Studies on the modification of ram spermatozoa by ejaculation and cryopreservation and the effects of Binder of Sperm Proteins

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Declaration

Apart from the assistance mentioned in the acknowledgements, the studies contained within this thesis were planned, executed, analysed and written by the author, and have not been previously submitted for any degree to a University or institution.

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Summary

Frozen thawed ram spermatozoa must be deposited directly into the uterus via laparoscopy as they are unable to traverse the cervix in sufficient numbers to achieve acceptable fertility. Seminal plasma has been heralded as a possible solution to this problem, as it appears to enhance sperm cervical transit. However, the mechanism by which seminal plasma, or indeed cryopreservation, alters spermatozoa to subsequently influence cervical transit is unknown. This thesis explores how seminal plasma and cryopreservation affect the structure and function of ram spermatozoa, with the ultimate aim of improving the in vivo performance of cryopreserved ram semen.

Results reported herein showed seminal plasma exposure and cryopreservation significantly altered the sperm proteome and glycocalyx, and interactions between spermatozoa and immune cells. Seminal plasma added two unique proteins (LEG1, EDIL3) and significantly increased the abundance of 39 proteins, including the Binder of Sperm Proteins (BSPs) 1 and 5. Ejaculated ram spermatozoa showed significant protein conservation (95%) compared to sperm proteomes from the human, bull, stallion, rooster and trout. Seminal plasma exposure significantly decreased available sialic acid, and increased N-acetylglucosamine on the sperm surface. Seminal plasma also protected spermatozoa from immune cells, significantly reducing the percentage of neutrophils bound to ejaculated spermatozoa (by 67.5%). However, it did not prevent opsonin mediated binding.

Egg yolk in freezing media was shown to contribute 15 proteins to ram spermatozoa, including vitellogenins, apolipoproteins and complement C3. Cryopreservation itself increased the abundance of 27 proteins (e.g. SERPINB1, FER) and decreased 24 proteins (e.g. TOM1L1, CSN1G2). Chaperones constituted 20% of the proteins lost following freezing, suggesting important functional consequences. Cryopreservation also reduced available galactose and N-acetylglucosamine and increased available mannose on the sperm surface. Freezing induced sugar changes were related to the modification, loss or gain of glycoproteins. The effects of cryopreservation on immune cell interaction were unclear due to inhibition of neutrophil binding by

cryodiluent. This was not due to egg yolk, which significantly enhanced binding (by 29.0%). Further investigation is required to determine whether cryopreservation itself alters phagocytic susceptibility of spermatozoa.

The physiological and potential cryoprotective effects of BSPs were investigated, given their high abundance in seminal plasma and demonstrated transfer to the sperm surface during ejaculation. Under significant cAMP upregulation, BSP1 showed both pro- and de-capacitating effects on fresh spermatozoa, including promotion of cholesterol efflux and the acrosome reaction, and limitation of membrane lipid disorder and protein tyrosine phosphorylation respectively. In contrast, BSP5 had limited effects on capacitation related processes. Pre freeze supplementation with BSPs improved post thaw motility (by 36.2%) and sperm kinematic parameters in an artificial mucus medium. Pre freeze supplementation with either BSP1 or BSP5 significantly improved post thaw progressive motility (by 15.6% and 15.0% respectively) and viability (by 11.2% and 10.4% respectively), and decreased freezing induced tyrosine phosphorylation. Supplementation with BSPs did not minimise loss of acrosome integrity or membrane lipid disorder. Further work is necessary to confirm translation of these positive outcomes to improvements in vivo.

The findings of this thesis demonstrate that the physiological process of ejaculation and the artificial practice of cryopreservation significantly alter the molecular profile and function of spermatozoa. Seminal plasma exposure confers additional proteins, alters the sperm glycocalyx, protects spermatozoa from immune cells and regulates the timing of capacitation. Cryopreservation also alters the sperm proteome and glycocalyx, possibly increasing susceptibility of this sperm type to dynamic environmental pressures within the female reproductive tract. Further investigation of the degree to which BSPs benefit ram spermatozoa in the female tract is warranted given their significant effects on sperm cryosurvival and physiology demonstrated herein. The importance of this protein family in ram sperm physiology offers a potential avenue for future improvements in the fertility of frozen thawed ram spermatozoa following cervical insemination.

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List of Abbreviations

°C	degrees Celsius
2DGE	two dimensional gel electrophoresis
ACN	acetonitrile
AI	artificial insemination
ALH	amplitude of lateral head displacement
ATP	adenosine triphosphate
BCF	beat cross frequency
BSA	bovine serum albumin
BSPs	Binder of Sperm Proteins
BSP1	Binder of Sperm Protein 1
BSP5	Binder of Sperm Protein 5
cAMP	cyclic adenosine 3′,5′-monophosphate
CASA	computer assisted sperm analysis
COC	cumulus-oocyte complex
ConA	concanavalin agglutinin
CRISP	cysteine-rich secretory protein
dbcAMP	dibutyryl cyclic adenosine 3′,5′-monophosphate
EY	egg yolk
FITC	fluorescein isothiocyanate
Fn-2	fibronectin type-II
g	gravity, acceleration due to
GAG	glycosaminoglycans
GlcNAc	N-acetylglucosamine
h	hour(s)

H_2DCFDA	dichlorodihydrofluorescein diacetate acetyl ester
H33342	Hoechst 33342
RP-HPLC	reverse phase high performance liquid chromatography
Hz	hertz
ICSI	intra cytoplasmic sperm injection
IVF	in vitro fertilisation
kDa	kilodalton
LIN	linearity
LPA	*Limulus polyphemus* agglutinin
LSD	least significant difference
m (prefix)	milli ($\times 10^{-3}$)
M	molar
min	minute(s)
M540	merocyanine 540
mg	milligram(s)
mL	millilitre(s)
LC-MS/MS	liquid chromatography tandem mass spectrometry
MW	molecular weight
mW	milli watt
n (prefix)	nano ($\times 10^{-9}$)
Neu5AC	N-acetylneuraminic acid (sialic acid)
OEC	oviductal epithelial cell(s)
PANTHER	Protein ANalysis THrough Evolutionary Relationships
PBS	phosphate buffered saline
pH	hydrogen ion concentration,-log 10 of
PI	propidium iodide
PMN	polymorphonuclear leucocyte
PNA	peanut agglutinin

ppm	parts per million
PVA	polyvinyl alcohol
PVDF	polyvinylidene difluoride
REML	residual maximum likelihood
ROS	reactive oxygen species
RSP	ram seminal plasma protein (binder of sperm)
RSVP	ram seminal vesicle protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SOD	superoxide dismutase
s	second(s)
SP	seminal plasma
STR	straightness
SWATH-MS	sequential windowed acquisition of all theoretical mass spectra
TLP	Tyrode's lactate pyruvate medium
TALP	Tyrode's lactate pyruvate medium with 0.3% (w/v) bovine serum albumin
TBS	tris buffered saline
TBS-TW	tris buffered saline with 0.1% (v/v) Tween-20
TCEP	*tris*(2-carboxyethyl)phosphine
TFA	trifluoroacetic acid
μ (prefix)	micro ($\times 10^{-6}$)
v/v	volume to volume ratio
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight line velocity
WGA	wheat germ agglutinin
w/v	weight to volume ratio

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List of Publications

Two of the chapters in this thesis have previously been published as the following references;

Chapter 2

Pini, T., Leahy, T., Soleilhavoup, C., Tsikis, G., Labas, V., Combes-Soia, L., Harichaux, G., Rickard, J.P., Druart, X., and de Graaf, S.P. (2016) Proteomic investigation of ram spermatozoa and the proteins conferred by seminal plasma. *J. Proteome Res.* **15**(10), 3700-3711

T. Pini was responsible for study co-design, data analysis and writing the manuscript.

Chapter 4

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Chapter 3

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T. Pini was responsible for study co-design, data collection, data analysis and writing the manuscript.

Chapter 6

Pini, T., Farmer, K.L., Druart, X., Teixeira-Gomes, A., Tsikis, G., Labas, V., Leahy, T., and de Graaf, S.P. Binder of Sperm Proteins protect ram spermatozoa from freeze-thaw damage. *Cryobiology*

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1. Review of the Literature

1.1. GENERAL INTRODUCTION

Spermatozoa are a unique cell type, not only in terms of their haploidy, but also due to their mobility, lifespan and tolerance of environmental conditions. Spermatozoa begin development in the testis, transit and are stored in the epididymis, and encounter the external world after mixing with seminal plasma from the accessory sex glands during ejaculation. Spermatozoa can be further 'processed' in vitro prior to insemination, allowing for short term storage with chilling or long term storage with cryopreservation. Spermatozoa from the epididymis and the ejaculate, and those which have undergone cryopreservation, are three significantly different sperm types. Epididymal spermatozoa are missing a key 'ingredient' of semen – seminal plasma. Cryopreserved spermatozoa have been taken to the point of freezing, then brought back to body temperature, a damaging process, and the implications of which continue to unravel.

The use of frozen thawed semen and artificial insemination has become widespread in many production industries, particularly for dairy cattle. In this species, insemination into the uterine body via the cervix is easily achieved, allowing for semen deposition close to the site of fertilisation. The same is not true for sheep; a tortuous cervix with misaligned cartilaginous rings largely bars direct entry to the uterus (Halbert *et al.* 1990; Kershaw *et al.* 2005). This anatomical quirk limits insemination options to cervical, where semen is deposited at the cervical os, or laparoscopic insemination, a minor surgical intervention which allows semen deposition directly into the uterine horns (Killen and Caffery 1982; Evans and Maxwell 1987). While cervical insemination with fresh ram semen produces acceptable pregnancy rates, neither frozen thawed (Maxwell and Hewitt 1986) nor epididymal spermatozoa (Rickard *et al.* 2014) are very successful when inseminated below the cervix. It is clear that for sheep, the cervix acts as a selective barrier, and only certain spermatozoa are capable of traversing the cervix to reach the site of fertilisation. The characteristics which afford this ability to transit the cervix are unknown, beyond a requirement for motility and viability. Interestingly, seminal plasma has been shown to confer significant enhancements in cervical transit (Rickard *et al.* 2014), yet the mechanism behind this advantage is undetermined. Clearly, while exposure to seminal plasma is highly beneficial, cryopreservation is detrimental to the progression of ram spermatozoa through the cervix.

While laparoscopic insemination is a viable alternative to employ frozen thawed ram semen, the cost, skill and labour requirements involved far exceed that of cervical insemination. Further, the invasive nature of laparoscopic insemination is likely to be unfavourable in bids to

improve animal welfare within the wool and sheep meat industries. Thus there is a considerable need for investigation into how seminal plasma alters spermatozoa and improves cervical transit, what makes frozen thawed ram spermatozoa vulnerable to failure in vivo, and whether elements of seminal plasma can be exploited to improve insemination outcomes using frozen thawed spermatozoa. As such, this review will explore changes to spermatozoa along three general themes; the changes which occur during epididymal maturation, those brought about by seminal plasma and the female tract, and finally the 'manmade' changes to spermatozoa which occur during semen freezing and how seminal plasma may be able to minimise cryopreservation damage. While focusing on sheep, this review will draw on complementary research from other species to demonstrate the importance of epididymal maturation, seminal plasma exposure and cryopreservation damage.

1.2. EPIDIDYMAL MATURATION

1.2.1. Changes to the sperm membrane

During the process of epididymal maturation, spermatozoa exit the rete testes and migrate through the caput, corpus and finally the cauda epididymis. There has been significant investigation into the changes which occur over this period of maturation, covered in many excellent reviews (Dacheux and Paquignon 1980; Aitken *et al.* 2007; Cornwall 2009; Dacheux *et al.* 2012; Dacheux and Dacheux 2014; Gervasi and Visconti 2017). Such a significant maturation event is beyond the scope of this review, and will only be covered in brief to provide some context of pre ejaculatory maturational changes. Apart from the development of motility and fertilising capacity, some of the most well studied changes to spermatozoa during this developmental period are those which alter the outer sperm membrane, typically involving changes to proteins and carbohydrates. During epididymal maturation, proteins are both gained and lost from the sperm surface (Belleannee *et al.* 2011) and the global quantity of particular proteins in spermatozoa may change (Labas *et al.* 2015b; Skerget *et al.* 2015). In addition, the frequency of protein post translational modifications may also be altered during epididymal transit (e.g. tyrosine phosphorylation (Lin *et al.* 2006; Fàbrega *et al.* 2011b)). Spermatozoa gain motility (Fournier-Delpech *et al.* 1979; Angrimani *et al.* 2014; Peña Jr *et al.* 2015) and fertilising ability (Hoppe 1975; Fournier-Delpech *et al.* 1979) during this period, which in some cases has been directly related to exposure to particular proteins originating from the epididymis (Acott *et al.* 1979; Vijayaraghavan *et al.* 1996; Focarelli *et al.* 1998; Fàbrega *et al.* 2011a). In addition, the activities of various enzymes such as antioxidants, glycosyltransferases and glycosidases have been shown to fluctuate across the regions of the epididymis (Tulsiani 2006; Angrimani *et al.* 2014).

Such fluctuations in carbohydrate altering enzymes have been linked to the significant changes in sperm surface carbohydrate groups which occur during epididymal maturation

(Gordon *et al.* 1975; Holt 1980; Fain-Maurel *et al.* 1984; Voglmayr *et al.* 1985; Young *et al.* 1986; Magargee *et al.* 1988; Sarkar *et al.* 1991; Toyonaga *et al.* 2011; Fàbrega *et al.* 2012b). These changes have also been associated with loss and gain of glycan bearing proteins (Toowicharanont and Chulavatnatol 1983; Fàbrega *et al.* 2012b). Changes to glycoconjugates and particular sugar residues have further been related to different patterns of anionic sites, electrophoretic mobility and isoelectric point of spermatozoa from various sections of the epididymis (Bedford 1963; Moore 1979; Stoffel *et al.* 2002). Another well established characteristic of epididymal transit is the alteration to lipid components of the membrane. Depending on the species, this may include changes to the cholesterol/phospholipid ratio, the various proportions contributed by subclasses of neutral lipids and phospholipids, overall amounts of each lipid class (Nikolopoulou *et al.* 1985; Rana *et al.* 1991) and an increase in lipid diffusion (Christova *et al.* 2002). It is theorised that these lipid based changes ultimately determine membrane fluidity and hence the ability to perform critical tasks such as capacitation and fertilisation. It is clear that during the period of epididymal transit, spermatozoa are transformed by complex alterations to various important structural and functional elements of the cell. As with all processes in spermatozoa, these changes are ultimately contributing to the fundamental task of the sperm cell, fertilisation.

1.2.2. Development of fertilising capacity during epididymal maturation

After entering the female tract, spermatozoa must complete several tasks and undergo a swathe of changes termed ‘capacitation’ (covered in section 1.3.1.2), which culminate in fertilisation of the oocyte. Depending on the site of semen deposition, spermatozoa must navigate some length of the female reproductive tract, form the oviductal sperm reservoir, navigate the upper reaches of the tract to locate the oocyte, develop hyperactivated motility to enter the cumulus-oocyte complex, complete capacitation, bind to the zona pellucida and undergo the acrosome reaction; all tasks which are prerequisite to successful fertilisation. Research into the more functional aspects of epididymal maturation have centred on the ability of spermatozoa to perform these processes. It has been demonstrated that in a capacitating environment, spermatozoa from the most developed region, the cauda, display significantly higher membrane disorder (Fàbrega *et al.* 2012a), hyperactivated motility and ability to bind to the zona (Kawakami *et al.* 2002), compared to spermatozoa from the caput and corpus. Further in vitro work has shown that both binding to oviductal explants (Peña Jr *et al.* 2015) and ability to acrosome react (Yeung *et al.* 1996; Sirivaidyapong *et al.* 2001; Lin *et al.* 2006) similarly develop in parallel with the progression of epididymal maturation.

The final act of fertilisation itself has likewise been confirmed to drastically improve with progression towards the cauda epididymis (Hoppe 1975). Put to the test in vivo, these results are unyielding; in a range of mammalian species, pregnancy rate following intrauterine

insemination significantly increases with epididymal transit, developing in the corpus and peaking in the cauda epididymis (Dacheux and Paquignon 1980). In some instances, this favourable development extends to increases in early embryo survival and litter size (Fournier-Delpech *et al.* 1979). Clearly, the time spent in transit through the epididymis is not wasted, with significant remodelling of surface components and critical changes culminating in the production of fertile spermatozoa. Yet the end of the epididymis is not the end of the story for sperm maturation, and the changes which occur after ejaculation are just as biologically relevant to the success of reproduction. However, the ability of cauda epididymal spermatozoa to successfully perform many of the crucial aspects involved in fertilisation begs the question of what role seminal plasma has to play.

1.3. EJACULATION AND SEMINAL PLASMA

1.3.1. Composition of seminal plasma and its manipulation of spermatozoa

Seminal plasma is the complex fluid produced by the accessory sex glands, including the ampulla, seminal vesicles, prostate and bulbourethral glands, which is mixed with spermatozoa at the time of ejaculation. It is composed of ions, sugars, salts, vitamins, lipids and proteins (Mann 1964), with the latter having received the most attention in the last few decades, particularly with the advent of mass spectrometry. Extensive seminal plasma proteomes have been produced for several species, including production animals (bull (Kelly *et al.* 2006; Druart *et al.* 2013; Rego *et al.* 2014), ram (Druart *et al.* 2013; Soleilhavoup *et al.* 2014; Rickard *et al.* 2015), boar (Druart *et al.* 2013; Vilagran *et al.* 2015; Perez-Patiño *et al.* 2016), stallion (Novak *et al.* 2010; Druart *et al.* 2013), goat, camel and alpaca (Druart *et al.* 2013)), fish (trout (Nynca *et al.* 2014b), carp (Dietrich *et al.* 2014), salmon (Gombar *et al.* 2017)) and humans (Intasqui *et al.* 2015). These studies identified anywhere from 46 to over 700 proteins in seminal plasma depending on the proteomic methods used and are a fitting resource for identifying proteins of interest for further investigation. Two of the major seminal plasma protein families identified across several species were the Binder of Sperm Proteins (BSPs, e.g. BSP1, BSP3, BSP5, BSPH1) and spermadhesins (e.g. spermadhesin Z13, BDH2, AQN1). Both of these protein families play important roles in sperm physiology (Töpfer-Petersen *et al.* 1998; Plante *et al.* 2015b), and the BSPs will be discussed further in the following section. While informative, a significant limiting factor of seminal plasma proteomes is that without accompanying sperm proteomes, a clear picture of all the proteins which bind tightly to the sperm surface at ejaculation cannot be built. There is evidence that epididymal and ejaculated spermatozoa have protein based differences due to the addition of proteins by seminal plasma (e.g. porcine spermadhesins (Dostàlovà *et al.* 1994), bovine Binder of Sperm Proteins (Manjunath *et al.* 2007)). However, there are no in depth proteomic studies directly comparing spermatozoa before and after exposure to seminal plasma.

As with spermatozoa, seminal plasma has been found to have some unique species differences, but is still believed to play many common roles. Seminal plasma from unrelated species is able to stimulate motility of caput spermatozoa, suggesting a common stimulatory action of this fluid, potentially conferred by the same active ingredient (Acott *et al.* 1979). Proteomic investigation of seminal plasma from a range of domesticated species has shown both considerable divergence, as well as some maintenance of proteins (Druart *et al.* 2013). While conservation of proteins between species can be high (e.g. 73% common proteins between ram and buck), some species appear to have relatively divergent seminal plasma proteomes (e.g. 13% common proteins between buck and stallion). The most likely explanations for this are evolutionary separation and differing reproductive strategies (e.g. site of semen deposition, spontaneity of ovulation, oestrus length). Unique functions of seminal plasma have been characterised, but by and large, seminal plasma performs a consistent set of actions across species. These have been elegantly summarised by Bedford (2014), and include transport, maintenance of viability and fertilising capability (Kawano *et al.* 2014; Araki *et al.* 2016) and improvement of post fertilisation developmental success (O'Leary *et al.* 2004; Shima *et al.* 2015). While there is no lack of evidence that seminal plasma alters sperm function, the mechanisms of these alterations are less clear.

On a molecular level, previous studies have shown that epididymal and ejaculated spermatozoa differ significantly, suggesting that exposure to seminal plasma is a key maturational step. Mirroring the process of epididymal maturation, exposure to seminal plasma elicits changes in sperm surface carbohydrates (Magargee *et al.* 1988) and potential differences in proteomic makeup (Voglmayr *et al.* 1983; Dostàlovà *et al.* 1994). In particular, a ‘masking’ of membrane components by seminal plasma factors has been demonstrated (Buttke *et al.* 2006), which could underlie its ability to reverse capacitation, the final stage of maturation prior to fertilisation (Chang 1957). Epididymal and ejaculated spermatozoa also differ physiologically; antioxidant capabilities (Angrimani *et al.* 2014) and sensitivity to osmotic and cooling stresses (Varisli *et al.* 2009; Monteiro *et al.* 2013) are significantly changed after contact with seminal plasma. These differences between epididymal and ejaculated spermatozoa highlight the fact that seminal plasma is more than just a transport medium; it actively contributes to modulation of the sperm surface. However, further research is required to exhaustively profile the effects of seminal plasma exposure.

1.3.1.1. Binder of Sperm Proteins

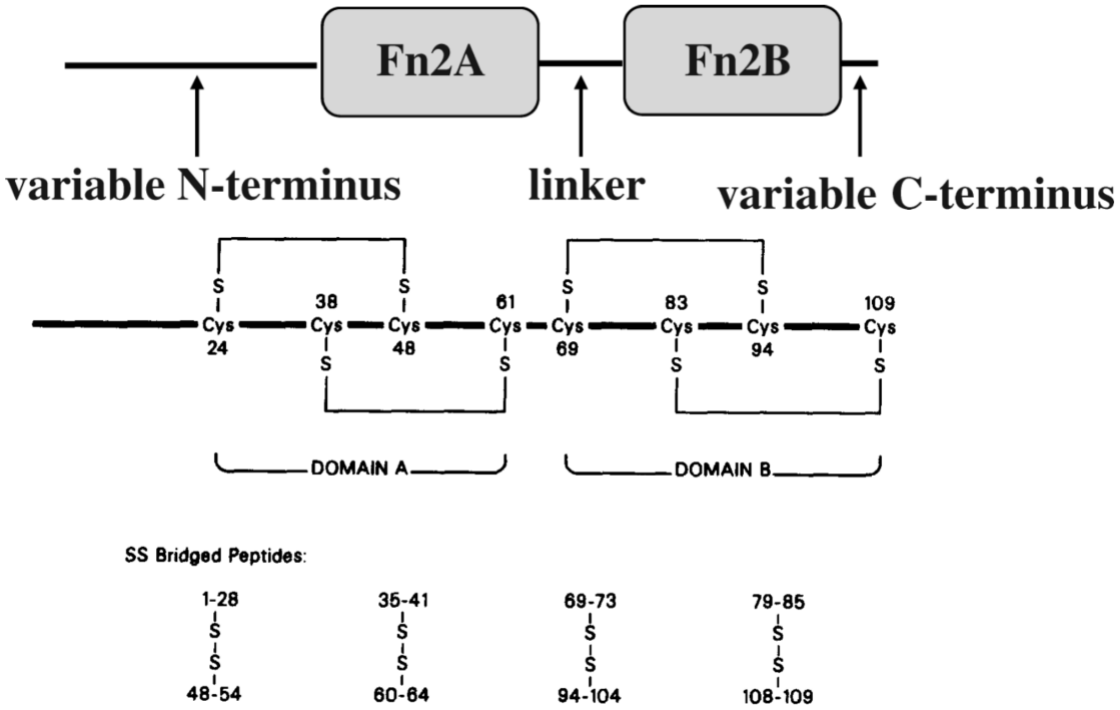

Figure 1.1 (**A**) Characteristic structure of Binder of Sperm Proteins, containing two fibronectin type II domains and variable termini (from Manjunath *et al.* (2009)). (**B**) Typical disulphide bridges present within fibronectin domains (from Esch *et al.* (1983)).

Several of the major seminal plasma protein families have been well characterised, but one family in particular has received significant attention. Binder of Sperm Proteins (BSPs) are a protein 'superfamily' consisting of several low molecular weight acidic proteins, characterised by the presence of variable N and C termini adjoining two tandem fibronectin type II domains, each containing two disulphide bridges (Esch *et al.* 1983; Manjunath and Sairam 1987; Calvete *et al.* 1995; Calvete *et al.* 1996) (Figure 1.1). Some family members are glycosylated and have multiple glycoforms (Manjunath *et al.* 1987; Calvete *et al.* 1995; Calvete *et al.* 1996; Gerwig *et al.* 1996), while others bare no glycans. Conserved structural components confer binding affinity for gelatin (Manjunath *et al.* 1987), heparin, glycosaminoglycans (Chandonnet *et al.* 1990), phosphorylcholine (Desnoyers and Manjunath 1992), apolipoprotein A1 (Manjunath *et al.* 1989), low density lipoprotein (Manjunath *et al.* 2002), casein (Lusignan *et al.* 2011) and other milk proteins (Plante *et al.* 2015a).

This family has now been phylogenetically identified in 30 species (Serrano *et al.* 2015) and physically characterised in at least 8 species (Leblond *et al.* 1993; Plante *et al.* 2015b). The characterisation of this family began with the isolation of three proteins from bovine seminal plasma (Esch *et al.* 1983; Manjunath and Sairam 1987; Manjunath *et al.* 1987), originally named PDC-109/BSP-A1/A2, BSP-A3 and BSP-30 kDa, now referred to as BSP1, BSP3 and

BSP5 respectively (Manjunath *et al.* 2009). While similar in sequence, these proteins bear different post translational modifications; BSP1 is phosphorylated at serine and threonine residues (Barrios *et al.* 2005) and BSP5 is significantly more glycosylated than BSP1 (Manjunath and Sairam 1987; Calvete *et al.* 1996; Gerwig *et al.* 1996; Barrios *et al.* 2005; Cardozo *et al.* 2008). These proteins account for as much as 57% of bull seminal plasma proteins (Nauc and Manjunath 2000) and BSP1 and 5 have also been identified as highly abundant in ram seminal plasma (Jobim *et al.* 2005; Soleilhavoup *et al.* 2014), accounting for up to 20% of ram seminal plasma proteins by weight (Bergeron *et al.* 2005). These proteins are predominantly produced in the seminal vesicles in the bull and ram (Scheit *et al.* 1988; Fernández-Juan *et al.* 2006), with protein homologues from other species being produced in the epididymis (Lefebvre *et al.* 2009). These proteins bind to the sperm membrane at ejaculation (Manjunath *et al.* 1988; Barrios *et al.* 2005) by interacting with phospholipids. BSP1 and BSP3 bind strongly to phosphatidylcholine and sphingomyelin via the phosphorylcholine head group (Desnoyers and Manjunath 1992; Therrien *et al.* 2013). While BSP5 similarly binds to phosphorylcholine containing phospholipids, it also has some affinity for phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol (Desnoyers and Manjunath 1992). This interaction with phospholipids leads to partial insertion into the outer leaflet of the membrane bilayer, forming a strong connection anchored by phosphorylcholine (Therrien *et al.* 2013; Le Guillou *et al.* 2016). BSPs may also interact with the long fatty acid chains of phospholipids upon insertion (Anbazhagan *et al.* 2008). Thus at least in the ram and bull, BSPs represent a major protein constituent added to the sperm surface at ejaculation and their established functions reveal the depth of their importance.

While abundant in both bull and ram seminal plasma, the functions of BSPs have only been thoroughly investigated in the bull. The interaction of BSPs with bull spermatozoa is dynamic, evolving over time as the physiological surroundings change. When BSPs first associate with epididymal bull spermatozoa (i.e. what would normally occur at ejaculation), there is a fast and progressive efflux of cholesterol (Thérien *et al.* 1998; Moreau *et al.* 1999) and phospholipids (Moreau *et al.* 1999; Tannert *et al.* 2007; Therrien *et al.* 2013) from the sperm membrane. However, the insertion of these proteins also results in significant membrane stabilisation (Greube *et al.* 2001; Swamy *et al.* 2002), potentially a major benefit for the journey to the site of fertilisation. While the bulk of seminal plasma is then left behind in the lower reproductive tract, BSPs remain tightly bound to the sperm surface. After several hours of exposure to BSPs, when spermatozoa would likely be high in the female reproductive tract, these proteins have caused significant loss of both membrane cholesterol and phospholipids (Moreau *et al.* 1998; Thérien *et al.* 1998; Moreau *et al.* 1999; Thérien *et al.* 1999). In addition, BSPs are able to interact with the cholesterol acceptor high density lipoprotein (HDL), via binding to ApoA1

(Manjunath *et al.* 1989), and further promote cholesterol efflux (Thérien *et al.* 1998). BSPs then confer the ability to bind to oviductal epithelial cells (Gwathmey *et al.* 2003), and extend the motile life span of spermatozoa in this reservoir (Gwathmey *et al.* 2006). It is unclear whether these various roles of BSPs in capacitation are specific to bovine spermatozoa or common to both ram and bull. Once spermatozoa have completed the process of capacitation, BSPs are either partially lost from the sperm surface (Barrios *et al.* 2005) or modified (Hung and Suarez 2012). In sum, these functions drive the final sperm maturation process of capacitation (Thérien *et al.* 1995; Thérien *et al.* 1997; Manjunath and Thérien 2002) which prepares spermatozoa to engage in fertilisation. This is reflected by enhanced in vitro fertilisation rates when BSPs are present (Rodríguez-Villamil *et al.* 2015). This dynamic and complex relationship between BSPs and bull spermatozoa underlines the importance of a singular group of seminal plasma proteins in modulating sperm function. Yet it also raises the question of how this protein family influences sperm function in other species, particularly in sheep. While studies are beginning to investigate the physiological roles of BSPs in mice (Plante *et al.* 2013; Plante and Manjunath 2015), humans (Plante *et al.* 2014), pigs (Lusignan *et al.* 2007) and sheep (Luna *et al.* 2015), the full extent of their actions in these species, particularly in terms of sperm capacitation, is yet to be resolved.

1.3.1.2. Modulation of capacitation by seminal plasma

Capacitation was first defined by Chang (1951) and Austin (1951) as a fundamental change which spermatozoa must undergo before gaining the capacity to fertilise, achieved by several hours of incubation in the female tract. Capacitation is now understood to be a multifaceted process of both subtle and obvious alterations to sperm structure and function which prepare the male gamete for the ultimate task of fertilisation. The intricate details of such a complex transformative process are outside the scope of this review, and the reader is referred to comprehensive published reviews on the subject (Rodriguez-Martinez 2007; Bailey 2010; Visconti *et al.* 2011; Signorelli *et al.* 2012; Aitken and Nixon 2013). For the purposes of this review, only the general process of capacitation and its relationship with seminal plasma will be discussed.

Capacitation is a highly regulated event, progressing via a signalling cascade which involves cyclic AMP/protein kinase A and protein tyrosine kinase pathways (Visconti *et al.* 1995a; Visconti *et al.* 1995b). In vivo, these pathways are initially stimulated by factors in the female tract, which can be used to capacitate spermatozoa in vitro. These factors can include bicarbonate (Suzuki *et al.* 1994; Tardif *et al.* 2003; Harrison and Gadella 2005; Grasa *et al.* 2006; Battistone *et al.* 2013), cholesterol acceptors such as bovine serum albumin (Visconti *et al.* 1995b) and calcium (Baldi *et al.* 2000; Navarrete *et al.* 2015). However, all of these

elements are not necessarily required depending on the species. Interestingly, ram spermatozoa require significant exogenous upregulation of cyclic AMP in order to demonstrate classical signs of capacitation in vitro (Colás *et al.* 2008; Leahy *et al.* 2016). The sperm membrane is a key location affected by capacitation; outcomes include increased disorder of membrane lipids, scrambling of membrane phospholipids between leaflets (Gadella and Harrison 2000) and redistribution of membrane sterol components (Flesch *et al.* 2001), supporting efflux of membrane cholesterol (Leahy and Gadella 2015). Capacitation also results in significant changes to sperm surface components, including loss and rearrangement of membrane bound proteins (Peknicova *et al.* 1994; Focarelli *et al.* 1998; Giovampaola *et al.* 2001; Baker *et al.* 2010) and sugars (Gordon *et al.* 1975; Bawa *et al.* 1993; Jiménez *et al.* 2003; Wu *et al.* 2012). These structural changes lead to significant functional differences, ultimately producing a spermatozoon that is capable of fertilisation. This complex sequence of events must progress in a timely manner, as capacitated spermatozoa are destined to 'expire' if they fail to meet an oocyte within a short allotment of time (Aitken 2011). This calls for fail proof mechanisms to regulate the initiation and progression of capacitation, to ensure that spermatozoa reach their full fertilising potential within the required window.

Spermatozoa must exist in a non-capacitated state for the vast majority of time prior to fertilisation, and seminal plasma performs key regulatory actions to achieve this. As soon as spermatozoa enter the female reproductive tract, they are assailed by high concentrations of bicarbonate (Zhou *et al.* 2005), calcium (Hugentobler *et al.* 2007), albumin and other cholesterol acceptors (Ehrenwald *et al.* 1990; Tunón *et al.* 1998; Alavi-Shoushtari *et al.* 2006). However, it takes spermatozoa several hours to capacitate and successfully fertilise (Chang 1951). Soon after Chang's initial discovery of capacitation, he published work demonstrating that seminal plasma is able to undo the effects of the female tract, effectively 'decapacitating' spermatozoa (Chang 1957; Bedford and Chang 1962). This effect is now recognised to be largely due to seminal plasma proteins including spermadhesins (Caballero *et al.* 2009), cysteine rich secretory proteins (Nixon *et al.* 2006), seminal vesicle proteins (Kawano and Yoshida 2006; Lin *et al.* 2008; Lu *et al.* 2010), β defensins (Tollner *et al.* 2004) and others yet to be identified (Martins *et al.* 2003). These proteins tend to significantly decrease hallmarks of capacitation such as tyrosine phosphorylation, acrosome reaction and zona binding (Tollner *et al.* 2004; Kawano and Yoshida 2006; Nixon *et al.* 2006; Lin *et al.* 2008; Caballero *et al.* 2009; Lu *et al.* 2010), and can abolish successful in vitro fertilisation by decapacitation (Suzuki *et al.* 2002). The mechanisms by which most of these proteins regulate capacitation are unclear. Disruption of key capacitation related processes (e.g. limiting cholesterol efflux (Lu *et al.* 2010)) and masking of receptors (Tollner *et al.* 2004; Tecle and Gagneux 2015) are possible explanations.

Interestingly, while seminal plasma proteins have largely been thought of as 'decapacitating' factors, there is a significant body of evidence describing seminal plasma proteins which promote capacitation. Apart from Binder of Sperm Proteins (described in section 1.3.1.1), calcitonin, angiotensin II, fertilisation promoting peptide (FPP) and CD38 are all examples of proteins which drive capacitation via a variety of mechanisms. While BSPs encourage cholesterol efflux (Thérien *et al.* 1998), calcitonin, angiotensin II and FPP upregulate cyclic AMP (Fraser *et al.* 2006) and CD38 initiates a phosphorylation induced capacitation cascade (Kim *et al.* 2015b). Thus while seminal plasma has often been painted as a fluid which prevents capacitation, the reality is more subtle, with synergistic impeding and promoting functions. Continuing characterisation of individual seminal plasma proteins is likely to identify those proteins which contribute to this manipulation of sperm capacitation.

1.3.2. Effects of seminal plasma on in vivo fertility

With the advent of advanced reproductive technologies such as IVF and ICSI, it has become clear that seminal plasma is not an absolute requirement for fertilisation (Silber *et al.* 1995; Rath and Niemann 1997; Blash *et al.* 2000). Further, many previous studies have demonstrated that even in vivo, inseminations with epididymal spermatozoa can achieve pregnancies (Fournier-Delpech *et al.* 1979; Morris *et al.* 2002; Soler *et al.* 2003; Tsutsui *et al.* 2003; Hori *et al.* 2011; Rickard *et al.* 2014; Stone *et al.* 2015). Such evidence seems to suggest that seminal plasma is largely superfluous for sperm function, yet studies comparing the in vivo fertility of spermatozoa before and after exposure to seminal plasma emphasise the importance of this fluid. Exposure of stallion epididymal spermatozoa to seminal plasma prior to direct uterine insemination significantly improved pregnancy rates (Heise *et al.* 2010), suggesting that seminal plasma has important roles in insemination outcome. The importance of this fluid in sheep is particularly intriguing; while seminal plasma did not affect the outcome of uterine insemination (Fournier-Delpech *et al.* 1979; Rickard *et al.* 2014), epididymal spermatozoa exposed to seminal plasma achieved a pregnancy rate five-fold that of the unexposed control after cervical insemination (Rickard *et al.* 2014). Thus the context of insemination, particularly the species and site of semen deposition, dictates the effects of seminal plasma on fertility outcomes.

It has been hypothesised that seminal plasma is able to enhance the ability of spermatozoa to transit the female reproductive tract, improving insemination outcome when semen is deposited far from the site of fertilisation. While there is evidence to support this hypothesis, the mechanism of seminal plasma 'rescue' is far from clear. However, the results of in vitro comparisons of epididymal and ejaculated spermatozoa provide some clues. Looking at the most basic and fundamental sperm functional parameters of motility and viability, the vast majority of previous research has shown no significant differences between epididymal and

ejaculated spermatozoa (Morris *et al.* 2002; Varisli *et al.* 2009; Gloria *et al.* 2011; Hori *et al.* 2011; Monteiro *et al.* 2011; Fàbrega *et al.* 2012a; Yeste *et al.* 2012; Monteiro *et al.* 2013; Angrimani *et al.* 2014; Rickard *et al.* 2014; Cunha *et al.* 2016). Similarly, various studies report no significant differences in spontaneous acrosome reactions (Varisli *et al.* 2009; Fàbrega *et al.* 2012a; Cunha *et al.* 2016) or DNA fragmentation index (Garcia-Macias *et al.* 2006). Thus the majority of functional parameters appear to be identical both before and after exposure to seminal plasma. However, scavenging of reactive oxygen species (ROS) is a means by which seminal plasma could contribute to improving success in the female reproductive tract. The accessory sex glands are a key source of the antioxidant enzymes catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Chen *et al.* 2003) and in particular, catalase appears to be almost solely produced by the prostate in a range of species (Jeulin *et al.* 1989; Ball *et al.* 2000; Koziorowska-Gilun *et al.* 2011). As a result, epididymal spermatozoa have significantly lower, and sometimes non-existent, catalase activity compared to ejaculated spermatozoa (Koziorowska-Gilun *et al.* 2011; Angrimani *et al.* 2014). Lower activity of these enzymes in spermatozoa and seminal plasma has been directly related to increased DNA damage susceptibility (Chen *et al.* 2003) and is correlated with human infertility (Jeulin *et al.* 1989; Atig *et al.* 2017), suggesting important functional consequences in vivo. Thus while epididymal and ejaculated spermatozoa demonstrate similar levels of functionality in vitro, their ability to respond to the production of ROS may underlie their capacity to undertake the costly process of migration through the lower female reproductive tract.

Beyond the intrinsic functionality of spermatozoa, there are a host of interactions between spermatozoa and the female reproductive tract which may benefit from seminal plasma. Cervical mucus forms a significant barrier to sperm progression (Cone 2009; Lai *et al.* 2009) and penetration of cervical mucus has been used as an important human fertility indicator for decades (Davajan *et al.* 1970; Katz *et al.* 1980). Interestingly, seminal plasma has been shown to significantly improve both entry of spermatozoa into mucus (Overstreet *et al.* 1980) as well as the distance spermatozoa penetrated into mucus within a given time (Rickard *et al.* 2014). The ability to penetrate cervical mucus has been linked both to enhanced formation of oviductal reservoirs (Cox *et al.* 2002) and heightened in vivo fertility (Taş *et al.* 2007b), highlighting the importance of such improvements by seminal plasma. In addition to cervical mucus, spermatozoa are met by an influx of phagocytic cells almost immediately post insemination. Seminal plasma is both a significant cause of and potential solution to this barrier; this is covered in greater detail in section 1.3.3.

Once in the upper female reproductive tract, spermatozoa form an oviductal reservoir by binding directly to oviductal epithelial cells (OEC), a key process for maintaining spermatozoa in a viable and ‘decapacitated’ state (Töpfer-Petersen *et al.* 2002). Boar, bull and tom cat

spermatozoa exposed to seminal plasma demonstrate a significantly higher OEC binding index compared to epididymal spermatozoa (Gwathmey *et al.* 2003; Yeste *et al.* 2012; Henry *et al.* 2015; Peña Jr *et al.* 2015). In addition, particular proteins from the accessory sex glands including BSP1 (bull; (Gwathmey *et al.* 2003)), AQN1 (boar; (Ekhlasi-Hundrieser *et al.* 2005)) and an unidentified high molecular weight protein (alpaca; (Apichela *et al.* 2014)) have been shown to be involved in the formation of the oviductal sperm reservoir. Further proteomic investigation of the proteins contributed by seminal plasma to the surface of ejaculated spermatozoa may help to identify those proteins involved in OEC binding in other species. Once spermatozoa are released from the oviductal reservoir, they complete the process of capacitation. As capacitation is a prerequisite for fertilisation, epididymal spermatozoa are clearly capable of completing this functional maturation step. However, as described in section 1.3.1.2, seminal plasma has significant impacts on capacitation. These effects may be key to regulating the timing of capacitation in vivo, and some evidence suggests that ejaculated spermatozoa are slower to capacitate than epididymal spermatozoa (Yeung *et al.* 1996). Thus if the timing of capacitation is brought into an appropriate window by seminal plasma, this could plausibly lead to significant improvements in in vivo fertility.

There is evidence to suggest that seminal plasma improves the ability of spermatozoa to transit the cervix in sheep, and improves insemination outcomes in other species. While aspects of sperm function remain similar after exposure to seminal plasma, the interaction between the male gamete and the female reproductive tract are clearly altered. What remains to be investigated are the mechanisms behind the influence of seminal plasma on this interaction. As described in section 1.3.1, there is a relatively limited knowledge of the physical changes which seminal plasma confers to the sperm surface, particularly in terms of addition of proteins and modulation of the complex glycocalyx. As membrane bound molecules are the means by which spermatozoa interact with the female reproductive tract in many different capacities, changes to the surface architecture of spermatozoa may have far reaching consequences for fertility. Thus investigation into the modulation of the sperm surface by seminal plasma could be key to understanding the observed in vivo benefits of this complex fluid.

1.3.3. Spermatozoa, seminal plasma and the female immune response

The female reproductive tract can be a harsh environment for spermatozoa, particularly for vaginal depositors such as the ram and bull, most rodents, rabbits and humans. Acidic pH (Linhares *et al.* 2011; Maddison *et al.* 2016) and mucin rich cervical mucus (Cone 2009) form an instant physiological barrier which spermatozoa must overcome. In addition, the female immune response represents a significant hurdle for spermatozoa, an invading ‘non-self’ cell. Semen deposition leads to production of several inflammatory cytokines (CSF2, IL1α, IL6 and

IL8) and increased expression of a range of genes associated with inflammatory and immune pathways within cervical tissue (Scott *et al.* 2009; Sharkey *et al.* 2012). Similar increases in production of inflammatory cytokines (GM-CSF, CSF2, CCL2/MCP-1, CCL5, CCL20, CXCL1, IL1β, IL6, IL8, TNFβ1) have also been attributed to seminal plasma (Robertson *et al.* 1996; Denison *et al.* 1999; O'Leary *et al.* 2004; Sharkey *et al.* 2007; Ochsenkühn *et al.* 2008; Scott *et al.* 2009; Introini *et al.* 2017). While the majority of these studies have looked at the impact of seminal plasma on cytokine production after an extended period (i.e. 12-24 hours), a recent study by Introini *et al.* (2017) demonstrated that seminal plasma can elicit significant inflammatory cytokine production by cervical explants within 2 hours of exposure. The components of seminal plasma suggested to be responsible for this strong and rapid inflammatory response include intrinsic cytokines (Tremellen *et al.* 1998), prostaglandins (Joseph *et al.* 2012) and proteins (Rodriguez-Martinez *et al.* 2010).

Production of these cytokines ultimately leads to accumulation of immune cells within the tissues of the female reproductive tract (Robertson *et al.* 2000). An influx of antigen presenting cells, T lymphocytes and polymorphonuclear granulocytes into the epithelial tissues (Bischof *et al.* 1994; Bischof *et al.* 1995; Fiala *et al.* 2007; Scott *et al.* 2009; Sharkey *et al.* 2012; Vilés *et al.* 2013) and lumen of the female reproductive tract (Austin 1957; Mattner 1969; Thompson *et al.* 1992; Kotilainen *et al.* 1994; Robertson *et al.* 1996; Rozeboom *et al.* 1998; Portus *et al.* 2005; Scott *et al.* 2006; Scott *et al.* 2009) in response to semen or seminal plasma has been widely observed. As with cytokine production, this cellular response to seminal plasma and spermatozoa is similarly rapid, occurring within 3 hours (Scott *et al.* 2006; Fiala *et al.* 2007) and lasting for days post insemination (Bischof *et al.* 1995). One study even reported the appearance of significantly increased numbers of luminal neutrophils in cervical mucus within 20 minutes of insemination (Thompson *et al.* 1992), demonstrating an almost immediate immune response from the female tract. Thus the outcome of semen deposition in the female tract is widespread upregulation of inflammatory cytokines and the rapid and sustained infiltration of immune cells, including those with phagocytic and antigen presenting capabilities. This post mating inflammation is considered physiological rather than pathological; it is believed to be important for clearance of spermatozoa from the female reproductive tract (Troedsson *et al.* 2001) and promotion of immune tolerance to any resulting foetuses (Robertson *et al.* 2009; Sharkey *et al.* 2012; Bromfield 2016).

While the idea that phagocytes in the lumen of the female tract post insemination are responsible for sperm clearance is both plausible and realistic, investigation into the interaction of these cells with spermatozoa has produced inconsistent theories. For many years, phagocytes in the female tract were typecast to the role of clearance of dead, dying or otherwise abnormal spermatozoa (Austin 1957; Austin 1960; Symons 1967; Moyer *et al.* 1970;

Vogelpoel and Verhoef 1985), as is the case for phagocytes in the male reproductive tract (Cooper and Hamilton 1977; Tomlinson *et al.* 1992). However, others have shown that live spermatozoa are phagocytosed just as readily as non-viable spermatozoa (D'Cruz and Haas 1995; Alghamdi *et al.* 2001; Li and Funahashi 2010), or even preferentially (Troedsson *et al.* 2005). In addition, while motile spermatozoa can be phagocytosed, some appear to be intrinsically resistant (Taylor 1982; Alghamdi *et al.* 2001); no in vitro studies report 100% phagocytosis of spermatozoa (Matthijs *et al.* 2000; Alghamdi *et al.* 2004). This raises an intriguing question; are particular subsets or types of spermatozoa preferentially 'targeted' for phagocytosis, based on something other than their viability?

While capacitation has been suggested as a potential process to increase targeting of spermatozoa by immune cells, results have been inconsistent (Bedford 1965; Matthijs *et al.* 2000; Oren-Benaroya *et al.* 2007). However, this theory presents an interesting concept on the relation of cell surface changes to phagocytic susceptibility. As alluded to in section 1.3.1.2, capacitation involves significant changes to the proteins and glycans present on the sperm surface. Alteration of surface components of somatic cells and pathogens has significant effects on their susceptibility to phagocytosis (Doolittle *et al.* 1983; Schauer *et al.* 1984; Fischer *et al.* 1991; Crestani *et al.* 1993; Sheth *et al.* 2011; Paris *et al.* 2012). This is likely due to the fact that amongst the wide range of receptor-ligand interactions employed by phagocytes for target recognition, many involve recognition of particular surface glycans and proteins (Athamna *et al.* 1991; Futosi *et al.* 2013; Thomas and Schroder 2013; van Rees *et al.* 2016). In this context, it is conceivable that any processes which could alter sperm surface components (e.g. exposure to seminal plasma, cryopreservation, capacitation), may make spermatozoa more or less likely targets for phagocytosis, but this remains to be confirmed. Given the considerable loss of spermatozoa through phagocytosis reported by in vitro studies (Alghamdi *et al.* 2009), it may be a significant contributor to poor in vivo fertility of particular sperm types (e.g. epididymal (Rickard *et al.* 2014) and cryopreserved spermatozoa (Maxwell and Hewitt 1986)) when inseminated far from the site of fertilisation.

Seminal plasma is evidently a significant contributor to the development of post mating inflammation, but it also has a substantial role in regulating and suppressing the female immune response. Seminal plasma has been shown to significantly limit binding and phagocytosis of equine, porcine and bovine spermatozoa by neutrophils (Dahms and Troedsson 2002; Alghamdi *et al.* 2004; Cropp 2006; Troedsson *et al.* 2006; Li *et al.* 2012). This has largely been attributed to proteins of seminal plasma, including DNAse, which limits entrapment in neutrophil extracellular traps (Alghamdi and Foster 2005) and CRISP3 (Doty *et al.* 2011). In addition, seminal plasma has strong 'anti-complement' function thanks to complement regulatory proteins CD59, CD55 and CD46, which limit effective complement

mediated attack (Rooney *et al.* 1993; Harris *et al.* 2006). Seminal plasma, even at relatively low concentrations, has also been shown to inhibit upregulation of neutrophil receptors targeting spermatozoa (D'Cruz and Haas 1995), reduce neutrophil chemotaxis (Rozeboom *et al.* 2001; Taylor *et al.* 2009; Li *et al.* 2012), inhibit the oxidative burst response of phagocytes (Schopf *et al.* 1984; Gilbert and Fales 1996; Binks and Pockley 1999) and protect spermatozoa by scavenging ROS produced by the oxidative burst (Kovalski *et al.* 1992; Gilbert and Fales 1996). In opposition to the inflammatory cytokine production it stimulates, seminal plasma interestingly also assists in inflammation resolution. Seminal plasma contains significant concentrations of transforming growth factor beta (TGF-β) (Tremellen *et al.* 1998; Robertson *et al.* 2002) and also promotes upregulation of this (Ochsenkühn *et al.* 2008) and other anti-inflammatory cytokines (e.g. IL10 (Denison *et al.* 1999)). Finally, the ability of seminal plasma to promote maternal tolerance of an allogenic conceptus (Robertson *et al.* 2002; Johansson *et al.* 2004; Robertson *et al.* 2009; Robertson *et al.* 2013; Kim *et al.* 2015a; Shima *et al.* 2015; Bromfield *et al.* 2017) and potentially influence the development of adulthood disease (Bromfield 2014) has received significant attention in the last two decades. Thus seminal plasma clearly has potent immunomodulatory effects, which may underlie its ability to protect spermatozoa within the female tract and promote development of the resulting conceptus.

1.4. CRYOPRESERVATION

1.4.1. Freezing induced changes to sperm structure and function

The ability to cryopreserve the male gamete has major benefits for artificial reproduction, making international semen transport, optimally timed inseminations and indefinite storage of genetic material entirely achievable. Yet these benefits come at a cost; the process of cryopreservation is a challenging treatment for spermatozoa, and reports of decreased viability and motility following freezing are ubiquitous. While this loss of fundamental sperm viability due to lethal damage is certainly a setback, it is the sublethal freezing damage which spermatozoa experience that underlies the significant issues with fertility covered in section 1.4.2. Sublethal freezing damage has gained a considerable amount of attention in the previous few decades and as such, not all aspects of this phenomenon can be covered here in depth. Oxidative stress, loss of DNA integrity and alterations to mitochondrial function will be addressed in brief but readers are referred to more comprehensive reviews on the subject for further information (Ball 2008; Fraser *et al.* 2011; Amidi *et al.* 2016).

While seminal plasma confers significant protection against reactive oxygen species (section 1.3.2), cryopreservation exceeds this antioxidant capacity, with negative consequences. Spermatozoa produce significantly higher amounts of reactive oxygen species (ROS) both during cooling to 5°C (Wang *et al.* 1997; Santiani *et al.* 2014) and following cryopreservation (Chatterjee and Gagnon 2001; Kim *et al.* 2010) compared to fresh spermatozoa. Frozen

thawed spermatozoa produce nitrous oxide, hydrogen peroxide and oxygen free radicals in significantly greater quantities than fresh spermatozoa (Chatterjee and Gagnon 2001; Kim *et al.* 2010). Interestingly, frozen thawed spermatozoa are also far more sensitive to the damaging effects of ROS (Garg *et al.* 2009) and show significantly higher levels of lipid peroxidation than fresh spermatozoa (de Andrade *et al.* 2012). These outcomes are likely due to significant loss of antioxidant activity following cryopreservation, particularly that of superoxide dismutase and its cofactor glutathione (Alvarez and Storey 1992; Lasso *et al.* 1994; Bilodeau *et al.* 2000). While there is yet to be undeniable proof of causality, many authors have suggested links between increased intracellular ROS and DNA damage (Novotny *et al.* 2013). What is known for certain is that freezing induces significant DNA damage, resulting in alterations to chromatin structure (Hammadeh *et al.* 1999) and significant increases in both the proportion of spermatozoa with DNA damage (Peris *et al.* 2007; Kim *et al.* 2010; Partyka *et al.* 2010; Zribi *et al.* 2012) and its severity (Peris *et al.* 2007; Kim *et al.* 2010). Further, freezing also causes significant disruption to mitochondrial function, resulting in decreased respiration and mitochondrial membrane potential (Schober *et al.* 2007; Partyka *et al.* 2010). These changes represent significant alterations to sperm function, yet they are only the tip of the iceberg that is sublethal freezing damage.

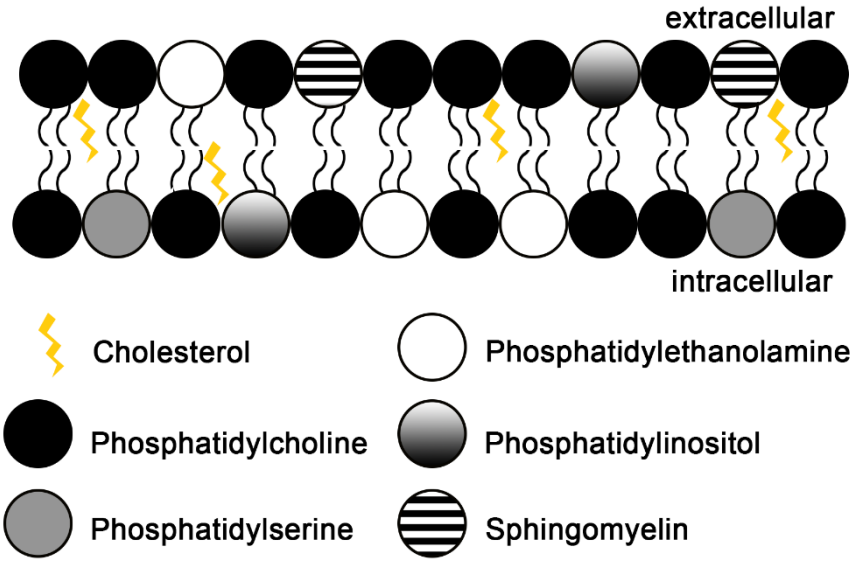

Figure 1.2 The sperm plasma membrane, showing asymmetric distribution of phospholipid species between the intra and extracellular leaflets

The sperm membrane is a typical phospholipid bilayer, consisting of a multitude of phospholipid species distributed asymmetrically between the intracellular and extracellular leaflets (Figure 1.2). In addition, sterols such as cholesterol and desmosterol are inserted into the hydrophobic interior of both leaflets. Cryopreservation causes significant changes to

sperm membrane phospholipids and sterols; following freezing, spermatozoa lose significant amounts of phosphatidylcholine, phosphatidylethanolamine and cholesterol (Hinkovska-Galcheva *et al.* 1989; Alvarez and Storey 1992; Chakrabarty *et al.* 2007; Kadirvel *et al.* 2009; Ushiyama *et al.* 2016). In addition, the amounts of a wide range of phospholipid fatty acid groups are altered (Alvarez and Storey 1992; Buhr *et al.* 1994; Schiller *et al.* 2000). As covered in section 1.3.1.2, the loss of cholesterol from the sperm membrane is a key event in capacitation, and thus this change alone represents an important functional alteration. The cause of these alterations to membrane structure during cryopreservation are yet to be resolved; some authors suggest that membrane lipids are shed to cope with freezing stress (Chakrabarty *et al.* 2007), via mechanisms such as lipid peroxidation (Alvarez and Storey 1992) and activation of hydrolytic enzymes (Schiller *et al.* 2000).

Further to overall loss of membrane phospholipids, freezing induced changes to their asymmetric distribution between the two leaflets of the membrane bilayer have also been documented. After either snap freezing or standard cryopreservation, extraction of membrane phospholipids revealed increased internalisation of phosphatidylserine and phosphatidylglycerol, and increased externalisation of phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylinositol (Hinkovska-Galcheva *et al.* 1989; Fang *et al.* 2016). However, looking at phospholipid translocation in individual spermatozoa presents slightly different results. Using the phosphatidylserine (PS) probe Annexin V, several authors found significantly higher proportions of live, frozen thawed spermatozoa demonstrating externalised PS compared to fresh spermatozoa (Duru *et al.* 2001a; Duru *et al.* 2001b; Schuffner *et al.* 2001; Anzar *et al.* 2002; Januskauskas *et al.* 2003; Guthrie and Welch 2005; Thomas *et al.* 2006; Vadnais and Althouse 2011; Kumar *et al.* 2016). Interestingly, others have found no differences between fresh and frozen spermatozoa in Annexin V binding (Glander and Schaller 1999; Peña *et al.* 2003; Kim *et al.* 2010), which may be due to the species studied or differences in semen processing. While the mechanism of changes to membrane phospholipid asymmetry has not been conclusively demonstrated, the similarity of these changes to those observed during capacitation make the involvement of phospholipid scramblase a distinct possibility (Gadella and Harrison 2002).

The sperm membrane is clearly disturbed by freezing, and as a result, proteins which are either intrinsic to or associated with the membrane undergo significant changes in their distribution and abundance. In early studies, changes to protein distribution were demonstrated using electron microscopy; a homogeneous distribution of ‘particles’ in the membrane shifted to particle clusters in frozen thawed spermatozoa (Ilieva *et al.* 1992). More recently, changes to protein distribution following freezing have been characterised using immunofluorescence, typically profiling the movement of a single protein (Miller *et al.* 2015;

Varghese *et al.* 2016). This redistribution does not appear to be consistent between different proteins, as SP22 staining was lost from the equatorial region (Miller *et al.* 2015), while HSP70 staining was lost from the apical region following freezing (Varghese *et al.* 2016). Changes to protein abundance as a result of freezing have been given far more attention. Performing SDS-PAGE of detergent membrane extracts, Ollero *et al.* (1998a) demonstrated the clear loss of 4 protein bands following sperm cryopreservation. Interestingly, these authors also suggested that 3 protein bands gained after freezing originated from egg yolk in the freezing diluent. Other studies have used western blotting to demonstrate the loss of proteins including HSP70 (Varghese *et al.* 2016), HSP90 (Zhang *et al.* 2015), P25b (Lessard *et al.* 2000) and CSNK2A2 (He *et al.* 2017). Interestingly, the only study to report an increase in a specific protein after freezing using western blotting was that of Ardon and Suarez (2013), which reported significantly higher levels of Binder of Sperm Proteins 1, 3 and 5 in frozen thawed compared to fresh bull spermatozoa, however this conflicts with other studies (Nauc and Manjunath 2000; Westfalewicz *et al.* 2015). While informative, these studies are limited by focusing on single proteins and lack the context provided by high throughput proteomic techniques.

While proteomic studies of spermatozoa exist in abundance, few quantitative proteomic comparisons of spermatozoa before and after cryopreservation have been published. Currently available are comparisons of human (Wang *et al.* 2014; Bogle *et al.* 2017), fish (Li *et al.* 2010; Zilli *et al.* 2014), rooster (Cheng *et al.* 2015), boar (Chen *et al.* 2014), bull (Westfalewicz *et al.* 2015) and ram (He *et al.* 2016) fresh and frozen thawed spermatozoa. The proteomic content of the extracellular medium following freezing of carp (Dietrich *et al.* 2015) and trout (Nynca *et al.* 2015a) spermatozoa has also been profiled to identify proteins lost due to 'shedding' or 'leakage'. Overall, the results of these studies have been highly variable, with the quantities of anywhere from 6 to nearly 100 proteins altered by cryopreservation. Few proteins were commonly identified across studies; those that were are detailed in Table 1.1. The majority of studies found that more proteins were significantly decreased following freezing than increased, with an average of 16 proteins decreasing (range 4-60) and an average of 12 proteins increasing (range 2-32). While some proteins identified in these studies are localised intracellularly, the vast majority are either secreted (e.g. PSPI (Chen *et al.* 2014), BSP1 (Westfalewicz *et al.* 2015), CLU (Westfalewicz *et al.* 2015; Bogle *et al.* 2017)), or have previously been identified in extracellular exosomes (e.g. TPI1 (Chen *et al.* 2014; Cheng *et al.* 2015), ANXA4 (Li *et al.* 2010; Bogle *et al.* 2017), VIM, UCHL3 (Wang *et al.* 2014)). Authors have reported that between 13 and 29% of proteins identified as altered in abundance after freezing were localised to the membrane.

Table 1.1 Proteins identified in multiple studies as significantly different in abundance in frozen thawed compared to fresh spermatozoa

Protein name	Gene symbol	Species	Function[a]	Proteomic method	Change in abundance[b]	References
Outer dense fiber of sperm tails 1/2	ODF1/ODF2	rooster, boar	Structural	2DE, MALDI-TOF[1] iTRAQ labelled LC-MS/MS[2]	increased	[1], [2]
ADAM metallopeptidase domain 2	ADAM2	ram, bull	Cell-cell interactions, sperm-oocyte binding	2DE, MALDI-TOF/TOF[3,4]	Increased	[3], [4]
ATP synthase subunit beta	ATP5B	human, boar	Oxidative phosphorylation	iTRAQ labelled LC-MS/MS[2] TMT 10plex labelled LC-MS/MS[5]	increased	[2], [5]
Adenosylhomocysteinase	AHCY	carp*, trout*	Intermediate metabolism	2DE, MALDI-TOF/TOF[6,7,8]	decreased	[6], [7], [8]
Cofilin 2	CFL2	carp*, trout*	Actin turnover	2DE, MALDI-TOF/TOF[6,7,8]	decreased	[6], [7], [8]
Heat shock protein 90 beta family member 1	HSP90B1	carp*, trout*	Chaperone	2DE, MALDI-TOF/TOF[6,7]	decreased	[6], [7]
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	sea bream, rooster	Glycolysis, nuclear functions	2DE, MALDI-TOF[1] 2DE, LC-MS/MS[9]	decreased	[1], [9]
Cytochrome B5 reductase 2	CYB5R2	human	Fatty acid and sterol modification	TMT 10plex labelled LC-MS/MS[5] 2DE, MALDI-TOF[10]	decreased	[5], [10]
Calmodulin 1	CALM1	human, trout*	Calcium dependent enzyme and transporter control	TMT 10plex labelled LC-MS/MS[5] 2DE, MALDI-TOF/TOF[7]	decreased	[5], [7]
Calreticulin 3B	CALR3B	carp*, trout*	Calcium binding chaperone	2DE, MALDI-TOF/TOF[6,7]	decreased	[6], [7]
Lactate dehydrogenase B	LDHB	rooster, carp*	Glycolysis	2DE, MALDI-TOF[1] 2DE, MALDI-TOF/TOF[6,8]	decreased	[1], [6], [8]
NME/NM23 family member 7	NME7	human, carp*	Structural, nucleoside kinase	TMT 10plex labelled LC-MS/MS[5] 2DE, MALDI-TOF/TOF[6]	decreased	[5], [6]
Transaldolase 1	TALDO1	carp*	Lipid metabolism, ROS protection	2DE, MALDI-TOF/TOF[6,8]	decreased	[6], [8]
Valosin containing protein	VCP	carp*, trout*	Structural	2DE, MALDI-TOF/TOF[6,7]	decreased	[6], [7]
Aldo-keto reductase family 1 member B1	AKR1B1	bull, carp*	NADPH dependent aldehyde reduction	2DE, MALDI-TOF/TOF[4,6]	decreased	[4], [6]
Actin beta	ACTB	carp*, trout*	Structural, motility	2DE, MALDI-TOF/TOF[6,7]	decreased	[6], [7]
Phosphoglycerate kinase 1	PGK1	human, carp*	Glycolysis	TMT 10plex labelled LC-MS/MS[5] 2DE, MALDI-TOF/TOF[6,8]	unclear	[5], [6], [8]
Enolase 1	ENO1	human, rooster, ram, carp*, trout*	Glycolysis, hypoxia tolerance	2DE, MALDI-TOF[1,10] 2DE, MALDI-TOF/TOF[3,6,7]	unclear	[1], [3], [6], [7], [10]
Superoxide dismutase 1	SOD1	boar, rooster, sea bream, trout*	ROS protection	2DE, MALDI-TOF[1] iTRAQ labelled LC-MS/MS[2] 2DE, MALDI-TOF/TOF[7]	unclear	[1], [2], [7], [9]

				2DE, LC-MS/MS[9]		
Triosephosphate isomerase 1	TPI1	boar, rooster, human, trout*	Glycolysis	2DE, MALDI-TOF[1] iTRAQ labelled LC-MS/MS[2] TMT 10plex labelled LC-MS/MS[5] 2DE, MALDI-TOF/TOF[6]	unclear	[1], [2], [5], [6]
Sperm equatorial segment protein 1	SPESP1	boar, human	Zona binding	iTRAQ labelled LC-MS/MS[2] TMT 10plex labelled LC-MS/MS[5]	unclear	[2], [5]
Acrosin	ACR	rooster, human	Acrosomal protease	2DE, MALDI-TOF[1] TMT 10plex labelled LC-MS/MS[5]	unclear	[1], [5]
Clusterin	CLU	bull, human	Extracellular chaperone	2DE, MALDI-TOF/TOF[4] TMT 10plex labelled LC-MS/MS[5]	unclear	[4], [5]
Creatine kinase B	CKB	rooster, human, trout*, carp*	Energy transduction and homeostasis	2DE, MALDI-TOF[1] TMT 10plex labelled LC-MS/MS[5] 2DE, MALDI-TOF/TOF[6,7]	unclear	[1], [5], [6], [7]
Annexin A4	ANXA4	human, carp	Calcium dependent phospholipid binding	TMT 10plex labelled LC-MS/MS[5] 2DE, MALDI-TOF/TOF[8]	unclear	[5], [8]
Pyruvate dehydrogenase beta	PDHB	human, ram	Glycolysis	2DE, MALDI-TOF/TOF[3] TMT 10plex labelled LC-MS/MS[5]	unclear	[5], [3]

[a] function based on annotations in Gene Cards database (www.genecards.org)

[b] 'unclear' – protein was identified as increased and decreased in abundance in different studies

*identified in extracellular fluid after freeze thawing, indicating loss from spermatozoa

[1] Cheng *et al.* 2015, [2] Chen *et al.* 2014, [3] He *et al.* 2015, [4] Westfalewicz *et al.* 2015, [5] Bogle *et al.* 2017, [6] Dietrich *et al.* 2015, [7] Nynca *et al.* 2015, [8] Li *et al.* 2010, [9] Zilli *et al.* 2014, [10] Wang *et al.* 2014.

Looking at the functions of some individual proteins begins to highlight important alterations caused by cryopreservation. Several authors reported decreases in proteins involved in antioxidant pathways (VIM (Wang *et al.* 2014), CYBR52 (Wang *et al.* 2014; Bogle *et al.* 2017), SOD1 (Cheng *et al.* 2015), TALDO1 (Li *et al.* 2010)), maintenance of plasma membrane integrity (TXNDC2, GSTM3 (Bogle *et al.* 2017)) and important structural proteins (CFAP45 (Bogle *et al.* 2017), tubulins (Cheng *et al.* 2015), TEKT1 (Wang *et al.* 2014)). Further, there were decreases reported for important capacitation related proteins including ROPN1 (Bogle *et al.* 2017), BSP1 and 5 (Westfalewicz *et al.* 2015), CALM1 (Nynca *et al.* 2015a; Bogle *et al.* 2017), and the known 'de-capacitation' protein PSP1 (Chen *et al.* 2014), as well as proteins with involvement in sperm-oocyte binding (SPACA3 (Bogle *et al.* 2017), AWN1 (Chen *et al.* 2014), ACRBP (He *et al.* 2016)). On the other hand, proteins which increased after cryopreservation included markers of apoptosis (IL4I1 (Chen *et al.* 2014)), proteins which promote phagocytosis (MFGE8 (Chen *et al.* 2014)), some antioxidants (GPX4 (Chen *et al.* 2014), PKM2 (Wang *et al.* 2014)) and proteins which promote tyrosine phosphorylation (PRKACA, ROPN1L (He *et al.* 2016)). Somewhat unexpectedly, several authors found that proteins directly involved in oocyte binding also increased after cryopreservation (ZPBP (Chen *et al.* 2014), ADAM2 (Westfalewicz *et al.* 2015; He *et al.* 2016), SPESP1, SPACA1 (He *et al.* 2016), PSMA1 (Wang *et al.* 2014)). While earlier work suggested that membrane bound proteins increased after freezing may originate from egg yolk in freezing media (Ollero *et al.* 1998a), this has not been investigated by any proteomic studies to date. Further, while the studies discussed here have provided valuable information about protein based changes to spermatozoa during freezing, most have employed less sensitive methods for distinguishing protein abundance, particularly densitometry of two dimensional electrophoresis gels. In order to comprehensively profile alterations to sperm proteins during cryopreservation, investigations into the proteins contributed by egg yolk and comparisons of whole sperm lysates using highly sensitive proteomic techniques including labelled (e.g. iTRAQ) and non-labelled (e.g. SWATH) quantification are required.

Glycan moieties are one component of the sperm surface which has received far less attention than others. While there has been significant profiling of surface sugars during epididymal maturation and capacitation, little is known about the effects of cryopreservation on carbohydrate elements of the sperm membrane. Talaei *et al.* (2010) reported that frozen thawed human spermatozoa bound significantly lower amounts of lectins specific for galactose and N-acetylgalactosamine than fresh spermatozoa. However, this study used fixed smears and did not differentiate on the basis of viability, thus it may not accurately reflect sublethal alterations. Peláez *et al.* (2011) on the other hand, demonstrated by lectin based flow cytometric analysis that cryopreservation significantly increased the amounts of N-

acetyllactosamine, N-acetylglucosamine and galactose on the surface of viable rooster spermatozoa. While evidence for changes to glycans is limited, the effects of ROS on cell glycocalyces in pathological states suggests that cryopreservation, and particularly freezing induced ROS production, may significantly alter the sperm glycocalyx. Ischemia-reperfusion injury (IRI) is associated with shedding of glycoconjugates from the surface of vascular endothelial cells (Rehm *et al.* 2007). Significant amounts of ROS are produced during IRI, and this has been directly related to endothelial cell glycocalyx damage (Constantinescu *et al.* 2001; Rubio-Gayosso *et al.* 2006; Singh *et al.* 2013). Further, damage to the endothelial cell glycocalyx has been successfully prevented using antioxidants (Vink *et al.* 2000; Rubio-Gayosso *et al.* 2006). While no such association has been investigated in spermatozoa, it presents a very interesting possibility. Clearly, modification of the sperm glycocalyx by cryopreservation requires further investigation and may provide important insights into sublethal freezing damage and mechanisms to prevent it.

In addition to changes to membrane phospholipids, proteins and sugars, cryopreservation results in a collection of changes often termed ‘cryocapacitation’. These changes mirror hallmarks of the normal capacitation process, but the pathways and signalling cascades involved may reflect cellular damage rather than normal physiological progression. Calcium dependent fluorescent chlortetracycline (CTC) staining has been employed for several decades to determine percentages of non-capacitated, capacitated and acrosome reacted spermatozoa, based on the pattern of fluorescence displayed (Saling and Storey 1979; Ward and Storey 1984). A significant increase in the percentage of spermatozoa displaying the capacitated CTC staining pattern after both cooling (Fuller and Whittingham 1997; Maxwell and Johnson 1997) and freeze thawing has been demonstrated in a wide range of species (ram (Pérez *et al.* 1996; Gillan *et al.* 1997), bull (Cormier *et al.* 1997; Cormier and Bailey 2003), stallion (Schembri *et al.* 2002), boar (Maxwell and Johnson 1997; Satorre *et al.* 2007), buffalo (Kadirvel *et al.* 2009; Kadirvel *et al.* 2011) and dog (Burgess *et al.* 2012)). An increase in membrane lipid disorder (i.e. decreased homogeneity of membrane lipid packing) is a key capacitation event (Gadella and Harrison 2000; Fàbrega *et al.* 2012a), particularly increased by cyclic AMP upregulation (Leahy *et al.* 2016). Similar significant increases in membrane lipid disorder of live spermatozoa have been observed following freezing of stallion (Thomas *et al.* 2006; Yeste *et al.* 2015), boar (Guthrie and Welch 2005; Vadnais and Althouse 2011), buffalo (Kadirvel *et al.* 2009) and trout semen (Purdy *et al.* 2016). Phosphorylation of tyrosine residues is a further important alternation which is characteristic of capacitation (Visconti *et al.* 1995a), regulated by a cyclic AMP dependent PKA pathway (Visconti *et al.* 1995b). Freezing has been shown to cause either an overall increase in tyrosine phosphorylation (Cormier and Bailey 2003; Kadirvel *et al.* 2011; Kumaresan *et al.* 2011; Vadnais and Althouse 2011; de Andrade

et al. 2012; Kumaresan *et al.* 2012; Wang *et al.* 2014) or tyrosine phosphorylation of particular protein bands (Pérez-Pé *et al.* 2002; Cormier and Bailey 2003; Satorre *et al.* 2007; Kumaresan *et al.* 2011; Vadnais and Althouse 2011; Kumaresan *et al.* 2012). The capacitation process culminates in two fundamental events; the acrosome reaction and fertilisation itself. Frozen thawed spermatozoa are significantly more responsive to promotion of the acrosome reaction by calcium ionophores compared to their fresh counterparts (Cormier and Bailey 2003; Vadnais and Althouse 2011). Further, cryopreserved spermatozoa are typically able to bind and fertilise oocytes in vitro at an earlier stage of co-incubation than fresh spermatozoa (Byers *et al.* 1989; Garde *et al.* 1993; Watson 1995; Fuller and Whittingham 1997; De los Reyes *et al.* 2009; Palomino and De los Reyes 2009; Clulow *et al.* 2010) or without further capacitation stimulants (Cormier *et al.* 1997), suggesting that frozen thawed spermatozoa are either partially or fully capacitated immediately post thaw. Overall, these freezing induced changes fundamentally alter the function of spermatozoa by propelling them to an advanced stage of maturity before the appropriate time, which may be a key contributor to the poor fertility of frozen thawed spermatozoa.

1.4.2. Effects of cryopreservation on fertility

The intended outcome of commercial semen cryopreservation is typically future use in advanced reproductive technologies. As such, the maintenance of fertilising capacity throughout the cryopreservation process is of utmost importance. However, the ability to fertilise does not necessarily define the fertility of a spermatozoon. Hence while cryopreservation may result in spermatozoa that are capable of fertilisation, the resulting fertility outcomes following insemination may still be quite poor. Insemination of frozen thawed semen has been shown to result in significantly fewer successful fertilisations (Lightfoot and Salamon 1970) and lower pregnancy rates than fresh, extended semen in sheep (Armstrong and Evans 1984; Maxwell and Hewitt 1986; Eppleston *et al.* 1994; Donovan *et al.* 2004), cats (Lambo *et al.* 2012) and dogs (Niżański 2006). This is the case even when inseminating the same number of total (Maxwell and Hewitt 1986; Donovan *et al.* 2004) or motile spermatozoa (Eppleston *et al.* 1994; Niżański 2006; Lambo *et al.* 2012). Interestingly however, this disparity in the pregnancy rates achieved by fresh and frozen thawed spermatozoa largely appears after inseminations into the lower female reproductive tract. In contrast, inseminations of fresh and frozen thawed spermatozoa directly into the uterus or oviduct appear to achieve similar pregnancy rates, at least in sheep (Lightfoot and Salamon 1970; Armstrong and Evans 1984; Maxwell *et al.* 1993).

These findings suggest that frozen thawed spermatozoa retain their fertilising capacity, evidenced further by equivalent rates of IVF (Gillan *et al.* 1997; Gomez *et al.* 1997) and ICSI success using fresh and frozen thawed spermatozoa (Ulug *et al.* 2005; Ohlander *et al.* 2014).

Thus rather than fertilisation itself, cryopreservation appears to have significant impacts on the ability of spermatozoa to transit the female tract and reach the site of fertilisation. From the results of many field studies, there is a clear relationship between proximity of semen deposition to the site of fertilisation and the insemination success of frozen thawed semen. This has been demonstrated most thoroughly in sheep, where laparoscopic intrauterine insemination consistently results in significantly higher pregnancy rates than cervical insemination when frozen thawed semen is used (Salamon and Lightfoot 1967; Gustafsson 1978; Armstrong and Evans 1984; Maxwell and Hewitt 1986; Maxwell *et al.* 1999; King *et al.* 2004; Fair *et al.* 2005; Leahy *et al.* 2010a; Prado *et al.* 2013; Masoudi *et al.* 2017). Similar results have also been reported in cats (Villaverde *et al.* 2009), dogs (Linde-Forsberg *et al.* 1999), horses (Govaere *et al.* 2014) and goats (Leboeuf *et al.* 2000; Salvador *et al.* 2005). Simply introducing an inseminating pipette further into the cervical canal in a 'deep cervical insemination' is enough to significantly improve pregnancy rates (Salamon and Lightfoot 1967; Eppleston *et al.* 1994; Salvador *et al.* 2005; Richardson *et al.* 2012). Thus, in stark contrast to the beneficial effects of seminal plasma (section 1.3.2), there is a clear impairment of cervical transit in cryopreserved spermatozoa, leading to significant reductions in pregnancy rates when employing cervical insemination.

1.4.3. Mechanisms of fertility failure in cryopreserved spermatozoa

The reasons why frozen thawed spermatozoa often perform poorly when inseminated may appear obvious on the surface, particularly when considering the lethality of cryopreservation. In almost all species studied, cryopreservation results in significant losses of motility and viability. Yet these obvious changes are not enough to explain fertility losses. While post thaw motility has certainly been correlated to in vivo fertility (Gillan *et al.* 2008; Furstoss *et al.* 2010), it does not completely account for it. Further, frozen thawed spermatozoa require not only more total, but also more motile spermatozoa in an inseminate in order to achieve similar pregnancy rates to fresh spermatozoa (Shannon and Vishwanath 1995; Bathgate *et al.* 2008). Such findings imply that the mechanisms behind in vivo failures of frozen thawed spermatozoa are not limited to decreases in motility (or the number of motile spermatozoa), but rather a host of complex and interacting factors.

Several studies have reported observing significantly lower numbers of frozen thawed spermatozoa at the utero-tubal junction (UTJ) and within the oviduct compared to fresh spermatozoa following cervical insemination (Salamon and Lightfoot 1967; Lightfoot and Salamon 1970; Pursel *et al.* 1978; Abad *et al.* 2007b). One of the factors which may be responsible for this is the rapid loss of frozen thawed spermatozoa from the female tract. It has been shown in sheep that only 30 minutes after cervical insemination with equal numbers of motile spermatozoa, there are significantly fewer frozen thawed than fresh spermatozoa

present at the cervix (Lightfoot and Salamon 1970). In addition, even after uterine insemination, the number of spermatozoa remaining in the tract decreased at a faster rate for frozen thawed compared to fresh spermatozoa (Gillan *et al.* 2000). This significant decline in sperm numbers may be as a result of a shorter lifespan leading to increased 'back flow' and expulsion of frozen thawed spermatozoa from the vagina, but this remains speculation.

Another potential explanation for the rapid loss of frozen thawed spermatozoa from the female tract is an increase in the number of spermatozoa being phagocytosed by immune cells involved in the inflammatory response to insemination (section 1.3.3). Studies on red blood cells have effectively demonstrated that alterations to membrane phospholipids, particularly loss of asymmetry, external exposure of phosphatidylserine and increased membrane lipid disorder, significantly increase phagocytosis by macrophages (Tanaka and Schroit 1983; McEvoy *et al.* 1986). Similar significant increases in phagocytosis have been shown in response to cells with high intracellular calcium (Miki *et al.* 2013) and alterations to surface sugars (Doolittle *et al.* 1983; Schauer *et al.* 1984; Fischer *et al.* 1991; Crestani *et al.* 1993; Sheth *et al.* 2011; Paris *et al.* 2012). As covered in section 1.4.1, cryopreservation of spermatozoa causes significant changes to membrane phospholipids, heightens membrane disorder, increases intracellular calcium concentrations (McLaughlin and Ford 1994; Kadirvel *et al.* 2009), and it is speculated that freezing may significantly alter the sperm glycocalyx. While it is conceivable that these significant cryopreservation induced alterations may lead to increased phagocytic targeting of frozen thawed compared to fresh spermatozoa, there is no evidence currently available to support this hypothesis.

Further to potential immune barriers, the mucus produced by the cervix offers a significant physical barrier to sperm progression into the upper reproductive tract. Interestingly, the ability of frozen thawed spermatozoa to penetrate either natural cervical mucus or a polyacrylamide substitute was significantly higher for bulls with high field fertility (Hamano *et al.* 2001; Taş *et al.* 2007a). Similarly, frozen thawed ram spermatozoa showed significantly better penetration in mucus from sheep breeds well known for high pregnancy rates following cervical insemination of frozen thawed semen (Richardson *et al.* 2011). Thus the ability to penetrate cervical mucus is clearly a key factor determining in vivo success. Yet despite maintaining high progressive motility, frozen thawed spermatozoa demonstrate poor mucus penetration in vitro (Tollner *et al.* 2011). Thus, as observed in studies on the effects of seminal plasma (section 1.3.2), the ability to penetrate mucus is not solely a function of motility (Rickard *et al.* 2014). This suggests that sublethal freezing damage has functional consequences beyond a loss of motility which may lead to reduced mucus penetration, limiting progression of frozen thawed spermatozoa to the upper reaches of the female reproductive tract.

Once spermatozoa have gained access to the upper reproductive tract, they quickly enter the oviduct and form a reservoir by binding to receptors on oviductal epithelial cells (Suarez 1998; Suarez 2001). Whilst bound in this reservoir, spermatozoa are maintained with low levels of intracellular calcium (Dobrinski *et al.* 1996), in a viable and motile state for up to several days (Kölle 2015). While an extended interval between insemination and ovulation tends to negatively impact fertility (Maxwell *et al.* 1983; Waberski *et al.* 1994), the formation of this reservoir gives spermatozoa the best chance of successful fertilisation. The oviductal reservoir maintains viability and manipulates sperm capacitation in order to provide a sustained release of cells, ensuring that viable, capacitated spermatozoa are available at the correct time to achieve fertilisation (Töpfer-Petersen *et al.* 2002). As discussed earlier in this section, fewer frozen thawed spermatozoa tend to reach the oviduct than fresh spermatozoa, and those that do are substantially less successful in binding to oviductal epithelial cells (OECs) (Dobrinski *et al.* 1995; Goldman *et al.* 1998; Ellington *et al.* 1999; Burgess *et al.* 2012), overall forming a smaller oviductal reservoir (Abad *et al.* 2007b). Interestingly, frozen thawed ram spermatozoa show a different OEC binding time course to fresh spermatozoa (Gillan *et al.* 2001). Frozen thawed spermatozoa initially bind to OECs in higher numbers than fresh spermatozoa, but are rapidly released after 2 hours. This is a possible consequence of the formational changes discussed in section 1.4.1. This suggests that while frozen thawed spermatozoa may still be able to form an oviductal reservoir, it is generally smaller than that of fresh spermatozoa, and premature, en masse release of frozen thawed spermatozoa may cause them to miss the appropriate window for fertilisation.

Artificial insemination is largely performed on animals in a synchronised oestrus, and timed to coincide with ovulation in order to limit the ‘waiting time’ for both spermatozoa and oocytes. However, response to oestrus synchronisation can vary, with an individual female’s time of ovulation spread anywhere over a 24 to 72 hour window depending on the synchronisation protocol (Ali *et al.* 2009). Thus there is significant potential for spermatozoa to be kept waiting for the arrival of an oocyte. This appears to be a sizable problem for frozen thawed spermatozoa, as pregnancy rates decline significantly with an increasing temporal gap between insemination and ovulation (Maxwell *et al.* 1983; Waberski *et al.* 1994; Spencer *et al.* 2010; Ringwelski *et al.* 2013; Richardson *et al.* 2017). A similar effect has been observed by pre incubating frozen thawed spermatozoa prior to in vitro fertilisation (Garde *et al.* 1993; Gillan *et al.* 1997). The reasons for poor longevity of cryopreserved spermatozoa likely relate back to important functional deficits such as increased ROS production combined with lowered antioxidant capacity (covered in section 1.4.1), a compromised response to osmotic stress (Khan and Ijaz 2008; Pinto and Kozink 2008) and poor formation of the oviductal sperm reservoir as described above. This poor longevity may be exacerbated by introduction lower

in the female reproductive tract, and compounded by low numbers of spermatozoa available for fertilisation due to rapid loss of progressive motility (Shannon and Vishwanath 1995; Batista *et al.* 2012).

A further issue with an extended time period from insemination to ovulation is that cryopreserved spermatozoa are more likely to be capacitated before an oocyte is available for fertilisation. The presence of capacitated spermatozoa in frozen thawed semen has been correlated to poor field fertility (Thundathil *et al.* 1999; Kuroda *et al.* 2007) and thus obviously presents a significant problem. The issue is two-fold; not only are more spermatozoa already in a capacitated state immediately post thaw, but frozen thawed spermatozoa capacitate at a faster rate than fresh spermatozoa (Pérez *et al.* 1996; Rota *et al.* 1999; Gillan *et al.* 2000; Suzuki *et al.* 2002). This is likely due to an increased sensitivity to capacitating agents (Pommer *et al.* 2003), and could also result from an altered membrane cholesterol/phospholipid ratio, which determines the speed of capacitation (Davis 1981). Loss of 'de-capacitating' seminal plasma proteins (section 1.3.1.2) during cryopreservation could also be a significant contributor to this phenomenon, but this remains unsubstantiated. These factors are once again compounded by insemination lower in the female tract, which reduces the time required for capacitation (Hunter and Rodriguez-Martinez 2004).

The shortcomings of frozen thawed spermatozoa in the female tract are numerous and complex; some of the processes which may be affected are not well understood themselves. While some spermatozoa in an inseminate of frozen thawed semen are clearly capable of achieving fertilisation, attempts to improve the in vivo fertility of cryopreserved spermatozoa are well warranted. From the angle of cryopreservation, this task largely centres around improving post thaw outcomes by minimising the lethal and sublethal damage discussed in section 1.4.1. In addition, any factors which could improve the motility, mucus penetration, OEC binding or longevity of frozen thawed spermatozoa, or limit freezing induced capacitation, have real potential to improve in vivo outcomes of inseminations using cryopreserved semen.

1.4.4. Using proteins to prevent and reverse cryopreservation damage

Despite its composition and the functions ascribed to seminal plasma, it is not required for successful freezing of spermatozoa. Processing of ejaculates to remove seminal plasma prior to freezing improves osmotic resilience and acrosome integrity of stallion spermatozoa (Barrier-Battut *et al.* 2013) and has no apparent negative effects on the motility, viability or subsequent fertilising ability of ruminant (Azerêdo *et al.* 2001; Ledesma *et al.* 2015) or macaque spermatozoa (Yang *et al.* 2011). While pre freeze washing does not affect the post thaw proportion of capacitated ram spermatozoa (Ledesma *et al.* 2015), it does increase cryopreservation related protein tyrosine phosphorylation in boar semen (Okazaki *et al.* 2009)

and spontaneous acrosome reactions in macaque spermatozoa (Yang *et al.* 2011). Overall, these results seem to suggest that the presence of seminal plasma is largely superfluous to freezing outcome. However, a large body of evidence has accumulated regarding the effects of seminal plasma supplementation on post thaw function in a range of species, and particularly in sheep (Table 1.2).

As can be seen in Table 1.2, the majority of studies report significant benefits of seminal plasma addition on post thaw motility, viability and acrosome integrity (Maxwell *et al.* 1996; Ben *et al.* 1997; Ollero *et al.* 1998b; de Graaf *et al.* 2007; Ghaoui *et al.* 2007; Leahy *et al.* 2009; Okazaki *et al.* 2009; Garcia *et al.* 2010; Leahy *et al.* 2010b; Bernardini *et al.* 2011; de Andrade *et al.* 2011; Okazaki *et al.* 2012; cold shock - Church and Graves 1976; Colás *et al.* 2009). In addition, seminal plasma has been shown to prevent and revert indicators of capacitation, increase IVF success rates and improve longevity of spermatozoa in vitro (Ben *et al.* 1997; Maxwell *et al.* 1999; Vadnais *et al.* 2005; Ghaoui *et al.* 2007; Domínguez *et al.* 2008; Okazaki *et al.* 2009; de Andrade *et al.* 2012; Gómez-Fernández *et al.* 2012; Okazaki *et al.* 2012; Martins *et al.* 2013; Casao *et al.* 2017; cold shock - Pérez-Pé *et al.* 2002). Interestingly, while there are no reported negative effects of adding seminal plasma to frozen thawed spermatozoa on subsequent in vivo fertility, studies have demonstrated that seminal plasma supplementation significantly increases (Maxwell *et al.* 1999; Nöthling *et al.* 2005; Okazaki *et al.* 2009; López-Pérez and Pérez-Clariget 2012; Okazaki *et al.* 2012), or has no effect on pregnancy rates following artificial insemination (Gunay *et al.* 2006; Abad *et al.* 2007a; O'Meara *et al.* 2007; Garcia *et al.* 2010; Leahy *et al.* 2010a; Prado *et al.* 2013). Thus while on one hand seminal plasma appears to be a highly beneficial supplement to improve the post thaw function of cryopreserved spermatozoa, the results both in vitro and, particularly in vivo, are inconsistent. The reasons for these inconsistencies are likely largely due to differences in methodology, including the final percentage of seminal plasma used for supplementation (ranging from 3% to 80%), the point of addition of seminal plasma during semen processing (pre or post thaw) and the source of seminal plasma (homologous (same individual/species) or heterologous (different individual/species)).

Variation in seminal plasma may be a considerable factor in the inconsistency of studies investigating its effects on cryopreserved sperm function. Seminal plasma content varies considerably between seasons, as does its protective effect (Domínguez *et al.* 2008; Leahy *et al.* 2010b). In addition, the individuals used for seminal plasma collection may play a significant role. Studies in the ram, stallion, boar and macaque indicate that seminal plasma from males with superior post thaw sperm function is able to 'rescue' motility, viability, acrosome integrity and oocyte penetration ability of spermatozoa from males with poor freezing outcomes (Aurich *et al.* 1996; Hernández *et al.* 2007; Yang *et al.* 2011; Rickard *et al.* 2016).

Table 1.2 Effects of the addition of seminal plasma on in vitro and in vivo characteristics of frozen thawed spermatozoa from various species

	POSITIVE EFFECT		NO EFFECT		NEGATIVE EFFECT	
Motility	Ram	Maxwell *et al.* 1996, Leahy *et al.* 2009, Ghaoui *et al.* 2007, de Graaf *et al.* 2007, Ollero *et al.* 1998	Ram	Gunay *et al.* 2006	Ram	Martins *et al.* 2016, Mataveia *et al.* 2010
	Boar	Maxwell *et al.* 1996, Garcia *et al.* 2010, Okazaki *et al.* 2012	Stallion	Moore *et al.* 2005	Donkey	Sabatini *et al.* 2014
	Human	Ben *et al.* 1997				
Viability	Ram	Maxwell *et al.* 1996, Leahy *et al.* 2009, Leahy *et al.* 2010b, Ollero *et al.* 1998, Bernardini *et al.* 2011, Colás *et al.* 2009 (cold shock)	Ram	Gunay *et al.* 2006	Ram	Martins *et al.* 2016
	Boar	Maxwell *et al.* 1996, Garcia *et al.* 2010	Stallion	Moore *et al.* 2005	Donkey	Sabatini *et al.* 2014
Acrosome integrity	Ram	de Graaf *et al.* 2007	Ram	Gunay *et al.* 2006, Rovegno *et al.* 2013	Ram	Martins *et al.* 2016
	Boar	Okazaki *et al.* 2012, Okazaki *et al.* 2009				
	Bull	Church and Graves 1976 (cold shock)	Stallion	Moore *et al.* 2005, Heise *et al.* 2011	Boar	Fernández-Gago *et al.* 2013
	Stallion	de Andrade *et al.* 2011				
Mitochondrial function	Ram	de Graaf *et al.* 2007			Ram	Martins *et al.* 2016
	Boar	Gómez-Fernández *et al.* 2012				
Osmotic tolerance	Ram	Martins *et al.* 2013, Domínguez *et al.* 2008			Donkey	Sabatini *et al.* 2014
Mucus penetration	Ram	Maxwell *et al.* 1999				
Minimisation or reversal of capacitation	Ram	Maxwell *et al.* 1999, Casao *et al.* 2017, Pérez-Pé *et al.* 2002 (cold shock)				
	Boar	Vadnais 2005, Suzuki 2002, Okazaki *et al.* 2009, Okazaki *et al.* 2012				
	Stallion	de Andrade *et al.* 2012				
IVF rate	Ram	Maxwell *et al.* 1999, Ghaoui *et al.* 2007, Casao *et al.* 2017				
Maintenance of motility/viability over time	Ram	Domínguez *et al.* 2008, Martins *et al.* 2013, Bernardini *et al.* 2011				
	Boar	Gómez-Fernández *et al.* 2012				
	Human	Ben *et al.* 1997				
Pregnancy rate	Ram	Maxwell *et al.* 1999, López-Pérez *et al.* 2012	Ram	Leahy *et al.* 2010, Prado *et al.* 2013, Gunay *et al.* 2006, O'Meara *et al.* 2007		
	Boar	Okazaki *et al.* 2009, Okazaki *et al.* 2012	Boar	Garcia *et al.* 2010, Abad *et al.* 2007		
	Dog	Nöthling *et al.* 2005				

In both the ram (Goularte *et al.* 2014; Rickard *et al.* 2015) and boar (Corcini *et al.* 2012), improved freezing success has been related to the presence or absence of particular proteins in seminal plasma. Overall, these results suggest that select proteins within seminal plasma may be responsible for its observed cryoprotective effects. Identification, isolation and subsequent application of these individual proteins may lend more consistent benefits to cryopreserved spermatozoa than crude seminal plasma.

Investigations into the cryoprotective effects of one particular subset of proteins from ram seminal plasma has produced promising results. Barrios *et al.* (2000) tested the effects of 8 different fractions of ram seminal plasma, separated by size exclusion chromatography, on the reversal of cold shock induced membrane damage. Interestingly, a single fraction (F6) had the same effect as whole seminal plasma, reversing membrane permeability to propidium iodide and membrane structural damage as visualised by scanning electron microscopy. This suggested that individual proteins within this fraction of seminal plasma had significant cryoprotective effects, and this was later demonstrated (Barrios *et al.* 2005). Two proteins from F6, named P14 and P20 in relation to their respective molecular weights, maintained significantly higher sperm viability under cold shock than an untreated control. Only P20 was as effective as the whole F6 fraction, suggesting a superior protective effect compared to P14. The same authors localised the binding of these proteins to the sperm head, acrosome and tail, and suggested that their protective effects may involve decapacitation due to their release from the sperm surface under capacitating conditions. P14 and P20 were later identified as RSVP14 and RSVP20 (Jobim *et al.* 2005; Cardozo *et al.* 2008), and have since been reclassified as Binder of Sperm Proteins (BSP) 1 and 5 respectively (Serrano *et al.* 2015).

Since these fundamental investigations into cold shock, BSPs have surfaced in several studies examining ram sperm function following cryopreservation. Ram seminal plasma collected during autumn contains significantly higher concentrations of BSPs than seminal plasma from other seasons, and improves the motility of frozen thawed ram spermatozoa (Domínguez *et al.* 2008). In addition, semen collected by electroejaculation also has higher concentrations of BSPs (Ledesma *et al.* 2014), and significantly better post thaw viability, acrosome integrity and IVF success than semen collected by artificial vagina (Ledesma *et al.* 2015). Further, seminal plasma isolated from electroejaculated semen also improves viability and reduces both membrane lipid disorder and tyrosine phosphorylation when added to frozen thawed spermatozoa (Ledesma *et al.* 2016). Similar improvements in motility and sperm ultrastructure have been observed when cryopreserved spermatozoa were supplemented with a mixture of membrane binding proteins, including BSP5, isolated from seminal plasma (Bernardini *et al.* 2011). These significant improvements in the survival and function of frozen thawed ram spermatozoa may be a reflection of the presence of BSPs. However, the direct and isolated

cryoprotective effects of BSP1 and BSP5 have not been examined. Interestingly, the theory of a cryoprotective role for BSPs in the ram is in polar contrast to the in vitro effects of BSPs on bull spermatozoa (Leahy and de Graaf 2012), and could reflect differences in their physiological actions in these two species (section 1.3.1.1). The protective effects of egg yolk and milk based diluents are believed to be due to the BSP sequestering properties of these biological substances (Bergeron and Manjunath 2006). In addition, direct sequestering of BSPs by specific antibodies prior to freezing has been shown to improve post thaw motility and acrosome integrity of bull spermatozoa, and minimise indicators of cryocapacitation (Srivastava *et al.* 2013). This discrepancy between the proposed effects of BSPs on spermatozoa of two closely related species is interesting, and deserves further attention. Overall, an in depth investigation into the effects of BSPs on ram spermatozoa, particularly in the context of industry standard freezing, is warranted to confirm their potential usefulness as a cryoprotective supplement.

Apart from those present in seminal plasma, a range of proteins have been tested for their potential to protect spermatozoa during cryopreservation. These include proteins isolated from oviductal fluid (Abe *et al.* 1995; Imam *et al.* 2008), milk (Gonçalves *et al.* 2008b), silk worm cocoon (Kumar *et al.* 2015) and fish blood plasma (Prathalingam *et al.* 2006; Beirão *et al.* 2011; Nishijima *et al.* 2014; Zilli *et al.* 2014), as well as a range of recombinant proteins (Holt *et al.* 2015; Dalal *et al.* 2016; Qadeer *et al.* 2016; Selvaraju *et al.* 2016; Zalazar *et al.* 2016). Proteins from the bovine oviduct, including oviduct specific glycoprotein (Abe *et al.* 1995) and heparin binding proteins (Imam *et al.* 2008), show great potential to improve freezing outcomes when incorporated into bovine cryopreservation media, resulting in substantial increases in post thaw motility, viability, acrosome integrity and osmotic tolerance. Fish antifreeze proteins (AFPs) have also had considerable investigation as a sperm cryoprotectant, with mixed results. While AFPs had little effect on the motility of rabbit spermatozoa (Nishijima *et al.* 2014) or viability of bull spermatozoa (Prathalingam *et al.* 2006), they significantly improved the viability of seabream spermatozoa (Beirão *et al.* 2011) and the osmotic tolerance of bull spermatozoa (Prathalingam *et al.* 2006). In addition, AFPs helped to maintain a normal level of phosphatidylserine in the plasma membrane and the same abundance of several proteins observed in fresh seabream spermatozoa (Beirão *et al.* 2011; Zilli *et al.* 2014). Other observed benefits of protein supplementation include increased activity of antioxidant enzymes leading to reduced lipid peroxidation (sericin (Kumar *et al.* 2015)) and improved in vitro mucus penetration (heparin binding oviduct proteins (Imam *et al.* 2008)). While these findings are encouraging, the only proteins assessed in terms of fertility failed to improve either in vitro or in vivo fertility outcomes (Gonçalves *et al.* 2008b; Qadeer *et al.* 2016). This suggests that perhaps due to the multifaceted nature of cryopreservation damage,

several proteins may be required to work in combination to positively affect in vivo fertility and as such, further investigation into cryoprotective proteins is required. The production of recombinant proteins, or isolation of proteins from easily sourced materials (e.g. milk, seminal plasma, blood) and their incorporation into freezing diluents would offer a logistically feasible means of introducing cryoprotective proteins for industry use.

1.5. CONCLUDING REMARKS AND OBJECTIVES

The use of frozen thawed spermatozoa is a goal for a variety of industries, including production animal breeding, human infertility treatment and conservation of endangered wildlife species. Yet there remain significant barriers to the use of cryopreserved semen, particularly in sheep, with poor fertility rates using conventional handling and insemination methods. While seminal plasma has often been heralded as the answer to many fertility related problems, the results of in vivo studies have proven contradictory. Moreover, the mechanisms behind the benefits which seminal plasma confers at ejaculation are not understood, let alone any benefits within the artificial environment of in vitro processing for cryopreservation. In order to make effective use of both fresh and cryopreserved semen, a better understanding of the 'natural' changes conferred by seminal plasma and the 'artificial' alterations unknowingly conferred by in vitro handling is required. With a better understanding of these processes, components of seminal plasma able to improve semen cryopreservation and male fertility may be identified and exploited.

The main aim of this thesis is to investigate the effects of seminal plasma exposure and cryopreservation on the ram sperm proteome and glycocalyx, and interactions between spermatozoa and phagocytic immune cells. A further aim is to profile the functions of the prominent seminal plasma protein family, the Binder of Sperm Proteins, both in a physiological capacity during capacitation and as protective agents during cryopreservation of ram spermatozoa. To this end, this thesis will include an examination of the following subjects;

- The proteomic content of epididymal and ejaculated ram spermatozoa, identifying proteins which are contributed by seminal plasma
- The proteomic content of fresh and frozen thawed ram spermatozoa, identifying proteins contributed by egg yolk and freezing induced changes to sperm proteins
- Changes to sugars of the ram sperm glycocalyx as a result of exposure to seminal plasma and cryopreservation, and the potential impacts of these processes on susceptibility to phagocytosis by neutrophils
- Effects of Binder of Sperm Proteins isolated from seminal plasma on the capacitation of ram spermatozoa under various levels of cAMP stimulation
- Effects of Binder of Sperm Proteins isolated from seminal plasma on freezing outcomes of ram spermatozoa

2. A proteomic investigation of ram spermatozoa and the proteins conferred by seminal plasma

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2.1. ABSTRACT

Sperm proteomes have emerged for several species, however the extent of species similarity is unknown. Sheep are an important agricultural species for which a comprehensive sperm proteome has not been produced. In addition, potential proteomic factors from seminal plasma which may contribute to improved fertility after cervical insemination are yet to be explored. Here we use GeLC-MS/MS to investigate the proteome of ejaculated ram spermatozoa, with quantitative comparison to epididymal spermatozoa. We also present a comparison to published proteomes of five other species. We identified 685 proteins in ejaculated ram spermatozoa, with the most abundant proteins involved in metabolic pathways. Only 5% of ram sperm proteins were not detected in other species, suggesting highly conserved structures and pathways. Of the proteins present in both epididymal and ejaculated ram spermatozoa, 7% were more abundant in ejaculated spermatozoa. Only two membrane bound proteins were detected solely in ejaculated sperm lysates; liver enriched gene 1 (LEG1/C6orf58) and epidermal growth factor-like repeats and discoidin I-like domains 3 (EDIL3). This is the first evidence that despite its relatively complex proteomic composition, seminal plasma exposure leads to few novel proteins binding tightly to the ram sperm plasma membrane.

2.2. INTRODUCTION

In order to achieve fertilisation, spermatozoa must interact with a range of elements in the female environment, including proteins, hormones, endometrial epithelial cells and immune cells. Many of these interactions are believed to involve proteins on the exterior membrane of spermatozoa (Tecle and Gagneux 2015), which are suggested to largely originate from protein rich seminal plasma (Caballero *et al.* 2012; Soleilhavoup *et al.* 2014). However, the

demonstrated fertility of epididymal spermatozoa, both in vitro (Chaveiro *et al.* 2015) and in vivo when inseminated directly into the uterus (Fournier-Delpech *et al.* 1979; Hori *et al.* 2011; Monteiro *et al.* 2011) demonstrates that seminal plasma is not required for fertilisation. Yet in sheep there is a confounding factor; when the barrier of the ewe's cervix is introduced, ejaculated spermatozoa achieve significantly higher pregnancy results than epididymal spermatozoa (Rickard *et al.* 2014). This suggests that ram seminal plasma plays an important role in aiding sperm transport through the female tract from the natural site of semen deposition in the anterior vagina. It is unknown if seminal plasma aids passage through the cervix by exposing spermatozoa to new proteins, altering the abundance of existing membrane bound proteins, or through other non-proteomic factors. While proteomics has begun to paint a picture of protein changes during epididymal maturation (Belleannee *et al.* 2011; Labas *et al.* 2015b) there are no proteomic profiles directly comparing epididymal and ejaculated spermatozoa.

Sperm proteins have many important functions including cellular regulation (Puri *et al.* 2008; Li *et al.* 2014), metabolism (Miki *et al.* 2004; Odet *et al.* 2011), cell-cell adhesion and fertilisation (Herrero *et al.* 2005; Gwathmey *et al.* 2006). Sperm proteomes have been published for both mouse (Baker *et al.* 2008; Guyonnet *et al.* 2012) and human (Johnston *et al.* 2005; Baker *et al.* 2007; Amaral *et al.* 2013; Sharma *et al.* 2013; Amaral *et al.* 2014) and those for the first wildlife (Kawase *et al.* 2015) and livestock species including the bull (Byrne *et al.* 2012), stallion (Swegen *et al.* 2015) and rooster (Labas *et al.* 2015a) have also recently emerged. While the proteins present in the seminal plasma (Bergeron *et al.* 2005; Soleilhavoup *et al.* 2014; Rickard *et al.* 2015) and other reproductive tract fluids (Souza *et al.* 2012) of the ram have been characterised previously, an all-encompassing proteome of the mature sperm cell has not been completed for this species. A comprehensive proteomic profiling of the sheep male gamete is important for the identification of potential proteins of interest, with the end goal of improving artificial breeding success in this agriculturally important animal.

Comparative proteomics has given us the ability to detect tangible differences between individuals that may contribute to differences in sperm function and fertility. A range of recent studies have compared proteomes between males with documented differences in fertility (Ashrafzadeh *et al.* 2013; Sharma *et al.* 2013), identifying both markers of performance and interesting candidates for further study. While such research suggests functional differences between individuals within the same species, it also prompts us to look at these differences at an interspecies level. Interestingly, the proteins in seminal plasma of closely related ungulates have been shown to be highly divergent, possibly mirroring differences in reproductive

strategies and success (Druart *et al.* 2013). While comprehensive proteomic comparisons of human and non-human primate spermatozoa and spermatozoa of various wild species have been published recently (Kawase *et al.* 2015; Zhou *et al.* 2015), the field lacks more species diverse comparisons of global sperm proteomes. Systematic comparison of sperm proteomes will allow for the identification of common proteins of interest and demonstrate the extent to which certain protein directed processes are comparable across species.

Our main objectives were to create a comprehensive ram sperm proteome, establish proteomic differences between epididymal and ejaculated ram spermatozoa and look at species based differences in sperm proteins. We employed GeLC-MS/MS to identify proteins found in lysates of ejaculated ram spermatozoa. In addition, a proteome of epididymal ram spermatozoa from the same individuals was also produced, allowing for identification of unique proteins contributed by seminal plasma from the accessory sex glands. Quantitative mass spectrometry data was used to compare the abundance of proteins which co-occurred in epididymal and ejaculated spermatozoa, highlighting those proteins which were more abundant following seminal plasma exposure. Subsequently, proteins identified in ejaculated ram spermatozoa were systematically compared to published sperm protein lists for a variety of species to determine the extent of protein conservation across species.

2.3. MATERIALS AND METHODS

2.3.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (St Louis, USA) unless otherwise stated.

2.3.2. Animals

Mature rams were kept in an animal house at the French National Institute for Agricultural Research (INRA), Nouzilly, France (n = 3). They were maintained on a chaff based diet supplemented with lupins, with an average body condition of 3.5. All animal procedures were carried out in accordance with welfare guidelines from the Ministry of French Agriculture and approved by the animal ethics committee at INRA.

2.3.3. Collection and preparation of semen

Semen was collected via artificial vagina and assessed immediately for quality by scoring wave motion. Only samples with a wave motion of ≥ 3 out of 5 were used. For seminal plasma extraction, ejaculates were centrifuged at 12, 000 x *g* for 20 min in a model 1-14 bench-top centrifuge (Sigma-Aldrich, St Louis, USA) at 4°C. The supernatant was further centrifuged at 12, 000 x *g* for 20 min at 4°C. The resulting supernatant was stored at -20°C until further use. Rams used for semen collection were euthanised and cauda epididymal spermatozoa were immediately collected by retrograde flushing of the vas deferens with a tris-citrate-fructose

diluent (300 mM tris, 95 mM citric acid (monohydrate), 28 mM fructose, pH 7.4) and assessed as above. To create a treatment of epididymal spermatozoa exposed to seminal plasma, seminal plasma from the same respective ram was added 1:1 (v/v) to epididymal flushings and incubated for 20 min at 37°C.

In preparation for SDS-PAGE and western blotting, all samples were diluted 10 fold in tris-citrate-fructose diluent and washed three times by room temperature centrifugation at 3, 000 x *g* for 3 min, then resuspended in 1 mL of tris-citrate-fructose diluent. The resulting cell pellet was resuspended to 200 x 10^6 spermatozoa/mL with tris-citrate-fructose diluent, before a further 2.5 fold dilution in lysis buffer (2% (w/v) SDS in 10 mM tris with a protease inhibitor diluted 1:20, pH 6.8). Samples were vortexed for 2 min, then left to stand for 3 min. The protein rich supernatant was separated from cellular debris by centrifuging at 15, 000 x *g* for 10 min at 4°C. The resulting supernatant was assessed for protein concentration using an Uptima BC Assay kit (Interchim, Montluçon, France) according to manufacturer's instructions, with bovine serum albumin as a standard. Samples were then diluted 5 fold with 5x loading buffer, heated to 90°C for 5 min and stored at - 20°C until further use.

2.3.4. Sample preparation for MS analysis

Sample preparation, LC-MS/MS and protein identification were performed as per Rickard (2014).1D SDS-PAGE was performed using Laemmli's method (Laemmli 1970) with 50 µg per lane on a 8–16% gradient gel (180 V, 60 min). The gel was stained with Coomassie blue (overnight, room temperature with agitation). Each lane was cut horizontally into 20 bands in preparation for digestion (Supplementary file 2.1).

2.3.5. In gel digestion

Gel pieces were washed separately in water and acetonitrile solution (1:1, 5 min) followed by 100% acetonitrile (10 min). Reduction and cysteine alkylation was performed by incubation with 10 mM dithiothreitol in 50 mM NH_4HCO_3 (30 min, 56°C), then 55 mM iodoacetamide in 50 mM NH_4HCO_3 (20 min, room temperature, in the dark). Pieces were then incubated with 50 mM NH_4HCO_3 and acetonitrile (1:1, 10 min) followed by acetonitrile (15 min). Proteolytic digestion was carried out overnight using 25 mM NH_4HCO_3 with 12.5 ng/µL trypsin (sequencing grade, Roche Diagnostics, Paris, France). Resultant peptides were extracted by incubation in 5% (v/v) formic acid (sonicated) with the supernatant removed and saved, followed by further incubation in acetonitrile and 1% (v/v) formic acid (1:1, 10 min). After a final incubation with acetonitrile (5 min), the supernatant was again removed and saved. These two peptide extractions were pooled and dried using a SPD1010 speedvac system (ThermoSavant, Thermofisher Scientific) and the resultant peptide mixture was analysed by liquid chromatography tandem mass spectrometry (GeLC-MS/MS).

2.3.6. Nano LC-MS/MS analysis

All experiments were performed on an LTQ Orbitrap Velos Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to an Ultimate 3000 RSLC chromatographer (Dionex, Amsterdam, The Netherlands). Samples were loaded on a trap column (Acclaim PepMap 100 C18 Nano-Trap, 100 µm i.d. x 2 cm long, 3 µm particles) and desalted for 10 min at 5 mL/min with 4% solvent B. Mobile phases consisted of (A) 0.1% formic acid, 97.9% water, 2% acetonitrile (v/v/v) and (B) 0.1% formic acid, 15.9% water, 84% acetonitrile (v/v/v). Separation was conducted using a nanocolumn (Acclaim PepMap 100 C18 Nano, 75 µm i.d. x 50 cm long, 3 µm particles) at 300 nL/min by applying a gradient consisting of 4–55% B over 90 min. The mass spectrometer was operated in data dependent scan mode. Survey full scan MS spectra (from 300–1800 *m/z*) were acquired with resolution set at 60, 000. The 20 most intense ions with charge states ≥ 2 were sequentially isolated (isolation width: 2 *m/z*; 1 micro scan) and fragmented using CID mode (normalised collision energy of 35% and wideband-activation enabled). Dynamic exclusion was active during 30 s with a repeat count of 1. Polydimethylcyclosiloxane (*m/z* 445.12) ions were used for internal calibration.

2.3.7. Protein identification and validation

MS/MS ion searches were performed using Mascot search engine (version 2.2, Matrix Science, London, UK) via Proteome Discoverer software (version 1.4, ThermoFisher Scientific, Bremen, Germany) against a local database (8, 000, 106 entries). From the NCBI database (downloaded 08/07/2015), a sub-database was generated using Proteome Discoverer software using keywords targeting mammalian taxonomy. The search parameters included trypsin as a protease with two allowed missed cleavages and carbamidomethylcysteine, methionine oxidation and acetylation of N-terminal proteins as variable modifications. The tolerance of the ions was set to 5 ppm for parent and 0.8 Da for fragment ion matches. Mascot results obtained from the target and decoy database searches were subjected to Scaffold software (version 4.4, Proteome Software, Portland, USA). Peptide and protein identifications were accepted if they could be established at greater than 95% probability as specified by the Peptide Prophet algorithm (Keller *et al.* 2002) and Protein Prophet algorithm (Nesvizhskii *et al.* 2003) respectively. Protein identifications were accepted if they contained at least two identified peptides.

2.3.8. Label-free protein quantification using spectral counting

For comparative analysis, we employed Scaffold software (version 4.4, Proteome Software, Portland, USA) using the protein cluster analysis option within the spectral counting quantitative module to cluster proteins based on shared peptide evidence and produce a single identification. Quantification was performed using the ‘Weighted Spectra’ method and

carried out on protein clusters. Thus, numbers of normalised weighted spectra (NWS) were tabulated using experiment-wide protein clustering. Significance between epididymal and ejaculated samples was determined using a Student's t test within the Scaffold software, where $p < 0.05$ was considered significant. Limits of an average normalised weighted spectra (NWS) of ≥ 5 and fold change of ≥ 2 were included to increase the validity of the quantitative comparison.

2.3.9. Gene ontology, localisation and network analysis

Proteins were assessed for GO terms using the PANTHER Classification System (version 10, www.pantherdb.org), with *Homo sapiens* as the organism to maximise classifications. Localisation of proteins was evaluated using a range of sources including UniProt (www.uniprot.org), GeneCards (www.genecards.org) and PANTHER analysis. To further characterise those proteins which were more abundant in lysates of ejaculated spermatozoa, FASTA sequences were assessed using SignalP 4.1 to predict signal peptides, SecretomeP 2.0 to predict non-classical secretion and TMHMM 2.0 to predict transmembrane domains (www.cbs.dtu.dk/services). Predicted interactions between proteins were evaluated using STRING (version 10, www.string-db.org).

2.3.10. Cross species comparison of common mammalian sperm proteins

To characterise cross species protein conservation, the entire proteome of ejaculated ram spermatozoa was compared to available proteomes for human (Amaral *et al.* 2013; Baker *et al.* 2013; Wang *et al.* 2013), bull (Peddinti *et al.* 2008; Byrne *et al.* 2012; Kasvandik *et al.* 2015), stallion (Swegen *et al.* 2015), rooster (Labas *et al.* 2015a) and trout (Nynca *et al.* 2014a) spermatozoa. Multiple human and bull sperm proteomes were analysed to ensure the most complete picture of conserved proteins was achieved; for other species, only one sperm proteome has been published. Proteins conserved across all evaluated mammals were subjected to STRING network analysis.

2.3.11. Western blotting of Binder of Sperm Proteins

BSP1 and BSP5, two members of the Binder of Sperm Protein (BSP) family (formerly known as RSP 15-16 kDa and RSP 20-22 kDa respectively (Bergeron *et al.* 2005)) were found to be present in ejaculated ram spermatozoa lysates. We carried out further investigation by western blotting epididymal and ejaculated sperm lysates and seminal plasma for these two proteins. Primary rabbit IgG antibodies against gelatin affinity purified ram BSPs (Plante *et al.* 2015a) (RRID AB_2715559) were kindly provided by P. Manjunath (Departments of Biochemistry and Medicine, Faculty of Medicine, University of Montreal). From this mixture, BSP1 and BSP5 antibodies were further blot-affinity purified (Olmsted 1981) by comparison to bands of 25 kDa and 15 kDa respectively from a ram seminal plasma blot. HRP conjugated goat anti-rabbit IgG

was purchased from Sigma-Aldrich (St Louis, USA). Samples were collected, washed and lysed as described above. Pooled samples from two rams were migrated (50 µg per lane) on a 12–20% gradient SDS-PAGE (180 V, 60 min). Liquid transfer of proteins was performed at 4°C (30 V, 16 h). Western blots were blocked with TBS-Tween-20 (0.5% w/v), supplemented with lyophilised low-fat milk (5% w/v). Membranes were incubated with primary antibodies under mild agitation at 4°C overnight or 37°C for 2 h and with secondary antibodies at 37°C for 1 h. The peroxidase was revealed with SuperSignal West Pico and West Femto Substrate (Thermo Fisher Scientific, Waltham, USA). Images were digitised with a cooled CCD camera (ImageMaster VDS-CL, Amersham Biosciences, GE HealthCare Lifesciences, Pittsburgh, PA).

2.4. RESULTS

2.4.1. Identification of ram sperm proteins

GeLC-MS/MS allowed for the identification of a total of 685 proteins in the lysate of ejaculated ram spermatozoa (Supplementary file 2.2) when using at least 2 peptides for identification. In comparison, 710 proteins were identified from lysates of epididymal ram spermatozoa applying the same limit (Supplementary file 2.3). Average protein identification probabilities were 96% and 97% and sequence coverage was 15% and 19% for ejaculated and epididymal spermatozoa respectively. Only 2 proteins had no gene symbol assigned. Of note are the presence of several important protein families, namely spermadhesins (spermadhesin z13, bodhesin 2), Binder of Sperm Proteins (BSP1, BSP5) and cysteine rich secretory proteins (CRISP2). Heat shock proteins were also well represented (HSP 10 kDa, HSP 60 kDa, HSP 70 mitochondrial, HSP 70 kDa 1, HSP 70 kDa 2, HSP 70 kDa 4, HSP 90α).

2.4.2. Gene ontology and network analysis of highly abundant ram sperm proteins

Highly abundant proteins in ejaculated spermatozoa were classified as those proteins with the 50 highest normalised weighted spectra averaged across 3 males. Gene ontology analysis using PANTHER suggested that these proteins are largely involved in catalytic activity (54%), with a sizeable proportion also involved in binding and structural support (26%). Up to 39% of these proteins are directly involved in metabolic processes, particularly primary metabolism involving carbohydrates and proteins. Almost half of these proteins fall into either oxidoreductase, hydrolase or transferase protein classes, with a wide variety of other classes represented. STRING network analysis revealed five distinct clusters of proteins according to their functions (Figure 2.1). Clustering largely revolved around sperm metabolism and particularly cellular respiration, with three clusters of proteins involved in glycolysis, the TCA cycle/oxidative phosphorylation and β oxidation of fatty acids. Of the two smaller clusters, one involved proteins from the dynein and tubulin families, suggesting an association of proteins

which are important for sperm locomotion and the other included proteins such as ZPBP and SPAM1, possibly involved in gamete interaction during fertilisation.

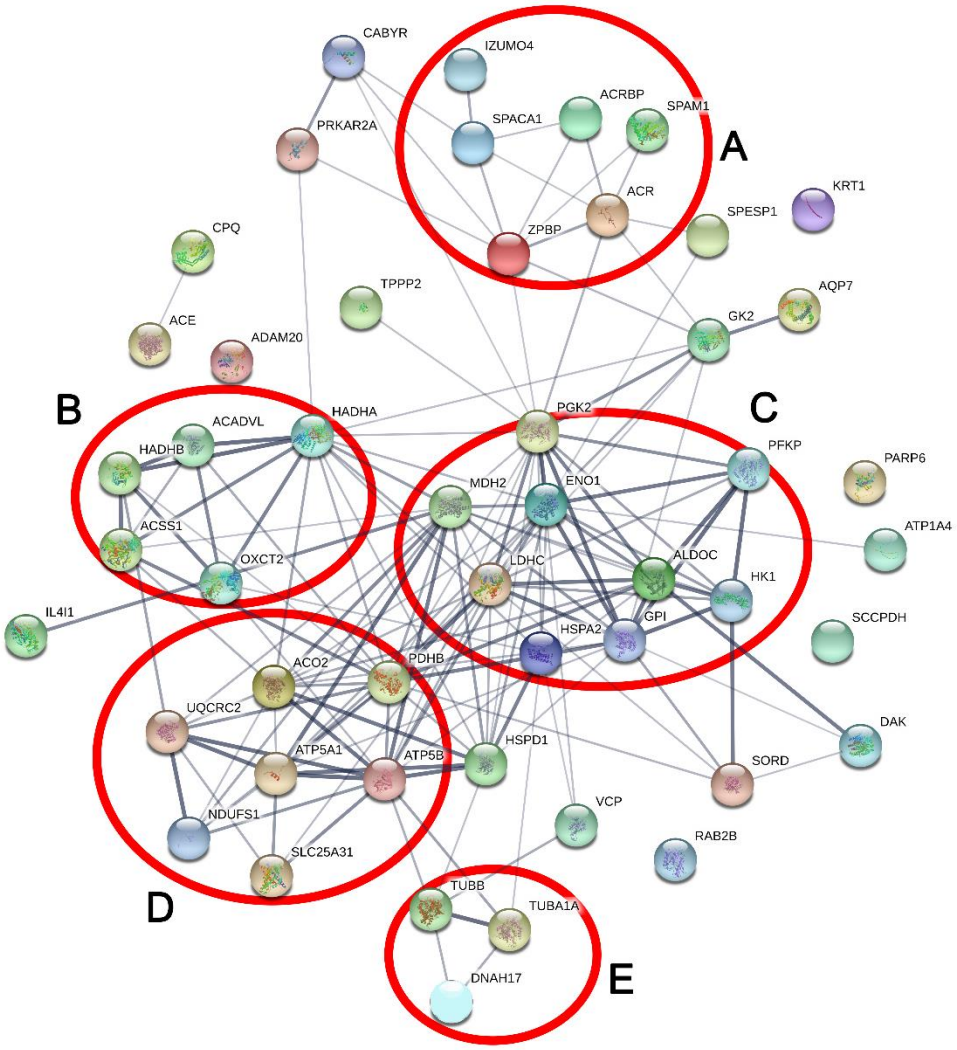

Figure 2.1 A network of protein-protein interaction between the 50 most abundant proteins in ejaculated spermatozoa by normalised total spectra was created using STRING version 10 (www.string-db.org). Connections between spheres, which represent individual proteins, are based on a variety of sources including experimental data, database and text mining and genome based predictions. The weight of these lines represents the confidence with which a predicted interaction occurs. Highlighted network interactions include proteins involved in fertilisation (**A**), β oxidation of fatty acids (**B**), glycolysis (**C**), oxidative phosphorylation (**D**) and locomotion (**E**)

2.4.3. Cross species comparison of sperm proteins

On the basis of comparison to published sperm proteomes, ram spermatozoa share a total of 604 (88%), 445 (65%), 408 (60%), 271 (40%) and 67 (10%) proteins with human, bull, stallion, rooster and trout spermatozoa respectively (Supplementary file 2.4). The vast majority (95%) of proteins in the proteome of ejaculated ram spermatozoa were found in at least one other species' proteome. A network analysis of the 299 proteins conserved across all evaluated mammals demonstrates strong and complex clustering of proteins including proteasome

complex proteins, chaperones and glycolytic and oxidative phosphorylation enzymes (Figure 2.2).

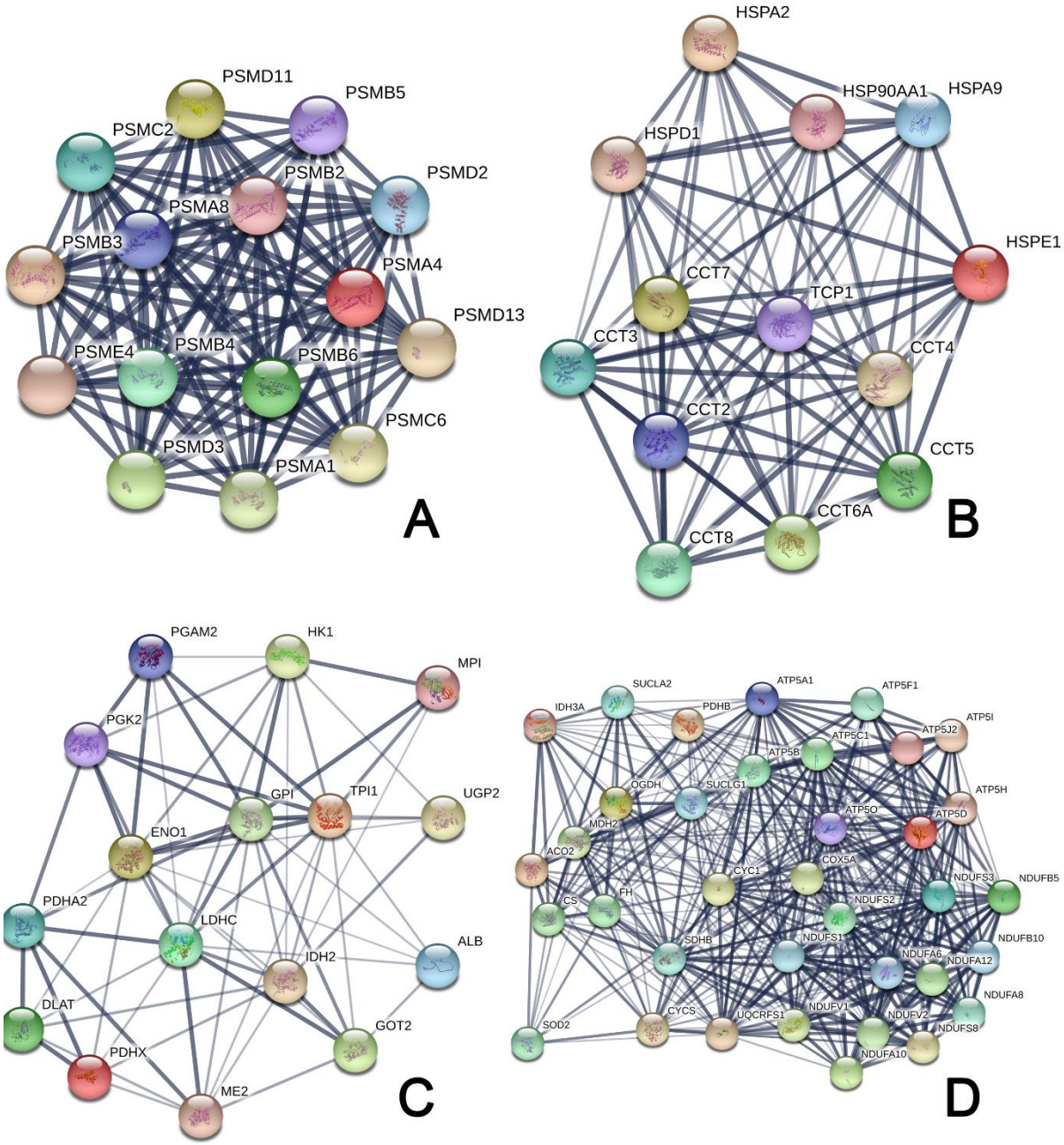

Figure 2.2 Isolated networks of protein-protein interaction between the proteins co-occurring in ram, human, bull and stallion sperm proteomes was created using STRING version 10 (www.string-db.org). Connections between spheres, which represent individual proteins, are based on a variety of sources including experimental data, database and text mining and genome based predictions. The weight of these lines represents the confidence with which a predicted interaction occurs. Clusters include proteins involved in proteasome formation and activity (**A**), chaperone activity (**B**), glycolysis (**C**) and oxidative phosphorylation (**D**)

Table 2.1 Proteins identified by LC-MS/MS which were significantly more abundant ($p<0.05$) in lysates of ejaculated ram spermatozoa compared to epididymal spermatozoa using a Student's *t* test.

Protein name	Gene symbol	Epi SPC	Ejac SPC	Fold change	Secreted‡
Binder of Sperm 5	BSP5	0	6.9	N/A	x
Liver Enriched Gene 1	C6orf58	0	15.21	N/A	x
EGF-like repeat and discoidon 1-like domain-containing protein 3	EDIL3	0	38.73	N/A	x
L-2-hydroxyglutarate dehydrogenase, mitochondrial	L2HGDH	0.92	8.26	9	-†
Cilia- and flagella-associated protein 70	CFAP70	1.19	9.41	7.9	-
Mitochondrial chaperone BCS1-like	BCS1L	0.87	6.85	7.9	-
Spermadhesin Z13-like	SPADH2	2.24	15.54	6.9	x
Presequence protease, mitochondrial	PITRM1	1.21	7.2	6	-
Saccharopine dehydrogenase-like oxidoreductase	SCCPDH	114.33	563.44	4.9	-
Coiled-coil domain-containing protein lobo homolog	DRC7	1.27	5.77	4.6	-
Retinal rod rhodopsin-sensitive cGMP 3',5'-cyclic phosphodiesterase subunit delta	PDE6D	1.5	6.51	4.3	-
Septin-7	SEPT7	1.21	5.04	4.2	-
Bodhesin-2	BDH2	48.74	156.99	3.2	x
Lon protease homolog, mitochondrial	LONP1	10.87	34.26	3.2	-
Spermatogenesis-associated protein 32	SPATA32	2.46	7.92	3.2	-
Dynein intermediate chain 2, axonemal	DNAI2	5.85	18.04	3.1	-
Acetyl-coenzyme A synthetase 2-like, mitochondrial	ACSS1	54.46	158.25	2.9	-
Hydroxysteroid dehydrogenase-like protein 2	HSDL2	13.38	39.28	2.9	x
Lamin A/C	LMNA	4.23	12.29	2.9	-
Nuclear pore complex protein Nup155	NUP155	1.86	5.4	2.9	-†
Isocitrate dehydrogenase (NADP), mitochondrial	IDH2	13.9	38.3	2.8	x
2-oxoglutarate dehydrogenase, mitochondrial	OGDH	51.64	141.28	2.7	-
Citrate synthase, mitochondrial	CS	32.58	87.28	2.7	x
Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADM	24.25	65.96	2.7	x
Probable threonine protease PRSS50	PRSS50	2.24	6.12	2.7	-†
Isoleucine-tRNA ligase, mitochondrial	IARS2	6.58	17.32	2.6	-
Glycerol-3-phosphate dehydrogenase, mitochondrial	GPD2	30.14	71.5	2.4	-
NAD(P) transhydrogenase	NNT	15.15	36.09	2.4	-
Cilia- and flagella-associated protein 69	CFAP69	3.4	8.3	2.4	-
Stress-70 protein, mitochondrial	HSPA9	27.56	62.06	2.3	-
Uncharacterised protein C3orf84	C3orf84	8.95	20.92	2.3	x

Protein phosphatase 1 regulatory subunit 7	PPP1R7	2.15	5.05	2.3	x
Trifunctional enzyme subunit beta, mitochondrial	HADHB	79.07	171.06	2.2	x
Inactive ribonuclease-like protein 9	RNASE9	7.72	16.94	2.2	x
Protein FAM154A	SAXO1	2.49	5.42	2.2	-†
3-ketoacyl-CoA thiolase, mitochondrial	ACAA2	54.01	111.26	2.1	x
Pyruvate dehydrogenase E1 component subunit alpha, testis-specific form, mitochondrial	PDHA2	27.43	53.87	2	-†
Calpain-11	CAPN11	16.34	32.07	2	-
Thioredoxin domain-containing protein 3	NME8	13.89	28.18	2	-
Ras GTPase-activating-like protein IQGAP2	IQGAP2	10.89	21.99	2	-

‡ A prediction of whether or not proteins are extracellular and thus likely to be secreted. Secretion prediction was based on results from SignalP 4.1, SecretomeP 2.0 and TMHMM 2.0 (www.cbs.dtu.dk/services) checking for signal peptides, probability of being secreted by non-classical pathways and predicted presence of transmembrane domains respectively.

†Predictions of secretion were discounted if literature evidence supported proteins being integral to sperm architecture or localised intracellularly.

Results are ranked in decreasing order of fold change.

2.4.4. Characterisation of proteins found only in ejaculated spermatozoa

A total of three proteins (BSP5, LEG1 (C6orf58) and EDIL3) were detected in ejaculated spermatozoa and not detected in epididymal spermatozoa by mass spectrometry (Table 2.1). These proteins are predicted to have no transmembrane domains and to produce signal peptides or undergo non-classical secretion. On the basis of these predictions and their previous identification in seminal plasma, these proteins are likely contributed by seminal plasma from the accessory sex glands. PANTHER analysis of gene ontology suggested that the key molecular functions of these three proteins are enzyme regulation, catalytic activity, binding and receptor activity. Of the proteins with known functions, involvement in metabolic and immune system processes, localisation, biological regulation, adhesion and response to stimulus were noted. STRING network analysis failed to predict any relationships between these three proteins.

2.4.5. Differences in protein abundance between epididymal and ejaculated spermatozoa

A total of 548 proteins co-occurred in epididymal and ejaculated spermatozoa lysates (Supplementary file 2.5) with average normalised weighted spectral counts of ≥ 5 in at least one sperm type. Of the proteins found in both treatments, 79% were present in similar amounts, 14% were more abundant in epididymal and 7% were more abundant in ejaculated spermatozoa. Only those proteins with a fold change of greater than 2 were considered, making a total of 112 proteins which differed significantly between the two treatments. 37 proteins were significantly more abundant in ejaculated spermatozoa ($p < 0.05$; Table 2.1, Supplementary file 2.6) and 75 proteins were more abundant in epididymal spermatozoa ($p < 0.05$). The vast majority of proteins (75%) which occurred in significantly different amounts in the two sperm types are involved in catalytic activity and binding. While the majority of proteins more abundant in epididymal spermatozoa show no network interaction, many of their individual functions are key to testicular development and epididymal maturation.

2.4.6. Binder of Sperm Proteins

Western blots confirmed the presence of BSPs 1 and 5 on ejaculated ram spermatozoa and showed an absence of BSP1 and very low levels of BSP5 on epididymal spermatozoa (Figure 2.3). In addition, we confirmed that BSP1 and BSP5 are present in seminal plasma and will bind to epididymal spermatozoa during in vitro incubation of epididymal spermatozoa with seminal plasma. The combination of our MS and western blotting results suggest that in the ram, BSP1 and 5 are present in extremely low amounts in epididymal spermatozoa and high amounts in ejaculated spermatozoa as a result of contact with BSP rich seminal plasma.

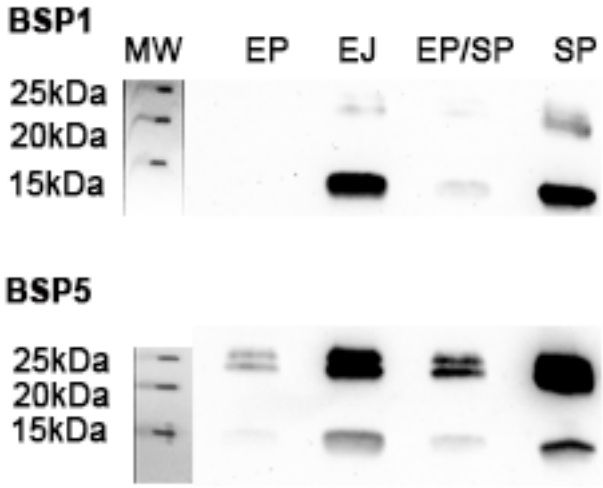

Figure 2.3 Western blotting against Binder of Sperm Proteins was carried out on epididymal (EP) and ejaculated spermatozoa (EJ), epididymal spermatozoa incubated with seminal plasma (EP/SP) and ram seminal plasma (SP), collected from the same individuals (n=2). EP/SP samples were created by incubating epididymal flushings with seminal plasma (1:1 v/v, 20 min, 37 °C) from the same respective ram. Prior to lysis, all samples were washed three times in tris-citrate-fructose diluent. Samples were pooled across individuals prior to running on SDS-PAGE. Antibodies used for detection targeted either BSP1 (top) or BSP5 (bottom)

2.5. DISCUSSION

2.5.1. Highly abundant proteins power the sperm cell

The proteins we have identified through GeLC-MS/MS of ejaculated ram spermatozoa include numerous members of thoroughly researched protein families, as well as apparently isolated and poorly characterised proteins. Assessing the 50 most abundant proteins identified based on normalised weighted spectra, the proteome of ejaculated ram spermatozoa is in many respects an intricate system of inter-related proteins, with the primary goal of energy production to power sperm transport. Spermatozoa have high energy demands for critical cellular processes, particularly motility (Allen *et al.* 2010), capacitation and the acrosome reaction. This is supported by our finding that many of the more abundant proteins in ram spermatozoa are involved in metabolism and can be roughly evenly divided into either glycolytic or TCA cycle/oxidative phosphorylation enzymes, including β oxidation enzymes which feed substrate into the TCA cycle. While these pathways may be redundant (Ruiz-Pesini *et al.* 2007) or their importance species specific (Windsor 1997; Miki *et al.* 2004; Plaza Davila *et al.* 2015), their abundance suggests that both play important roles in ram spermatozoa, even if not equally so. On the basis of spectral counting, few proteins contribute the quantitative bulk of the proteome and these are largely integral structural elements, particularly those of the tail. Both microtubule components and their associated motor proteins were the most common proteins observed, with the former being identified almost twice as much as the next most abundant protein. The correct folding and function of these proteins is as important

as the supply of ATP (Ishijima *et al.* 2002) in ensuring adequate motility to navigate the female tract and penetrate the zona pellucida prior to fertilisation. Few of the most abundant proteins are involved directly in fertilisation (e.g. SPAM1, ZPBP), however those that are have important functions including adhesion to (Hanqing *et al.* 1991) and cooperative proteolytic digestion of the cumulus (Zhou *et al.* 2012) and zona pellucida (Ferrer *et al.* 2012). The most abundant proteins in ejaculated ram spermatozoa evidently play key roles, allowing for vast amounts of energy to be produced and consumed in the race towards fertilisation.

2.5.2. Conserved and species specific sperm proteins

95% of ejaculated sperm lysate proteins were common to the other species analysed, heightened even further when comparing protein homologues (e.g. the BSP superfamily is also represented in stallions with the protein BSP2 (Plante *et al.* 2015a)). Overall, this suggests that many sperm proteins (or at least protein families) are conserved across multiple mammalian taxa, in agreement with a recent cross-species comparison of epididymal spermatozoa (Bayram *et al.* 2016). Of the few proteins which were not found in any of the other proteomes to which ram spermatozoa was compared, several stand out and some have not yet been described in spermatozoa. Bodhesin 2 (BDH2) has previously been identified on the plasma membrane of ram spermatozoa (van Tilburg *et al.* 2013) and in goat seminal plasma (Melo *et al.* 2008). Interestingly, it is highly abundant in ram seminal plasma (Soleilhavoup *et al.* 2014) and was one of the most abundant proteins we identified in lysates of ejaculated ram spermatozoa. However, increased levels of seminal plasma BDH2 has previously been linked to below average (< 80%) sperm motility (Rodrigues *et al.* 2013). C reactive protein (CRP) has previously been identified in the semen of men with chronic prostatitis (Girgis *et al.* 1983), but was not present in sperm lysates from healthy donors (Amaral *et al.* 2013; Baker *et al.* 2013; Wang *et al.* 2013). CRP is a blood serum protein produced by hepatocytes and has pleiotropic effects on the immune system, with contextual pro and anti-inflammatory activity (Black *et al.* 2004), which could indicate an immune based role when present on ejaculated spermatozoa. CEACAM21 is an ortholog of CEACAM1 which is present in human spermatozoa (Dráberová *et al.* 2000), immune cells (Gray-Owen and Blumberg 2006), Sertoli cells and epididymal epithelial cells and is believed to be involved in cell-cell adhesion (Lauke *et al.* 2004). Another protein with no detection in other sperm proteomes, α-S1 casein (CSN1S1), has been shown to have immunomodulatory effects (Vordenbäumen *et al.* 2013; Vordenbäumen *et al.* 2016). In addition, we have reported for the first time several proteins which have not previously been found in lysates of spermatozoa (e.g. LEG1 (C6orf58 gene), C3orf84, LOC100763131, LOC101122400, LOC105608858, MGC137036). While very few of the identified proteins appear to be unique to ram spermatozoa, those which are may provide an avenue for identification of species specificity

in important processes such as sperm based immune defence or manipulation, and binding to various cell types of the female reproductive tract.

It is interesting to note that while ram spermatozoa share a considerable proportion of proteins with that of a non-mammalian internal fertiliser (e.g. rooster, 40% (Labas *et al.* 2015a)), comparing ram to an external fertiliser such as trout yields low similarity (10% (Nynca *et al.* 2014a)). In addition, rams share more common proteins with fellow vaginal depositors (human and bull) than the stallion, a uterine depositor. This suggests that male/female interactions during the reproductive process, particularly the nature and site of semen deposition, act as selection pressures for sperm evolution. Proteins conserved across all evaluated mammals showed four distinct network clusters, largely consistent with a previous comparison of rodent and ungulate spermatozoa (Bayram *et al.* 2016). Several conserved proteins are involved in the formation and regulation of proteasome complexes, which are likely to be key in mammalian sperm capacitation, acrosome reaction and fertilisation (Sutovsky 2011). Chaperone protein families also form a modest network cluster, namely consisting of CCT subunits and heat shock proteins. Members of these chaperone families appear to be key both in early stages of spermatogenesis (Dix *et al.* 1996; Souès *et al.* 2003) and also in preparing the sperm surface for interaction with the zona pellucida (Asquith *et al.* 2005; Dun *et al.* 2011). The remaining larger clusters include proteins involved in energy production pathways of glycolysis and oxidative phosphorylation. This again highlights that while oxidative phosphorylation is likely to be the most important pathway for ATP generation (Ruiz-Pesini *et al.* 2007), glycolysis is well conserved and thus likely to play some role in acquiring and/or sustaining motility (Ford 2006). These similarities between the protein components of spermatozoa from different mammalian species clearly reinforce that in addition to cellular metabolic pathways, many proteins responsible for interaction with the female environment have also been conserved to ensure successful fertilisation.

2.5.3. Do seminal plasma proteins prepare spermatozoa for cervical transit and fertilisation?

Interestingly, only 0.5% of proteins identified in ejaculated spermatozoa were not detected in epididymal spermatozoa. While ram seminal plasma contains many proteins (Soleilhavoup *et al.* 2014), it appears that very few of the proteins which become tightly bound to the sperm surface at ejaculation are being introduced for the first time. Additionally, those proteins which are unique to ejaculated spermatozoa were detected at levels below the average for other identified proteins. It is possible that membrane proteins are under-represented in this proteome due to the sample complexity and dynamic range of whole cell lysates (Brewis and

Gadella 2010). Future LC-MS/MS of membrane enriched samples, as previously characterised for bulls (Byrne *et al.* 2012), would be a useful confirmation of the current results.

Field fertility trials have suggested that ejaculated ram spermatozoa are better able to traverse the cervix post insemination compared to epididymal spermatozoa (Rickard *et al.* 2014). While the authors suggest that perhaps membrane bound proteins contributed to spermatozoa by seminal plasma could be responsible for these differences in cervical migration, the current results suggest that these two sperm types may differ by as few as three unique membrane bound proteins (BSP5, LEG1 (C6orf58), EDIL3). The effects of seminal plasma on the female tract and developing embryos (McGraw *et al.* 2015) fail to explain the low level of fertility obtained by epididymal spermatozoa when inseminated at the cervix and the high level of fertility when introduced directly into the uterus (Rickard *et al.* 2014). This forces us to consider the possibility that a few novel proteins binding to spermatozoa at ejaculation may cause significant changes to sperm function. The demonstrated and proposed functions of these few proteins may provide clues as to their role in the scheme of successful cervical transit and subsequent fertility.

LEG1 protein (Liver Enriched Gene 1, also known as UPF0762 Protein, C6orf58 gene) has poor end function characterisation and research of its roles may help to distinguish its level of importance. LEG1 protein has been identified both in trout seminal plasma (Nynca *et al.* 2014b) and ovarian fluid (Nynca *et al.* 2015b), but was not found in trout sperm lysate (Nynca *et al.* 2014a). It has not been identified in sperm lysates of any species other than sheep to date. While it was found to be relatively abundant in a recent ram seminal plasma proteome (Rickard *et al.* 2015), we did not find it bound to ejaculated ram spermatozoa in very large amounts. While LEG1 protein has had no investigation in a reproductive context, homologous genes LEG1a and LEG1b are believed to be important in normal liver development of zebrafish (Chang *et al.* 2011).

Epidermal growth factor-like repeats and discoidin I-like domains 3 (EDIL3, also known as Del-1) was the most abundant protein that we detected only on ejaculated spermatozoa. To date, EDIL3 has largely been researched as a marker of, and important player in, hepatocellular carcinomas (Sun *et al.* 2010). This protein has been shown to act on αvβ5 integrin, inducing αvβ3 integrin expression in endothelial cells and promoting angiogenesis (Zhong *et al.* 2003). Interestingly, sperm-bound αvβ3 integrin has been shown to be involved in the fusion of gametes from multiple species, with the αv subunit being of most importance for mice (Boissonnas *et al.* 2010) and β3, as well as various other integrin subunits, important for cattle fertilisation (Gonçalves *et al.* 2008a). While we failed to find any integrin subunits, previous proteomic work has identified the β5 integrin subunit in ram seminal plasma (Rocha

et al. 2015) and αvβ3 integrin in the ovine endometrium (Wan *et al.* 2011), suggesting possible targets for sperm-bound EDIL3. EDIL3 has further interesting properties of immune evasion and manipulation, particularly protection of normal endothelial cells from neutrophil and monocyte attachment, and limiting leucocyte recruitment during inflammation (Choi *et al.* 2008). These anti-inflammatory and immune modulatory functions may have important natural applications for ram spermatozoa, who find themselves a 'non-self' cell in the heavily immune-armed environment of the ewe's cervix (Scott *et al.* 2006). These kinds of protein based interaction with the female tract and ultimately the oocyte are undoubtedly important for the success of insemination, and deserve further investigation.

BSPs (BSP1, BSP5) presented contradictory mass spectrometry and western blotting results, but hold promise for interesting future investigation. BSP1 was identified in both sperm types by mass spectrometry, but not picked up in western blots of epididymal sperm lysates. Conversely, BSP5 was only identified in ejaculated spermatozoa by mass spectrometry, but faint antibody signal was detected in epididymal samples. We believe these contradictory results are a shortfall in the sensitivity of our preparative and mass spectrometry techniques, in particular as a result of glycan chains causing decreased detection sensitivity and incomplete tryptic digestion due to glycan induced steric hindrance on the glycosylated BSPs (Manjunath and Sairam 1987; Segu *et al.* 2010; Jois *et al.* 2015). BSPs are by far the most well studied of the proteins we found to be contributed largely by seminal plasma, with exciting implications. In the bull, BSPs from seminal plasma have been suggested to be important in creation of the oviductal sperm reservoir (Gwathmey *et al.* 2003) and maintenance of sperm motility during this period prior to fertilisation (Gwathmey *et al.* 2006). In addition, both BSP1 and BSP5 have been linked to bull sperm capacitation, during which they act as a bridge between heparin and the sperm membrane, encouraging cholesterol efflux (Moreau *et al.* 1998; Thérien *et al.* 1998). A study using recombinant proteins determined that heparin mediated capacitation of bull spermatozoa is directly related to the tandem fibronectin domains of BSP5 (Jois *et al.* 2015), the structural feature which defines the BSP family. BSP1 has further demonstrated activity as a chaperone which directs protein folding under physiological conditions (Sankhala and Swamy 2010). These important biological functions of BSPs, along with their widespread occurrence across different species (Manjunath *et al.* 2009), suggest that they are key players in the manipulation of mature spermatozoa by seminal plasma to afford fertility post ejaculation. Both BSP1 and 5 have previously been identified in ram seminal plasma (Jobim *et al.* 2005; Soleilhavoup *et al.* 2014) and have been shown to afford protection during cold stress (Barrios *et al.* 2005; Bernardini *et al.* 2011), yet their biological relationship with ram spermatozoa has not been clearly elucidated. While there are clear differences in the action of BSPs on ram and bull spermatozoa during in vitro handling (Leahy and de Graaf

2012), any positive, or potentially negative, effects of this protein family on ram spermatozoa in vivo remain to be seen.

While previous articles have described seminal plasma as the source of many important membrane binding proteins to which spermatozoa have no exposure prior to ejaculation, these results suggest that this is not the case for ram spermatozoa. But the question remains whether it is a few novel proteins in seminal plasma or significant changes to quantities of proteins already present in epididymal plasma which could explain observed differences in cervical transit of epididymal and ejaculated spermatozoa (Rickard *et al.* 2014). Those proteins which we found increased in abundance due to seminal plasma exposure are an important avenue of investigation to better understand in vivo fertility. Some of these proteins, such as SPADH2, PPP1R7, BDH2 and RNASE9 have had some characterisation in relation to sperm function. Spermadhesin Z13 (SPADH2) is highly abundant in ram seminal plasma (Soleilhavoup *et al.* 2014) and was detected as 7 times more abundant in ejaculated spermatozoa. Interestingly, spermadhesin z13 is higher in abundance in the seminal plasma of bulls with lower fertility (Moura *et al.* 2006) and poorer semen quality (Sarsaifi *et al.* 2015), however this may be a case of species specific roles for the same protein (Coy and Yanagimachi 2015) or simply correlation without causation. Further investigation into the interaction of this protein with ram spermatozoa and possible subsequent effects on interaction with the female are needed.

As its name suggests, protein phosphatase 1 regulatory subunit 7 (PPP1R7, also known as sds22) regulates protein phosphatase 1 (PP1) and it has demonstrated inhibitory activity on testis specific PP1γ2 during epididymal maturation (Huang *et al.* 2002; Mishra *et al.* 2003). Inhibition of PP1γ2 leads to stimulation of progressive motility, as well as heightened velocity in already progressively motile epididymal spermatozoa (Vijayaraghavan *et al.* 1996). The effect of PP1 inhibition on ejaculated sperm motility has never been investigated, however inhibition by a number of the 50 documented interacting proteins (Cohen 2004) rapidly brings on capacitation (Signorelli *et al.* 2013) and hyperactivation (Si and Okuno 1999). These results suggest that serine/threonine phosphatases may have a role in capacitation and that their inhibition accelerates its completion. While there are obviously many redundancies in terms of sperm protein phosphatase inhibitors, PPP1R7 may contribute to a natural build-up of PP1 inhibitory factors as a pre-requisite for capacitation and hyperactivation. While this goes against the classical picture of seminal plasma being rich in ‘decapacitating factors’, it encourages the idea that carefully timed binding of proteins in certain amounts is required for maximal sperm function.

BDH2 and RNASE9 have been studied in relation to fertility, but with limited results. Bodhesin 2 (BDH2) has not been studied directly, but shares the presence of a CUB domain and 70% sequence homology with porcine AQN-1, which is involved in oviductal epithelial cell binding (Töpfer-Petersen *et al.* 1998). RNASE9 is believed to be released by epididymal epithelial cells, after which it localises to the sperm plasma membrane during epididymal transit (Cheng *et al.* 2009). While RNASE9 knockout mice show impaired motility of corpus epididymal spermatozoa in situ, there is no impact on overall maturation or in vivo fertility (Westmuckett *et al.* 2014). Interestingly, recombinant RNASE9 has demonstrated antibacterial activity against *E. coli*, suggesting a possible role in defence against the hostile female environment (Cheng *et al.* 2009). The remaining proteins which were more abundant in ejaculated spermatozoa have a variety of metabolic roles including β-oxidation of fatty acids (HADHB, ACADM) (Carpenter *et al.* 1992; Prunotto *et al.* 2013) and participation in the TCA cycle (CS) (Buschow *et al.* 2010). Other interesting functions include involvement in capacitation (GPD2) (Kota *et al.* 2009) and demonstrated anti-fungal properties (ACAA2) (Lee *et al.* 2009). These varied roles both highlight the complexity of the interaction between spermatozoa and seminal plasma proteins and call for further investigation into their usefulness in improving artificial breeding.

Spermatozoa must be a sum of their many parts if they are to successfully complete the taxing journey from ejaculation to fertilisation. We identified 685 proteins in ejaculated ram spermatozoa, many of which are involved in cellular metabolism and some with yet to be discovered functions. These results shed light on the variety and sources of proteins within this unique cell type and demonstrate the complexity of their bioenergetics and interaction with the surrounding environment. Comparison to published proteomes of other species revealed conservation of metabolic enzymes and proteins associated with sperm development, maturation and fertilisation. Those proteins which are not conserved between species may reflect differences in the physical act of mating and other species specific reproductive characteristics. Finally, we detected by mass spectrometry only two extracellular, membrane bound proteins (LEG1, EDIL3) which were present in ejaculated spermatozoa but not epididymal spermatozoa. Our results suggest that Binder of Sperm Proteins BSP1 and BSP5 are present on epididymal ram spermatozoa and that exposure to seminal plasma significantly increases the amount of these proteins bound to the surface of ejaculated ram spermatozoa. We also observed increased levels of a number of other proteins (e.g. SPADH2, BDH2, RNASE9) on ejaculated spermatozoa, with potential impacts on the success of cervical transit and fertilisation. Further research into these proteins in particular may yield information relevant to the improved function of spermatozoa for application in cryopreservation of semen and artificial insemination of sheep, and potentially other agricultural species.

2.6. ACKNOWLEDGEMENTS

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3. Cryopreservation and egg yolk medium alter the proteome of ram spermatozoa

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3.1. ABSTRACT

Cryopreservation causes significant lethal and sublethal damage to spermatozoa. In order to improve freezing outcomes, a comprehensive understanding of sublethal damage is required. Cryopreservation induced changes to sperm proteins have been investigated in several species, but few have employed currently available state of the art, targeted data independent acquisition mass spectrometry (MS) methods. We used the SWATH-MS method to quantitatively profile proteomic changes to ram spermatozoa following exposure to egg yolk and cryopreservation. Egg yolk contributed 15 proteins to spermatozoa, including vitellogenins, apolipoproteins and complement component C3. Cryopreservation significantly altered the abundance of 51 proteins. Overall, 27 proteins increased (e.g. SERPINB1, FER) and 24 proteins decreased (e.g. CCT subunits, CSNK1G2, TOM1L1) in frozen thawed ram spermatozoa, compared to fresh spermatozoa. Chaperones constituted 20% of the proteins lost from spermatozoa following cryopreservation. These alterations may interfere with both normal cellular functioning and the ability of frozen thawed spermatozoa to appropriately respond to stress. This is the first study to apply SWATH mass spectrometry techniques to characterise proteins contributed by egg yolk based freezing media and to profile cryopreservation induced proteomic changes to ram spermatozoa.

3.2. INTRODUCTION

The process of cryopreservation has been shown to inflict considerable damage to the sperm cell through cellular dehydration, osmotic stress and intracellular ice formation (Parks and Graham 1992; Muldrew and McGann 1994; Gao and Critser 2000; Johnson *et al.* 2000). As a result, frozen thawed spermatozoa have reduced membrane and acrosome integrity (Salamon and Maxwell 1995; Gillan *et al.* 1997), in addition to exhibiting reorganisation and disruption of important lipid-protein associations within the plasma membrane (De Leeuw *et al.* 1991; Parks and Graham 1992; Holt 2000; Watson 2000; Leahy and Gadella 2011). During cooling, freezing and rewarming, spermatozoa undergo important alterations to membrane phospholipids (Fang *et al.* 2016), sustain significant DNA damage (Partyka *et al.* 2010),

produce high amounts of reactive oxygen species (Kim *et al.* 2010) and often show hallmarks of capacitation (Naresh and Atreja 2015). Cryopreservation also significantly decreases tolerance to stressors such as reactive oxygen species (Neild *et al.* 2005; Garg *et al.* 2009) and osmotic shock (Khan and Ijaz 2008; Pinto and Kozink 2008). While cryoprotectants such as egg yolk generally help to minimise damage, the full scope of their effects on spermatozoa are not well characterised. Thus while cryopreservation can clearly be lethal, there is an element of sublethal damage which may significantly affect those spermatozoa which remain viable post thaw.

While frozen thawed semen is currently employed in a range of animal industries, as well as human reproductive medicine, improvements in freezing outcomes would no doubt be welcomed. This is of particular interest in sheep, where the use of cryopreserved spermatozoa is limited due to its reduced fertility following cervical insemination (Maxwell and Hewitt 1986; King *et al.* 2004), a symptom of the diminished ability of frozen thawed ram spermatozoa to transit the ovine cervix. Minimising both lethal and sublethal damage to spermatozoa during cryopreservation is key to improving insemination outcomes, but in order to do this, we must comprehensively understand what this damage entails. In particular, the freezing induced loss of functionally important proteins is of significant interest. In addition, the modification of the sperm proteome by cryoprotective agents, particularly ubiquitously used egg yolk, requires further investigation. With the advent of comparative proteomics, there has been investigation into protein based changes caused by cryopreservation of spermatozoa from man (Wang *et al.* 2014; Bogle *et al.* 2017), bull (Westfalewicz *et al.* 2015), ram (He *et al.* 2016), boar (Chen *et al.* 2014), rooster (Cheng *et al.* 2015), carp (Li *et al.* 2010), sea bream (Zilli *et al.* 2014) and trout (Nynca *et al.* 2014a). However despite these encouraging developments, many of these studies have used mass spectrometry to identify differentially abundant spots from 2D gel electrophoresis (2DGE). While an effective approach, 2DGE is not without its limitations, such as limited sensitivity of the densitometry analysis, poor detection of proteins with very high or low pI, under representation of small (< 10 kDa) or large (> 100 kDa) proteins, and limited detection of hydrophobic proteins. Labelled (e.g. iTRAQ) or label free quantification of whole sperm lysates, as previously published for boar (Chen *et al.* 2014) and human (Bogle *et al.* 2017) spermatozoa, offers a more comprehensive assessment of proteomic changes due to cryopreservation, including changes to low abundance proteins not detectable by 2DGE. However, these methods are yet to be applied to ram spermatozoa to compare proteomic changes due to cryopreservation, or to investigate proteins which may be conferred by freezing media containing egg yolk.

One such method is a non-labelled, quantitative acquisition technique, involving data independent sequential window acquisition of all theoretical mass spectra (SWATH-MS; (Gillet *et al.* 2012)). This technique has been employed in recent years to investigate proteomic changes in bodily fluids and somatic cells from a wide range of species with high success (Anjo *et al.* 2017). SWATH based data-independent acquisition facilitates simultaneous high throughput scanning and fragmentation in specified *m/z* blocks, identifying all peptides within a given mass range. Spectra are then compared back to an ion library which computationally generates objective and reliable quantification (Vowinckel *et al.* 2013). To date, Perez-Patiño *et al.* (2016) is the only study to apply SWATH-MS in a reproductive context, investigating proteomic differences between portions of the boar ejaculate. Therefore, applying this technique to ram spermatozoa offers a novel, accurate method of quantifying potentially small but biologically relevant proteomic differences between fresh and frozen thawed ram spermatozoa.

The aim of the current study is to utilise LC-MS/MS employing SWATH acquisition to develop a quantitative picture of the proteomic differences between fresh and frozen thawed ram spermatozoa, as well as any proteins which are contributed to spermatozoa by an egg yolk based medium. We hypothesise that the sperm proteome will be significantly altered both by exposure to egg yolk and cryopreservation, and that proteins which are lost or gained may have important functional roles, including altering the ability of frozen thawed ram spermatozoa to successfully traverse the ovine cervix.

3.3. MATERIALS AND METHODS

3.3.1. Chemicals

Unless otherwise stated, all reagents were sourced from Sigma Aldrich (Castle Hill, Australia).

3.3.2. Animals

Mature merino rams (*n* = 3) and ewes (*n* = 2) used for collection were housed at the University of Sydney, Camperdown campus. Animals were maintained on a chaff based diet, supplemented with lupins. All procedures were approved by the University of Sydney animal ethics committee (approval 2013/5854).

3.3.3. Collection and preparation of spermatozoa

Ejaculates (*n* = 2/ram) were collected by artificial vagina (June, 2016) from Merino rams (*n* = 3) in the presence of a teaser ewe. Ejaculates were immediately assessed for wave motion (data not shown), and only accepted if wave motion scored ≥ 4 out of 5. Samples were slowly extended 1 + 3 (semen + diluent; v/v) with either warmed (37°C) tris-citrate-fructose solution (‘fresh’; 308 mM tris, 104 mM citric acid (monohydrate), 28 mM D-fructose, pH 7.3), a tris-

citrate-fructose solution supplemented with 15% (v/v) egg yolk ('fresh + EY') or tris-citrate-glucose solution supplemented with 15% (v/v) egg yolk and 5% (v/v) glycerol ('frozen', (Evans and Maxwell 1987)). Frozen samples were chilled to 5°C and frozen by the pellet method (250 µL; Evans and Maxwell, 1987). Briefly, 250 µL of sample was deposited onto a block of dry ice for 3 min, after which the pellet was submerged in liquid nitrogen. Pellets were thawed in a dry glass tube by agitating for 2 min in a 37°C water bath.

Immediately post dilution (fresh and fresh + EY) or thawing (frozen), samples were washed free of seminal plasma and freezing diluent by a 'swim up' procedure. Briefly, 500 µL aliquots of sample were layered under 3 mL of warmed phosphate buffered saline (PBS) and incubated for 1 h at 38.5°C. Post incubation, the top 2 mL was removed and centrifuged (900 × *g*; 10 min; room temperature). The supernatant was discarded and the concentration of the resultant pellet was determined using a haemocytometer (Neubauer Improved, Precicolor HBG; Giessen-Lützellinden, Germany) before being resuspended to 500 × 10^6 spermatozoa/mL with PBS. An aliquot was taken to assess sperm viability. Briefly, samples stained for 10 min with SYBR-14 (final concentration 100 nM) and propidium iodide (final concentration 6 µM) were assessed using an Accuri C6 flow cytometer (Becton Dickson) equipped with a standard argon laser (488 nm) and suitable detectors (533/30 nm BP, > 670 nm LP), reading a minimum of 10, 000 spermatozoa per sample.

The remainder of each sample was centrifuged again (900 × *g*; 10 min; room temperature) before resuspension in lysis buffer (62.5 mM tris, 2% (w/v) sodium dodecyl sulphate (SDS) and cOmplete protease inhibitor cocktail; 1:1.5 v/v). Lysates were standardised to contain approximately 200-300 x 10^6 total spermatozoa. Samples were vortexed for 2 min before being left to stand at room temperature for 1 hour, vortexing every 15 min. Lysed samples were then centrifuged (7, 500 × *g*; 15 min; room temp), the supernatant collected and stored at - 80°C until further use.

3.3.4. Digestion and preparation of samples for mass spectrometry

SDS was removed from sperm lysates using a chloroform/methanol precipitation as previously described by Wessel and Flügge (1984). Protein concentration was determined using a Qubit protein assay (2.0 fluorometer; Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions and was consequently standardised to 100 µg of total protein with 50 mM ammonium bicarbonate. Samples were reduced (10 mM TCEP; 1 h; 37°C), alkylated (50 mM iodoacetamide; 30 min; room temperature in the dark) and digested overnight with trypsin (final ratio 1:50 (v/v) trypsin: substrate; 37°C; Promega; Madison, WI, USA). Digested samples were then desalted using a C18 Oasis HLB column (Waters; Elstree, Herts, UK) and vacuum dried prior to mass spectrometry. Samples for LC-MS/MS were resuspended in 3% (v/v)

acetonitrile, 0.1% (v/v) formic acid (1 µg injection). A global standard was created to enable the generation of an ion spectral library. Equal amounts of protein from each sample (*n* = 9) were pooled, dried down and resuspended in 90% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid (10 µg injection; 2D LC-MS/MS).

3.3.5. Generation of an ion spectral library using 2D LC-MS/MS

The global standard was processed using 2D LC-MS/MS to obtain superior protein separation and generate a comprehensive ion spectral library for quantitative analysis of SWATH acquisition data. Peptides were first fractionated using an Agilent 1200 HPLC system coupled to an in house built fritless TSK-Amide 80 HILIC column (4.5 mm (i.d.) × 17 cm column, 5 µm particle size). LC mobile phase buffers were comprised of A: 0.1% (v/v) trifluoroacetic acid (TFA) and B: 90% (v/v) acetonitrile, 0.1% (v/v) TFA. A total of 12 fractions were eluted into a V bottom 96 well polypropylene plate (Griener, Sigma Aldrich, Castle Hill, Australia) using 100% B for 26.5 min, followed by a linear gradient of 60- 30% B over 11 min and then 100% B wash over 2 min at a flow rate of 6 µL/min. The resultant fractions were dried down and resuspended in 3% (v/v) acetonitrile, 0.1% (v/v) formic acid ready for LC-MS/MS.

Approximately 1 µg/5 µL of each global standard fraction was separated by nano-LC using an Eksigent 415 UHPLC system (Sciex, Foster City, USA) coupled to an in-house built fritless nano column (75 µm (i.d.) × 20 cm column) packed with ReproSil Pur 120 C18 stationary phase (1.9 µm particle size, Dr Maisch GmbH, Germany). LC mobile phase buffers were comprised of A: 0.1% (v/v) formic acid and B: 80% (v/v) acetonitrile, 0.1% (v/v) formic acid. Peptides were eluted using a linear gradient of 5% to 40% B over 90 min and then 95% B wash over 1 min at a flow rate of 300 nL/min. Mass spectra were acquired in the mass-to-charge ratio (*m/z*) range of 350 – 1, 500 using a 6600 TripleTOF mass spectrometer (Sciex, Foster City, USA). Up to 50 of the most abundant ions, with charge states between + 2 to + 5 were sequentially isolated and fragmented, and a product ion scan collected over 100 to 1800 *m/z*. Ions selected for MS/MS were dynamically excluded for 20 seconds.

3.3.6. LC-MS/MS of spermatozoa employing SWATH acquisition

One microgram of each sample was separated by nano-LC using the same 90 min gradient conditions described above. MS/MS runs were conducted using a 6600 TripleTOF mass spectrometer (Sciex, Foster City, USA) in data independent acquisition mode, using SWATH acquisition. A total of 34 windows at a width of 54 Da covered the mass range 300 – 1,500 Da. An overlap of 1 Da between each SWATH window was used. An accumulation time of 96 ms was used for each fragment ion scan and for the survey scans acquired at the beginning of each cycle, resulting in a total cycle time of 3.3 s. The rolling collision energy for each window was 2 +. The mass spectrometer was operated in high sensitivity mode.

3.3.7. Peptide identification by comparison to ion spectral library

An ion spectral library was generated by searching 2D LC results (.wiff format) of the global standard in ProteinPilot (version 5.0, Sciex, Foster City, USA), using the Paragon search algorithm. Search parameters included iodoacetamide as an alkylating agent, trypsin as a protease, thorough ID search effort and a detected protein threshold of 0.05. Two unique ion spectral libraries were produced; one by searching the global standard against an NCBI database for all mammals (downloaded August 2016), with *Ovis aries* as the 'species' search parameter*,* and the other against an NCBI database specific to the domestic chicken *Gallus gallus* (downloaded September 2017). The ion spectral library searched against *Ovis aries* was used for identification of proteins in fresh and frozen samples, while the ion spectral library searched against the domestic chicken was used for identification of proteins in fresh and fresh + EY samples.

Identification of proteins was performed in the SWATH microapp within PeakView (version 1.2.0.4; Sciex, Foster City, USA). The ion spectral library of interest was imported (.group format), excluding shared peptides. Retention time calibration was performed by selecting house-keeping peptides spaced equally along the 90 min gradient, with high intensity (> 2500) of the fragment ion and no modifications. Fresh and fresh + EY samples were compared to the chicken ion spectral library using the following processing parameters; 5 peptides per protein, 6 transitions per peptide, 90% peptide confidence, exclusion of shared peptides, XIC extraction window 5 min and XIC width 0.1 Da. Fresh and frozen samples were compared to the sheep ion spectral library using the following processing parameters; 4 peptides per protein, 4 transitions per peptide, 99% peptide confidence, exclusion of shared peptides, XIC extraction window 5 min and XIC width 0.1 Da.

3.3.8. Statistical analysis

Proteins with a false discovery rate below 1% were exported into MarkerView (version 1.3.1, Sciex, Foster City, USA) for statistical analysis. A t-test (α of 0.05) was used to compare fresh versus fresh + EY samples and fresh versus frozen samples respectively. High fold change (> 1.5 or < 0.5) was applied as a further cut off to ensure statistical significance and p values were corrected for multiple testing by controlling for a 1% false discovery rate during analysis using the Benjamini-Hochberg method. A principal component analysis of significantly different proteins was used to visualise variation due to replicate and treatment.

Sperm viability data was statistically analysed in Genstat (version 18, VSN International). Data were analysed for normality to ensure test assumptions were met. Viability data was assessed using a linear mixed model (α of 0.05), accounting for treatment as a fixed effect and replicate

and ram as random effects. Means were compared by least significant difference and are reported as the mean ± standard error of the mean.

3.3.9. Gene ontology and functional protein associations

Proteins of interest which were found to be significantly different between treatment groups were assessed for molecular function and biological process Gene Ontology (GO) terms using the PANTHER Classification System (version 12; www.pantherdb.org), with *Homo sapiens* or *Gallus gallus* as the organism to maximise classifications. Protein interactions and associations were also assessed using STRING (version 10.5; www.string-db.org).

3.4. RESULTS

3.4.1. Viability of samples used for proteomic analysis

Following swim up and prior to sperm lysis, there were no significant differences in the percentage of viable spermatozoa between fresh (59.1 ± 10.3%), fresh + EY (58.3 ± 8.6%) and frozen (39.0 ± 8.6%) treatments ($p > 0.05$)

3.4.2. Proteins conferred to ram spermatozoa by chicken egg yolk

2D LC-MS/MS analysis of a global standard containing equal amounts of all samples resulted in identification of 8, 170 distinct peptides, which were matched to a total of 1, 713 protein groups from the domestic chicken *Gallus gallus* at a 1% FDR (Supplementary table 3.1). Following comparison to this ion spectral library, 442 proteins were confidently identified and quantified in all samples (Supplementary table 3.2), and 20 of these proteins were found to significantly increase after exposure of spermatozoa to egg yolk, on the basis of p value ($p < 0.05$) and fold change (> 1.5). Of these proteins, 15 have previously been identified in proteomes of hen's egg yolk (Mann and Mann 2008; Farinazzo *et al.* 2009; Gao *et al.* 2017), including vitellogenins, apolipoproteins, complement C3 and ovotransferrin (Table 3.1). The remaining 5 proteins (C1orf167, RAB5B, COPS3, AFG3L2 and MUT; Supplementary table 3.2) have not previously been identified in any published egg yolk proteome. Clustering of samples within a principal component analysis showed that variation between significant proteins in fresh and fresh + EY samples was largely due to treatment (Figure 3.1).

Table 3.1 Proteins of egg yolk origin* which were significantly increased in lysates of spermatozoa after exposure to hen's egg yolk identified by SWATH LC-MS/MS and sorted by fold change

Gene symbol	Protein name	Mean fresh	Mean fresh + EY	p-value	Fold change
VMO1	Vitelline membrane outer layer protein 1 homolog	26,482	5,075,452	0.002	191.7
VTG2	Vitellogenin-2 precursor	354,926	32,130,459	0.004	90.5
APOVLDLII	Apovitellenin-1 isoform X1	84,711	5,446,670	0.001	64.3
LOC417848	Cathepsin E-A isoform X1	36,153	1,641,658	0.039	45.4
APOA1	Apolipoprotein A-I preproprotein	44,896	1,885,410	0.007	42.0
VTG1	Vitellogenin-1 precursor	46,156	839,571	0.002	18.2
VTG3	Vitellogenin-3	119,508	2,114,586	0.000001	17.7
APOB	Apolipoprotein B precursor	48,093	790,094	0.004	16.4
APOH	Beta-2-glycoprotein 1 precursor	11,978	164,451	0.033	13.7
C3	Complement C3 precursor	62,645	430,355	0.00002	6.9
TF	Ovotransferrin precursor	52,722	283,307	0.017	5.4
CP	Ceruloplasmin	11,610	56,729	0.006	4.9
ALB	Serum albumin precursor	979,391	3,489,738	0.017	3.6
HSPA8	Heat shock cognate 71 kda protein	46,189	126,727	0.010	2.7
HPX	Hemopexin	56,453	127,343	0.034	2.3

*Proteins were identified by comparison to an ion spectral library produced by Paragon search against a *Gallus gallus* NCBI database. Only those proteins which have been previously identified in proteomes of hen's egg yolk (Mann and Mann 2008; Farinazzo et al. 2009; Gao et al. 2017) were included.

3.4.3. Proteins which differed significantly after cryopreservation of ram spermatozoa

2D LC-MS/MS analysis of a global standard containing equal amounts of all samples resulted in identification of 19, 302 distinct peptides, which were matched to a total of 1, 154 protein groups from all mammals, with an identification preference for *Ovis aries* at a 1% FDR (Supplementary table 3.3). Following comparison to this ion spectral library, 1, 082 proteins were confidently identified and quantified in all samples (Supplementary table 3.4), with tubulins, zona pellucida-binding protein 1 (ZPBP1), izumo sperm-egg fusion protein 4 (IZUMO4), ATP synthase subunit β (ATP5B) and hyaluronidase PH-20-like (SPAM1) being the most abundant proteins. A total of 51 proteins were found to differ significantly between fresh and frozen treatments on the basis of p value ($p < 0.05$) and fold change (> 1.5 or < 0.5).

Table 3.2 Proteins identified by LC-MS/MS and SWATH which were present in significantly different quantities in lysates of ram spermatozoa after cryopreservation

Gene symbol	Protein name	Mean fresh[1]	Mean frozen[2]	p-value	Fold change[3]
BTD	Biotinidase isoform X1 [Ovis aries]	16,385	144,316	0.02	8.81
NFS1	Cysteine desulfurase, mitochondrial [Ovis aries]	14,358	64,853	0.03	4.52
SPCS2	Signal peptidase complex subunit 2 [Ovis aries]	7,071	27,695	0.001	3.92
SMPD4	Sphingomyelin phosphodiesterase 4 isoform X2 [Ovis aries musimon]	11,918	40,617	0.001	3.41
SLC25A32	Mitochondrial folate transporter/carrier [Ovis aries musimon]	120,949	359,449	0.02	2.97
MARS2	Methionine-tRNA ligase, mitochondrial isoform X2 [Ovis aries musimon]	13,471	39,469	0.001	2.93
FER	Tyrosine-protein kinase Fer isoform X2 [Ovis aries musimon]	20,514	57,035	0.03	2.78
ARMC3	Armadillo repeat-containing protein 3 isoform X2 [Ovis aries]	79,851	216,047	0.004	2.71
SERPINB1	Leukocyte elastase inhibitor [Ovis aries]	60,465	156,951	0.03	2.60
LOC101113819	ATP-binding cassette sub-family A member 3-like isoform X1 [Ovis aries]	228,077	559,403	0.03	2.45
HMGCL	Hydroxymethylglutaryl-CoA lyase, mitochondrial [Ovis aries]	39,857	93,351	0.0004	2.34
CASC1	Protein CASC1 isoform X1 [Ovis aries]	45,338	105,904	0.0001	2.34
WDR66	WD repeat-containing protein 66 isoform X2 [Ovis aries musimon]	19,782	45,817	0.01	2.32
RSPH6A	Radial spoke head protein 6 homolog A isoform X2 [Ovis aries musimon]	164,649	366,996	0.04	2.23
NUP58	Nucleoporin p58/p45 isoform X1 [Ovis aries]	31,283	69,240	0.02	2.21
DNAH2	Dynein heavy chain 2, axonemal isoform X2 [Ovis aries musimon]	82,943	180,970	0.02	2.18
PRSS37	Probable inactive serine protease 37 isoform X2 [Ovis aries]	4,116	8,779	0.04	2.13
PDK3	Pyruvate dehydrogenase kinase, isozyme 3 isoform X2 [Ovis aries]	53,679	108,171	0.01	2.02
ENO4	Enolase-like protein ENO4 isoform X2 [Ovis aries musimon]	34,349	67,418	0.04	1.96
LRRD1	Leucine-rich repeat and death domain-containing protein 1 [Ovis aries musimon]	23,745	45,558	0.03	1.92
C3H2orf61	Uncharacterized protein C2orf61 homolog isoform X2 [Ovis aries]	6,736	12,779	0.04	1.90
LOC101103112	Phospholipid scramblase 1 isoform X1 [Ovis aries]	3,334	6,291	0.04	1.89
LOC105603539	Protein piccolo-like [Ovis aries musimon]	7,814	14,584	0.02	1.87
MROH9	Maestro heat-like repeat-containing protein family member 9 isoform X1 [Ovis aries musimon]	8,897	16,074	0.04	1.81
PNPT1	Polyribonucleotide nucleotidyltransferase 1, mitochondrial isoform X1 [Ovis aries]	16,559	28,615	0.02	1.73

MAN2A1	Alpha-mannosidase 2 isoform X2 [Ovis aries]	20,973	34,680	0.01	1.65
IQCA1L	IQ and AAA domain-containing protein 1-like isoform X2 [Ovis aries musimon]	55,264	88,952	0.04	1.61
ALDH3A2	Fatty aldehyde dehydrogenase isoform X1 [Ovis aries]	39,833	17,847	0.03	0.45
TSTA3	GDP-L-fucose synthase isoform X1 [Ovis aries]	28,518	12,669	0.04	0.44
HIP1	Huntingtin-interacting protein 1 isoform X3 [Ovis aries musimon]	322,900	138,475	0.02	0.43
LOC101123216	Disintegrin and metalloproteinase domain-containing protein 20-like [Ovis aries musimon]	17,909	7,445	0.02	0.42
SYPL1	Synaptophysin-like protein 1 isoform X2 [Ovis aries musimon]	12,781	4,905	0.03	0.38
NPEPPS	Puromycin-sensitive aminopeptidase isoform X4 [Ovis aries musimon]	224,158	84,942	0.002	0.38
IDH1	Isocitrate dehydrogenase [NADP] cytoplasmic [Ovis aries]	112,278	39,941	0.02	0.36
ATP6V1C1	V-type proton ATPase subunit C 1 isoform X2 [Ovis aries]	93,030	33,007	0.01	0.35
HEXB	Beta-hexosaminidase subunit beta-like isoform X1 [Ovis aries]	38,875	13,692	0.03	0.35
APEH	Acylamino-acid-releasing enzyme isoform X1 [Ovis aries]	113,338	39,565	0.02	0.35
LOC101111911	D-dopachrome decarboxylase isoform X2 [Ovis aries musimon]	169,588	58,087	0.01	0.34
HSPA4L	Heat shock 70 kDa protein 4L isoform X1 [Ovis aries]	386,639	132,030	0.001	0.34
CUL3	Cullin-3 isoform X1 [Ovis aries]	210,181	69,011	0.04	0.33
CCT4	T-complex protein 1 subunit delta [Ovis aries musimon]	2,723,485	843,621	0.04	0.31
CLMN	Calmin isoform X6 [Ovis aries musimon]	77,419	21,974	0.01	0.28
TCP1	T-complex protein 1 subunit alpha [Ovis aries]	391,737	110,011	0.03	0.28
ART5	ART5 protein, partial [Macaca speciosa]	49,771	13,040	0.04	0.26
ICA	Inhibitor of carbonic anhydrase-like isoform X3 [Ovis aries musimon]	84,589	21,715	0.002	0.26
VAT1	Synaptic vesicle membrane protein VAT-1 homolog isoform X2 [Ovis aries musimon]	817,262	194,375	0.04	0.24
TRAP1	Heat shock protein 75 kDa, mitochondrial isoform X3 [Ovis aries]	865,914	186,078	0.02	0.21
TEPP	Testis, prostate and placenta-expressed protein [Bison bison bison]	26,406	5,571	0.03	0.21
TOM1L1	TOM1-like protein 1 isoform X2 [Ovis aries]	109,974	18,620	0.03	0.17
CCT8	T-complex protein 1 subunit theta isoform X1 [Bos taurus]	830,033	123,201	0.04	0.15
CSNK1G2	Casein kinase I isoform gamma-2 isoform X2 [Ovis aries musimon]	209,399	14,396	0.04	0.07

[1]Fresh – fresh spermatozoa diluted in tris-citrate-fructose medium [2]Frozen – frozen thawed spermatozoa diluted pre-freeze in tris-citrate-glucose medium with 15% (v/v) egg yolk [3]Fold change >1 = increased after freezing, <1 = decreased after freezing

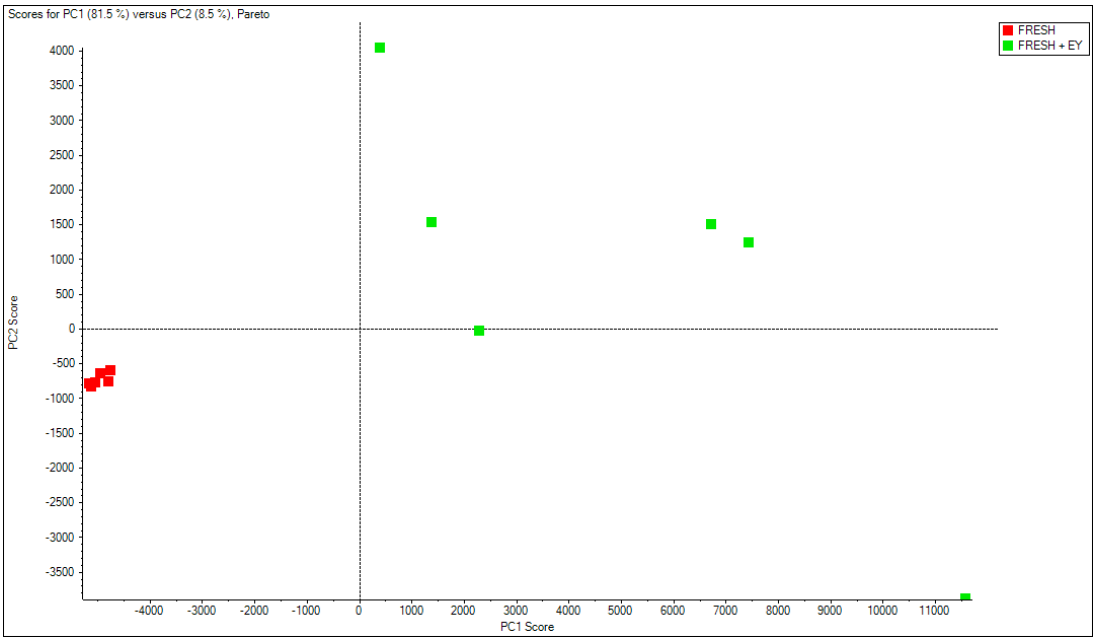

Figure 3.1 Principal component analysis of variation in proteins which were significantly different after exposure to 15% (v/v) egg yolk

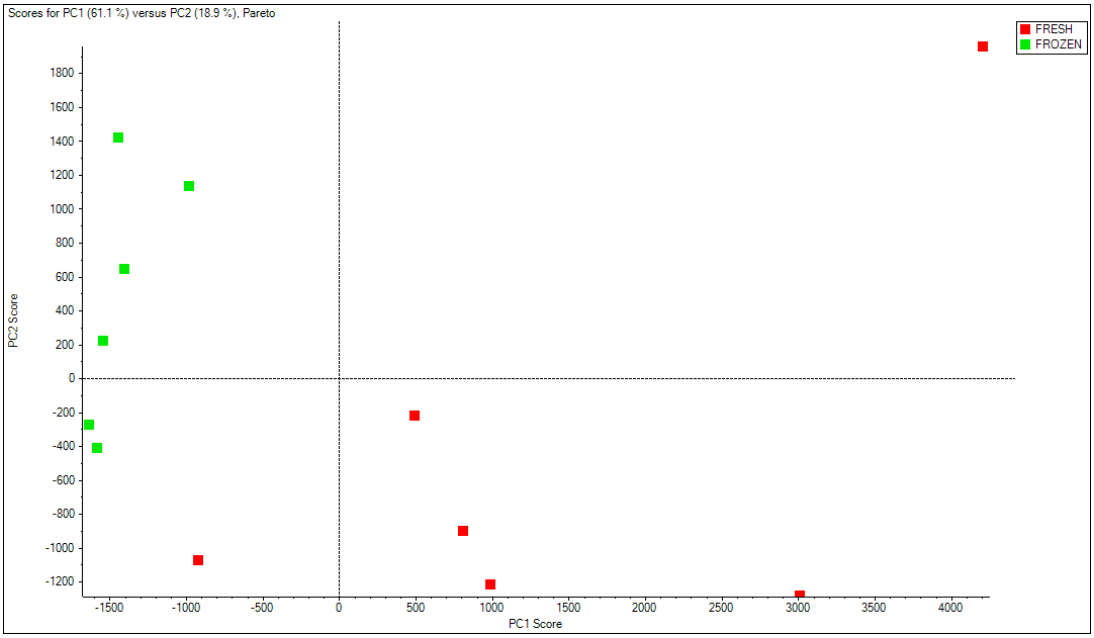

Figure 3.2 Principal component analysis of variation in proteins which were significantly different after cryopreservation

Of these 51 proteins (Table 3.2), 27 proteins increased ($p < 0.05$, fold change > 1.5) when ram spermatozoa were cryopreserved. The proteins which recorded the greatest fold change were biotinidase (BTD), cysteine desulfurase (NFS1) and signal peptidase complex subunit 2 (SPCS2). Additionally, 24 proteins were found to decrease ($p < 0.05$, fold change < 0.5) when ram spermatozoa were cryopreserved (Table 3.2). The proteins which recorded the greatest

fold change were TOM1-like protein (TOM1L1), T complex protein 1 subunit theta (CCT8) and casein kinase 1 subunit γ 2 (CSNK1G2). Clustering of samples within a principal component analysis showed that variation between significant proteins in fresh and frozen samples was largely due to treatment (Figure 3.2).

3.4.4. Gene ontology and STRING pathways

Of the proteins which significantly increased after exposure to egg yolk, the majority were classified as having catalytic (27.8%, GO: 0003824, e.g. MUT, AFG3L2, LOC417848) or binding (16.7%, GO: 0005488, e.g. APOA1, C3, TF) molecular functions and being involved in metabolic (33.3%, GO: 0008152, e.g. APOB, COPS3, AFG3L2) or cellular processes (44.4%, GO: 0009987, e.g. RAB5B, APOH, APOA1). Interestingly, several proteins were identified as performing immune functions (GO: 0002376, APOH, HSPA8, C3) and having lipid transporter activity (GO: 0005319, APOA1, APOB). Of the proteins significantly altered by freezing, the majority were classified as having either a catalytic (54.8%; GO: 0003824, e.g. PNPT1, VAT1, MAN2A1) or binding (19.4%; GO: 0005488, e.g. CLMN, CUL3, FER) molecular function and being involved in a metabolic (32.3%; GO: 0008152, e.g. CCT8, CSNK1G2, PDK3) or cellular (24.6%; GO: 0009987, e.g. TOM1L1, RSPH6A, HIP2) biological process, with a wide variety of protein classes represented.

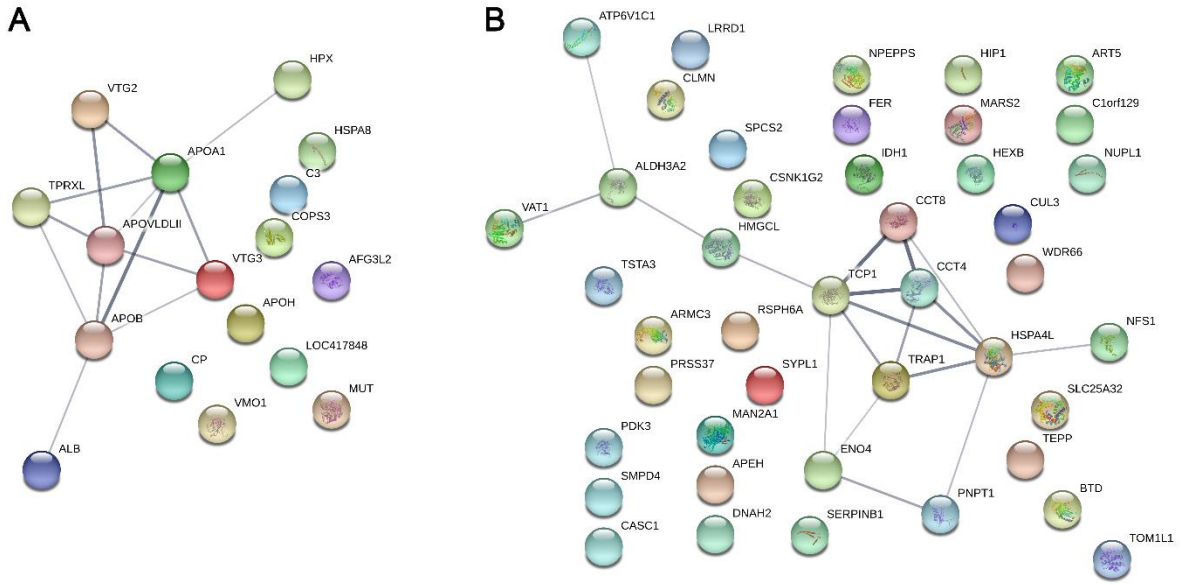

Figure 3.3 Protein network association determined by STRING (www.string-db.org) for proteins contributed to spermatozoa by hen's egg yolk (**A**) or significantly altered after cryopreservation of ram spermatozoa (**B**)

STRING analysis of the proteins contributed by egg yolk formed networks which were largely based on co-expression and similarity of function (Figure 3.3A). STRING confidently identified one protein association network in proteins altered by freezing, based on chaperone activity (TCP1, CCT4, CCT8, HSP4AL and TRAP1; Figure 3.3B).

3.5. DISCUSSION

Using LC-MS/MS and employing SWATH acquisition, we have successfully identified 15 proteins which are conferred to spermatozoa during incubation in media containing hen's egg yolk and 51 proteins which were significantly altered following industry standard cryopreservation of ram spermatozoa. While the addition of egg yolk proteins to frozen thawed spermatozoa has certainly been previously suggested (Ollero *et al.* 1998a), to our knowledge this is the first time that the transfer of proteins from egg yolk based media to spermatozoa has been both confirmed and profiled using a highly sensitive, quantitative mass spectrometry method. We have further applied these tools to characterise the gain and loss of proteins following sperm cryopreservation. As spermatozoa are transcriptionally silent, it is likely that proteins which were detected as more highly abundant in frozen thawed spermatozoa were taken up from the surrounding fluid during cryopreservation, having originated from seminal plasma or potentially its extracellular vesicles (Piehl *et al.* 2013). A second explanation, particularly relevant to typically intracellular proteins, is that these proteins were made more available in lysates of frozen thawed spermatozoa due to increased membrane sensitivity to detergents (Schweisguth and Hammerstedt 1992). In contrast, those proteins which were lost from spermatozoa following cryopreservation may be lost by shedding, degradation or active cleavage, however such mechanisms have not been investigated. Previous studies have identified anywhere from 6 (Zilli *et al.* 2014) to over 90 (Bogle *et al.* 2017) sperm proteins in a range of species, which were significantly altered by cryopreservation, with the majority identifying between 10 and 30 significantly different proteins (Chen *et al.* 2014; Wang *et al.* 2014; Cheng *et al.* 2015; Westfalewicz *et al.* 2015; He *et al.* 2016). Thus this study represents a relative advancement in the label free detection of protein based changes due to cryopreservation. In addition, we have identified proteins of interest linked to sperm function and fertility which offer direction for future study.

Cryopreservation of spermatozoa involves many separate processes, and each may contribute to the alteration of the sperm proteome. For example, the addition of the cryoprotectant glycerol has previously been shown to alter 10 sperm proteins (Yoon *et al.* 2016). To date, there have been no in-depth proteomic studies on the impact of the cryoprotectant egg yolk on the sperm proteome, in ram or any other species. The proteome of hen's egg yolk is well established (Mann and Mann 2008; Farinazzo *et al.* 2009; Gao *et al.* 2017), and contains a variety of egg specific proteins (e.g. vitellogenins 1, 2 and 3), as well as more common protein families (e.g. apolipoproteins; APOA1, APOB). Our results indicated that a total of 15 proteins previously identified in egg yolk proteomes were present at significantly higher levels in spermatozoa after exposure to egg yolk. Apart from a few proteins with similar structure and function (e.g. vitellogenins, apolipoproteins), these 15 proteins did

not form any significant networks. The cryoprotective nature of egg yolk is largely attributed to low density lipoprotein (LDL; (Moussa *et al.* 2002; Hu *et al.* 2010)), however it is possible that other proteins we have identified in this study may play some role in cryoprotection, for example the antioxidant capacity of metal chelators hemopexin (Tolosano and Altruda 2002) and transferrin (Pacht and Davis 1988). Further investigation is required to determine how these egg yolk proteins affect sperm function, particularly during cryopreservation. An additional 5 proteins (C1orf167, RAB5B, COPS3, AFG3L2 and MUT) were also significantly increased after exposure to egg yolk, however at present we are unable to confirm their origin due to their absence from published studies of the egg yolk proteome. While only AFG3L2 has previously been identified in lysates of ram spermatozoa (Pini *et al.* 2016), these could alternatively be differentially abundant sperm proteins. Several proteins were reported as significantly decreased following exposure to egg yolk. As a number of these proteins have previously been identified in a proteome of fresh ram spermatozoa (Pini *et al.* 2016) and none have ever been identified in an egg yolk proteome (Mann and Mann 2008; Farinazzo *et al.* 2009; Gao *et al.* 2017), we suggest that these are intrinsic sperm proteins which are altered in abundance by exposure to egg yolk.

Interestingly, some of the proteins conferred by egg yolk may have negative effects on spermatozoa post thaw. APOA1, the main protein component of high density lipoprotein (HDL), is central to cholesterol efflux (Takahashi and Smith 1999), which may have negative effects on sperm function. Cholesterol efflux is a hallmark of sperm capacitation (Osheroff *et al.* 1999), and premature cholesterol loss from the plasma membrane leads to membrane destabilisation (Leahy and Gadella 2015) and loss of acrosome integrity (Iborra *et al.* 2000). Such disturbance to the sperm membrane could reduce the longevity of spermatozoa in the female tract, potentially impacting in vivo fertility. Spermatozoa exposed to egg yolk in this study also had a 7 fold increase in the level of the complement component C3, the most abundant complement protein and a key player in both the promotion of inflammation and opsonisation of cells (Carroll and Sim 2011). Increased phagocytosis of both red blood cells and yeast has been directly attributed to opsonisation by the C3 protein (Matsuyama *et al.* 1992), and as such spermatozoa exposed to egg yolk may be more susceptible to phagocytosis following insemination. This is supported by in vitro immune cell binding to spermatozoa, which is increased by exposure to media containing egg yolk (Li *et al.* 2012; Pini *et al.* 2017). Such an increase in phagocytosis could be detrimental to the passage of frozen thawed spermatozoa through the ovine cervix, however this relationship warrants further investigation. The effects of other egg yolk proteins have not been characterised, and further research in this area could improve our understanding of both the protective mechanisms and potential downsides of this widely used cryoprotective agent.

While the mechanisms by which proteins are lost from spermatozoa during cryopreservation are not yet clear, the loss itself may result in sublethal sperm damage and be responsible for poor sperm function post thaw. Molecular chaperones, including subunits of the chaperonin containing TCP1 (CCT) complex and various heat shock proteins (HSPs), constituted 20% of the proteins significantly reduced by cryopreservation. Further, these proteins represented some of the most substantial fold changes observed. This is in agreement with previous studies which have shown loss of these chaperone proteins from spermatozoa during cooling and cryopreservation (Huang *et al.* 1999; Dietrich *et al.* 2015; Nynca *et al.* 2015a; Varghese *et al.* 2016; Bogle *et al.* 2017). While the reason for the significant reduction in the abundance of chaperone proteins is unclear, these results demonstrate that HSP and CCT proteins are particularly impacted by the cryopreservation process. Both HSPs and the CCT complex fulfil important roles, including ensuring correct protein folding (Naletova *et al.* 2011), regulating capacitation related events (Li *et al.* 2014) and assembly of adhesion proteins into functional zona pellucida receptor complexes (Dun *et al.* 2011; Nixon *et al.* 2015). HSPs in particular also exhibit important stress response functions, including stabilisation of membrane lipid rafts (Chen *et al.* 2005), clearance of damaged proteins, refolding of aggregated proteins and suppression of apoptosis pathways (Kalmar and Greensmith 2009), particularly in response to oxidative stress. The response to stress is an important mechanism which may be compromised by reduced levels of chaperone proteins, exemplified by the increased susceptibility of frozen thawed spermatozoa to osmotic (Khan and Ijaz 2008; Pinto and Kozink 2008) and oxidative stress (Neild *et al.* 2005; Garg *et al.* 2009). Overall, the loss of proteins with chaperone roles may create significant problems for normal cellular functioning of frozen thawed spermatozoa, and may limit their ability to respond to environmental stress. Supplementation of these lost chaperone proteins may offer a significant therapeutic benefit to cryopreserved spermatozoa and deserves further investigation. Several other interesting proteins were altered by the cryopreservation process. Casein kinase 1 subunit γ 2, a membrane bound serine/threonine protein kinase with phosphorylation activity, and inhibitor of carbonic anhydrase were both significantly reduced by freezing. These proteins may have some role in capacitation due to their involvement in phosphorylation (Visconti *et al.* 2011) and bicarbonate metabolism (Flesch *et al.* 2001; Harrison and Gadella 2005) respectively. In addition, two other proteins which were decreased after cryopreservation, ADAM20-like and β hexosaminidase (aka β-N-acetylglucosaminidase), are believed to play important roles in oocyte binding (Hooft van Huijsduijnen 1998; Miranda *et al.* 2000) and penetration (Miller *et al.* 1993). While mechanisms of zona pellucida binding may be redundant, disruption of ADAMs results in total infertility of mice due to a lack of zona binding (Okabe and Cummins 2007). On the other hand, while β hexosaminidase is not solely responsible for the zona

binding capacity of human spermatozoa (Miranda *et al.* 2000), its loss may still reduce overall binding success rates.

Those proteins which underwent the largest positive fold changes, including biotinidase (BTD), cysteine desulfurase (NFS1) and signal peptidase complex subunit 2 (SPCS2), have divergent roles which have largely been investigated in somatic cells. BTD plays a key role in the metabolism of biotin (Wolf 2005), a vitamin found in relatively high concentrations in egg yolk (Romanoff and Romanoff 1949), and as such the significant increase in this protein may be connected to the presence of egg yolk during cryopreservation. NFS1 is involved in the regulation of cellular iron homeostasis through its participation in the iron-sulfur cluster assembly complex (Kispal *et al.* 1999). SPCS2, also known as SPC25, forms part of a signal peptidase complex responsible for co- and post-translational cleavage of signal peptides from secretory proteins (Green *et al.* 2002). Interestingly, this particular subunit has been identified previously in microsomes isolated from hen oviduct (Miles 2002), again implicating egg yolk as a possible source. While the consequences of the observed increases in BTD, NFS1 and SPCS2 are unclear, further investigation into their specific functions in spermatozoa may help to elucidate any such effects. Aside from those proteins with the highest fold changes, several other proteins which increased following cryopreservation may help to explain some of the observed properties of cryopreserved spermatozoa. Cryopreservation causes a significant increase in the protease activity of the surrounding cryoprotective medium, likely due to loss of membrane integrity of many cells (Gurupriya *et al.* 2014). This presents a serious threat to those spermatozoa which remain viable, however we detected a significant increase in the level of SERPINB1 in spermatozoa following freezing, possibly originating from seminal plasma (Soleilhavoup *et al.* 2014). SERPINB1 is a potent inhibitor of a range of proteases, acting as a ‘suicide substrate’ to spare cellular proteins from degradation, and also shows some anti-apoptotic properties (Torriglia *et al.* 2017), which could potentially benefit surviving cells. We also found a significant increase in the tyrosine protein kinase FER, which is responsible for murine capacitation associated protein tyrosine phosphorylation (Alvau *et al.* 2016), and could be linked to cryopreservation induced development of tyrosine phosphorylation (Naresh and Atreja 2015). As the exact mechanism of ‘cryocapacitation’ related tyrosine phosphorylation is not currently understood, further investigation into the activity of tyrosine protein kinase FER during cryopreservation is warranted. Even solely on the basis of these few proteins, cryopreservation clearly induces important changes in the sperm proteome which may have far reaching impacts during various stages of sperm capacitation and fertilisation.

In the present study we have identified a large number of proteins altered by cryopreservation, spanning a range of protein classes and functions. Interestingly, few of the proteins which we identified as significantly altered in abundance following cryopreservation have been identified in other studies. This may be as a result of differences in methods used for determining quantitative differences (e.g. 2DGE versus whole sperm lysates, unlabelled versus labelled quantification (Neilson *et al.* 2011)) or species specificity in response to cryopreservation (Thurston *et al.* 2002). Compared to a previous 2DGE based investigation of protein changes in cryopreserved ram spermatozoa, we were able to identify changes to more than double the number of proteins (He *et al.* 2016). While all of the proteins identified by He *et al.* (2016) were also identified in the current study, these proteins were not determined to be significantly different. This likely reflects the increased sensitivity of MS based quantification compared to 2DGE, densitometry based quantification. Using SWATH-MS, we were able to overcome peptide identification bias due to high sample complexity associated with traditional data dependent acquisition (Doerr 2015). In addition, using a label-free quantification method reduces the cost and labour intensity of sample preparation, as well as the time for acquisition method optimisation (Anjo *et al.* 2017). While slightly less sensitive than other acquisition methods, SWATH-MS is superior in terms of consistent detection and quantification of low abundance peptides in complex samples (Gillet *et al.* 2012). This highlights the benefit of using a whole lysate, label free SWATH-MS quantification approach, particularly for the identification of subtle but significant differences in protein abundance within high complexity samples such as spermatozoa and seminal plasma.

Using a variety of proteomic methods, we have now profiled ram seminal plasma (Soleilhavoup *et al.* 2014), the proteins conferred by seminal plasma at ejaculation (Pini *et al.* 2016), changes due to egg yolk exposure and cryopreservation, and the seminal plasma of rams with contrasting freezing tolerance (Rickard *et al.* 2015). The sum of these proteomic investigations has highlighted important proteins for normal sperm physiology, as well as proteins which may confer significant benefits to spermatozoa during cryopreservation and subsequent insemination. Future studies should aim to put these large scale data sets to practical use, analysing candidate proteins of interest for their ability to improve ram sperm cryopreservation outcomes both in vitro and in vivo. We have successfully shown that ram spermatozoa have a significantly different proteome following cryopreservation, both due to the addition of proteins from hen's egg yolk in the freezing medium, as well as freezing itself. The loss of chaperone proteins may represent a significant hindrance to the normal functioning and stress response competence of frozen thawed spermatozoa. Overall, the consequences of these changes, as well as the potential therapeutic benefit of supplementing proteins lost during cryopreservation require further investigation.

3.6. ACKNOWLEDGEMENTS

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4. Seminal plasma and cryopreservation alter ram sperm surface carbohydrates and interactions with neutrophils

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4.1. ABSTRACT

Spermatozoa deposited vaginally must navigate the physical, chemical and immune barriers of the cervix to reach the site of fertilisation. Characteristics that favour successful cervical transit remain largely unknown beyond the obvious factors of motility and viability. Epididymal and cryopreserved ram spermatozoa demonstrate poor cervical transit, for unknown reasons. We hypothesised that seminal plasma exposure and cryopreservation alter the surface sugars of these sperm populations and consequently, their interaction with immune cells, both potential factors for successful cervical transit. The carbohydrate profiles of epididymal, ejaculated and frozen thawed ram spermatozoa were assessed by flow cytometry and western blotting using lectins for galactose, sialic acid, N-acetylglucosamine and mannose. Seminal plasma exposure and cryopreservation caused significant changes to the relative amounts of surface sugars detected by flow cytometry and lectin blotting. Immune cell interaction was characterised using a neutrophil binding assay. Seminal plasma acted as a robust protective mechanism, limiting binding of spermatozoa, whereas the media used for cryopreservation caused a significant disruption to opsonin mediated binding. We were unable to demonstrate a link between changes to surface sugars and neutrophil susceptibility. Seminal plasma and cryopreservation clearly alter the sperm glycocalyx, as well as the interaction of spermatozoa with immune cells.

4.2. INTRODUCTION

The journey of a spermatozoon to the site of fertilisation is a perilous one, with the female tract presenting a host of physical, chemical and immune obstacles to overcome. In the ewe, the physical anatomy of the cervix presents a barrier in itself; a complex series of misaligned folds minimises straightforward passage towards the uterine body (Halbert *et al.* 1990). Once inside the cervix, spermatozoa face a dynamic barrier in the form of cervical mucus (Cone 2009),

which causes significant alterations to motility (Eriksen *et al.* 1998) and mitochondrial function (Martínez-Rodríguez *et al.* 2014). Finally, introduction of spermatozoa, and even cell free seminal plasma in humans and other species, causes increased production of inflammatory cytokines (Sharkey *et al.* 2007), leading to a significant influx of immune cells into the epithelial tissues (Sharkey *et al.* 2012) and lumen of the female tract (Mattner 1969; Kotilainen *et al.* 1994; Robertson *et al.* 1996), particularly at the site of the cervix in sheep (Scott *et al.* 2006). The presence of activated phagocytes can lead to significant decreases in sperm motility due to high concentrations of reactive oxygen species (ROS) (Kovalski *et al.* 1992; Baumber *et al.* 2002) and active phagocytosis of spermatozoa has been observed in vitro (Matthijs *et al.* 2000; Alghamdi *et al.* 2004). In addition to maintaining adequate motility, viability and fertilising capacity, spermatozoa must be successful in overcoming these various barriers in order to transit the length of the female tract and achieve fertilisation. Yet the specific characteristics that make a spermatozoon capable of successful cervical transit are not well understood. From studies of applied reproduction, it is clear that the absence of seminal plasma (Rickard *et al.* 2014) or undergoing the process of cryopreservation (Maxwell and Hewitt 1986; Maxwell *et al.* 1999) significantly reduces the ability of spermatozoa to transit the cervix and effect fertilisation.

The exact nature of these failures is far from certain. Epididymal, ejaculated and cryopreserved spermatozoa have previously been compared on a wide range of characteristics, particularly in terms of developmental changes due to seminal plasma exposure and damaging alterations due to freezing. These comparisons have included proteomic make up (Dostàlovà *et al.* 1994; Westfalewicz *et al.* 2015; He *et al.* 2016; Pini *et al.* 2016), membrane stability (Pérez *et al.* 1996), oviduct epithelial cell binding (Gwathmey *et al.* 2003) and fertilizing ability (Maxwell and Hewitt 1986; Rickard *et al.* 2014). Interestingly, in and ex vivo studies have shown that both epididymal and cryopreserved ram spermatozoa struggle with cervical migration compared to fresh, ejaculated spermatozoa (Lightfoot and Salamon 1970; Rickard *et al.* 2014). While these studies provide evidence that there are significant differences between these sperm types, the ‘golden ticket’ for successful cervical transit remains a mystery. Changes to the outer carbohydrate rich coat of spermatozoa, the glycocalyx, have been well studied in the context of epididymal maturation (Voglmayr *et al.* 1983; Magargee *et al.* 1988), but less attention has been given to alterations caused by seminal plasma exposure and freezing. The sperm glycocalyx is a roughly 60 nm border extending from the cell membrane, which is rich in O- and N-glycans forming glycolipids, glycoproteins and glycosaminoglycans (Figure 4.1, (Tecle and Gagneux 2015)). As the outermost component of the cell, the glycocalyx is crucial for cell-environment interactions and alterations to its structure can modulate interaction with cervical mucus (Tollner *et al.* 2008b),

epithelial cells (Tollner *et al.* 2008a) and immune cells (Schauer *et al.* 1984; Athamna *et al.* 1991; Toshimori *et al.* 1991), possibly making it a key factor affecting cervical transit.

Figure 4.1 Reprinted from Tecle & Gagneux, 2015 (creative commons BY-NC-ND licence). The structure of the sperm glycocalyx, demonstrating the major classes of glycoconjugates on the sperm surface

The female immune response represents a significant challenge for successful cervical migration. Neutrophils, or polymorphonuclear leucocytes (PMN), have long been known to phagocytose spermatozoa, and in many instances have been typecast to the role of clearance of dead or abnormal spermatozoa (Tomlinson *et al.* 1992). However, widespread reports of the phagocytosis of live, motile spermatozoa (Matthijs *et al.* 2000; Oren-Benaroya *et al.* 2007; Li and Funahashi 2010) suggest that there are more factors at play than simply cell viability. Research on somatic cells and pathogenic microorganisms suggests that changes in cell surface glycosylation (Schauer *et al.* 1984; Athamna *et al.* 1991; Crestani *et al.* 1993; Sheth *et al.* 2011; Paris *et al.* 2012), expression of heat shock proteins (Vega and De Maio 2005) and alterations to membrane outer leaflet phospholipids (López-Revuelta *et al.* 2007) are associated with increases in phagocytosis. Such changes may well occur when spermatozoa are cryopreserved, which leaves spermatozoa apparently functional but 'sub-clinically' damaged (Yeste 2016). Further, seminal plasma has been demonstrated to have immunomodulatory functions in a range of species (Gilbert and Fales 1996; Binks and Pockley 1999; Alghamdi *et al.* 2004; O'Leary *et al.* 2004; Harris *et al.* 2006) and likely protects spermatozoa from immune cell attack. Modulation of phagocytosis by seminal plasma and cell surface changes, whether by maturation or in vitro handling, presents a possible influential factor for cervical transit.

We hypothesised that the sperm glycocalyx is significantly modified both by exposure to seminal plasma and the process of cryopreservation, thereby altering the susceptibility of these sperm populations to immune cell attack in the female reproductive tract.

4.3. MATERIALS AND METHODS

4.3.1. Experimental design

Two studies were designed to investigate the changes to sperm carbohydrates caused by exposure to seminal plasma and freezing, and how such procedures may influence interaction between spermatozoa and the female immune system. As such, fresh ejaculated spermatozoa were compared to fresh epididymal and frozen thawed ejaculated spermatozoa. Due to ethical restrictions, different rams were used to obtain epididymal and ejaculated spermatozoa. To investigate carbohydrate based changes, both viable, intact spermatozoa and sperm cell lysates were probed with lectins specific for mannose (Concanavalin A; ConA

primarily recognising α-mannose), galactose (peanut agglutinin; PNA recognising β-galactose), N-acetylglucosamine (wheat germ agglutinin; WGA, primarily recognising GlcNAc) and sialic acid (*Limulus polyphemus* agglutinin; LPA, recognising sialic acid, also known as neuraminic acid (Neu5Ac)). In order to investigate how seminal plasma and freezing may influence immune based interactions, epididymal, ejaculated and frozen thawed spermatozoa were compared on the basis of binding by isolated PMNs. To further explore the mechanisms involved in sperm-neutrophil binding, these assays were performed with and without heat treated ewe serum. This allowed for the assessment of non-opsonin binding (e.g. via selectins, lectins, integrins) in a serum free environment and opsonin binding (e.g. via immunoglobulins, C reactive protein) with serum, excluding complement by heat treating. Further experiments investigated the effects of diluent on these interactions and the importance of sperm surface carbohydrates as a PMN binding mechanism. Each experiment included a minimum of 8 replicates.

4.3.2. Chemicals

All chemicals were purchased from Sigma-Aldrich (Castle Hill, Australia) unless otherwise stated. Lectin-fluorescein isothiocyanate conjugates were purchased from Sigma (ConA, WGA, PNA) and EY Labs (San Mateo, USA; LPA). Ewe serum was purchased from Sigma and heat treated at 56°C for 30 min to inactivate complement, then frozen in aliquots and stored at -20°C until use.

4.3.3. Animals

Mature rams (n = 3) used for semen collection and ewes (n = 2) used for blood collection were kept on a chaff based diet supplemented with lupin in an animal house at the University of Sydney, Camperdown, NSW, Australia. Abattoir material was used for epididymal sample collection. All work was approved by the University of Sydney animal ethics committee (Project No: 2016/1106).

4.3.4. Semen collection and dilution

Semen was collected via artificial vagina and assessed immediately for quality by scoring wave motion. Only samples with a wave motion of ≥ 3 out of 5 were used. A single ejaculate was split to create fresh and frozen thawed ejaculated spermatozoa treatments. Testes, including epididymides, were collected immediately after slaughter, transported to the laboratory on ice and flushed within 4 h of collection. Epididymal spermatozoa were collected by retrograde flushing of the cauda epididymis via the vas deferens, using Tyrode's medium (TLP; 10 mM HEPES, 0.4 mM $MgCl_2$, 100 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 0.3 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM sodium pyruvate, 5 mM glucose, 21.6 mM sodium lactate, pH 7.3) and assessed as above.

For lectin experiments, fresh spermatozoa samples were diluted 1:4 (v/v) in Tyrodes albumin lactate pyruvate (TALP) medium (TLP, containing 0.3% (w/v) bovine serum albumin (BSA) and 1 mM D-penicillamine to minimise agglutination (Leahy *et al.* 2016)) and frozen thawed spermatozoa samples were diluted 1:4 (v/v) in AndroMed diluent (Minitube, Tiefenbach, Germany) adjusted to pH 7.4. For neutrophil experiments, samples were diluted to 100 x 10^6 spermatozoa/mL either in TALP (epididymal, ejaculated) or Salamon's cryodiluent (300 mM tris, 28 mM glucose, 104 mM citric acid (monohydrate), 15% (v/v) egg yolk, 5% (v/v) glycerol, pH 7.3; frozen thawed). Frozen thawed samples were chilled to 4°C over 2 h, frozen on dry ice in pellets of 250 µL and stored in liquid nitrogen. Pellets were thawed in a 37°C water bath for 2 min.

4.3.5. Lectin binding

4.3.5.1. Flow cytometric analysis

Fluorescein isothiocyanate (FITC) conjugated lectins were used to determine the relative abundance and distribution of various carbohydrates on the sperm surface. To avoid differences due to dilution media, all samples were washed by swim up prior to staining. Briefly, 500 µL sample was reverse layered under 3.5 mL TALP and incubated at 38.5°C for 1 h. The top 2 mL were extracted and samples were resuspended to 50 x 10^6 spermatozoa/mL. In order to prevent agglutination and facilitate flow cytometric analysis, aliquots were fixed at room temperature with 0.4% (v/v) paraformaldehyde (WGA) or 0.1% (v/v) gluteraldehyde (ConA) for 30 min and 45 min respectively. FITC conjugated lectins were subsequently incubated for 30 min either at room temperature (WGA, ConA) or 37°C (PNA, LPA) at various final staining concentrations (ConA 2 µg/mL, PNA 4 µg/mL, WGA 0.75 µg/mL, LPA 50 µg/mL) previously determined to limit agglutination while displaying adequate fluorescence signal. Samples were counterstained with propidium iodide (PI; 6 µM) at room temperature (WGA, ConA) or 37°C (PNA, LPA) for the final 10 min of incubation to allow for gating of the viable cell population.

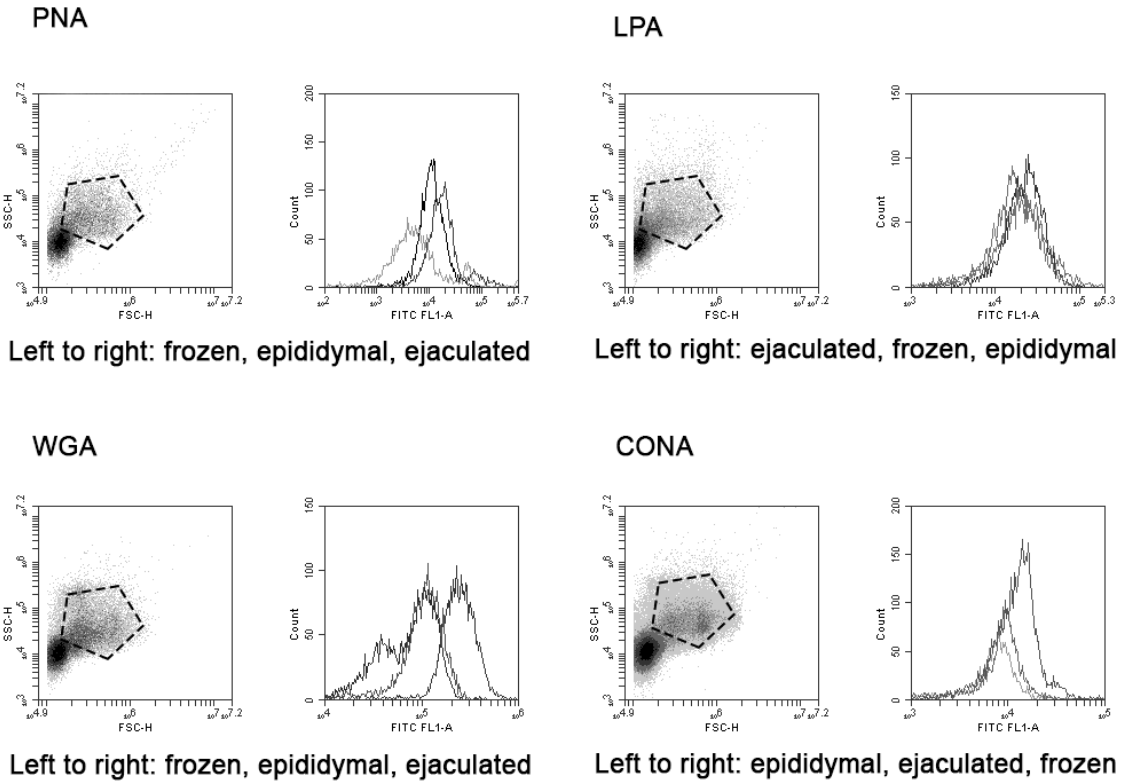

Figure 4.2 Forward versus side scatter plots (left of each panel) and 533/30 nm channel histograms (right of each panel) of samples stained with fluorescein isothiocyanate (FITC) conjugated lectins specific for galactose (PNA), sialic acid (LPA), N-acetylglucosamine (WGA) and mannose (ConA). Dashed line polygons on forward versus side scatter plots represent gating used to separate spermatozoa from background debris for analysis. Representative histograms for each treatment are overlayed, with text indicating treatment position from left to right

Samples were analysed on a C6 Accuri flow cytometer (Becton Dickinson, New Jersey, USA) with a 20 mW 488 nm laser. Fluorescence detection used a 533/30 nm band pass filter for FITC and a > 670 nm long pass filter for PI. Instrument calibration was performed each day using Spherotech 8-peak validation beads (Becton Dickinson, New Jersey, USA). Initial sample gating was based on forward/side scatter to eliminate debris (Figure 4.2), then PI fluorescence to select viable spermatozoa. 5000 events were collected within the viable population. Overlapping of emission spectra from FITC and PI were minimised by computed compensation. Samples were compared on the basis of median FITC fluorescence of the viable population only. Randomly selected representative samples were also assessed using an Olympus BX51 (Tokyo, Japan) fluorescent microscope to determine any qualitative changes to sugar distribution after freezing.

4.3.5.2. Lectin blotting

Lectin blotting was used to visualise overall changes to the total amount of sperm glycoproteins. Samples were prepared by swim up as for flow cytometry, resuspended to 50

x 10^6 spermatozoa/mL and then washed twice in TLP (600 x *g*, 10 min, room temperature). The resulting cell pellet was resuspended 1:1 with lysis buffer (cOmplete, mini protease inhibitor cocktail, 1% (w/v) sodium dodecyl sulphate (SDS), 2 M urea) and incubated at room temperature for 1 h. Lysates were centrifuged at 7, 500 x *g* for 15 min at room temperature to remove cellular debris and the supernatant was stored at - 80°C until use. Reduced samples containing a total of 10 µg of protein were separated by SDS-PAGE (10% Bio-Rad TGX stain free gel (Bio-Rad, California, USA)) at 200 V for 40 min. Gels were blotted onto Immun-Blot low fluorescence PVDF membrane (Bio-Rad, California, USA) at 100 V for 75 min at 4°C. Blots were blocked with 1% (w/v) BSA and 0.1% (v/v) Tween-20 in tris buffered saline (TBS; 20mM tris, 120mM NaCl, pH 7.6) for 1 h at room temperature. FITC conjugated lectins (PNA, WGA, ConA; final concentration 1 µg/ml) were incubated for 1 h at room temperature, then blots were washed 5 times with 0.1% (v/v) Tween-20 in TBS. Blots were visualised immediately using a ChemiDoc MP (Bio-Rad, California, USA) and images were analysed using Image Lab software (version 6.0, Bio-Rad, California, USA). Normalisation of lectin blots was performed using the 'stain free' method developed by Bio-Rad. Briefly, trihalo compounds within Bio-Rad stain free gels bind to tryptophan residues of proteins and react under UV stimulation to produce detectable fluorescence, allowing for the imaging of protein bands post transfer. The density of bands on each fluorescent lectin blot was normalised against a corresponding stain free image of the same blot.

4.3.6. Neutrophil isolation

Blood was collected from mature ewes (*n* = 2) into EDTA coated vacutainers (Becton Dickinson, New Jersey, USA) and pooled. Blood was layered onto a two-phase gradient of Histopaque-1119 and Histopaque-1077 and centrifuged (1, 200 x *g*, 30 min, room temperature). The PMN layer was recovered and red blood cells removed by 30 s hypotonic lysis with ultra-pure water. PMNs were washed twice in phosphate buffered saline (PBS; 137mM NaCl, 3mM KCl, 8mM Na_2HPO_4, 1mM KH_2PO_4) and resuspended to 2 x 10^6 PMN/mL. Cell viability was determined by trypan blue exclusion and was consistently high (> 95%). Immediately prior to mixing with spermatozoa, 15% (v/v) heat treated ewe serum was added to an aliquot of cells.

4.3.7. Sperm-PMN binding assay

4.3.7.1. Phagocytosis assay

Spermatozoa were diluted 1:1 with PMNs in PBS, with or without heat treated ewe serum, resulting in final concentrations of 50 x 10^6 spermatozoa/mL, 1 x 10^6 PMN/mL and 7.5% (v/v) serum respectively. Prior to PMN addition, an aliquot of fresh ejaculated spermatozoa was treated by repeated freeze thaw in liquid nitrogen, which served as a non-viable control in

order to investigate the contribution of viability to neutrophil binding susceptibility. Samples were incubated for 1 to 3 h at 37°C. After 30, 60, 120 and 180 min, samples were mixed vigorously with a pipette to disassociate large cell clumps and a 10 µL aliquot was smeared on a microscope slide and thoroughly air dried.

4.3.7.2. Effects of diluent and free sugars

The importance of semen dilution media and the presence of free sugars were assessed using the assay as described above. To test dilution media, semen was collected as above and diluted to 100 x 10^6 spermatozoa/mL in TALP, Salamon's cryodiluent or TALP + 15% (v/v) fresh egg yolk. To investigate the effect of free sugars, PMNs were incubated in PBS containing either 15 mM galactose, N-acetylglucosamine or sodium chloride at 37°C for 20 min prior to their addition to spermatozoa.

4.3.7.3. Microscopic determination of cell binding

Air dried slides were stained with modified Wright's stain according to manufacturer's instructions. Slides were examined using a combination of bright field and phase contrast microscopy (Olympus CX41, Tokyo, Japan) at 200 x magnification. A total of 200 PMNs were evaluated on each slide and classified as free or bound to ≥ 1 spermatozoon. The percentage of PMNs binding spermatozoa rather than spermatozoa binding PMNs was used for assessment, as spermatozoa were present in far higher numbers and this measure allowed us to obtain a clear indication of immune cell function.

4.3.8. Statistical analysis

All data were analysed using Genstat (version 16, VSN International). Data were assessed for normality and homogeneity of variances; if necessary, data were transformed to a normal distribution using the appropriate transformation and models were modified to allow for unequal variances. Data were fitted to linear mixed models accounting for treatment as a fixed factor and ram and technical replicate as random factors. Differences were considered to be significant when $p < 0.05$, with mean comparison by least significant difference, confirmed by Bonferroni adjustment. All values are reported as mean ± standard error of the mean, back transformed as appropriate.

4.4. RESULTS

4.4.1. Alterations to sperm surface carbohydrates and glycoproteins

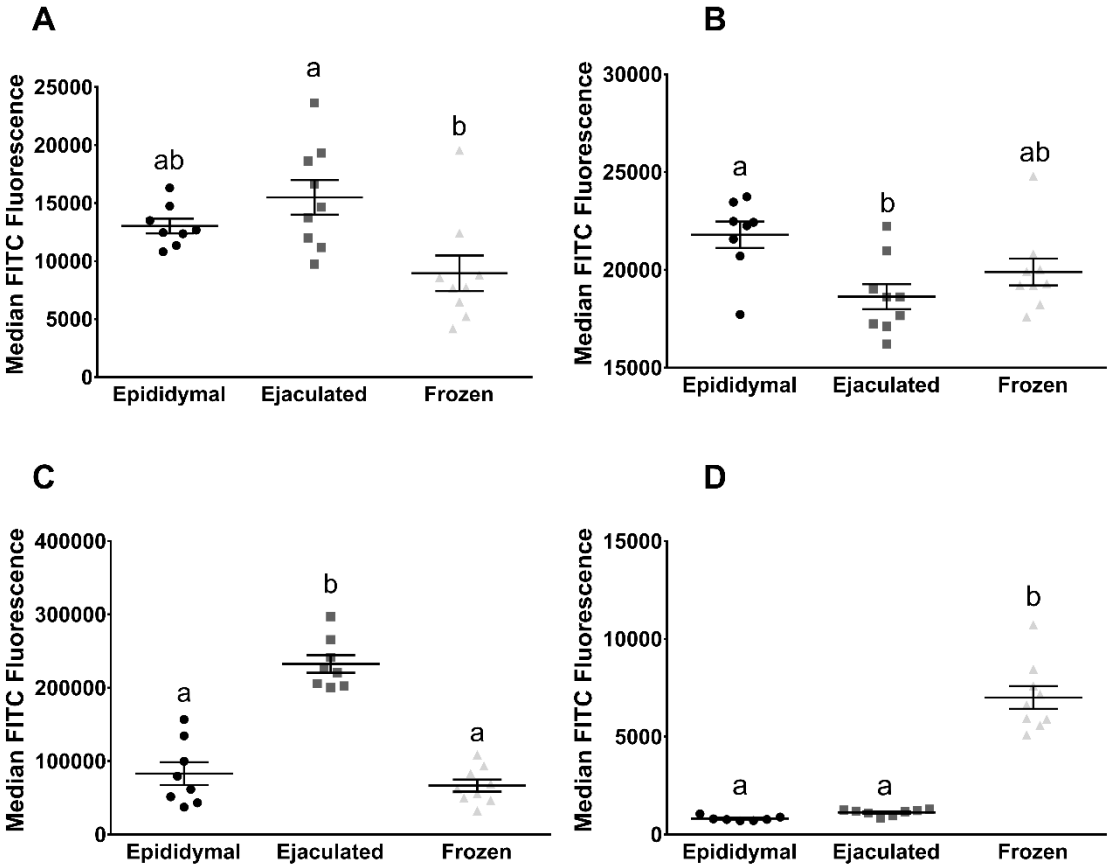

Figure 4.3 Epididymal (black circles, *n* = 9), ejaculated (dark grey squares, *n* = 8) and frozen thawed (light grey triangles, *n* = 9) spermatozoa were isolated by swim up and incubated with fluorescein isothiocyanate (FITC) conjugated lectins specific for **A**) galactose (PNA), **B**) sialic acid (LPA), **C**) N-acetylglucosamine (WGA) and **D**) mannose (ConA). Samples were compared by median FITC fluorescence (arbitrary units) after flow cytometric analysis. Data are presented as individual values, with a line indicating the mean ± SEM. Values without common superscripts denote significant differences ($p < 0.05$) between treatments, within each lectin

Mixing with seminal plasma and freezing both significantly changed the available carbohydrates on the sperm surface as measured by flow cytometry (Figure 4.2, Figure 4.3). Exposure to seminal plasma significantly increased availability of N-acetylglucosamine (WGA; $p = 0.009$) and decreased availability of sialic acid (LPA; $p = 0.011$) on the sperm membrane. Conversely, freezing led to a significant decrease in available galactose (PNA; $p = 0.004$) and N-acetylglucosamine (WGA; $p = 0.009$), as well as increased availability of mannose (ConA; $p < 0.001$). Analysis of randomly selected representative samples from the ejaculated and frozen thawed spermatozoa treatments by fluorescence microscopy suggested that there

were no significant changes to the distribution of sugars on the sperm membrane after freezing (Figure 4.4).

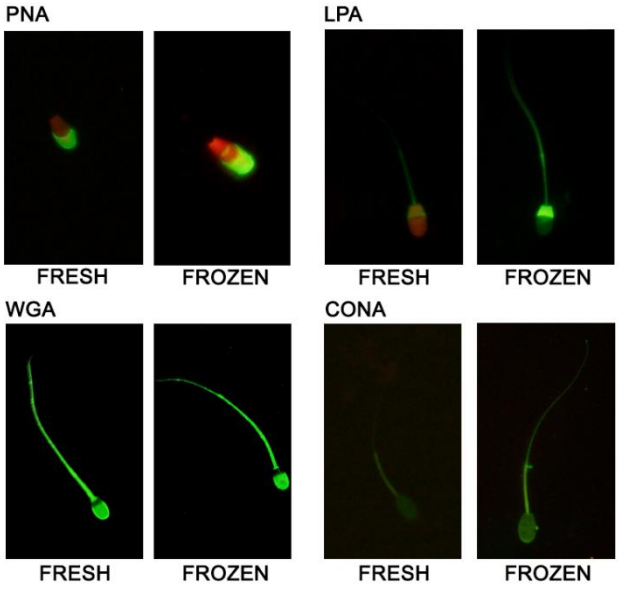

Figure 4.4 Fresh (left panel) and frozen thawed (right panel) ejaculated spermatozoa were isolated by swim up and incubated with fluorescein isothiocyanate (FITC; green fluorescence) conjugated PNA (galactose), WGA (N-acetylglucosamine) or ConA (mannose) as indicated, counterstained with propidium iodide (PI; red fluorescence). The distribution of sugars was qualitatively assessed by the lectin binding pattern for a randomly selected sample from each treatment, and each image is representative of the lectin binding pattern observed throughout the sample

The total density of each lane did not differ significantly between epididymal, ejaculated and frozen thawed spermatozoa in western blots stained with PNA, WGA or ConA. A WGA-reactive band of approximately 100 kDa was significantly decreased in frozen thawed compared to fresh ejaculated spermatozoa (Figure 4.5, $p < 0.01$). ConA-reactive bands of approximately 35, 28 and 25 kDa were significantly increased in frozen thawed compared to ejaculated spermatozoa (Figure 4.5, $p = 0.036$). There were no significant differences in any individual PNA-reactive bands between treatments (Figure 4.5).

4.4.2. Effect of sperm type, diluent and free sugars on neutrophil binding

There were significant differences in PMN binding to spermatozoa from the cauda epididymis, fresh ejaculated spermatozoa and frozen thawed ejaculated spermatozoa (Figure 4.6, $p < 0.001$). There was no significant effect of time over the 3 h incubation period. In the absence of serum, almost all PMNs were bound to epididymal spermatozoa (95.2% ± 0.8%). In comparison, ejaculated (27.7% ± 1.4%) and frozen thawed spermatozoa (27.6% ± 1.8%) were bound by significantly fewer PMNs. In the presence of serum however, the vast majority of

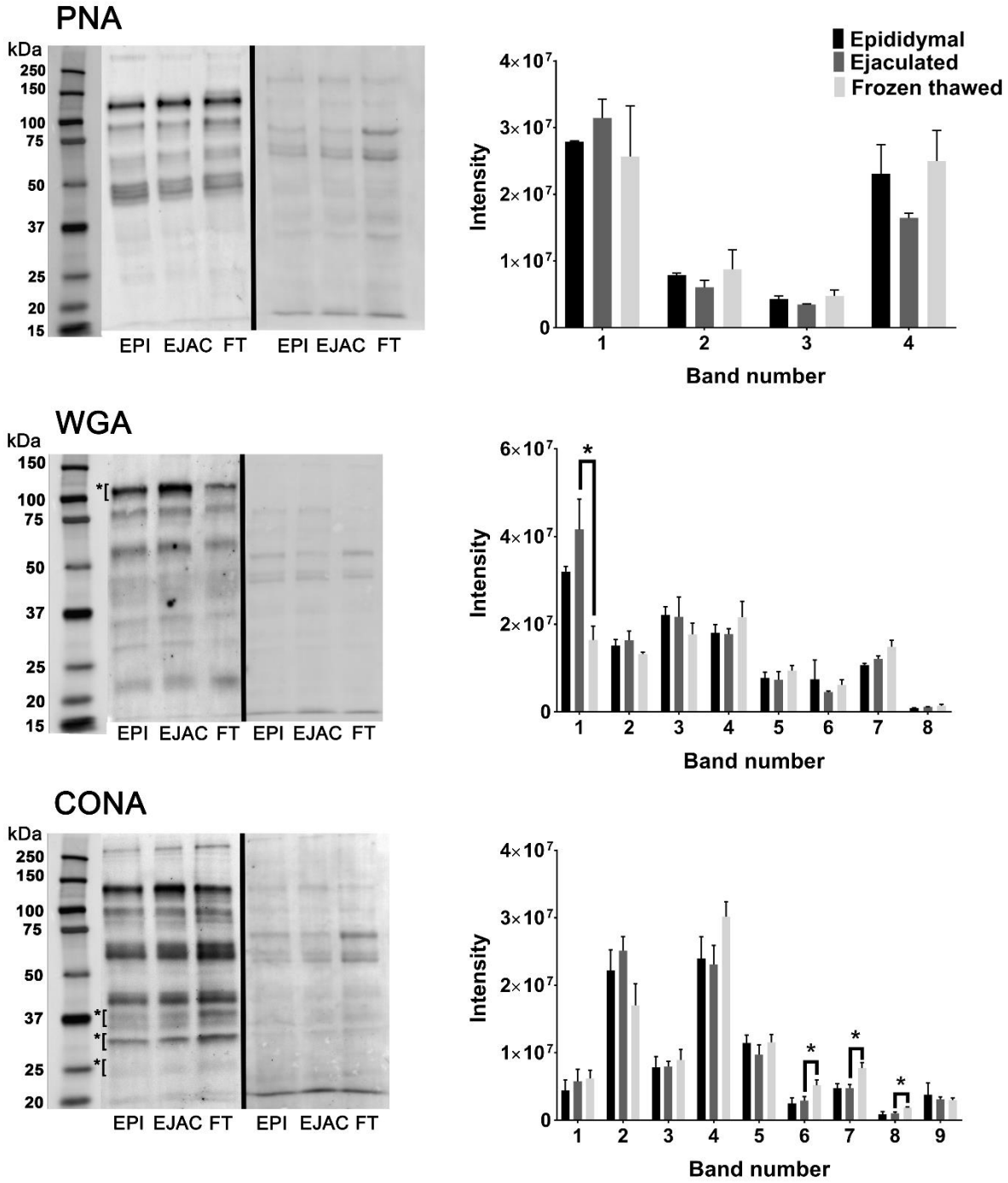

Figure 4.5 Representative western blot (10 µg total protein) of epididymal (EPI; left), ejaculated (EJAC; centre) and frozen thawed (FT; right) sperm lysates probed with fluorescein isothiocyanate (FITC) conjugated PNA (galactose), WGA (N-acetylglucosamine) or ConA (mannose) as indicated (left panel); fluorescent blots were normalised using Image Lab software (Bio-Rad) against a stain free image of the same blot using total lane protein (right panel). Corresponding bar graphs depict the intensity of the brightest bands from each blot in arbitrary units (data are pooled over 4 replicate blots per lectin and presented as mean ± SEM). *indicates bands which had significantly ($p < 0.05$) different intensity in frozen thawed spermatozoa compared to ejaculated spermatozoa

PMNs were bound to both epididymal and ejaculated spermatozoa (94.5% ± 0.6%, 95.9% ± 0.5%), while binding of frozen thawed spermatozoa remained low (31.9% ± 2.2%). As frozen thawed spermatozoa typically have significantly lower viability than fresh spermatozoa, the effect of sperm viability on binding was assessed. There was no significant difference between PMN binding to viable or non-viable fresh ejaculated spermatozoa in the presence of serum (95.9% ± 0.5% vs 93.3% ± 1.6%), thus viability is unlikely to be responsible for the observed difference in PMN binding to fresh and frozen thawed spermatozoa.

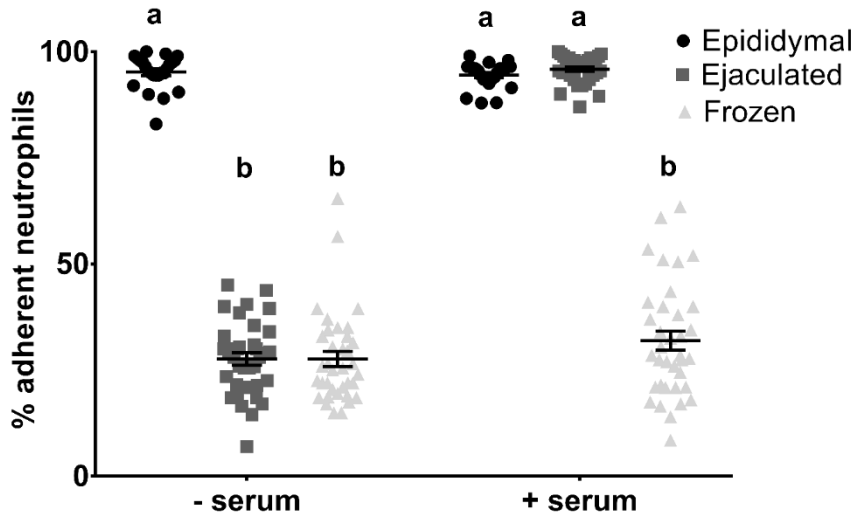

Figure 4.6 Percentage of neutrophils bound to ≥ 1 spermatozoon. Epididymal (black circles, *n* = 8), ejaculated (dark grey squares, *n* = 9) and frozen thawed (light grey triangles, *n* = 9) spermatozoa were incubated at 50 x 10^6 spermatozoa/mL with PMNs at 1 x 10^6 cells/mL isolated from ewe blood, either in the absence (left) or presence (right) of 7.5% (v/v) heat treated ewe serum at 37°C for 180 min. Data are pooled over 4 time points, and presented as individual values, with a line indicating the mean ± SEM. Values without common superscripts denote significant differences ($p < 0.05$) between treatments, within serum status

Dilution of fresh, ejaculated spermatozoa with Salamon's cryodiluent (i.e. the diluent used for cryopreservation containing glycerol and egg yolk) resulted in significantly lower binding by PMNs (26.2% ± 5.5% without serum, 37.5% ± 6.1% with serum) compared to TALP, while dilution in TALP containing 15% (v/v) egg yolk either increased (77.6% ± 7.1% without serum) or maintained (94.9% ± 1.5% with serum) the same level of binding as TALP (Figure 4.7, $p < 0.001$). As in the initial experiment, exposure to 7.5% (v/v) serum had no effect on binding of PMNs to spermatozoa diluted in Salamon's cryodiluent, but significantly increased binding of PMNs to spermatozoa diluted in TALP.

Addition of free sugars was used to investigate the importance of sperm carbohydrates as a binding target. 'Blocking' of PMNs with 15 mM galactose or N-acetylglucosamine for 20 min prior to commencement of the assay, followed by exposure to 7.5 mM of these sugars during incubation with spermatozoa, did not significantly alter their ability to bind to spermatozoa compared to a control with the same concentration of sodium chloride ($p > 0.05$, Table 4.1).

The presence of 7.5% (v/v) heat treated ewe serum during incubation significantly increased the binding of PMNs in all treatments ($p < 0.001$).

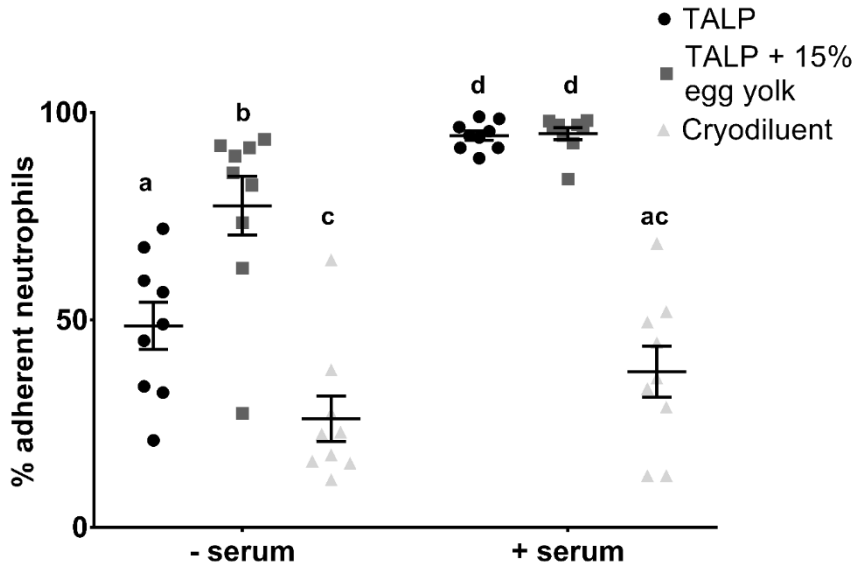

Figure 4.7 Percentage of neutrophils bound to ≥ 1 spermatozoon. Fresh ejaculated semen (*n* = 9) was diluted to 100 x 10^6 spermatozoa/mL in either Tyrodes albumin lactate pyruvate (TALP) media containing 0.3% (w/v) BSA and 1 mM penicillamine (black circles), TALP containing 15% (v/v) egg yolk (dark grey squares) or Salamon's cryodiluent containing 15% (v/v) egg yolk (light grey triangles). Samples were diluted 1:1 with isolated PMNs and incubated at 37°C for 60 min. Data are presented as individual values, with a line indicating the mean ± SEM. Values without common superscripts denote significant differences ($p < 0.05$) between treatments, across serum status

Table 4.1 Percentage of neutrophils bound to ≥1 spermatozoon after 1 h of incubation at 37°C in the presence or absence of heat-treated ewe serum, after pre-incubation with 15 mM NaCl (control), galactose or N-acetylglucosamine

	Blocking treatment		
	15 mM NaCl	**15 mM Gal**	**15 mM GlcNAc**
No serum	34.7 ± 7.1[a]	38.2 ± 7.0[a]	40.2 ± 6.2[a]
7.5% (v/v) Heat-treated ewe serum	90.4 ± 2.9[b]	92.7 ± 3.3[b]	90.3 ± 3.7[b]

Gal, galactose; GlcNAc, N-acetylglucosamine.

[ab]Values without common superscripts denote significant differences ($p < 0.05$) between treatments. Data are presented as mean ± s.e.m.

4.5. DISCUSSION

4.5.1. Fundamental changes to sperm carbohydrates

We have shown that when spermatozoa undergo the natural process of mixing with seminal plasma at ejaculation or the artificial process of cryopreservation, there are substantial and significant quantitative changes to sperm surface carbohydrates. On the basis of fluorescent lectin probe binding, we found significantly different relative amounts of available galactose, sialic acid, N-acetylglucosamine and mannose on live epididymal, ejaculated and cryopreserved ram spermatozoa. These results support the hypothesis that seminal plasma exposure and cryopreservation both significantly alter the sperm glycocalyx. The present results are supported by previous qualitative measures of sugars on ram spermatozoa from the epididymis and ejaculate (Holt 1980; Magargee *et al.* 1988). While our investigation has uncovered significant differences in several key carbohydrates on the sperm surface, a lectin microarray could be useful in further profiling each sperm type (Xin *et al.* 2014). Overall, the changes caused by seminal plasma exposure represent a meaningful natural manipulation of the sperm surface upon ejaculation, whereas those changes caused by freezing may be an unnatural deviation from normal cellular development as a result of significant damage to the sperm plasma membrane during freezing and thawing. Variation of individual simple sugars highlights the subtle yet important processes that manipulate sperm carbohydrates.

N-acetylglucosamine (GlcNAc) is an amino derivative of glucose and contributes to both core and antennary elements of hybrid and complex carbohydrates (Figure 4.8). While exposure to seminal plasma caused a significant increase in available GlcNAc, cryopreservation resulted in a significant decrease. Increased GlcNAc on ejaculated spermatozoa may be due to uptake of significant amounts of GlcNAc rich products from seminal plasma, for example hyaluronic acid (HA), a product of the accessory sex glands in the bull (Tammi *et al.* 1994). The lack of changes in WGA-reactive protein bands between epididymal and ejaculated spermatozoa lends support to the idea that it is not necessarily a glycoprotein based difference but instead may be due to other sugar rich macromolecules such as glycosaminoglycans. Interestingly, the significant decrease in GlcNAc observed due to freezing mirrors the change observed when spermatozoa reach the final stage of maturation by in vitro capacitation (Bawa *et al.* 1993; Mahmoud and Parrish 1996; Jiménez *et al.* 2002; Taitzoglou *et al.* 2007). Lectin blotting further revealed a significant decrease in a 100 kDa WGA-reactive glycoprotein band after freezing. Shedding and rearrangement of surface proteins is similarly a hallmark of capacitation (Gadella and Boerke 2016), lending support to the occurrence of ‘cryocapacitation’ (Pérez *et al.* 1996; Gillan *et al.* 1997; Schembri *et al.* 2002; Naresh and Atreja 2015) and highlighting one way in which cryopreserved spermatozoa may be compromised by in vitro handling.

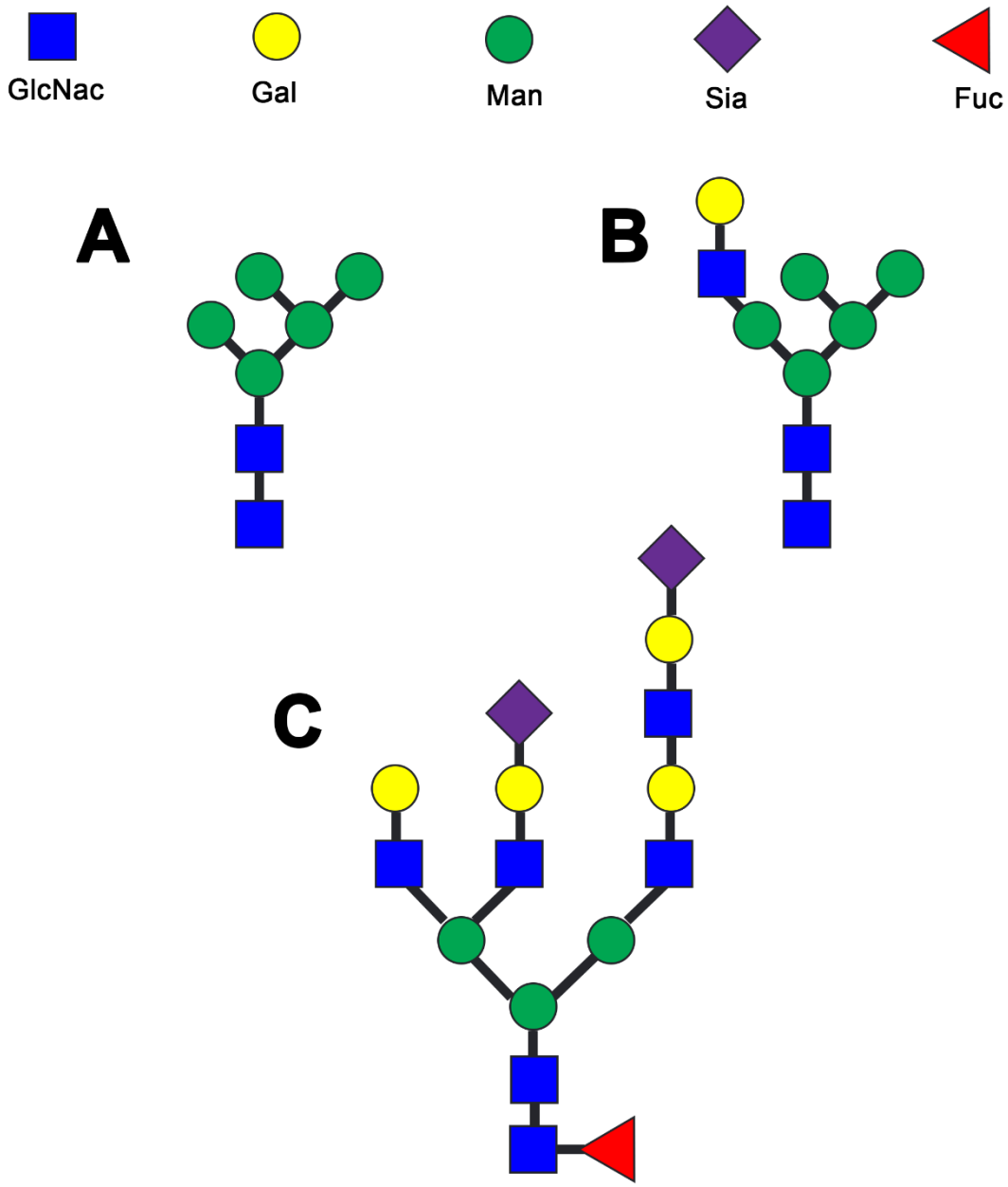

Figure 4.8 Example 2 dimensional structures of (**A**) high mannose, (**B**) hybrid and (**C**) complex N-linked carbohydrates. Blue square, N-acetylglucosamine (GlcNAc); yellow circle, galactose (Gal); green circle, mannose (Man); red triangle, fucose (Fuc); purple diamond, sialic acid (Sia)

Galactose is a simple sugar that forms part of the antennae of hybrid and complex carbohydrates, often in the terminal position (Figure 4.8). The only significant change observed in surface galactose was a significant decrease due to cryopreservation. We hypothesise that in addition to potential loss of whole glycoconjugates, the observed decrease in binding of both galactose and GlcNAc by their relevant lectins on frozen thawed spermatozoa may be due to the action of reactive oxygen species (ROS). Cooling and thawing of semen has been shown to significantly increase both oxygen free radicals and nitric oxide (Chatterjee and Gagnon 2001; Santiani *et al.* 2014). ROS production in pathological

circumstances has been implicated in modification of the cell glycocalyx (Vink *et al.* 2000; Constantinescu *et al.* 2001; Rubio-Gayosso *et al.* 2006; Rehm *et al.* 2007), but the mechanism of damage remains unclear. Disturbances due to ROS have included decreases in terminal glycocalyx GlcNAc and heparan sulphate (Singh *et al.* 2013), resulting in an increase in free sugars, suggesting direct cleavage of sugar residues from glycoconjugates.

Sialic acid (neuraminic acid or Neu5Ac) is a monosaccharide that typically takes the terminal position in hybrid and complex carbohydrates (Figure 4.8). Surface Neu5Ac was only altered by exposure to seminal plasma, which caused a significant decrease in the terminal sugar. This change may be accounted for by exposure to seminal plasma glycosidases which are not as abundant in the epididymis. Ram seminal plasma contains neuraminidase 1 (Soleilhavoup *et al.* 2014), an enzyme capable of cleaving terminal sialic acid. While the relative activities of this enzyme in ram epididymal and seminal plasma have not been investigated, previous studies have reported similar findings of lower amounts of sialic acid on ejaculated versus epididymal ram spermatozoa (Holt 1980).

Mannose is a monosaccharide that can form either the core (complex), or both the core and antennae (high mannose or hybrid types) of carbohydrates (Figure 4.8). One of the major changes to ram spermatozoa after being cryopreserved was a significant increase in available mannose. High mannose N-linked glycans contain only α-mannose as terminal sugars, and hence represent a possible source of significantly increased ConA binding. Our results from lectin blotting of sperm cell lysates suggest that glycoproteins of around 35, 28 and 25 kDa may be an important source of increased mannose on frozen thawed spermatozoa. Additionally, removal of terminal galactose and N-acetylglucosamine may also contribute to increased availability of mannose at the core of complex carbohydrates. Whether this increase in mannose is due to ‘unmasking’, the action of soluble glycosyltransferases present in seminal plasma (Tulsiani 2006) or the addition of complete high mannose bearing glycoproteins is unclear.

4.5.2. Altered interactions between spermatozoa and neutrophils

When spermatozoa first enter the female tract, they encounter a significant leucocytic response, consisting primarily of neutrophils (Thompson *et al.* 1992; Scott *et al.* 2006). We have shown that seminal plasma and cryopreservation significantly alter the interactions of spermatozoa with neutrophils, however these differences may not necessarily be due to changes in sperm surface sugars. There has been significant in vitro investigation into the effects of seminal plasma on the female immune response, with conflicting conclusions about its action. Insemination of seminal plasma or its proteins elicits a strong cytokine response in many species, including humans, mice and sheep (Robertson *et al.* 1996; Robertson *et al.*

2002; Scott *et al.* 2009), resulting in an influx of leucocytes into the tissues and lumen of the female tract (Robertson *et al.* 1996; O'Leary *et al.* 2004; Portus *et al.* 2005; Rodriguez-Martinez *et al.* 2010), lasting from hours to days. However, both pro and anti-inflammatory cytokine production can be attributed to seminal plasma and its immunomodulatory constituents (e.g. prostaglandins, transforming growth factor-β (TGF-β) (Denison *et al.* 1999; Robertson *et al.* 2002; Clark and Schust 2013)). Studies into how seminal plasma affects leucocytes are similarly inconsistent; while studies in cattle and mice report increases in leucocyte binding and phagocytosis (Alghamdi *et al.* 2009; Ma *et al.* 2016), the vast majority describe the beneficial effect of seminal plasma that we have observed, down to the effect of individual proteins (Doty *et al.* 2011) and enzymes (Alghamdi and Foster 2005). Evidence from horses, donkeys, cattle and humans supports the idea that seminal plasma interferes with neutrophil binding (D'Cruz and Haas 1995; Alghamdi *et al.* 2004; Cropp 2006; Oren-Benaroya *et al.* 2007; Miró *et al.* 2013), as well as producing direct cytotoxic effects in PMNs, reducing their viability, ROS production and phagocytic activity (Gilbert and Fales 1996; Binks and Pockley 1999; Aloé *et al.* 2012).

These studies have largely been conducted in the absence of blood serum, yet in this study we have clearly demonstrated a significant effect of heat treated ewe serum on the interaction between spermatozoa and neutrophils. Our observation of extensive binding in the presence of heat treated serum suggests binding mechanisms involving opsonins such as immunoglobulins and C reactive protein. Ejaculated spermatozoa were ‘protected’ from neutrophil binding by seminal plasma, but this protective effect subsided when heat treated serum was introduced. Interestingly however, studies have shown that this protective effect of seminal plasma is active in the presence of serum that is not heat treated and retains intact complement function (Troedsson *et al.* 2005; Li *et al.* 2012). Seminal plasma has significant anti-complement activity (see Harris *et al.* (2006) for review), and thus would likely be responsible for diminished neutrophil binding to spermatozoa in serum with intact complement. The sum of these findings suggest that for many species, seminal plasma is a powerful protective mechanism, providing defence against both complement mediated binding and also binding which is not reliant on serum components. This may explain in part why we see significantly improved cervical transit when epididymal spermatozoa are supplemented with seminal plasma (Rickard *et al.* 2014).

While our initial investigation showed significant differences between neutrophil binding to fresh and cryopreserved spermatozoa in the presence of serum, follow up experiments indicated that these differences were in fact due to the diluent used for cryopreservation. There was no significant increase in neutrophil binding when serum was introduced if cryodiluent

was present. This suggests that cryodiluent causes a failure of opsonin mediated binding by neutrophils, likely due to failure of this receptor pathway in neutrophils, rather than failed opsonisation itself. Egg yolk has been shown to stimulate antibody production following repeated inseminations (Griffin *et al.* 1971; Coulter *et al.* 1976) and has been linked to both increased neutrophil chemotaxis and phagocytosis of porcine spermatozoa (Li *et al.* 2012), which reflects our results of a significant increase in binding due to the addition of 15% egg yolk to TALP. Our own pilot studies further suggest that 5% (v/v) glycerol is not responsible for this difference, and that neutrophil viability remains high (99%) after 1 hour of incubation in either TALP or cryodiluent. Barring egg yolk and glycerol, the high concentration of citric acid, a strong calcium chelator, in cryodiluent offers a potential explanation for the suppression of opsonin mediated binding. Trisodium citrate, a salt of citric acid, has been shown to inhibit phagocytosis of opsonised particles by up to 90%, and significantly decreases neutrophil chemotaxis in vitro (Pfister *et al.* 1984; Taylor *et al.* 2009). These phenomena are believed to be due to interference with the function of neutrophil receptors, specifically caused by calcium chelation. These findings suggest that the presence of a calcium chelator may cause significant disruption to opsonin mediated binding and may be responsible for the lack of response to serum in samples diluted with cryodiluent.

4.5.3. Are carbohydrate changes responsible for differences in non-opsonin neutrophil binding?

Neutrophils contain a variety of cell surface receptors involved in target recognition (Futosi *et al.* 2013), the initial step involved in phagocytosis. Non-opsonin mediated target recognition, such as lectin-carbohydrate interaction (Ofek and Sharon 1988), offers an explanation for the significant phagocyte binding activity we observed in the absence of serum components such as immunoglobulins and complement. There has been evidence that changes to cell surface carbohydrates may decrease or enhance the ability of phagocytes to recognise or successfully phagocytose them (Schauer *et al.* 1984; Fischer *et al.* 1991; Crestani *et al.* 1993; Sheth *et al.* 2011; Paris *et al.* 2012), an event which has been previously documented in spermatozoa (Toshimori *et al.* 1991). We have shown that the surface carbohydrates of spermatozoa change when they come into contact with seminal plasma, and it is tempting to hypothesise that such changes could explain the lessened affinity of neutrophils for ejaculated spermatozoa. While we attempted to elucidate the importance of carbohydrate mediated binding by incubating neutrophils with competing simple sugars, we were unable to observe any effects on binding and thus could not find support for the hypothesis that changes to surface sugars were a key factor for susceptibility to neutrophil binding. Our findings may be due to the use of simple sugars rather than oligosaccharides, or an insufficient concentration of sugars. Alternatively, non-opsonin mediated binding of neutrophils to spermatozoa may

instead rely heavily or solely on another receptor, for example toll-like receptors (TLRs). Further investigations using competitive complex carbohydrates, sugar specific glycosidases or antibodies to target particular carbohydrate ligands on the sperm surface may bring to light the importance of carbohydrate mediated binding in neutrophil phagocytosis of spermatozoa, and any relationship between changes to sperm surface sugars and phagocytic susceptibility.

4.5.4. Implications of changes due to seminal plasma exposure and freezing

The requirements for successful cervical transit are complex and varied, yet we know that an absence of seminal plasma and the process of cryopreservation give spermatozoa limited chance of success. We observed significant changes to the cell glycocalyx after seminal plasma exposure and freezing. Cell surface glycoconjugates are involved in a range of functions, and for spermatozoa these may include key functions related to successful cervical transit, including functioning as a ligand (Kurpisz and Alexander 1995) and 'cloaking' of antigens (Toshimori *et al.* 1992; Yudin *et al.* 2005). Our investigation into the interactions between neutrophils and spermatozoa further highlights how changes to spermatozoa may alter their chances of effective cervical passage. Seminal plasma limits non-opsonin mediated binding, and has additional inhibitory effects on complement mediated phagocytosis (Harris *et al.* 2006). This could explain why spermatozoa exposed to seminal plasma have a major advantage in cervical transit (Rickard *et al.* 2014). While we initially believed that poor fertility of frozen thawed spermatozoa may be linked to increased phagocytosis, our results suggest that instead cryodiluent limits in vitro opsonin mediated binding. This is an example of how in vitro handling of spermatozoa may influence the outcomes of natural challenges from the female reproductive tract, whether for better or worse. These differences we have observed between epididymal, ejaculated and cryopreserved spermatozoa may well underpin what arms a spermatozoon to deal with the trials of migration through the cervix and the subsequent challenge of fertilisation.

4.6. ACKNOWLEDGEMENTS

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5. Binder of Sperm Proteins 1 and 5 have contrasting effects on the capacitation of ram spermatozoa

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5.1. ABSTRACT

Binder of Sperm Proteins (BSPs) are the most abundant seminal plasma protein family in the ram and bull. They have been extensively studied in the bull but less is known about their function in ovine seminal plasma and current knowledge suggests BSPs may have different effects in these two species. In the bull they facilitate capacitation and destabilise the sperm membrane during in vitro handling, whereas in the ram, they appear to stabilise the sperm membrane and prevent cryopreservation induced capacitation-like changes. Further investigation into the effects of BSPs on ram spermatozoa under capacitating conditions is required to further clarify their physiological roles in the ram. We investigated the effects of BSP1 and BSP5 on epididymal ram spermatozoa in conditions of low, moderate and high cAMP. BSPs had minimal effects on sperm function in low cAMP conditions, but caused significant changes under cAMP upregulation. BSP1 stabilised the membrane and qualitatively reduced protein tyrosine phosphorylation, but significantly increased cholesterol efflux and induced spontaneous acrosome reactions. BSP5 slightly increased spontaneous acrosome reactions and caused sperm necrosis. However, BSP5 had minimal effects on membrane lipid order and cholesterol efflux and did not inhibit protein tyrosine phosphorylation. These findings demonstrate that under maximal cAMP upregulation, BSP1 affected ram spermatozoa in a manner comparable to bull spermatozoa, while BSP5 did not.

5.2. INTRODUCTION

Bedford and Chang described the ‘decapacitation’ effect of seminal plasma over 50 years ago (Chang 1957; Bedford and Chang 1962) and a significant amount of research since has focused on how this fluid can be exploited to prevent detrimental capacitation-like changes in bull, boar and ram spermatozoa, caused by semen handling and storage (Leahy and Gadella

2011; Caballero *et al.* 2012). We now know that seminal plasma contains proteins which are able to suppress or reverse capacitation (e.g. murine SERPINE2 (Lu *et al.* 2010)) and proteins which promote capacitation (e.g. human CD38 (Kim *et al.* 2015b)). Proteomic studies have highlighted the complex makeup of ram seminal plasma, identifying over 700 proteins and the most abundant protein families (e.g. Binder of Sperm Proteins and spermadhesins) (Soleilhavoup *et al.* 2014). Binder of Sperm Proteins (BSPs) 1 and 5 are particularly interesting, as they are highly abundant in ram seminal plasma, bind to the sperm membrane in large amounts at ejaculation (Pini *et al.* 2016), are well conserved across a range of species (Manjunath *et al.* 2009) and their homologues play roles in the capacitation of bull (Thérien *et al.* 1997), mouse (Plante and Manjunath 2015), human (Plante *et al.* 2014) and boar (Lusignan *et al.* 2007) spermatozoa.

BSPs account for over 50% of bull seminal plasma proteins (Nauc and Manjunath 2000), and their effects have been well characterised in this species. BSPs have powerful cholesterol efflux potential (Thérien *et al.* 1998), interact with other capacitation promoters such as high density lipoprotein (Thérien *et al.* 1997) and promote the acrosome reaction (Thérien *et al.* 1999), making them an important stimulator of capacitation for bull spermatozoa. However, because of these roles in capacitation, extended exposure of bull spermatozoa to BSP rich seminal plasma during in vitro handling can be detrimental (Manjunath *et al.* 2007). Our knowledge of how BSPs affect ram spermatozoa is more limited, however previous research suggests that they are abundant in ram seminal plasma (Soleilhavoup *et al.* 2014), and the main constituent of seminal plasma which significantly protects ram spermatozoa during in vitro handling (Leahy and de Graaf 2012) and cold shock (Barrios *et al.* 2000), by stabilising the sperm membrane. One previous study has looked at the effect of BSPs on ram spermatozoa under capacitating conditions, and suggests that they act as decapacitation factors (Luna *et al.* 2015), a potential benefit during transit through the ewe's convoluted cervix (Kershaw *et al.* 2005). Thus there is potentially a considerable divergence in the 'natural' roles of BSPs in the ram and bull, and their effects during in vitro sperm processing. These differences may reflect species specific sperm membrane make up (Darin-Bennett and White 1977) and post translational protein modifications (e.g. glycosylation (Gerwig *et al.* 1996)), however the effects of BSPs are not yet well characterised enough in the ram for a fair comparison. A more in depth assessment of capacitation related parameters is required in order to fully elucidate the in vitro and likely in vivo roles of ram BSPs.

Capacitation involves a swathe of changes, including promotion of hyperactivated motility, increased disorder of membrane lipids, cholesterol efflux, changes to sperm glycoconjugates and development of tyrosine phosphorylation (Naresh and Atreja 2015; Gadella and Boerke 2016; Liu 2016). These changes can be replicated in vitro using a medium which mimics

oviductal fluid (Yanagimachi 1994), and typically contains bicarbonate, calcium, and delipidated albumin. This base medium is then further modified to include species specific capacitation stimuli, which in the ram includes cyclic AMP analogues (e.g. dibutyryl (db) cAMP), and phosphodiesterase inhibitors (caffeine, theophylline). These chemicals serve to significantly upregulate cAMP levels, a phenomenon observed in response to physiological capacitating agents such as oviductal fluid (Uguz *et al.* 1992) and allow for the development of capacitation associated high molecular weight protein tyrosine phosphorylation and increased lateral fluidity of membrane phospholipids (Colás *et al.* 2008; Leahy *et al.* 2016). Ram spermatozoa do not display these capacitation hallmarks without such additional cAMP upregulation when compared to other mammalian species (for instance pigs, cattle, rodents and humans) and the reason for this is not well understood. The moieties responsible for the promotion of ram sperm capacitation in vivo are still unknown. Seminal plasma proteins may be a key factor affecting the responsiveness of ram spermatozoa to capacitation induction under low cAMP stimulatory conditions, however this requires further investigation.

BSPs appear to have contrasting effects on ram and bull spermatozoa (Leahy and de Graaf 2012), but information on their action in ram spermatozoa is limited. Further, profiling of the effects of BSPs on ram spermatozoa may provide avenues to improve in vitro capacitation and to better understand their roles in vivo. Consequently, we have investigated the effects of isolated Binder of Sperm Proteins 1 and 5 on ram sperm functional parameters in both basal and stimulatory conditions.

5.3. MATERIALS AND METHODS

5.3.1. Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Castle Hill, Australia). Fluorescent probes were purchased from Life Technologies (Scoresby, Australia). Primary rabbit IgG antibody against gelatin affinity purified ram BSPs (Plante *et al.* 2015a) (RRID AB_2715559) was kindly provided by P. Manjunath (Departments of Biochemistry and Medicine, Faculty of Medicine, University of Montreal).

5.3.2. Animals and semen collection

Rams used for seminal plasma collection (n = 50) were housed at the commercial CEDEO AI centre in Ordiarp, France and maintained on pasture. Semen for seminal plasma isolation was collected from mature rams (n = 50) via artificial vagina (1 ejaculate per ram). All ejaculates were assessed for wave motion and were of sufficient quality (≥ 4 out of 5). Ejaculates were pooled across rams and centrifuged twice (14, 000 x g, 20 min, 4°C) to isolate seminal plasma, which was stored at - 80°C until further use.

Testes with epididymides were collected from a local slaughterhouse, transported to the laboratory on ice, stored at 4°C and flushed within 24 h. A set of epididymides from a single ram (n = 3) was considered a biological replicate, with each epididymis acting as a technical replicate, giving a 3 x 2 experimental design. Epididymal spermatozoa were collected by retrograde flushing of the cauda epididymis via the vas deferens using warm Tyrode lactate pyruvate (TLP) medium (10 mM HEPES, 0.4 mM $MgCl_2$, 100 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 0.3 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM sodium pyruvate, 5 mM glucose, 21.6 mM sodium lactate, pH 7.3) and assessed as above.

5.3.3. Binder of Sperm Protein isolation

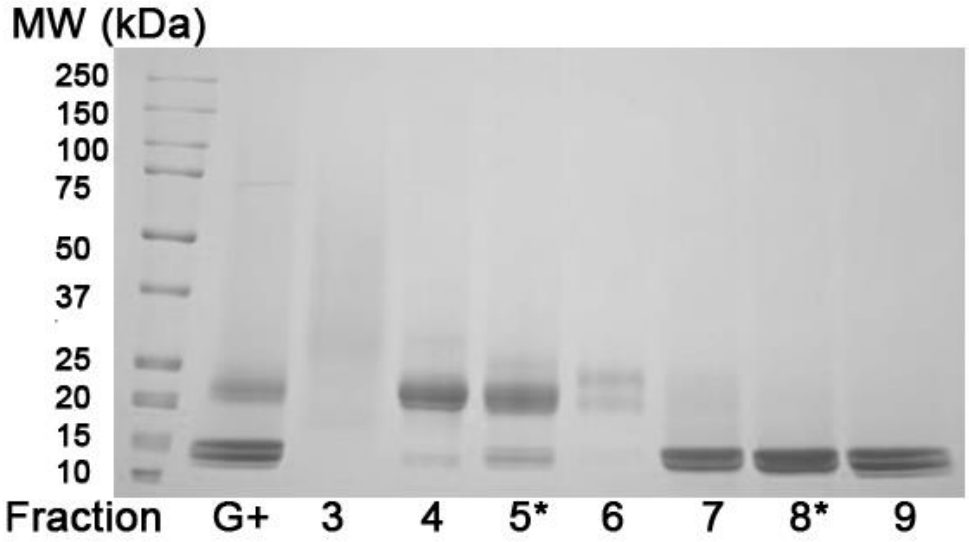

Figure 5.1 Fractions collected after gelatin affinity chromatography, followed by RP-HPLC of ethanol precipitated ram seminal plasma proteins. Coomassie brilliant blue stained 8-16% SDS PAGE of gelatin binding fraction (G+) (10 µg) and sequentially eluted RP-HPLC fractions (fractions 3 and 6; 5 µg, fractions 4, 5, 7, 8, 9; 10 µg). The fractions employed as 'purified BSP1' (8) and 'purified BSP5' (5) are indicated*

320 mg of ethanol precipitated seminal plasma proteins were loaded onto a 12 mL gelatin affinity column (gelatin was previously coupled to Affi-Gel 15 resin (Bio-Rad, Marnes-la-Coquette, France)). Bound proteins were eluted with phosphate buffered saline (PBS) containing 5 M urea. 1 mL fractions were collected and pooled relative to absorbance at 280 nm, desalted three times with a PD10 column and lyophilised. A total of 64 mg of the gelatin absorbed proteins were subjected to multiple runs of reversed phase high performance liquid chromatography (RP-HPLC) on a Waters XBridge BEH C18 OBD Prep column (250 mm × 10 mm i.d., particle size 5 µm, pore size 130 Å; Waters, Guyancourt, France). A linear 28 to 45% acetonitrile gradient with 0.1% trifluoroacetic acid (TFA) was used at a constant flow rate of 3 mL/min for 29 min. BSP1 and BSP5 were isolated in two separate fractions (Figure 5.1), which were desalted by dialysing against 50 mM ammonium bicarbonate and lyophilised. Western blotting confirmed binding of the isolated proteins to epididymal spermatozoa (Figure 5.2).

Purified proteins were resuspended in physiological saline at high concentration (> 2 mg/mL) and stored at - 80°C. Prior to use, proteins were thawed on ice and warmed to 37°C.

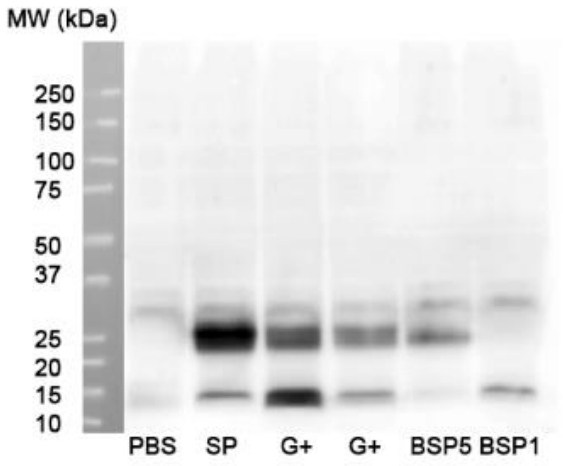

Figure 5.2 Binding of purified BSP1 and BSP5 to epididymal ram spermatozoa (1 h, 37°C). Western blot of epididymal spermatozoa (490 x 10^6 total) incubated 1:1 (v/v) with (1) phosphate buffered saline, (2) seminal plasma, (3,4) gelatin binding fraction (13 mg/mL or 1 mg/mL), (5) purified BSP5 (0.4 mg/mL), (6) purified BSP1 (1 mg/mL), probed with anti-BSP

5.3.4. LC-MS/MS of purified proteins

Six reversed phase chromatography fractions (Figure 5.1) were in-solution digested with bovine trypsin. Briefly, proteins in 50 mM NH_4HCO_3 were reduced in 5 mM dithiothreitol (30 min, 56°C) and alkylated in 12.5 mM iodoacetamide (20 min, room temperature in the dark). Proteins were digested overnight with 12.5 ng/µL trypsin (sequencing grade, Roche, France) with a ratio of 1:40 enzyme:substrate. 5 µL of peptides was directly injected onto a trap column and separated on a nano-column as previously described (Labas *et al.* 2015a), using a 4 – 55% B 90 min gradient at a flow rate of 300 nL/min on an Ultimate 3000 RSLC UHPLC system (Dionex, Netherlands). Eluate was ionised using a Thermo Finnigan Nanospray Ion Source 1 and MS/MS was carried out on an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Germany). Data were acquired in positive data dependent mode, with sequential isolation (isolation width 2 *m/z*) and fragmentation (collision induced dissociation) of the 20 most intense peptide ions (charge state 2 +, *m/z* range 300 – 1, 800). Proteins were identified by Mascot search engine (version 2.3, Matrix Science) against the NCBInr database using mammalian taxonomy (downloaded December 2016). Database search criteria included trypsin as a protease with two missed cleavages allowed, variable modifications (carbamidomethylcysteine, methionine oxidation, acetylation of N-terminal proteins) and 5 ppm/0.8 Da parent/fragment ion match tolerance. Scaffold software (version 3.6, Proteome Software, USA) was used to validate protein identifications using the Peptide and Protein Prophet algorithms. Protein identifications were accepted if they contained at least two

peptides and had > 95% probability. The abundance of identified proteins was estimated by calculating the emPAI using Scaffold Q+ software (Proteome Software, USA).

5.3.5. Treatment with capacitation stimulants and isolated protein

All experiments were repeated six times, including three biological replicates (rams) and two technical replicates (epididymides). Epididymal spermatozoa were diluted with TALP (TLP with 0.3% (w/v) fatty acid free bovine serum albumin (BSA), fraction V) to 58 x 10^6 spermatozoa/mL. Aliquots were then further diluted to 50 x 10^6 spermatozoa/mL with TALP containing 0, 75 or 150 µg/mL of isolated BSP1 or BSP5 and cAMP upregulators, as appropriate. The concentrations of BSPs used are slightly lower than the total concentration of BSPs in a ram ejaculate (roughly 200 µg per 50 x 10^6 spermatozoa as per Manjunath *et al.* (2007), assuming ejaculate concentration of 4 x 10^9 spermatozoa/mL). Three different cAMP stimulation levels were used across three independent experiments; basal (TALP alone), moderate (TALP with 1 mM caffeine) and high (TALP with cAMP upregulators 1 mM caffeine, 1 mM theophylline, 1 mM dbcAMP). All treatments were incubated for 20 min at 37°C immediately after protein/cAMP upregulator addition to allow for protein binding to the sperm membrane. Samples were then held at 37°C, with assessment at 0, 3 and 6 h. For flow cytometry and motility analyses, aliquots taken at each time point were treated with 1 mM D-penicillamine to prevent agglutination and allow for accurate analysis (Leahy *et al.* 2016).

5.3.6. Motility analysis

Objective computer assisted motility analysis was performed using an IVOS II (Hamilton Thorne, operating Animal Breeder software, version 1.8), with settings appropriate for ram spermatozoa (head size 10 – 42 µm^2, progressive motility thresholds of straightness 80% and average path velocity 75 µm/s). Samples were diluted to a final concentration of 25 x 10^6 spermatozoa/mL immediately prior to assessment and loaded onto a CELL-VU slide. 8 screen captures recording ≥ 200 spermatozoa were obtained for each sample.

5.3.7. Flow cytometry

Flow cytometry analysis was performed using a C6 Accuri flow cytometer (Becton Dickinson, New Jersey, USA) with a 20 mW 488 nm laser source for scatter detection of spermatozoa and excitation of spermatozoa-associated fluorescent probes. Instrument calibration was performed each day using Spherotech 8-peak and 6-peak validation beads (Becton Dickinson, New Jersey, USA). Probes were used to assay viability (propidium iodide, PI, 6 µM), acrosome integrity (fluorescein isothiocyanate conjugated to peanut agglutinin, FITC-PNA, 0.4 µg/mL), early changes in membrane permeability (YO-PRO-1, 25 nM) and membrane lipid disorder (merocyanine 540, M540, 0.83 µM). Stains were run in combination (FITC-PNA/PI and M540/YO-PRO-1) to allow for viability gating. Probes were incubated with samples in the dark

for 10 min at 37°C prior to analysis. Fluorescence detection employed a 533/30 nm band pass filter for FITC-PNA and YO-PRO-1 and a > 670 nm long pass filter for PI and M540. Forward/side scatter was used to eliminate debris and select spermatozoa, with further gating based on viability as measured by appropriate probes (PI or YO-PRO-1). A minimum of 10, 000 events within the initial population of spermatozoa were analysed and samples were either compared on the basis of percentage of probe-positive spermatozoa (FITC-PNA, PI) or median channel fluorescence of the relevant fluorophore within the viable population (M540).

5.3.8. Amplex Red cholesterol assay

Aliquots at 50 x 10^6 spermatozoa/mL were extended with TLP, washed (14, 000 x *g*, 10 min, room temp), and the supernatant was retained. The supernatant was filtered (0.22 µm) to remove any contaminating spermatozoa and stored at - 80°C. Thawed supernatants were assessed for cholesterol content using an Amplex Red cholesterol assay kit (Thermofisher, Waltham, USA), according to manufacturer's instructions. Briefly, cholesterol is oxidised by cholesterol oxidase, producing H_2O_2, which in turn reacts with the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine). In the presence of horse radish peroxidase, this reaction produces fluorescent resorufin (ex/em maxima 571/585 nm). Fluorescence intensity was measured at 590 nm on an Infinite M-1000 pro spectrophotometer (Tecan, Mannedorf, Switzerland) and cholesterol concentration calculated against standards.

5.3.9. Tyrosine phosphorylation western blotting

A total of 7.5 x 10^6 spermatozoa were washed twice with TLP (14, 000 x *g*, 10 min and 600 x *g*, 10 min). The supernatant was discarded and the pellet diluted 1:1 (v/v) with lysis buffer (cOmplete EDTA free protease inhibitor cocktail (Sigma), 1% (w/v) sodium dodecyl sulphate (SDS), 1 mM sodium orthovanadate, TLP). Spermatozoa were lysed at room temperature for 60 min with regular vortexing, then centrifuged (7, 500 x *g*, 15 min). The supernatant was retained and stored at - 80°C. Cell lysates were measured for protein content using a Qubit protein assay (Life technologies, California, USA), according to manufacturer's instructions. 10 µg of protein was separated on a 10% TGX stain free gel (Bio-Rad) by SDS-PAGE (200 V, 40 min) using a mini-PROTEAN tetra cell (Bio-Rad) and blotted onto a PVDF membrane (100 V, 75 min, 4°C) using a mini Trans-Blot cell (Bio-Rad). The membrane was blocked with tris buffered saline (TBS) with 0.1% Tween-20 (TW) and 1% (w/v) BSA at room temp for 60 min. The blocked membrane was probed with 1:2000 HRP-anti-phosphotyrosine (Merck-Millipore, Billerica, USA, RRID AB_310779) in TBS-TW with 0.1% (w/v) BSA at room temp for 60 min and washed 5 times with TBS-TW. Signal was visualised using 1:1 (v/v) luminol and peroxidase (Bio-Rad Immun-star western chemiluminescence kit), incubated at room temp for 5 min. Images were captured using a ChemiDoc XRS+ (Bio-Rad, California, USA) and qualitatively analysed using Image Lab software (version 5.1, Bio-Rad, California, USA). Due

to sample limitations for basal and moderate cAMP stimulation, only western blots of BSP1 treated spermatozoa were produced.

5.3.10. Statistical analysis

Statistical analysis was carried out using Genstat (version 18, VSN International). Collections from a single epididymis were considered an experimental unit, giving a total of 6 replicates. Data were assessed for normality using the Shapiro-Wilk test and homogeneity of variances by fitted value residual plots, and transformed if necessary (by $\log_{10}$, square root or power as appropriate) to meet the requirements of a linear mixed model. Outcomes were assessed using a linear mixed model incorporating treatment, time (as applicable), technical replicate and ram, with an α of 0.05. Means were compared on the basis of least significant difference and all values are reported as the mean ± standard error of the mean, back transformed if applicable.

5.4. RESULTS

5.4.1. Confirmation of purity of isolated BSPs

Mass spectrometry confirmed that the isolated fractions of interest contained BSP1 and BSP5, at 99% and 92% purity respectively (Supplementary Table 5.1).

5.4.2. Effects of BSPs under various levels of capacitation stimulation

5.4.2.1. <u>The effects of BSPs on sperm motility and viability</u>

Compared to plain TALP medium (basal cAMP stimulation), moderate cAMP stimulation (1 mM caffeine) did not alter total motility, progressive motility or the proportion of necrotic spermatozoa (Table 5.1).

Table 5.1 Total and progressive motility of epididymal ram spermatozoa incubated in TALP, TALP plus 1 mM caffeine or TALP plus cAMP upregulators* at 0, 3 and 6 hours of incubation at 37°C

	Total motility (%)			**Progressive motility (%)**			**Necrotic sperm (%)****		
Time (hours)	**0**	**3**	**6**	**0**	**3**	**6**	**0**	**3**	**6**
TALP	68.1 ± 5.3[a]	66.9 ± 6.1[a]	55.8 ± 10.7[a]	50.7 ± 4.3[a]	53.3 ± 6.4[a]	44.7 ± 9.7[a]	12.6 ± 1.4[a]	15.7 ± 2.7[a]	23.5 ± 4.7[a^]
TALP + 1 mM caffeine	78.4 ± 1.7[a]	78.0 ± 3.7[a]	66.8 ± 6.7[a]	65.7 ± 2.4[a]	67.0 ± 4.1[a]	56.0 ± 6.5[a]	14.9 ± 1.3[a]	18.2 ± 2.4[a]	26.1 ± 3.3[a^]
TALP + cAMP upregulators*	63.8 ± 10.7[a]	30.9 ± 4.4[b^]	22.9 ± 5.7[b^]	51.4 ± 10.4[a]	17.1 ± 2.2[b^]	12.5 ± 3.7[b^]	24.0 ± 2.2[b]	33.8 ± 3.5[b^]	48.6 ± 1.2[b^]

[a] different superscript letters denote significant differences ($p < 0.05$) within column ^denotes significant differences ($p < 0.05$) within row, compared to 0 hours ***** cAMP upregulators included 1 mM caffeine, theophylline and dbcAMP ** 'necrotic' sperm were defined as non-viable (PI positive) but acrosome intact (FITC-PNA negative)

Table 5.2 Motility parameters and viability of epididymal ram spermatozoa incubated in TALP, TALP plus 1 mM caffeine or TALP plus cAMP upregulators, with or without isolated BSPs, pooled over 6 hours of incubation

	TALP					TALP + 1 mM caffeine					TALP + cAMP upregulators*				
	Control	75 µg/mL BSP1	150 µg/mL BSP1	75 µg/mL BSP5	150 µg/mL BSP5	Control	75 µg/mL BSP1	150 µg/mL BSP1	75 µg/mL BSP5	150 µg/mL BSP5	Control	75 µg/mL BSP1	150 µg/mL BSP1	75 µg/mL BSP5	150 µg/mL BSP5
Total motility (%)	63.6 ± 7.9^{a}	56.0 ± 4.0^{a}	50.6 ± 3.3^{a}	63.8 ± 5.2^{a}	59.4 ± 4.7^{a}	74.4 ± 2.8^{a}	68.1 ± 3.6^{b}	61.5 ± 4.5^{c}	68.8 ± 3.1^{a,b}	70.1 ± 4.1^{a,b}	39.2 ± 5.9^{a}	48.5 ± 5.8^{a}	41.4 ± 6.0^{a}	65.0 ± 5.0^{b}	65.4 ± 4.3^{b}
Progressive motility (%)	49.6 ± 3.8^{a}	41.0 ± 3.4^{a}	36.1 ± 2.9^{a}	46.1 ± 5.5^{a}	42.8 ± 4.9^{a}	62.9 ± 2.8^{a}	57.2 ± 3.3^{b}	52.8 ± 4.0^{b}	58.1 ± 3.1^{b}	57.8 ± 4.0^{b}	27.0 ± 5.5^{a}	41.2 ± 5.4^{b}	35.8 ± 5.4^{b}	53.8 ± 4.9^{c}	53.5 ± 4.6^{c}
VAP (µm/s)	165.7 ± 7.5^{a}	165.3 ± 11.7^{a}	162.9 ± 11.5^{a}	158.2 ± 11.3^{a}	165.1 ± 10.3^{a}	136.8 ± 2.6^{a}	148.1 ± 3.1^{b,c}	153.3 ± 3.8^{c}	142.4 ± 3.6^{a,b}	137.6 ± 3.1^{a}	103.8 ± 4.8^{a}	133.4 ± 4.3^{b,c}	141.1 ± 3.5^{b}	125.2 ± 4.5^{c,d}	123.2 ± 5.0^{d}
VCL (µm/s)	212.9 ± 10.5^{a}	224.6 ± 16.2^{a}	217.6 ± 12.7^{a}	204.3 ± 12.4^{a}	211.7 ± 11.4^{a}	173.8 ± 4.9^{a}	194.0 ± 6.6^{b}	199.6 ± 7.1^{b}	174.8 ± 6.1^{a}	169.8 ± 5.9^{a}	122.3 ± 5.3^{a}	154.5 ± 5.5^{b}	169.3 ± 5.0^{c}	141.1 ± 5.0^{d}	139.6 ± 5.6^{d}
VSL (µm/s)	151.6 ± 7.0^{a}	147.6 ± 10.6^{a}	146.3 ± 11.3^{a}	142.1 ± 11.3^{a}	147.9 ± 10.9^{a}	128.2 ± 2.3^{a}	137.2 ± 2.8^{b}	142.9 ± 3.4^{b}	133.5 ± 3.4^{a}	128.9 ± 2.6^{a}	96.4 ± 5.0^{a}	127.3 ± 4.3^{b,c}	132.9 ± 3.4^{b}	119.2 ± 4.5^{c,d}	117.6 ± 4.8^{d}
LIN (%)	71.5 ± 1.0^{a}	66.5 ± 0.7^{a}	67.2 ± 1.8^{a}	69.5 ± 1.5^{a}	69.5 ± 1.8^{a}	75.9 ± 1.3^{a}	72.8 ± 1.8^{b}	73.4 ± 1.6^{b}	78.5 ± 1.4^{c}	78.1 ± 1.4^{a,c}	81.9 ± 1.8^{a,b}	83.7 ± 1.5^{a,c}	80.0 ± 1.7^{b}	85.6 ± 1.1^{c}	85.5 ± 1.2^{c}
ALH (µm)	6.3 ± 0.2^{a}	7.0 ± 0.5^{b}	6.8 ± 0.4^{b}	6.2 ± 0.2^{a}	6.3 ± 0.3^{a}	5.2 ± 0.2^{a}	5.9 ± 0.3^{b}	5.8 ± 0.3^{b}	4.8 ± 0.2^{a}	4.8 ± 0.2^{a}	4.0 ± 0.2^{a,b}	4.4 ± 0.2^{b,c}	4.6 ± 0.2^{c}	3.8 ± 0.2^{a}	3.8 ± 0.2^{a}
BCF (Hz)	35.9 ± 0.9^{a}	35.8 ± 0.8^{a}	34.7 ± 0.6^{a}	34.2 ± 1.0^{a}	35.1 ± 1.0^{a}	33.2 ± 0.8^{a,b}	33.9 ± 0.6^{a}	34.7 ± 0.7^{a}	31.1 ± 1.1^{b}	30.8 ± 1.3^{b}	25.3 ± 1.2^{a}	27.5 ± 1.4^{a}	32.3 ± 1.3^{b}	26.6 ± 0.8^{a}	26.9 ± 1.1^{a}
Necrotic sperm (%)**	17.3 ± 2.1^{a}	15.2 ± 1.7^{a}	12.5 ± 1.3^{b}	20.9 ± 2.0^{c}	22.4 ± 2.0^{c}	19.7 ± 1.8^{a}	14.1 ± 1.5^{b}	15.8 ± 1.9^{b}	28.1 ± 2.5^{c}	25.9 ± 2.3^{c}	35.5 ± 2.8^{a}	37.6 ± 4.1^{a}	30.8 ± 3.6^{a}	34.2 ± 2.9^{a}	37.3 ± 3.0^{a}

[a] different superscript letters denote significant differences ($p < 0.05$) across treatments, within media, for each kinetic parameter

*cAMP upregulators included 1 mM caffeine, theophylline and dbcAMP

** 'necrotic' sperm were defined as non-viable (PI positive) but acrosome intact (FITC-PNA negative)

In comparison, high cAMP stimulation significantly altered these parameters, reducing motility and causing deterioration of spermatozoa, particularly after extended incubation (Table 5.1).

In plain TALP, motility parameters including total and progressive motility, velocity, linearity and beat cross frequency were not significantly altered by the presence of 75 – 150 µg/mL of BSP1 or BSP5 (Table 5.2). BSP5, but not BSP1, caused a slight increase in sperm necrosis. Several motility parameters were altered by the presence of 75 – 150 µg/mL of BSP1 or BSP5 under moderate cAMP stimulation (Table 5.2). While BSP1 reduced sperm necrosis at this level of stimulation, BSP5 promoted it. Interestingly, inclusion of either BSP1 or BSP5 under high cAMP stimulation diminished the negative effects of cAMP upregulators on a range of motility parameters (Table 5.2). However, addition of BSPs was unable to combat the significant deterioration of sperm viability under high cAMP stimulation. In general, compared to samples with no BSPs present, BSP1 consistently increased velocity, whilst BSP5 only showed improvements with all cAMP upregulators present. Linearity and straightness were decreased by BSP1 but increased by BSP5. BSP1 caused a consistent increase in the amplitude of lateral head displacement, and also increased beat cross frequency under high cAMP stimulation.

5.4.2.2. The effects of BSPs on the induction of acrosome reactions

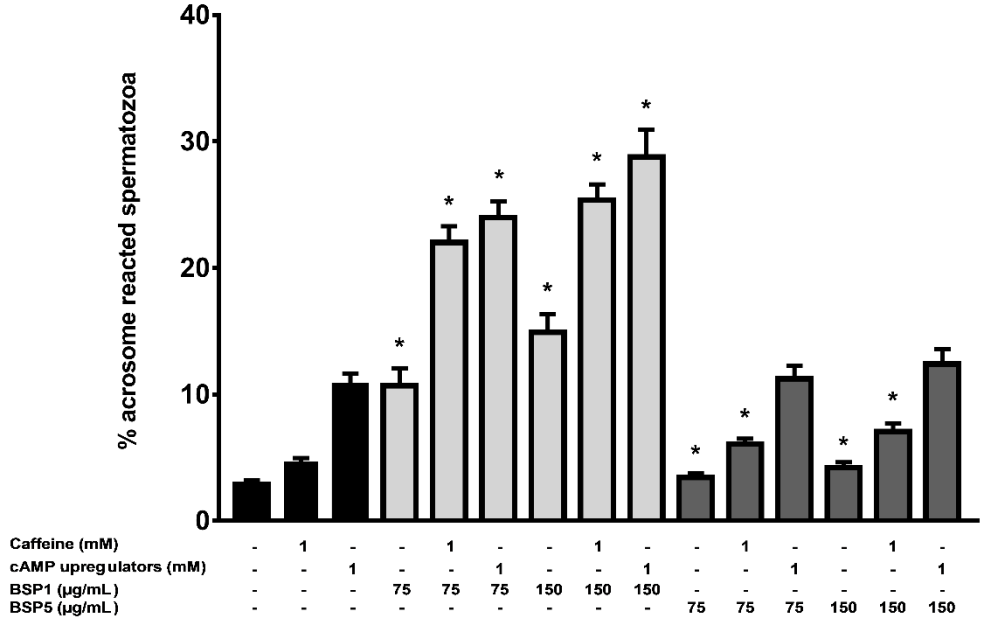

Figure 5.3 Acrosome integrity assessed by FITC-PNA fluorescence. Percentage of acrosome reacted spermatozoa, pooled across a 6 hour incubation with 0 or 1 mM of caffeine or all cAMP upregulators (caffeine, theophylline, dbcAMP) to promote capacitation, and with 0, 75 or 150 µg/mL of BSP1 or BSP5. *$p < 0.05$ relative to relevant control (with 0 or 1 mM of caffeine or cAMP upregulators)

Spontaneous acrosome reactions were measured following 0, 3 or 6 hours of incubation in basal, moderate and high cAMP stimulating conditions. The acrosome reaction in the absence of BSPs was < 5% in both basal and moderate cAMP stimulating conditions and approximately 10% under high cAMP stimulation (Figure 5.3). BSP1 addition significantly increased acrosome reactions compared to the control under all cAMP stimulation levels, with a further significant effect of dose (75 versus 150 µg/mL). In contrast, BSP5 caused a slight but significant increase in acrosome reactions only under basal and moderate cAMP stimulation, with no effects of dose.

5.4.2.1. The effects of BSPs on sperm membrane lipid disorder responses

Membrane lipid disorder significantly increased in control samples with stimulation by exogenous cAMP upregulators. Membrane lipid disorder was not significantly different in BSP exposed spermatozoa compared to the control without cAMP stimulation (Figure 5.4). However, when caffeine was introduced, all BSP treatments had slightly but significantly lower membrane lipid disorder than the control, and this effect was not dose dependent. When all cAMP upregulators were present, 150 µg/mL BSP1 significantly minimised membrane lipid disorder in relation to the control, however membrane disorder was still significantly higher than that observed in basal conditions (Figure 5.4).

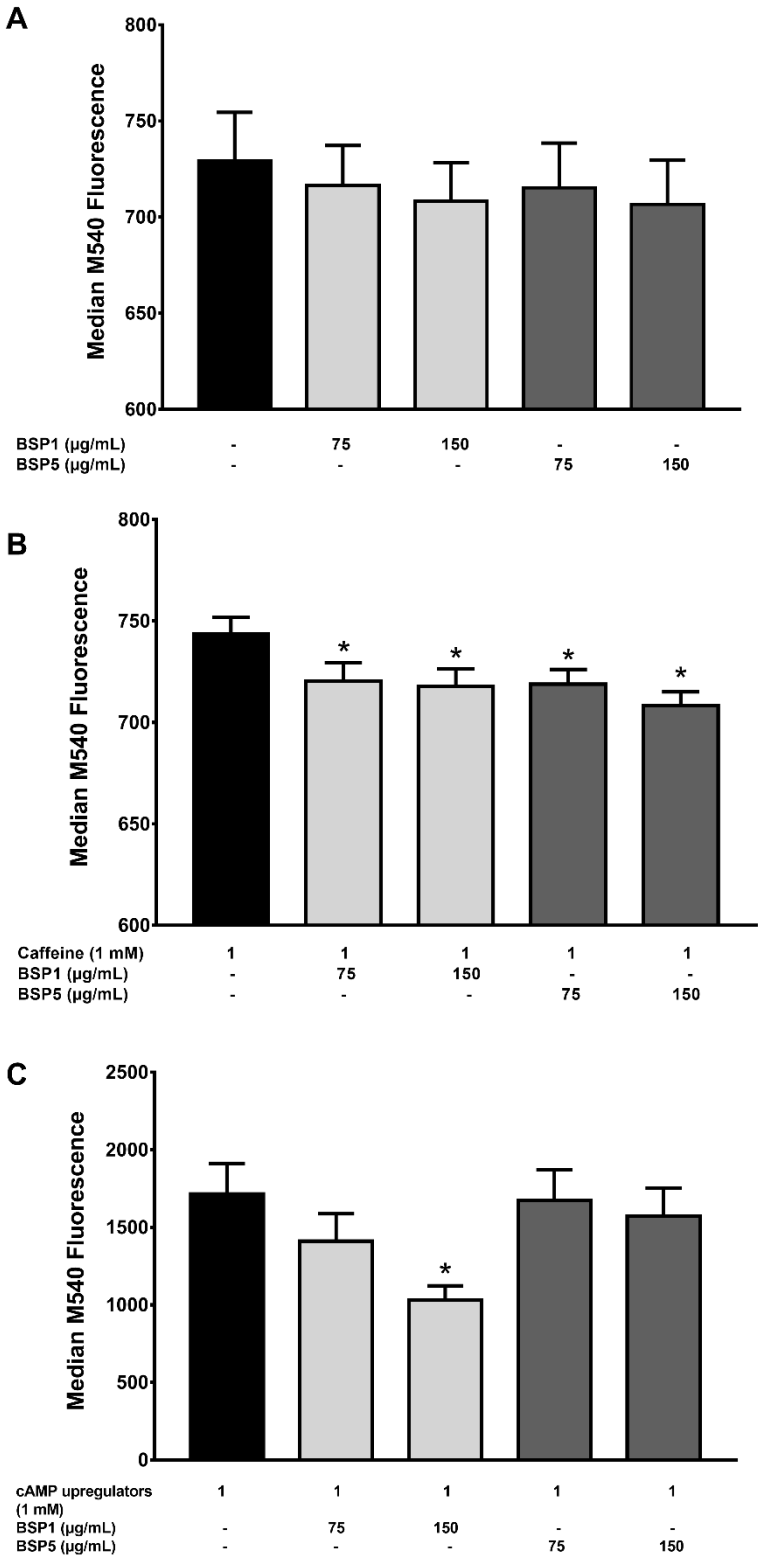

Figure 5.4 Membrane lipid disorder, assayed as median M540 fluorescence (arbitrary units) of the YO-PRO-1 negative ('viable') population, pooled across a 6 h incubation in basal TALP (**A**), or with 1 mM of caffeine (**B**) or all cAMP upregulators (caffeine, theophylline, dbcAMP, **C**), and with 0, 75 or 150 µg/mL of BSP1 or BSP5. *$p < 0.05$ relative to relevant BSP free control

5.4.2.2. The effects of BSPs on protein tyrosine phosphorylation responses

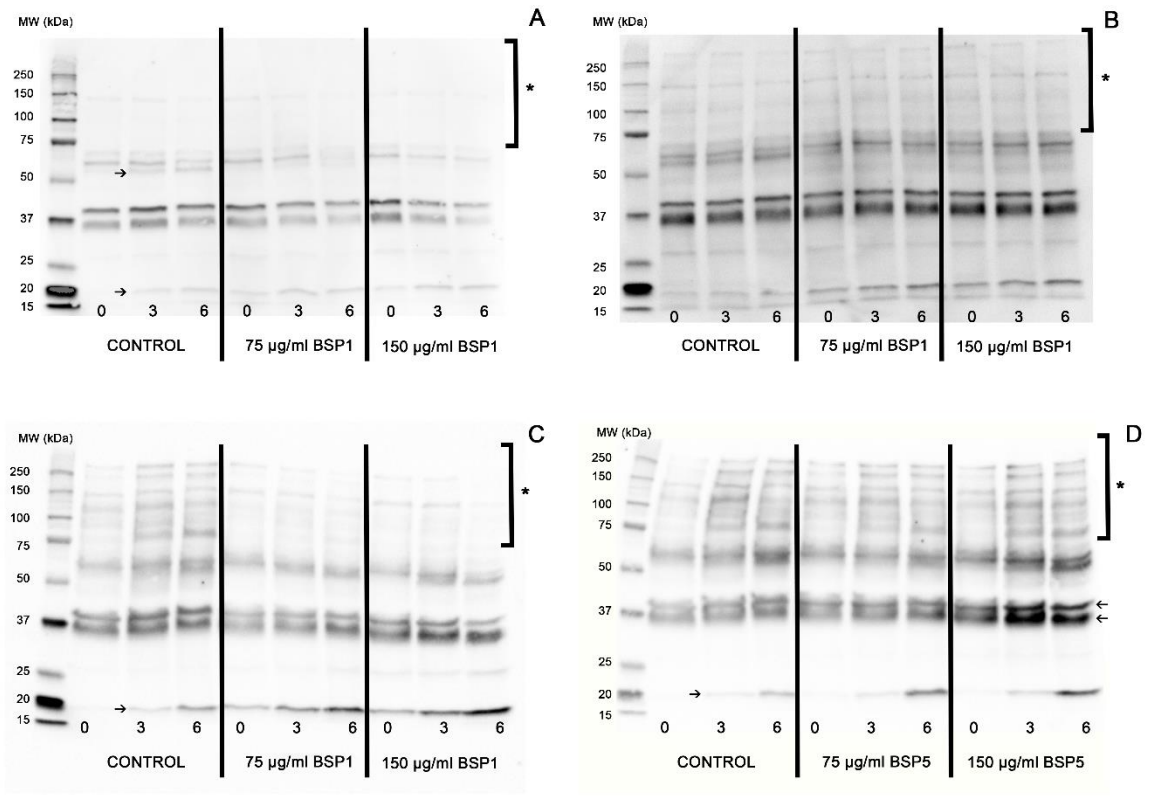

Figure 5.5 Western blots against tyrosine phosphorylation at 0, 3 and 6 h of incubation, from lysates of epididymal spermatozoa (10 µg total) exposed to 0, 75 or 150 µg/mL BSP1 (**A, B, C**) or BSP5 (**D**) in TALP (**A**), with 1 mM caffeine (**B**) or with 1 mM cAMP upregulators (caffeine, theophylline, dbcAMP, **C, D**). *Indicates high molecular weight region of interest, arrows indicate other bands of interest

In basal cAMP conditions, there was time dependent phosphorylation of a 55 kDa band in the control, which was not observed in spermatozoa treated with 75 – 150 µg/mL BSP1 (Figure 5.5A). This 55 kDa band was present with moderate cAMP stimulation, but its development was not time dependent. However, this band was again not observed in spermatozoa treated with 75 – 150 µg/mL BSP1 (Figure 5.5B). There was no observable, time dependent development of tyrosine phosphorylation of high molecular weight (> 75 kDa) proteins in any treatments under basal or moderate cAMP stimulation. Presence of cAMP upregulators was required for the development of time dependent tyrosine phosphorylation of high molecular weight proteins in the control (Figure 5.5C) as has been previously shown (Colás *et al.* 2008). Both 75 and 150 µg/mL BSP1 appeared to inhibit to some degree the tyrosine phosphorylation of high molecular weight (> 75 kDa) proteins in response to high cAMP stimulation, while also inducing somewhat stronger time dependent protein tyrosine phosphorylation of an 18 kDa band compared to the control (Figure 5.5C). Such an inhibitory response was not evident for BSP5, which did not alter tyrosine phosphorylation of high molecular weight proteins at 75 or 150 µg/mL compared to the control (Figure 5.5D). However, at 150 µg/mL, BSP5 appeared to

increase tyrosine phosphorylation of four moderate molecular weight protein bands (35, 37, 52, 55 kDa), as well as the same 18 kDa band, albeit at a slower rate than BSP1.

5.4.2.3. The effects of BSPs on cholesterol efflux

There was no significant efflux of cholesterol from any treatment over 6 h of incubation with low or moderate cAMP stimulation (Figure 5.6A,B). With all cAMP upregulators present, the control showed minimal cholesterol efflux, compared to significant cholesterol efflux in the presence of 150 µg/mL BSP1 (Figure 5.6C).

5.5. DISCUSSION

BSPs have been shown to play intricate roles in the capacitation of bull spermatozoa. In this study, we have investigated the effects of BSP1 and BSP5 on ram spermatozoa in environments ranging from minimal to maximal promotion of capacitation. Epididymal ram spermatozoa were used for these experiments, as they have had very little contact with BSPs, compared to ejaculated spermatozoa which contain high levels of BSPs originating from seminal plasma (Pini *et al.* 2016). Epididymal spermatozoa were obtained from the most distal region of the cauda epididymis to ensure maximal maturity and adequate response to capacitation stimulants (Lewis and Aitken 2001; Baker *et al.* 2003; Fàbrega *et al.* 2011b). Epididymal spermatozoa has previously been used in investigations of capacitation (Williams *et al.* 1991; Si and Olds-Clarke 2000; Navarrete *et al.* 2015) and for both in vitro (Nagai *et al.* 1984; Karja *et al.* 2013) and in vivo fertilisation (Dacheux and Paquignon 1980; Rickard *et al.* 2014), confirming that epididymal spermatozoa are fully capable of undergoing capacitation. Caffeine, theophylline and db cAMP were used in this study to create varying levels of cAMP upregulation, a requirement of ram spermatozoa to demonstrate the classical hallmarks of capacitation in vitro (Grasa *et al.* 2006; Colás *et al.* 2008). While the phosphodiesterase inhibitor activity of caffeine leads to significant increases in intracellular cAMP (Colás *et al.* 2010), addition of caffeine has also been shown to significantly increase intracellular calcium (Ho and Suarez 2001; Colás *et al.* 2010). However, as the main impact of caffeine driven increase in calcium appears to be hyperactivation (Ho and Suarez 2001; Colás *et al.* 2010), which was not observed in this study, we suggest that the observed effects of caffeine are likely due to its role as a cAMP upregulator.

The results presented in this report demonstrate that the two BSPs tested (BSP1 and BSP5) each affected specific responses to basal, moderate and high cAMP stimulating conditions. We have shown that in the ram, overall both BSPs have both pro- and de-capacitating effects on epididymal spermatozoa. BSP1 caused significantly higher loss of acrosome integrity than BSP5, and only BSP1 induced the efflux of cholesterol. Under the highest level of cAMP stimulation, BSP1 was able to limit membrane lipid disorder, while BSP5 had no such effects.

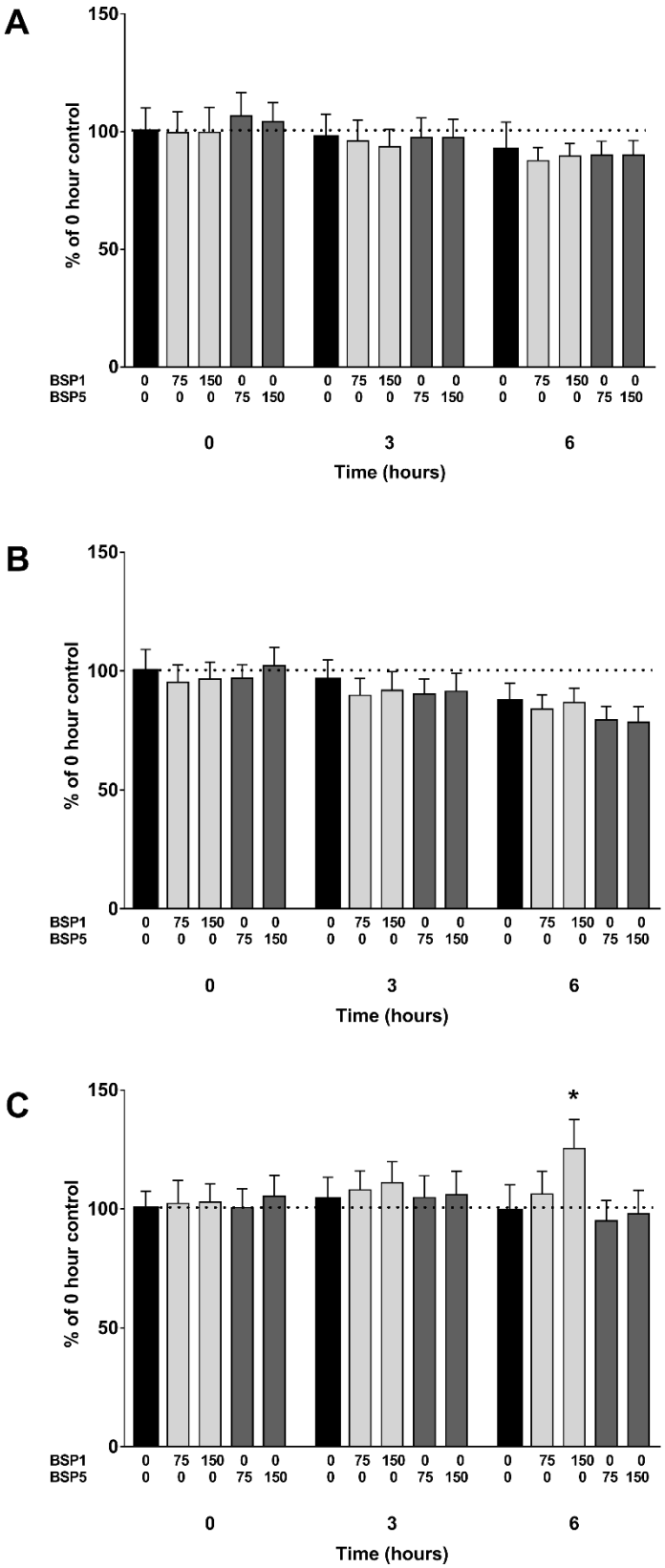

Figure 5.6 Supernatant cholesterol as a percentage of the 0 h control measurement (indicated by dotted line) from samples containing epididymal spermatozoa incubated in TALP (**A**), TALP with 1 mM caffeine (**B**) or TALP with cAMP upregulators (caffeine, theophylline, dbcAMP, **C**) and 0, 75 or 150 µg/mL of BSP1 or BSP5. Supernatant cholesterol was assessed using an Amplex Red assay. *$p < 0.05$ relative to the control

Finally, BSP1 qualitatively appeared to reduce the cAMP dependent tyrosine phosphorylation response of high molecular weight proteins (> 75 kDa), which was not observed in samples treated with BSP5. In contrast, BSP5 qualitatively appeared to increase the phosphorylation of moderate weight bands (35-55 kDa) compared to the control. Overall, the unique effects of BSP1 and BSP5 may be caused by differences in the structure of these two proteins. While their tandem fibronectin domains are very similar, BSP5 contains an extended N-terminal and is more highly glycosylated than BSP1 (Calvete *et al.* 1996). In addition, BSP5 has fewer hypothetical cholesterol interacting 'CRAC' domains than BSP1 (Scolari *et al.* 2010), which is in line with our observations.

BSPs are largely contributed by seminal plasma at ejaculation, after which they bind tightly to the sperm membrane (Pini *et al.* 2016). There is a significant body of evidence which describes how BSPs interact with plasma membranes. BSP1 binds specifically to the phosphorylcholine head group of phosphatidylcholine (PC), while BSP5 also interacts with phosphatidylethanolamine (PE), phosphatidylserine (PS) and several other phospholipids (Desnoyers and Manjunath 1992). BSPs are able to penetrate into the membrane, becoming partially embedded in the outer leaflet and remaining strongly adhered via PC (Le Guillou *et al.* 2016). While much research has focused on the interaction between BSPs and phospholipid head groups, there is evidence that BSP1 also interacts with the acyl chains of PC upon insertion (Anbazhagan *et al.* 2008). Of most relevance to the current results are previous studies showing that insertion of BSPs into the sperm membrane or analogous lipid vesicles results in rapid and significant immobilisation of phospholipids (Greube *et al.* 2001), as well as cholesterol (Swamy *et al.* 2002). Such interactions with the membrane can significantly influence lipid ordering of the outer leaflet. BSP1 was able to inhibit membrane lipid disorder to some degree, and significantly more so than BSP5, possibly because its structure allows for deeper penetration into the outer lipid leaflet, rendering it more lipid ordered. This is the first report of the effects of BSPs on membrane disorder using the fluorescent probe merocyanine 540, so there are no instances with which to compare our findings. However, our results may help to explain the previously observed beneficial effects of BSPs on ram spermatozoa in 'challenging' in vitro conditions (Barrios *et al.* 2000; Barrios *et al.* 2005; Bernardini *et al.* 2011), and encourages further investigation into their potential exploitation to reduce handling induced changes to ram spermatozoa.

In contrast to its stabilizing effect, consistent, long term exposure to BSP1 has been shown to cause significant phospholipid (Thérien *et al.* 1999) and cholesterol efflux (Thérien *et al.* 1998) from epididymal bull spermatozoa when incubated in basal capacitating medium (e.g. Tyrode's medium). Interestingly, these processes lead to the formation of BSP-phospholipid and BSP-cholesterol efflux particles. While BSPs are not able to directly bind cholesterol

(Desnoyers and Manjunath 1992), these authors have previously suggested that complexes may be formed between aggregated BSPs with hydrophobic pockets and effluxed sterols, effectively mitigating the requirement for a cholesterol acceptor (e.g. albumin, HDL). However, the only situation in which we were able to observe significant efflux of cholesterol was when epididymal ram spermatozoa were exposed to both BSP1 and cAMP upregulators (caffeine, theophylline, dbcAMP) in a highly stimulatory environment. Bull spermatozoa do not require cAMP regulation to efflux cholesterol after exposure to BSP1 (Thérien *et al.* 1998) and this difference is most likely due to the different conditions required to stimulate capacitation of ram spermatozoa (Colás *et al.* 2008; Leahy *et al.* 2016). This aligns with the idea that while extended exposure to BSP rich seminal plasma may be beneficial for ram spermatozoa during in vitro handling (i.e. without cAMP upregulation) it is detrimental for bull spermatozoa (Leahy and de Graaf 2012). Interestingly, while BSP1 caused significant cholesterol efflux, no such response was observed for BSP5. Apart from a lack of cholesterol interacting domains in BSP5 (Scolari *et al.* 2010) and a slightly different structure to BSP1 (Calvete *et al.* 1996), this could possibly be due to interference with concomitant cholesterol efflux caused by the phospholipid binding preferences of BSP5. Partial scrambling of phospholipids to the sperm surface during capacitation is suggested to facilitate cholesterol efflux (Gadella and Harrison 2000; Flesch *et al.* 2001), however binding of BSP5 to external PE and PS may hinder this process.

Upregulation of protein tyrosine phosphorylation by a cAMP/PKA pathway was first linked to mouse sperm capacitation (Visconti *et al.* 1995b) and has since been documented in other species (bull (Galantino-Homer *et al.* 1997), ram (Colás *et al.* 2008), human (Aitken *et al.* 1995)). While some BSP homologues have been shown to play a role in the development of capacitation associated protein tyrosine phosphorylation (Plante and Manjunath 2015), others have no apparent effect (Plante *et al.* 2014). There has been no investigation into the effects of BSPs on bull sperm tyrosine phosphorylation during capacitation. However, in vitro studies have demonstrated that bovine BSP1 has a strong, dose dependent inhibitory action on protein tyrosine kinase (PTK) activity (Yu *et al.* 2003), a group of enzymes responsible for tyrosine phosphorylation. This inhibitory activity may explain why in the current study we only observed limited high molecular weight (> 75 kDa) tyrosine phosphorylation when BSP1 was present in addition to cAMP upregulators, which drive the cAMP/PKA pathway, promoting PTK activation and subsequent tyrosine phosphorylation (Galantino-Homer *et al.* 1997; Baldi *et al.* 2000). Interestingly however, ejaculated ram spermatozoa, which have had exposure to BSP1 through seminal plasma, are able to develop strong high molecular weight tyrosine phosphorylation in response to cAMP upregulators (Colás *et al.* 2008). This suggests that in

a physiological situation, the effects of BSP1 on this pathway are tempered by other seminal plasma constituents.

We found that BSP1, and to a lesser extent BSP5, significantly increased spontaneous acrosome reactions in ram spermatozoa, in a time, stimulation and dose dependent manner. BSP1 in particular caused a significant increase in spontaneous acrosome reactions compared to the control after just 20 min of incubation, and this value also rose significantly after 3 and 6 h of incubation. In line with our findings, prolonged exposure of epididymal bull spermatozoa in basal capacitation medium to purified bovine BSP1 or BSP5 has been shown to significantly increase both lyso-PC induced and spontaneous acrosome reactions (Thérien *et al.* 1999). Similar results have been documented in humans, mice and pigs, with isolated or recombinant BSPs promoting ionophore induced acrosome reactions (Lusignan *et al.* 2007; Plante *et al.* 2012; Plante *et al.* 2014). These results suggest that BSPs, particularly BSP1, are able to trigger a non-physiological acrosome reaction (i.e. not preceded by capacitation) in ram spermatozoa, which involves a pathway that is sensitive to the direct or indirect effects of cAMP upregulators. Further investigation into how BSPs interact with other sperm membrane proteins and lipids is needed to guide our understanding of the exact biochemical physiology of this protein family. Until we understand more about their physiological relationship with acrosome integrity in ram spermatozoa, use of these proteins to improve in vitro handling outcomes should be treated with caution.

While cholesterol efflux and the acrosome reaction are important endpoints of sperm capacitation, the maintenance of motility is just as crucial for successful fertilisation. There has been limited investigation into how BSPs impact sperm motility, particularly when sperm are challenged by stimulatory conditions to induce capacitation. While some studies report no changes in motility after BSP addition (Lusignan *et al.* 2007; Plante *et al.* 2014), this is likely due to the use of washed, ejaculated spermatozoa. As BSPs physically insert into the sperm membrane (Le Guillou *et al.* 2016), washing is unlikely to totally remove these proteins and as such, ‘control’ spermatozoa would still have BSPs present. In addition, previous studies have largely carried out subjective motility analysis, which is not as comprehensive as CASA. While there were limited differences in basal conditions, we found that under high cAMP stimulation, BSPs could preserve total and progressive motility, with BSP1 significantly increasing velocity and the amplitude of lateral head displacement and BSP5 increasing linearity. These findings suggest that BSPs have a profound effect on the patterns and maintenance of motility exhibited by spermatozoa undergoing capacitation, as demonstrated previously in the presence of oviduct explants (Gwathmey *et al.* 2006). Epididymal spermatozoa exposed to BSP1 swim faster and more vigorously in stimulating environments, a potential advantage for penetrating cervical mucus, transiting the female tract and entering the cumulus-oocyte

complex (COC). This may be a contributing factor in the improved ability of seminal plasma exposed ram spermatozoa to transit the cervix (Rickard *et al.* 2014) and the increased cleavage rates observed when epididymal bull spermatozoa were exposed to BSP1 during in vitro fertilisation of bovine COCs (Rodríguez-Villamil *et al.* 2015).

Finally, it is worth considering the likely responses to seminal plasma derived BSPs in more physiological circumstances. Previous proteomic studies have confirmed that both BSP1 and BSP5 are present in ram seminal plasma (Soleilhavoup *et al.* 2014; Rickard *et al.* 2015), and on ejaculated spermatozoa (Pini *et al.* 2016). As this study investigated the effects of each protein in isolation, it would be interesting for future studies to use BSP1 and BSP5 in combination, which more closely resembles the physiological situation. Interestingly, immunofluorescence work has established that BSP1 and BSP5 show different patterns of localisation on ejaculated ram spermatozoa (Barrios *et al.* 2005). Roughly one third of spermatozoa exhibit binding of BSP1 and BSP5 over the whole sperm surface. However, of the remainder, more spermatozoa show binding of BSP5 than BSP1 on the tail and more show binding of BSP1 than BSP5 within the surface area covering the acrosome. These differences in localisation align with our findings of relatively different effects of BSP1 and BSP5, and suggest that in vivo, the two BSPs likely act synergistically, performing slightly different roles.

In conclusion, BSPs have been investigated for decades in the bull, and more recently in the mouse and human, highlighting their important roles in sperm capacitation. This is the first report of BSPs showing both pro and de-capacitation properties in ram spermatozoa. BSPs were shown to cause spontaneous acrosome reactions and could promote cholesterol efflux under capacitating conditions. Their maintenance of membrane lipid order, disruption of tyrosine phosphorylation and push towards higher motility and velocity under this stimulation shows their alternative potential for preservation of sperm quality. This study highlights the potential in vivo roles of BSPs in the ram, and clarifies the differences in their action on ram spermatozoa in comparison to their well established effects on bull spermatozoa.

5.6. ACKNOWLEDGEMENTS

The authors extend thanks to Francis Fidelle from CEDEO AI centre for provision of seminal plasma, Professor P. Manjunath from the University of Montreal for provision of BSP antibody and Lucie Combes-Soia for nanoLC-MS/MS analyses.

6. Binder of Sperm Proteins protect ram spermatozoa from freeze thaw damage

At the time of submission, this chapter was submitted for publication as; Pini, T., Farmer, K.L., Druart, X., Teixeira-Gomes, A., Tsikis, G., Labas, V., Leahy, T., and de Graaf, S.P. Binder of Sperm Proteins protect ram spermatozoa from freeze-thaw damage. *Reprod., Fert. Dev.*

6.1. ABSTRACT

Cryopreservation causes sublethal damage which limits the fertility of frozen thawed spermatozoa. Seminal plasma has been investigated as a cryoprotective agent, but has yielded inconsistent results due to considerable variation in its constituents. Individual seminal plasma proteins offer an ideal alternative to whole seminal plasma, and several have been correlated with freezing success. Binder of Sperm Proteins (BSPs) are abundant ram seminal plasma proteins which have been suggested to have a significant protective effect on ram spermatozoa during cold shock. This is in direct opposition to bull spermatozoa, where BSPs cause sperm deterioration during in vitro handling. We investigated the potential of BSP1 and BSP5 to prevent freezing associated damage to important functional parameters of ram spermatozoa. BSPs purified by size exclusion chromatography improved post thaw motility and penetration through artificial mucus. Highly purified BSP1 and BSP5, isolated by gelatin affinity and RP-HPLC, improved motility and viability, and reduced post thaw protein tyrosine phosphorylation. Exposure to BSP5 before freezing increased the amount of phosphatidylethanolamine on the sperm surface after thawing. Neither BSP1 nor BSP5 prevented freezing associated membrane lipid disorder. These results suggest that BSPs significantly improve freezing outcomes of ram spermatozoa, and could be exploited for commercial semen freezing.

6.2. INTRODUCTION

The ability to cryopreserve the male gamete is highly beneficial in animal production industries, as it creates a convenient, transportable material for artificial insemination. During early investigations into cryopreservation, assessment of post thaw sperm quality was based on easily observable characteristics such as subjective motility and insemination outcome. However, advanced in vitro assessment technologies have uncovered a plethora of sublethal freezing effects. Apart from reductions in motility and viability (Peris *et al.* 2007), cryopreservation leads to production of damaging reactive oxygen species (ROS) (Chatterjee and Gagnon 2001; Gürler *et al.* 2016), DNA damage (Peris *et al.* 2007; Fraser *et al.* 2011),

capacitation like changes (Thomas *et al.* 2006; Naresh and Atreja 2015), scrambling of membrane phospholipids (Fang *et al.* 2016) and altered abundance and distribution of proteins (Miller *et al.* 2015; Westfalewicz *et al.* 2015). The impacts of these changes surface as a commercially relevant end point; a significant reduction in insemination success in many species, particularly when inseminated far from the site of fertilisation (Lightfoot and Salamon 1970; Maxwell *et al.* 1999; Donovan *et al.* 2004; Heise *et al.* 2010; Masoudi *et al.* 2017). This is especially challenging in sheep, where the options for insemination are limited due to the difficulty in accessing the uterine body via the cervix (Halbert *et al.* 1990; Kershaw *et al.* 2005). While readily performable, cervical insemination in sheep fails to achieve commercially viable pregnancy rates using cryopreserved semen (Maxwell *et al.* 1999; Donovan *et al.* 2004). These limitations call for further improvements in the quality of frozen thawed ram semen, both on the fundamental level of motility and viability, as well as in the reduction of sublethal freezing damage.

The most obvious detrimental effects of freezing have largely been minimised by the development of suitable freezing media. Penetrating and non-penetrating cryoprotectants, namely glycerol and egg yolk respectively, have formed the basis of most commercial semen freezing media. Seminal plasma has also had significant investigation as a 'natural' cryoprotective agent, with inconsistent results. While several studies in sheep report no beneficial effects in vitro (Rovegno *et al.* 2013; Ledesma *et al.* 2015) or in vivo (O'Meara *et al.* 2007; Leahy *et al.* 2010a; Prado *et al.* 2013), others have reported significant benefits of adding seminal plasma either pre freeze or post thaw (García-López *et al.* 1996; Maxwell *et al.* 1999; Pérez-Pé *et al.* 2001; Domínguez *et al.* 2008; Leahy *et al.* 2009). These differences may be due to the amount, timing and composition of seminal plasma added (Domínguez *et al.* 2008; Rickard *et al.* 2015; Rickard *et al.* 2016). While seminal plasma is a variable and undefined additive, previous studies have associated individual seminal plasma proteins with the ability of ram semen to withstand cryopreservation and in vitro processing (Goularte *et al.* 2014; Soleilhavoup *et al.* 2014; Rickard *et al.* 2015). This suggests that particular proteins in ram seminal plasma may be capable of protecting spermatozoa during freezing. Addition of a select few proteins rather than bulk seminal plasma to freezing protocols would be an ideal alternative, as it is likely to reduce the variability of results and could offer a commercially viable improvement in freezing outcomes.

Several seminal plasma proteins show promise for the improvement of freezing success, including SPINK3 (Zalazar *et al.* 2016), heat shock proteins (Holt *et al.* 2015; Rickard *et al.* 2015) and Binder of Sperm Proteins (BSPs). BSPs are one of the most abundant protein families in bull (Nauc and Manjunath 2000) and ram seminal plasma (Soleilhavoup *et al.* 2014)

and bind to the sperm membrane in large amounts at ejaculation (Manjunath *et al.* 1988; Pini *et al.* 2016). BSP1 and BSP5 have previously been isolated from seminal plasma by chromatographic separation (Manjunath and Sairam 1987; Plante *et al.* 2015a) offering a simple method for purification using a readily available raw material. BSPs have been well characterised in the bull, and contribute to significant reductions in semen quality during extended in vitro handling (Bergeron *et al.* 2004; Leahy and de Graaf 2012) due to their promotion of cholesterol efflux (Thérien *et al.* 1998), a key capacitation process. In addition, the cryoprotective effect of egg yolk in bovine freezing media has been related to its ability to sequester BSPs (Bergeron and Manjunath 2006). In contrast, BSP1 and 5 demonstrate significant positive effects on ram spermatozoa undergoing cold shock (cooling to 5°C for 10 min), including reversal of membrane structural damage (Barrios *et al.* 2000) and improvements in viability (Barrios *et al.* 2005). The fact that these experiments were performed in the absence of egg yolk suggests that BSPs have direct protective effects on ram spermatozoa during cooling. These proteins have also been implicated in the reversal of freezing damage (Bernardini *et al.* 2011) and 'decapacitation' of ram spermatozoa (Luna *et al.* 2015), likely due to their stabilisation of the sperm membrane (Desnoyers and Manjunath 1992; Greube *et al.* 2001). These apparent differences between the effects of BSPs on bull and ram spermatozoa may be due to species differences in the membrane cholesterol to phospholipid ratio (Darin-Bennett and White 1977). However, the full extent of the effects of BSPs on ram spermatozoa during in vitro processing are not clear. While cold shock studies suggest that BSPs have great potential for cryoprotection of ram spermatozoa, their efficacy in preventing freezing damage has never been investigated.

There is a significant need to improve the post thaw outcomes of ram spermatozoa in order to more successfully employ cervical insemination for frozen thawed semen. Whilst BSPs have shown potential for the protection of ram spermatozoa, no studies have investigated their isolated effects under freezing conditions. In the present study, we sought to characterise the effects of BSP addition to ram spermatozoa prior to cryopreservation, using a range of in vitro assessments.

6.3. MATERIALS AND METHODS

6.3.1. Experimental design

A pilot experiment was first conducted using a seminal plasma size exclusion chromatographic fraction enriched in BSP1 and BSP5 to ascertain any potential beneficial effects of pre freeze supplementation on post thaw motility, as well as an appropriate protein concentration. A second experiment used highly purified BSPs, isolated by gelatin affinity chromatography coupled with reversed phase high performance liquid chromatography (RP-HPLC). Proteins

were supplemented at the most beneficial concentration established from the first experiment. This experiment characterised post thaw effects of BSP1 and BSP5 individually and in more detail, using a variety of stains assessed by flow cytometry, as well as western blotting of tyrosine phosphorylation. Epididymal spermatozoa were used in order to isolate the effects of our added BSPs from those of BSPs already present on the sperm membrane, which are at very low levels in epididymal ram spermatozoa (Pini *et al.* 2016). Experiments were replicated with 6 rams, split evenly across 2 technical replicates (i.e. 3 rams per technical replicate).

6.3.2. Chemicals

All chemicals were of the highest purity available, sourced from Sigma-Aldrich (Castle Hill, Australia) unless otherwise stated. Fluorescent probes were purchased from Life Technologies (Scoresby, Australia). Primary rabbit IgG antibodies against gelatin affinity purified ram BSPs (RRID AB_2715559) were kindly provided by P. Manjunath (Departments of Biochemistry and Medicine, Faculty of Medicine, University of Montreal).

6.3.3. Animals, semen collection and seminal plasma isolation

All experiments were performed using epididymal spermatozoa flushed from testes of recently slaughtered rams (n = 6) from a local abattoir. Testes were excised, transported at 4°C and processed within 5 h of collection. Spermatozoa were collected from the cauda epididymis via retrograde flushing with warm tris-citrate-fructose medium (308 mM tris, 104 mM citric acid (monohydrate), 28 mM D-fructose, pH 7.3). Collected samples were assessed for wave motion and processed if scored ≥ 3 out of 5 (Evans and Maxwell 1987).

Rams used for seminal plasma collection were either housed at University of Zaragoza, Spain (n = 8, size exclusion purification), or CEDEO AI centre in Ordiarp, France (n = 50, gelatin affinity, RP-HPLC purification). Animals were maintained in an animal house (University of Zaragoza) or on pasture (CEDEO AI) and all animal procedures followed the European Union directive 2010/63/EU for animal care and welfare. Seminal plasma was extracted by double centrifugation of ejaculates at 14, 000 x g for 20 min. For size exclusion purification, seminal plasma was concentrated using Amicon 3 kDa molecular weight cut off centrifugal filters (Millipore, Madrid, Spain) and stored at - 80°C until further use. For gelatin affinity, RP-HPLC purification, seminal plasma proteins were ethanol precipitated, lyophilised and stored at 4°C until further use.

6.3.4. Isolation of Binder of Sperm Proteins

6.3.4.1. Size exclusion purification

Up to 100 mg of concentrated ram seminal plasma was loaded onto a Sephacryl 16/100 s-100 gel chromatography column (GE Healthcare Life Sciences, Barcelona, Spain). Samples were run at a flow rate of 0.1 - 0.2 mL/min, collecting fractions of 1.5 - 2 mL. Fractions were pooled based on their absorbance at 280 nm. The fractions which corresponded to the BSP enriched 'fraction 6' and 'fraction 7' as previously characterised (Barrios *et al.* 2000; Barrios *et al.* 2005) were pooled together, dialysed against 1:100 (v/v) column buffer to ultra-pure water, concentrated using Amicon 3 kDa molecular weight cut off centrifugal filters and stored at -80°C (Figure 6.1A). Binding of the pooled BSP enriched fractions to epididymal spermatozoa was confirmed by western blotting (Figure 6.1B). The purity of BSPs in this pooled fraction has not been determined by any quantitative measures, and as such, this fraction will be referred to as a 'BSP enriched fraction'.

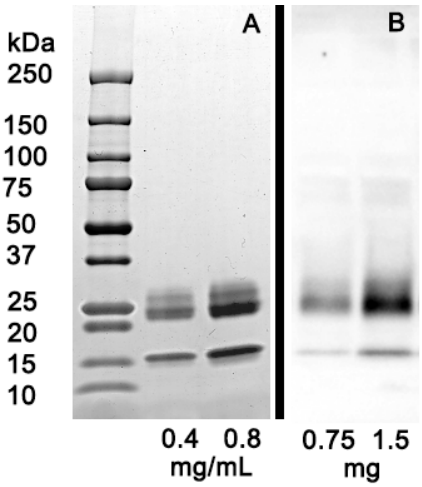

Figure 6.1 (**A**) Pooled, concentrated fraction of interest (0.4 mg/mL, 0.8 mg/mL) from size exclusion chromatography of seminal plasma on a Sephacryl S-100 column, run on a 4-20% SDS PAGE and stained with coomassie brilliant blue. Pooled fractions contain a clean band at approximately 15 kDa and doublets at 22-26 kDa (**B**) 30 µg of epididymal spermatozoa lysate following 20 min exposure to 0.75 mg or 1.5 mg of the fraction pictured in A, probed with a BSP antibody (with affinity for both BSP1 and BSP5). BSA exposed controls produced no detectable signal (not shown)

6.3.4.2. Gelatin affinity and RP-HPLC purification

320 mg of ethanol precipitated seminal plasma proteins were loaded onto a 12 mL gelatin affinity column (gelatin was previously coupled to Affi-Gel 15 resin (Bio-Rad, Marnes-la-Coquette, France)). Bound proteins were eluted with phosphate buffered saline (PBS) containing 5 M urea. 1 mL fractions were collected and pooled based on their absorbance at 280 nm, desalted three times with a PD10 column and lyophilised. A total of 64 mg of the gelatin absorbed proteins were subjected to multiple runs of RP-HPLC on a Waters XBridge BEH C18 OBD Prep column (250 mm × 10 mm i.d., particle size 5 µm, pore size 130 Å; Waters, Guyancourt, France). A linear 28 to 45% acetonitrile gradient with 0.1% trifluoroacetic

acid was used at a constant flow rate of 3 mL/min for 29 min. BSP1 and BSP5 were isolated in two separate fractions (Figure 6.2), which were desalted by dialysing against 50 mM ammonium bicarbonate, lyophilised and stored at 4°C. Western blotting confirmed binding of the isolated proteins to epididymal spermatozoa (Figure 6.3). Purified proteins were resuspended in tris buffered saline (20 mM tris, 150 mM NaCl, pH 7.6) at high concentration (> 12 mg/mL) and stored at - 80°C. These isolated fractions are referred to as 'isolated BSP1 and BSP5'.

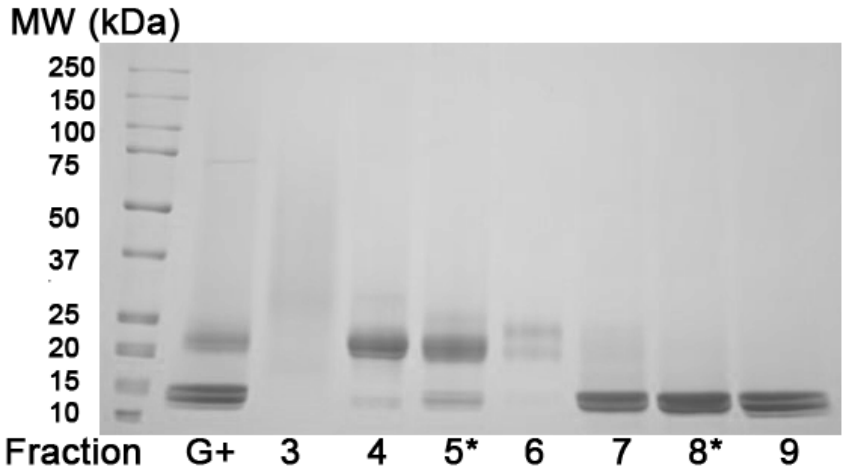

Figure 6.2 Fractions collected after gelatin affinity chromatography, followed by RP-HPLC of ethanol precipitated ram seminal plasma proteins. Coomassie brilliant blue stained 8-16% SDS PAGE of gelatin binding fraction (G+) (10 µg) and sequentially eluted RP-HPLC fractions (fractions 3 and 6; 5 µg, fractions 4, 5, 7, 8, 9; 10 µg). The fractions employed as 'purified BSP1' (8) and 'purified BSP5' (5) are indicated*

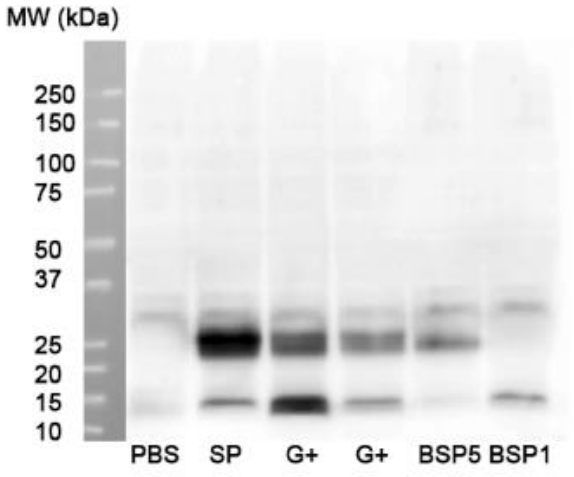

Figure 6.3 Binding of purified BSP1 and BSP5 to epididymal ram spermatozoa (1 h, 37°C). Western blot of epididymal spermatozoa (490 x 10^6 total) incubated 1:1 (v/v) with (1) phosphate buffered saline, (2) seminal plasma, (3,4) gelatin binding fraction (13 mg/mL or 1 mg/mL), (5) purified BSP5 (0.4 mg/mL), (6) purified BSP1 (1 mg/mL), probed with anti-BSP

6.3.4.3. LC-MS/MS to assess purity of gelatin affinity and RP-HPLC purified proteins

Six reversed phase chromatography fractions (Figure 6.2) were in-solution digested with bovine trypsin. Briefly, proteins in 50 mM NH_4HCO_3 were reduced in 5 mM dithiothreitol (30 min, 56°C) and alkylated in 12.5 mM iodoacetamide (20 min, room temperature in the dark). Proteins were digested overnight with 12.5 ng/µL trypsin (sequencing grade, Roche, France) with a ratio of 1:40 enzyme:substrate. 5 µL of peptides was directly injected onto a trap column and separated on a nano-column as previously described (Labas *et al.* 2015), using a 4 – 55% B 90 min gradient at a flow rate of 300 nL/min on an Ultimate 3000 RSLC UHPLC system (Dionex, Netherlands). Eluate was ionised using a Thermo Finnigan Nanospray Ion Source 1 and MS/MS was carried out on an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Germany). Data were acquired in positive data dependent mode, with sequential isolation (isolation width 2 *m/z*) and fragmentation (collision induced dissociation) of the 20 most intense peptide ions (charge state 2 +, *m/z* range 300 – 1800). Proteins were identified by Mascot search engine (version 2.3, Matrix Science) against the NCBInr database using mammalian taxonomy (downloaded December 2016). Database search criteria included trypsin as a protease with two missed cleavages allowed, variable modifications (carbamidomethylcysteine, methionine oxidation, acetylation of N-terminal proteins) and 5 ppm/0.8 Da parent/fragment ion match tolerance. Scaffold software (version 3.6, Proteome Software, USA) was used to validate protein identifications using the Peptide and Protein Prophet algorithms. Protein identifications were accepted if they contained at least two peptides and had > 95% probability. The abundance of identified proteins was estimated by calculating the emPAI using Scaffold Q+ software (Proteome Software, USA).

6.3.5. Treatment of epididymal spermatozoa with BSPs

All treatments were applied prior to dilution in freezing media to ensure adequate binding of BSPs prior to sequestering by egg yolk low density lipoprotein (Manjunath *et al.* 2002). Proteins were thawed on ice and warmed to 37°C prior to use.

6.3.5.1. BSP enriched fraction

Epididymal spermatozoa were diluted with tris-citrate-fructose medium containing 0.3% (w/v) bovine serum albumin (BSA) to a concentration of 100 x 10^6 spermatozoa/mL. Aliquots containing a total of 5 x 10^6 spermatozoa were diluted with tris-citrate-fructose medium containing 1.5 mg BSA (control), 0.75 or 1.5 mg of the BSP enriched fraction. Samples were incubated at 37°C for 20 min to allow for protein binding, then further diluted to 9 x 10^6 spermatozoa/mL with 15% (v/v) egg yolk cryodiluent (Evans and Maxwell 1987). Diluted

samples were chilled at 4°C for 1.5 h, frozen in 270 µL pellets on dry ice and stored in liquid nitrogen until further use.

6.3.5.2. Isolated BSP1 and BSP5

The results of the first experiment suggested that the lower dose of 0.75 mg per 5 x 10^6 spermatozoa was the most beneficial, and as such this dose was used for the second experiment. Epididymal spermatozoa were treated as above, with 0.75 mg of BSA, isolated BSP1 or isolated BSP5 per 5 x 10^6 spermatozoa. Further dilution and freezing was carried out as above.

6.3.6. In vitro assessment of post thaw sperm characteristics

Pellets were thawed in a glass tube for 2 min in a 37°C water bath, then immediately diluted 1:1 (v/v) with tris-citrate-fructose 0.3% (w/v) BSA medium. The following assessments were carried out immediately post thaw (0 h) and after a prolonged incubation at 37°C (3 h).

6.3.6.1. Motility

Objective motility was assessed using computer assisted sperm analysis (Hamilton Thorne IVOS II, Animal Breeder software, version 1.8), capturing a minimum of 200 cells, with settings appropriate for ram spermatozoa.

6.3.6.2. Mucus penetration

The ability of spermatozoa to penetrate mucus was tested using objective motility when incubated for 3 h, diluted 1:1 (v/v) with an artificial mucus medium (2% (w/v) porcine gastric mucins reconstituted at 4°C for 24 h in 60 mM NaCl, 3mM $Ca(OH)_2$, 25 mM KOH, 22 mM lactic acid, 17 mM acetic acid, 7 mM urea, 28 mM glucose, 15 mM $NaHCO_3$, 0.1% (v/v) glycerol, 0.002% (w/v) BSA, 2% (v/v) MEM amino acid 50x solution, pH 6.3). Objective motility was assessed using computer assisted sperm analysis as above.

6.3.6.3. Flow cytometry

Advanced flow cytometric assessment in the second experiment was conducted using a CytoFLEX flow cytometer calibrated prior to use with CytoFLEX daily QC Fluorospheres (Beckman Coulter, Lane Cove, Australia). Stain panels conducted included fluorescein isothiocyanate (FITC)-PNA (0.4 µg/mL final)/PI (6 µM final), Cy5-duramycin (0.4 µM final)/FITC-annexin V (0.3 µg/mL final)/PI, merocyanine 540 (M540; 0.8 µM final)/YO-PRO-1 (25 nM final) and H_2DCFDA (5 µM final)/PI. All samples were also stained with the DNA probe Hoechst 33342 (1 µg/mL final) in order to gate out debris. Three lasers were employed; 50 mW 488 nm, 50 mW 638 nm and 80 mW 405 nm. Fluorescence detection used 450/45 BP

(Hoechst 33342), 525/40 BP (FITC, H_2DCFDA, YO-PRO-1), 585/42 BP (PI, M540) and 660/10 BP (Cy5-duramycin) filters. Initial gating based on 488 nm forward and side scatter was used to eliminate debris and isolate the sperm population. Within this, a minimum of 10, 000 Hoechst 33342 positive events were recorded for each sample.

6.3.6.4. Western blotting of tyrosine phosphorylation

After 3 h of incubation at 37°C post thaw, aliquots at 4.5 x 10^6 spermatozoa/mL were centrifuged at 14, 000 x *g* for 10 min and the supernatant removed. The cell pellet was resuspended to the original volume with BSA free tris-citrate-fructose medium and the wash repeated. The resulting cell pellet was resuspended 1:2 (v/v) in lysis buffer (1% (w/v) sodium dodecyl sulphate (SDS), 1 mM sodium orthovanadate, cOmplete protease inhibitor tablet, tris-citrate-fructose medium) and incubated for 1 h at room temperature with vortexing every 15 min. Samples were centrifuged at 7, 500 x *g* for 15 min and the resulting supernatant was stored at - 80°C until use. Lysate protein content was determined using a Qubit fluorometer (Thermo Fisher Scientific, North Ryde, Australia) and samples were reduced with Laemmli sample buffer by boiling for 5 min at 95°C. 10 µg of protein were loaded on a 10% TGX stain free gel (Bio-Rad, Gladesville, Australia) and separated by SDS-PAGE (200 V, 40 min) using a mini-PROTEAN tetra cell (Bio-Rad). Separated proteins were blotted onto PVDF membrane (100 V, 75 min, 4°C) using a mini Trans-Blot cell (Bio-Rad) and blocked with 5% (w/v) BSA in tris buffered saline (TBS) with 0.1% (v/v) Tween-20 (TW) for 60 min at room temp. Blots were incubated for 60 min at room temp with HRP-anti-phosphotyrosine diluted 1:2000 (Merck Millipore, Billerica, USA, RRID AB_310779) in TBS-TW with 0.1% (w/v) BSA. Blots were washed five times with TBS-TW and revealed using the Bio-Rad Immun-star western chemiluminescence kit (luminol and peroxidase diluted 1:1 (v/v)), incubated for 5 min with agitation. Images were visualised on a Chemi Doc XRS+ and processed using Image Lab software (version 5.1, Bio-Rad). Bands were quantified using Image Lab tools. Normalisation was performed using the 'stain free' method developed by Bio-Rad. Briefly, trihalo compounds within Bio-Rad stain free gels bind to tryptophan residues of proteins and react under UV stimulation to produce detectable fluorescence, allowing for the imaging of protein bands post transfer. The density of bands on the blot was normalised against a corresponding stain free image of the same blot and compared across treatments (n = 2 blot replicates).

6.3.7. Statistical analysis

Statistical analysis was performed using Genstat (version 18, VSN International). Data were assessed for normality and transformed if necessary to meet test assumptions. Outcomes were assessed using a linear mixed model incorporating treatment and time (as applicable) as the fixed model and technical replicate and ram as the random model, with an α of 0.05.

Means were compared by least significant difference. All values are reported as the mean ± standard error of the mean, back transformed as appropriate.

6.4. RESULTS

6.4.1. Effects of a size exclusion chromatographic fraction enriched in BSP1 and BSP5 at different doses

Pre freeze addition of a BSP enriched fraction significantly improved a range of post thaw motility parameters (Table 6.1). The BSP enriched fraction significantly increased post thaw total motility, all measures of velocity, straightness, amplitude of lateral head displacement and beat cross frequency. In the majority of parameters, the low dose of 0.75 mg resulted in significantly larger improvements than the high dose of 1.5 mg, and as such this dose was used for supplementation with highly purified proteins.

Addition of a BSP enriched fraction significantly altered motility patterns of epididymal ram spermatozoa in an artificial mucus medium. While the low dose of BSPs had more pronounced effects than the high dose, their addition generally resulted in increased motility, velocity and amplitude of lateral head displacement, as well as decreased linearity when incubated in the presence of a 2% (w/v) mucin containing medium (Figure 6.4).

6.4.2. Purity of BSP1 and BSP5 isolated by gelatin affinity and RP-HPLC

Mass spectrometry confirmed the isolated fractions contained 99% BSP1 and 92% BSP5 respectively (Supplementary Table 6.1).

6.4.3. Effects of highly purified BSP1 and BSP5 isolated by gelatin affinity and RP-HPLC

6.4.3.1. Post thaw motility parameters are improved by BSPs, but this effect is time sensitive

Addition of highly purified BSP1 or BSP5 produced significantly different results to supplementation with a BSP enriched fraction in terms of motility. Most parameters were improved immediately post thaw, but were significantly lower than the control by 3 h (Table 6.2). While BSPs did not alter total motility, the percentage of progressively motile spermatozoa was almost double that of the control immediately post thaw (Figure 6.5). However, BSP supplemented spermatozoa did not show longevity in their motility; total motility

Table 6.1 Post thaw motility parameters of epididymal ram spermatozoa immediately post thaw (0 h) or after extended incubation at 37°C (3 h), treated with 1.5 mg bovine serum albumin (BSA; control), 0.75 or 1.5 mg of a size exclusion chromatographic fraction enriched in Binder of Sperm Proteins (BSPs), prior to cryopreservation

	0 h			3 h		
	1.5 mg BSA (control)	**0.75 mg BSPs**	**1.5 mg BSPs**	**1.5 mg BSA (control)**	**0.75 mg BSPs**	**1.5 mg BSPs**
Total motility (%)	46.8 ± 3.98[a]	83.0 ± 2.79[b]	60.2 ± 7.94[c]	5.5 ± 2.70[a]	42.9 ± 6.28[b]	23.2 ± 6.93[c]
Average path velocity (µm/s)	41.6 ± 3.53[a]	89.3 ± 3.02[b]	79.0 ± 5.90[c]	22.2 ± 2.33[a]	44.0 ± 3.48[b]	37.1 ± 2.51[c]
Curvilinear velocity (µm/s)	96.8 ± 6.54[a]	165.0 ± 7.02[b]	144.9 ± 10.38[c]	50.9 ± 7.87[a]	111.9 ± 6.91[b]	94.8 ± 5.35[c]
Straight line velocity (µm/s)	31.3 ± 3.37[a]	67.7 ± 2.99[b]	60.6 ± 4.10[b]	13.4 ± 0.83[a]	33.8 ± 3.16[b]	29.5 ± 2.04[b]
Straightness (%)	73.7 ± 2.31[a]	77.3 ± 2.92[b]	78.5 ± 0.99[c]	66.2 ± 6.02[a]	75.9 ± 2.22[b]	80.2 ± 2.28[c]
Amplitude of lateral head displacement (µm)	5.8 ± 0.31[a]	6.9 ± 0.45[b]	6.1 ± 0.44[a]	3.6 ± 0.58[a]	5.6 ± 0.33[b]	4.5 ± 0.36[a]
Beat cross frequency (Hz)	24.0 ± 0.93[a]	33.3 ± 1.04[b]	32.0 ± 0.38[b]	21.3 ± 1.50[a]	30.1 ± 0.88[b]	35.2 ± 2.68[c]

[a]different superscript letters within the same row and time point denote significant differences ($p < 0.05$)

Table 6.2 Post thaw parameters of epididymal ram spermatozoa immediately post thaw (0 h) or after extended incubation at 37°C (3 h), treated with 0.75 mg bovine serum albumin (BSA; control), 0.75 mg gelatin affinity and RP-HPLC purified Binder of Sperm Protein 1 (BSP1) Binder of Sperm Protein 5 (BSP5) prior to cryopreservation

	0 h			3 h		
	0.75 mg BSA (control)	**0.75 mg BSP1**	**0.75 mg BSP5**	**0.75 mg BSA (control)**	**0.75 mg BSP1**	**0.75 mg BSP5**
Average path velocity (μm/s)	94.7 ± 2.80[a]	102.3 ± 4.21[a]	115.8 ± 3.03[b]	56.4 ± 4.24[a]	38.9 ± 5.00[b]	44.4 ± 3.31[b]
Curvilinear velocity (μm/s)	190.6 ± 6.82[a]	190.9 ± 7.92[a]	222.7 ± 4.88[b]	114.9 ± 6.49	106.6 ± 6.07	113.2 ± 4.44
Straight line velocity (μm/s)	66.2 ± 2.92[a]	77.9 ± 2.65[b]	87.4 ± 2.32[c]	46.6 ± 3.47[a]	30.9 ± 4.72[b]	35.9 ± 2.72[b]
Straightness (%)	72.0 ± 1.71[a]	77.6 ± 1.05[b]	76.6 ± 0.43[b]	82.9 ± 0.42[a]	77.9 ± 2.56[b]	80.3 ± 1.59[c]
Linearity (%)	38.5 ± 1.75[a]	44.2 ± 0.85[b]	42.1 ± 0.60[a,b]	42.0 ± 1.35[a]	28.6 ± 3.28[b]	31.7 ± 2.04[b]
Amplitude of lateral head displacement (μm)	8.4 ± 0.31[a]	8.1 ± 0.39[a]	9.3 ± 0.29[b]	5.1 ± 0.24[a]	5.3 ± 0.32[a]	5.6 ± 0.33[a]
Beat cross frequency (Hz)	31.4 ± 1.08	32.3 ± 0.28	31.6 ± 1.22	32.7 ± 0.96	37.5 ± 1.71	34.6 ± 1.53
Membrane disorder[1]	63, 456 ± 1390[ab]	65, 152 ± 1315[a]	61, 404 ± 1478[b]	62, 787 ± 1844[a]	66, 864 ± 1427[b]	65, 051 ± 1021[ab]

[a]different superscript letters within the same row and time point denote significant differences ($p < 0.05$)

[1]Membrane disorder was measured as the median fluorescence of M540 (arbitrary units) in the YO-PRO-1 negative population

declined significantly compared to the control, whilst progressive motility was on par by 3 h. BSP5 improved all measures of velocity and increased the amplitude of lateral head displacement. BSP1 significantly increased straight line velocity, linearity and straightness.

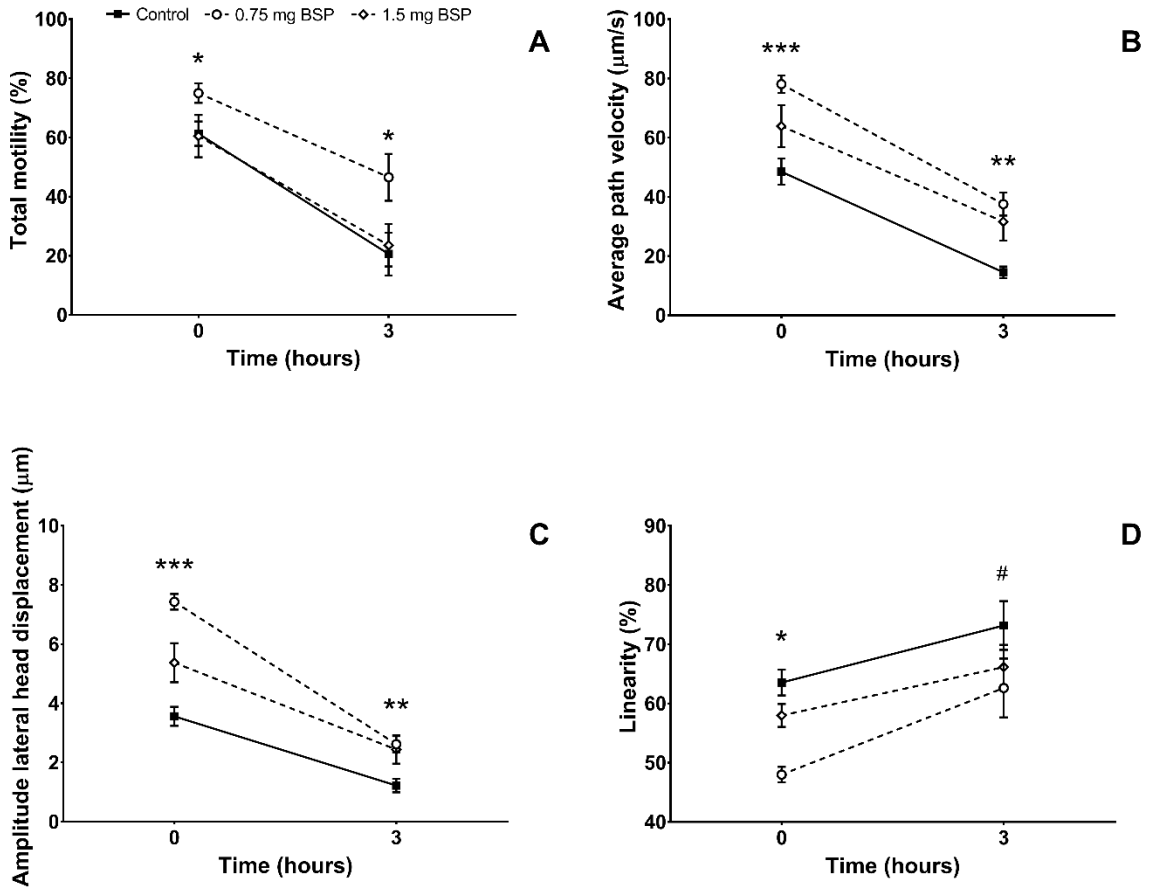

Figure 6.4 Motility parameters of frozen thawed epididymal ram spermatozoa in an artificial mucus medium (see methods for formulation), with pre freeze exposure to 1.5 mg bovine serum albumin (control; filled square, solid line), 0.75 mg (open circle, dashed line) or 1.5 mg (open diamond, dashed line) of a chromatographic fraction enriched in BSP1 and BSP5. Total motility (**A**), average path velocity (**B**), amplitude of lateral head displacement (**C**) and linearity (**D**) were measured immediately after dilution in mucus medium (0 h) and after extended incubation at 37°C (3 h). # one BSP treatment $p < 0.05$ compared to the control, * one BSP treatment $p < 0.05$ compared to the control and the other BSP treatment ** both BSP treatments $p < 0.05$ compared to the control, *** both BSP treatments $p < 0.05$ compared to the control, and to one another

6.4.3.2. BSPs alter viability, acrosome integrity and ROS production

Both BSP1 and BSP5 addition significantly improved post thaw viability (Figure 6.5). The percentage of acrosome reacted spermatozoa was significantly increased by BSP1 and decreased by BSP5 compared to the control (Figure 6.5), however such small differences are unlikely to be physiologically relevant. On the basis of median H_2DCFDA fluorescence, spermatozoa exposed to BSP1 and BSP5 pre freeze produced significantly higher amounts of reactive oxygen species (ROS) at both 0 h and 3 h (Figure 6.6). However, median H_2DCFDA

fluorescence had a significant positive correlation (ρ= 0.61, p < 0.05) with progressive motility at 0 h when assessed by Spearman's rank correlation.

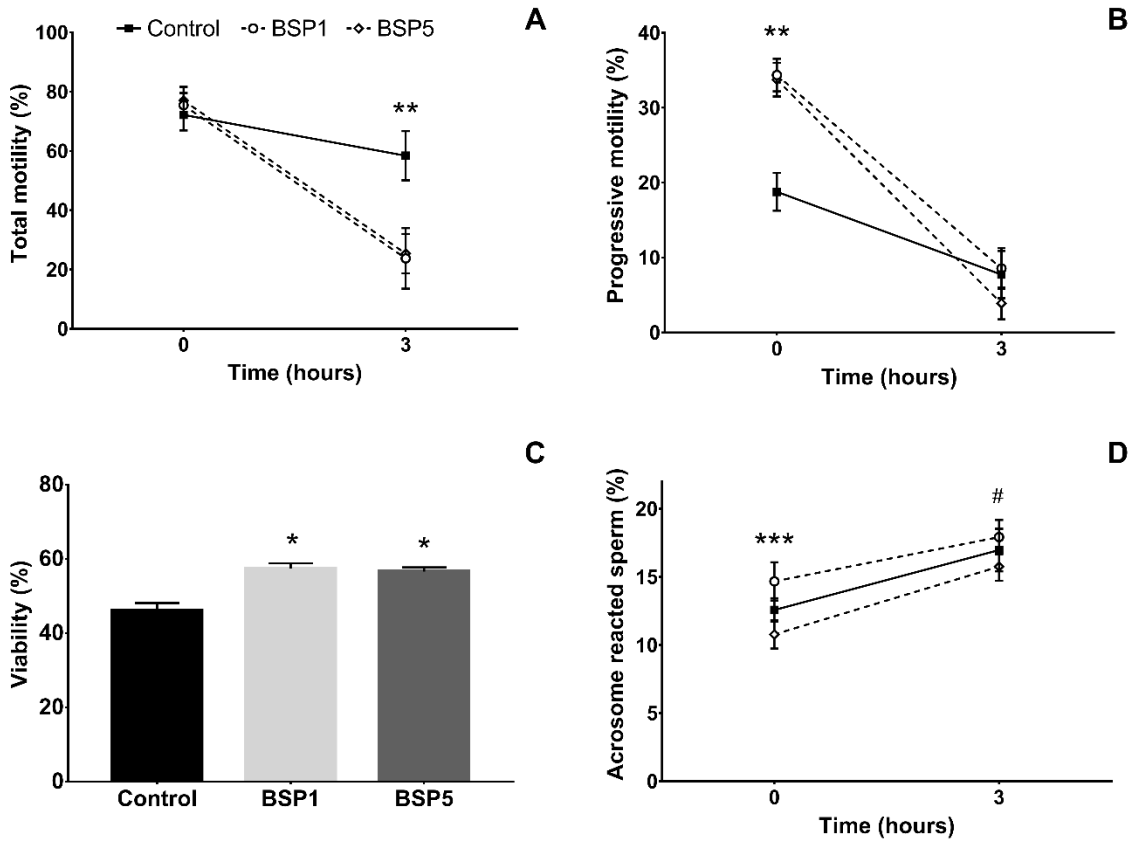

Figure 6.5 Post thaw parameters of frozen thawed epididymal ram spermatozoa, with pre freeze exposure to 0.75 mg bovine serum albumin (control; filled square, solid line), 0.75 mg gelatin affinity and RP-HPLC purified Binder of Sperm Protein 1 (BSP1; open circle, dashed line) or Binder of Sperm Protein 5 (BSP5; open diamond, dashed line). Total motility (**A**), progressive motility (**B**), viability (**C**) and acrosome integrity (**D**) were measured immediately after thawing (0 h) and after extended incubation at 37°C (3 h). #one BSP treatment p < 0.05 compared to the control and the other BSP treatment, *one BSP treatment p < 0.05 compared to the control, **both BSP treatments p < 0.05 compared to the control, ***both BSP treatments p < 0.05 compared to the control, and to one another

6.4.3.3. BSPs do not minimise membrane disorder and BSP5 causes changes in membrane phosphatidylethanolamine

The lipid probe merocyanine 540 (M540) is able to intercalate with membrane phospholipids only when the lipid bilayer becomes disordered, creating gaps to accommodate the fluorescent probe. On the basis of M540 median fluorescence, addition of BSPs did not significantly decrease membrane lipid disorder resulting from the cryopreservation process (Table 6.2).

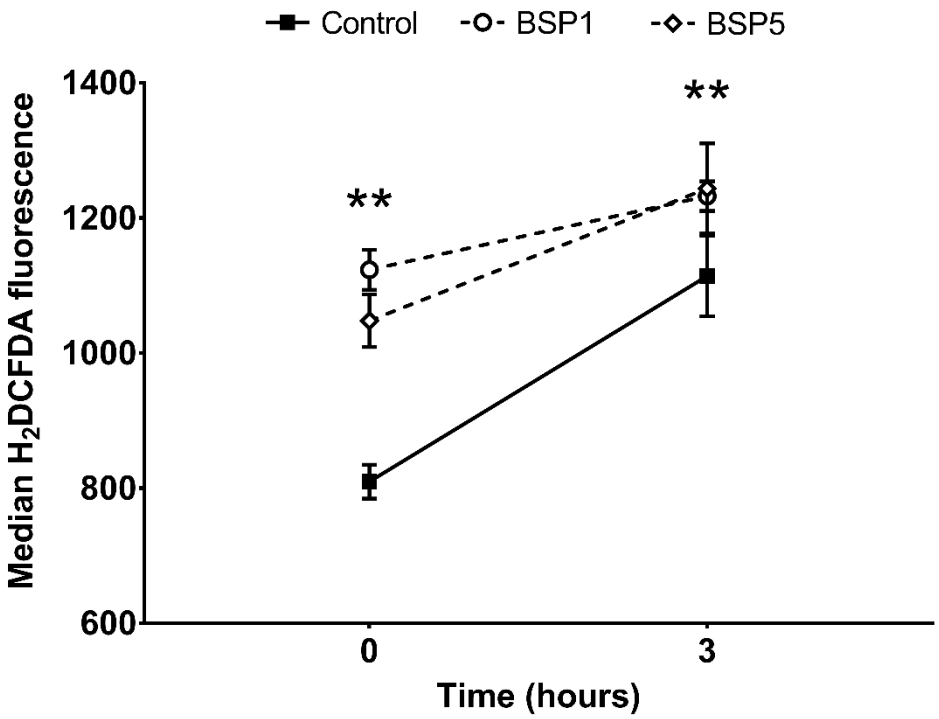

Figure 6.6 Reactive oxygen species production, measured by median H_2DCFDA fluorescence (arbitrary units), of viable frozen thawed epididymal ram spermatozoa with pre freeze exposure to 0.75 mg bovine serum albumin (control; filled square, solid line), 0.75 mg gelatin affinity and RP-HPLC purified Binder of Sperm Protein 1 (BSP1; open circle, dashed line) or Binder of Sperm Protein 5 (BSP5; open diamond, dashed line). **both BSP treatments $p < 0.05$ compared to control

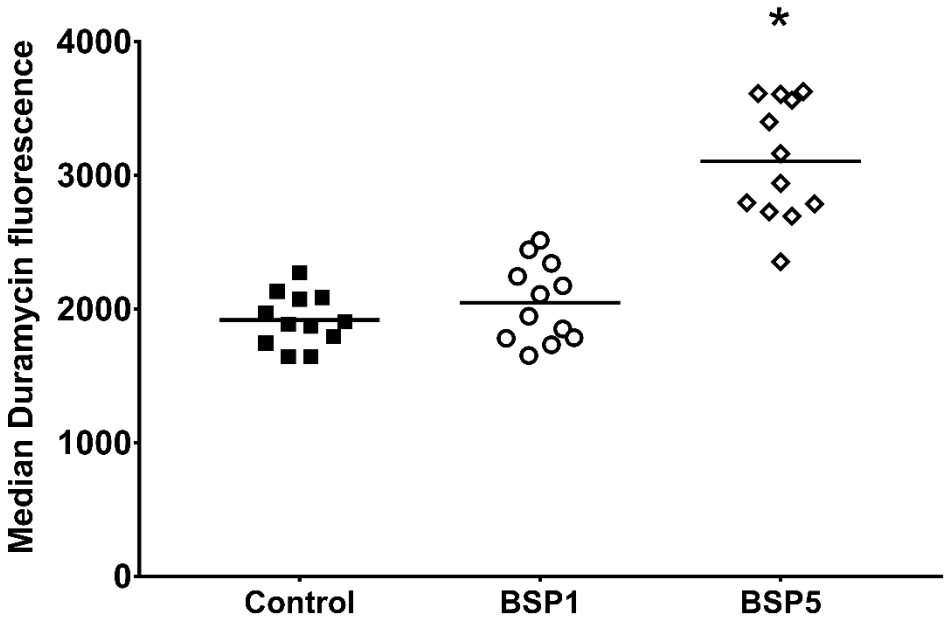

Figure 6.7 The relative level of phosphatidylethanolamine present on the outer membrane leaflet, measured by median Duramycin fluorescence (arbitrary units), of frozen thawed epididymal ram spermatozoa with pre freeze exposure to 0.75 mg bovine serum albumin (control; filled square), BSP1 (open circle) or BSP5 (open diamond). Measurements pooled over 0 and 3 h time points.*$p < 0.05$ compared to control and BSP1

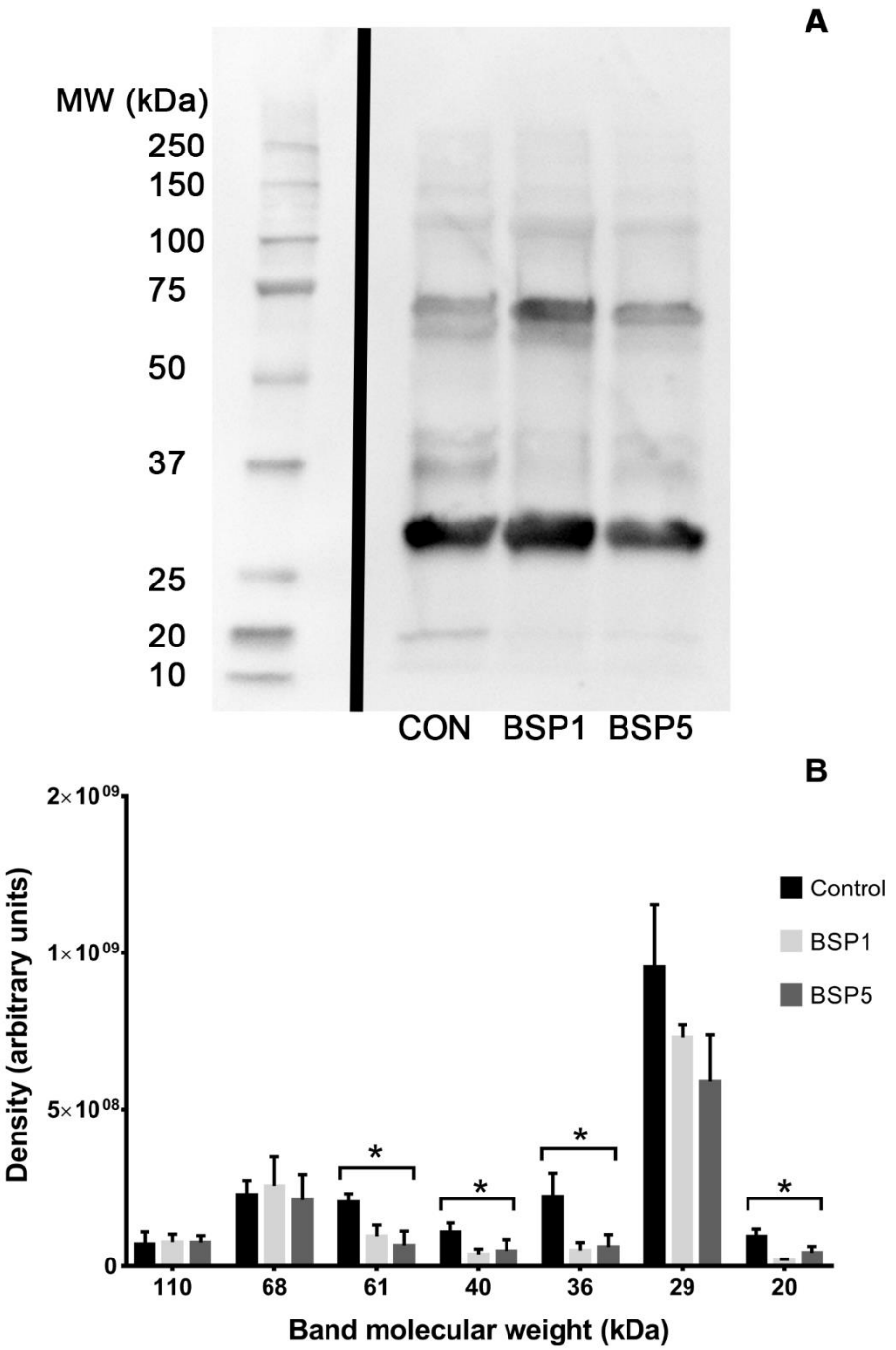

Figure 6.8 (**A**) Western blot against tyrosine phosphorylation from lysates of frozen thawed epididymal ram spermatozoa (pooled from 3 rams) with pre freeze exposure to 0.75 mg bovine serum albumin (control; lane 2), BSP1 (lane 3) or BSP5 (lane 4). 10 µg of protein were separated by SDS-PAGE, blotted onto PVDF membrane and probed with 1:2000 anti-phosphotyrosine. (**B**) Corresponding densitometry results (n = 2 blot replicates). Blots were normalised against a stain free image prior to band density analysis in Image Lab software. * indicates bands with significantly lower density in BSP1 and BSP5 compared to the control ($p < 0.05$)

The bilayer membrane is made up of phospholipids, some of which are preferentially located on the inner (cytoplasmic) or outer leaflet, and can become scrambled to the opposite leaflet during certain processes (e.g. apoptosis). Phosphatidylethanolamine (PE) and phosphatidylserine (PS) are two phospholipids which are largely found on the inner leaflet of the membrane bilayer. We used duramycin, a probe for PE, and annexin V, a probe for PS (Larson *et al.* 2012), to characterise the phospholipid makeup of the accessible outer leaflet in spermatozoa frozen with or without BSP exposure. While there were no significant changes in PS (data not shown), pre freeze addition of BSP5 resulted in significantly higher levels of PE detected on the sperm surface compared to the control after thawing (Figure 6.7).

6.4.3.4. Tyrosine phosphorylation is reduced in spermatozoa exposed to BSPs

Pre-freeze exposure of epididymal spermatozoa to either BSP1 or BSP5 significantly decreased tyrosine phosphorylation of bands at approximately 61, 40, 36 and 20 kDa compared to the control (Figure 6.8).

6.5. DISCUSSION

We have investigated the effects of BSP1 and BSP5 on cryopreservation outcomes of epididymal ram spermatozoa in an effort to understand their physiological actions during freezing and explore their potential for exploitation to improve ram spermatozoa cryopreservation success. A preliminary experiment, conducted using a BSP enriched fraction (Barrios *et al.* 2000; Barrios *et al.* 2005), showed that BSPs improved the motility of frozen thawed spermatozoa in a standard tris-citric acid-fructose medium, as well as a mucin rich medium. A subsequent study using highly purified BSP1 and BSP5 and a wider range of functional assays found that BSPs improved progressive motility, velocity, linearity and straightness of ram spermatozoa immediately after thawing. In addition, BSP supplementation increased viability and reduced cryopreservation induced tyrosine phosphorylation over an extended incubation period. Both BSP1 and BSP5 are abundant in ram seminal plasma (Soleilhavoup *et al.* 2014) and their physiological roles may be unique rather than redundant, due to subtle differences in their structure (Calvete *et al.* 1996). While we observed many similar effects of the proteins when applied independently, it is unclear whether their individual effects would be additive if applied together. As such, further investigation into the combined effects of these proteins is warranted.

Binder of Sperm Proteins were originally isolated from bovine seminal plasma (Manjunath and Sairam 1987) and have since been thoroughly studied in the bull. Their involvement in capacitation (Thérien *et al.* 1997; Moreau *et al.* 1998), sperm reservoir formation (Gwathmey *et al.* 2003), in vitro oocyte penetration (Moura *et al.* 2007), zygote cleavage and blastocyst

formation (Rodríguez-Villamil *et al.* 2015) suggest important roles in sperm function and fertility. However, BSPs have also been shown to have significant negative impacts on cryopreserved bull spermatozoa, including correlation with reduced post thaw motility (Singh *et al.* 2014; Magalhães *et al.* 2016) and lower conception rates (Moura *et al.* 2006; Somashekar *et al.* 2015). Comprehensive studies by Srivastava *et al.* (2012; 2013) established that immediate sequestering of BSPs at ejaculation led to improved post thaw viability, acrosome integrity, membrane integrity and oocyte penetration, as well as significant reductions in freezing induced capacitation-like changes of bull spermatozoa. The sum of these studies suggest that BSPs in the bull are a double edged sword; important physiologically, but detrimental in the context of extended in vitro processing for artificial insemination.

We have shown for the first time that in stark contrast to the bull, the presence of BSPs confers significant advantages to ram spermatozoa during freezing and thawing. Using epididymal spermatozoa, we found that pre freeze exposure to either a BSP enriched chromatography fraction, or highly purified BSP1 or BSP5 significantly improved important motility parameters. These findings are in agreement with previous studies characterising the protective effects of BSPs during cooling of ram spermatozoa to 5°C without cryoprotectants (Barrios *et al.* 2000; Barrios *et al.* 2005). Incubation of spermatozoa with BSPs for 20 min prior to dilution in egg yolk based freezing media allowed time for association of supplemented proteins with the sperm membrane (Manjunath *et al.* 2002), ensuring that we observed the direct consequences of BSP exposure. While addition of highly purified BSPs resulted in higher progressive motility than the control immediately post thaw, this level could not be maintained after extended incubation as we observed with the BSP enriched fraction produced by size exclusion chromatography. This divergence in effects could potentially be due to several factors, namely the effects of ‘contaminating’ proteins in the BSP enriched fraction, the difference of effects when BSPs are present in isolation rather than together and potential impacts of more thorough chromatographic purification on protein function. The short lived improvement in progressive motility by isolated BSP1 and BSP5 may also reflect more rapid energy expenditure, leading to an earlier ‘burn out’ in motility parameters. While reports on the direct effects of BSPs on bull sperm motility are scarce, continued exposure to these proteins clearly causes deterioration of bovine spermatozoa due to significant cholesterol and phospholipid efflux (Thérien *et al.* 1998; Moreau *et al.* 1999). Whether or not this same efflux is occurring in frozen thawed ram spermatozoa is unclear, however the maintenance of viability over time suggests that this is not the case. Complementary additives such as antioxidants and other cryoprotective proteins (e.g. SPINK3 (Zalazar *et al.* 2016)) may help to maintain progressive motility in spermatozoa treated with isolated BSPs for longer periods. Overall, these results

suggest that pre freeze supplementation of BSPs has significant positive effects on post thaw motility of ram spermatozoa.

In addition to basic motility parameters, we have also shown that pre freeze addition of the BSP enriched fraction, isolated by size exclusion chromatography, grants maintenance of high total motility, velocity and increased amplitude of lateral head displacement in an artificial mucus medium. Frozen thawed spermatozoa may struggle to penetrate cervical mucus, even when retaining high progressive motility post thaw (Tollner *et al.* 2011), and this penetration ability has been correlated with in vivo fertility (Cox *et al.* 2002; Taş *et al.* 2007a). As such, potential improvements in the ability of frozen thawed ram spermatozoa to penetrate cervical mucus could be a significant advantage in vivo. It is tempting to speculate that the observed improvements in post thaw sperm characteristics would be beneficial for in vivo fertility. However, pause is warranted given the variability of previous studies involving seminal plasma supplementation (Maxwell *et al.* 1999; O'Meara *et al.* 2007; Leahy *et al.* 2010a), and as such, artificial insemination trials are required to investigate any potential in vivo benefits.

While previous studies have demonstrated that BSPs can maintain sperm viability during cooling to 5°C (Barrios *et al.* 2005), this is the first direct evidence that purified BSP1 and 5 are both capable of improving post thaw viability of ram spermatozoa. In addition, and similarly to studies employing whole seminal plasma (Pérez-Pé *et al.* 2002; de Andrade *et al.* 2012) and BSP containing protein mixtures (Ledesma *et al.* 2016), we found that supplementation of BSPs leads to significant reductions in protein tyrosine phosphorylation. As protein tyrosine phosphorylation is a marker of freezing induced capacitation-like changes (Thomas *et al.* 2006; Kumaresan *et al.* 2011), this represents a significant positive effect of pre freeze supplementation with purified BSPs, which could potentially extend the fertilising lifespan of cryopreserved ram spermatozoa in vivo. While pre freeze supplementation of BSPs resulted in significantly higher ROS production immediately post thaw, this was directly related to the higher proportion of progressively motile spermatozoa. In addition, BSPs reduced the rate of ROS production over time, suggesting potential mitigating effects. A damaging effect of cryopreservation is increased membrane lipid disorder of viable spermatozoa (Guthrie and Welch 2005; Yeste *et al.* 2015). We observed that BSPs were not able to mitigate this increase in lipid disorder, and as such, their use in combination with any component which minimises freezing induced lipid disorder may offer more comprehensive cryoprotection. On the basis of these results, we propose that both BSP1 and BSP5 are generally beneficial for ram sperm cryopreservation, and could potentially be exploited to improve commercial freezing success.

An interesting observation in this study was the significant increase in phosphatidylethanolamine (PE) detected on the sperm surface following pre freeze treatment

with BSP5. Unlike BSP1, BSP5 has affinity for PE (Desnoyers and Manjunath 1992) and as such, there are multiple explanations for this observation. In spermatozoa, PE is predominantly located on the inner membrane leaflet, however scrambling of PE to the outer leaflet is a hallmark of freezing damage (Fang *et al.* 2016). As such, there is a possibility that BSP5 may cause increased scrambling of PE to the external leaflet, which could have negative consequences for membrane function. Alternatively, due to the presence of two fibronectin type II binding domains (Desnoyers and Manjunath 1993; Moreau *et al.* 1998), BSP5 could simultaneously bind to the sperm membrane and PE originating from egg yolk low density lipoproteins (Anton *et al.* 2003), increasing the amount of PE on the sperm surface. At this point it is unclear which mechanism is responsible for the observed increase in PE on the outer membrane leaflet of BSP5 treated spermatozoa, however the lack of negative effects on post thaw parameters overall suggests that BSP5 does not have a detrimental effect on membrane structure.

While shown to have important physiological functions in vivo, BSPs have earned a negative reputation in vitro due to their detrimental effects on bull spermatozoa following extended in vitro processing. In synergy with previous reports, our results are proof of concept of the beneficial actions of BSPs on frozen thawed ram spermatozoa when added prior to freezing. Apart from increasing our understanding of how seminal plasma proteins interact with spermatozoa during freezing, these findings present an exciting opportunity for the improvement of in vitro processing of ram semen. Further investigation is required to extend these benefits to in vivo fertility outcomes and determine the most commercially viable application of these findings.

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7. General discussion

Exposure to seminal plasma and cryopreservation are two contrasting processes, one being a physiological event and the other a human intervention. They have opposite effects on spermatozoa inseminated far from the site of fertilisation in sheep, with seminal plasma exposure significantly increasing pregnancy rates (Heise *et al.* 2010; Rickard *et al.* 2014) and cryopreservation significantly reducing reproductive success (Armstrong and Evans 1984; Maxwell and Hewitt 1986; Donovan *et al.* 2004). The reasons for these respective effects have been investigated, but have remained unclear for several decades. Nevertheless, seminal plasma has been employed in several in vivo studies in an attempt to improve the success of cervically inseminated, frozen thawed ram spermatozoa (Maxwell *et al.* 1999; O'Meara *et al.* 2007; Leahy *et al.* 2010a). The results of these trials have been frustratingly inconsistent, highlighting the need for a better understanding of how both seminal plasma and cryopreservation affect spermatozoa. From this, a more consistent and reliable way to exploit seminal plasma to improve cryopreservation outcomes may be discovered. The findings reported in the thesis shed light on these as yet unanswered questions, providing evidence of how seminal plasma and cryopreservation alter ram sperm structure and function. Both of these process were shown to significantly alter the sperm proteome, and the contribution of egg yolk proteins to spermatozoa was profiled using mass spectrometry for the first time. Further, both seminal plasma exposure and cryopreservation were shown to modify the sperm glycocalyx. The immunomodulatory effects of seminal plasma were demonstrated and the mechanism of sperm-neutrophil binding was investigated. Studies on an abundant seminal plasma protein family, the Binder of Sperm Proteins (BSPs), highlighted their important roles in the regulation of capacitation and their potential as cryoprotective agents for ram spermatozoa.

The studies reported in chapters 2 and 3 demonstrated the effects of both seminal plasma exposure and cryopreservation on the proteomic makeup of spermatozoa. The findings from chapter 2 challenge the classical theory that seminal plasma contributes an array of novel proteins to the sperm surface. Sensitive LC-MS/MS identified only two novel proteins contributed at ejaculation, LEG1 and EDIL3. A total of 39 other proteins, including BSP1 and BSP5 were present on epididymal spermatozoa, but significantly increased in abundance after exposure to seminal plasma. The lack of agreement between western blotting and mass spectrometry results in regards to the BSPs suggests that these proteins are not readily identified by LC-MS/MS, a finding of note for further proteomic research. While the roles of

BSPs were further pursued in this thesis, investigation into the functions of both LEG1 and EDIL3 is warranted given the lack of information on their effects in a reproductive context. As a relatively small group of proteins were increased in abundance by seminal plasma, further characterisation of their individual functions in spermatozoa may be a realistic goal. In particular, looking at their effects both alone and in combination, may help to identify any proteins which are likely to underpin the ability of seminal plasma to enhance cervical transit (Rickard *et al.* 2014).

The studies detailed in chapter 3 provided the first comprehensive assessment of proteomic changes to ram spermatozoa induced both by exposure to egg yolk and cryopreservation. A total of 15 proteins previously identified in egg yolk proteomes were found to significantly increase after exposure to an egg yolk based medium. While not exhaustive, these results confirm that proteins are transferred from egg yolk to spermatozoa, confirming previously unsubstantiated theories (Ollero *et al.* 1998a). There is no information available regarding the impacts, if any, of egg yolk specific proteins such as vitellogenins and vitelline membrane outer layer protein 1 on sperm function. In comparison, while the effects of apoplipoproteins have been well characterised, their specific effects during cryopreservation have not been profiled and would be of great interest. Overall, further investigation into how proteins originating from egg yolk alter spermatozoa would help to solidify our understanding of how in vitro processing modifies male gametes.

In comparison to seminal plasma, cryopreservation did not result in the absolute loss or gain of proteins, but rather more subtle adjustments to protein abundance. A total of 51 proteins were altered by freezing, with 27 increased and 24 decreased. At present, there is no experimental evidence to explain the mechanism by which proteins would increase or decrease in abundance in transcriptionally silent spermatozoa. However, it could be suggested that while increases may reflect absorption of proteins from the extracellular fluid, decreases may be due to cleavage or proteolytic degradation of proteins. The proteins which decreased in abundance following cryopreservation were particularly interesting, as proteins with chaperone function constituted 20% of this loss. The loss of these important regulatory proteins may underpin the lessened capability of frozen thawed spermatozoa to deal with stressors (Pinto and Kozink 2008; Garg *et al.* 2009), and could negatively impact cervical transit. Supplementation of these regulatory proteins either during the freezing or thawing processes could potentially benefit the function of frozen thawed ram spermatozoa, however further work is necessary to confirm this. These findings demonstrate the significant proteomic changes which spermatozoa undergo at various stages during the freezing process, producing a cell with fundamental sublethal changes which may affect in vivo performance.

Studies in chapter 4 investigating simple sugars on the sperm surface similarly confirmed that the glycocalyx is significantly altered by both seminal plasma exposure and cryopreservation. These results confirmed earlier qualitative studies comparing epididymal and ejaculated ram spermatozoa (Magargee *et al.* 1988), and are the first record of freezing induced changes to surface sugars in a species other than human (Talaei *et al.* 2010) or fowl (Peláez *et al.* 2011). The changes caused by freezing did not mirror those caused by seminal plasma exposure, highlighting the unique impacts which these processes have on the molecular makeup of spermatozoa. While these results are informative, further in depth analysis of the sperm glycocalyx using advanced techniques such as a lectin microarray (Xin *et al.* 2014) or surface glycomics by mass spectrometry (Montacir *et al.* 2017) would provide a more comprehensive assessment of these changes. As sugars on the sperm surface form a glycocalyx extending well beyond the membrane itself, the observed alterations to sugars have the potential to impact interactions between spermatozoa and various elements of the female tract. The functional consequences of this could include changes to penetration of cervical mucus, formation of sperm reservoirs, evasion of phagocytic cells and capacitation. However, further work is necessary to demonstrate a causative link between these alterations to surface sugars and any functional consequences.

Evidence in chapter 4 further supports the hypothesis that in addition to modifying sperm proteins and sugars, seminal plasma plays important immunomodulatory roles to directly protect spermatozoa. Significantly fewer neutrophils bound to ejaculated compared to epididymal spermatozoa. These results suggest that seminal plasma disrupts sperm-neutrophil binding, a possible mechanism by which this fluid enhances sperm transit through the cervix. Certain seminal plasma proteins have previously been identified as disruptors of sperm-neutrophil binding (Alghamdi and Foster 2005; Doty *et al.* 2011), and investigation into particular protective proteins from ram seminal plasma would be worthwhile. While seminal plasma had significant effects on sperm-neutrophil interaction, the effects of cryopreservation were not as clear. Due to interference by the cryoprotective medium, it remains unclear whether cryopreservation alters the affinity of neutrophils for spermatozoa. As spermatozoa would likely swim out of medium quickly after insemination, incubation of neutrophils with washed cryopreserved spermatozoa may more closely emulate the in vivo situation and provide evidence to answer this question.

While the results presented in chapter 4 are intriguing, several important questions remain unanswered. Given the significant alterations to the glycocalyx observed in the first experiments of chapter 4 and the recognised importance of glycans in immune cell-target interaction (Schauer *et al.* 1984; Athamna *et al.* 1991; Paris *et al.* 2012), an attempt was made

to investigate surface sugars as a key mechanism for sperm binding by neutrophils. No such relationship was observed, possibly due to ineffectiveness of the blocking treatment. Further studies employing more sophisticated models, such as glycosidase or antibody treatment, may help to solidify important receptor-ligand partners in this interaction. In addition, the finding in chapter 3 that exposure to egg yolk based media significantly increases the level of complement C3 on the sperm surface is particularly pertinent to interaction with immune cells. Increased opsonisation with C3 has been directly related to increased phagocytosis (Matsuyama *et al.* 1992), thus this alteration may cause cryopreserved spermatozoa to be more readily targeted and cleared by phagocytic immune cells in the female tract. Clearly, further studies are required to establish the mechanism of interaction between spermatozoa and immune cells of the female reproductive tract, and the impacts of both seminal plasma exposure and cryopreservation. A better understanding of these interactions will assist in utilising seminal plasma as an immunomodulatory agent to benefit artificial insemination outcomes of fresh and frozen thawed spermatozoa.

Given that in chapter 2 the BSPs were found to be primarily contributed at ejaculation, this protein family was chosen as the basis for further studies in chapters 5 and 6. Studies in chapter 5 drew on the known roles which BSPs play in bovine sperm capacitation (Moreau *et al.* 1998; Thérien *et al.* 2001; Manjunath and Thérien 2002). A main goal of this work was to compare the effects of BSP1 and BSP5 on ram spermatozoa to those observed in bull spermatozoa. Results from chapter 5 highlight the unique requirement of significant exogenous cAMP upregulation to capacitate ram spermatozoa. In addition, unlike in the bull, the effects of the BSPs on capacitation processes were only pronounced in this high cAMP environment. Overall, the results from these experiments suggest that BSP1 is an important regulator of the timing of capacitation. BSP1 may minimise premature membrane destabilisation during transit and subsequently encourage cholesterol efflux under adequate capacitating conditions near the site of fertilisation. In comparison, BSP5 did not show any major effects under various capacitating conditions, and as such, may fulfil different roles to BSP1 in ram spermatozoa. Further studies into the combined effects of BSP1 and 5 and their interaction with other substances (e.g. high density lipoprotein) are required in order to develop a working model of the physiological roles of these proteins in ram sperm capacitation. Overall, chapter 5 highlights how one major constituent of seminal plasma modulates sperm function, potentially regulating the onset and progression of capacitation within the female reproductive tract to ensure mature spermatozoa are available at the appropriate time for fertilisation.

The observation in chapter 5 that BSP1 could stabilise the sperm membrane supported previous investigations demonstrating the protective effects of BSPs on ram spermatozoa

during cooling stress (Barrios *et al.* 2000; Barrios *et al.* 2005). This suggested that BSPs may improve freezing outcomes, and this cryoprotective potential was comprehensively investigated in chapter 6. Both BSP1 and BSP5 had significant beneficial effects on post thaw motility, viability and mucus penetration, and minimised freezing induced tyrosine phosphorylation. These findings indicate that in contrast to the bull, the presence of BSPs is advantageous for in vitro processing of ram spermatozoa, potentially related to the decapacitating roles of these proteins. No differences in the abundance of BSPs were observed following cryopreservation in the proteomic study detailed in chapter 3. This implies that while BSPs are not lost from the sperm surface during freezing, supplementation with these proteins above physiological concentrations may still offer significant benefits. An interesting finding was the increase in membrane surface phosphatidylethanolamine (PE) following cryopreservation with BSP5. This could either be due to translocation of PE across membrane leaflets, or interaction of egg yolk derived PE with membrane bound BSP5. Phospholipid translocation was not investigated in chapter 5, thus it would be interesting to investigate its potential occurrence under capacitating conditions when BSPs are present. While promising, the experiments presented in chapter 6 are simply proof of concept that constituents of seminal plasma can be exploited to improve freezing outcomes. As the desired goal is an improvement in pregnancy rates following cervical insemination of frozen thawed semen, artificial insemination trials are required to confirm the translation of these results to beneficial effects in vivo.

The processes of ejaculation and cryopreservation both significantly alter spermatozoa, with divergent outcomes. It is unsurprising that their effects differ; while exposure to seminal plasma plays a physiological role in preparing spermatozoa for fertilisation, changes due to cryopreservation overwhelmingly result from cellular damage and dysfunction. This thesis has characterised important alterations to spermatozoa as a result of these processes, providing a more complete context to understand why epididymal, ejaculated and frozen thawed ram spermatozoa perform differently in vivo. In addition, studies herein have exploited important elements from seminal plasma to prevent injurious changes to ram spermatozoa during cryopreservation and improve their fertility. Further work is required to comprehensively understand many of the relationships described, however these findings represent exciting advances in our understanding of how seminal plasma and cryopreservation modify spermatozoa.

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Appendix 1: Supplementary Files

Chapter 2: A proteomic investigation of ram spermatozoa and the proteins conferred by seminal plasma

Supplementary file 2.1 SDS-PAGE of ejaculated and epididymal sperm lysates, noting separation of bands for in gel digestion

Supplementary file 2.2 Qualitative proteome of ejaculated ram spermatozoa

Supplementary file 2.3 Qualitative proteome of epididymal ram spermatozoa

Supplementary file 2.4 Cross species comparison of sperm proteomes

Supplementary file 2.5 Quantitative comparison of epididymal and ejaculated ram spermatozoa

Supplementary file 2.6 SDS-PAGE band localisation of proteins that were more abundant in ejaculated ram spermatozoa

Chapter 3: Cryopreservation and egg yolk medium alter the proteome of ram spermatozoa

Supplementary file 3.1 All proteins identified in a global standard searched against a *Gallus gallus* NCBI database

Supplementary file 3.2 All proteins identified in fresh and fresh + EY treatments when compared against an ion spectral library generated from a *Gallus gallus* NCBI database

Supplementary file 3.3 All proteins identified in a global standard searched against an *Ovis aries* targeted NCBI database

Supplementary file 3.4 All proteins identified in fresh and frozen treatments when compared against an ion spectral library generated from an *Ovis aries* targeted NCBI database

Chapter 5. Binder of Sperm Proteins 1 and 5 have contrasting effects on the capacitation of ram spermatozoa

Supplementary file 5.1 Relative normalised emPAI and contribution (%) of proteins identified by LC-MS/MS from fractions collected following gelatin affinity chromatography and RP-HPLC of ram seminal plasma proteins.

Chapter 6. Binder of sperm Proteins protect ram spermatozoa from freeze-thaw damage

Supplementary file 6.1 Relative normalised emPAI and contribution (%) of proteins identified by LC-MS/MS from fractions collected following gelatin affinity chromatography and RP-HPLC of ram seminal plasma proteins.

Appendix 2: Conference Proceedings

I. CHANGES TO SPERM SURFACE CARBOHYDRATES FOLLOWING EXPOSURE TO SEMINAL PLASMA AND FREEZING

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Epididymal, ejaculated and frozen-thawed spermatozoa are exceptionally different sperm types. When placed directly into the uterus all sperm types display high fertility. However, when forced to traverse the ovine cervix (following vaginal or cervical AI), epididymal and frozen-thawed ram spermatozoa display dramatically lower fertility than fresh ejaculated spermatozoa. We suggest that changes to the outer carbohydrate-rich glycoprotein coat of spermatozoa represent a potential reason for altered cervical transit ability, as this is the primary interface with the female environment. As such, we used lectin binding to investigate modifications of the carbohydrate portion of sperm glycoconjugates in response to seminal plasma exposure and freezing. Fresh and frozen ejaculated spermatozoa and fresh epididymal spermatozoa were subjected to a swim up to remove loosely associated glycoproteins and remnants of freezing diluent. Spermatozoa were stained with fluorescein isothiocyanate (FITC) conjugated lectins (LPA, PNA, WGA and ConA), specific for the sugars of interest (sialic acid, galactose, N-acetylglucosamine and mannose, respectively) and counter-stained with propidium iodide (PI). Median FITC fluorescence of the PI negative (live) population was compared amongst treatments using a restricted maximum likelihood approach. The results provide strong evidence that both exposure to seminal plasma and freezing are processes which alter the outermost surface of spermatozoa. Seminal plasma exposure significantly increased N-acetylglucosamine (WGA; $p< 0.001$) on the sperm membrane. Conversely, freezing led to a significant decrease in available galactose (PNA; $p<0.01$) and N-acetylglucosamine (WGA; $p<0.001$) as well as increased availability of mannose (ConA; $p <0.001$), typically a carbohydrate located proximal to the membrane in glycoconjugates. The observed changes lend support to the theory that while exposure to seminal plasma may orchestrate meaningful alteration of the sperm surface, the freezing process further modifies this important region, possibly to its detriment. This is the first evidence that industry standard freezing of ram semen leads to large and significant changes in selected sperm surface carbohydrates. Further research is required to fully elucidate the consequence of these changes and establish possible means to prevent or repair the damage to the glycoprotein coat of ram spermatozoa.

II. A QUANTITATIVE PROTEOMIC COMPARISON OF EPIDIDYMAL AND EJACULATED RAM SPERMATOZOA

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Sheep are an important agricultural animal, yet a comprehensive sperm proteome has not been produced for this species. In addition, while ram seminal plasma can significantly improve field fertility by supporting cervical transit, the basis for this is unknown. Sperm proteomes have been published for a range of species, however the extent of species similarity is a topic of debate. Here we employ liquid chromatography paired with tandem mass spectrometry to investigate the proteome of ejaculated ram spermatozoa, with quantitative comparison to epididymal spermatozoa by spectral counting and Student's T-test. We also present a comparison to other mammalian species using published proteomes. We identified a total of 493 proteins in ejaculated ram spermatozoa, with the most abundant proteins involved in glycolysis and oxidative phosphorylation. 26% of proteins found in ram spermatozoa were also found in human, mouse and horse spermatozoa, while 16% were unique to ram. Exposure to seminal plasma resulted in 5% of proteins increasing in abundance compared to epididymal spermatozoa. Only 4 membrane bound proteins were concluded to be contributed solely by seminal plasma (SPADH2, C6orf58, EDIL3 and PPP1R7). This is the first evidence that the seminal plasma of rams confers few novel proteins to spermatozoa at ejaculation, despite being a complex, protein rich fluid. Identification of these proteins provides direction for further research on the role of specific proteins contributed by seminal plasma.

III. SEMINAL PLASMA AND CRYODILUENT ALTER THE INTERACTION OF NEUTROPHILS WITH RAM SPERMATOZOA

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Neutrophils are the main immune cell infiltrate following insemination, and were originally believed to preferentially phagocytose dead or abnormal spermatozoa to facilitate their clearance from the female reproductive tract. Widespread reports of the phagocytosis of live, motile spermatozoa suggest that there are more factors involved in sperm-neutrophil interaction than simply viability, but these factors and the binding receptors involved are not well understood. We investigated the interaction of neutrophils with spermatozoa from the ram epididymis (epididymal) and ejaculate (ejaculated), as well as cryopreserved ejaculated spermatozoa (frozen-thawed). These treatments facilitated investigation of the importance of exposure to seminal plasma from the accessory sex glands and the cryopreservation process for targeting by phagocytic cells. Neutrophils ($1x10^6$ cells/ml) isolated from the blood of mature ewes (n=2) were incubated 1:1 (v/v) with epididymal, ejaculated and frozen-thawed ram spermatozoa (n=9) at 50 x 10^6 spermatozoa/ml with or without 7.5% (v/v) heat treated ewe serum. This allowed for the assessment of non-opsonin binding (e.g. via selectins, lectins, integrins) in a serum-free environment and opsonin binding (e.g. via immunoglobulins, C-reactive protein) with serum, excluding complement by heat treating. Binding was assessed by microscopic examination of Wright's stained smears, counting 200 neutrophils and expressing results as percentage of neutrophils bound to ≥ 1 spermatozoon. In the absence of serum, almost all neutrophils were bound to epididymal spermatozoa (95.2% ± 0.8%), whereas a significantly lower proportion of neutrophils bound to ejaculated spermatozoa (27.7 ± 1.4%). In the presence of serum however, the vast majority of neutrophils were bound to both epididymal and ejaculated spermatozoa (94.5% ± 0.6%, 95.9% ± 0.5%). These results show that seminal plasma acted to protect spermatozoa from non-opsonin mediated phagocytosis, but failed to limit opsonin mediated phagocytosis. While this may represent an avenue for phagocytosis in the presence of seminal plasma, previous research has demonstrated additional strong anti-complement functions of seminal plasma. Such immunomodulatory effects may help to explain how seminal plasma increases the ability of spermatozoa to successfully transit the female tract post insemination, leading to significantly higher pregnancy rates. Ejaculated and frozen-thawed spermatozoa were similarly bound in the absence of serum (27.7 ± 1.4% and 27.6% ± 1.8% respectively). When heat treated serum was present, binding to ejaculated spermatozoa significantly increased (95.9% ± 0.5%), while

binding of frozen-thawed spermatozoa remained low (31.9% ± 2.2%). These results were not related to sperm viability (viable 95.9% ± 0.5% vs non-viable 93.3% ± 1.6%). Further experiments showed that simply diluting ejaculated spermatozoa in cryodiluent significantly reduced opsonin-mediated binding (cryodiluent 37.5% ± 6.1% vs control 94.4% ± 1.1%).The exact mechanism by which cryodiluent interferes with opsonin mediated binding of spermatozoa is not known, however we suggest that it may be due to the chelating action of citric acid in the freezing media. While further investigation *in vivo* is required, this could potentially be useful in limiting neutrophilic infiltrates into the female reproductive tract at the time of insemination.