
BOLA-DQB	ENSBTAG00000021077	major histocompatibility complex, class II, DQ beta	Immune response, antigen processing and presentation	-22.652410
AHSG	ENSBTAG00000000522	alpha 2-HS glycoprotein	regulation of inflammatory response positive regulation of phagocytosis	-29.6964674
CADPS2	ENSBTAG00000005110	calcium dependent secretion activator 2	positive regulation of exocytosis synaptic vesicle exocytosis	-1.288826673
BoLA	ENSBTAG00000005182	Bos taurus major histocompatibility complex, class I, A	antigen processing and presentation of endogenous peptide antigen via MHC class Ib	-2.005123331
GALNT17	ENSBTAG00000008718	polypeptide N-acetylgalactosaminyltransferase 17	protein glycosylation	-1.841893142
ANKRD22	ENSBTAG00000013368	ankyrin repeat domain 22	Protein coding	-1.853158972
TRAPPC9	ENSBTAG00000013955	trafficking protein particle complex 9	positive regulation of NF-kappaB transcription factor activity	-1.368959275

	ENSBTAG00000039289	Novel gene		-9.850251615
ALOX12	ENSBTAG00000021933	arachidonate 12-lipoxygenase	arachidonate 12(S)-lipoxygenase activity	-3.736116419
HSH2D	ENSBTAG00000038154	hematopoietic SH2 domain containing	protein-macromolecule adaptor activity	-2.264241458

4.4.3 Spermatozoa induce a significant increase in PMN populations in the endometrium

There were more PMNs in the epithelial layer of the endometrium in both the HF and LF treatments (average of 1.2) compared to the control group (average of 0.33; $P < 0.05$; Figure 5). However, there was no difference between the HF and LF treatments.
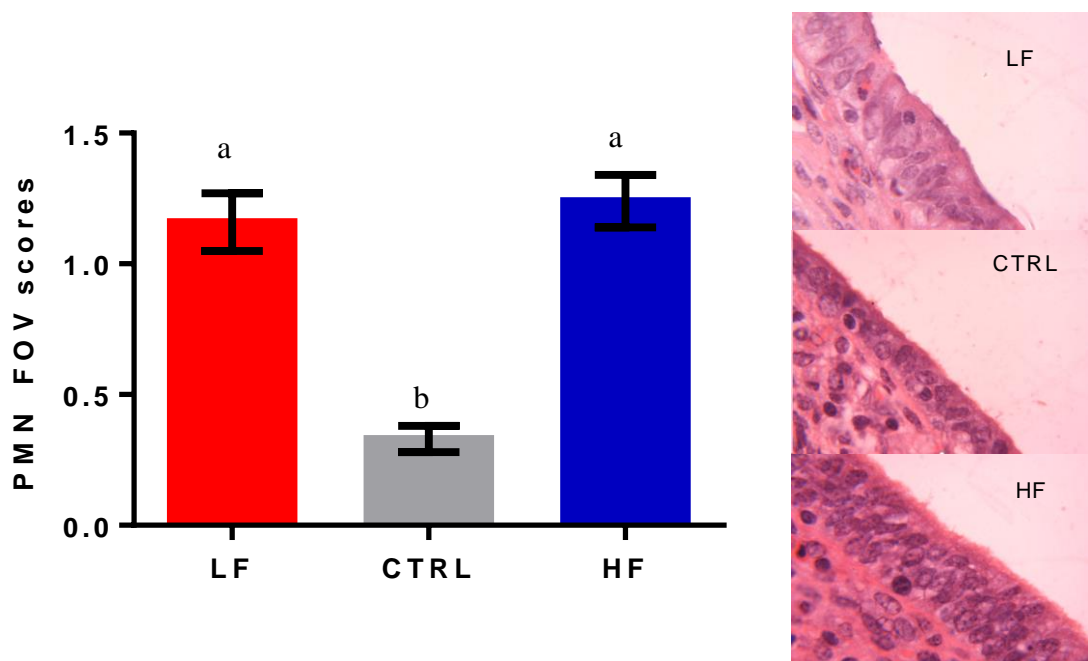
Figure 4.5 Field of view (FOV) scores of PMNs in the endometrium of heifers inseminated with frozen-thawed semen from HF bulls, LF (LF) bulls and control (CTRL) treatment. Histological analysis of endometrium samples were performed on a subset of heifers (n=3 per treatment). Tissue was stained with haematoxylin and eosin and assessed at 60x. Representative images from each treatment group are shown to the right. Data are presented as mean ± s.e.m. [abc] Different superscripts denote a significant difference between the treatments (p<0.05).

4.5 Discussion

The main findings of this study were that not only are spermatozoa immunogenic in the uterus but there was a differential transcriptomic response in the uterus in response to spermatozoa from bulls of high and low field fertility. When compared to the CTRL treatment, HF bulls had a more active transcriptomic response (845 DEG's) compared to the LF treatment (4 DEG's). When directly compared, the HF relative to LF treatment had 67 DEG's, primarily immune genes and genes associated with the inflammatory response.

The top genes with increased expression in the HF treatment compared to CTRL include *PTGDS, CCD73* and *SLC7A3. PTGDS* also known as *L-PGDS*, has been shown to have an important role in the development of spermatozoa and sperm maturation (Rossitto et al., 2015). The top genes with increased expression in the CTRL compared to HF group included *ALOX12* and *INHBA. ALOX12* is known to regulate platelet aggregation and cell migration as well as inflammation while *INHBA* is a key regulator of follicle stimulating hormone (Zheng et al., 2020, Namwanje and Brown, 2016). Multiple genes from the solute carrier protein had significantly lower expression in the HF treatment. These are membrane integral proteins and are known facilitative and secondary active transporters (Cesar-Razquin et al., 2015). Gene ontology analysis showed a role for cell adhesion molecule binding and receptor regulator binding as well as biosynthetic processes, cholesterol biosynthetic and metabolic processes.

The top DEGs between the HF and LF groups directly, with increased expression in the HF group, include *IL1A,* collagen type IX alpha 3 chain *(COL9A3)* and protocadherin 9 (*PCDH9). PCDH9* gene is from the photocadherin family with its main role to mediate cell-to-cell adhesion and in the recognition of the presence of calcium (Wang et al., 2017). *COL9A3* is a collagen type IX alpha 3 chain which is an extracellular matrix structural constituent (Huang et al., 2018).

While a proportion of the DEGs are not linked directly with fertility, through stringent analysis of gene families there is a clear prevalence of genes related to immunity. *IL1A* is a potent inflammatory marker and previous *in vitro* studies have shown spermatozoa are capable of eliciting an inflammatory response in the uterus with an increase in pro-inflammatory molecules *CXCL8, TNFA* and *IL1B* (Elweza et al., 2018, Ezz et al., 2019).

This inflammatory response is associated with the recruitment of PMNs into the uterine glands where spermatozoa interact. The role of this sperm-PMN interaction is for the rapid removal of spermatozoa from the endometrium in preparation for pregnancy (Hansen, 2011, Katila, 2012). The increased inflammatory response from the HF bulls in this study could be associated with clearing and preparing the endometrium more effectively for pregnancy compared to LF bulls. Although, there was no difference in the number of PMNs recruited into the epithelial layer from heifers inseminated with HF or LF semen 12 h post insemination, perhaps each PMN binds more spermatozoa from LF bulls as reported in Chapter 3 which could impede sperm transport to the site of fertilisation.

This study represents similarities to other bovine and murine *in vivo* studies where spermatozoa plus SP but not SP alone altered the transcriptomic response *in vivo* (Recuero et al., 2020, Schjenken et al., 2021). The top DEGs with decreased expression in HF compared to LF groups also include immune genes namely, *BOLA-DQB* and *BOLA,* alpha 2-HS glycoprotein *(AHSG)* and trafficking protein particle complex 9 (*TRAPPC9)*. MHC molecules have been shown to be essential to the female's tolerance to pregnancy establishment and although the genes expressed in this study are from 12 h post insemination, the initial crosstalk between spermatozoa and the female reproductive tract has been shown to be mediated by MHC class members (Rapacz-Leonard et al., 2014, Melo et al., 2017). The decreased expression of *BOLA-DQB* was also observed in pregnant heifers after natural mating, similar to its downregulation in the HF group in this study. *AHSG* (known as Feutin) was also increased and has been shown to be an antagonist of the pro-inflammatory molecule *TNF* (Ombrellino et al., 2001). *TRAPPC9* gene is known to play a key role in the NF-κB transcription factor which is involved a number of cellular processes such as innate and adaptive immunity, cellular proliferation, apoptosis, and development (Zhang et al., 2017).

The extent of this immune response and its benefit on subsequent fertility is not exactly known, however, recent studies in mice show sperm signalling the adaptive immune response *in vivo* with the up-regulation of T-regulatory cells (Schjenken et al., 2021) which are important mediators of maternal immune tolerance and regulates embryo implantation and fetal survival (Samstein et al., 2012). Similarly in this study, some genes in the wider dataset were significant and had strong read counts showing an increased expression of *IL11RA* and *IL16* in the HF compared to CTRL group. IL16 is an immunomodulatory chemokine which is associated with activating T-regulatory cells

and uses the CD4 molecule as it's receptor (Huang et al., 2019). Indeed, *Il11ra* null mutant female mice are infertile as decidualization of the endometrium is not possible (Robb et al., 1998, Dimitriadis et al., 2003). However, *IL11* and *IL6* had higher expression in the CTRL group signifying that perhaps this adaptive response was just beginning to switch on in the endometrium of heifers with HF semen 12 h post-insemination.

In conclusion, this study has shown for the first time a distinct divergent transcriptomic response in the endometrium of heifers inseminated with frozen-thawed semen from HF and LF bulls with a pivotal role of the inflammatory response. There was a clear effect of spermatozoa in recruiting PMNs to the endometrium with a more active transcriptomic response in the HF group suggesting a more rapid clearing of the endometrium as well as preparing the endometrium for implantation and subsequent pregnancy. This study not only furthers our understanding of the etiology of bull fertility but also of pregnancy establishment.

Chapter 5 Identification of differentially expressed mRNAs and miRNAs in spermatozoa of bulls of varying fertility

5.1 Abstract

Bulls used in artificial insemination, with apparently normal semen quality, can vary significantly in their field fertility. Detailed assessments of sperm function using computer-assisted sperm analysis and flow cytometry have had limited success in accurately predicting field fertility. The sperm transcriptome contains a rich population of messenger mRNAs, and long non-coding and small RNAs, such as miRNAs, which are fundamental for sperm function, successful fertilization, and embryo development. This study aimed to characterize the transcriptome of spermatozoa from HF and LF fertility bulls at the mRNA and miRNA level in order to identify potential novel markers of fertility. Holstein-Friesian bulls were assigned to either the HF or LF group (n=10 per group) based on adjusted fertility scores calculated from a minimum of 500 inseminations per bull. Total RNA was extracted from a pool of frozen-thawed spermatozoa from three different ejaculates per bull, following which mRNA-seq and miRNA-seq were performed. Six mRNAs and 13 miRNAs were found differentially expressed (P<0.05, log2FC>1.5) between HF and LF bulls. Of particular interest, the gene pathways targeted by the 13 differentially expressed miRNAs were related to embryonic development and gene expression regulation. Previous studies reported that disruptions to protamine 1 mRNA (*PRM1*) had deleterious consequences for sperm chromatin structure and fertilising ability. Notably, *PRM1* exhibited a higher expression in spermatozoa from LF than HF bulls. In contrast, Western Blot analysis revealed a decrease in PRM1 protein expression for spermatozoa from LF bulls; this was not associated with defects in chromatin compaction or increased DNA fragmentation, as assessed by flow cytometric analyses. This could suggest that LF bulls exhibit an abnormal sperm chromatin structure. This study has identified potential biomarkers that could be used for improving semen quality assessment of bull fertility.

5.2 Introduction

Due to the relatively recent advent of genomic selection (Hayes et al., 2009), elite bulls can now be reliably used as sires for use in AI programs as soon as they reach puberty. However, one of the problems associated problems is that it provides little time for test inseminations prior to widespread use of the bull's semen. While AI centres perform robust semen quality controls, primarily based on motility and morphological based parameters of spermatozoa post-thawing; bulls can still vary significantly in their field fertility. Indeed, there can be up to 25% variation in conception rates among bulls used commercially for AI, all of which have passed routine semen quality controls (Larson and Miller, 2000). This has led to more detailed assessments of sperm kinematics using computer-assisted sperm analysis (CASA) as well as cellular function using flow cytometry. However, despite various multivariate and statistical approaches being taken, no single test nor combination of tests can reliably and consistently predict field fertility. This is evident from the work of both Sellem et al. (2015) and Bernecic et al. (2021) which could explain approximately 40% and 47%, respectively of the variation in Holstein bull fertility through the assessment of flow cytometry and CASA based parameters. A recent study by Narud et al. (2020) increased the explained variation in bull field fertility to 59% by adding the measurement of intracellular metabolites and traces elements (such as amino acids, Fe and Zn) in the spermatozoa of Norwegian Red Bulls (Narud et al., 2020). This suggests that other factors, such as other biochemical and/or molecular characteristics of spermatozoa could explain some of the remaining variation, and their assessments could improve bull fertility prediction.

The acquisition of the full fertility potential of males post puberty requires a complex reorganization of the genomic and epigenomic architectures of sperm cell precursors during spermatogenesis and spermiogenesis, which involves the sequential transcription of thousands of genes. Recent advances in transcriptomics have revealed that mature spermatozoa not only carry the paternal haploid genome, but also deliver an abundant cargo of various types of RNAs into the oocyte, including messenger mRNAs, as well as long and small non-coding RNAs, tRNAs, rRNAs, piRNAs, and miRNAs (Selvaraju et al., 2017, Sellem et al., 2020).

While they used to be only considered as remnant transcripts produced during spermatogenesis, evidence now suggests that mRNAs are involved in capacitation, fertilization, and early embryogenesis (Card et al., 2013). Numerous studies have been

conducted to decipher the role of mRNAs in bull fertility, and they found associations with the expression of certain mRNAs to the quality of semen and sire conception rate, such as the transcripts from genes *CRISP2*, *CCT8* and *PEBP1* (Arangasamy et al., 2011), *AK1, IB5*, *TIMP2* and *PLCz1* (Kasimanickam et al., 2012) *PRM1* (Ganguly et al., 2013), Adiponectin, C1Q And Collagen Domain (*ADIPOQ)*, aldehyde reductase *(AR1* and *AR2)* (Kasimanickam et al., 2013), Bone Morphogenetic Protein 2 (*BMP2)* and NFRSF1A Associated Via Death Domain (*TRADD)* (Parthipan et al., 2017), *AQP7* (Kasimanickam et al., 2017), or *CB1* and Fatty Acid Amide Hydrolase *(FAAH)* (Kumar et al., 2018). In a recent study using a RNA-seq approach, Card and colleagues respectively identified 3227 and 5366 transcripts differentially expressed in spermatozoa between high and low bull fertility populations (Card et al., 2017).

While the transcription of mRNA is arrested by the time developing germs cells reach the late spermatid stage (Reilly et al., 2016), accumulation of non-coding RNAs occurs both during spermatogenesis and during epididymal transit through the incorporation of epididymosomes (Reilly et al., 2016), and it has been demonstrated that they play a fundamental role in regulating gene expression during early embryo development (Yuan et al., 2016, Gross et al., 2019). In particular, miRNAs function in RNA silencing and post-transcriptional regulation of gene expression. Similarly, to mRNAs, differential expression of miRNAs has been identified between bulls which differ in terms of fertility, such as bta-miR-502-5p and bta-miR-1249-3p (Fagerlind et al., 2015), or bta-miR-15a and bta-miR-29b (Menezes et al., 2020). Capra et al. (2017) identified 83 differentially expressed miRNAs between high and low motile bull spermatozoa, with these miRNAs targeting biological pathways related to apoptosis (Capra et al., 2017). Recently, Turri et al. (2021) reported 13 differentially expressed miRNAs between HF and LF with miR-423-3p highly expressed among LF bulls associated with severe asthenozoospermia in human spermatozoa. In the same study miR-191, had increased expression in HF bulls and has previously been positively associated with the fertilization rate of blastocysts (Xu et al., 2020).

While numerous studies have been conducted to catalogue mRNAs and miRNAs which are involved in bull fertility, to our knowledge none have conducted both analyses on the same bull population. Moreover, in many studies, the fertility phenotype is unreliable as bull field fertility was not sufficiently divergent and/or was based on too few inseminations, or RNA was extracted from a single ejaculate and did not take account of ejaculate-to-ejaculate variation (Amann and DeJarnette, 2012). The aim of this study was

to examine the disparity in sperm mRNA and miRNA fingerprints and associated biological pathways between HF and LF bulls.

5.3 Material and methods

5.3.1 Ethical approval

All protocols were in accordance with the Cruelty to Animals Act (Ireland 1876, as amended by European Communities regulations 2002 and 2005) and the European Community Directive 86/609/EC.

5.3.2 Animals and semen collection

Mature Holstein-Friesian bulls with high (HF; n = 10) or low (LF; n = 10) fertility were selected from a population of 1665 AI bulls. Bull fertility was based on adjusted fertility scores (Berry et al., 2011), calculated from a record of at least 500 inseminations (mean = 13,292, min = 519, max = 100,288). HF bulls showed an average adjusted fertility score of +6.5%, whereas LF bulls showed an average fertility score of -6.6% (Table 5.1). The mean of the population was 0. Semen was collected at two AI centers in Ireland *via* an artificial vagina, frozen in 0.25 ml French straws using a programmable freezer and stored in liquid nitrogen pending further analysis.

Table 5.1 Fertility data from the AAM (%) for 10 HF and 10 LF bulls

Bull	Number of inseminations	AAM (%)	Fertility
1	8637	6 %	High
2	37849	6.7 %	High
3	11459	5.8 %	High
4	3912	6.5 %	High
5	12424	6.8 %	High
6	100288	6.8 %	High
7	17441	6.2 %	High

8	5119	6.2 %	High
9	34973	7.1 %	High
10	1041	6.7 %	High
11	23811	- 4.3 %	Low
12	740	- 9.1 %	Low
13	519	- 9.3 %	Low
14	1034	-8.5 %	Low
15	1477	-12.3 %	Low
16	1772	-7.3 %	Low
17	1195	- 3.5 %	Low
18	568	- 3.0 %	Low
19	597	- 4.6 %	Low
20	980	- 3.9 %	Low

5.3.3 Total RNA extraction

From each bull, two straws from three different ejaculates were pooled (i.e. 6 straws in total)to minimize transcript expression profile biases. The pools were centrifuged at 2400 g for 7 min at room temperature, and the supernatant discarded. The sperm pellet was resuspended in 1.5 ml somatic cell lysis buffer (SCLB, 0.1% SDS + 0.5% Triton X-100 in RNase-free H_2O) to obtain a pure sperm cell population (Goodrich et al., 2007). After centrifugation and SCLB supernatant removal, the pellet was washed with PBS. Total RNA was then extracted as described previously (Sellem et al., 2020), with modifications. After washing, the pellet was resuspended in 98 µl RLT buffer (Qiagen, Hilden, Germany) with 2-mercaptoethanol. Then, 1 ml of TRIzol was added to the sperm pellet and homogenized by vortexing for 1 min, and incubated for 5 min at RT. To the lysate, 100 µl chloroform was added and mixed vigorously by hand for 15 s, and tubes were allowed to stand at RT for 3 min. The mixture was centrifuged at 12,000 g for 15 min at

4°C. After centrifugation, the upper aqueous layer containing RNA was transferred to a new 1.5 ml tube, to which 100 µl chloroform was added and the same process redone. An equal volume of isopropanol + glycogen was added to the aqueous solution and mixed gently. The mixture was kept overnight at -20°C. After centrifugation at 12,000 g for 15 min at 4°C, the supernatant was discarded, and 1.5 ml of 75% ethanol was added to the RNA pellet and centrifuged at 12,000 g for 15 min, 4°C. Ethanol was removed, and the RNA pellet was air-dried for 10 min to remove traces of ethanol. The pellet was dissolved in 44 µl of RNase-free H_2O. Then, removal of traces of gDNA was performed by treatment with DNase (TURBO DNase; Ambion, Austin, TX, USA) for 30 min at 37°C. To remove the enzyme and its buffer, the extraction process was performed again. In the end, and after two washing steps with 75% ethanol, total RNA was eluted in 12 µl RNase-free H_2O. RNA concentration was determined using the Qubit RNA HS Assay kit on the Qubit 4.0 Fluorometer (Invitrogen, Waltham, MA, USA).

5.3.4   Quality controls before sequencing by RT-PCR

Studying sperm RNA primarily relies on extracting non-biased and good quality RNAs, which still presents several challenges (Parthipan et al., 2015, Bianchi et al., 2018). As the quantity of RNA in spermatozoa is exceptionally low compared to somatic cells, on the femtogram scale , the analysis of sperm RNAs is prone to bias. Potential sources of cellular contamination, such as epithelial cells, leucocytes, and immature diploid spermatocytes, need to be removed from the samples. Among the various methods available (El Fekih et al., 2017) we choose the somatic cells lysis using SCLB treatment, since its efficacy is known (Ostermeier et al., 2002). Sperm RNA purity was confirmed by RT-PCR, a method which we also employed to assess RNA quality after extraction, as spermatozoa do not contain the 18S and 28S ribosomal RNA, preventing calculation of the RNA Integrity Number (RIN).

Three nanograms of total RNA was reverse-transcribed and amplified using the OneStep RT-PCR kit (Qiagen) according to the manufacturer's instructions and using 40 amplification cycles. Primers were designed using Primer-BLAST (Ye et al., 2012), with the bovine reference genome ARS-UCD1.2. Genomic DNA contamination was tested using intron spanning primers specific to the bovine protamine 1 (*PRM1*) gene. The positive control was a purified bovine genomic DNA extracted from sperm cells, following a protocol previously described (Perrier et al., 2018). Absence of leucocytes, epithelial and germ cell contamination was tested using primers targeting protein tyrosine

phosphatase receptor type C (*PTPRC*), cadherin-1 (*CDH1*) and mast/stem cell growth factor receptor Kit (*KIT*), respectively, as these genes are not expressed in sperm cells. RNA extracted from bovine uterine samples was used as positive control. For each RT-PCR run, negative controls were added and consisted of RT-PCR mix without template RNA. All primers were supplied by Sigma (St. Louis, MO, USA), and their sequences are listed in Appendix II The PCR products were separated by electrophoresis with 1.5% agarose, 0.5X Tris-acetate EDTA gel stained with SYBR safe gel stain (Invitrogen).

5.3.5   mRNA and miRNA sequencing

Library preparations from total RNA and sequencing were carried out by GenomeScan (Leiden, The Netherlands). RNA concentration and the size distribution of the RNAs were determined using a Fragment Analyzer system (Agilent, Santa Clara, CA, USA). For mRNA-seq, library preparation was carried out using the NEBNext® Single Cell/Low Input RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA). For small RNA-seq, library preparation was performed using the NEBNext® Multiplex Small RNA Library Prep kit for Illumina (NEB). Possible adapter dimers were removed using the Blue Pippin size selection. Both mRNA and small RNA libraries were sequenced on a NovaSeq6000 (Illumina, San Diego, CA, USA) according to manufacturer's instructions, using the following parameters: paired-end and 150 bp read length.

5.3.6   mRNA-seq bioinformatics and data analysis

Raw sequencing reads were first checked for sequencing quality using FASTQC (version 0.11.8, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequencing reads were then trimmed using Trimmomatic V0.30 (Bolger et al., 2014) of Illumina TruSeq adapter sequences. Following removal of sequencing adapters, reads were aligned to the *Bos taurus* reference genome (ARS-UCD1.2 including the Y chromosome from Btau_5.1 assembly), using the read aligner TopHat (v2.0.14; (Kim et al., 2013)) with default settings. Based on the mapped locations in the read alignment files the frequency of how often a read was mapped to a transcript was determined through HTSeq (v0.6.1.pl; (Anders et al., 2015)). The number of read counts mapping to each annotated gene from HTSeq was then collated into a single file to be used for subsequent differential gene expression analysis. The R (version 2.14.1; R Core Team, 2020) Bioconductor package,

EdgeR (version 3.26.7, (Robinson et al., 2010)) was employed to undertake differential gene expression analysis of sequencing data. For this, mRNA reads were first converted to counts per million within EdgeR, any mRNA within samples that had less than one count per million (CPM) in at least half of the samples was subsequently removed from the analyses. For the retained mRNAs, their counts were normalized using the trimmed mean of M values (TMM) method. To test for differential mRNA expression between treatment groups, the normalized counts were modelled using a generalized linear model under a binomial distribution using moderated tagwise dispersions.

5.3.7   miRNA-seq bioinformatics and data analysis

Raw sequence reads were firstly assessed for sequencing quality using FASTQC (version 0.11.8;(Andrews, 2010). The Illumina sequencing adaptor was clipped off all the raw read sequences using Cutadapt (version 1.18, (Martin, 2011)). Reads of lengths shorter than 15 bp, and longer than 28 bp were subsequently removed as short and long reads, respectively. The retained reads were then additionally filtered for other bovine short RNA species including ribosomal rRNAs, transfer tRNAs, small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) downloaded from https://rnacentral.org/. To profile miRNA expression in each sample, the miRDeep2 package (version 2.00.8, (Friedlander et al., 2012)) modules were used, together with the bovine reference genome (ARS1.2+y) and the known bovine mature miRNA sequences and their precursor sequences from the miRBase database (release 22.1, (Kozomara et al., 2019)). The miRDeep2 mapper module (mapper.pl) was then used with default parameters to collapse reads of the sequences into clusters. Bowtie 1 (version 1.1.1, (Langmead et al., 2009) was then employed to align the collapsed reads to the indexed reference genome. Using default parameters, and input files including the reference genome, collapsed reads versus reference genome alignment, known bovine mature miRNAs and their precursors sequences (including the hairpin structures), and *Bos taurus* (bta) as the species of interest, the miRDeep2 module (miRDeep2.pl) was used to quantify bovine miRNAs. Through this the miRDeep2 quantifier module was used to quantify all known expressed miRNAs in the sequence data, producing read counts for each individual sample.

Resultant read counts for each sample were merged into one file and subsequently assessed for differentially expressed miRNA using the R (v2.14.1) Bioconductor package, EdgeR (version 3.26.7) as previously described for mRNA. Target genes for

differentially expressed miRNA were predicted using TargetScan (release 7.2, (Agarwal et al., 2015)), and the biological pathways in which the target genes are involved was revealed using the KEGG pathway analysis

5.3.8 Validation of the mRNA and miRNA-seq data by Reverse-Transcription - quantitative PCR (RT-qPCR)

To obtain sufficient RNA template for performing the validations of the mRNA-seq and miRNA-seq data by RT-qPCR, total RNA was extracted from a separate batch of straws, originating from the same ejaculates from the same bulls as for the mRNA-seq experiment.

RT-qPCR was carried out for five of the six DEGs that were identified by mRNA-seq: *PRM1*, SCP2 Sterol Binding Domain Containing 1 (*SCP2D1*), and RB Binding Protein 6 (*RBBP6*), and the 2 novel genes (no primers could be successfully designed for *SLC24A1*). RT reactions were performed using 20 ng of template RNA and the high-capacity cDNA reverse transcription Kit (Applied Biosystems, Waltham, MA, USA), following the manufacturers' instructions. Each sample reaction contained 1 µl of multiScribe reverse transcriptase, 2 µl of 10X RT random primers, 0.8 µl of 25X dNTP mix (100mM), 2 µl of 10X RT buffer, 4.2 µl of nuclease free water and 10 µl of total RNA (at 2 ng/µl). The following program was used in a 2720 Thermal Cycler (Applied Biosystems): 10 min. at 25°C, 120 min. at 37°C, and finally 5 min. at 85°C;. For real-time quantitative PCR (qPCR) reactions; primers were designed using Primer-BLAST (Ye et al., 2012) and with the bovine reference genome ARS-UCD1.2 (primers sequences can be found in Appendix II). Primer efficiencies were assessed using serial dilutions of pooled cDNA samples and were calculated to be > 80 % and < 120%. qPCR reactions were performed in triplicate using the TaqMan Fast Advanced Master Mix (Applied Biosystems), following the manufacturer's instructions. Each sample reaction contained 10 µl of 2X TaqMan Fast Advanced Master Mix, 1 µl of 10µM primer mix, 7 µl of nuclease free water and 2 µl cDNAs. The following program was used: 20 sec at 95°C, followed by 40 cycles of 3 sec at 95°C and 30 sec at 60°C; in a 7500 Fast Real-Time PCR System v2.0.1 (Applied Biosystems). For qPCR data, Ct values were imported into GenEx software (v.5.2.7.44). Ct values were adjusted for primer efficiency and replicate values were averaged. Data were normalized using the geometric mean of the reference gene yrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

(YWHAZ) and fold change values were calculated using the delta-delta Ct method ($\Delta\Delta$Cq; also known as$\Delta\Delta$CT).

Due to low levels of expression, it was not possible to directly validate the differentially expressed miRNAs by RT-qPCR. Spermatozoa have low levels of miRNAs and the differentially expressed miRNAs were not the most abundant ones. Therefore, a technical validation of the miRNA-seq data was carried out by RT-qPCR on 2 miRNAs that were identified as highly expressed in all samples (bta-miR-100 and bta-miR-34c). bta-miR-125 was used as a reference miRNA. This approach was similar to that taken by Sellem et al. (2020) and its aim was to demonstrate that the relative abundance of miRNAs is broadly consistent across miRNA seq and RT-qPCR. RT reactions were performed using 10 ng of template RNA and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems), following the manufacturers' instructions. Each sample reaction contained 1 µl of multiScribe reverse transcriptase, 3 µl of 5X stem-loop RT primer, 0.15 µl of 100mM dNTPs, 1.5 µl of 10X RT buffer, 0.19 µl of RNase inhibitor (20 U/µl), 4.16 µl of nuclease free water and 5 µl of total RNA (at 2 ng/µl). The following PCR program was used: 30 min at 16°C, 30 min at 42°C, and finally 5 min at 85°C; in a 2720 Thermal Cycler (Applied Biosystems). For qPCR reactions, primers were retrieved from TaqMan MicroRNA assay (Applied Biosystems). Primer efficiencies were assessed using serial dilutions of pooled cDNA samples and were calculated > 80 % and < 120 %. qPCR reactions were performed in triplicate using the TaqMan Fast Advanced Master Mix (Applied Biosystems) following manufacturer's instructions for TaqMan MicroRNAs Assays. Each sample reaction contained 10 µl of 2X TaqMan Fast Advanced Master Mix, 1 µl 20 X TaqMan MicroRNA Assay primers, 4.5 µl of nuclease free water and 4.5 µl cDNAs. The following qPCR program was used in a 7500 Fast Real-Time PCR System v2.0.1 (Applied Biosystems): 20 sec at 95°C, followed by 40 cycles of 3 sec at 95°C and 30 sec at 60°C;. For the analysis, bta-miRNA-125 was used to normalize the data. Statistical analysis was carried out using the Kruskal-Wallis test (R version 2.14).

5.3.9 Sperm nuclear protein acid extraction and Western blotting

The most expressed sperm specific gene in the RNAseq analysis was *PRM1,* which had higher expression in spermatozoa from LF than HF bulls. Therefore, the objective was to assess the protein PRM1 expression level in spermatozoa from the HF and LF bulls. Sperm nuclear proteins were extracted according to de Yebra and Oliva (1993). For each bull, semen from three separate ejaculates was pooled and the sperm concentration was

determined prior to processing. Semen samples were washed twice in cold PBS containing 6 mM EDTA and 1 mM PMSF, followed by hypotonic wash in ddH$_2$O with 6 mM EDTA and 1 mM PMSF. Sperm nuclear proteins were denatured for 30 min at RT in PBS containing 6 mM EDTA, 1 mM PMSF, 2.4 M guanidine hydrochloride and 100 mM DTT. One ml of 100% ethanol (-20°C) was then added and the sample was centrifuged at 12,000 g for 10 min at 4°C. The supernatant was then removed and discarded, and the gel-like pellet was washed twice in 1 ml of 100% ethanol. The pellet was then resuspended in 0.5 M hydrochloric acid and the nuclear proteins were acid solubilized under agitation for 15 min at 37 °C. The samples were centrifuged at 12,000 g for 10 min and the supernatant containing the solubilized nuclear proteins was retained. Trichloroacetic acid was added to a final concentration of 40% and precipitated nuclear proteins were collected by centrifugation at 20,000 g for 20 min at RT. The pellet was washed twice in 100% acetone containing 1% 2-mercaptoethanol and then air-dried. Pellets were snap frozen in liquid nitrogen and stored at -80°C. Purified nuclear proteins were resuspended in methylene blue sample buffer containing 6 M urea and 5% acetic acid and incubated for 1 h at RT prior to loading onto 16% poly-acrylamide gels containing 6 M urea and 5 % acetic acid. For each bull, a volume corresponding to 110,000 sperm cells was analyzed. Gels were run under reverse polarity at 120 – 150 V for 100 min. Separated proteins were transferred to 0.2 µm pore size PVDF membrane in a 0.7% acetic acid transfer buffer for 25 min at 340 mA. Membranes were blocked in TBS-Tween (0.1%) containing 5% milk for 1 h at RT followed by overnight incubation at 4°C with anti-PRM1 antibody (mAb Hup-1N, BriarPatch Biosciences, 1/2000 dilution). The signal intensities of PRM1 protein bands from each bull were quantified using Bio-1D Analysis Software (Vilbur Lourmat Fusion Fx6 EDGE imaging system). Following background subtraction, the sum of pixel intensities in a fixed area surrounding each band was determined. Data were pooled from two biological replicates, each with three technical repeats.

5.3.10 Flow cytometric assessment of protamine deficiency and DNA fragmentation

The assessment of protamine deficiency and DNA fragmentation were performed on a CytoFLEX flow cytometer from Beckman Coulter (Labplan; Dublin, Ireland). CytoFLEX daily quality control fluorospheres (Beckman Coulter) were used prior to each experiment to verify the optical alignment. A sperm-specific population was gated

following identification with side and forward scatter. For all assessments, 10,000 events were recorded (unless otherwise stated) and the area of the signal pulse was used during data collation. The collection and preparation of data for analysis was performed using the CytExpert software. For those parameters measured, an ejaculate from a single bull (reference sample) was included for each assessment to monitor day-to-day variation. Chromomycin A3 (CMA3) and Acridine Orange (AO) were sourced from Sigma Aldrich and Invitrogen, respectively.

Chromomycin A_3 labelling of G-C regions of DNA was employed as an indirect assessment of protamine deficiency (as measured by the degree of chromatin compaction) (Lolis et al., 1996). For this assessment, a protocol adapted from Fortes et al. (2014) was used. Briefly, frozen-thawed spermatozoa were washed twice with PBS free of Ca^{2+} and Mg^{2+} (PBS; pH 7.0) via centrifugation (500 x g, 5 min). As a positive control, a reference sample was incubated with 5 mM DTT for 15 min at 37˚C prior to washing with PBS. Following washes, samples were resuspended to 50 x 10^6 sperm/mL with 0.25 mg/mL CMA3 in McIlvaine's buffer (17 mM citric acid, 164 mM Na_2HPO_4, 10 mM $MgCl_2.6H_2O$; pH 7.0) and incubated at RT, in the dark for 1 h. Prior to flow cytometry assessment, spermatozoa were washed with PBS (500 x g, 5 min) to remove excess CMA3 and diluted to 5 x 10^6 sperm/mL. CMA3 was excited by a 405 nm laser and detected with 525/40 nm band-pass filter. Three populations were detected with this fluorophore as observed in previous studies, low, medium and high CMA3 labelling (Fortes et al., 2014), where high CMA3 labelling is indicative of protamine deficiency. The susceptibility of sperm chromatin to DNA fragmentation was assessed using AO. As a positive control for DNA fragmentation, a reference sample was incubated with 0.8 M HCl for 5 min at 37˚C prior to assessment. Using one straw from the same ejaculates as used for RNA extraction (20 bulls, 3 ejaculates per bull), samples were prepared and stained with AO according to the protocol described by Evenson and Jost (2000). AO was excited using a 488 nm laser and green and red fluorescence was detected with a 525/40 nm or 690/50 nm band-pass filter, respectively. During data acquisition, the flow rate was adjusted to approximately 200 events/s and 5000 events (in the sperm-specific gate) were recorded for analysis. The population with high red and low green fluorescence was identified as spermatozoa with high DNA fragmentation.

5.3.11 Statistical analysis of the western blot and flow cytometry data

In all experiments, bull, was the experimental unit. Western blot data were normalized using MIN-MAX scaling and statistical analysis was carried out using the students unpaired t-test (PRISM 8 software). All statistical analyses for CMA3 and DNA fragmentation were performed using R. Fertility was set as a fixed effect in the model, whereas bull and ejaculate were included as nested random effects. Normality and homoscedasticity of the residuals were assessed for all models by use of Shapiro-Wilk test and Bartlett's test, respectively. Identification of statistical outliers was assessed by Cooks Distance and observations were removed when necessary. Pairwise comparisons between high and low fertility were determined using a Tukey adjustment. To determine the relationship between CMA3 and DNA fragmentation, a Pearson's product-moment correlation was performed using all data from individual ejaculates collected from HF and LF bulls. With the exception of the correlation, all data are presented as the mean ± s.e.m.

5.4 Results

5.4.1 Sperm RNA yield, quality, and purity
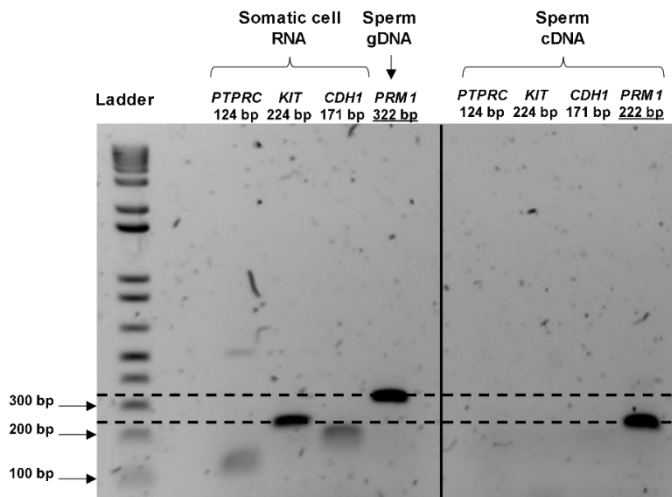
Figure 5.1 RNA purity control and validation of the presence of transcripts in bull spermatozoa. The cDNA from the control (cow uterine RNA) amplifies all the primers at the expected product size, whereas the cDNA synthesized from the sperm RNA amplified only for the sperm specific primer (*PRM1*). PRM1 primers were designed to span a 100 bp intron, therefore the gDNA signal appeared at 322 bp for the control (gDNA extracted from spermatozoa), whereas mRNA appeared at 222 bp. Overall, these indicated the purity of the sperm RNA from other contaminating cells and sperm genome DNA.

Total RNA extraction from the pooled bovine straws resulted in a recovery of an average RNA concentration of 62.6 ± 42.7 ng per sample, from $83.0 \times 10^6 \pm 32.4 \times 10^6$ sperm cells. Contamination with genomic DNA was not detected by RT-PCR in any of the tested samples, nor was any RNA contamination arising from leukocytes, germ cells or epithelial cells (as demonstrated by the lack of amplification of *PRM1* at 322 bp, *KIT*, *PTPRC*, and *CDH1* in spermatozoa, respectively; Figure 5.1). Electrophoretic profiles obtained before library preparation exhibited an absence of 18S and 28S rRNA peaks, and a peak fragment size at 115 bp (data not shown). Together, this demonstrated that only RNAs originating from sperm cells were present in the samples, and that there was an absence of gDNA contamination.

5.4.2 Identification of the differentially expressed mRNAs between HF and LF bulls

Sequencing of the 20 libraries generated an average of 20.2 ($\pm$ 9.2) Gb of data, and an average of 67.4 ($\pm$ 30.6, with a minimum of 48.0 and a maximum of 194) million reads per sample (Appendix II). On average, 91.9 ($\pm$ 0.7) % of the data showed a Q-score over 30 (i.e., a base-calling accuracy of 99.9%). There was no difference for any of the above parameters when comparing the HF and LF groups of bulls ($P > 0.05$). This eliminated the possibility of technical bias during RNA library preparation or sequencing that could affect subsequent results and indicated that the data were of overall good quality.

A list of the 20 most highly expressed mRNAs, unrelated to the fertility group, is given in Appendix II, the most highly expressed being the sperm specific gene *PRM1*. In total, we identified six differently expressed mRNAs between HF and LF, corresponding to the *SCP2D1*, *PRM1*, solute carrier family 24 member 1 (*SLC24A1*), *RBBP6*, and 2 novel genes, *ENSBTAG00000048468* and *ENSBTAG00000054826* (Table 5.1). Genes *PRM1*, *SCP2D1*, and the two novel genes were found more highly expressed in the LF group, whereas *SLC24A1* and *RBBP6* were more highly expressed in the HF group.

Table 5.2 List of the differentially expressed mRNAs between HF and LF bulls.

Gene symbol (Ensembl ID)	Gene name	Fold change	Expression in HF group	pvalue

Gene	Description	Fold change	Expression	P value
PRM1 (ENSBTAG00000021493)	sperm protamine P1	2.22	↓	0.018
SCP2D1 (ENSBTAG00000005202)	SCP2 sterol binding domain containing 1	2.32	↓	0.020
Novel gene 1 (ENSBTAG00000048468)	-	2.19	↓	0.024
SLC24A1 (ENSBTAG00000025826)	solute carrier family 24 member 1	2.04	↑	0.040
RBBP6 (ENSBTAG00000009441)	retinoblastoma binding protein 6	1.65	↑	0.041
Novel gene 2 (ENSBTAG00000054826)	-	5.41	↓	0.049

5.4.3 Identification of differentially expressed miRNAs

Sequencing of the 20 libraries generated an average of 4.4 (± 1.0) Gb of data, and an average of 14.8 (± 3.2) million reads per sample (Appendix II) On average, 81.0 (± 1.8) % of the data showed a Q-score over 30. As for the mRNA-seq data, there was no significant difference for any of the parameters when comparing the HF and LF bull, eliminating the possibility of technical bias.

The identification of the differentially expressed miRNAs was carried out on 458 miRNAs that were found expressed across the samples. A list of the 20 most highly expressed miRNAs, unrelated to the fertility group, is given in Appendix II. In total, we identified 13 miRNAs where expression differed significantly between HF and LF ($p<0.05$, FC>1.5), which are listed in Table 5.3. Among them, six were found upregulated in the HF group, while the seven remaining were found downregulated in the HF group.

Table 5.3 List of the differentially expressed miRNAs between HF and LF bulls.

miRNA name	Fold change	Expression in HF group	P value
bta-miR-1298	5.52	↑	0.001
bta-miR-155	2.03	↓	0.002
bta-miR-374b	1.77	↓	0.003
bta-miR-146a	1.57	↓	0.012
bta-miR-885	2.41	↑	0.013

bta-miR-98	1.52	↗	0.014
bta-miR-2285p	2.18	↗	0.015
bta-miR-338	3.11	↘	0.016
bta-miR-486	1.64	↗	0.022
bta-miR-2342	2.17	↗	0.036
bta-miR-655	2.15	↘	0.037
bta-miR-502a	1.53	↘	0.047
bta-miR-2285t	2.44	↗	0.050

5.4.4 RT-qPCR validation of mRNA and miRNA RNA-seq

Validation of the data was carried out by RT-qPCR on five of the six DEGs: *PRM1*, *SCP2D1*, *RBBP6* and ENSBTAG00000048468 and ENSBTAG00000054826. Results are presented in Figure 5.2. For 4 out of 5 DEGs, RT-qPCR results showed the same trend than for the mRNA-seq results represented by a downregulation of *PRM1*, *CP2D1* and *ENSBTAG00000048468* as well as an upregulation for *RBBP6* expression, for the HF bulls. Concerning ENSBTAG00000054826, results were discordant with the mRNA-seq results.
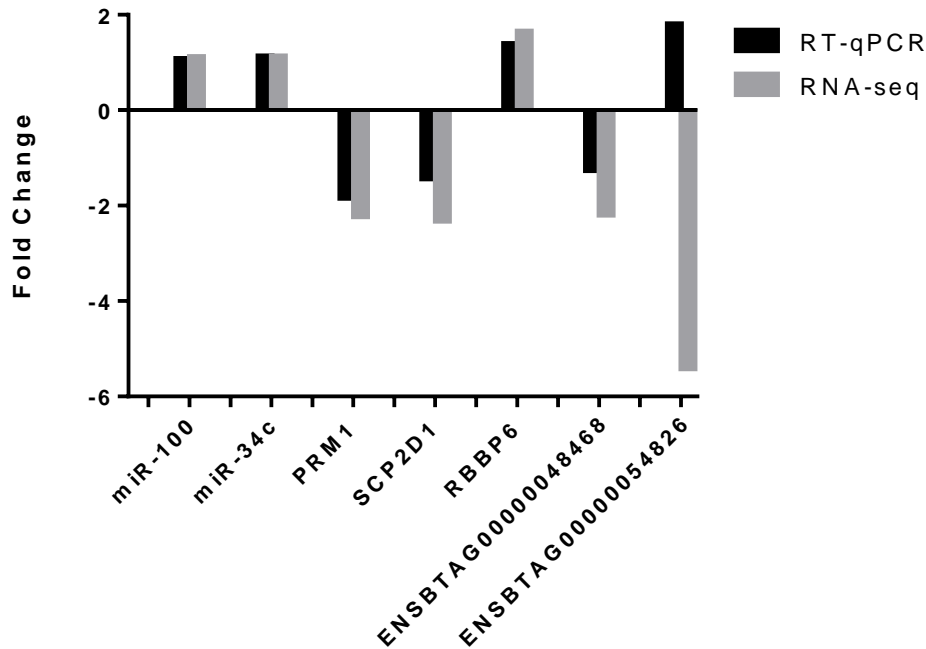
Figure 5.2 Validation of the mRNA-seq and miRNA-seq differentially expressed genes by RT-qPCR. Both miRNAs, miR-100 and miR-34c exhibited the same trend (an upregulation in the HF group) for RNA-seq and RT-qPCR. For four out of the five differentially expressed genes identified by mRNA-seq and assessed by RT-qPCR, the same trend was observed i.e. a downregulation of *PRM1*, *SCP2D1* and *ENSBTAG00000048468* as well as an upregulation for *RBBP6*. *ENSBTAG00000054826* did not observe the same trend.

To perform a technical validation of the miRNA-seq data, RT-qPCR was carried out on two miRNAs (bta-miR-100 and bta-miR-34c) which are highly expressed in the samples and are known to be highly expressed in bull spermatozoa (Govindaraju et al., 2012). Results are also presented in Figure 5.2. Both miRNAs were expressed in all samples, which is in agreement with the miRNAseq data.

5.4.5   Comparison between mRNA-seq and miRNA-seq results

As only six differentially expressed mRNAs were identified, pathway analysis could not be performed. For the differentially expressed miRNAs, an analysis of the transcripts they target was carried out using TargetScan. Three of them, bta-miR-655, bta-miR-2285p and bta-miR-2285t, have RBBP6 as a common target, a DEG identified by mRNA-seq. A KEGG pathway analysis (Kanehisa and Goto, 2000) was also carried out using the list of transcripts targeted by each differentially expressed miRNA, to identify if these targets participate in common biological pathways. It appeared that 5 of the differentially

expressed miRNAs (bta-miR-2285p, bta-miR-98, bta-miR-155, bta-miR-374b, and bta-miR-486) target transcripts which are significantly involved in the same 3 biological pathways: signalling pathways regulating pluripotency of stem cells, mTOR signalling, and FoxO signalling (Table 5.4). For the mTOR and FoxO signalling pathways, two miRNAs are upregulated and two downregulated for HF bulls, but for the pathway regulating the pluripotency of stem cells, three out of four miRNAs are downregulated for HF bulls.

Table 5.4 Biological pathways targeted by the differentially expressed miRNAs between HF and LF bulls.

Biological pathways	miRNA name	Expression in HF bulls	Number of targets involved in the pathway	Corrected p value (Benjamini)
Signaling pathways regulating pluripotency of stem cells	bta-miR-2285p	↑	62	1.30E-07
	bta-miR-98	↓	26	9.00E-07
	bta-miR-155	↓	15	1.90E-04
	bta-miR-374b	↓	14	5.00E-02
mTOR signaling pathway	bta-miR-155	↓	9	8.70E-04
	bta-miR-486	↑	6	2.20E-03
	bta-miR-98	↓	11	6.20E-03
	bta-miR-2285p	↑	25	6.60E-03
FoxO signaling pathway	bta-miR-2285p	↑	62	1.90E-08
	bta-miR-98	↓	21	1.50E-04
	bta-miR-486	↑	7	2.20E-03
	bta-miR-155	↓	10	1.90E-02

5.4.6 Comparison of PRM1 transcription and protein expression levels

A western blot analysis was carried out to verify PRM1 protein expression levels. On average, PRM1 protein expression for the LF group was downregulated compared to the HF group ($p < 0.05$; Figure 5.3), with 9 out of 10 LF bulls having PRM1 protein levels less than the HF average. The downregulation in the LF group was mainly driven by four bulls, which presented a significant decrease in PRM1 expression compared to the HF group. PRM1 protein levels were also much more variable in the LF than the HF group.
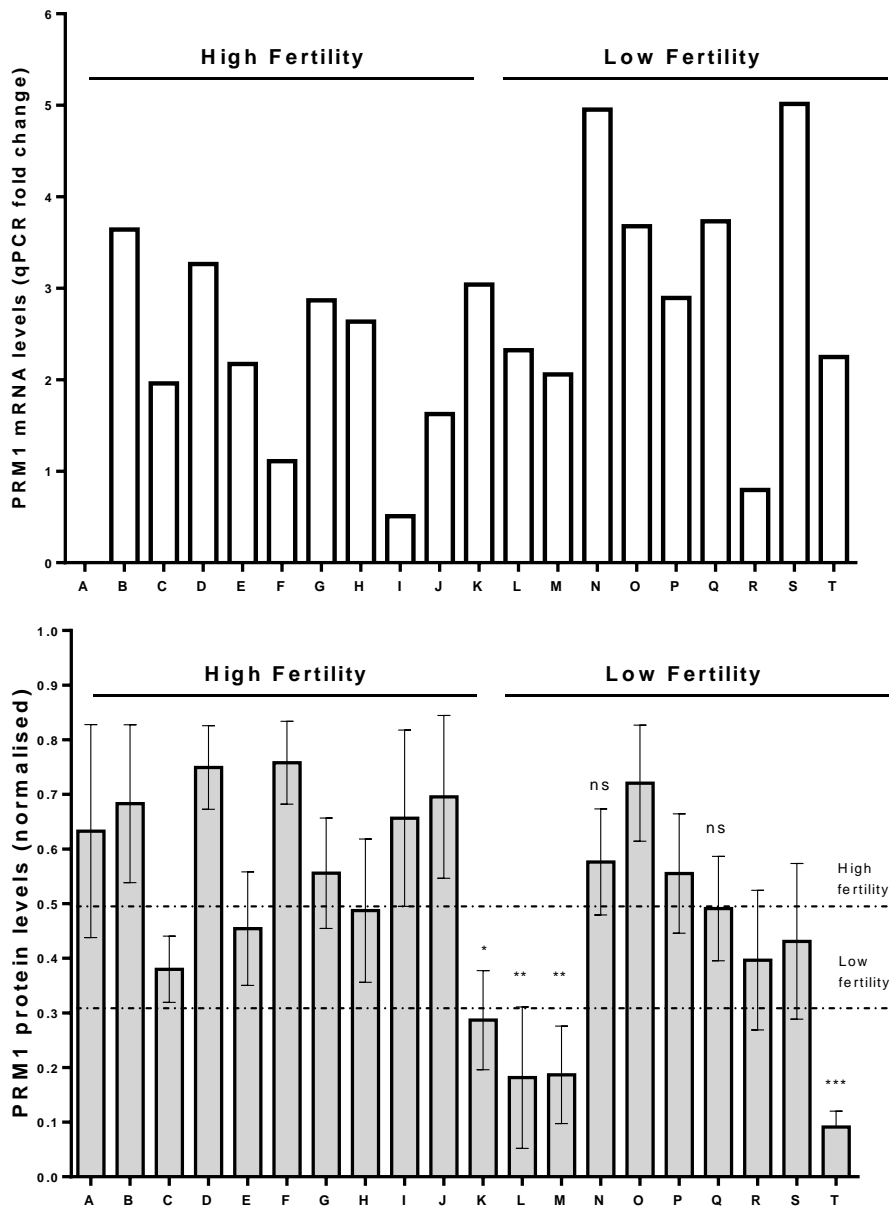
Figure 5.3 Comparison on PRM1 transcription and protein expression levels. Upper panel. PRM1 mRNA levels as revealed by qPCR. Lower panel. PRM1 protein levels assessed by Western Blot. LF (LF) bulls presented on average, a decrease in the PRM1 protein expression compared to HF (HF) bulls ($p < 0.05$, unpaired t-test). The decrease in

expression was particularly evident in 4 LF bulls (K,L,M and T), while the others were similar to HF bulls.

5.4.7   Evaluation of protamine deficiency and DNA fragmentation

To further investigate sperm protamine deficiency as well as the incidence of DNA fragmentation, HF and LF bulls were subjected to the flow cytometry assessment of CMA3 and AO, respectively. Overall, there were no differences in the percentage of spermatozoa with protamine deficiency (high CMA3 staining) or DNA fragmentation between HF and LF bulls ($p > 0.05$; Figure 5.4). A consistent finding across all attributes assessed was the level of variability for both HF and LF (Figure 5.4). Aside from the absence of differences in these attributes, there was a significant and positive correlation between spermatozoa with protamine deficiency and DNA fragmentation for bulls (and ejaculates), irrespective of fertility group ($p < 0.01$; Figure 5.5).
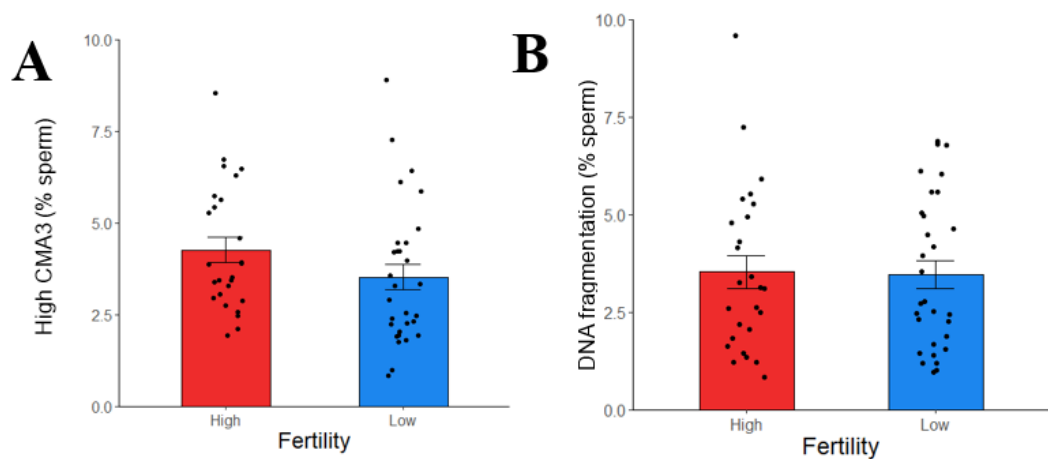
Figure 5.4 Protamine deficiency (high CMA3) (A) and DNA fragmentation (B) in frozen-thawed spermatozoa from HF (n = 10 bulls; exception of high CMA3, where n = 9) and LF (n = 10) bulls were assessed with CMA3 and AO, respectively. Each data point represents an individual ejaculate from a bull. No differences between HF and LF was observed for these attributes ($p > 0.05$).
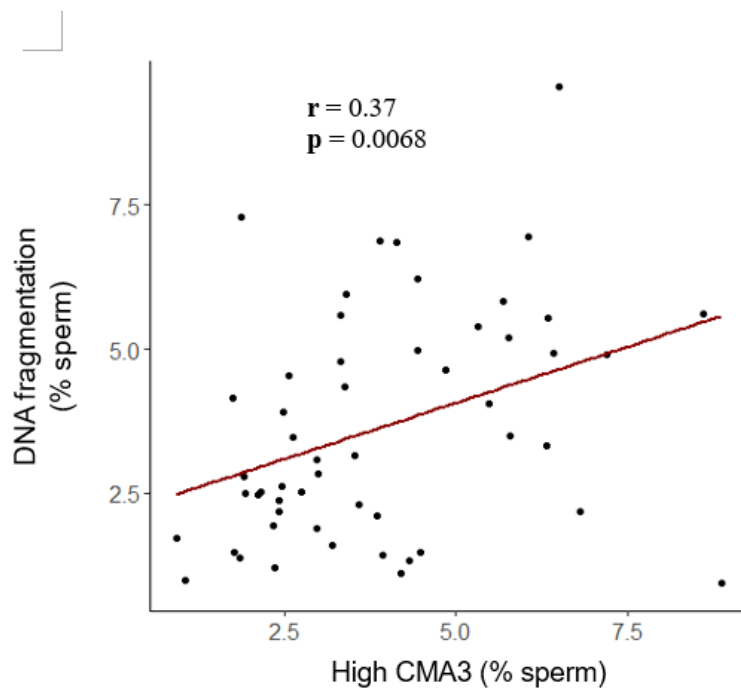
Figure 5.5 Correlation between the percentage of spermatozoa from bulls with protamine deficiency (high CMA3) and DNA fragmentation. Based on these data, as the percentage of spermatozoa with protamine deficiency increased so too did the incidence of DNA fragmentation ($r = 0.37$; $p < 0.01$; Pearson's product-moment correlation). Each dot represents an individual ejaculate from a bull of HF (n = 9) and LF (n = 10).

5.5   Discussion

The aim of the present study was to identify differentially expressed mRNAs and miRNAs in the spermatozoa of a population of bulls with reliable divergent fertility. Sequencing allowed us to catalogue a population of mRNAs present within the bull sperm samples. Among the list of the most highly expressed mRNAs, five of them had already been reported in previous publications: transcripts from genes *PRM1*, Charged Multivesicular Body Protein 5 (*CHMP5)*, coiled-coil domain containing 181 (*CCDC181)*, high mobility group box 4 (*HMBG4)* and kinesin family member 5C (*KIF5C)* (Selvaraju et al., 2017, Card et al., 2013, Card et al., 2017). Nevertheless, only six differentially expressed mRNAs were identified in total, which could be explained by several factors. First, the adjusted fertility score used to categorize bulls as HF or LF, is based on an average of 13,292 AIs, carried out across the bulls' career, and using multiple different ejaculates. Pooling three different ejaculates for each bull prior to RNA extraction was an attempt to be more representative of an individual bull's fertility by erasing inter-ejaculate variability in terms of semen quality. Secondly, considering that the LF phenotype may have a wide range of underlying causes for each individual bull, combining bulls as two groups is a convenient method for analysis but solely permits the revelation of major differences, and not the most subtle ones. This phenomenon is further strengthened by the relatively high number of bulls in each group for this type of study (n=10 per group) compared to others.

The most highly expressed miRNAs identified in the current study are similar to those described by Sellem et al. (2020), with bta-miR-100 being the most highly expressed miRNA, and 13 out of the 20 miRNAs being common to both studies. This overlap between two studies using completely independent groups of bulls adds validity to the findings. As for the mRNA-seq, the miRNA analysis revealed only 13 differentially expressed miRNAs. Nevertheless, several of the miRNAs are particularly relevant for the context of fertility. In particular, bta-miR-155, had increased expression in the testis of chickens with low sperm motility (Liu et al., 2018). Similarly in our study, this miRNA was down-regulated in HF bulls. It is likely to be expressed and brought to the sperm cells by the epithelial cells of the caput epididymis during sperm maturation (Browne et al., 2018) and is also involved in multiple inflammatory pathologies (O'Neill et al., 2011). Tsatsanis et al. (2015)showed that miR-155 was a potential marker of human subfertility which was in line with its decreased expression in the HF group in this study. In comparison, miR-146a, which was also decreased in the HF group, has been described as

a beneficial target for improving bull fertility (Khan et al., 2017), potentially by its action on the low-density lipoprotein receptor (*LDLR*) transcripts. Bta-mir-98, another inflammatory-related miRNA found differentially expressed in our study, was downregulated following infection with bacterial LPS and immune activation in rat testis (Parker and Palladino, 2017). This miRNA, which plays a role in the inhibition of the anti-apoptotic agent B-cell lymphoma-extra large (*Bcl-xl*) translation, induces an increased cell proliferation during the rat peri-implantation embryo development (Xia et al., 2014). The miRNA, bta-miR-486, which exhibited increased expression in the HF group has been described as controlling spermatogonial sperm cells stemness gene expression and growth properties by targeting an activator of the β-catenin signaling pathway (Liu et al., 2019), furthermore it has already been identified as being differentially expressed between high and low motile bull sperm fractions (Capra et al., 2017). Another miRNA, bta-miR-374b, with decreased expression in the HF group had a stable expression in the spermatozoa of fertile men (Salas-Huetos et al., 2014) but its expression may be altered in the seminal plasma of infertile men (Wang et al., 2011). Interestingly, its expression was markedly decreased in azoospermia, but increased in asthenozoospermia cases. This miRNA is also considered as a good potential biomarker of human prostate cancer diagnosis (Barcelo et al., 2019), being differentially expressed in human prostate cancer tumors (He et al., 2013).

Notably, three miRNA including bta-miR-655, bta-miR-2285p and bta-miR-2285t were listed as having a target mRNA gene *RBBP6* with increased expression in the HF group. While bta-miR-2285p and bra-miR-2285t have been reported to have roles in the bovine estrous cycle (Gad et al., 2020, Salilew-Wondim et al., 2014), *RBBP6* sperm transcripts were reportedly involved in utero embryonic development (Prakash et al., 2021). Analysis of the transcripts targeted by the differentially expressed miRNAs showed that five of them (bta-miR-2285p, bta-miR-98, bta-miR-155, and bta-miR-374b, and bta-miR-486) appear to specifically target elements involved in three major biological pathways. The first one is the signaling pathway regulating pluripotency of stem cells, which encompass the transcription factors and their downstream target gene that promote pluripotent stem cells self-renewal and pluripotency (Kuijk et al., 2011). The second one, mammalian target of rapamycin (mTOR) signaling pathway, which exists in two mTOR complexes (mTORC1 and mTORC2), is a central regulator of cell metabolism, growth, proliferation, and survival, and plays a key role during gamete production as well as early embryo development (Correia et al., 2020). Finally, the FoxO signaling pathway involves a family

of transcription factors that regulates the expression of genes implicated in apoptosis, cell-cycle control, glucose metabolism, oxidative stress resistance, and longevity. Knockdown of *FoxO1, FoxO2* and *FoxO4* genes has been shown to impair mouse preimplantation embryonic development (Kuscu et al., 2019). In the current study, it is difficult to decipher the collective actions of the differentially expressed miRNAs, but from previous studies it is likely that these miRNAs have an effect on early embryo development, prior to embryonic genome activation (Gross et al., 2017, Turri et al., 2021). Further analyses, carried out on embryos generated with the semen from HF and LF bulls, is needed to corroborate this. Indeed the integration with other molecular component of the cell, such as DNA methylation or histone posttranslational modifications (Gross et al., 2020) would also be useful.

PRM1 is the most highly expressed mRNA in bovine spermatozoa (Card et al., 2013, Selvaraju et al., 2017, Prakash et al., 2020). Terminally replacing the histone DNA binding proteins during spermatogenesis, PRM1 is a core element for the establishment of the highly condensed state of mammalian sperm chromatin. Numerous studies have already shown that perturbation in PRM1 implementation in mature spermatozoa can have deleterious consequences on sperm chromatin structure. For example, in bovine spermatozoa, reduced protamination has been linked to an increase in sperm DNA fragmentation and a decrease in fertility potential (Dogan et al., 2015). However, strong overexpression of PRM1 protein during spermatogenesis leads to complete sterility in mice due to impaired spermatid maturation, affecting sperm viability and motility (Haueter et al., 2010). In our study, we found that, on average, LF bulls showed significantly reduced PRM1 protein expression, as revealed by western blotting. This decrease in PRM1 protein expression was associated with a significant increase in PRM1 mRNA expression, showed by the RNA-seq and RT-qPCR data. In comparison to our study, poor quality bull spermatozoa had significantly lower levels of *PRM1* mRNAs expression as compared to bulls with good quality spermatooa (Ganguly et al., 2013). Similar results were obtained in human spermatozoa, with *PRM1* mRNA and protein levels found to be positively correlated with sperm concentration, motility, fertilization potential and embryo quality (Kempisty et al., 2007, Depa-Martynow et al., 2012). Nevertheless, in other studies, similar results to the current study were obtained, such as an increase in *PRM1* transcripts in low motility human sperm fractions compared to high motile ones (Lambard et al., 2004), or an aberrant *PRM1* transcript retention associated

with abnormal protein synthesis in cases of infertile men (Aoki et al., 2006b, Aoki et al., 2006a).

One hypothesis to explain this transcript retention phenomenon is that if there is an inefficient translation of the *PRM1* transcripts into proteins during the later stages of spermatogenesis, the transcripts could remain within the spermatozoa, resulting in greater transcript abundance and reduced protein production in the fully mature cell, which would impair fertility. However, the disparity between mRNA and protein expression of PRM1 could be due to many post-transcriptional modifications (such as miRNA regulation), post-translational modifications (such as methylation, acetylation, etc.), and differential protein degradation (such as proteasome-mediated or autophagy-mediated protein breakdown), the mRNA and protein levels are rarely in line with each other. Moreover, PRM1 content by Western blotting alone is not predictive of bull fertility, because there is not always an adequate amount of PRM1 protein in spermatozoa from LF bulls, but rather an inadequate localization of the protein, in the acrosomal region of the head, which is associated with a distorted nuclear shape (Dogan et al., 2015). More investigation on the nuclear localization of PRM1 proteins, as well as its implementation during the different steps of spermatogenesis, is warranted, but the present result is a good clue for a deeper understanding of bull fertility at the molecular level.

As a means to further investigate the identification of *PRM1* as a biomarker of fertility, we employed the fluorophore, CMA3, to indirectly assess protamine deficiency owing to its ability to bind to available G-C sites. These sites are assumed to increase as the level of protamines, and therefore chromatin compaction, decreases (Lolis et al., 1996). Whilst the percentage of cells positive for CMA3 labelling has been used as a predictive tool to evaluate fertility in humans (Lolis et al., 1996, Iranpour et al., 2000) this was not the case for the population of bulls in this study. However, a frequently observed consequence of reduced protamination is the susceptibility of DNA to fragmentation (Hamilton and Assumpcao, 2020), which is primarily associated with an increased production of ROS (Aitken, 2017, Hamilton and Assumpcao, 2020). The incidence of high sperm DNA fragmentation has been linked with reduced embryo quality and implantation in humans (Virro et al., 2004, Simon et al., 2014). Based on our results, protamine deficiency was positively and moderately correlated with the percentage of spermatozoa with DNA fragmentation, irrespective of fertility group.

In conclusion, by assessing mRNAs and miRNAs from the spermatozoa of the same individuals, our results underline the important involvement of PRM1 and miRNAs in the fertility of bulls. Integrating these as biomarkers will permit to further reduce the proportion of variation in bull fertility still unexplained by functional and morphological characteristics of spermatozoa and will help us gain a better understanding as to how sire fertility status regulates the establishment of pregnancy.

Chapter 6　General discussion and future work

6.1 Discussion

Using AI, semen from one bull can be used to inseminate thousands of cows and the impact of reduced fertility can have significant economic losses for farmers, especially in seasonal grass based production systems. To protect against this, animal breeding centres worldwide use a range of microscopy-based assessments including sperm motility and morphology before releasing semen into the field. Despite these stringent tests, a number of bulls with apparently normal semen quality vary significantly in their field fertility by up to 25%. There has been a plethora of studies to-date that have focused on *in vitro* markers of fertility including sperm motility, kinematics as well as flow cytometry assessments including, mitochondrial activity, DNA integrity and sperm acrosomal status to name a few (Holden et al., 2017a, Sellem et al., 2015, Odhiambo et al., 2014). A combination of these parameters have been shown to account to up to 47% of variation in fertility (Bernecic et al., 2021). However, this thesis aimed to further our understanding of the etiology of sire subfertility and identify where along the developmental axis through the female reproductive tract does reproductive wastage due to sire subfertility occur. Specifically, it sought to (i) assess differential sperm transport in the female reproductive tract *ex vivo* (ii) compare the inflammatory response in the uterus *ex vivo* to spermatozoa from HF and LF bulls (iii) characterise the transcriptomic response in the uterus *in vivo* to spermatozoa from HF and LF bulls (iv) to identify the differentially expressed mRNAs and miRNAs in spermatozoa from HF and LF bulls.

It is clear from the aforementioned literature that bulls vary in their fertility for different reasons and therefore we took a multifactorial approach to experimental analysis of HF and LF bulls. From the assessments of frozen-thawed semen from HF and LF bulls, both groups had similar *ex vivo* sperm functional characteristics that aid sperm transportation to the site of fertilisation. There were no differences in sperm attributes including viability, acrosomal integrity, membrane fluidity, motility, VAP, LIN, STR, or AHL, however, spermatozoa from HF bulls had higher curvilinear velocity compared to the LF group (P<0.05). In the main, this is in agreement with previous studies that reported no significant differences in overall motility (Kutchy et al., 2019) and a study in Holstein bulls that reported a strong positive correlation between VCL and non-return rates (Shojaei et al., 2012). Contrastingly, differences in motility STR, LIN and viability have been reported as well as VAP and ALH between HF and LF bulls (Gliozzi et al., 2017, Kutchy et al., 2019, Bernecic et al., 2021). The values obtained for acrosome integrity are in line with previous studies of bull spermatozoa (Gurler et al., 2015, Sellem et al., 2015).

Our study is also in agreement with Garcia-Macias et al. (2007) with no significant difference between acrosomal integrity and fertility but this was in disagreement with Sellem et al. (2015). Similarly, there is conflicting literature regarding viability and fertility with no correlation reported by Garcia-Macias et al. (2007) to a positive correlation reported by (Januskauskas et al., 2003, Bernecic et al., 2021). There was no difference in the ability of spermatozoa from HF and LF bulls to penetrate cervicovaginal mucus which is in agreement with some studies (Verberckmoes et al., 2002) but not with others (Al Naib et al., 2011, Tas et al., 2007).To our knowledge, this was the first time the rheotactic response from HF and LF bulls has been reported. There were no significant differences in the rheotactic response between HF and LF bulls there were strong correlations between the rheotactic response and VSL with 18.9% of the variation in the rheotactic response accounted for by VSL. A higher VSL was observed from spermatozoa from HF bull although it did not reach statistical significance (P=0.08). Further studies should be carried out as a proxy for sperm transport in the female reproductive tract. A 21% difference in the ability of spermatozoa to bind to the oviductal epithelium were reported between treatment groups. However, further studies are required to establish the exact source of these differences. Perhaps future studies could include a further characterisation of the sperm surface proteins (Mahe et al., 2021) including BSPs and integrin α5β1 where defects or an absence of these surface proteins could be preventing the formation of a sufficient sperm reservoir.

Due to subtle differences in sperm functional charactersitcs *ex vivo*,the next hypothesis focused on the uterine environment, which is normally the site of semen deposition during AI, to further understand sperm transport through the uterus and implications of uterine interactions on priming the endometrium for pregnancy. The immune response to spermatozoa and SP in the uterus has been previously described (Ezz et al., 2019, Rizo et al., 2019) however, this was the first study to characterise the immune response from frozen-thawed semen from bulls with divergent fertility. There were no significant differences in the the inflammatory gene expression between high and low fertility treatments for *IL1A, IL1B, IL6, CXCL8 and TNFA*. Moreover, there was a significant upregualtion of *IL1A, IL1B* and *TNFA* which was also confirmed by ELISA for IL1-β and IL-8. These results are in agreement with previous *in vitro* and *ex vivo* studies (Elweza et al., 2018, Ezz et al., 2019, Akthar et al., 2019).

To shed light on the significane of this immune reponse, the results demonstrated an up regulation of mRNA expression for *CXCL8* and a significant up regulation at the protein

level indicating the recruitment of leukocytes specifically PMNs, the most abundant leukocyte at 38% in adult cows (Lumsden et al., 1980), into the uterine lumen (Alghamdi et al., 2009). A previous *in vivo* study reported a moderate recruitment of PMNs to the uterine lumen was associated with higher conception rates (Kaufmann et al., 2009). We hypothesised potential differences in sperm binding from bulls of divergent fertilities with *in vitro* cultured PMNs. Interestingly, there was an increase in the number of spermatozoa bound per PMN from spermatozoa from low fertility bulls. This increase in binding could be preventing LF bulls from reaching the site of fertilisation where previous studies have reported a decrease in sperm motility following the formation of neutrophil extracellular traps (Zambrano et al., 2016, Alghamdi et al., 2009).(Zambrano et al., 2016, Alghamdi et al., 2009). It is thought this PMN-sperm binding selects the fittest or most-compatble spermatozoa increasing the chances of a male siring a healthy offspring (Firman et al., 2017). Hong et al. (2017) identified nine inhibitors that were active against phosphatidylinositol 3-kinase and reduced sperm-PMN interactions however, the exact mechanism underpinning this is unknown. There are a number of factors that have been reported as having contrasting effects on spermatozoa namely, PMN binding including the contact of SP, cryodiluents and cryopreservation. An increase effect of SP was reported for bovine sperm - PMN binding while cryodiluents reported contrasting effects depending on the type of cryodiluent used (Alghamdi et al., 2009, Fichtner et al., 2020) and cryopreserved ram spermatozoa adhered less to PMNs (Pini et al., 2017).

Murine studies reported a population of PMNs recruited at mating are retained in the endometrium and regulate various aspects of embryo implantation and tissue remodelling (Finn and Pope, 1991, McMaster et al., 1993, Schjenken et al., 2021). A bovine study reported significant differences in PMN expressions taken from blood samples between pregnant and non-pregnant cows in the peri-implantation period where IL-8 showed a higher expression from PMNs in non-pregnant cows with significant differences emerging on Day 14 post AI (Manjari et al., 2016). There was a significantly higher expression of pro-inflammatory markers from PMNs expressed in non-pregnant cows 40 days post AI, (Manjari et al., 2016). This illustrates that the immune reponse is important long after insemination and a further study perhaps where the PMN expressions were characterised from heifers inseminated with HF and LF bulls 40 days post AI could further our understanding of the impact of sire fertility on regulating this immune reponse towards a successful pregnancy. Indeed in the sire fertility model used for this thesis it is not clear if the pregnancy loss is prior to Day 21 or after.

To comprehend the source of the uterine inflammatory response to spermatozoa, we amalysed gene expressions of uterine tissue treated with CES and CES plus SP to represent the contents of the semen straw used for AI. Contrastingly, to washed frozen-thawed spermatozoa, CES or CES+SP did not alter inflammatory gene expressions except for *IL6* CES+SP resulted in an up-regulation of *IL6*. Similarly, Ibrahim et al. (2019) showed that 5% SP in the presence of spermatozoa or not, elicited changes to the abundance of the inflammatory genes *IL6* and *IL1B*. This provides evidence that perhaps spermatozoa need to be coated, or present in a small amount of SP, to initiate an inflammatory response. However, due to the more subtle effects compared to frozen-thawed spermatozoa, it is plausible that cryopreservation and/or the addition of cryodiluents may be altering the sperm membrane, contributing to the inflamamtory response, however further experimentation directly comparing frozen-thawed and fresh semen is required.

To further identify the biological significance and implications of the uterine response from spermatozoa, Chapter 4 focused on characterising the uterine transcriptomic response as well as PMN populations in the uterus of heifers inseminated with frozen-thawed semen from HF and LF bulls. The RNA-sequencing identified a more active transcriptomic response in the uterus 12 h post AI and this was more pronounced in heifers inseminated with HF semen with 845 DEGs between the HF and the control group as oppose to just 4 DEGs between the LF and control group. Semen from HF bulls induced a higher expression of *PTGDS* compared to the control group which is associated with sperm development and maturation (Rossitto et al., 2015) along with *ALOX12* which is associated with cell migration as well as inflammation (Zheng et al., 2020). The majority of genes differentially expressed between the HF and LF groups directly were immune genes inlcuding; *BOLA-DQB* and *BOLA* (MHC class II and I respectively), *AHSG* and *TRAPPC9*. This strongly confirms a role of the immune response as observed from our *in vitro* experiments. In particular, the MHC genes are important for pregnancy establishment and strengthens the hypothesis that the initial interaction of spermatozoa with the endometrium could have downstream consequences on subsequent implantation and pregnancy (Melo et al., 2017). A decrease in *BOLA-DQB* was observed in the endometrium of heifers inseminated with HF semen which is in line with previous studies that observed this trend in pregnant heifers after mating (Melo et al., 2017). The top DEG between the high and low treatment groups was *IL1A* which is in agreement with previous *in vitro* studies, (Elweza et al., 2018, Akthar et al., 2019) however, this is the first *in vivo*

study to identify a difference in the inflammatory response in the endometrium between sires. As a potent inflammatory molecule that is associated with the recruitment of PMNs into the uterine lumen, we characterised the number of PMNs in the epithelial layer of the endometrium post-insemination. There was a significant increase in the number of PMNs in the uterine epithelium in response to frozen-thawed semen although there was no siginificant differences between high and low fertility treatments. However, the differences could lie with the number of spermatozoa bound to each PMN as previously reported in Chapter 3.

There were also a number of genes in the wider data-set linked to the adaptive immune response including *IL16* and *IL11RA* with increased expression in the HF group. *IL16* is a chemokine associated with activating T-reg cells where previous studies have reported T-reg cells as important mediators in regulating maternal immune tolerance and embryo implantation and survival (Samstein et al., 2012).While *IL11RA* knockout mice are infertile due to the failure of the decidualization of the endometrium (Dimitriadis et al., 2003). However, *IL11* and *IL6* had increased expression in the control group compared to the HF group which suggest perhaps this adaptive response was just being switched on however, further research is required over a number of time-points to determine the timeline of this immune reponse post-insemination and it effect on potential pregnancy outcome.

Another key factor that contributes to post-fertilisation events is the carrying of miRNAs and mRNAs into the oocyte which have been reportedly involved in fertilisation and early embryogenesis (Card et al., 2013). The highly expressed miRNAs including bta-miR-100 were also found in a similar study by Sellem et al. (2020). Particular miRNAs that were relevant to the study of fertility included bta-miR-155 which had decreased expression in HF bulls was associated with low sperm motility in chickens (Liu et al., 2018) and has been identified as a potential marker of subfertility in humans (Tsatsanis et al., 2015). The miRNA, bta-miR-486, which had increased expression in the HF group has been described as controlling spermatogonial cells stemness gene expression and growth properties by targeting an activator of the β-catenin signaling pathway (Liu et al., 2019). The same miRNA has been identified as differentially expressed between high and low motile bull sperm fractions (Capra et al., 2017). Comparison of miRNA and mRNA results identified three miRNA including bta-miR-655, bta-miR-2285p and bta-miR-2285t were reported as having a target mRNA gene *RBBP6* with increased expression in the HF group. *RBBP6* sperm transcripts have reported functions in utero embryonic

developemnt (Prakash et al., 2021). Analysis of transcripts targeted by differentially expressed miRNA showed five of them are linked to three major biological pathways. Two of these pathwyas which were of particular interest to fertility included mTOR, which reportedly plays a role in cell metabolism, growth and proliferation and played a key role in gamete formation as well as early embryo development (Correia et al., 2020). The FoxO pathway involved a family of trasncription factors that regulate the expression of genes implicated in apoptosis. Knockout murine models for FoxO1, FoxO2 and FoxO4 have been shown to impair preimplantation and embryo development (Kuscu et al., 2019).

One of the most highly abundant mRNAs, *PRM1* (Prakash et al., 2020), had a significant increase in mRNA expression amongst low fertility bulls while contrastingly protein anlysis reported a significant decrease in PRM1 amongst low fertility bulls. PRM1 is a core element of the condensed state of sperm chromatin and a disruption to PRM1 could have detremental consequences on sperm chromatin structure. The results reported in this study are in conflict with some previous studies that stated poor quality spermatozoa had significantly lower levels of *PRM1* mRNA (Ganguly et al., 2013). In addition, results in human spermatozoa have reported *PRM1* as positively correlated with motility, fertilisation ability and embryo quality (Kempisty et al., 2007, Depa-Martynow et al., 2012). However, other studies are in agreement with the results observed here with an increase in *PRM1* transcripts in low motility human spermatozoa (Lambard et al., 2004) and an abnormal retention of the PRM1 trasncript associated with atypical protein synthesis in cases of infertile men (Aoki et al., 2006b, Aoki et al., 2006a). There are several reasons behind the disparity between mRNA and protein expression including the inefficient translation of PRM1 transcripts at the later stages of spermatogenesis or it could be due to post-transcriptional modifications and protein degradation.

To further investigate PRM1 we utilised the fluorophore CMA3 to indirectly assess protamine definiciency due to its ability to bind to G-C bindign sites. While previous studies reported CMA3 labelling as a indicator of fertility in humans (Iranpour et al., 2000) we found no such relationship. A frequently observed consequence of reduced protamination is the susceptibility of DNA fragmentation (Hamilton and Assumpcao, 2020). In our study there was no difference in DNA fragmentation levels between high and low fertility groups however, there an overall moderate positive correlation between protamine deficiency and the percentage of spermatozoa with DNA fragmentation.

In summary, this thesis has reported significant advances in the understanding of bull fertility variation and the molecular mechanisms underpinning sire sub-fertility. Although minor differences were observed relating to sperm transport to the site of fertilisation, there was a clear role of the immune response to spermatozoa in the endometrium reported both *ex vivo* and *in vivo,* with more LF spermatozoa bound to PMNs and a more active transcriptomic response in the uterus from HF semen with genes relating to immunity thay have key roles in embryo developemnt and implantation. Moreover, this thesis further identified differential expression between HF and LF sperm miRNA's and mRNA's that deliver an abundance of cargo to the oocyte that have roles in implantation and embryo developement as well the important involvement of *PRM1*. Integrating these results has progressed our understanding of the molecular differences behind bull fertility variation and allows us to narrow the focus of future experimental analysis.

6.2   Future work

The novel work in this thesis has revealed new oportunities for both *in vitro* and *in vivo* experiments. The inflammatory response to frozen-thawed spermatozoa in the endometrium was characterised with a more active response from HF bulls *in vivo,* however, the exact molecular link between spermatozoa and the TLR signalling pathways have yet to be characterised. More research is needed in how spermatozoa elicit an immune response as most of the work to date is on the effects of SP on the uterine inflammatory response. This could provide a more rounded depiction of the differences in the inflammatory response between bulls of divergent fertilities.

Futher *in vivo* studies are required over additioanl time-points to determine the effect frozen-thawed spermatozoa from sires of divergent fertiltiy have on pregnancy establishment.While although the logistics of this are difficult, previous work has reported no difference in PMNs isolated from peripheral blood vs the uterus (Alghamdi et al., 2009) and therefore, a trial where immune cells were monitored from peripheral blood samples of heifers inseminated with semen from HF and LF bulls (from 0 to 40 days post insemination) would develop an accurate comparable inflammtory profile and its relationship with pregnancy success. In additon, it would be interesting to inseminate heifers with dead (to avoid fertilisaiton) frozen-thawed spermatozoa from HF and LF bulls and on Day 7 transfer in an embryo (or multiple embryos) and either track their development during pregnancy or slaughter the heifers on Day 16 (or other time points such as Day 40) and assess embryo development, interferon tau production *in vitro*, as well as gene expression.

In relation to miRNAs and mRNAs and their effect on early embryo development, further analysis is required on *in vitro* produced embryos generated from spermatozoa of HF and LF bulls to assess the impact on gene expression in Day 7 blastocysts as well as embryos transferred and recovered on Day 16. These studies would help to gain a more comprehensive understanding of how sire fertility can regulate pregnancy establishement and thus the ability to improve the efficiency of AI.

Appendix I

A Gene Ontology (GO) and molecular function for heifers inseminated with frozen-thawed semen from HF bulls relative to Control (CTRL) heifers. **B** Gene Ontology and biological process results for heifers inseminated with frozen-thawed semen from HF bulls relative to CTRL heifers.

A

ID	Description	Gene ratio	BgRatio	p.adjust	Gene ID	Count
GO:0050839	cell adhesion molecule binding	42/685	383/11927	0.0263311	ACTN2/OLFM4/NRG1/ESM1/BZW2/CIB2/ITGA5/JAML/THY1/HSPA8/CD2AP/EIF4G2/TWF1/YKT6/SLC9A3R2/CAPG/EZR/PTN/RSL1D1/RAB10/SH3GLB1/IGF1/CRKL/MSN/ESYT2/KIF5B/UBFD1/LRRC59/ADAMTS13/MAPRE1/PICALM/FASN/HDLBP/BZW1/PTPN1/NRXN2/YWHAZ/IGF2/THBS1/CLINT1/ACTN1/H1FX	42
GO:0030545	receptor regulator activity	30/685	249/11927	0.0275109	FGF18/NRG1/HMGB2/OSGIN1/IL6/IL16/NTS/APOA1/GDF10/TNFSF18/WNT2/CMTM3/INHBA/IL1A/STC1/CSF1/C1QTNF12/PTN/IGF1/MDK/CDK5/CDNF/ANGPT4/IL11/CXCL11/CCL25/INHA/EBI3/NPPC/IGF2	30
GO:0048018	receptor ligand activity	28/685	229/11927	0.0275109	FGF18/NRG1/HMGB2/OSGIN1/IL6/IL16/NTS/APOA1/GDF10/TNFSF18/WNT2/CMTM3/INHBA/IL1A/STC1/CSF1/C1QTNF12/PTN/IGF1/MDK/CDNF/IL11/CXCL11/CCL25/INHA/EBI3/NPPC/IGF2	28

Gene ID = list of genes assigned to a given pathway
BgRatio = the ratio of the number of genes that are not differentially expressed in a pathway to the number of expressed genes with GO identifier
GeneRatio – the ratio of the number of differentially expressed genes in a given pathway to the number of differentiating genes with GO identifier
p. adjust = adjusted p value

B

ID	Description	Gene ratio	BgRatio	p.adjust	Gene ID	Count
GO:0016126	sterol biosynthetic process	15/684	59/11865	0.0020259	HMGCS1/PMVK/CFTR/CYB5R2/INSIG1/SREBF1/DHCR24/KPNB1/IDI1/GPAM/SREBF2/FASN/SQLE/HMGCR/DHCR7	15
GO:0019218	regulation of steroid metabolic process	19/684	95/11865	0.0020259	HMGCS1/APOA1/PMVK/DHH/ATP1A1/CLCN2/DGAT2/INSIG1/SREBF1/KPNB1/NR1D1/IDI1/GPAM/SREBF2/FASN/LDLR/SQLE/HMGCR/DHCR7	19
GO:0006695	cholesterol biosynthetic process	14/684	56/11865	0.0020259	HMGCS1/PMVK/CFTR/INSIG1/SREBF1/DHCR24/KPNB1/IDI1/GPAM/SREBF2/FASN/SQLE/HMGCR/DHCR7	14
GO:1902653	secondary alcohol biosynthetic process	14/684	56/11865	0.0020259	HMGCS1/PMVK/DHH/ATP1A1/CLCN2/INSIG1/SREBF1/KPNB1/NR1D1/IDI1/GPAM/SREBF2/FASN/SQLE/HMGCR/DHCR7	16
GO:0050810	regulation of steroid biosynthetic process	16/684	72/11865	0.0020259	HMGCS1/PMVK/DHH/ATP1A1/CLCN2/INSIG1/SREBF1/KPNB1/NR1D1/IDI1/GPAM/SREBF2/FASN/SQLE/HMGCR/DHCR7	16
GO:0090181	regulation of cholesterol metabolic process	13/684	49/11865	0.0020259	HMGCS1/PMVK/DGAT2/SREBF1/KPNB1/IDI1/GPAM/SREBF2/FASN/LDLR/SQLE/HMGCR/DHCR7	13

Gene ID = list of genes assigned to a given pathway
BgRatio = the ratio of the number of genes that are not differentially expressed in a pathway to the number of expressed genes with GO identifier
GeneRatio – the ratio of the number of differentially expressed genes in a given pathway to the number of differentiating genes with GO identifier
p. adjust = adjusted p value

Appendix II

A Library characterization and mapping efficiency on the bovine genome (ARS-UCD1.2) of in mRNA-seq and miRNA-seq libraries. **B** List of the 20 most highly expressed mRNAs. **C** List of the 20 most highly expressed miRNAs **D** RT-PCR primers for quality controls after RNA extraction E RT-PCR primers for quality controls after RNA extraction

A

Group		High Fertility	Low Fertility
mRNA-seq data	Yield (Gb)	21.3 ± 13.1	19.2 ± 1.8
	Number of read pairs analyzed (million)	71.1 ± 43.7	63.8 ± 6.0
	Number of bases with a Q-score >30 (%)	91.7 ± 0.7	92.1 ± 0.7
	Uniquely mapped reads (%)	67.0 ± 8.5	66.8 ± 11.3
	Ambiguous reads (%)	2.4 ± 0.3	2.3 ± 0.4
	Unmapped reads (%)	30.6 ± 8.7	30.9 ± 11.7
miRNA-seq data	Yield (Gb)	4.6 ± 1.3	4.3 ± 0.5
	Number of read pairs analyzed (million)	15.2 ± 4.3	14.4 ± 1.8
	Number of bases with a Q-score >30 (%)	81.7 ± 1.2	80.2 ± 2.0
	Reads <15bp (%)	21.2 ± 6.6	21.8 ± 7.3
	Mapped reads (%)	66.8 ± 8.4	66.4 ± 7.5
	Unmapped reads (%)	33.2 ± 8.4	33.6 ± 7.5

B

Gene symbol (Ensembl ID)	Gene name	Mean count
PRM1 (ENSBTAG00000021493)	protamine 1	816,382
GOLGA4 (ENSBTAG00000016563)	golgin A4	554,717
- (ENSBTAG00000040318)	novel gene	528,827
BAZ2B (ENSBTAG00000020654)	bromodomain adjacent to zinc finger domain 2B	492,269
EEA1 (ENSBTAG00000000421)	early endosome antigen 1	488,231
CHMP5 (ENSBTAG00000012383)	charged multivesicular body protein 5	407,796
CEP295 (ENSBTAG00000001902)	centrosomal protein 295	360,010
KIF5C (ENSBTAG00000018125)	kinesin family member 5C	315,901
HMGB4 (ENSBTAG00000000335)	high mobility group box 4	311,026
CCDC181 (ENSBTAG00000020264)	coiled-coil domain containing 181	303,303
CHD5 (ENSBTAG00000040477)	chromodomain helicase DNA binding protein 5	222,768
CHD2 (ENSBTAG00000051831)	chromodomain helicase DNA binding protein 2	178,802
SSRP1 (ENSBTAG00000000375)	structure specific recognition protein 1	170,963
RBBP6 (ENSBTAG00000009441)	RB binding protein 6, ubiquitin ligase	159,104
TRA2B (ENSBTAG00000001697)	transformer 2 beta homolog	154,430
CHD4 (ENSBTAG00000014734)	chromodomain helicase DNA binding protein 4	154,060
CCDC174 (ENSBTAG00000038298)	coiled-coil domain containing 174	150,409
BRD9 (ENSBTAG00000006971)	bromodomain containing 9	142,487
MYH10 (ENSBTAG00000021151)	myosin heavy chain 10	135,613
BAZ1A (ENSBTAG00000020164)	bromodomain adjacent to zinc finger domain 1A	131,847

C

miRNA name	Mean counts	% of miRNAs
bta-miR-100	338,795	39.3
bta-miR-30d	36,838	5.9
bta-miR-21-5p	33,096	5.6
bta-miR-99a-5p	27,961	4.3
bta-miR-34c	32,641	3.6
bta-miR-191	19,618	3.1
bta-miR-27a-3p	17,983	2.8
bta-miR-2284x	15,428	2.3
bta-miR-186	15,159	2.1
bta-miR-148a	11,916	1.9
bta-miR-125b	16,198	1.7
bta-miR-22-3p	10,464	1.6
bta-miR-27b	10,235	1.6
bta-miR-3432a	8,904	1.4
bta-miR-375	7,175	1.1
bta-miR-128	7,422	1.1
bta-miR-16b	5,960	1.0
bta-miR-7	6,847	0.9
bta-miR-449a	7,931	0.8
bta-miR-204	7,619	0.8

D

Primer name	Gene symbol (Ensembl ID)	Accession number	Primer sequence (5'-3')
PRM1_F1	PRM1	NM_174156.2	AAGATGTCGCAGACGAAGGAG
PRM1_R1	(ENSBTAG00000021493)		GTGGCATTGTTCGTTAGCAGG
SCP2D1_F1	SCP2D1	NM_001040507.2	GGCAAGTTCAAAGTGAGCGG
SCP2D1_R1	(ENSBTAG00000005202)		TTCCTGATATTCCGGCAGGC
SLC14A1_F1	SLC14A1	NM_174655.2	CACGAGCAGACCCACTCTT
SLC14A1_R1	(ENSBTAG00000025826)		AGAGGGTAACCACAAACCTTCA
SLC14A1_F2	SLC14A1	NM_174655.2	CACGAGCAGACCCACTCTTTG
SLC14A1_R2	(ENSBTAG00000025826)		CACAAACCTTCAAACCTGGACAT
RBBP6_F1	RBBP6	NM_001304554.1	ACAAGCACCACCTTTGTCCA
RBBP6_R1	(ENSBTAG00000009441)		TGGACTTTTTCTTTTCCTCTTCTTT
CHD9_F1	CHD9	NM_001205650.2	CCTCTAGGGCGCAGACGAAA
CHD9_R1	(ENSBTAG00000002287)		ATGAAGTGTCAGAATCCTTGGCT
NovelGene1_F1	Novel Gene 1	/	ACCCTTCTCCCAACCAGTGA
NovelGene1_R1	(ENSBTAG00000048468)		CTGAAGCTTGGCCCTTCTTG
NovelGene1_F2	Novel Gene 1	/	CGTTGCCTTAACAGGTGGTG
NovelGene1_R2	(ENSBTAG00000048468)		GAAGCTTGGCCCTTCTTGTTC
NovelGene2_F1	Novel Gene 2	/	TAGGGTGATGGTGGCCTCATA
NovelGene2_R1	(ENSBTAG00000054826)		CAGACCCATCACAAGCATGG
NovelGene2_F2	Novel Gene 2	/	TCAGCTGTGAAGCCGTCTG
NovelGene2_R2	(ENSBTAG00000054826)		AGACCCATCACAAGCATGGAA

E

Primer name	Gene symbol (Ensembl ID)	Accession number	Primer sequence (5'-3')
PRM1_F	PRM1 (ENSBTAG00000021493)	NM_174156.2	AAGATGTCGCAGACGAAGGAG
PRM1_R			GTGGCATTGTTCGTTAGCAGG
PTPRC_F	PTPRC (ENSBTAG00000023144)	NM_001206523.1	ACCCAACCTTCTACTCAAGATG
PTPRC_R			CGTATTTGTTCTCACATGGTGG
CDH1_F	CDH1 (ENSBTAG00000015991)	NM_001002763.1	CTGCATTCCTGGCTTTGGTG
CDH1_R			GTAAGCACGCCATCTGTGTG
KIT_F	KIT (ENSBTAG00000002699)	NM_001166484.1	GAATAGCTGGCATCAGGGTG
KIT_R			CCAGATCCACATTCTCTCCATC

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