REPRODUCTION
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Seminal plasma aids the survival and cervical transit of epididymal ram spermatozoa

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Abstract

Seminal plasma purportedly plays a critical role in reproduction, but epididymal spermatozoa are capable of fertilisation following deposition in the uterus, calling into question the biological requirement of this substance. Through a combination of direct observation of spermatozoa *in utero* using probe-based Confocal Laser Endomicroscopy, *in vivo* assessment of sperm fertility and *in vitro* analysis of various sperm functional parameters, this study investigated the role of seminal plasma in spermatozoa transit through the cervix of the ewe. Following deposition in the cervical os, epididymal spermatozoa previously exposed to seminal plasma displayed an enhanced ability to traverse the cervix as evidenced by both significantly higher pregnancy rates and numbers of spermatozoa observed at the utero-tubal junction when compared with epididymal spermatozoa not previously exposed to seminal plasma. The beneficial effect of seminal plasma on sperm transport was clearly localised to transit through the cervix as pregnancy rates of spermatozoa deposited directly into the uterus were unaffected by exposure to seminal plasma. This phenomenon was not explained by changes to sperm motion characteristics, as seminal plasma had no effect on the motility, kinematic parameters or mitochondrial membrane potential of spermatozoa. Rather, *in vitro* testing revealed that seminal plasma improved the ability of epididymal spermatozoa to penetrate cervical mucus recovered from ewes in oestrus. These results demonstrate that the survival and transport of ram spermatozoa through the cervix of the ewe is not linked to their motility or velocity but rather the presence of some cervical penetration trait conferred by exposure to seminal plasma.

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Introduction

During ejaculation, spermatozoa from the tail of the epididymis mix with secretions of the major accessory sex glands and are deposited into the female tract together as semen (Mann 1964). These secretions (in addition to relatively minor contributions from the testes and epididymides) are referred to as seminal plasma and comprise a complex mixture of inorganic ions, citric acid, sugars, organic salts, prostaglandins and particularly proteins (Mann 1964). Initially thought to act simply as a medium for efficient transfer of spermatozoa to the female, seminal plasma has since been implicated in a variety of functional roles important to successful reproduction (Leahy & Gadella 2011). Sperm capacitation (Manjunath & Therien 2002, Maxwell *et al*. 2007), sperm storage in the female tract (Talevi & Gualtieri 2010) and conditioning of the female immune system (Robertson 2005) are each influenced by seminal plasma, suggesting that this substance (and the presence of the accessory sex glands) is necessary for normal sperm function and fertility. However, other evidence suggests that this is not necessarily the case. Epididymal spermatozoa are fertile when used for ICSI (human: (Silber *et al*. 1995), IVF (Songsasen & Leibo 1998, Stout *et al*. 2012) and even following intrauterine insemination (Fournier-Delpech *et al*. 1979, Hori *et al*. 2005, Ehling *et al*. 2006, Monteiro *et al*. 2011). Moreover, when inseminated *in vivo*, epididymal spermatozoa have usually achieved pregnancy rates similar to those of ejaculated spermatozoa (Fournier-Delpech *et al*. 1979, Ehling *et al*. 2006, Monteiro *et al*. 2011). Such normal fertility of spermatozoa which have never come into contact with seminal plasma raises the question of whether exposure to seminal plasma from the accessory sex glands is a biological requirement vital for the *in vivo* function and fertility of spermatozoa or whether this substance is largely superfluous to reproductive success.

Reports exist of a beneficial effect of seminal plasma on the ability of liquid stored (López-Pérez & Pérez-Clariget 2012) and cryopreserved (Maxwell *et al*. 1999)

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ram spermatozoa to navigate the tortuous ovine cervix and improve pregnancy rates following intracervical artificial insemination (AI). Similarly, through the use of sperm penetration tests, seminal plasma and its individual proteins have been shown to enhance the ability of human (Overstreet *et al*. 1980), ram (Maxwell *et al*. 1999), buffalo (Arangasamy *et al*. 2005) and macaque (Tollner *et al*. 2008) spermatozoa to migrate through cervical mucus. While not definitive, these results suggest that seminal plasma may play a role in the migration of ram spermatozoa through the cervix. However, given that no studies exist reporting the fertility of epididymal spermatozoa deposited before the highly selective barrier of the ewe's cervix, this hypothesis remains untested. As such, the aim of the current study was to examine the effect of seminal plasma on the transit of epididymal ram spermatozoa through the cervix and whether potential effects were mediated by alteration of objectively measurable sperm parameters.

Materials and methods

Experimental design

Procedures herein were approved by the University of Sydney's Animal Ethics Committee. Unless otherwise stated, all chemicals were supplied by Sigma–Aldrich. Three experiments were conducted to investigate the role of seminal plasma in the transit of spermatozoa through the cervix of the ewe. In the first experiment, ejaculated spermatozoa, collected via artificial vagina, and epididymal spermatozoa, harvested from the same ram, were deposited directly into the cervical os following fluorescent staining. Probe-based Confocal Laser Endomicroscopy (pCLE) was used to count *in utero* the number of spermatozoa within the genital tract at the utero-tubal junction (UTJ) over a 2 min period. In the second experiment, epididymal spermatozoa harvested from the testes of culled rams, epididymal spermatozoa exposed to seminal plasma and ejaculated spermatozoa were deposited directly into the cervical os or directly into the uterine horns of ewes via laparoscopy. Cervical transit ability of each treatment was assessed by successful fertilisation as measured by presence of a foetus at ultrasound on day 60 after insemination and wet/dry assessment 2 weeks after lambing. In the final experiment *in vitro*, the functions of epididymal spermatozoa, epididymal spermatozoa exposed to seminal plasma and ejaculated spermatozoa were compared. Spermatozoa were assessed for motility, capacitation status, mitochondrial membrane potential and their ability to penetrate cervical mucus immediately after collection and over a 6 h incubation period.

Animals

Rams (3-years old; body condition score 3–4) were kept on a chaff-based diet (oaten:lucerne chaff, 1:1) supplemented with lupin grain in an animal house either at the Faculty of Veterinary Science, University of Sydney, Camperdown, NSW, Australia ($n=3$; *in vitro* assessment), or at the Department of Physiology of Reproduction and Behavior, National Institute of Agronomic Research (INRA) Nouzilly, France ($n=3$; *in utero* assessment). Rams ($n=3$; mature; body condition score 3–4) and androgenised wethers ($n=12$; <1-year old; body condition score 3–4) used during the *in vivo* fertility trial were kept on a pasture-based diet at 'Arthursleigh' (Marulan, NSW, Australia). The *in vivo* fertility trial was conducted during the breeding season (April, 2013) at 'Arthursleigh'. Mature Merino ewes ($n=303$; 2–4 years old; body condition score 2–3) used for insemination were kept on a pasture-based diet supplemented with lupin grain, while mature Romanov ewes ($n=19$; 2–4 years old, body condition score 2–3) were kept on a chaff-based diet supplemented with lupin grain in an animal house at the Station of Physiology of Reproduction and Behavior, National Institute of Agronomic Research (INRA) Nouzilly, France.

Collection and preparation of seminal plasma

Seminal plasma was obtained from several ejaculates (collected during the breeding season in 2013) from Merino rams ($n=3$). Following collection (artificial vagina), ejaculates were immediately assessed for wave motion (scored on a scale of 0–5) and appearance (thick and creamy, milky or watery). Only ejaculates with a wave motion score of three or more, a thick and creamy appearance and if free of blood and urine contamination were accepted for the experiment. Semen was then centrifuged at 16 000 ***g*** Model 1–14 Centrifuge; Sigma–Aldrich), once for 30 min, the supernatant aspirated and spun for a further 30 min at 16 000 ***g*** to remove any remaining spermatozoa and cell debris. Seminal plasma samples were then separated into aliquots and stored at −80 °C. Individual aliquots were thawed on ice and vortexed as needed.

Direct observation of spermatozoa in utero *using pCLE*

Ejaculated spermatozoa (EJAC) were collected from Romanov rams ($n=3$ rams$\times$1 ejaculate) using an artificial vagina in the presence of a teaser ewe. Ejaculates were immediately assessed for wave motion (data not shown) and pooled. Epididymal spermatozoa (EP) were obtained from the testes of Romanov rams ($n=3$; the same rams from which ejaculated samples had previously been collected) at slaughter via microperfusion (Dacheux 1980) using a warmed (37 °C) PBS solution. Both ejaculated and epididymal samples were immediately assessed for wave motion and concentration determined using a spectrophotometer (Evans & Maxwell 1987). Samples were then slowly diluted to 3×10^9 spermatozoa/ml with a skim-milk powder extender (10%; w/v; Régilait; Brest Cedex, France).

Samples were then supplemented with Octadecyl rhodamine B chloride (R18, O 246; final concentration 300 μM; Molecular Probes, Eugene, OR, USA) and Mito-Tracker Green FM (M-7514; final concentration 20 μM; Molecular Probes) and incubated at 37 °C for 5 min. The use of R18 and Mitotracker Green FM for pCLE cell imaging was validated by Druart *et al*. (2009), using flow cytometry.

Before cervical insemination, the motility of both EJAC and EP samples was assessed using computer-assisted sperm analysis (CASA; Hamilton-Thorne, Beverly, MA, USA) to ensure that all treatments had similar motility and velocity characteristics.

Mature Romanov ewes were synchronised for oestrus using a combination of progestagen-impregnated intravaginal sponges (20 mg fluorogestone acetate; Centravet, Dinan, France) for 12 days, followed by 200 IU of i.m. pregnant mare serum gonadotrophin (PMSG; Centravet). Cervical insemination was performed as per industry standards (Colas 1984) on 19 ewes (n=11 EJAC; n=8 EP) at 55 h after sponge removal with an insemination dose of 750×10^6 total spermatozoa (250 µl sample loaded into 0.25 ml straws; IMV Technologies, L'Aigle, France).

Observation of spermatozoa *in utero* was conducted by pCLE (Cellvizio DualBand; Mauna Kea Technologies, Paris, France; www.cellviziodualband.com) at 7–9 h after cervical insemination. The Cellvizio DualBand system comprises a dual-band laser-scanning unit (λ=500–630 nm), which sequentially scans each optic fibre within the miniaturised fibre-based optical microprobe (for this study, an S1500 with 1.5 mm diameter, 3.3 µm lateral resolution and 500×600 µm field of view was used) constructing a real-time (12 frames/s) image (Fig. 1A). Operation of the Cellvizio DualBand to visualise spermatozoa *in situ* was conducted as described previously by Druart *et al.* (2009) with some minor modifications. Briefly, ewes were placed under general anaesthesia and their genital tract, including uterine horns, oviducts and ovaries were exteriorised after laparotomy. The pCLE microprobe was inserted into the tip of the uterine horn following electrocautery of the uterine wall (Fig. 1B illustrates probe insertion and placement). Image acquisition was standardised and video sequences of 2 min duration were recorded in both the left and right horns of each ewe. Quantification of spermatozoa at each UTJ was performed by manual counting using the software provided with the imagery system (Druart *et al.* 2009). Figure 1C displays an image of ram spermatozoa visualised at the UTJ of a ewe's genital tract using pCLE.

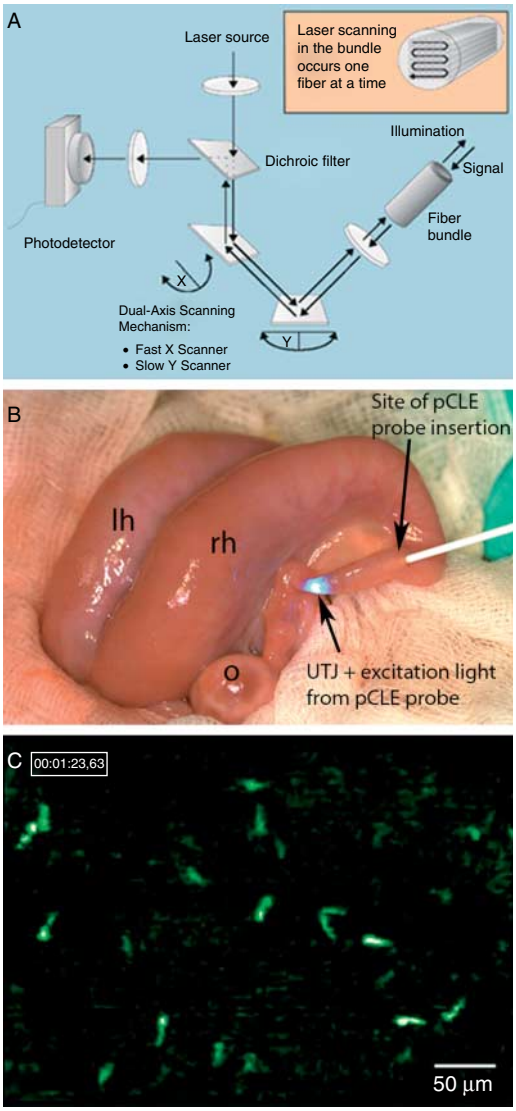

Figure 1 Probe-based Confocal Laser Endomicroscopy (pCLE) for imaging of spermatozoa *in utero*. (A) pCLE was conducted using a Cellvizio DualBand system, which sequentially scans each optic fibre using a dual-axis scanning mechanism, illuminating each fibre within the optical microprobe one at a time. The signal detected by the microprobe is returned to a photodetector for analysis and image reconstruction. (B) Site of microprobe insertion into the tip of the uterine horn for imaging of the utero-tubal junction (UTJ). lh, left uterine horn; rh, right uterine horn; o, ovary. (C) ram spermatozoa stained with R18 and Mitotracker Green observed using pCLE at the UTJ at 7–9 h following intracervical artificial insemination.

Assessment of in vivo *sperm fertility following AI*

Ejaculated spermatozoa (EJAC) were collected from mature Merino rams (n=3 rams×3 ejaculates) using an artificial vagina, in the presence of a teaser ewe. Ejaculates were immediately assessed for wave motion (data not shown) and slowly diluted 1:2 (semen:diluent, v/v) with warmed (37 °C) ultra heat treated (UHT) milk. Epididymal spermatozoa (EP) were obtained from the testes of Merino rams (n=3) at slaughter via microperfusion (Dacheux 1980) using a warmed (37 °C) Tris–citrate–fructose solution (Evans & Maxwell 1987) and subsequently centrifuged (800 ***g***; 10 min) to concentrate the sample and remove any contaminants. Sperm concentration was determined for all samples using a haemocytometer as described by Evans & Maxwell (1987). Epididymal samples were then split with one half being undiluted epididymal spermatozoa (EP) and the remainder undergoing a 1:1 dilution with seminal plasma previously collected from one of the same three rams that gave an ejaculated sample (EP+SP). EJAC, EP and EP+SP samples were then further diluted to 500×10^6 spermatozoa/ml with UHT milk and maintained at 30 °C until cervical or laparoscopic intrauterine AI. Samples destined for laparoscopic intrauterine AI were diluted 1:1 to 250×10^6 spermatozoa/ml with warmed (37 °C) UHT milk immediately before insemination. The motility of spermatozoa was subjectively assessed in all samples to ensure that all treatments had similar sperm function characteristics before insemination (data not shown).

Mature Merino ewes (n=303) were synchronised for oestrus using a combination of intravaginal progestagen-impregnated

sponges (30 mg Fluogestone Acetate; Bioniche Animal Health Australasia, Armidale, NSW, Australia) for 12 days followed by an i.m. injection of 400 IU PMSG (1 ml Pregnecol; Bioniche Animal Health Australasia) at sponge removal. Androgenised wethers (400 mg administered at sponge insertion and a further 150 mg at sponge removal; 50 mg/ml Testosterone propionate; Jurox, Rutherford, NSW, Australia) were introduced to the ewes at sponge removal at a proportion of 1:30 to encourage the onset of oestrus and ovulation. All ewes were fasted 24 h before insemination and were left undisturbed for ~3 h following insemination.

Ewes were inseminated by either intracervical AI ($n=164$) at 53 h after sponge removal with 100×10^6 motile EP ($n=46$), EP+SP ($n=41$) or EJAC ($n=77$) spermatozoa/ewe (insemination volume of 0.2 ml) or laparoscopic intrauterine AI ($N=139$) at 55 h after sponge removal with 25×10^6 motile EP ($n=53$), EP+SP ($n=46$) or EJAC ($n=40$) spermatozoa/ewe (insemination volume of 0.1 ml) using standard industry techniques (Evans & Maxwell 1987). Those ewes undergoing laparoscopic insemination were given pre-operative pain relief and sedation via an i.m. injection of Ketamil (150 mg; Troy Ilium, Glendenning, NSW, Australia) and Acetylpromazine (ACP2; 2 mg; Delvet, Seven Hills, NSW, Australia), followed by a s.c. injection of local anaesthetic (2 ml of 2% Lignocaine; Mavlab, Logan City, QLD, Australia) to the site of abdominal puncture.

Pregnancy status of inseminated ewes was determined on day 60 after insemination using real-time cutaneous ultrasound to detect the presence of foetuses. Approximately 2 weeks following the start of lambing, all ewes previously designated as pregnant by ultrasound were examined for evidence of parturition, mammary gland development and presence of suckling to determine the number of ewes which either gave birth or experienced foetal loss (Evans & Maxwell 1987).

In vitro *assessment of sperm function*

Ejaculated spermatozoa (EJAC) were collected from mature Merino rams ($n=3$ rams$\times6$ ejaculates) using an artificial vagina, in the presence of a teaser ewe. Ejaculates were immediately assessed for wave motion (data not shown) and slowly diluted 1:1 (semen:diluent, v/v) with a Tris–citrate–fructose diluent (Evans & Maxwell 1987). Testes from mature Merino rams were obtained at slaughter from Southern Meats abattoir (Goulburn, NSW, Australia) and transported at 5 °C to the University of Sydney, where they remained chilled at 5 °C for 24 h. Epididymal spermatozoa (EP) were collected via microperfusion (Dacheux 1980) using a warmed (37 °C) Tris–citrate–fructose solution. Sperm concentration was determined for all samples using a haemocytometer as described by Evans & Maxwell (1987). Epididymal samples were then split with one half being undiluted epididymal spermatozoa (EP) and the remainder undergoing a 1:1 dilution with seminal plasma previously collected from the same ram which gave an ejaculated sample (EP+SP). EJAC, EP and EP+SP samples were then further diluted to 50×10^6 spermatozoa/ml with UHT milk and incubated in a water bath at 37 °C. Aliquots were taken at 0, 3 and 6 h after collection and diluted 1:1 (semen: diluent, v/v) with Androhep (AH; Minitube Australia, Smythes Creek, VIC, Australia) to 25×10^6 spermatozoa/ml for assessment.

EJAC, EP and EP+SP were assessed for motility, mitochondrial membrane potential and ability to penetrate cervical mucus. Motility was assessed using computer-assisted sperm analysis (HT CASA IVOS II (Animal Breeder) Version 1.4; Hamilton-Thorne). Semen samples (5.5 µl) were placed on pre-warmed slides (Cell Vu, Millenium Sciences Corp., New York, NY, USA) and immediately transferred to the CASA. Motility and kinematic characteristics including progressive motility, average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN) were determined by the assessment of several microscopic fields (200–300 cells/sample) using factory CASA (ram) settings.

The mitochondrial membrane potential of spermatozoa was assessed by staining spermatozoa with the mitochondrial probe 5,5′, 6,6′-tetra-chloro-1, 1′, 3, 3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1; final concentration 4 ng/ml: Molecular Probes). Following incubation for 10–15 min at 37 °C, an aliquot (10 µl) was loaded onto a clean, pre-warmed slide and viewed under an Olympus BHS fluorescent microscope (200× magnification, 200 cells/sample) comprising a 520–550 nm band pass filter and a supplementary 515 nm exciter filter. Emissions were observed through a 565 nm dichroic mirror with an additional 610 nm barrier filter (Garner & Thomas 1999). A number of spermatozoa observed with fluorescent orange mid pieces (aggregates) were considered to have high membrane potential while spermatozoa exhibiting green fluorescence (monomers) were of low membrane potential.

The ability of spermatozoa to migrate through cervical mucus *in vitro* was assessed using a cervical migration test (Kremer 1965). Briefly, cervical mucus was collected from synchronised Merino ewes in oestrus during the breeding season, centrifuged (1300 ***g***; 15 min) to remove contaminants and stored at −80 °C in 500 µl aliquots. Aliquots were thawed on ice as needed, slowly warmed to 37 °C and loaded into glass capillary tubes ($0.3\times0.3\times100$ mm; Microslides, Mountain Lakes, NJ, USA). Spermatozoa were incubated with a DNA-specific stain for 10 min (final working concentration 40 µg/µl; IDENT; Hamilton-Thorne) at 37 °C, then transferred to a polyethylene capsule (BEEM; ProSciTech, Thuringawa, QLD, Australia). The mucus-filled capillary tube was sealed at one end with CristaSeal (Hawksley, London, UK), immersed in the stained sperm sample and co-incubated for 1 h at 37 °C. Following incubation, the distance reached by the furthest spermatozoon (Vanguard distance) was recorded under an Olympus BHS fluorescent microscope (400× magnification) comprising a 270–380 nm band pass filter. Emissions were observed through a 380 nm dichroic mirror (Richardson *et al.* 2011).

Statistical analysis

Data recorded from all experiments were analysed using a restricted maximum likelihood model (REML) in Genstat (Version 15, VSN International, Hemel Hempstead, UK). Data collected during observation of spermatozoa *in utero* had sperm treatment (EP or EJAC) and horn (ipsilateral or contralateral) specified as fixed effects and ewe number as the

random effect. Data collected on the motility of treatments before insemination were assessed using a two-tailed, paired *t*-test. Data collected during the *in vivo* assessment of sperm fertility had AI technique (cervical or intrauterine laparoscopic) and sperm treatment (EP, EP+SP and EJAC) specified as fixed effects, while day of insemination and sperm treatment (EP, EP+SP and EJAC) were specified as random effects. Data collected during assessment of sperm function *in vitro* had ram, repetition and sperm treatment (EP, EP+SP and EJAC) as fixed effects, while the time of assessment (PF, 0, 3, 6 h) and sperm treatment (EP, EP+SP and EJAC) were specified as random effects. Correlations were conducted in Genstat (Version 15, VSN International) between motility kinematic parameters and mucus penetration ability of each treatment over the 6 h incubation period. For all experiments, means were reported with ±S.E.M. and $P<0.05$ was considered statistically significant. Differences were determined by least significant differences.

Results

Direct observation of spermatozoa in utero *using pCLE*

There was no significant difference between the progressive motility and velocity of EP and EJAC spermatozoa following fluorescent labelling or before insemination ($P>0.05$; Fig. 2). Fluorescence microscopy *in utero* revealed significantly higher numbers of spermatozoa at the UTJ of ewes inseminated with EJAC spermatozoa when compared with those ewes inseminated with EP spermatozoa ($P<0.05$; Fig. 2). There was no significant difference between treatments in the number of spermatozoa observed at the ipsilateral or contralateral UTJs ($P>0.05$; data not shown).

Assessment of in vivo *sperm fertility following AI*

There was no difference in the pre-insemination motility of spermatozoa in each treatment group (data not shown). In each treatment group, spermatozoa were inseminated with a motility score of 70–80%. Pregnancy rates at day 60 for each sperm and insemination treatment group combination are shown in Table 1. There was no significant difference between treatment groups when spermatozoa were deposited directly into the uterus by laparoscopic AI (Table 1; $P>0.05$). However, when spermatozoa were deposited in the cervical canal, pregnancy rates were significantly higher for treatment groups containing seminal plasma (EJAC and EP+SP) than for those that did not (EP; $P<0.05$).

When treatments were compared between insemination methods, pregnancy rates were higher for EJAC inseminated laparoscopically compared with EJAC inseminated cervically ($P<0.05$). Similarly, EP inseminated laparoscopically had a significantly higher pregnancy rate than EP inseminated cervically ($P<0.05$). However, there was no significant difference in the pregnancy rate between ewes inseminated cervically or laparoscopically with EP+SP ($P>0.05$). There was no significant difference between pregnancy rate and lambing rate for any treatment, with only a single ewe aborting her foetus during gestation ($P>0.05$; Table 1).

Assessment of in vitro *sperm function*

Motility characteristics

As expected, total motility decreased over time ($P<0.001$), but there were no significant differences in total motility between treatment groups ($P>0.05$; Fig. 3a). When spermatozoa from each treatment group were incubated at 37 °C for over 6 h, EJAC had significantly higher progressive motility (Fig. 3b), VAP (Fig. 3c), VSL (Fig. 3d), BCF (Fig. 3e), STR (Fig. 3f) and LIN (Fig. 3f) than EP or EP+SP, and this was maintained over the entire incubation period (0–6 h; $P<0.05$). There was no significant difference between EP and EP+SP for the above-mentioned kinematic parameters ($P>0.05$). At 0 h

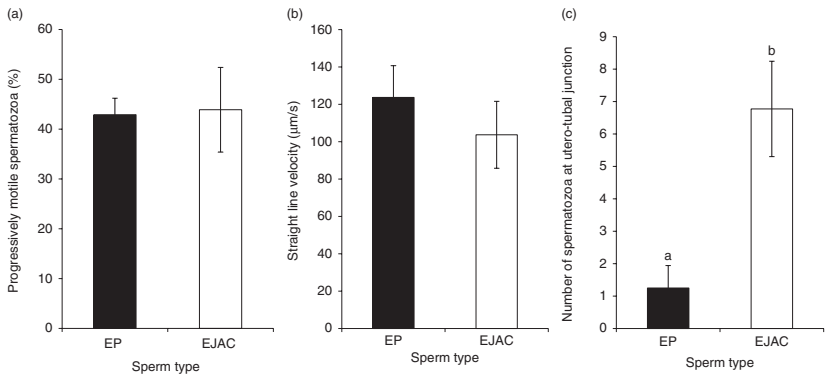

Figure 2 Average progressive motility (a) and straight line velocity (b) of epididymal (EP; black columns) and ejaculated (EJAC; white columns) spermatozoa before intracervical insemination. (c) The mean number of epididymal (EP; black column) and ejaculated (EJAC; white column) spermatozoa observed over a 2-min period at the utero-tubal junction of ewes at 7–9 h after intracervical insemination. Spermatozoa were labelled with R18 and Mitotracker Green before insemination and visualised *in situ* using pCLE. Columns without common superscripts differ significantly within each panel ($P<0.05$).

Table 1 Pregnancy and lambing rates after intracervical or laparoscopic intrauterine artificial insemination of synchronised ewes with epididymal spermatozoa (EP), epididymal spermatozoa exposed to seminal plasma (EP+SP) or ejaculated spermatozoa (EJAC).

	Cervical AI			Laparoscopic intrauterine AI		
Trt	Ewes inseminated	Ewes pregnant at day 60 (%)[a]	Lambed (%)[b]	Ewes inseminated	Ewes pregnant at day 60 (%)[a]	Lambed (%)[b]
EP	41	3 (7.3)*	3 (7.3)*	46	23 (50.0)[‡]	22 (47.8)[‡]
EP+SP	46	17 (37.0)[†,‡]	17 (37.0)[†,‡]	53	31 (58.5)[‡]	31 (58.5)[‡]
EJAC	77	16 (20.8)[†]	16 (20.8)[†]	40	17 (42.5)[†,‡]	17 (42.5)[†,‡]

Within column, values without common superscripts differ significantly ($P<0.05$).
[a]Pregnancy was determined by real-time cutaneous ultrasound. [b]Lambing data were collected at ~2 weeks after lambing commenced and determined by examination of ewes for signs of parturition and lactation (Evans & Maxwell 1987).

only, EJAC had significantly higher VCL ($P<0.05$; Fig. 3d) than EP and EP+SP and, surprisingly, at 6 h, EP had significantly higher VCL than EP+SP and EJAC ($P<0.05$; Fig. 3d). There was no significant difference in VCL between treatments at 3 h ($P>0.05$). Similarly, there was no significant difference in ALH between treatments over the incubation period ($P>0.05$; data not shown).

Mitochondrial membrane potential

Sperm type did not affect the percentage of spermatozoa with a high mitochondrial membrane potential at any measured time point over 6 h of incubation at 37 °C ($P>0.05$; data not shown).

Cervical migration test

The ability of spermatozoa to migrate through natural cervical mucus, as measured by the distance travelled by the vanguard spermatozoon, was affected by treatment only when spermatozoa had already been incubated at 37 °C ($P<0.05$; Fig. 4; data presented pooled over time points). The vanguard spermatozoon travelled significantly further for EP+SP than for the EP and EJAC treatments ($P<0.05$; Fig. 4), the distance for the latter two treatments being similar ($P>0.05$).

Correlation of mucus-penetrating ability with motility kinematic parameters

Table 2 displays correlations between the distances reached by the vanguard spermatozoon and motility parameters (progressive motility, VAP, VSL, VCL, ALH, BCF, LIN and STR). Very weak correlations were found between the kinematic parameters of the spermatozoa and the distance reached by the vanguard spermatozoon and all were found to be non-significant ($P>0.05$; Table 2).

Discussion

This study demonstrated that seminal plasma enhances the transit of ram spermatozoa through the cervix of the ewe. The addition of seminal plasma to spermatozoa, either through manual supplementation or by natural exposure at ejaculation, significantly increased both the number of spermatozoa observed at the UTJ and pregnancy rate following insemination within the cervical os. By contrast, exposure to seminal plasma had no effect on pregnancy rate when spermatozoa were deposited directly into the uterus. This demonstrated effect of seminal plasma on sperm transport is localised to the site of the cervix. Further analysis of the *in vitro* characteristics of spermatozoa exposed to seminal plasma showed that it enhances their ability to penetrate cervical mucus despite unchanged motility characteristics, suggesting that cervical transit in the female reproductive tract is not linked to the motility or velocity of

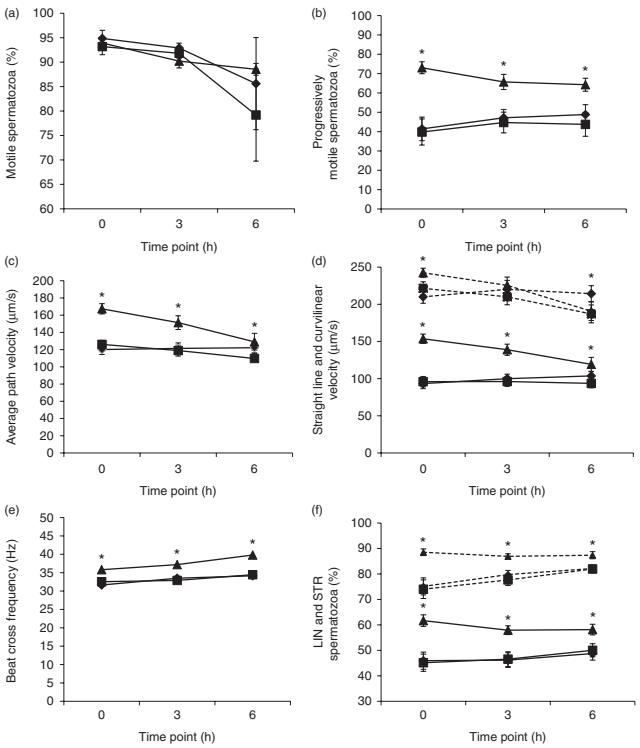

Figure 3 Average motility percentage (a), progressive motility percentage (b), average path velocity (c), straight line velocity (solid line; d), curvilinear velocity (dotted line; d), BCF (e), LIN (solid line; f) and STR (dotted line; f) determined by CASA for epididymal spermatozoa (EP; filled diamond), epididymal spermatozoa exposed to seminal plasma (EP+SP; filled square) and ejaculated spermatozoa (EJAC; filled triangle) at 0, 3 and 6 h after incubation at 37 °C. Data are actual means±S.E.M. *Indicates that treatments within each time point are significantly different ($P<0.05$).

spermatozoa but rather an unknown cervical penetration trait conferred by exposure to seminal plasma.

To date, the only other studies which have specifically investigated the role of seminal plasma on the cervical transit of spermatozoa have been on frozen–thawed ejaculated ram spermatozoa, rather than those sourced from the epididymis. Maxwell *et al.* (1999) demonstrated that frozen–thawed ram spermatozoa were able to recover their ability to traverse the cervix and fertilise ewes at a synchronised oestrus, when supplemented with seminal plasma after thawing. This result has been inconsistently achieved in similar experiments (Leahy *et al.* 2010), leading some authors to suggest that variation in the composition of seminal plasma may be responsible for the differences observed between each study (Rickard *et al.* 2014). While the result of Maxwell *et al.* (1999) was achieved with a markedly different sperm type to that used in this study (namely, a heavily processed cell that had previously been exposed to seminal plasma), it still supports the findings presented here that seminal plasma can dramatically alter the ability of spermatozoa to traverse the ewe's cervix. In terms of epididymal spermatozoa, it is remarkable that their ability to transit the cervix has not been described previously in sheep and relatively little is known in other species. Nonetheless, what little information is available supports the findings of this study that transit of epididymal spermatozoa through the cervix is in some way compromised compared with ejaculated spermatozoa. For example, in the dog, poor pregnancy rates were achieved following intravaginal inseminations with epididymal spermatozoa (Thomassen & Farstad 2009). It would be interesting to duplicate the present experiment in other vaginal depositors, such as the cow, to determine whether the effect of seminal plasma on cervical transit is consistent across species with similar mating strategies.

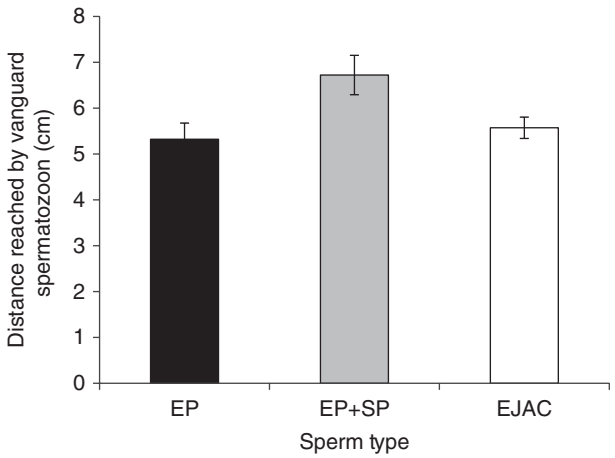

Figure 4 Distance reached by the vanguard spermatozoon of epididymal spermatozoa (EP; black column), epididymal spermatozoa exposed to seminal plasma (EP+SP; grey column) and ejaculated spermatozoa (EJAC; white column). Data are pooled over 0, 3 and 6 h ± S.E.M. Columns without common superscripts differ significantly ($P<0.05$).

Table 2 Correlation coefficient between progressive motility, average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), beat cross frequency (BCF), amplitude head displacement (ALH), linearity (LIN), straightness (STR) of spermatozoa and the distance travelled by the vanguard spermatozoon through oestrous cervical mucus.

Kinematic parameters	Correlation coefficient (r) to vanguard distance
Progressive motility	0.03 ($P=0.78$)
VAP	0.04 ($P=0.73$)
VCL	0.10 ($P=0.38$)
VSL	0.01 ($P=0.93$)
ALH	0.18 ($P=0.12$)
BCF	−0.22 ($P=0.06$)
LIN	−0.04 ($P=0.73$)
STR	−0.05 ($P=0.68$)

The fertility of epididymal spermatozoa deposited directly into the uterus is much better described. Results in the current study are similar to those achieved by Fournier-Delpech *et al.* (1979) who found no significant difference between pregnancy rates of ejaculated and epididymal ram spermatozoa following laparoscopic intrauterine insemination. Similarly, Ehling *et al.* (2006) and Álvarez *et al.* (2012) achieved pregnancy rates as high as 87 and 55.8% respectively, when frozen–thawed epididymal ram spermatozoa were inseminated by intrauterine laparoscopic AI.

One limitation of this study was the use of epididymal and ejaculated spermatozoa from different rams as part of the *in vivo* fertility and *in vitro* experiments. Ideally, as occurred in the *in utero* imaging trial, epididymal and ejaculated spermatozoa would have been obtained from the same ram to eliminate any inter-male variation affecting the results of the trial. Unfortunately, this was not possible for logistical reasons. However, inter-male variation was accounted for through supplementation of epididymal spermatozoa with seminal plasma (the EP+SP treatment) in lieu of the 'seminal plasma treatment' consisting simply of ejaculated spermatozoa from a different ram. Additionally, the seminal plasma used for the supplementation of epididymal spermatozoa was obtained from the same rams as those used for the ejaculated sperm treatments. Nonetheless, variation between the males used for the ejaculated and epididymal sperm treatments could explain the differences in fertility observed between the two types of spermatozoa when those from the epididymis were exposed to seminal plasma. Though not statistically significant, the pregnancy rates obtained for ejaculated spermatozoa were lower than that of the comparable epididymal+seminal plasma treatment and certainly lower than those reported for commercial AI programmes (Evans & Maxwell 1987); though similar to

pregnancy rates achieved in previous trials on the same property (Hollinshead *et al*. 2003, El-Hajj Ghaoui *et al*. 2007, Beilby *et al*. 2009). While identical numbers of motile spermatozoa were inseminated for each of these treatment groups, it is possible that some unknown male effect may have been at play. However, this is of little ultimate consequence, given the fact that testing of the primary hypothesis in both the *in vitro* and *in vivo* experiments was fulfilled by comparison between epididymal spermatozoa with and without exposure to seminal plasma, i.e. without the influence of inter-male variation between treatments that were or were not exposed to seminal plasma.

In the current study, the presence or absence of seminal plasma had no effect on the motility of epididymal spermatozoa immediately after collection or when maintained at 37 °C for up to 6 h. These results agree with those of Graham (1994) who only saw a beneficial effect of ram seminal plasma on the motility of epididymal spermatozoa after thawing. Numerous studies have documented the protective effect of seminal plasma during cryopreservation, particularly in the ram and bull (Leahy & de Graaf 2012). However, the non-significant effect of seminal plasma on epididymal sperm motility observed in the current study suggests that seminal plasma acts to ameliorate handling-induced stressors caused during freezing rather than stimulate or enhance sperm function (at least under more 'natural' conditions) through some form of physiological mechanism. By contrast, the results from the current study and that of Graham (1994) differ from Dott *et al*. (1979) who showed supplementation of epididymal ram spermatozoa with seminal plasma had first a stimulatory then a detrimental effect on motility following incubation for 22 h at 30 °C (Dott *et al*. 1979). These differing results could be attributed to variation in the presence or absence of critical components within seminal plasma and highlight the potential negative effect of prolonged exposure to seminal plasma on sperm function *in vitro*.

Overall, the ability of epididymal spermatozoa to penetrate cervical mucus was significantly improved with exposure to seminal plasma, despite having relatively similar motility and velocity characteristics. Maxwell *et al*. (1999) also showed that cryopreserved ejaculated ram spermatozoa thawed and resuspended in the presence of 30% (v/v) seminal plasma travelled significantly further in natural ovine mucus obtained from ewes in oestrus, than frozen–thawed spermatozoa without seminal plasma. The lack of a significant correlation between the ability of epididymal spermatozoa to penetrate cervical mucus and its kinematic motility parameters suggests that in the current study, the mucus-penetrating ability of spermatozoa was not influenced by its motility or velocity but rather by some unknown means conferred by exposure to seminal plasma. This contradicts published reports in the ram (Suttiyotin *et al*. 1992, 1995, Robayo *et al*. 2008) and human (Keel & Webster 1988), who linked increased mucus penetration with increased velocity and motility. However, in the latter study, 10–15% of patients with otherwise normal semen parameters demonstrated poor mucus-penetrating ability, suggesting that mucus penetration tests provide information about sperm function not attainable by motility analysis alone (Keel & Webster 1988). The fact that artificial mucus was used in several of the above-mentioned papers instead of natural mucus (as was used in the current study) could also explain some of the discrepancies between trials.

This study has shown that seminal plasma enhances the ability of spermatozoa to penetrate cervical mucus *in vitro* and traverse the cervix *in vivo*. In the current study, this was not attributed to changes in the motility or velocity of epididymal spermatozoa. The means by which seminal plasma confers this effect is yet to be established in sheep, but results from other species have demonstrated that individual seminal plasma proteins increase sperm penetration of cervical mucus (heparin- and gelatine-binding proteins in buffalos (Arangasamy *et al*. 2005) and glycoprotein β defensin 126 in macaques (Tollner *et al*. 2008)) possibly through modification of the negative net charge of the sperm membrane (Tollner *et al*. 2008). Clearly, considerable further research is required to investigate individual proteins within ram seminal plasma that may promote mucus penetration, not to mention the nature of the sperm–mucus interaction itself.

In conclusion, the current study strongly suggests that seminal plasma plays a vital role in the normal survival and transit of spermatozoa through the cervix of the ewe. Despite having similar motility characteristics, epididymal spermatozoa not exposed to seminal plasma reached the UTJ in fewer numbers, displayed a reduced ability to penetrate cervical mucus and resulted in lower pregnancy rates (when forced to navigate the cervix following intracervical AI) compared with ejaculated spermatozoa or epididymal spermatozoa supplemented with seminal plasma. From these results, it is clear that seminal plasma did not confer this ability through alteration of sperm motility or velocity but rather by facilitating increased cervical mucus penetration of spermatozoa through some unknown means. Further research is warranted to identify the components within seminal plasma which are responsible for assisting the transit of spermatozoa through the cervix and the mechanisms by which they act.

Declaration of interest

The authors declare that there are no conflicts of interest, which could affect the fairness and objectivity of the work submitted.

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