

The biological mechanisms regulating sperm selection by the ovine cervix

S Fair¹, K G Meade², K Reynaud³, X Druart³ and S P de Graaf⁴

¹Laboratory of Animal Reproduction, School of Natural Sciences, Faculty of Science and Engineering, University of Limerick, Limerick, Ireland, ²Animal & Bioscience Research Department, Animal & Grassland Research and Innovation Centre, Teagasc, Grange, Co Meath, Ireland, ³UMR PRC, INRA 85, CNRS 7247, Université de Tours, IFCE, Physiologie de la Reproduction et des Comportements, Institut National de la Recherche Agronomique, Nouzilly, France and ⁴The University of Sydney, School of Life and Environmental Sciences, Faculty of Science, Sydney, New South Wales, Australia

Abstract

In species where semen is deposited in the vagina, the cervix has the unique function of facilitating progress of spermatozoa towards the site of fertilisation while also preventing the ascending influx of pathogens from the vagina. For the majority of species, advances in assisted reproduction techniques facilitate the bypassing of the cervix and therefore its effect on the transit of processed spermatozoa has been largely overlooked. The exception is in sheep, as it is currently not possible to traverse the ovine cervix with an inseminating catheter due to its complex anatomy, and semen must be deposited at the external cervical os. This results in unacceptably low pregnancy rates when frozen-thawed or liquid stored (>24 h) semen is inseminated. The objective of this review is to discuss the biological mechanisms which regulate cervical sperm selection. We assess the effects of endogenous and exogenous hormones on cervical mucus composition and discuss how increased mucus production and flow during oestrus stimulates sperm rheotaxis along the crypts and folds of the cervix. Emerging results shedding light on the sperm-cervical mucus interaction as well as the dialogue between spermatozoa and the innate immune system are outlined. Finally, ewe breed differences in cervical function and the impact of semen processing on the success of fertilisation, as well as the most fruitful avenues of further investigation in this area are proposed.

Reproduction (2019) **158** R1–R13

Introduction

In vaginal depositors, the cervix has the unique function of preventing the influx of pathogens from the vagina to the uterus while serving to regulate sperm passage. Of the millions to billions (species-dependent) of spermatozoa deposited in the female reproductive tract (FRT), less than 100 spermatozoa arrive at the site of fertilisation and the cervix plays a critical role in the selection of the successful few (see review by [Sakkas *et al.* 2015](#)). The ability to bypass the cervix during assisted reproduction in most species, including humans, has meant that much of the recent research has focused on the interaction of sperm with the utero-tubal junction and oviduct (see review by [Holt & Fazeli 2016](#)). Despite intensive research efforts, the sheep is the only large domestic animal species in which it remains currently impossible to traverse the cervix during artificial insemination (AI) and therefore the furthest semen can usually be deposited is at the external cervical os (opening). While pregnancy rates following cervical AI with liquid semen inseminated on the day of collection are generally acceptable (~60%) ([O'Hara *et al.* 2010](#)),

pregnancy rates fall to below 30% when frozen-thawed (F/T) spermatozoa are inseminated ([Salamon & Maxwell 1995](#)). The reasons for this dramatic decline in pregnancy rates with F/T semen remains elusive. However, with the deposition of F/T spermatozoa laparoscopically into each uterine horn, pregnancy rates recover to approximately 70%, identifying the cervix as the principal site of sperm transport inhibition ([Salamon & Maxwell 1995](#)). Laparoscopic AI is labour intensive, requires specialist veterinary expertise and is not considered welfare friendly. Therefore, for the extensive uptake of sheep AI, which provides widespread access to elite genetics, an improved approach for routine cervical deposition of semen must be devised. However, the understanding of the factors regulating sperm selection in the cervix remain elusive, and this precludes the development of sheep AI. This review takes a systematic approach to the problem by reviewing the salient scientific literature on the anatomy and micro-anatomy of the cervix. Focusing on sheep, we describe the sperm interactions within the cervix, not only with its secretions which change according to hormonal influences, but also with the immune system. Interesting observations can be made

through the study of ewe breed differences and lessons can be learned from studies on the impact of semen processing and seminal plasma (SP) on the number and function of spermatozoa crossing the cervix. A summary of these factors are presented in Fig. 1. We conclude by proposing likely fruitful avenues of future investigation.

Anatomy of the cervix

Gross anatomy limits cervical penetration

The cervix is a long fibrous structure composed primarily of connective tissue (fibrillar collagen and high-molecular-weight proteoglycan complexes), an outer serosal layer and inner epithelial layer. The ectocervix protrudes into the vagina, the opening of which is referred to as the external cervical os, while the endocervix acts as the passageway to the uterus. The ovine cervix is approximately 4–7 cm in length but this varies between individual ewes as well as with parity, age, breed and physiological state (Kershaw *et al.* 2005). The inner lumen is dominated by the presence of 4–7 angular folds, which point caudally but are not concentrically aligned and thus obscure the central lumen (Fig. 1). It is these angular folds that pose the challenge to the use of

an AI catheter in sheep. The number of folds and their level of interdigitation is directly correlated to the depth of penetration of an inseminating pipette. The lumen is at its narrowest (2–3 mm) nearest the vagina and the second and third fold is out of alignment with the first so that the inseminating pipette is misdirected away from the central lumen, typically resulting in less than 1 cm penetration into the cervical canal in the majority of ewes. While under the influence of oestrogen, there is some softening of the cervix during oestrus, through the rearrangement of collagen bundles within the cervical extracellular matrix (Kershaw *et al.* 2007), but this is not enough to allow passage of an insemination pipette. When deeper penetration is achieved, there is a 7–12% per cm increase in fertility as depth of insemination is increased (Salamon & Maxwell 2000). There have been reports of successful cervical penetration through redesigning the insemination catheters (Wulster-Radcliffe *et al.* 2004) or clamping the external cervical os and physically retracting it into the vagina allowing for deeper cervical penetration which has led to marginally improved fertility (Halbert *et al.* 1990). However, cervical penetration rates were influenced by inseminator skill and experience, ewe parity, interval from previous lambing and season of the year and due to inconsistent

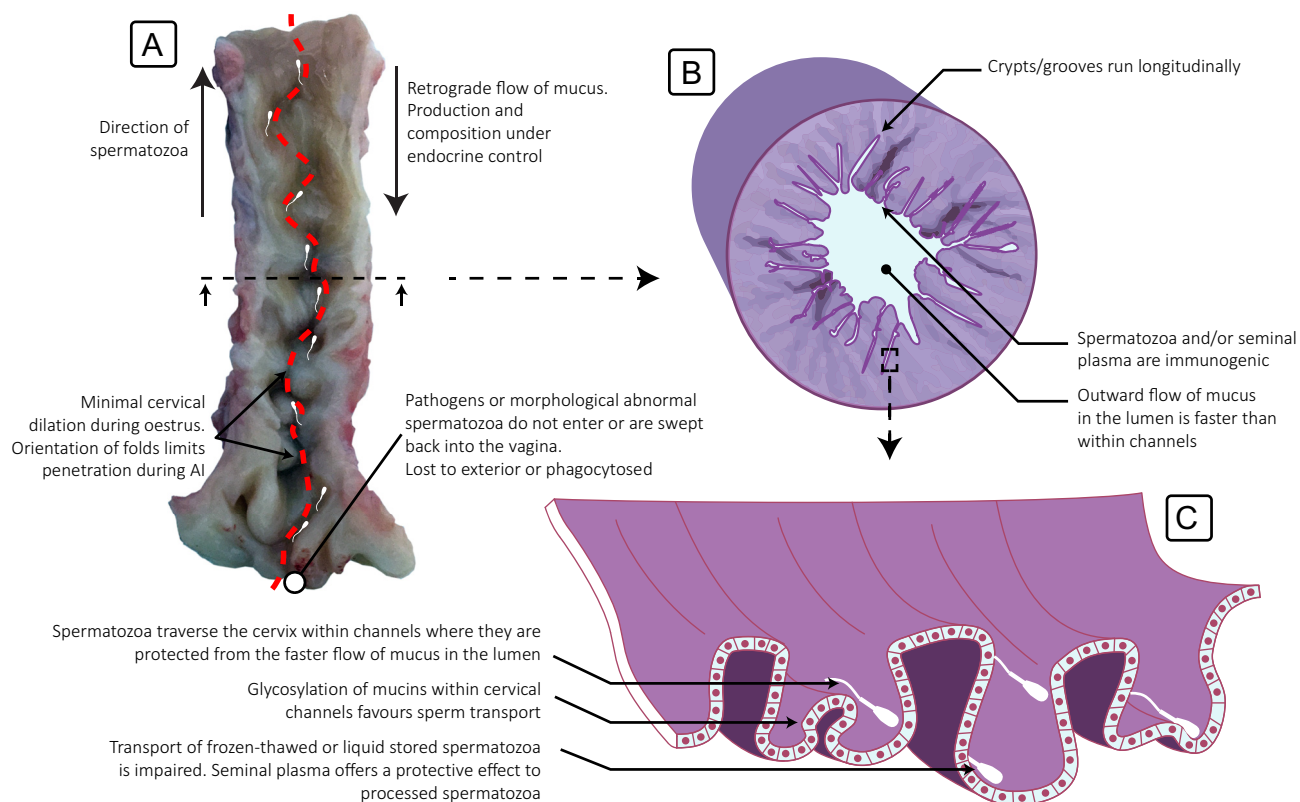


Figure 1 Graphical illustration of (A) the gross anatomy of the ovine cervical canal (B), a transverse section showing secondary grooves and (C) the micro-anatomy demonstrating the channels within which spermatozoa progress. Also detailed are some of the key physiological processes regulating sperm transport through the cervix. Not to scale.

fertility results, as well as welfare concerns, is not widely used. Other studies have focused on understanding the mechanisms of remodelling of the extracellular matrix leading to cervical dilation. A plethora of studies have used pharmacological approaches toward cervical dilation through the use of prostaglandin E₁, FSH and LH, oxytocin, 17 β -oestradiol and hyaluronan. While cervical softening has been achieved, and led in some cases to deeper penetration of the cervix, none have resulted in acceptable levels of fertility following cervical AI with F/T semen.

Cervical micro-anatomy and its role in sperm transport

In cows, Mullins and Saacke (1989) characterised the anatomy of the bovine cervix. They described folds which run the length of the cervix and apparently provide 'privileged pathways' for spermatozoa to migrate the full length of the endocervical canal to the uterus. These folds have also been described in women (Kessel 1979) but not in other species studied to date. Assessment of the folds following mating using transmission electron microscopy demonstrated that beating cilia were orientated in the direction of the vagina, while spermatozoa were orientated towards the uterus, indicating that spermatozoa swim against waves created by the ciliary beating (Mullins & Saacke 1989). In Fig. 2, representative scanning electron microscopy images of the luminal epithelium of the sheep cervix are presented. These demonstrate the crypts and folds of the ovine cervix are similar to that reported in other species, although it is not clear if these run continuously all the way from the cervical os to the endocervix. The presence of ciliated epithelial cells in the ovine cervix (Fig. 2F) was unexpected and to the best of our knowledge has not been previously reported. It is not known if spermatozoa interact with these cilia in a similar manner to the ciliated epithelial cells in the oviduct. The presence of folds along the FRT suggests that they may have evolved to accommodate sperm transit as evidenced by the presence of spermatozoa deep within the cervical channels in cervices from ewes recovered shortly after insemination with F/T semen (Richardson *et al.* 2019). The flow of mucus within these crypts and folds is thought to differ in composition and be slower than the stronger flow in the lumen of the cervix, which is critical for the protection of the upper FRT against the infiltration of pathogens (Cone 2009). Indeed when ewes were inseminated with immotile spermatozoa, these spermatozoa were found in the lumen and not in the crypts of the cervix (Mattner & Braden 1969).

A number of studies have focused on the biophysical benefits of fluid flow on human, mouse and bull sperm transit using *in vitro* microfluidics approaches (see review by Suarez & Wu 2017). Motile spermatozoa orientate and swim against a flow (positive rheotaxis), which has been proposed as a major determinant of

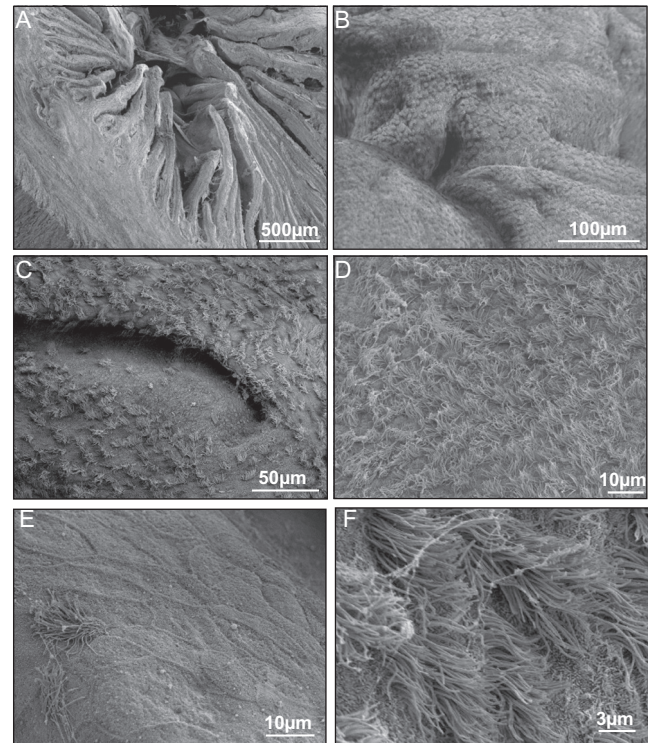


Figure 2 Representative scanning electron microscopy images of the luminal epithelium of the sheep cervix. The images are arranged in increasing magnification (A, B, C, D, E and F) and illustrate the folds of the cervix (A), the undulating surface (B), the presence of crypts, (C) the mucin mesh covering the epithelium (D and E) as well as ciliated epithelial cells (F). The cervixes of animal 1 (A, D and E) and animal 2 (B and C) were both collected 1 day after ovulation.

sperm guidance over long distances in the mammalian FRT (Miki & Clapham 2013). Conversely, mechanisms such as chemotaxis and thigmotaxis are likely to only be effective once spermatozoa are in close proximity within the oviducts. Given the fibrous nature of the cervix, smooth muscle contractions are likely to play less of a role than in the uterus and oviducts and thus rheotaxis may be a major determinant of cervical sperm transport.

It has been suggested that rheotaxis is the result of the flagella beating forming a conical surface behind the sperm head, directing the sperm head upstream (Miki & Clapham 2013). However, others have proposed that sperm orientation against a flow is governed by near-surface hydrodynamic interactions (wall tracking behaviour), via the interaction of a front-back asymmetric microswimmer with a solid boundary. Both human and bull spermatozoa not only swim against the fluid flow, but tend to swim upstream in spiral-shaped trajectories along the walls of the microchannel. This wall-tracking behaviour is known as thigmotaxis, although it is yet to be established if this is only a feature of *in vitro* systems or indeed regulates sperm rheotaxis *in vivo*. Tung *et al.* (2014) studied the migration of bull spermatozoa against fluid flow in a microfluidic device that recreated the

biophysical environment of mammalian spermatozoa with microgrooves (20 µm in width) embedded on a microchannel surface. They reported that microgrooves allow spermatozoa to swim faster and more efficiently in the presence of the flow which suggests that the grooves present along the FRT have evolved, in part at least, to accommodate spermatozoa transport. Interestingly, research investigating the pathogenesis of *Tritrichomonas foetus*, a puller swimmer pathogen, was swept back against a flow (Tung *et al.* 2015) suggesting that motility alone does not lead to positive rheotaxis but the faster outward flow of mucus in the cervical lumen during oestrus has the ability to prevent pathogens from crossing the cervix. Taken together, the evidence suggests that cervical sperm migration is stimulated by rheotaxis and occurs deep within the crypts and folds of the cervix in an environment which is structurally distinct to that of the lumen. The outward flow of mucus in the lumen aids in the removal of pathogens, white blood cells as well as defective spermatozoa and provides protection to the upper FRT suggesting a coevolution of females and males to support fertilisation while suppressing infection.

Cervical mucus

Mucus production and its mechanical properties

Cervical mucus is a complex non-Newtonian viscoelastic bodily fluid comprised of secretions from the oviducts, endometrium and cervix. It is primarily composed of water (95–99%) but also includes cellular material, ions, plasma proteins, bactericidal proteins, enzymes and mucins (Curlin & Bursac 2013). In vaginal depositors such as sheep and humans, it regulates sperm migration to the upper FRT while, at the same time, acts as a protective barrier against infection. It has also been reported to limit the progression of immotile and membrane-damaged spermatozoa as well as DNA-fragmented spermatozoa (Bianchi *et al.* 2004) towards the oviduct, suggesting that the sperm surface morphology may reflect their DNA status.

Sperm transport from the vagina to the oviducts is dependent on the properties of cervical mucus in which spermatozoa must travel, including mucus quantity, viscosity and hydration – all of which are regulated by ovarian steroids. Cervical mucus production is continuous and its production is under the influence of oestrogen, whereas progesterone has a modifying effect. As a result, its composition varies across the reproductive cycle. In humans, the cervix produces approximately 20–60 mg of mucus per day during the luteal phase and increases 10–20 fold, up to 700 mg per day, in the peri-ovulatory period (Moghissi & Syner 1976). Similar increases in mucus production during oestrus have been demonstrated in sheep (Maddison *et al.* 2016). These increases are likely due to increased

para-cellular permeability of the ectocervical cells as has been shown in *in vitro* culture systems using human ectocervical cells supplemented with oestrogen (Gorodeski 2000). In both sheep and humans, the levels of hydration peaks in the follicular phase and is inversely related to the protein content, with a less proteinaceous, viscoelastic mucus produced in the follicular phase (Maddison *et al.* 2016). This is essential for progression of spermatozoa with normal mobility and morphology. This natural variation in cervical mucus production and composition seems to facilitate sperm transport during the follicular phase (oestrogen dominant) while during the luteal phase (progesterone dominant), it acts as an antimicrobial barrier as well as priming the FRT for an impending pregnancy.

For sperm transport, the viscoelastic properties of mucus appear to be more influential than the viscosity alone (Tung *et al.* 2017). Mucus viscoelasticity is largely regulated by mucins, which are large polymeric glycosylated proteins that are widely expressed within the oviduct, endometrium, cervix and vagina and make up approximately 45% of all proteins in cervical mucus. These large complex glycoproteins consist of a central core protein domain, rich in the amino acids serine, threonine and proline, which provide a high number of attachment points for branching oligosaccharide side chains and are terminated with either sialic acid (NeuAc) or fucose (Fuc). Unlike the majority of glycoproteins, the oligosaccharides of mucins are predominantly O-linked. It is this complex arrangement of side chains that give mucin its filamentous properties and a bottle-brush-like appearance. The carbohydrate side chains can be neutral, sulphated or sialyated (Andersch-Bjorkman *et al.* 2007), with the latter two being partly responsible for conferring a net-negative charge to mucus. Mucins can be characterised into three classes (i) the secreted mucins, (ii) membrane-associated mucins and (iii) small soluble mucins. Secreted mucins can be further classified into either gel forming or non-gel forming.

The mucin core proteins are produced in the rough endoplasmic reticulum of the endocervix epithelial cell where a small amount of N-glycosylation is required for normal processing following which they are then shuttled to the Golgi apparatus in which O-glycosylation takes place. The highly condensed mucin is then transported to the mucin granule of goblet cells, in which high intergranular levels of calcium and hydrogen ions shield negatively charged sites on mucins from electrostatic repulsion (Verdugo 2012). This allows the highly condensed polyanionic macromolecular mucins to be packaged into mucin storage vesicles within goblet cells (Muchekehu & Quinton 2010). Mucin secretion can be modulated by pathogens, hormones and neurotransmitters, while, after leaving the vesicles, bicarbonate (HCO_3^-) appears to have the greatest modulating effect. Extracellular HCO_3^- removes

the cationic shields from mucins via sequestering of Ca^{2+} and buffering of H^+ . This allows for rapid expansion of the mucin via electrostatic repulsion into an extracellular network of 'tangled strings' as is evident in Fig. 2. In the process, the volume increases by as much as 1000-fold in less than a couple of seconds upon exposure to high concentrations of HCO_3^- (Muchekehu & Quinton 2010). The structure of mucins are modified during the cycle from globular-ovulatory to fibrous-pre-ovulatory mucus which appears to be regulated by pH changes and, combined with a reduction in viscosity, allows sperm penetration in the peri-ovulatory period (Baumber *et al.* 2002).

Cervical mucus proteome

Characterisation of gene expression changes in the endocervix as well as the quantification of proteins in its secretions over the duration of the oestrous cycle are key to understanding the relationship between mucus proteome, mechanical properties of mucus and sperm interaction with it. While over 800 proteins have been identified in cervical mucus (Soleilhavoup *et al.* 2016), its viscosity during oestrus is mainly due to the elevated amounts of secreted mucins as well as their level of glycosylation. Biochemical changes in cervical mucins, such as dramatic changes in O-glycosylation at ovulation, may also contribute to the hydration of cervical mucins, as well as promote sperm penetration and survival in the FRT due to altered sialic acid content (Ma *et al.* 2016). Five mucins have been identified in the FRT (Andersch-Bjorkman *et al.* 2007) of which three are gel forming (MUC 5B, MUC 5AC, MUC 6) and two are transmembrane proteins (MUC 1 and MUC 16). MUC 5B is the main gel-forming mucin responsible for the viscoelastic properties of cervical mucus (Portal *et al.* 2017). It has been shown to be more abundant in cervical mucus collected during the follicular than the luteal phase in both sheep (Soleilhavoup *et al.* 2016, Maddison *et al.* 2017) and humans (Gipson *et al.* 2001) when progesterone is at its lowest. The degradation of mucins is partly controlled by the enzymatic action of sialidase (NEU1) which cleaves terminal sialic acid residues from the carbohydrate end chains, leading to a decrease in the viscoelastic properties of mucus. Indeed recently, NEU1 was found to be present in sheep cervical mucus during oestrus and absent during the mid-luteal phase (Maddison *et al.* 2017).

Several transcripts associated with mucin biosynthesis and intracellular transport as well as their post secretory modifications have been shown to be upregulated in bovine cervical epithelium during oestrus (Pluta *et al.* 2012). Histological staining revealed that sialylated mucins dominate at the bases of bovine cervical folds, within the so-called privileged pathways, whereas sulphated mucins were more abundant at their apices. Interestingly, mucus consists mainly

of sialomucins with lesser amounts of sulfomucins present. This balance is under endocrine control as when ewes were ovariectomised, mucus production declined and consisted mainly of sulfomucins but following supplementation with oestradiol benzoate, mucus production was restored as were the levels of sialomucins mucus (Adams & Tang 1986). The core mucin glycans also appears to be altered during the transition from follicular to luteal phases, whereas terminal glycans change mainly in the peri-ovulatory period and are associated with changes in glycosidase activity (Pluta *et al.* 2011).

Despite anatomical barriers, 55% of proteins have been shown to be common in luminal fluid from the cervix, uterus and oviducts of sheep (Soleilhavoup *et al.* 2016). In the same study, the cervix and the oviduct had an increased number of proteins during oestrus, while the luteal phase was characterised by a higher abundance of proteins in uterine fluid associated with the preparation for supporting embryonic development. This pattern of protein abundance in the fluids along the cycle is most likely a result of the regulation of secretion by the endocrine and immune systems (Lee *et al.* 2015). The next phase of this work must be to identify which proteins are important for sperm transit across the cervix and to characterise their interaction with spermatozoa. Currently the only example of this is in the macaque, where the glycoprotein beta-defensin 126 (DEFB126) binds to the sperm surface in the corpus and cauda epididymis (Yudin *et al.* 2003) forming part of the complex sperm glycocalyx. The addition of sialic acid moieties to defensin peptides confers a negative charge to spermatozoa, thereby repelling the negatively charged mucus and facilitating migration of non-capacitated sperm through electronegative mucus. It plays a major role in immune recognition and its release during capacitation is required for spermatozoa to interact effectively with the zona pellucida (Tollner *et al.* 2004). Spermatozoa from men with *DEFB126* mutations have lower lectin-binding which is associated with fewer O-linked oligosaccharides, altered ability to penetrate synthetic mucus and reduced fertility (Tollner *et al.* 2011). Beta-defensins are conserved across mammalian species and are expressed within both male and female reproductive tissues (Equine; Narciandi *et al.* 2011, Johnson *et al.* 2015, Ovine, Hall *et al.* 2017). In cattle, DEFB126 is preferentially expressed in the caudal epididymis (Narciandi *et al.* 2016) with similar binding patterns on the sperm surface to macaque (Fernandez-Fuertes *et al.* 2016) and increases sperm motility and mucus penetration (Fernandez-Fuertes *et al.* 2016) as well as sperm binding to oviductal epithelial explants *in vitro* (Lyons *et al.* 2018). Further detailed characterisation of the functional role of proteins within the wider beta-defensin cluster, and others, both on spermatozoa and within mucus are needed to better understand sperm-cervical mucus interaction.

Sperm transport across the cervix

Characterising the problem

Establishment of a population of functional spermatozoa in the oviduct occurs over a period of 4–9 h with sperm numbers peaking in the oviducts approximately 24 h post insemination. Significant numbers of spermatozoa have been found in the ovine cervix within 1 h of insemination indicating that sperm entry into the cervix is relatively quick and there is a relationship between the numbers of spermatozoa in the cervix 1–2 h post insemination and the numbers in the oviducts at 24 h and resulting fertility (Crocker *et al.* 1975). Irrespective of whether fresh or frozen-thawed semen is inseminated, the majority of spermatozoa are lost to the exterior through the vagina. Phagocytosis of spermatozoa also plays a major role in the elimination of sperm from the FRT due to the infiltration of leukocytes into the uterine lumen, and cervix (Pini *et al.* 2017) which, through the interaction of L-selectin, bind sialic acid on the sperm surface (Yu *et al.* 2018). The production of reactive oxygen species by phagocytes has also been demonstrated to decrease sperm motility, which is likely to adversely affect sperm progression (Shi *et al.* 2012).

Cervical immunological response to spermatozoa

Multiple studies have now reliably established that SP is immunogenic in the FRT across multiple species including humans, mice and cattle. These studies have identified a common inflammatory profile of innate immune transcripts including recruitment of immune cells and activation of cytokines and chemokines (Sharkey *et al.* 2012). Rather than be detrimental to fertility, this physiological inflammation is associated with improved reproductive outcomes, and the orchestration of the immune response is now thought to be a key factor in establishing local adaptations that promote the tolerance of the allogenic foetus during pregnancy (reviewed by Schjenken & Robertson 2014).

In the assessment of the immune response in the FRT, the focus traditionally has been on the uterus, the site where semen is deposited during natural mating in some species or when AI is used, and the detail of the cervix has been overlooked. However, given that the anatomy of the ovine cervix precludes the successful use of cervical AI, the immune response to semen in this region is of critical importance in the ewe. What analysis has been performed to date suggests that the response to SP is site specific and varies between cervical and vaginal epithelial cells (Sharkey *et al.* 2007). Few studies, in any species, have examined the impact of the cervical immune system on fertility, and therefore, it is timely that a reappraisal of the role of the cervix is now beginning (Martyn *et al.* 2014). Even within the cervix, distinct regions have been identified, where the endocervix, which together with the uterus and oviduct

comprise the upper FRT, is composed of a single layer of columnar epithelial cells. The lower FRT consisting of the vagina and ectocervix are made up of keratinised, stratified squamous epithelium. Multiple studies have shown that it is the ectocervix that represents the primary site of responsiveness in terms of immune activation (Sharkey *et al.* 2007), which is logical, given its critical defence role to preventing ascending infection in the FRT. However, studies establishing the responsiveness and regional variation, particularly after the deposition of spermatozoa, are limited in the ewe.

Due to the aforementioned focus on SP, as the usual transport medium for spermatozoa during natural mating, it is still unclear if spermatozoa alone are directly immunogenic. One interesting study found that immune cells were recruited to the uterus of female mice mated with vasectomised males but the response was absent after mating with males from which seminal glands were removed (Johansson *et al.* 2004). This clearly indicates that, at least in mice, the predominant driver of the immune response in the FRT is SP and not spermatozoa. In contrast, however, the presence of antisperm antibodies (ASAs), both in circulation and in tissues of the FRT across multiple species, suggest otherwise. The identification of ASA in cervical mucus has also been reported, and interestingly, intrauterine insemination has been found to be an effective method to achieve pregnancy in humans. The fact that ASAs are generated against spermatozoa in the first place confirms that spermatozoa can be immunogenic, and it is possible that antigenic peptides are exposed on the sperm surface from some males and are reported to account for a significant proportion of unexplained infertility cases in humans (Cui *et al.* 2015). It is thought that impaired sperm–mucus interactions could contribute to the generation of ASA, and result in what is known as immunological infertility. Antisperm IgA on spermatozoa or in cervical mucus can severely inhibit sperm penetration of cervical mucus and migration through it (Kremer & Jager 1992).

The processing of ram semen prior to AI dilutes the immunomodulatory peptides usually resident in SP and may contribute to higher, and breed-specific inflammatory responses in cervical tissue of the ewe, which may ultimately preclude passage of spermatozoa through, and survival in the cervix. The sperm glycocalyx is known to be composed of highly glycosylated peptides (Teclé & Gagneux 2015), one important family of which are β -defensins. Small cationic peptides secreted in the epididymis to expansively coat spermatozoa – these multifunctional effector proteins – not only contribute to the charge-mediated passage of spermatozoa through mucus (discussed earlier) but also prevent immunorecognition and protect spermatozoa through prevention of the binding of ASA (Yudin *et al.* 2005).

Ewe breed differences in the immune response have been well established in response to infectious

agents, and there is no reason to suspect that similar differences in local FRT responsiveness to spermatozoa and/or SP also do not exist. Considerable variation in the inflammatory potential of SP samples from different humans has been reported (Sharkey *et al.* 2007), and this may explain why spermatozoa from the same ram can transverse the cervix of some ewe breeds better than others. It is possible therefore that inherent basal or induced immune response differences between breeds contribute to the success or otherwise of sperm passage, but this theory remains to be investigated in sheep.

The cervix possesses a potent ability to produce antimicrobial defence molecules and increased lysozyme activity after intercourse has been shown. Increased β -defensin (*HBD2* and *HBD3*) expression has also been documented with inflammation of the cervix (Meng *et al.* 2013), supporting an important role for peptides in mucus in defence of the FRT. This specific gene family is copy number variable across the genomes of multiple species, including in cattle (Bickhart *et al.* 2012). A lower *DEFB4* copy number was associated with susceptibility to cervical cancer in humans (Abe *et al.* 2013) and variation in the transcript content between breeds will undoubtedly contribute to the functional differences in fertility in sheep. Some of these transcripts are also expressed in the FRT leading to speculation that their roles may firstly contribute to regulation of immune system in the FRT as well as affecting sperm survival and transport. The advent of complete and accurately annotated genome sequences in sheep means that this is a fertile area for future research.

Exogenous hormones impair cervical sperm transport

One of the most pertinent examples of impaired cervical sperm transport is in ewes grazing oestrogenic pastures. These ewes have altered cervical morphology, significantly increased mucus production and severely reduced numbers of spermatozoa in the oviducts 24 h post mating, leading to impaired fertility (Lightfoot *et al.* 1967). In women, it is known that exogenous hormones impact mucus characteristics such as viscosity and protein content and that these changes negatively impact sperm penetration through mucus (Lewis *et al.* 2010). Impaired sperm transport due to cervical mucus thickening is the major contraceptive action of the levonorgestrel-releasing intrauterine system as well as an important secondary mechanism of the combined oral contraceptive pill. The use of exogenous hormones in the synchronisation of oestrus in sheep is essential for AI in most countries, but this has been long associated with reduced fertility. Several studies have reported reduced fertility rates and reduced numbers of spermatozoa in the FRT of the progestagen-treated ewe compared to naturally cycling animals. The precise cause of impaired sperm transport in hormonally treated ewes is unknown, although it is presumably due to an

altered endocrinological balance within the animal, but other factors such as neutrophil recruitment in response to the physical presence of a synchronisation device may also play a role (Mitchell *et al.* 2005). Observed phenomena in progestagen-treated ewes include increased production of cervical mucus (Maddison *et al.* 2016), altered cervical mucus proteome (Maddison *et al.* 2017), increased sperm breakage and loss (Gillan *et al.* 1999) as well as reduced functionality and viability of spermatozoa *in vitro*, when suspended in mucus from progestagen-treated ewes (Manes *et al.* 2016). In addition, synchronised ewes have higher enzymatic activity and protein content of the endometrium (Murdoch & White 1968), downregulation of *Interleukin-8* in the cervical epithelium (Mitchell *et al.* 2002), earlier infiltration of leucocytes (Quinlivan 1967), delayed secretion from the oviducts (Murdoch & White 1968) as well as increased degeneration of the glandular epithelium of the FRT (Hawk & Conley 1971). Similar issues of impaired sperm transport have been shown following superovulation in cattle where most fertilised oocytes have no accessory spermatozoa (Hawk *et al.* 1988).

Ewe breed differences in cervical function

The exception to the poor fertility achieved following cervical AI with frozen-thawed semen is in Norway, where lambing rates following insemination into the external cervical os or indeed the vagina (so-called 'shot-in-the-dark') with frozen-thawed semen to a natural oestrus have been reported to be greater than 70% (Paulenz *et al.* 2007). This success clearly demonstrates that cervical penetration is not essential for successful sheep AI; Donovan *et al.* (2004) evaluated the procedures used in Norway under Irish conditions and reported higher pregnancy rates using fresh compared to frozen-thawed semen but found no significant difference in pregnancy rate following a natural or synchronised oestrus. While there is variation between individual rams (O'Meara *et al.* 2005), as well as between ejaculates of the same ram, there is no difference between rams of Irish and Norwegian origin; however, there is a significant effect of ewe breed on pregnancy rate (Donovan *et al.* 2004) with values of 8, 28, 44 and 77% reported for Suffolk, Texel, Belclare and Finnish Landrace, respectively. Parallel, albeit higher, ewe breed differences in pregnancy rates have also been reported following cervical insemination with liquid-stored semen (O'Hara *et al.* 2010) and following natural mating (Hanrahan 2003). Further studies examining breed differences, gross anatomy of the cervix as well as in-timing of ovulation and endocrinological profiles failed to explain the ewe breed differences in fertility (Donovan *et al.* 2004, Fair *et al.* 2007). Using a combination of fertilisation rates and accessory sperm number, significantly more spermatozoa were shown to have reached the site of fertilisation in Belclare than in Suffolk ewes following

cervical insemination with frozen-thawed semen, and this difference was eliminated following laparoscopic insemination (Fair *et al.* 2005). This illustrates that it is the inability of spermatozoa to traverse the cervix of low (Suffolk) compared to high (Belclare) fertility breeds and is in agreement with other studies which have reported that the migration of spermatozoa through the cervix appears to be the critical limiting factor.

An investigation into the rheology of the mucus between the breeds found that Suffolk ewes tended to have higher elastic and complex moduli than that from Belclare ewes leading to greater mucus penetration, as assessed *in vitro* (Richardson *et al.* 2011). Glycosylation of cervical mucins also varied between breeds, with low fertility breeds (Suffolk) containing a significantly higher sialic acid content in the cervical channels than high fertility breeds (Belclare), while *in vitro*, the addition of sialic acid to spermatozoa increased mucus penetration (Richardson *et al.* 2019). The immunoprotection of spermatozoa against immune recognition in the female uterus has been shown to be mediated by sialic acid (Alkhodair *et al.* 2018), and therefore, differential glycosylation levels may mediate higher immunoreactivity and lower fertility *in vivo*. The larger variation in ewe breed cervical function is certainly a useful biological model to better understand sperm transport across the cervix in both animals and women.

Processing of semen reduces cervical penetration

Over the last 50 years, semen preservation methods for both frozen-thawed and liquid storage of semen have improved so as to yield acceptable levels of sperm quality for insemination. While mass motility of fresh semen has been shown to be correlated with field fertility (David *et al.* 2015, 2018), there is a poor correlation between post-thaw *in vitro* parameters and *in vivo* fertility in sheep (O'Meara *et al.* 2005). It is clear that processing of semen such as freezing, thawing or liquid storage reduces the longevity of spermatozoa in the FRT. Where possible, intrauterine insemination aids in overcoming this such as in humans (Kop *et al.* 2015) as well as cattle (Murphy *et al.* 2017). In sheep, when semen is collected, diluted and stored for more than 24 h, fertility declines rapidly when cervically inseminated (O'Hara *et al.* 2010). A meta-analysis by Maxwell and Salamon (1993) reported a reduction in fertility of 10–35% per day of storage following cervical AI. This decline occurred irrespective of the sperm number, semen diluent, storage temperature or conditions employed. Using fibered confocal microscopy, Druart *et al.* (2009) observed in excess of three times less spermatozoa in the body of the uterus 4 h following cervical insemination of ram semen stored for 24 h compared to fresh semen despite identical motility and velocity between sperm populations at insemination. When frozen-thawed semen is cervically inseminated, fertility rates can fall to

10–30%; however, when laparoscopically inseminated fertility rates of >60% are the norm (Salamon & Maxwell 2000). Modifications, such as double cervical inseminations of frozen-thawed semen a number of hours apart, have resulted in increased fertility, but this is due to increased sperm number rather than widening the time spermatozoa are present in the FRT (Salamon 1977). Similarly, increasing the number of frozen-thawed spermatozoa cervically inseminated increased the fertility but was still lower than the fertility achieved following laparoscopic insemination of frozen-thawed or cervical insemination of fresh semen (Maxwell & Hewitt 1986). Collectively, this demonstrates that the fertilising potential of frozen-thawed spermatozoa is maintained (albeit with sublethal damage; Pini *et al.* 2018a), but similar to that of liquid-stored semen, it is the inability of a sufficient number of spermatozoa to pass the cervical barrier into the uterus that results in low fertility following cervical AI.

Seminal plasma supports cervical sperm transport

Seminal plasma (SP) is a complex assortment of inorganic ions, organic salts, citric acid, sugars, prostaglandins, hormones, proteins and bioactive agents secreted mainly from the accessory glands but with contributions from the testes and epididymis (Mann 1964). Recently, studies have demonstrated that bioactive signalling agents in SP interact with the FRT, across a range of species, irrespective of the site of semen deposition (see review by Robertson & Sharkey 2016). As outlined earlier, SP has been shown to evoke gene expression and cellular changes in the innate immune system, aid in the protection from pregnancy disorders, improve embryo implantation following *in vitro* fertilisation (IVF) and even offspring health. In addition to these benefits, its role in nourishing spermatozoa and supporting their transit in the FRT is well established. Despite this, caudal epididymal spermatozoa of a wide range of species which have not been coated with SP are fertile when used in intracytoplasmic sperm injection (Human; Silber *et al.* 1995) and IVF (Cattle; Holden *et al.* 2017) and has yielded similar fertility to ejaculated spermatozoa, when deposited into the uterus in sheep (Rickard *et al.* 2014). It should be noted that in all of these cases, spermatozoa did not have to cross the cervical barrier and when vaginal insemination of epididymal spermatozoa was performed in dogs, it led to poor pregnancy rates (Thomassen & Farstad 2009). In sheep, a number of studies have shown addition of SP to liquid stored (Lopez-Perez & Perez-Clariget 2012) and cryopreserved (Maxwell *et al.* 1999) ram spermatozoa prior to cervical AI led to improved pregnancy rates, while others found no effect (O'Meara *et al.* 2007) or an inconsistent effect (Leahy *et al.* 2010). These conflicting results may be due to individual ram effects from which the SP was collected (Rickard *et al.* 2016). Rickard *et al.* (2014) exposed caudal

epididymal ram spermatozoa to SP prior to cervical and intrauterine insemination. Using a combination of probe-based Confocal Laser Endomicroscopy and *in vivo* fertility data, they reported more spermatozoa at the utero-tubal junction and higher pregnancy rates when epididymal spermatozoa were pre-exposed to SP prior to being deposited at the external cervical os. Exposure of epididymal spermatozoa to SP had no effect when laparoscopically deposited into the uterus, clearly demonstrating that the beneficial effect of SP is localised to spermatozoa traversing the tortuous ovine cervix. Seminal plasma appears to have a protective effect on liquid semen stored for 24 h especially when SP is from rams of predetermined high preservation ability (Soleilhavoup *et al.* 2014). When SP from rams deemed to be good or 'poor freezers' was added back to spermatozoa, the SP from the 'good freezers' improved post-thaw sperm motility (Rickard *et al.* 2015, 2016), although similar effects were not observed with bull spermatozoa (Holden *et al.* 2017). When exposed for a short period of time, SP seems to act as a protective medium during *in vitro* processing of ram spermatozoa and may ameliorate the processing induced stressors of cryopreservation (Leahy & de Graaf 2012), while this does not seem to be the case in other species (reviewed by Bromfield (2016)). It is unknown if the protective effects of ram SP during processing and cervical transit is by exposing spermatozoa to new proteins, altering the abundance of existing membrane-bound proteins, protection of spermatozoa in the FRT or indeed through other non-proteomic factors.

The continuous progression in methods of protein identification using high resolution strategies have provided extensive information about the human SP proteome, which can be used as a tool to identify biomarkers of reproductive function. A comparative study of the protein composition of goat buck, boar, ram, bull, stallion, alpaca and camel SP revealed considerable divergence of SP proteomes between the species (Druart *et al.* 2013), while ram sperm proteins appear to be highly conserved across species with 95% proteins reported in other species (Pini *et al.* 2016). As with all 'Omics' studies, the major challenge now is to characterise the biological functions of these proteins. There have been more than 700 proteins identified in ram SP (of which 40 were identified as being of sperm origin) including a high abundance of Binder of Sperm family proteins (BSP1, BSP5, SPADH1, SPADH2), the spermadhesin family (bodhesin2), lactoferrin and newly identified proteins like UPF0762 (*C6orf58* transcript; Soleilhavoup *et al.* 2014). Under *in vitro*-capacitating conditions, BSP1 has recently been shown to stabilise the ram sperm membrane, reduce protein tyrosine phosphorylation while increasing cholesterol efflux and induced spontaneous acrosome reactions, while BSP5 had minimal effects on capacitated ram spermatozoa (Pini *et al.* 2018b).

The process of cryopreservation as well as the use of egg yolk extenders have recently been shown to alter the proteome of ram spermatozoa (Pini *et al.* 2018c). SP proteins have been reported to protect ram sperm from cold shock damage prior to cryopreservation (Pini *et al.* 2018d). Some studies have suggested that spermadhesins are responsible for these protective properties (Barrios *et al.* 2005), while others found no association of this protein family with liquid preservation of semen (Soleilhavoup *et al.* 2014). Zinc alpha 2-glycoprotein (ZAG) in the SP increased ram sperm motility initially but was negatively associated with sperm motility after 24 h of semen preservation in liquid state, indicating a biphasic effect (Soleilhavoup *et al.* 2014). When the proteome of epididymal and ejaculated ram spermatozoa were compared, surprisingly, only two membrane-bound proteins were detected solely in ejaculated sperm lysates: liver-enriched transcript 1 (*LEG1/C6orf58*) and epidermal growth factor-like repeats and discoidin I-like domains 3 (*EDIL3*; Pini *et al.* 2016). This illustrates that despite its relatively complex composition, SP exposure leads to a very limited number of proteins binding tightly to the ram sperm plasma membrane. Further investigation of the effect of these three proteins on the function of ram spermatozoa are warranted.

Conclusions and future avenues of investigation

The problem of impaired cervical transit in sheep is well characterised and represents an ideal model of fertilisation failure due to impaired sperm transport in humans. Given the complexity of the problem, it is clear that elucidating the reasons for this requires an inter- and multi-dimensional approach. Advances in 'Omics' technologies have provided biologists with the toolbox to complete in-depth characterisation of changes in the cervix in the lead up to ovulation as well as the variation in SP composition and the effects of processing on the sperm membrane. It is likely that a combination of these changes which alter the delicate balance of sperm interaction with the cervix, and its secretions, is responsible for the impaired cervical sperm transit. Interpreting how these changes relate to cervical sperm transport and ultimately fertility is a difficult task, but one which must be undertaken. In the absence of achieving cervical dilation, the use of experimental models which have the highest levels of variation in cervical sperm transit are critical to advancing our knowledge and solving this enigma. The most promising of these is the interrogation of differences in cervical function in ewe breeds known to have divergent fertility as well as the factors within SP which increase epididymal sperm penetration of the cervix. Taking this approach will enable us to identify the molecular markers that mediate the dynamic sperm signalling responses which are crucial for cervical sperm transport similar to those already identified as being required by spermatozoa

to cross the utero-tubal junction and interact with the oviduct. This intriguing area of reproductive biology will not only lead to the development of protocols for effective cervical AI of sheep but also increase our basic understanding of sperm interaction with the cervix, applicable to all vaginal depositors.

Declaration of interest

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

Funding

S. Fair, X. Druart and K. Meade are supported by funding under the European Research Area Network on Sustainable Animal Production (Grant no. 16/RD/SusAn/ERA-NET). SP de Graaf is supported by funding from Australian Wool Innovation (Grant no. ON-00252) and NSW Stud Merino Breeders' Association Trust.

Author contribution statement

S. Fair drafted the manuscript with the assistance of K. Meade, S. de Graaf and X. Druart. K Reynaud provided the images for Fig. 2. All authors contributed to the content of the manuscript and proof read it.

Acknowledgments

The authors thank Thierry Meylheuc, from the imaging facility at the microscopy and imaging platform MIMA2 (INRA, Jouy-en-Josas, France) for his help with Fig. 2. They acknowledge the contribution of Dr Eoin White in making Figure 1.

References

- Abe S, Miura K, Kinoshita A, Mishima H, Miura S, Yamasaki K, Hasegawa Y, Higashijima A, Jo O, Sasaki K *et al.* 2013 Copy number variation of the antimicrobial-gene, defensin beta 4, is associated with susceptibility to cervical cancer. *Journal of Human Genetics* **58** 250–253. (<https://doi.org/10.1038/jhg.2013.7>)
- Adams NR & Tang BY 1986 Changed control of cervical secretion from infertile ewes previously exposed to oestrogenic clover pasture. *Journal of Reproduction and Fertility* **76** 147–152. (<https://doi.org/10.1530/jrf.0.0760147>)
- Alkhodair K, Almhanna H, McGetrick J, Gedair S, Gallagher ME, Fernandez-Fuertes B, Tharmalingam T, Larsen PB, Fitzpatrick E, Lonergan P *et al.* 2018 Siglec expression on the surface of human, bull and ram sperm. *Reproduction* **155** 361–371. (<https://doi.org/10.1530/REP-17-0475>)
- Andersch-Bjorkman Y, Thomsson KA, Larsson JM, Ekerhovd E & Hansson GC 2007 Large scale identification of proteins, mucins, and their O-glycosylation in the endocervical mucus during the menstrual cycle. *Molecular and Cellular Proteomics* **6** 708–716. (<https://doi.org/10.1074/mcp.M600439-MCP200>)
- Barrios B, Fernandez-Juan M, Muino-Blanco T & Cebrian-Perez JA 2005 Immunocytochemical localization and biochemical characterization of two seminal plasma proteins that protect ram spermatozoa against cold shock. *Journal of Andrology* **26** 539–549. (<https://doi.org/10.2164/jandrol.04172>)
- Bamber J, Vo A, Sabeur K & Ball BA 2002 Generation of reactive oxygen species by equine neutrophils and their effect on motility of equine spermatozoa. *Theriogenology* **57** 1025–1033. ([https://doi.org/10.1016/S0093-691X\(01\)00710-5](https://doi.org/10.1016/S0093-691X(01)00710-5))
- Bianchi PG, De Agostini A, Fournier J, Guidetti C, Tarozzi N, Bizzaro D & Manicardi GC 2004 Human cervical mucus can act in vitro as a selective barrier against spermatozoa carrying fragmented DNA and chromatin structural abnormalities. *Journal of Assisted Reproduction and Genetics* **21** 97–102. (<https://doi.org/10.1023/B:JARG.0000029492.54243.3c>)
- Bickhart DM, Hou Y, Schroeder SC, Alkan C, Cardone MF, Matukumalli LK, Song J, Schnabel RD, Ventura M, Taylor JF *et al.* 2012 Copy number variation of individual cattle genomes using next-generation sequencing. *Genome Research* **22** 778–790. (<https://doi.org/10.1101/gr.133967.111>)
- Bromfield JJ 2016 A role for seminal plasma in modulating pregnancy outcomes in domestic species. *Reproduction* **152** R223–R232. (<https://doi.org/10.1530/REP-16-0313>)
- Cone RA 2009 Barrier properties of mucus. *Advanced Drug Delivery Reviews* **61** 75–85. (<https://doi.org/10.1016/j.addr.2008.09.008>)
- Crocker KP, Robinson TJ & Shelton JN 1975 The effect of oestrogen administered during the progestational phase of the cycle on transport of spermatozoa in ewes. *Journal of Reproduction and Fertility* **44** 11–23. (<https://doi.org/10.1530/jrf.0.0440011>)
- Cui D, Han G, Shang Y, Liu C, Xia L, Li L & Yi S 2015 Antisperm antibodies in infertile men and their effect on semen parameters: a systematic review and meta-analysis. *Clinica Chimica Acta; International Journal of Clinical Chemistry* **444** 29–36. (<https://doi.org/10.1016/j.cca.2015.01.033>)
- Curlin M & Bursac D 2013 Cervical mucus: from biochemical structure to clinical implications. *Frontiers in Bioscience (Scholar Edition)* **5** 507–515. (<https://doi.org/10.2741/S386>)
- David I, Kohnke P, Lagriffoul G, Praud O, Plouraboue F, Degond P & Druart X 2015 Mass sperm motility is associated with fertility in sheep. *Animal Reproduction Science* **161** 75–81. (<https://doi.org/10.1016/j.anireprosci.2015.08.006>)
- David I, Kohnke P, Fehrenbach J, Lopes Simoes AR, Debreuve E, Descombes X, Plouraboue F, Degond P & Druart X 2018 New objective measurements of semen wave motion are associated with fertility in sheep. *Reproduction, Fertility and Development* **30**: doi:10.1071/RD17472.
- Donovan A, Hanrahan JP, Kummel E, Duffy P & Boland MP 2004 Fertility in the ewe following cervical insemination with fresh or frozen-thawed semen at a natural or synchronised oestrus. *Animal Reproduction Science* **84** 359–368. (<https://doi.org/10.1016/j.anireprosci.2003.12.014>)
- Druart X, Cognie J, Baril G, Clement F, Dacheux JL & Gatti JL 2009 In vivo imaging of in situ motility of fresh and liquid stored ram spermatozoa in the ewe genital tract. *Reproduction* **138** 45–53. (<https://doi.org/10.1530/REP-09-0108>)
- Druart X, Rickard JP, Mactier S, Kohnke PL, Kershaw-Young CM, Bathgate R, Gibb Z, Crossett B, Tsikis G, Labas V *et al.* 2013 Proteomic characterization and cross species comparison of mammalian seminal plasma. *Journal of Proteomics* **91** 13–22. (<https://doi.org/10.1016/j.jpro.2013.05.029>)
- Fair S, Hanrahan JP, O'Meara CM, Duffy P, Rizos D, Wade M, Donovan A, Boland MP, Lonergan P & Evans AC 2005 Differences between Belclare and Suffolk ewes in fertilization rate, embryo quality and accessory sperm number after cervical or laparoscopic artificial insemination. *Theriogenology* **63** 1995–2005. (<https://doi.org/10.1016/j.theriogenology.2004.09.005>)
- Fair S, Hanrahan JP, Donovan A, Duffy P, O'Meara CM, Lonergan P & Evans AC 2007 Hormonal relationships during the periovulatory period among ewe breeds known to differ in fertility after cervical artificial insemination with frozen thawed semen. *Animal Reproduction Science* **97** 284–294. (<https://doi.org/10.1016/j.anireprosci.2006.02.006>)
- Fernandez-Fuertes B, Narciandi F, O'Farrelly C, Kelly AK, Fair S, Meade KG & Lonergan P 2016 Cauda epididymis-specific beta-defensin 126 promotes sperm motility but not fertilizing ability in cattle. *Biology of Reproduction* **95** 122. (<https://doi.org/10.1095/biolreprod.116.138792>)
- Gillan L, Skovgold K, Watson PF, Evans G & Maxwell WMC 1999 Fate and functional integrity of fresh and frozen-thawed ram spermatozoa following intrauterine insemination. *Reproduction, Fertility, and Development* **11** 309–315. (<https://doi.org/10.1071/RD99074>)
- Gipson IK, Moccia R, Spurr-Michaud S, Argueso P, Gargiulo AR, Hill JA, Offner GD & Keutmann HT 2001 The amount of MUC5B

- mucin in cervical mucus peaks at midcycle. *Journal of Clinical Endocrinology and Metabolism* **86** 594–600. (<https://doi.org/10.1210/jcem.86.2.7174>)
- Gorodeski GI** 2000 Effects of menopause and estrogen on cervical epithelial permeability. *Journal of Clinical Endocrinology and Metabolism* **85** 2584–2595. (<https://doi.org/10.1210/jcem.85.7.6671>)
- Halbert GW, Dobson H, Walton JS, Sharpe P & Buckrell BC** 1990 Field-evaluation of a technique for transcervical intrauterine insemination of ewes. *Theriogenology* **33** 1231–1243. ([https://doi.org/10.1016/0093-691X\(90\)90041-Q](https://doi.org/10.1016/0093-691X(90)90041-Q))
- Hall TJ, McQuillan C, Finlay EK, O'Farrelly C, Fair S & Meade KG** 2017 Comparative genomic identification and validation of beta-defensin genes in the *Ovis aries* genome. *BMC Genomics* **18** 278. (<https://doi.org/10.1186/s12864-017-3666-x>)
- Hanrahan JP** 2003 Pregnancy rate in Suffolk and Texel sheep to natural mating at either synchronized or natural oestrus. *Reproduction in Domestic Animals* **38** 358.
- Hawk HW & Conley HH** 1971 Loss of spermatozoa from the reproductive tract of the ewe and intensification of sperm "breakage" by progestagen. *Journal of Reproduction and Fertility* **27** 339–347. (<https://doi.org/10.1530/jrf.0.0270339>)
- Hawk HW, Conley HH, Wall RJ & Whitaker RO** 1988 Fertilization rates in superovulating cows after deposition of semen on the infundibulum, near the uterotubal junction or after insemination with high numbers of sperm. *Theriogenology* **29** 1131–1142. ([https://doi.org/10.1016/S0093-691X\(88\)80038-4](https://doi.org/10.1016/S0093-691X(88)80038-4))
- Holden SA, Fernandez-Fuertes B, Murphy EM, Lonergan P & Fair S** 2017 Effect of seminal plasma from high- and low-fertility bulls on cauda epididymal sperm function. *Reproduction, Fertility, and Development* **29** 2457–2465. (<https://doi.org/10.1071/RD17136>)
- Holt WV & Fazeli A** 2016 Sperm selection in the female mammalian reproductive tract. Focus on the oviduct: hypotheses, mechanisms, and new opportunities. *Theriogenology* **85** 105–112. (<https://doi.org/10.1016/j.theriogenology.2015.07.019>)
- Johansson M, Bromfield JJ, Jasper MJ & Robertson SA** 2004 Semen activates the female immune response during early pregnancy in mice. *Immunology* **112** 290–300. (<https://doi.org/10.1111/j.1365-2567.2004.01876.x>)
- Johnson GP, Lloyd AT, O'Farrelly C, Meade KG & Fair S** 2015 Comparative genomic identification and expression profiling of a novel beta-defensin gene cluster in the equine reproductive tract. *Reproduction, Fertility and Development* **28** 1499–1508. (<https://doi.org/10.1071/RD14345>)
- Kershaw CM, Khalid M, McGowan MR, Ingram K, Leethongdee S, Wax G & Scaramuzzi RJ** 2005 The anatomy of the sheep cervix and its influence on the transcervical passage of an inseminating pipette into the uterine lumen. *Theriogenology* **64** 1225–1235. (<https://doi.org/10.1016/j.theriogenology.2005.02.017>)
- Kershaw CM, Scaramuzzi RJ, McGowan MR, Wheeler-Jones CP & Khalid M** 2007 The expression of prostaglandin endoperoxide synthase 2 messenger RNA and the proportion of smooth muscle and collagen in the sheep cervix during the estrous cycle. *Biology of Reproduction* **76** 124–129. (<https://doi.org/10.1095/biolreprod.106.054049>)
- Kessel RG** 1979 *Tissues and Organs: A Text-Atlas of Scanning Electron Microscopy*. San Francisco, CA: Freeman.
- Kop PA, van Wely M, Mol BW, de Melker AA, Janssens PM, Arends B, Curfs MH, Kortman M, Nap A, Rijnders E *et al.*** 2015 Intrauterine insemination or intracervical insemination with cryopreserved donor sperm in the natural cycle: a cohort study. *Human Reproduction* **30** 603–607. (<https://doi.org/10.1093/humrep/dev004>)
- Kremer J & Jager S** 1992 The significance of antisperm antibodies for sperm-cervical mucus interaction. *Human Reproduction* **7** 781–784. (<https://doi.org/10.1093/oxfordjournals.humrep.a137737>)
- Leahy T & de Graaf SP** 2012 Seminal plasma and its effect on ruminant spermatozoa during processing. *Reproduction in Domestic Animals* **47** (Supplement 4) 207–213. (<https://doi.org/10.1111/j.1439-0531.2012.02077.x>)
- Leahy T, Evans G, Maxwell WMC & Marti JJ** 2010 Seminal plasma proteins do not consistently improve fertility after cervical insemination of ewes with non-sorted or sex-sorted frozen-thawed ram spermatozoa. *Reproduction, Fertility, and Development* **22** 606–612. (<https://doi.org/10.1071/RD09207>)
- Lee SK, Kim CJ, Kim DJ & Kang JH** 2015 Immune cells in the female reproductive tract. *Immune Network* **15** 16–26. (<https://doi.org/10.4110/in.2015.15.1.16>)
- Lewis RA, Taylor D, Natavio MF, Melamed A, Felix J & Mishell D, Jr** 2010 Effects of the levonorgestrel-releasing intrauterine system on cervical mucus quality and sperm penetrability. *Contraception* **82** 491–496. (<https://doi.org/10.1016/j.contraception.2010.06.006>)
- Lightfoot RJ, Croker KP & Neil HG** 1967 Failure of sperm transport in relation to ewe infertility following prolonged grazing on oestrogenic pastures. *Australian Journal of Agricultural Research* **18** 755–765. (<https://doi.org/10.1071/AR9670755>)
- Lopez-Perez A & Perez-Clariget R** 2012 Ram seminal plasma improves pregnancy rates in ewes cervically inseminated with ram semen stored at 5 degrees C for 24 hours. *Theriogenology* **77** 395–399. (<https://doi.org/10.1016/j.theriogenology.2011.08.013>)
- Lyons A, Narciandi F, Donnellan E, Romero-Aguirregomezcorta J, Farrelly CO, Lonergan P, Meade KG & Fair S** 2018 Recombinant beta-defensin 126 promotes bull sperm binding to bovine oviductal epithelia. *Reproduction, Fertility, and Development*. doi:10.1071/RD17415.
- Ma X, Pan Q, Feng Y, Choudhury BP, Ma Q, Gagneux P & Ma F** 2016 Sialylation facilitates the maturation of mammalian sperm and affects its survival in female uterus. *Biology of Reproduction* **94** 123. (<https://doi.org/10.1095/biolreprod.115.137810>)
- Maddison JW, Rickard JP, Mooney E, Bernecic NC, Soleilhavoup C, Tsikis G, Druart X, Leahy T & de Graaf SP** 2016 Oestrus synchronisation and superovulation alter the production and biochemical constituents of ovine cervicovaginal mucus. *Animal Reproduction Science* **172** 114–122. (<https://doi.org/10.1016/j.anireprosci.2016.07.008>)
- Maddison JW, Rickard JP, Bernecic NC, Tsikis G, Soleilhavoup C, Labas V, Combes-Soia L, Harichaux G, Druart X, Leahy T *et al.*** 2017 Oestrus synchronisation and superovulation alter the cervicovaginal mucus proteome of the ewe. *Journal of Proteomics* **155** 1–10. (<https://doi.org/10.1016/j.jprot.2017.01.007>)
- Manes J, Rios G, Fiorentino MA & Ungerfeld R** 2016 Vaginal mucus from ewes treated with progestogen sponges affects quality of ram spermatozoa. *Theriogenology* **85** 856–861. (<https://doi.org/10.1016/j.theriogenology.2015.10.033>)
- Mann T** 1964 *The Biochemistry of Semen*. London: Methuen and CO LTD.
- Martyn F, McAuliffe FM & Wingfield M** 2014 The role of the cervix in fertility: is it time for a reappraisal? *Human Reproduction* **29** 2092–2098. (<https://doi.org/10.1093/humrep/deu195>)
- Mattner PE & Braden AWH** 1969 Comparison of distribution of motile and immotile spermatozoa in ovine cervix. *Australian Journal of Biological Sciences* **22** 1069–1070. (<https://doi.org/10.1071/BI9691069>)
- Maxwell WMC & Hewitt LJ** 1986 A comparison of vaginal, cervical and intrauterine insemination of sheep. *Journal of Agricultural Science* **106** 191–193. (<https://doi.org/10.1017/S0021859600061906>)
- Maxwell WMC & Salamon S** 1993 Liquid storage of ram semen - a review. *Reproduction, Fertility, and Development* **5** 613–638. (<https://doi.org/10.1071/RD9930613>)
- Maxwell WMC, Evans G, Mortimer ST, Gillan L, Gellatly ES & McPhie CA** 1999 Normal fertility in ewes after cervical insemination with frozen-thawed spermatozoa supplemented with seminal plasma. *Reproduction, Fertility, and Development* **11** 123–126. (<https://doi.org/10.1071/RD99046>)
- Meng W, Du R, Wang Y, Chen Z & Ding Y** 2013 Human beta-defensin messenger RNA is overexpressed in the cervical epithelia of patients with nongonococcal cervicitis. *Journal of Lower Genital Tract Disease* **17** 440–445. (<https://doi.org/10.1097/LGT.0b013e318281f1a0>)
- Miki K & Clapham DE** 2013 Rheotaxis guides mammalian sperm. *Current Biology* **23** 443–452. (<https://doi.org/10.1016/j.cub.2013.02.007>)
- Mitchell SE, Robinson JJ, King ME, McKelvey WA & Williams LM** 2002 Interleukin 8 in the cervix of non-pregnant ewes. *Reproduction* **124** 409–416. (<https://doi.org/10.1530/rep.0.1240409>)
- Mitchell SE, Robinson JJ, King ME & Williams LM** 2005 Proteinase-activated receptors in ovine cervical function. *Reproduction, Fertility, and Development* **17** 693–699. (<https://doi.org/10.1071/RD05032>)
- Moghissi KS & Syner FN** 1976 Cyclic changes in the amount and sialic acid of cervical mucus. *International Journal of Fertility* **21** 246–250.
- Muchekehu RW & Quinton PM** 2010 A new role for bicarbonate secretion in cervico-uterine mucus release. *Journal of Physiology* **588** 2329–2342. (<https://doi.org/10.1113/jphysiol.2010.187237>)

- Mullins KJ & Saacke RG 1989 Study of the functional anatomy of bovine cervical mucosa with special reference to mucus secretion and sperm transport. *Anatomical Record* **225** 106–117. (<https://doi.org/10.1002/ar.1092250205>)
- Murdoch RN & White IG 1968 Activity of enzymes in the endometrium, caruncles, and uterine rinsings of progesterone-treated and naturally cycling ewes. *Australian Journal of Biological Sciences* **21** 123–131. (<https://doi.org/10.1071/B19680123>)
- Murphy EM, Murphy C, O'Meara C, Dunne G, Eivers B, Lonergan P & Fair S 2017 A comparison of semen diluents on the in vitro and in vivo fertility of liquid bull semen. *Journal of Dairy Science* **100** 1541–1554. (<https://doi.org/10.3168/jds.2016-11646>)
- Narciandi F, Lloyd AT, Chapwanya A, Farrelly CO & Meade KG 2011 Reproductive tissue-specific expression profiling and genetic variation across a 19 gene bovine beta-defensin cluster. *Immunogenetics* **63** 641–651. (<https://doi.org/10.1007/s00251-011-0551-7>)
- Narciandi F, Fernandez-Fuertes B, Khairulzaman I, Jahns H, King D, Finlay EK, Mok KH, Fair S, Lonergan P, Farrelly CO *et al.* 2016 Sperm-coating beta-defensin 126 is a dissociation-resistant dimer produced by epididymal epithelium in the bovine reproductive tract. *Biology of Reproduction* **95** 121. (<https://doi.org/10.1095/biolreprod.116.1.38719>)
- O'Hara L, Hanrahan JP, Richardson L, Donovan A, Fair S, Evans AC & Lonergan P 2010 Effect of storage duration, storage temperature, and diluent on the viability and fertility of fresh ram sperm. *Theriogenology* **73** 541–549. (<https://doi.org/10.1016/j.theriogenology.2009.10.009>)
- O'Meara CM, Hanrahan JP, Donovan A, Fair S, Rizos D, Wade M, Boland MP, Evans AC & Lonergan P 2005 Relationship between in vitro fertilisation of ewe oocytes and the fertility of ewes following cervical artificial insemination with frozen-thawed ram semen. *Theriogenology* **64** 1797–1808. (<https://doi.org/10.1016/j.theriogenology.2005.04.009>)
- O'Meara CM, Donovan A, Hanrahan JP, Duffy P, Fair S, Evans ACO & Lonergan P 2007 Resuspending ram spermatozoa in seminal plasma after cryopreservation does not improve pregnancy rate in cervically inseminated ewes. *Theriogenology* **67** 1262–1268. (<https://doi.org/10.1016/j.theriogenology.2007.01.012>)
- Paulenz H, Adnoy T & Soderquist L 2007 Comparison of fertility results after vaginal insemination using different thawing procedures and packages for frozen ram semen. *Acta Veterinaria Scandinavica* **49** 26. (<https://doi.org/10.1186/1751-0147-49-26>)
- Pini T, Leahy T, Soleilhavoup C, Tsikis G, Labas V, Combes-Soia L, Harichaux G, Rickard JP, Druart X & de Graaf SP 2016 Proteomic investigation of ram spermatozoa and the proteins conferred by seminal plasma. *Journal of Proteome Research* **15** 3700–3711. (<https://doi.org/10.1021/acs.jproteome.6b00530>)
- Pini T, Leahy T & Paul de Graaf S 2017 Seminal plasma and cryopreservation alter ram sperm surface carbohydrates and interactions with neutrophils. *Reproduction, Fertility, and Development* **30** 689–702. (<https://doi.org/10.1071/RD17251>)
- Pini T, Leahy T & de Graaf SP 2018a Sublethal sperm freezing damage: manifestations and solutions. *Theriogenology* **118** 172–181. (<https://doi.org/10.1016/j.theriogenology.2018.06.006>)
- Pini T, de Graaf SP, Druart X, Tsikis G, Labas V, Teixeira-Gomes AP, Gadella BM & Leahy T 2018b Binder of Sperm Proteins 1 and 5 have contrasting effects on the capacitation of ram spermatozoa. *Biology of Reproduction* **98** 765–775. (<https://doi.org/10.1093/biolre/i0y032>)
- Pini T, Rickard JP, Leahy T, Crossett B, Druart X & de Graaf SP 2018c Cryopreservation and egg yolk medium alter the proteome of ram spermatozoa. *Journal of Proteomics* **181** 73–82. (<https://doi.org/10.1016/j.jprote.2018.04.001>)
- Pini T, Farmer K, Druart X, Teixeira-Gomes AP, Tsikis G, Labas V, Leahy T & de Graaf SP 2018d Binder of Sperm Proteins protect ram spermatozoa from freeze-thaw damage. *Cryobiology* **82** 78–87. (<https://doi.org/10.1016/j.cryobiol.2018.04.005>)
- Pluta K, Irwin JA, Dolphin C, Richardson L, Fitzpatrick E, Gallagher ME, Reid CJ, Crowe MA, Roche JF, Lonergan P *et al.* 2011 Glycoproteins and glycosidases of the cervix during the peri-estrous period in cattle. *Journal of Animal Science* **89** 4032–4042. (<https://doi.org/10.2527/jas.2011-4187>)
- Pluta K, McGettigan PA, Reid CJ, Browne JA, Irwin JA, Tharmalingam T, Corfield A, Baird A, Loftus BJ, Evans ACO *et al.* 2012 Molecular aspects of mucin biosynthesis and mucus formation in the bovine cervix during the peri-estrous period. *Physiological Genomics* **44** 1165–1178. (<https://doi.org/10.1152/physiolgenomics.00088.2012>)
- Portal C, Gouyer V, Magnien M, Plet S, Gottrand F & Desseyn JL 2017 In vivo imaging of the Muc5b gel-forming mucin. *Scientific Reports* **7** 44591. (<https://doi.org/10.1038/srep44591>)
- Quinlivan TD 1967 Studies in Ovine Reproduction: Sperm Transport and Fertilisation in Normal and Progesterone Treated Ewes. PhD Thesis: University of Sydney.
- Richardson L, Hanrahan JP, O'Hara L, Donovan A, Fair S, O'Sullivan M, Carrington SD, Lonergan P & Evans AC 2011 Ewe breed differences in fertility after cervical AI with frozen-thawed semen and associated differences in sperm penetration and physicochemical properties of cervical mucus. *Animal Reproduction Science* **129** 37–43. (<https://doi.org/10.1016/j.anireprosci.2011.10.012>)
- Rickard JP, Hanrahan JP, Tharmalingam T, Carrington S, Lonergan P, Evans ACO & Fair S 2019 Cervical mucus sialic acid content determines the progression of thawed ram sperm through the cervix. *Reproduction* **157** 259–271. (<https://doi.org/10.1530/REP-18-0547>)
- Rickard JP, Pini T, Soleilhavoup C, Cognie J, Bathgate R, Lynch GW, Evans G, Maxwell WM, Druart X & de Graaf SP 2014 Seminal plasma aids the survival and cervical transit of epididymal ram spermatozoa. *Reproduction* **148** 469–478. (<https://doi.org/10.1530/REP-14-0285>)
- Rickard JP, Leahy T, Soleilhavoup C, Tsikis G, Labas V, Harichaux G, Lynch GW, Druart X & de Graaf SP 2015 The identification of proteomic markers of sperm freezing resilience in ram seminal plasma. *Journal of Proteomics* **126** 303–311. (<https://doi.org/10.1016/j.jprote.2015.05.017>)
- Rickard JP, Schmidt RE, Maddison JW, Bathgate R, Lynch GW, Druart X & de Graaf SP 2016 Variation in seminal plasma alters the ability of ram spermatozoa to survive cryopreservation. *Reproduction, Fertility, and Development* **28** 516–523. (<https://doi.org/10.1071/RD14123>)
- Robertson SA & Sharkey DJ 2016 Seminal fluid and fertility in women. *Fertility and Sterility* **106** 511–519. (<https://doi.org/10.1016/j.fertnstert.2016.07.1101>)
- Sakkas D, Ramalingam M, Garrido N & Barratt CL 2015 Sperm selection in natural conception: what can we learn from Mother Nature to improve assisted reproduction outcomes? *Human Reproduction Update* **21** 711–726. (<https://doi.org/10.1093/humupd/dmv042>)
- Salamon S 1977 Fertility following deposition of equal numbers of frozen-thawed ram spermatozoa by single and double insemination. *Australian Journal of Agricultural Research* **28** 477–479. (<https://doi.org/10.1071/AR9770477>)
- Salamon S & Maxwell WMC 1995 Frozen storage of ram semen .2. Causes of low fertility after cervical insemination and methods of improvement. *Animal Reproduction Science* **38** 1–36. ([https://doi.org/10.1016/0378-4320\(94\)01328-J](https://doi.org/10.1016/0378-4320(94)01328-J))
- Salamon S & Maxwell WM 2000 Storage of ram semen. *Animal Reproduction Science* **62** 77–111. ([https://doi.org/10.1016/S0378-4320\(00\)00155-X](https://doi.org/10.1016/S0378-4320(00)00155-X))
- Schjenken JE & Robertson SA 2014 Seminal fluid and immune adaptation for pregnancy—comparative biology in mammalian species. *Reproduction in Domestic Animals* **49** (Supplement 3) 27–36. (<https://doi.org/10.1111/rda.12383>)
- Sharkey DJ, Macpherson AM, Tremellen KP & Robertson SA 2007 Seminal plasma differentially regulates inflammatory cytokine gene expression in human cervical and vaginal epithelial cells. *Molecular Human Reproduction* **13** 491–501. (<https://doi.org/10.1093/molehr/gam028>)
- Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K & Robertson SA 2012 Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. *Journal of Immunology* **188** 2445–2454. (<https://doi.org/10.4049/jimmunol.1102736>)
- Shi TY, Chen G, Huang X, Yuan Y, Wu X, Wu B, Li Z, Shun F, Chen H & Shi H 2012 Effects of reactive oxygen species from activated leucocytes on human sperm motility, viability and morphology. *Andrologia* **44** 696–703. (<https://doi.org/10.1111/j.1439-0272.2011.01252.x>)
- Silber SJ, Nagy Z, Liu J, Tournaye H, Lissens W, Ferenc C, Liebaers I, Devroey P & Van Steirteghem AC 1995 The use of epididymal and testicular spermatozoa for intracytoplasmic sperm injection: the genetic implications for male infertility. *Human Reproduction* **10** 2031–2043. (<https://doi.org/10.1093/oxfordjournals.humrep.a136231>)
- Soleilhavoup C, Tsikis G, Labas V, Harichaux G, Kohne PL, Dacheux JL, Guerin Y, Gatti JL, de Graaf SP & Druart X 2014 Ram seminal plasma

- proteome and its impact on liquid preservation of spermatozoa. *Journal of Proteomics* **109** 245–260. (<https://doi.org/10.1016/j.jprot.2014.07.007>)
- Soleilhavoup C, Riou C, Tsikis G, Labas V, Harichaux G, Kohnke P, Reynaud K, de Graaf SP, Gerard N & Druart X** 2016 Proteomes of the female genital tract during the oestrous cycle. *Molecular and Cellular Proteomics* **15** 93–108. (<https://doi.org/10.1074/mcp.M115.052332>)
- Suarez SS & Wu M** 2017 Microfluidic devices for the study of sperm migration. *Molecular Human Reproduction* **23** 227–234. (<https://doi.org/10.1093/molehr/gaw039>)
- Teclé E & Gagneux P** 2015 Sugar-coated sperm: unraveling the functions of the mammalian sperm glycocalyx. *Molecular Reproduction and Development* **82** 635–650. (<https://doi.org/10.1002/mrd.22500>)
- Thomassen R & Farstad W** 2009 Artificial insemination in canids: a useful tool in breeding and conservation. *Theriogenology* **71** 190–199. (<https://doi.org/10.1016/j.theriogenology.2008.09.007>)
- Tollner TL, Yudin AI, Treece CA, Overstreet JW & Cherr GN** 2004 Macaque sperm release ESP13.2 and PSP94 during capacitation: the absence of ESP13.2 is linked to sperm-zona recognition and binding. *Molecular Reproduction and Development* **69** 325–337. (<https://doi.org/10.1002/mrd.20132>)
- Tollner TL, Venners SA, Hollox EJ, Yudin AI, Liu X, Tang G, Xing H, Kays RJ, Lau T, Overstreet JW et al.** 2011 A common mutation in the defensin DEFB126 causes impaired sperm function and subfertility. *Science Translational Medicine* **3** 92ra65. (<https://doi.org/10.1126/scitranslmed.3002289>)
- Tung CK, Ardon F, Fiore AG, Suarez SS & Wu M** 2014 Cooperative roles of biological flow and surface topography in guiding sperm migration revealed by a microfluidic model. *Lab on a Chip* **14** 1348–1356. (<https://doi.org/10.1039/c3lc51297e>)
- Tung CK, Hu L, Fiore AG, Ardon F, Hickman DG, Gilbert RO, Suarez SS & Wu M** 2015 Microgrooves and fluid flows provide preferential passageways for sperm over pathogen *Trichomonas foetus*. *PNAS* **112** 5431–5436. (<https://doi.org/10.1073/pnas.1500541112>)
- Tung CK, Lin C, Harvey B, Fiore AG, Ardon F, Wu M & Suarez SS** 2017 Fluid viscoelasticity promotes collective swimming of sperm. *Scientific Reports* **7** 3152. (<https://doi.org/10.1038/s41598-017-03341-4>)
- Verdugo P** 2012 Supramolecular dynamics of mucus. *Cold Spring Harbor Perspectives in Medicine* **2**. (<https://doi.org/10.1101/cshperspect.a009597>)
- Wulster-Radcliffe MC, Wang S & Lewis GS** 2004 Transcervical artificial insemination in sheep: effects of a new transcervical artificial insemination instrument and traversing the cervix on pregnancy and lambing rates. *Theriogenology* **62** 990–1002. (<https://doi.org/10.1016/j.theriogenology.2003.12.031>)
- Yu L, Zheng Y, Feng Y & Ma F** 2018 Role of L-selectin on leukocytes in the binding of sialic acids on sperm surface during the phagocytosis of sperm in female reproductive tract. *Medical Hypotheses* **120** 4–6. (<https://doi.org/10.1016/j.mehy.2018.08.008>)
- Yudin AI, Tollner TL, Li MW, Treece CA, Overstreet JW & Cherr GN** 2003 ESP13.2, A member of the beta-defensin family, is a macaque sperm surface-coating protein involved in the capacitation process. *Biology of Reproduction* **69** 1118–1128. (<https://doi.org/10.1095/biolreprod.103.016105>)
- Yudin AI, Generao SE, Tollner TL, Treece CA, Overstreet JW & Cherr GN** 2005 Beta-defensin 126 on the cell surface protects sperm from immunorecognition and binding of anti-sperm antibodies. *Biology of Reproduction* **73** 1243–1252. (<https://doi.org/10.1095/biolreprod.105.042432>)

Received 20 November 2018

First decision 4 February 2019

Revised manuscript received 8 March 2019

Accepted 28 March 2019