Ram seminal plasma and its functional proteomic assessment

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Abstract

Ejaculation results in the confluence of epididymal spermatozoa with secretions of the accessory sex glands. This interaction is not a prerequisite for fertilisation success, but seminal factors do play a crucial role in prolonging the survival of spermatozoa both *in vitro* and *in vivo* by affording protection from handling induced stress and some selective mechanisms of the female reproductive tract. Reproductive biologists have long sought to identify specific factors in seminal plasma that influence sperm function and fertility in these contexts. Many seminal plasma proteins have been identified as diagnostic predictors of sperm function and have been isolated and applied *in vitro* to prevent sperm damage associated with the application of artificial reproductive technologies. Proteomic assessment of the spermatozoon, and its surroundings, has provided considerable advances towards these goals and allowed for greater understanding of their physiological function. In this review, the importance of seminal plasma will be examined through a proteomic lens to provide comprehensive analysis of the ram seminal proteome and detail the use of proteomic studies that correlate seminal plasma proteins with ram sperm function and preservation ability.

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The importance of extracellular proteins to sperm form and function

Understanding the influence of exogenous factors in the spermatozoon's environment has been of great interest because sperm development and survival is heavily regulated by its external environment. Most cells develop and survive a changing environment through intracellular and extracellular signals that drive gene expression and the production of new proteins (Davies 2016). For example, heat stress may induce the expression of heat shock proteins that help to refold proteins damaged by an increase in temperature. Spermatozoa are unique in this regard because they are rendered transcriptionally and translationally silent well before they become temporally or spatially competent to fertilise. The final post-meiotic phases of spermatogenesis involve DNA compaction and the removal of ribosomes. These processes seize gene expression before spermatozoa are released from their nursing Sertoli cell in the seminiferous tubules in the testis (Baker 2011). These testicular spermatozoa are not yet mobile or capable of traversing the female tract and fertilising the oocyte. Therefore, sperm maturation in the male (epididymal maturation) and female tract (capacitation in the oviduct), and the sperm cell's response to *in vitro* handling stressors and physiological stressors transiting the female tract, is mediated without the involvement of sperm gene expression. Sperm

adaptation, maturation and survival are entirely reliant on extrinsic factors surrounding the spermatozoa during transit through the epididymis, at ejaculation and upon entry and passage through the female tract.

Ejaculation results in the confluence of spermatozoa, which are in a mixture of testicular and epididymal fluids, with secretions from the seminal vesicles, prostate and bulbourethral glands (collectively termed the accessory sex glands). This complex fluid remodels the sperm surface and modifies the physiological response of spermatozoa *in vitro*, during processing associated with assisted reproductive technologies, and upon entering the female genital tract. For this reason, understanding the intricate influence of seminal plasma proteins on sperm cell physiology and their role in guiding the spermatozoa during their travel from the male to the female tract has been a key focus of reproductive scientists.

The physiological function of seminal plasma

The secretions of the epididymides and accessory sex glands modulate the function of spermatozoa and the female tract through the provision of signalling factors and glycoproteins with sperm-binding properties. The physiological importance of seminal plasma has been questioned (Bedford 2015) because epididymal

spermatozoa, which have not been exposed to seminal factors, are capable of fertilisation both *in vitro* and *in vivo* (Davis *et al.* 1991, Rickard *et al.* 2014). However, sperm survival and fertilising potential is hampered if they are not exposed to seminal factors, and these effects are particularly noticeable if epididymal spermatozoa is deposited in the lower reproductive tract and has to migrate to the site of fertilisation. Reduced fertility has been noted in mice (Pang *et al.* 1979, Peitz 1988), rats (Queen *et al.* 1981) and sheep (Rickard *et al.* 2014) under such conditions and the mouse seminal vesicle protein SVS2 is required for sperm survival in the uterus (Kawano *et al.* 2014). The prolonged survival and fertility afforded by seminal factors is a combination of its ability to stimulate motility (Maxwell *et al.* 2007), regulate capacitation (Manjunath *et al.* 2008, Muino-Blanco *et al.* 2008), influence sperm storage in the female tract (Manjunath *et al.* 2007, Talevi & Gualtieri 2010) and modulate the female immune system to tolerate spermatozoa and the conceptus (Robertson 2007).

Pronounced variability in seminal plasma composition across species has made it difficult to attribute clear physiological effects to the fluid as a whole and to delineate the individual importance of seminal proteins to sperm function and fertility. Variations in the amount and relative contribution of individual accessory sex glands result in dramatic physical and biochemical differences in seminal plasma between eutherian species. For example, it alters whether the ejaculate is expelled in fractions (e.g. dog, boar, stallion) or as one phase (e.g. human, ram, bull) and if these secretions exhibit a gel (human, alpaca) or fluid form (ram, bull). Accessory sex gland secretions also dictate ejaculate volume, which varies according to the site of semen deposition. Ejaculates which are deposited in the vagina are low (bull, ram) in volume and high in concentration to prevent semen backflow. Uterine depositors (boar, stallion) exhibit larger ejaculate volumes with lower sperm concentration. These adaptations of the accessory sex glands to suit evolutionary divergent mating strategies result in pronounced proteomic diversity of seminal fluid between species (Druart *et al.* 2013, Meslin *et al.* 2015). Even major protein families which are highly conserved across ungulates (i.e., pig, bull, ram, stallion), such as spermadhesins and binder of sperm proteins (BSP), appear to function in a species-specific manner. Differences in the structure, abundance and expression patterns of homologous proteins may underlie the functional divergences that are evident in even closely related species such as the bull and the ram (Leahy & de Graaf 2012). Due to these inherent variabilities, we have narrowed the focus of this review to allow for a comprehensive proteomic assessment of the effect of seminal plasma proteins from that of a single species, the ram.

The use of seminal plasma to improve ram sperm function during processing for artificial reproductive technologies

A goal of animal reproductive biologists has been to ascertain what the physiological functions of seminal plasma are and examine if this complex fluid could be used as a biotechnological tool to improve sperm function during processing for artificial reproductive technologies (ARTs). *In vitro* processing of spermatozoa for liquid storage, cryopreservation or sex sorting dramatically alters the extracellular fluid environment of the sperm sample. The most obvious effect is a dramatic dilution of seminal plasma proteins in the sperm environment from extension with diluents optimised for reproductive technologies. Wherever possible, *in vitro* handling attempts to mimic the signals and protective aspects of the *in vivo* environment to maintain sperm form and function. Semen processing also results in fluctuations of temperature, pressure, osmolality and pH that can damage the sperm plasma membrane and limit the fertilising lifespan of processed spermatozoa. These *in vitro* changes share many similarities to *in vivo* capacitation, such as alterations in lipid packing, cholesterol efflux and tyrosine phosphorylation. Because they occur *in vitro*, prior to deposition in the female tract, rather than at the site of *in vivo* fertilisation (the oviduct), the fertilising window of processed spermatozoa is considerably shortened (Bailey *et al.* 2000). As seminal plasma is removed or diluted during processing, and it contains factors which prevent premature capacitation (Chang 1957, Tseng *et al.* 2013), it has long been viewed as a physiological fluid that could protect spermatozoa from handling-induced stress.

Indeed, the vast majority of studies investigating the influence of ram seminal plasma on ram sperm function report improved sperm parameters (Muino-Blanco *et al.* 2008, Leahy & Gadella 2011, Leahy & de Graaf 2012) and this has translated to improved *in vivo* fertility (Maxwell *et al.* 1999b, Rickard *et al.* 2014). The inclusion of ram seminal plasma into sperm-handling protocols has been shown to have a decapacitation effect on frozen-thawed ram spermatozoa (assessed by an increased proportion of non-capacitated spermatozoa following chlortetracycline staining) and improve a range of other sperm quality characteristics including sperm heterogeneity, viability, motility and ability to penetrate cervical mucus *in vitro* (Graham 1994, Garcia-Lopez *et al.* 1996, Maxwell *et al.* 1999a, Barrios *et al.* 2000, Perez-Pe *et al.* 2001a, 2002, El-Hajj Ghaoui *et al.* 2007a,b, Leahy *et al.* 2009, 2010c). Glycoproteins in seminal plasma have been proposed to bind to the sperm membrane and have a stabilising effect on the membrane structure, as it adapts to changes in the temperature gradients during freezing and thawing

(Muino-Blanco *et al.* 2008). In part, this positive effect on sperm functioning is also due to anti-oxidant effects elicited by seminal plasma (Marti *et al.* 2007). In line with this, the addition of seminal plasma proteins has also been shown to protect sex-sorted ram spermatozoa from oxidative stress (Leahy *et al.* 2010a).

As seminal plasma is a complex biological fluid, intra-species variation in its effect on spermatozoa has also been reported. Extracellular interactions of spermatozoa and seminal plasma vary at the animal level due to physiological, pathological and exogenous (e.g. hormones, disease, season) factors (Maxwell *et al.* 2007, Muino-Blanco *et al.* 2008, Leahy *et al.* 2010c, Rickard *et al.* 2016). *In vitro* processing of spermatozoa and seminal plasma further influences the final outcome with noted differences in effect of seminal plasma due to the washing of spermatozoa (Ollero *et al.* 1997, Perez-Pe *et al.* 2001b), the point at which seminal plasma is introduced, and the protein concentration applied (Leahy *et al.* 2009). Such variability is thought to account for studies which find no influence of seminal plasma on ram sperm survival (Morrier *et al.* 2003) or a detrimental effect (Dott *et al.* 1979, de Graaf *et al.* 2007). Such inherent variability also contributes to the inconsistent effect of seminal plasma supplementation on the *in vivo* fertility of liquid-stored and frozen-thawed ram spermatozoa (Maxwell *et al.* 1999b, El-Hajj Ghaoui *et al.* 2007b, O'Meara *et al.* 2007, Leahy *et al.* 2010b, López-Pérez & Pérez-Clariget 2012, Rickard *et al.* 2014). To better delineate the seminal factors influencing sperm function, andrologists have utilised the latest advances in mass spectrometry (MS) technology to identify and quantify the entire protein complement (proteome) of seminal plasma and correlate protein abundance to ram sperm preservation ability.

From seminal proteins to the ram seminal proteome

Identification of the protein makeup of ram seminal plasma was initially performed by selecting bands or spots of interest from 1 or 2D SDS-PAGE and performing mass spectrometry (MS) on these targeted areas of interest.

Such techniques identified the highly abundant, low-molecular-weight (14–30 kDa) proteins in ram seminal plasma as members of the spermadhesin and binder of sperm protein (BSP) families (Bergeron *et al.* 2005, Cardozo *et al.* 2008). Over the next decade, the rapid development of mass spectrometry technology allowed for the global identification and quantification of proteins in complex biological fluids and tissues to identify close to the entire protein complement of a sample. These technological advances were enthusiastically adopted in the reproductive community and led to a rapid increase in the number of proteins identified in reproductive tissues, fluids and cells. Seminal plasma proteomes have now been reported in most species including, the boar (González-Cadavid *et al.* 2014, Perez-Patino *et al.* 2016, Pérez-Patiño *et al.* 2018), turkey (Slowinska *et al.* 2017), stallion (Novak *et al.* 2010) and bull (Kelly *et al.* 2006, Viana *et al.* 2018) as well as humans and other non-mammalian species (reviewed by Druart & de Graaf 2018). Dramatic increases in the identification of ram seminal plasma proteins were evidenced over sequential years from 61 in 2012 (Souza *et al.* 2012) to 109 in 2013 (Druart *et al.* 2013) and 727 protein identifications the following year (Soleilhavoup *et al.* 2014). The rapid increase in the number of seminal plasma proteins identified was largely due to improvements in mass spectrometry technology, particularly in the initial separation of peptides in the liquid chromatography phase prior to mass characterisation (Byrne *et al.* 2012).

Characterisation of the global seminal plasma proteome allowed for the system-wide modelling of this complex biological fluid to better understand biological clusters within seminal plasma, and potential interactions between seminal plasma and ram spermatozoa (Soleilhavoup *et al.* 2014). Relative protein quantification by spectral analysis revealed the protein composition of ram seminal plasma was highly unbalanced, as 5% of the proteins (39 out of 727 proteins) constituted 50% of the total spectra (top 10 most abundant proteins shown in Table 1) (Soleilhavoup *et al.* 2014). The proteins of high abundance included previously identified seminal proteins such as BSP proteins (BSP5) (Cardozo *et al.* 2008)

Table 1 The major proteins identified in ram seminal plasma after LC-MS/MS, based on total spectra counts (Soleilhavoup *et al.* 2014).

Protein name	Gene symbol	MW (kDa)	Function
Binder of sperm 5 precursor	<i>BSP5</i>	17.8	Binder of sperm (BSP) glycoprotein characterised by a fibronectin type-2 domain. Binds sperm
UPF0762 protein C6orf58	<i>LEG1</i>	40.9	Protein of unknown function
Clusterin	<i>CLU</i>	51.0	Ubiquitous glycoprotein with chaperone and anti-apoptotic functions
Bodhesin-2	<i>BDH2</i>	11.7	Spermadhesin protein characterised by a CUB domain. Binds sperm
Alpha-2-macroglobulin	<i>A2M</i>	164.2	Protease inhibitor
Carboxylesterase 5A	<i>CESSA</i>	64.2	Enzyme involved with lipid transfer processes
Lactoferrin	<i>LTF</i>	77.2	Antimicrobial activity and serine-type endopeptidase activity. Iron binding
EGF-like repeat and discoidin I-like domain-containing protein 3	<i>EDIL3</i>	54.9	Cell adhesion ligand that interacts with the alpha-v/beta-3 integrin receptor. Calcium binding

and spermadhesins (BDH-2) (Bergeron *et al.* 2005) as well as common serum proteins (albumin, clusterin, lactoferrin). A more unexpected finding was that the second most abundant seminal plasma proteins identified by spectral counting had not previously been reported in ram seminal plasma, nor in the seminal plasma of any mammalian species. Liver-enriched gene 1 (LEG1, also known as UPF0762 or c6orf58 protein) is a glycosylated, secretory protein, which contains a domain of unknown function (DUF781) (Chang *et al.* 2011). In the zebrafish, LEG1 has been shown to protect liver development from oxidative stress by mediating a novel anti-stress pathway that involves FGFR3 binding and the enhancement of Erk activity (Lin *et al.* 2014, Hu *et al.* 2016).

The function of LEG1 in the reproductive context is unknown, but it has been identified in the proteome of ejaculated ram spermatozoa, and not epididymal ram spermatozoa, suggesting that this protein is secreted by an accessory male sex gland and is mixed with epididymal sperm at ejaculation (Pini *et al.* 2016). The protein is abundantly present in seminal plasma (Soleilhavoup *et al.* 2014) but only low amounts of this protein were found to be interacting with the ram sperm membrane (Pini *et al.* 2016). Interestingly, out of the 700 plus proteins identified in seminal plasma, LEG1 was only one of three proteins that were present on ejaculated spermatozoa and absent on epididymal spermatozoa (Pini *et al.* 2016). The other two proteins were BSP5 and a cell adhesion protein termed EGF-like repeat and discoidin I-like domain-containing protein 3 (EDIL3). The interaction of BSP5 with the sperm membrane has been well documented and shown to occur in a rapid and specific manner through interactions with choline phospholipids (Desnoyers & Manjunath 1992). The association of EDIL3 with the sperm membrane has not been directly studied but EDIL3 is an integrin ligand, which binds the α -v/ β -3 integrin receptor, suggesting a possible target for sperm-bound EDIL3. In endothelial cells, EDIL3 has interesting properties of immune evasion and manipulation, by limiting leukocyte recruitment during inflammation (Choi *et al.* 2008). This interesting immune modulatory function may assist spermatozoa in evading the female immune system. Collectively, these results show that the confluence of spermatozoa with seminal plasma at ejaculation re-models the ram sperm plasma membrane in a highly specific way, but this does not occur through the addition of a significant number of new proteins. Suggesting that the proteomic interaction of spermatozoa and its seminal environment is perhaps not as complex as previously thought. The relevance of the binding of EDIL-3 to integrin receptors on the sperm surface for sperm survival in the female tract remains to be determined in future studies.

Further functional enrichment assessments of the proteomic datasets revealed that the global ram SP proteome contained proteins involved in three main protein pathways; proteins involved in metabolism (predominantly glycolytic enzymes), the ubiquitination pathway (heat shock proteins, 26s proteasome proteins and ubiquitination proteins) and chaperone-related proteins (heat shock proteins, proteins of the chaperonin-containing TCP1 complex). These pathways are typically cellular in origin and are the predominate functional pathways of mammalian spermatozoa (Baker *et al.* 2008, Byrne *et al.* 2012, Nixon *et al.* 2017). The arrest of spermatogenesis by scrotal insulation caused the loss of these proteins from seminal plasma, confirming that there are a high number of proteins in ram seminal plasma that are of sperm origin (Soleilhavoup *et al.* 2014).

The correlation of protein quantity to biological function

Advances in the qualitative and quantitative power of mass spectrometry technology allowed for variations in sperm preservation ability to be correlated to the relative amount of seminal plasma proteins. The balance of this review article provides an in-depth assessment of three quantitative proteomic studies that correlate the relative quantities of ram seminal plasma proteins to ram sperm function at ejaculation (Rodrigues *et al.* 2013), after liquid storage for 24 h at 15°C (Soleilhavoup *et al.* 2014) and after freezing and thawing (Rickard *et al.* 2015). Proteins that were statistically more abundant in the seminal plasma of animals which displayed high or low motility *in vitro* is shown in Fig. 1. Liquid chromatography MS/MS and spectral counting was used for the assessment of seminal proteins related to sperm function following liquid or frozen storage. This technique allowed for the quantification of all proteins in the sample and their statistical correlation with either high or low preservation ability. The study correlating seminal proteins to sperm motility patterns at ejaculation used densitometry of spots on a 2D gel to identify potential proteins overexpressed in high- or low-motility samples. These spots were then isolated and identified by mass spectrometry. Accordingly, this technique resulted in a reduced number of proteins ($n=4$) being correlated to sperm function compared to the proteomic studies which identified all the complete seminal plasma proteome (Fig. 1).

The purpose of the detailed assessment of these three studies is to better understand the biological function of ram seminal plasma and to identify the common and divergent responses of seminal proteins under varied handling conditions. One of the key benefits of proteomics is that it allows you to take a systems biology,

Figure 1 Proteomic studies correlating protein quantification to ram spermatozoa function *in vitro*. Seminal plasma proteins were separated by 2D SDS-PAGE and densitometry and mass spectrometry was used to quantify and identify, respectively, protein abundance. Seminal plasma proteins were identified and quantified by LC-MS/MS and spectral counting. Proteins that were statistically overexpressed in sperm samples showing high (blue arrow) or low (red arrow) motility at ejaculation (Rodrigues *et al.* 2013), following 24 h of liquid storage (Soleilhavoup *et al.* 2014) or after freeze-thawing (Rickard *et al.* 2015) are shown.

or holistic, approach to complex biological traits. For example, rather than identifying individual proteins that are correlated to fertility, you can assess which biological networks may influence sperm function or fertility. Global assessment of these three comparative proteomic investigations (fresh, liquid, frozen) identified five key proteins groupings that were positively or negatively associated with sperm preservation ability and these will be discussed below.

Secreted or extracellular proteins correlated with ram sperm function *in vitro*

Many of the abundant proteins that are known to be secreted from the accessory sex glands were negatively correlated to sperm function or preservation ability in the comparative proteomic studies (Table 2). This finding was somewhat surprising as many of these 'classical' seminal plasma proteins have been shown to have beneficial effects on ram sperm function when added in isolation in functional *in vitro* studies (Muino-Blanco *et al.* 2008). One of the most abundant, and well-characterised proteins in ram seminal plasma (BSP5), which belongs to the binder of sperm protein (BSP) family, was significantly correlated with lower ram spermatozoa motility following ejaculation (Rodrigues *et al.* 2013) (Table 2), whereas BSP1 and five showed no correlation to sperm survival following liquid or frozen storage. Such a result is unexpected, as BSP proteins isolated from ram seminal plasma have been shown to be beneficial to ram sperm survival *in vitro*, particularly following freezing and thawing. Binder of sperm proteins are small acidic proteins which originate in the seminal vesicles and are designated by the presence of two tandemly repeated fibronectin type 2 (Fn-2) modules. Ejaculation causes rapid and specific binding to the ram sperm membrane (Pini *et al.* 2016) through the direct association of BSP proteins with choline phospholipids (Manjunath *et al.* 2007). This association is thought to restrict the mobility of the lipid phase and result in a rigidification of the sperm membrane that provides stability during sperm transit through the reproductive tract (Manjunath *et al.* 2007). In the ram, this effect was attributed to the improved sperm viability and reduced tyrosine phosphorylation noted in spermatozoa supplemented with BSP proteins before exposure to cold shock conditions (Perez-Pe *et al.* 2001a, 2002) or in standard ram freezing protocols (Pini *et al.* 2018a). Upon reaching the oviduct, BSP proteins may also assist in the establishment of a bovine oviductal sperm reservoir as *in vitro* supplementation of BSP proteins to epididymal spermatozoa enhances sperm-oviductal epithelial cell binding (Gwathmey *et al.* 2003, 2006). It is proposed that when the oocyte arrives, secondary messengers in the oviductal fluid, such as high-density lipoprotein and heparin-like glycosaminoglycan's may bind to BSP proteins to coordinate the onset and progression of capacitation (Lane *et al.* 1999). In the bull, extended association of BSP proteins with spermatozoa *in vitro* has been well documented to cause capacitation-like changes, such as cholesterol efflux, which result in a loss of sperm viability (Manjunath *et al.* 2007) but such a pronounced effect has not been observed in the ram. This result highlights an important consideration of *in vitro* sperm storage. That while it may dilute seminal factors, it also significantly extends the amount of time spermatozoa associate with such proteins from a few minutes, at most, following ejaculation and deposition

Table 2 Secretory or extracellular-localised proteins that are statistically more abundant in ram sperm samples showing higher (↑) or lower (↓) motility following ejaculation (fresh (Rodrigues *et al.* 2013)), or after liquid (Soleilhavoup *et al.* 2014) or frozen storage (Rickard *et al.* 2015).

Protein name	Gene name	Fresh	Liquid	Frozen
Disintegrin and metalloproteinase domain protein 20	ADAM20			↓
Disintegrin and metalloproteinase domain protein 32	ADAM32			↓
Cartilage acidic protein 1	CRTAC1		↓	↓
Extracellular matrix protein 1 isoform 1	ECM1		↓	↓
Binder of sperm 5	BSP5	↓		
Bodhesin 2	BDH2	↓		
Angiogenin-2-like	ANG			↓
UPF0762 protein C6orf58 homolog	LEG1		↓	
Ribonuclease 4 isoform 1	RNASE4		↑	↓
Zinc-alpha-2-glycoprotein-like	AZGP1	↑	↓	↓
EGF-containing fibulin-like extracellular matrix 1	EFEMP1		↑	↑
Caltrin-like	PYY2			↑

in the female tract to days, months or years when spermatozoa are processed and stored in a liquid or frozen form. In the bull, it is thought that this extended association of BSP proteins with spermatozoa disrupts the natural balance of inhibition and stimulation of capacitation and leads to a loss of sperm function and fertility (Manjunath *et al.* 2008, Leahy & Gadella 2011). Accordingly, quantitative proteomic studies have associated both high and low levels of BSP proteins with poor fertility outcomes following artificial insemination of frozen-thawed bull spermatozoa (Moura *et al.* 2006, Somashekar *et al.* 2015). In the ram, the lack of quantitative association of BSP proteins with sperm preservation ability may relate to their sheer abundance in ram seminal plasma masking clear quantitative effects. This, however, does not fully explain why higher levels of BSP5 were correlated to reduced motility at ejaculation (Rodrigues *et al.* 2013). Nor why many other abundant secreted seminal plasma proteins were also negatively correlated to motility following ejaculation (the spermadhesin BDH2) or liquid storage (LEG1) (Table 2).

Another secretory seminal protein of interest is zinc-alpha-2-glycoprotein-like (AZGP1, ZAG). This protein showed an interesting biphasic effect of being beneficial in the short term but detrimental under long-term preservation conditions (Table 2). As the amount of AZGP1 in ram seminal plasma was positively correlated with ram sperm motility following ejaculation (Rodrigues *et al.* 2013) but negatively correlated with preservation ability following liquid (Soleilhavoup *et al.* 2014) or frozen (Rickard *et al.* 2015) storage. In humans, AZGP1 is secreted by the prostate (Hale *et al.* 2001) and binds to the surface of spermatozoa at ejaculation where it stimulates sperm motility through the cyclic AMP pathway (Qu *et al.* 2007). As successful sperm preservation relies on the depression or cessation of sperm movement and metabolic activity, it is clear why a stimulatory factor that aids sperm transit through the female tract would not be beneficial under prolonged *in vitro* storage conditions. Indeed, when recombinant AZGP1 was tested on ram spermatozoa, a stimulatory

effect prior to liquid storage and a detrimental effect after storage was found (Soleilhavoup *et al.* 2014).

One of the only small, secreted proteins of seminal origin that was positively correlated (Table 2) with improved ram sperm motility following freezing and thawing was caltrin (calcium transport inhibitor; PYY2). Caltrin is secreted by the seminal vesicles (Coronel *et al.* 1992, Chen *et al.* 1998) and was one of the first decapacitation factors to be characterised in bovine seminal plasma (Lewis *et al.* 1985). The serine-protease inhibitor binds to spermatozoa at ejaculation and inhibits extracellular Ca²⁺ uptake to prevent premature acrosomal exocytosis and hyperactivation as spermatozoa ascend through the female reproductive tract (Dematteis *et al.* 2008, Grasso & Coronel 2017). The influence of caltrin on ram spermatozoa has not been investigated *in vivo* or *in vitro* but a related serine-protease inhibitor, termed SPINK3, was able to partially prevent or revert ram spermatozoa freeze-thaw damage (Zalazar *et al.* 2016). Further investigation of the ability of PYY2 to prevent capacitation-like changes caused by freeze-thaw damage is warranted.

Finally, many extracellular matrix proteins (Table 2), of testicular origin, which are involved in cell adhesion (e.g. a disintegrin and metalloproteinase proteins; ADAM proteins) were correlated with poor motility following liquid or frozen storage (ADAM20, ADAM32, CRTAC1, ECM1). The exception to this was EGF-containing fibulin-like extracellular matrix 1 (EFEMP1), which was positively correlated with storage resilience in liquid or frozen form.

Immune-related proteins correlated with ram sperm function *in vitro*

Several proteins of the humoral (cell-free bodily fluid or serum) immunity system showed both positive and negative correlations with motility following liquid or frozen storage (Table 3). Secreted antimicrobial proteins of the BPI fold-containing family (BPIFA1 and BPIFB1) were negatively correlated to frozen sperm storage, but the concentration of BPIFB1 in seminal plasma was

Table 3 Immune-related proteins that are statistically more abundant in ram sperm samples showing higher (↑) or lower (↓) motility after liquid (Soleilhavoup *et al.* 2014) or frozen storage (Rickard *et al.* 2015).

Protein name	Gene name	Liquid	Frozen
BPI fold-containing family A member 1	<i>BPIFA1</i>		↓
Cystatin C	<i>CST3</i>		↓
Immunoglobulin alpha heavy chain	<i>IGHA1</i>		↓
Polymeric immunoglobulin receptor isoform 1	<i>PIGR</i>	↓	↓
C-reactive protein-like	<i>CRP</i>		↑
Cathelicidin-1 precursor	<i>CATHL1B</i>	↑	
Immunoglobulin V lambda chain	<i>Vlambda16.1</i>	↑	
Ig gamma-1 chain C region, partial	<i>IGHG1</i>	↑	
Immunoglobulin gamma-1 chain	Ig gamma-1	↑	
BPI fold-containing family B member 1	<i>BPIFB1</i>	↑	↓

positively correlated to motility following liquid sperm storage. BPI-containing proteins bind lipids, display antibacterial activity against gram-negative bacteria, and are involved in inflammatory responses at the cell surface (Britto & Cohn 2015). Other antimicrobial proteins (C-reactive protein (CRP)) and peptides (Cathelicidin-1 precursor (CATHL1B)) were also positively correlated with frozen and liquid storage, respectively.

Immunoglobulins, or antibodies, of the adaptive immune response were also both positively and negatively associated with sperm function *in vitro*. For example, immunoglobulin A (IGHA1) and the polymeric immunoglobulin receptor (PIGR) were negatively correlated with sperm preservation ability. Receptor proteins with transmembrane domains, such as PIGR, would not normally be expected in plasma fluids. The PIGR protein is a special case because it binds IgA at the basolateral surface of epithelial cells and transport the complex to the apical surface for secretion (Johansen & Kaetzel 2011). Secretion cleaves the extracellular component of the PIGR protein from the transmembrane segment where it can be detected in seminal plasma (Phalipon & Corthésy 2003, Kaetzel 2005) and provide a quantitative measure of immunoglobulin secretion levels. Other immunoglobulins, IgG (IGHG1 and the Immunoglobulin V lambda chain (Vlambda16.1)), showed the opposite response and were positively correlated with motility following liquid storage. Immunoglobulins (particularly IgG and IgA) and antimicrobial proteins and peptides are part of the normal humoral immunity system present in seminal plasma (Bier *et al.* 1977, Dondero *et al.* 1984). The abundance of these immune proteins is clearly correlated to sperm function, but the relationship is unlikely to be a simple linear one. Immune competency at the humoral level relies on having an established baseline of proteins involved in innate (antimicrobial proteins/peptides) and adaptive (immunoglobulins) immunity. Therefore, a depression of immune-related proteins may indicate

an immune-compromised state. On the other hand, the secretion of these proteins greatly increases during acute phase response to inflammatory stimuli caused by injury or infection so elevated levels of immune-related proteins may indicate an inflammatory state (Peterson & Artis 2014). Therefore, the quantitative relationship of immune proteins to sperm function and fertility is likely to be a quadratic one where too little expression or too great expression of immune proteins is associated with poor sperm function.

Chaperone-related proteins correlated with ram sperm function *in vitro*

Three functional cell-based pathways were previously reported in the ram seminal proteome (Soleilhavoup *et al.* 2014). In the comparative proteomic studies, all three of these pathways showed clear correlations with sperm preservation ability (Soleilhavoup *et al.* 2014, Rickard *et al.* 2015). The vast majority of the proteins in these functional pathways (Fig. 2) were of sperm origin and their abundance in seminal plasma was predominantly positively associated with sperm preservation ability.

The first of these is proteins with chaperone-like activity, which assist in the folding and unfolding, and the assembly or disassembly, of macromolecular structures. All the chaperone proteins that were associated with preservation ability were positively correlated with high motility following liquid or frozen storage (Fig. 2 and Table 4). The majority of chaperone proteins identified were part of the chaperonin-containing TCP1 complex (CCT complex), which is composed of eight unique subunits that are stacked like two donuts on top of each other to create a barrel. It is thought to primarily fold and stabilise cytoskeletal proteins due to the continuous presence of tubulin and actin in the CCT complex (Sarkar *et al.* 2011). The CCT complex shows a unique plasma membrane localisation (bull; Byrne *et al.* 2012, boar; Belleanne *et al.* 2011, human; Redgrove *et al.* 2011 and mouse; Dun *et al.* 2011) in spermatozoa and has been shown to associate with proteins of zona-binding affinity. Subunits of the CCT complex have previously been identified in ram seminal plasma, but they were not detectable following scrotal insulation, indicating they were of sperm origin (Soleilhavoup *et al.* 2014). It is therefore unlikely that the quantity of these proteins in the extracellular environment is having a direct effect on sperm quality *in vitro* but their abundance does appear to be a reliable diagnostic marker of sperm quality as all eight CCT subunits showed the same positive trend with preservation ability. The mechanism by which these cellular proteins enter the extracellular environment is unclear, but it may be caused by cell turnover in the epididymis or ejaculate.

Secretory chaperone proteins, termed heat shock proteins, were also positively associated with sperm

Figure 2 Visualisation of the network of (A) chaperone (Table 4), (B) proteolytic (Table 5) and (C) metabolism-related proteins (Table 6) in ram seminal plasma that are correlated to *in vitro* sperm function. Protein connections were established using STRING (<http://string-db.org>).

survival *in vitro* (HSPA1L, HSP90AA1). In spermatozoa, chaperone proteins have also been shown to assist in the assembly and delivery of zona-binding proteins to the outer surface of the sperm plasma membrane (Redgrove *et al.* 2011, Bromfield *et al.* 2016). Heat shock proteins are ubiquitous proteins that prevent protein aggregation

Table 4 Chaperone-related proteins that are statistically more abundant in ram sperm samples shower higher (↑) or lower (↓) motility after liquid (Soleilhavoup *et al.* 2014) or frozen storage (Rickard *et al.* 2015).

Protein name	Gene name	Liquid	Frozen
Large proline-rich protein BAG6	BAG6		↑
T-complex protein 1 subunit alpha	CCT1		↑
T-complex protein 1 subunit beta	CCT2		↑
T-complex protein 1 subunit gamma	CCT3		↑
T-complex protein 1 subunit delta	CCT4		↑
T-complex protein 1 subunit theta	CCT8		↑
Heat shock 70kDa protein 1-like	HSPA1L		↑
T-complex protein 1 subunit epsilon	CCT5	↑	↑
T-complex protein 1 subunit zeta	CCT6A	↑	↑
T-complex protein 1 subunit eta	CCT7	↑	↑
Heat shock protein HSP 90-alpha	HSP90AA1	↑	↑

and may be induced by stress, constitutively expressed or both (Rupik *et al.* 2011). Increased HSPs could therefore be a marker of physiological stressors in the male tract or they could indicate a high-functioning chaperone system, which may afford protection from stressors associated with *in vitro* handling and aid sperm-zona binding. In this case, the latter appears to be more correct as these markers were positively associated with sperm preservation ability (Table 4). Moreover, multiple HSP proteins were more abundant in the seminal plasma of men with high seminal reactive oxygen species levels but the two proteins more abundant in the medium and low ROS groups (HSPA1L and HSP90AA1, respectively, Agarwal *et al.* 2015) were the same HSP proteins correlated with ram sperm preservation ability. Higher abundance of the HSP90AA1 protein in the spermatozoa of good freezer boars has also been reported in the boar (Casas *et al.* 2010) and chaperone proteins have been identified as the functional group most susceptible to freeze-thaw damage as there abundance in ram spermatozoa is greatly reduced following freeze-thawing (Pini *et al.* 2018b). This makes HSPs good candidates for future research into bioactive cryoprotectants. Recombinant HSPA8 proteins has been shown to prolong ram sperm survival *in vitro* at 39°C (Lloyd *et al.* 2009) and further testing of HSP90AA1 supplementation during liquid or frozen storage is warranted.

Proteolysis-related proteins correlated with ram sperm function *in vitro*

All of the proteins involved in proteolysis through the multi-catalytic cellular complex known as the Ubiquitin Proteasome System (UPS) were positively correlated with motility following liquid or frozen storage (Fig. 2 and Table 5). The UPS system targets proteins for destruction by tagging them with a ubiquitin chain to direct them to the 26S proteasome complex for destruction. When proteasomal activity was first identified in spermatozoa, it was expected to coordinate the intracellular

Table 5 Proteolysis-related proteins that are statistically more abundant in ram sperm samples showing higher (↑) or lower (↓) motility after liquid (Soleilhavoup *et al.* 2014) or frozen storage (Rickard *et al.* 2015).

Protein name	Gene name	Liquid	Frozen
Ubiquitin-like modifier-activating enzyme 1	<i>UBA1</i>		↑
Polyubiquitin	<i>UBB</i>		↑
Cullin-3	<i>CUL3</i>		↑
Kelch-like protein 10	<i>KLHL10</i>		↑
Tripeptidyl-peptidase 2	<i>TPP2</i>		↑
26S proteasome non-ATPase regulatory subunit 2	<i>PSMD2</i>		↑
Proteasome subunit alpha type, 2	<i>PSMA2</i>	↑	
Proteasome subunit alpha type-7-like	<i>PSMA8</i>	↑	
Transitional endoplasmic reticulum ATPase	<i>VCP</i>	↑	↑

reorganisation and degradation of proteins occurring during spermatogenesis (Tipler *et al.* 1997). However, high levels of proteasomal activity have since been found in mature spermatozoa and found to regulate later events such as capacitation, the acrosome reaction and zona pellucida penetration (Arcelay *et al.* 2008, Kong *et al.* 2009, Yi *et al.* 2010, Sanchez *et al.* 2011, Zimmerman *et al.* 2011). Similar to the CTT complex, in spermatozoa, the 26 proteasome shows a unique sperm surface localisation (Redgrove *et al.* 2011, Byrne *et al.* 2012) rather than a traditional intracellular localisation. Proteasomal proteins have been reported in seminal plasma but scrotal insulation to arrest spermatogenesis resulted in their loss from ram seminal plasma suggesting they were of sperm origin (Soleilhavoup *et al.* 2014). Three proteins of the 26S proteasome (PSMD2, PSMA2, PSMA8) were higher abundant in spermatozoa which showed greater resistance to *in vitro* preservation (liquid or frozen storage). While these showed a consistent trend within and between the proteomes the large multi-protein 26S complex consists of more than 66 proteins (Bedford *et al.* 2010) and just under half of these (32 proteins) have been identified in ram seminal plasma (Soleilhavoup *et al.* 2014). As only three of these were correlated with preservation ability, this represents a relatively small proportion of the complex as a whole. However, a larger number of proteins which provide upstream or downstream support to the 26S proteasome were also found to be positively correlated with good preservation ability (Table 5). This includes enzymes that mediate the ubiquitin pathway (UBA1, CUL3, KLHL10), ubiquitin itself (UBB), proteins involved in the transport of ubiquitinated proteins to the 26S proteasome (VCP) and a peptidase which acts downstream of the 26S proteasome (TPP2). The tagging of spermatozoa with ubiquitin is thought to mark the cell as defective and target it for degradation (Sutovsky 2003). Subsequent assessment of sperm ubiquitin levels and sperm function has revealed a more complicated relationship. Increased sperm ubiquitin has been inversely correlated with

sperm function in bull (Zhang *et al.* 2018) and human (Sutovsky *et al.* 2004) spermatozoa, but ubiquitin levels were high in some human sperm samples with good clinical semen parameters. Ubiquitination has also been positively correlated to normal morphology in human semen (Muratori *et al.* 2005) and shown to be positively correlated to sperm survival following freeze-thawing in the boar (Purdy 2008). Because the proteasome is a large multi-protein complex, it is unlikely that the positive correlation of UPS proteins in seminal plasma to good sperm preservation ability is due to these proteins functioning in the extracellular medium and having a direct effect on spermatozoa at ejaculation or during *in vitro* storage. Similar to the subunits identified in the CCT complex, it is expected that the increased abundance of proteins from the UPS pathway in seminal plasma is a diagnostic marker of improved sperm quality due to differences in sperm production and turnover in the testis or epididymis. For example, stressors associated with freezing and thawing such as temperature or osmotic changes could damage sperm proteins, and increased ubiquitination may be indicative of a highly functioning UPS system that is degrading damaged proteins that are detrimental to sperm survival.

Metabolism-related proteins correlated with ram sperm function *in vitro*

The majority of metabolic enzymes identified in ram seminal plasma were positively correlated with preservation success (Fig. 2 and Table 6). The maintenance of motility during liquid preservation was particularly correlated with enzymes belonging to the glycolysis pathway that are of sperm origin and located in the cytoplasm (e.g. GPI, TPI, GAPDH, LDHC, FBPI, ENO1). It is unknown how they are released into the extracellular fluid, but the release of cytoplasmic droplets with remnant cytosol into the luminal fluid is a probable route. The higher concentration of glycolytic enzymes in seminal plasma of sperm samples that show high motility *in vitro* may be related to an increased abundance of glycolytic machinery in the spermatozoon or may represent higher sperm production. Liquid storage at reduced temperatures (15°C, 24 h) provides some suppression of sperm metabolism compared to physiological temperatures, but the maintenance of energy metabolism following storage is still the most important criteria for successful preservation. As glucose is a source of energy for spermatozoa, higher efficiency energy metabolism would support sperm mobility during preservation. However, metabolic enzymes show a more complicated correlation with fertility than simply more metabolic enzymes equals better sperm function. For example, the amount of enolase (ENO1) in bull spermatozoa was previously reported as a positive marker of bull sperm fertility (Park *et al.* 2012) and its abundance in seminal plasma is positively associated

Table 6 Metabolism-related proteins that are statistically more abundant in ram sperm samples showing higher (↑) or lower (↓) motility after liquid (Soleilhavoup *et al.* 2014) or frozen storage (Rickard *et al.* 2015).

Protein name	Gene name	Liquid	Frozen
Neutral alpha-glucosidase AB	GANAB		↓
ATP-citrate synthase	ACLY		↑
Acylamino-acid-releasing enzyme	APEH		↑
Betaine-homocysteine S-methyltransferase 1	BHMT		↑
Galactokinase	GALK1		↑
Alpha mannosidase 2C1	MAN2C1		↑
Sorbitol dehydrogenase	SORD		↑
Fructose-1,6-bisphosphatase 1	FBP1	↑	
Glucose-6-phosphate isomerase	GPI	↑	
Inositol monophosphatase 1	IMPA1	↑	
L-Lactate dehydrogenase C chain	LDHC	↑	
5'-Nucleotidase	NT5E	↑	
Triosephosphate isomerase	TPI1	↑	
Alpha-enolase	ENO1	↑	↓
Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	GAPDHS	↑	↓

with ram sperm preservation during liquid storage, but it is negatively correlated with ram sperm freezing success (Table 6). In the boar, the amount of triosephosphate isomerase (TPI) was negatively correlated with sperm membrane integrity, morphology and motility (Vilagran *et al.* 2016), the ability of boar spermatozoa to survive cryopreservation (Vilagran *et al.* 2013) and litter size following artificial insemination (Kwon *et al.* 2015). Such differences could be species related or due to the measurement of TPI in spermatozoa compared to seminal plasma. The source of the glycolytic enzymes and the mechanism of release into the luminal fluid are also likely to have a significant effect on their correlation to sperm function. In addition to the possibility that these cytosolic glycolytic enzymes are originating from cytoplasmic droplets, it is also possible that they are released from damaged spermatozoa or are not of sperm origin at all. Extracellular vesicles are released along the male reproductive tract from the epithelium of the epididymis or accessory sex glands into the extracellular fluid (Gatti *et al.* 2005, Aalberts *et al.* 2014). These small membranous structures are highly enriched in metabolic enzymes (Choi *et al.* 2015, Yang *et al.* 2017). Interestingly, since these metabolic complexes are embedded in a membrane, they are functional in the luminal environment and can even produce extracellular ATP (Ronquist *et al.* 2013a,b). However, when these enzymes are not present in membrane-embedded structures and are free soluble proteins in seminal plasma, membrane lysis either in cells or in extracellular structures has occurred. Recently, we isolated extracellular vesicles from ram seminal plasma using density gradient centrifugation and quantitatively compared protein abundance between vesicles and the whole seminal plasma. Many metabolic enzymes showed no quantitative differences (e.g. ENO1, GPI,

LHDC, FBPI) but TPI was 20.4-fold more enriched in the extracellular vesicle prep when compared to whole seminal plasma indicating that this protein does reside in membrane-embedded cytosolic structures in ram seminal plasma and that membrane lysis was minimal (Leahy *et al.* unpublished data).

Implications and future direction

Reproductive biologists have long sought to identify specific factors in seminal plasma that are highly correlated with sperm form and function. These proteins could be used as diagnostic predictors of sperm function and fertility or isolated and applied *in vitro* to prevent sperm damage associated with the application of artificial reproductive technologies. Proteomic assessment of the spermatozoon and its surroundings has provided considerable advances towards these goals and allowed for greater understanding of the physiological function of spermatozoa.

Proteomic characterisation of ram seminal plasma identified over 700 proteins (Soleilhavoup *et al.* 2014) with the most abundant seminal proteins being secreted from the accessory sex glands (e.g. BSP, BDH2). However, scrotal insulation to arrest spermatogenesis also highlighted many seminal proteins of lower abundance which originate from spermatozoa, most notably proteins involved in large cellular complexes such as the CCT complex, the 26s proteasome and metabolism enzymes. Comparative proteomic analysis showed that these sperm-associated protein complexes were predominantly associated with high sperm preservation ability. Proteins of the CCT complex appear to be particularly robust markers of preservation ability as all eight proteins in this complex were positively associated with high motility following liquid or frozen storage.

Despite decades of research showing whole seminal plasma, and its abundant secretory components (e.g. BSPs), are beneficial to ram sperm function *in vitro* and *in vivo*, relatively few classical seminal plasma proteins (e.g. secreted from the accessory sex glands) were positively associated with sperm preservation *in vitro*. Such a result highlights the limitations of comparing proteomic and molecular biology studies. The correlation of sperm function to protein abundance requires simplified groupings such as high or low motility or preservation ability and high or low protein abundance. Such discrete grouping can lead to distorted characterisation of protein action as being positive or negative for sperm function. Such a designation is oversimplified as protein effect is highly dependent on environmental conditions. For example, proteins that may be positively correlated to success following natural mating may not be beneficial under extended sperm storage conditions. In addition, many proteins would not be expected to show a linear physiological relationship with sperm function but a more complex

quadratic association where too much, or too little, of the protein negatively influence sperm function (e.g. immunoglobulins). Assessment of seminal plasma alone also sheds no real light on the amount and nature of seminal proteins that have bound the sperm membrane. It is this interaction that is expected to have the greatest influence on sperm transport and sperm membrane stabilisation *in vitro* and *in vivo*. Finally, recent seminal plasma proteomes, such as that of the bull (Viana *et al.* 2018), have identified 1000+ proteins, and it is expected that a significant proportion of ram seminal plasma proteins remain to be identified and characterised.

While the application of quantitative comparative proteomics is yet to fully explain why the addition of seminal plasma is beneficial to the function of processed ram spermatozoa, it has provided great insight into the function of seminal plasma and identified key proteins that require further investigation. Of note is the functional role of the introduction of EDIL3 to the sperm membrane at ejaculation and the potential *in vitro* application of the decapacitation factor caltrin (PYY2) or heat shock proteins (e.g. HSP90AA1) as protective factors to prevent handling-induced ram sperm damage.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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