The role of seminal plasma in the function, transport and fertility of ram spermatozoa

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Abstract. To date, there have been no studies investigating the role seminal plasma plays in cervical transit of epididymal ram spermatozoa. As such, epididymal spermatozoa were assessed in the presence and absence of seminal plasma both *in vitro* and *in vivo*. Experiment 1 examined the effect of seminal plasma on fresh epididymal spermatozoa with and without subsequent cryopreservation, measuring motility variables and the ability to penetrate cervical mucus. Motility parameters of fresh epididymal spermatozoa did not improve with exposure to seminal plasma. Only the total motility of cryopreserved epididymal spermatozoa significantly ($p<0.001$) improved with pre-freeze exposure to seminal plasma (average exposed 31.9% ± 4.9% vs unexposed 20.4% ± 4%). However, adding seminal plasma significantly improved mucus penetration by fresh and cryopreserved epididymal ram spermatozoa ($p<0.05$). Experiment 2 investigated the fertility of epididymal spermatozoa with and without exposure to seminal plasma after cervical and intrauterine insemination. While epididymal spermatozoa performed poorly when inseminated cervically without seminal plasma (7.3%), exposure to seminal plasma yielded significantly ($p=0.05$) higher pregnancy rates (37.0%). Treatment had no significant effect on pregnancy rates following intrauterine insemination. These results suggest that exposure to seminal plasma during ejaculation is necessary for normal survival and transit of spermatozoa through the cervix.

Additional keywords: epididymal spermatozoa, cervix, cervical mucus, sheep

Introduction

Seminal plasma is the complex fluid secreted by the major accessory sex glands and while it has many proposed functions, its exact role in reproduction remains unclear. Epididymal spermatozoa, which have never before had contact with seminal plasma from the major accessory sex glands, have been shown to have surprisingly high fertility. This has been demonstrated both *in vitro* through techniques such as intracytoplasmic sperm injection and IVF (Silber *et al.*, 1995; Stout *et al.*, 2012) and *in vivo* when inseminated directly into the uterus (Fournier-Delpech *et al.*, 1977; Hori *et al.*, 2004; Hori *et al.*, 2005; Ehling *et al.*, 2006; Monteiro *et al.*, 2011), in many cases achieving pregnancy rates similar to

those of uterine inseminated ejaculated spermatozoa (Fournier-Delpech *et al.*, 1977; Ehling *et al.*, 2006; Monteiro *et al.*, 2011). 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 for normal sperm function *in vivo* or whether this substance is largely superfluous to reproductive success.

Despite these observations, evidence also exists that seminal plasma plays a beneficial role in reproductive processes and fertility. It has been demonstrated that addition of seminal plasma post thaw can improve the *in vitro* fertilising ability of cryopreserved ejaculated ram spermatozoa (El-Hajj Ghaoui *et al.*, 2007). Furthermore, it has also been suggested that seminal plasma may improve the ability of cryopreserved ejaculated ram spermatozoa to navigate the tortuous ovine cervix *in vivo* (Maxwell *et al.*, 1999). While not definitive, this fosters the idea that one of the main roles of seminal plasma could be assisting cervical migration of spermatozoa. The cervical migration model in sheep offers a novel means of testing the effect of seminal plasma on the ability of spermatozoa to interact with the female reproductive tract, as it is well established as a site of high sperm selectivity (see Druart, 2012 for review).

No studies have yet reported the fertility of epididymal spermatozoa in the presence and absence of seminal plasma after cervical insemination in the ewe. In addition, while beneficial effects of seminal plasma have been demonstrated *in vitro* using washed, ejaculated ram spermatozoa, they have yet to be replicated using epididymal spermatozoa. As such, the aim of the current study was to examine the effect of seminal plasma on the *in vitro* function of epididymal spermatozoa with and without subsequent cryopreservation and its influence on epididymal spermatozoa transport and fertility *in vivo*. We hypothesise that exposure to seminal plasma will have beneficial effects on important functional characteristics and *in vivo* fertility.

Materials & Methods

Equipment and reagents

All chemicals used were laboratory grade and manufactured by Sigma-Aldrich (Castle Hill, Australia) unless otherwise stated. Eppendorfs and pipette tips were sourced from Eppendorf South Pacific Pty. Ltd (North Ryde, Australia). Ultra-heat treated (UHT) milk was obtained from retail sources on the day of assessment. All experiments were assessed and approved by the University of Sydney Animal Ethics Committee.

Experiment 1: The effect of seminal plasma on the in vitro function of epididymal spermatozoa, with and without subsequent cryopreservation

Collection of ejaculated semen and seminal plasma

Semen was collected from mature merino rams (*N*=3) housed at the Gunn Building, University of Sydney, Camperdown via artificial vagina in the presence of a teaser ewe. Ejaculates were immediately assessed for colour, volume and wave motion (data not shown). After assessment, samples were diluted 1:1 with Salamon's Sheath Fluid (SSF; tris-citric acid, fructose) and concentration determined using a haemocytometer as described by Evans and Maxwell (1987). Collections were subsequently equally divided between fresh (F-EJAC) and cryopreserved (C-EJAC) treatments.

Seminal plasma was obtained from several ejaculates (collected during the breeding season in 2013) of the same three rams by centrifuging at 4000 × *g*, once for 20 minutes, with the supernatant then collected and spun for a further 30 minutes at 4000 × *g* (Sigma-Aldrich, Castle Hill, Australia). Seminal plasma samples were then separated into aliquots and stored at -80°C. Individual aliquots were thawed on ice as needed.

Collection of epididymal spermatozoa

Ram testes (*N*=9 rams) were obtained at slaughter from Goulburn abattoir and transported on ice to the University of Sydney, Camperdown where they remained chilled at 5°C for 24 hours. Epididymal spermatozoa were obtained by microperfusion (Dacheux, 1980) using SSF. Collections were assessed for wave motion (data not shown) and the concentration determined using a haemocytometer as described by Evans and Maxwell (1987). Epididymal collections were then halved, with one half being undiluted epididymal spermatozoa (EP) and the remainder undergoing a 1:1 dilution with seminal plasma previously collected from an analogous ram (EPSP). Treatments were subsequently subjectively assessed for motility (data not shown). These two treatments were again halved, with one half remaining fresh (F-EP, F-EPSP), and the remainder being subsequently cryopreserved (C-EP, C-EPSP).

Fresh sample preparation

Fresh treatments were diluted with UHT milk to stock solutions of 50×10^6 spermatozoa/mL, which were kept in a 37˚C water bath over a period of 6 hours. At the time of collection (0 hours), 3 hours and 6 hours after collection, a 500µL aliquot of each sample was diluted with Androhep (Minitube, Ballarat, Australia) to 25×10^6 spermatozoa/mL for assessment.

95 *Cryopreserved sample preparation*

96 Cryopreserved treatments were diluted to 100×10^6 spermatozoa/mL with Salamon's
97 cryodiluent (tris-citric acid, glucose, egg yolk). Samples were chilled to 5˚C over a period of 2 hours
98 and aliquots taken for a pre-freeze assessment. Chilled samples were subsequently loaded into pre-
99 cooled straws (0.25mL; IMV Technologies, Germany), which were suspended above liquid nitrogen
100 for 8 minutes before being submerged. Straws were then stored in liquid nitrogen until use. Straws
101 were thawed by agitating in a 37˚C water bath for 2 minutes. A 500µL aliquot of each sample was
102 diluted with Androhep to 25×10^6 spermatozoa/mL at 0, 3 and 6 hours post thaw for assessment.

103 *Motility*

104 A 5.5µL aliquot of 25×10^6 spermatozoa/mL sample was assessed for motility parameters by
105 Computer Assisted Sperm Analysis (CASA; IVOS II Animal, Hamilton Thorne, Beverly, USA),
106 using CELL-VU slides (Millennium Sciences, Mulgrave, Australia; pre-warmed to 37˚C) with a
107 22×22mm cover slip (chamber depth 20µm). Motility parameters were determined on an average of at
108 least three random fields (200-300 cells per sample) using factory settings for ram, with a sampling
109 frequency of 60 Hz. Recorded variables included motility, progressive motility, average path,
110 curvilinear and straight line velocities, amplitude of lateral head displacement, beat cross frequency,
111 linearity and straightness.

112 *Cervical migration test*

113 Natural cervical mucus was collected from synchronised merino ewes in oestrus, separated
114 into 500µL aliquots and stored at -80°C. Individual aliquots were thawed on ice as needed. A vial of
115 DNA-specific stain (IDENT; Hamilton Thorne, Beverly, USA) was diluted with 500µL of UHT milk
116 for fresh samples or Salamon's cryodiluent for cryopreserved samples, giving a stock solution of
117 80µg/µL. Spermatozoa were stained 1:1 with IDENT (final working concentration 40µg/µL). Samples
118 were incubated for 10 minutes then transferred to a polyethylene capsule (BEEM; ProSciTech,
119 Thuringawa, Australia). A glass capillary tube (0.3×0.3×100 mm; Microslides, Mountain Lakes,
120 USA) filled with natural cervical mucus and sealed with Cristaseal (Hawksley, London, UK) was
121 immersed in the stained sample and co-incubated (37°C, 1 h). Following incubation, capillary tubes
122 were read under fluorescent microscopy (200×; Olympus BX51) and the number of spermatozoa at
123 1cm and the vanguard distance (furthest spermatozoon) recorded.

124

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Experiment 2: The effect of seminal plasma on the in vivo fertility of fresh epididymal spermatozoa

Ewe synchronisation

Oestrus was synchronised in mature merino ewes (*N*=303), using a combination of intravaginal progesterone pessaries (30mg; Ova-Gest; Bioniche, Armidale, Australia) for 12 days, followed by 400 IU of intramuscular PMSG (1mL; Pregnecol; Bioniche, Armidale, Australia) at sponge removal. Testosterone supplemented wethers (400mg administered at sponge insertion and a further 150mg at removal; Duramate; Intervet, Australia) were introduced to the flock at sponge removal, at a ratio of 1 wether: 25 ewes.

Collection of ejaculated semen

Ejaculates of mature merino rams (*N*=3; F-EJAC) were collected via artificial vagina immediately prior to insemination and diluted 1:2 with warmed UHT milk. Diluted samples were transported a short distance to the insemination site, where they were assessed for concentration and motility (data not shown). Samples were kept at 30˚C prior to further dilution with UHT milk for insemination.

Collection of epididymal spermatozoa

Testes were removed from culled Merino rams (*N*=3 rams) and epididymal spermatozoa collected into a sterile petri dish via microperfusion with SSF (Dacheux, 1980). Following collection, epididymal spermatozoa were centrifuged at 800 × *g* for 10 minutes to concentrate the sample and remove contaminants. The pellet was subsequently resuspended in SSF. Epididymal treatments (F-EP, F-EPSP) were subsequently assessed and prepared as per experiment 1. Treatments were kept at 30˚C prior to further dilution with UHT milk for insemination.

Insemination

Inseminations occurred over two days in April 2013 at the University of Sydney property Arthursleigh in the New South Wales southern highlands, with all animals held on site. In order to minimise animal stress and maximise insemination success, no working dogs were used and ewes were given time to settle before and after insemination. Ewes were inseminated by cervical or intrauterine laparoscopic AI with F-EJAC, F-EP and F-EPSP spermatozoa (with motility ≥ 70%). 164 ewes were inseminated cervically (F-EJAC=77, F-EP=41 and F-EPSP=46) and 139 laparoscopically (F-EJAC=40, F-EP=46 and F-EPSP=53).

Prior to cervical insemination, all treatments were diluted to a stock solution of 500×10^6 spermatozoa/mL with UHT milk. Cervical insemination pipettes were loaded with 0.2mL of semen with a 0.2mL cushion of air either side, giving a cervical insemination dose of 100×10^6 spermatozoa/ewe. Ewes were cervically inseminated to industry standards. Ewes were prepared for laparoscopic insemination with intramuscular injections of Ketamil (150mg; Troy Ilium, Glendenning, Australia) and ACP 2 (acetylpromazine, 2mg; Delvet, Seven Hills, Australia), followed by a subcutaneous injection of local anaesthetic (2mL of 2% Lignocaine; Mavlab, Logan City, Australia). Prior to laparoscopic insemination, stock solutions were diluted 1:1 with UHT milk to give a concentration of 250×10^6 spermatozoa/mL. Laparoscopic insemination pipettes were loaded with 0.05mL of semen with a 0.2mL cushion of air either side, giving a laparoscopic insemination dose of 12.5×10^6 spermatozoa/horn/ewe. Ewes were laparoscopically inseminated by experienced professionals to industry standards.

60 days after insemination, ewes were subjected to ultrasound in order to determine pregnancy status. After lambing, ewes were assessed for mammary gland development and suckling to determine the number of ewes which had lambed and foetal loss as per Evans and Maxwell (1987).

Statistical Analyses

Statistical analyses were carried out using GENSTAT (15th Edition; VSN International, Hemel Hempstead, UK). Experiment 1 was assessed using a linear mixed model and experiment 2 using a generalised linear mixed model, both accounting for fixed and random effects. Transformations were used to attain data normality where required. Means are reported with ± standard error of the mean.

Results

Experiment 1: The effect of seminal plasma on the in vitro function of epididymal spermatozoa, with and without subsequent cryopreservation

Motility parameters

The total percentage of fresh motile spermatozoa decreased significantly over time as expected ($p<0.001$), but treatment had no significant effect. F-EJAC had significantly higher progressive motility than F-EP and F-EPSP treatments at all time points ($p<0.001$; average 67.7% ± 3.5%; 45.8% ± 5.2%; 42.8% ± 6.1%; figure 1a). The interaction of time and treatment significantly influenced average path velocity ($p<0.05$; figure 1b), with both F-EP and F-EPSP having a lower average path velocity (average 120.8μm/s ± 6μm/s; 122.5μm/s ± 5.5μm/s) than the F-EJAC treatment (average 159.2μm/s ± 7μm/s) at 0 and 3 hours. Similar results were obtained for both straight line ($p<0.05$) and curvilinear velocity ($p=0.05$). Amplitude of lateral head displacement was significantly

lower in the F-EJAC treatment (average 7µm ± 0.3µm) versus F-EP and F-EPSP (average 8.1µm ± 0.4µm; 7.9µm ± 0.4µm) overall (p<0.001), with all treatments decreasing in displacement over time (p<0.05). F-EJAC demonstrated a significantly higher beat cross frequency than both F-EP and F-EPSP at all time points (p<0.001; average 37.6Hz ± 1.2Hz; 33Hz ± 1.2Hz; 33.3Hz ± 1.2Hz). F-EJAC similarly had greater linearity than both F-EP and F-EPSP across the 6 hour incubation (p<0.001; average 87.6% ± 1.2%; 79% ± 2.1%; 77.8% ± 2.5%). Straightness yielded similar results, with F-EJAC having significantly higher straightness on average (p<0.001) and all treatments increasing in straightness over time (p=0.005).

There was a small but significant difference in motility (p<0.001) between C-EJAC (92.7% ± 1.3%) and both C-EP and C-EPSP (85% ± 2.7%; 85.6% ± 2.6%) in the pre-freeze assessment. Nevertheless, during the 6 hour post thaw incubation, all three cryopreserved treatments had significantly different motilities (p<0.001; average C-EJAC 74.9% ± 1.7%;C-EP 20.4% ± 4%; C-EPSP 31.9% ± 4.9%; figure 3a). Progressive motility was significantly affected by the interaction between treatment and time (p<0.05; figure 3b), however during the pre-freeze and post thaw assessments, overall significant differences only existed between C-EJAC (average 42.4% ± 2.8%) and both cryopreserved epididymal treatments (average C-EP 9.3% ± 2.6%; C-EPSP 11.7% ± 3.2%). Overall, C-EJAC had a significantly higher average path velocity (average 107.7µm/s ± 7µm/s ; figure 3c) than both C-EP and C-EPSP (average 62.1µm/s ± 5.2µm/s; 67.3µm/s ± 6.3µm/s) over the pre-freeze assessment and post thaw incubation (p<0.001), with similar results for curvilinear velocity (p<0.001). The interaction between treatment and time significantly affected straight line velocity with C-EJAC moving significantly faster than epididymal treatments at all time points (p<0.05; average C-EJAC 91µm/s ± 6µm/s; C-EP 46.7µm/s ± 5.1µm/s; C-EPSP 49.5µm/s ± 5.5µm/s). C-EJAC had significantly higher amplitude of lateral head displacement than C-EP and C-EPSP overall (p<0.001; average C-EJAC 6.6µm ± 0.4µm; C-EP 4.9µm ± 0.4µm; C-EPSP 5.1µm ± 0.4µm). While all treatments were similar in the pre-freeze assessment, C-EJAC had a significantly higher beat cross frequency than epididymal treatments post thaw (p<0.05; average C-EJAC 37.8Hz ± 0.6Hz; C-EP 33.8Hz ± 1.3Hz; C-EPSP 32.8Hz ± 0.7Hz; figure 3d). C-EJAC had significantly higher linearity than epididymal treatments post thaw (p<0.05; average C-EJAC 54.4% ± 1.8%; C-EP 49.6% ± 2.7%; C-EPSP 49.5% ± 2.3%) with linearity increasing significantly over time (p<0.001). Straightness measures yielded similar results, with C-EJAC (average 85.1% ± 1.1%) performing significantly better than C-EP and C-EPSP (average 80.4% ± 2.8%; 79.1% ± 2.5%) over the post thaw assessment (p<0.05) and straightness increasing significantly (p<0.001) over time.

222 *Cervical migration test*

223 Time had a significant impact on the number of fresh spermatozoa which progressed to 1cm
224 (p<0.001). Treatment was also significant (p<0.05; figure 2a), with the F-EPSP treatment having a
225 significantly larger population of spermatozoa at 1cm than the F-EP treatment at 0 hours (F-EPSP
226 37.4 ± 9.4; F-EP 14.5 ± 5.3) and 3 hours (F-EPSP 24.5 ± 5.4; F-EP 9.6 ± 3.2). In addition, the
227 vanguard spermatozoon from the F-EPSP treatment penetrated significantly further through cervical
228 mucus than that of the F-EP treatment at 0 hours (p<0.05; F-EPSP 2.8cm ± 0.3cm; F-EP 1.9cm ±
229 0.3cm; figure 2b). Time significantly reduced the vanguard distance of both F-EJAC and F-EPSP
230 (p<0.001), but not F-EP.

231 Contrary to the results from fresh spermatozoa, the interaction between treatment and time
232 significantly affected the number of cryopreserved spermatozoa which reached 1cm (p<0.05; figure
233 4a). Across the pre-freeze assessment and 6 hour incubation, C-EJAC and C-EPSP treatments had
234 similar results (average 9.6 ± 3; 9.9 ± 3.6), and both had significantly greater numbers of spermatozoa
235 than the C-EP treatment at 1cm (average 3.4 ± 1.3). Vanguard distance was significantly influenced
236 by the interaction of treatment and time (p<0.05), with C-EJAC and C-EPSP penetrating significantly
237 further through cervical mucus than C-EP at 0 hours (C-EJAC 2.25cm ± 0.2cm; C-EP 1.1cm ± 0.1cm;
238 C-EPSP 1.6cm ± 0.1cm) and 3 hours (C-EJAC 2cm ± 0.2cm; C-EP 1.4cm ± 0.2cm; C-EPSP 2cm ±
239 0.3cm ; figure 4b).

240 *Correlation between motility and mucus penetration*

241 There were very weak positive correlations ($R^2 < 0.1$) between motility parameters of total
242 motility, progressive motility and average path velocity and mucus penetration variables of number of
243 spermatozoa at 1cm and vanguard distance for fresh and cryopreserved treatments.

244 *Experiment 2: The effect of seminal plasma on the in vivo fertility of fresh epididymal*
245 *spermatozoa*

246 Results for pregnancy rate, lambing rate and foetal loss are shown in Table I. The interaction
247 between treatment and method of insemination significantly affected pregnancy at 60 days (p=0.05).
248 F-EP had significantly poorer fertility when inseminated cervically (7.3%) versus laparoscopically
249 (50.0%) and compared to both F-EJAC and F-EPSP treatments (20.8%; 37.0%) when all were
250 inseminated cervically. There were no significant differences between treatments inseminated
251 laparoscopically. There were no significant differences between pregnancy rates and lambing rates.
252 Only a single ewe (F-EP, laparoscopically inseminated) aborted her foetus between ultrasound and
253 lambing.

Discussion

This study has shown that while seminal plasma does not alter the overall motility characteristics of epididymal spermatozoa, it does improve their ability to penetrate cervical mucus and traverse the ovine cervix. While ram seminal plasma has been shown to have beneficial effects *in vitro*, this is the first known report of a beneficial effect of seminal plasma on the transport and survival of epididymal ram spermatozoa after cervical insemination in an ovine model. Furthermore, this study has demonstrated that seminal plasma was able to improve *in vitro* mucus penetration in the absence of improvements in motility. This may suggest that the ability of spermatozoa to penetrate cervical mucus is not linked to motility, but rather an unknown trait conferred by exposure to seminal plasma. These results are encouraging, as they have helped to establish the importance of seminal plasma in different elements of cervical transit of spermatozoa, lending support to its application in advanced reproductive technologies.

Unexpectedly, the majority of motility parameters of both fresh and cryopreserved epididymal spermatozoa did not appear to improve with the addition of seminal plasma, with epididymal spermatozoa generally yielding poorer results than ejaculated spermatozoa regardless of exposure. These results agree with a study by Dott *et al.* (1979), who found that the addition of undiluted seminal plasma to ram epididymal spermatozoa resulted in a short lived increase, followed by a significant decline in motility by 3 hours after exposure. Furthermore, Dott *et al.* (1979) found that even exposing epididymal spermatozoa to 30% seminal plasma for just 15 minutes caused a clear decline in motility, hypothesising that while seminal plasma is initially stimulatory, it is ultimately detrimental to motility. Similarly, Heise *et al.* (2010) found that seminal plasma stimulated equine epididymal spermatozoa progressive motility to the same level of ejaculated spermatozoa when immediately assessed. Yet after freezing, supplemented epididymal spermatozoa had progressive motility equivalent to unsupplemented epididymal spermatozoa and far lower than that of ejaculated spermatozoa. Overall, these results and those of the current study lend support to the idea that exposing epididymal ram spermatozoa to undiluted seminal plasma has no long term benefits for motility.

While the poor progressive motility of the epididymal treatments in this study may be due to extended cold storage prior to collection, a lack of positive response may also be explained by the different reactions of ejaculated and epididymal spermatozoa to seminal plasma. It has been demonstrated that post thaw addition of seminal plasma to cryopreserved ejaculated ram spermatozoa may increase motility (El-Hajj Ghaoui *et al.*, 2007; Bernardini *et al.*, 2011). In opposition, when Thuwanut and Chatdarong (2009) supplemented feline epididymal spermatozoa post thaw, they found that seminal plasma significantly decreased motility compared to a control supplemented with a Tris buffer. This lack of a positive effect of seminal plasma could possibly be due to the inability of

epididymal spermatozoa to bind motility driving seminal plasma proteins. While not a protein affecting motility, the bovine BSP 3 protein is produced in the epididymis, but can only be bound by ejaculated and not epididymal spermatozoa (Souza *et al.*, 2011). This suggests that the interaction between spermatozoa and seminal plasma may be more complex than simply being in each other's presence and that optimal sperm function is possibly a combined result of epididymal maturation and ejaculation.

Overall, the ability of epididymal spermatozoa to penetrate cervical mucus was significantly improved with exposure to seminal plasma, supporting the hypothesis that seminal plasma may aid the passage of both fresh and cryopreserved spermatozoa through cervical mucus. In some cases, epididymal spermatozoa exposed to seminal plasma outperformed ejaculated spermatozoa, which is likely to be due to individual variations in male fertility. These results agree with the work of Arangasamy *et al.* (2005), who reported that epididymal buffalo spermatozoa exposed to isolated heparin and gelatin binding seminal plasma proteins progressed significantly further in buffalo cervical mucus than the unexposed control. Similar results were presented by Maxwell *et al.* (1999), with cryopreserved ejaculated ram spermatozoa showing a significant positive response in cervical mucus penetration to post thaw supplementation of 30% v/v seminal plasma in DBPS, compared to a DPBS supplemented control. The interaction between seminal plasma and cervical mucus remains somewhat of a mystery and to date, β defensin 126, a seminal plasma glycoprotein which facilitates the penetration of cervical mucus, is the only studied example of a biochemical interaction between the two (Tollner *et al.*, 2008). It was demonstrated that while the addition of β defensin 126 to washed macaque spermatozoa restored cervical mucus penetration, adding all seminal plasma proteins resulted in a slight inhibition of mucus penetration. While the results of this study are not conclusive and further investigation into this complex relationship is required, this evidence does encourage the idea that cervical transit of spermatozoa is driven and heavily influenced by the proteomic components of seminal plasma.

None of the measured motility variables were significantly correlated to the ability of fresh or cryopreserved spermatozoa to penetrate natural cervical mucus. These results conflict with the findings of several previous studies in humans (Keel and Webster, 1988; Ford *et al.*, 1992) and sheep (Suttiyotin *et al.*, 1995; Robayo *et al.*, 2008; Martínez-Rodríguez *et al.*, 2012), which found that parameters including total motility, progressive motility, amplitude of lateral head displacement and average and curvilinear velocity were significantly positively correlated to various measures of mucus penetration. The comparison between the current results and these previous studies raises the question of what other factors are possibly influencing the interaction between spermatozoa and cervical mucus. While seminal plasma failed to greatly improve motility parameters, it significantly improved

the ability of exposed epididymal spermatozoa to pass through cervical mucus, highlighting its potential importance in assisting spermatozoa to successfully traverse the ovine cervix.

As hypothesised, when inseminated cervically, epididymal spermatozoa had minimal fertility compared to both ejaculated spermatozoa and epididymal spermatozoa exposed to seminal plasma. Furthermore, when inseminated laparoscopically, all three treatments yielded relatively high, equivalent pregnancy rates at 60 days. Given the negligible abortion rates, it was concluded that there was no significant influence of treatment on foetal loss. The below average pregnancy rates for the ejaculated treatment are believed to be due to poor quality ejaculates from the rams used for collection. Nevertheless, these results suggest that epididymal spermatozoa struggled to overcome the cervical barrier, and were only able to do so effectively following exposure to seminal plasma. In a similar study using ejaculated ram spermatozoa, Maxwell *et al.* (1999) demonstrated that while frozen thawed ram spermatozoa had improved *in vivo* fertilisation capacity when resuspended in seminal plasma and inseminated cervically, there was no effect on pregnancy rates of laparoscopically inseminated ewes. A previous study of laparoscopic insemination with unsupplemented, cryopreserved epididymal ram spermatozoa yielded pregnancy rates as high as 87% (Ehling *et al.*, 2006), supporting the current finding of comparably high epididymal fertility when inseminated directly into the uterus. The use of epididymal spermatozoa rather than washed, ejaculated spermatozoa in this study has helped to confirm the beneficial effect of seminal plasma on cervical transit of spermatozoa. The consistency in these results gives substance to the idea that seminal plasma plays a key role in successful migration through the female reproductive tract to reach the end goal of fertilisation.

Factors that limit the extent to which these results can be generalised include variation between males and the possibility of breed based differences. Innate variations exist in the fertility of rams, with ram seminal plasma containing proteins of both beneficial and detrimental natures and the amount of each correlated to the fertility of the individual (Yue *et al.*, 2009). While several replicates accounted for variation in the quality of epididymal collections, a different male was used for the ejaculated and epididymal treatments. Ideally, ejaculates and seminal plasma would be collected from a ram which is then culled for an epididymal collection, but this was not logistically possible in this study. In order to ensure statistical validity and reasonable reliability, seminal plasma was instead individually pooled from several ejaculates of each ram and applied to an epididymal collection from a single ram. The second limiting factor in this study is the influence of breed, which has been shown to impact both *in vitro* mucus penetration (Richardson *et al.*, 2011) and pregnancy rates following cervical insemination (Donovan *et al.*, 2004). As this study used 100% purebred Australian merinos, the effects seen here may not be implicit across all sheep breeds.

This study has shown that exposure to seminal plasma is vital for normal migration of spermatozoa through the ovine cervix. Furthermore, it has demonstrated that *in vitro* measures of sperm function, such as motility, may not give an accurate representation of *in vivo* outcomes. Most importantly, this study has shown that seminal plasma significantly impacts both *in vitro* mucus penetration and *in vivo* fertilising ability following cervical insemination, supporting the idea that a key role of seminal plasma is assisting spermatozoa during the initial stages of transport through the female reproductive tract. Judging from the results of the current study, continued research into how seminal plasma supports successful cervical transit of spermatozoa may be the key to improving cervical insemination success rates using both fresh and cryopreserved ram semen.

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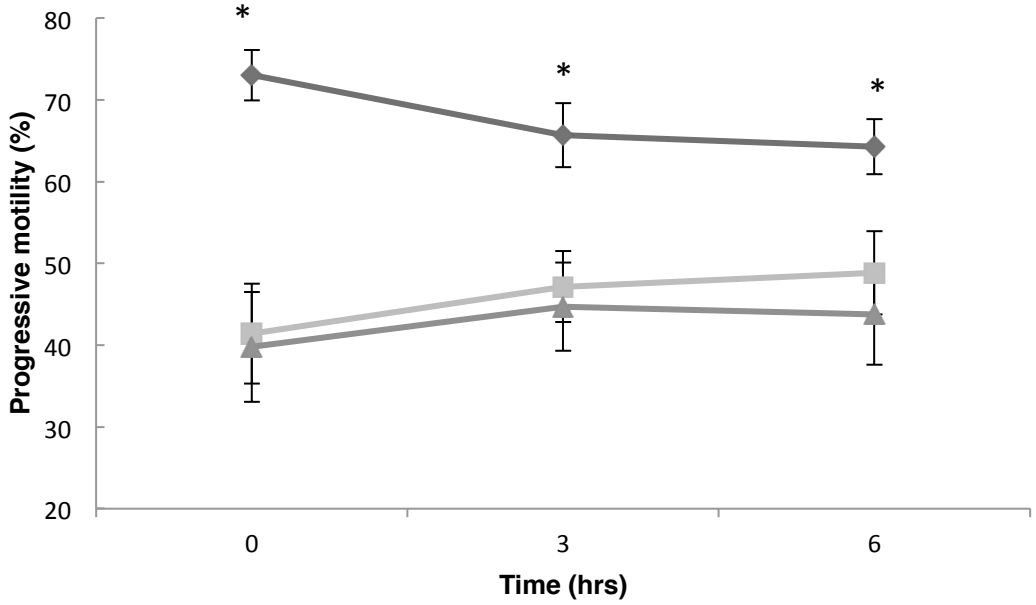
Figures

a)



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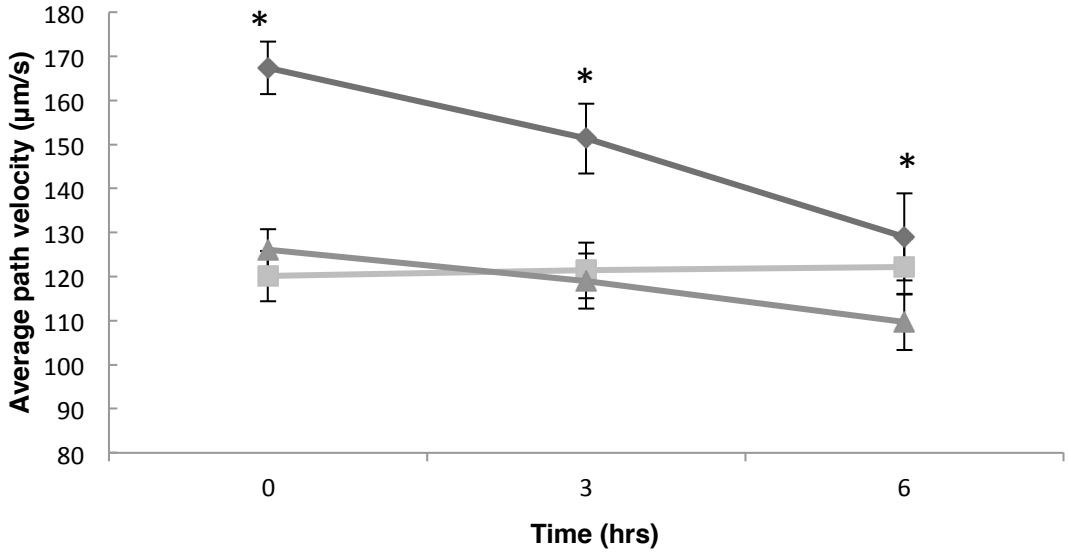
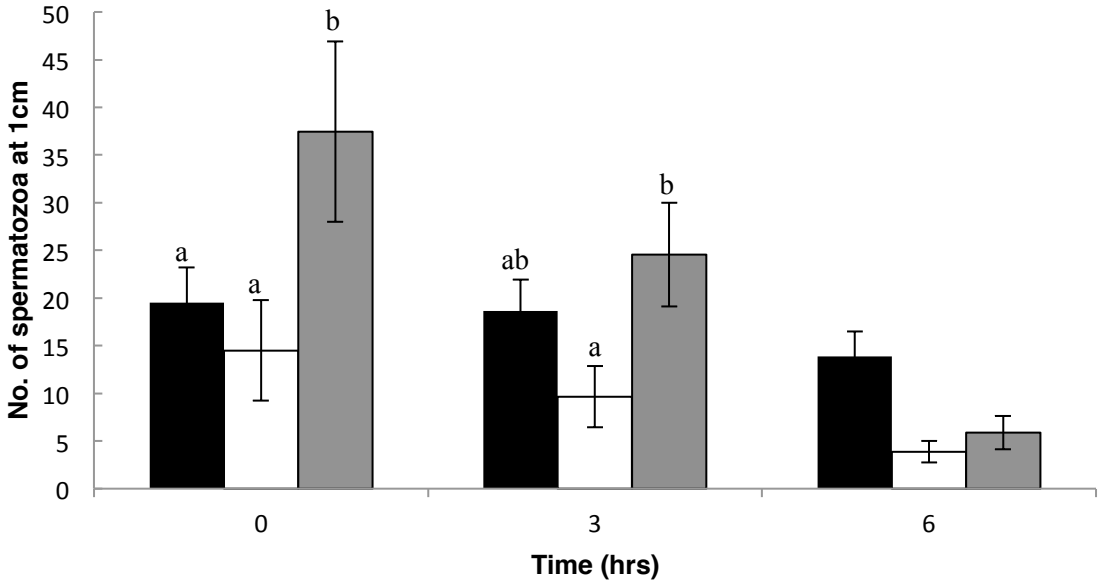


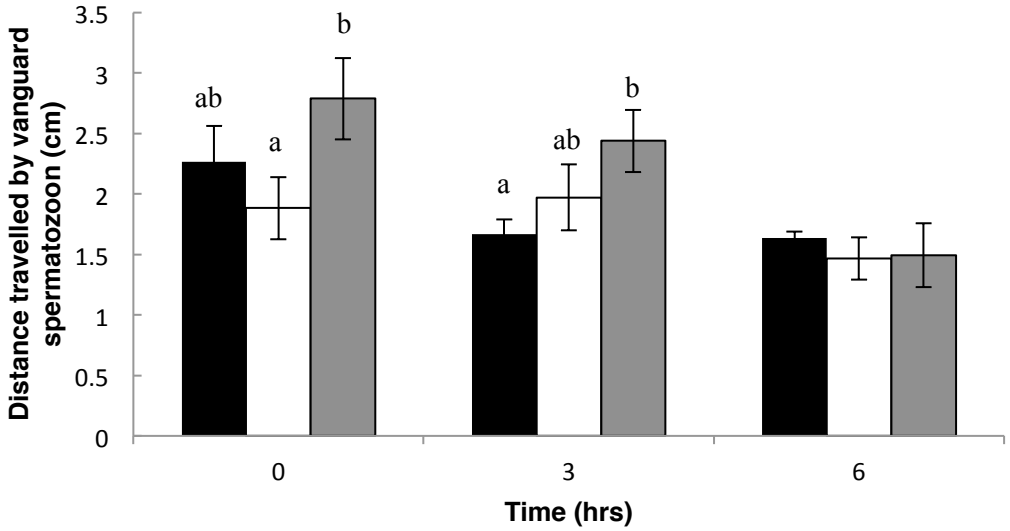
Fig 1. a) Progressive motility and b) average path velocity of F-EJAC (◆), F-EP (■) and F-EPSP (▲) treatments over a 6 hour incubation period. Values are means ± SEM. Within time points, * denotes significant differences (p<0.05).

401 a)



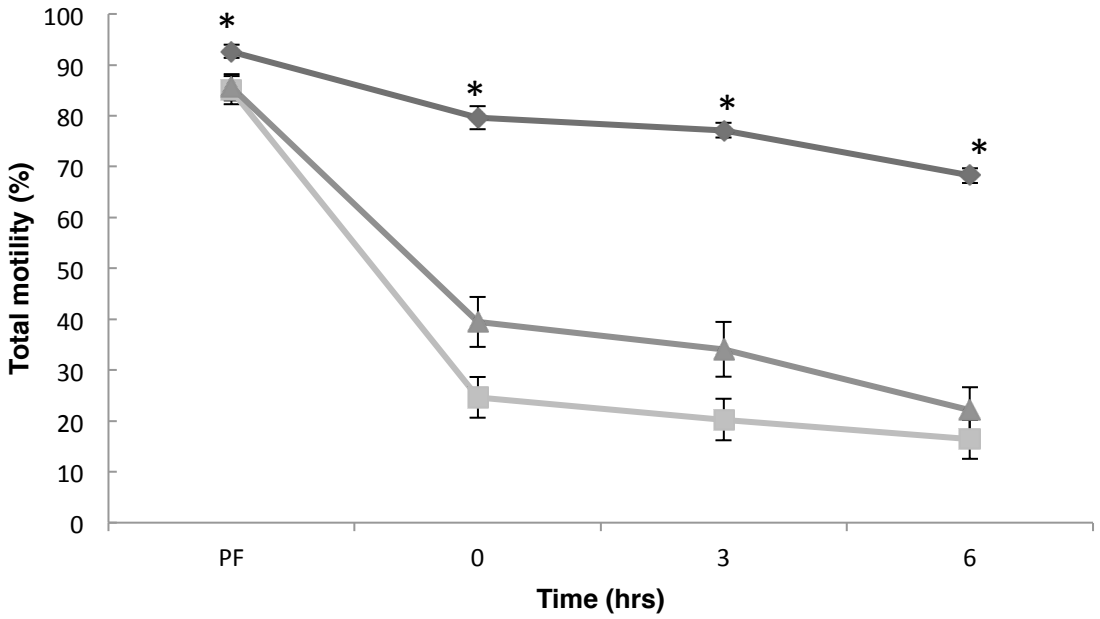
402

403 b)

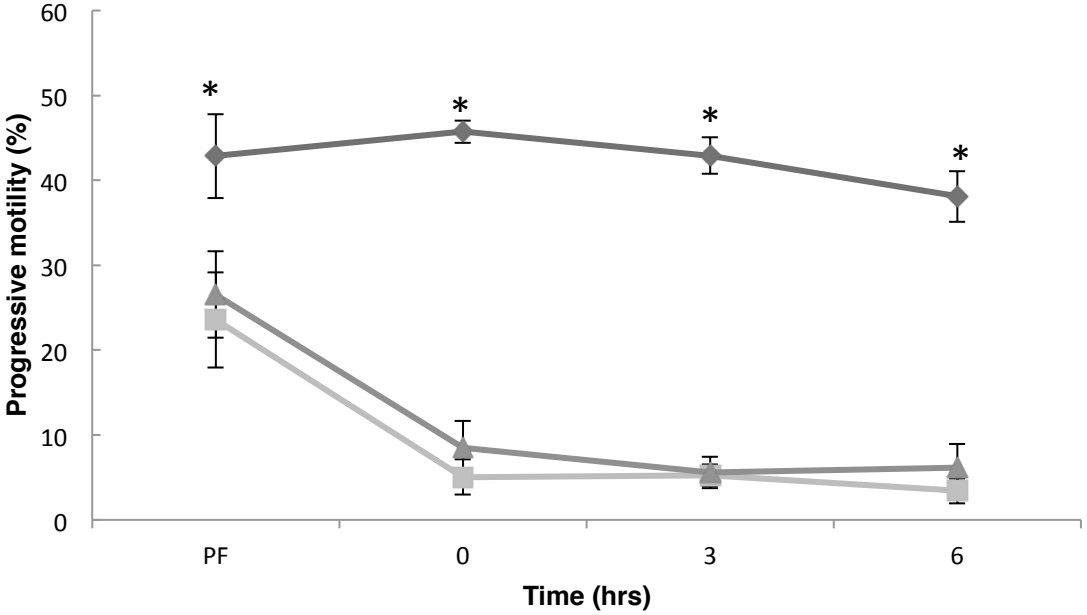


404 **Fig. 2.** a) Mean number of spermatozoa travelling 1cm through natural cervical mucus and b)
405 distance (cm) of the furthest spermatozoon after a 1 hour incubation for F-EJAC (black), F-EP (white)
406 and F-EPSP (grey) treatments over a 6 hour assessment. Values are means ± SEM. Within time
407 points, different letters denote significant differences (p<0.05).

408 a)



409 b)



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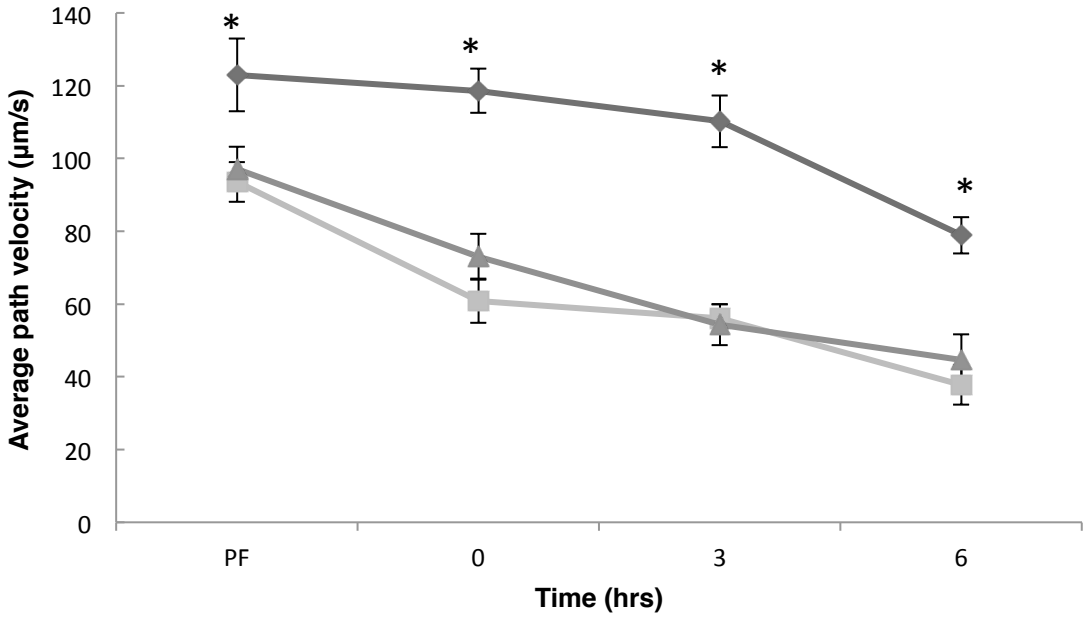


c)

d)

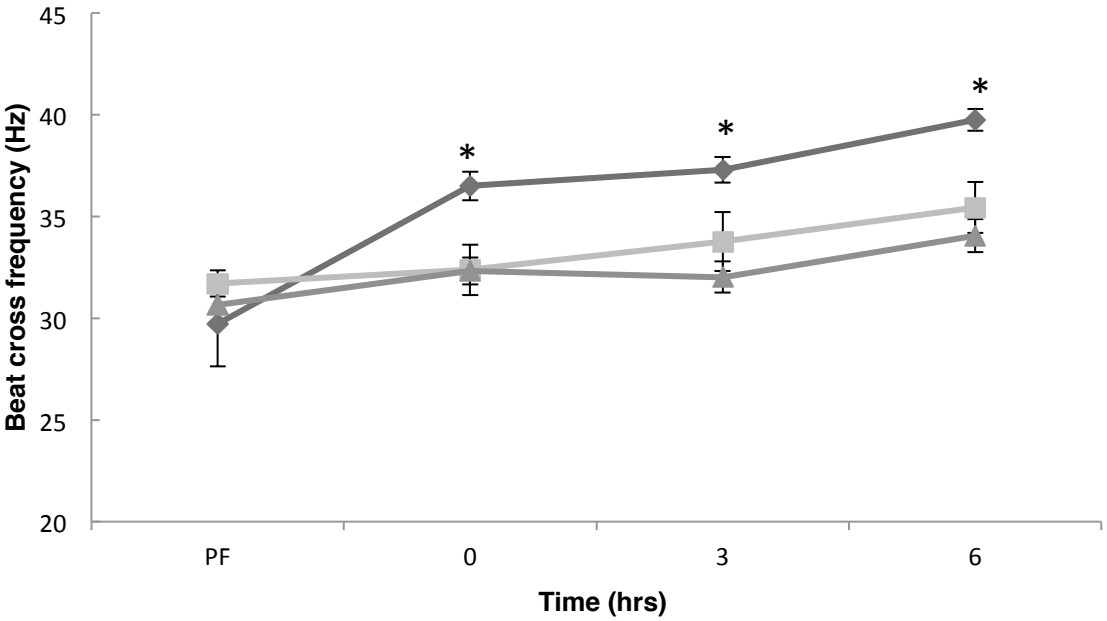
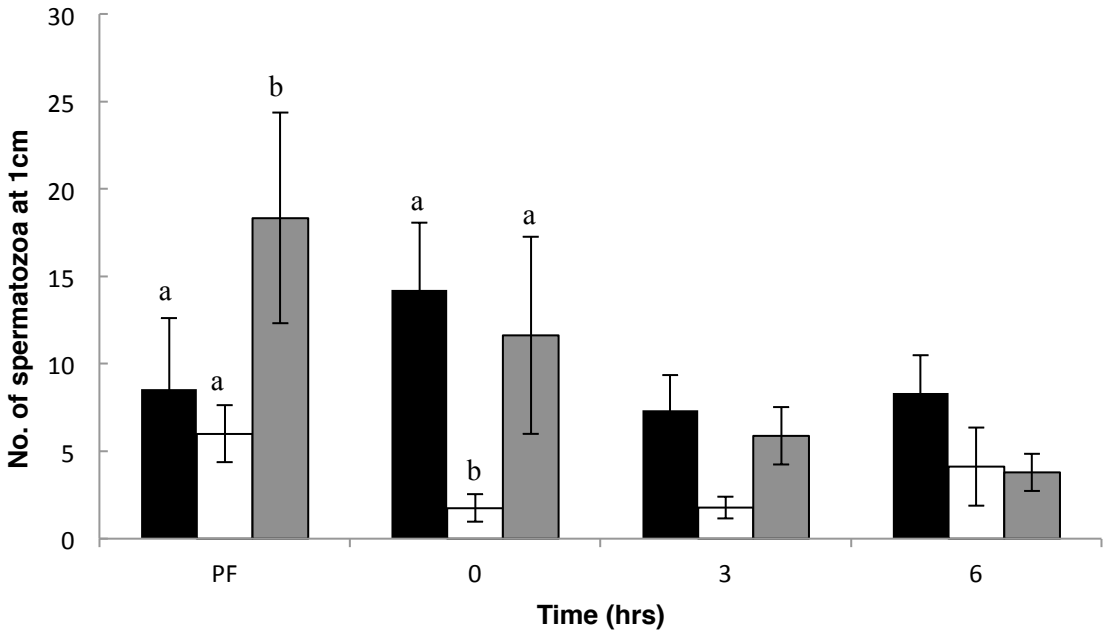
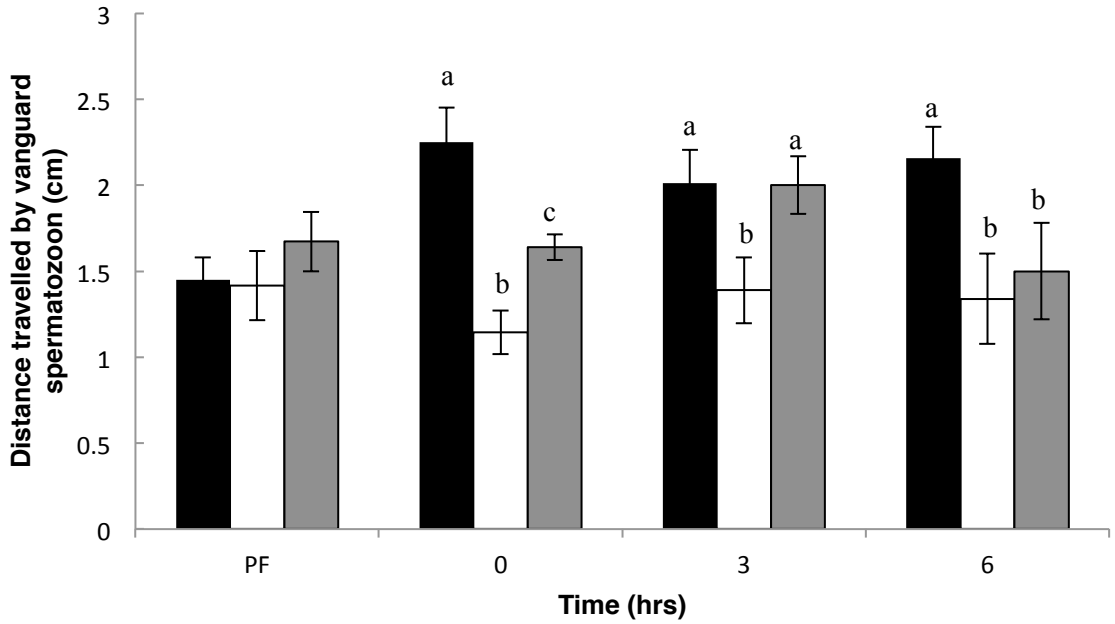


Fig. 3. a) Total motility b) progressive motility c) average path velocity d) beat cross frequency of C-EJAC (◆), C-EP (■) and C-EPSP (▲) treatments over a pre-freeze and 6 hour incubation period. Values are means ± SEM. Within time points, * denotes significant differences (p<0.05).

420 a)



421 b)



422 **Fig. 4.** a) Mean number of spermatozoa travelling 1cm through natural cervical mucus and b)
423 distance (cm) of the furthest spermatozoon after a 1 hour incubation for C-EJAC (black), C-EP
424 (white) and C-EPSP (grey) treatments over the pre-freeze assessment and 6 hour post thaw
425 assessment. Values are means ± SEM. Within time points, different letters denote significant
426 differences (p<0.05).

Table I. Pregnancy and lambing rates and foetal loss after cervical and laparoscopic intrauterine insemination of synchronised mature merino ewes with fresh ejaculated ram spermatozoa (F-EJAC), epididymal ram spermatozoa (F-EP) and epididymal ram spermatozoa exposed to ram seminal plasma (F-EPSP)

Treatment	Insemination method	No. ewes inseminated	No. ewes pregnant at day 60 (%)*	No. ewes lambed (%)**	Foetal loss (%)
F-EJAC	Cervical	77	16 (20.8) [a]	16 (20.8) [a]	0
	Laparoscopic	40	17 (42.5)	17 (42.5)	0
F-EP	Cervical	41	3 (7.3) [b]	3 (7.3) [b]	0
	Laparoscopic	46	23 (50)	22 (47.8)	2.2
F-EPSP	Cervical	46	17 (37) [a]	17 (37) [a]	0
	Laparoscopic	53	31 (58.5)	31 (58.5)	0

* Pregnancy rates determined by ultrasound at day 60 after insemination

**Lambing rates determined by mammary gland assessment after the expected lambing date as per Evans and Maxwell (1987)

[a,b]Within insemination method, different superscripts denote significant differences ($p<0.05$).

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