# The effect of exogenous hormones on ovine cervical mucus assessed through the penetrability by spermatozoa *in vitro* and pH *in vivo*.

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## Abstract

The influence of exogenous hormones on ovine cervical mucus was investigated through the assessment of sperm migration in a validated Cervical Migration Test (CMT) and monitoring the pH of cervical mucus *in vivo*. The preparation and storage methods for cervical mucus were first validated in order to determine the optimal conditions for maintaining mucus quality for CMTs. Sperm vanguard measurements in fresh and frozen cervical mucus were similar ($P>0.05$), however, these measurements were significantly reduced in chilled mucus ($P<0.05$). The validated CMT was then used to assess the ability for frozen-thawed ram spermatozoa to migrate through mucus obtained from ewes that were naturally cycling (NAT), synchronised (P4), or superovulated (SOV). The effect of these hormones used for controlled breeding purposes on mucus pH was concurrently investigated. Hormone treatment had no effect on the distance travelled by the vanguard spermatozoon ($P>0.05$), but the probability for spermatozoa to reach 1 cm in cervical mucus was significantly lower in SOV ewes compared to NAT ewes ($P<0.05$). In addition, there was no difference observed in the mucus pH between hormone treatments however, mucus obtained during the follicular phase had a significantly lower pH than luteal phase mucus ($P<0.05$). These results indicate that natural mucus for use in CMTs can be previously frozen for extended periods without compromising mucus quality.

Furthermore, although hormone administration did not appear to alter the pH of cervical mucus, treatment for superovulation may cause other changes in mucus that result in reduced sperm migration through the cervix, especially when utilising frozen-thawed spermatozoa.

## Introduction

Cervical mucus is a fundamental component of the female reproductive tract due to its ability to act as a physical and chemical barrier to pathogens and spermatozoa entering the uterus (Senger 2003; Curlin and Bursac 2013). However, during ovulation, cervical mucus becomes more receptive to spermatozoa and permits transport through the tract to enable fertilisation (Sujan *et al*. 1963). Nonetheless, the cervix remains a challenging barrier for frozen-thawed ram spermatozoa as evidenced by extremely low fertility following cervical artificial insemination (AI) of ewes, but acceptable fertility following intrauterine AI (Salamon and Maxwell 1995). The exact cause of this phenomenon is unknown, but an abnormal interaction between frozen-thawed spermatozoa and cervical mucus is implicated.

Variation in the penetrability of cervical mucus is driven by the fluctuation of endogenous hormones, which has been demonstrated in a number of species (Morales *et al.* 1993; Katz *et al.* 1997 [humans]; Heydon and Adams 1979 [sheep]; Haas *et al.* 1987 [rabbit]). The fluctuation in the properties of mucus is clearly illustrated during ovulation, where oestrogen dominance promotes abundant, low viscosity mucus, whereas under progesterone dominance in the luteal phase, mucus is scant and viscous (Curlin and Bursac 2013). In sheep, the administration of exogenous hormones for controlled breeding purposes, has been shown to alter endogenous hormone levels (Pearce and Robinson 1985) and as a result, this may have a negative effect on the delicate balance between the influence of endogenous hormones and mucus penetrability for spermatozoa.

The effect of exogenous hormones on cervical mucus and the resulting impact on sperm migration in sheep has been demonstrated in several studies. Rexroad and Barb (1977) observed that the protein concentration of cervical os and vaginal mucus obtained

from ewes treated with higher doses of progestagen, was increased and negatively correlated with the Spinnbarkheit or mucus elasticity. The study additionally found that the treatment of ewes with progestagens caused significant increases in cervical mucus production during oestrus, potentially leading to the reduction of spermatozoa in the cervical canal (Rexroad and Barb 1977). This conclusion was supported by a similar study, in which a decreasing trend in sperm numbers was discovered in the anterior cervix of progestagen treated ewes, 12 hours after insemination with fresh spermatozoa (Quinlivan and Robinson 1969). Furthermore, Hawk *et al.* (1987) investigated the impact of utilising Follicle Stimulating Hormone (FSH) for superovulation in ewes, and found that sperm numbers were significantly lower in the middle and anterior segments of the cervix, as well as the upper reproductive tract, when compared to untreated ewes 23 hours after insemination with fresh spermatozoa.

Although this previous research provides evidence for alterations in cervical mucus as a result of administrating exogenous hormones, current knowledge in this area is limited. In particular, further investigation is required on the influence of these hormones on the properties of mucus and the resulting impact on sperm migration. It has been reported that the pH of cervical mucus varies over the reproductive cycle (Eggert-Kruse *et al.* 1993) and that it has a strong bearing on sperm migration through the tract (Peek and Matthews 1986; Bartoov *et al.* 1980). Due to the close relationship between endogenous hormones and the variation in mucus properties, the use of exogenous hormones in sheep may result in changes to the pH of cervical mucus, which could have a detrimental effect on the ability for spermatozoa to migrate through the cervix.

Moreover, as many of these previous studies were conducted *in vivo*, it would be useful to investigate changes in cervical mucus through *in vitro* methods, such as Cervical

Migration Tests (CMTs). CMTs provide a means of assessing the ability of spermatozoa to migrate through mucus, which can be related to the migration capacity *in vivo* (Mortimer *et al.* 1990; Cox *et al.* 2002; Martinez-Rodriguez *et al.* 2012). In addition, the use of CMTs is favourable for sperm-mucus investigations as this method is less invasive as well as being an applicable and practical option in comparison to *in vivo* methods of measurement. However, the validation of appropriate preparation and storage methods for ovine cervical mucus used in CMTs has not been extensively investigated. Therefore, the optimisation of these methods ensures mucus quality is maintained for CMTs, in order to accurately identify the effect of cervical mucus on sperm migration.

In addition, the majority of previous studies in this field have utilised fresh spermatozoa to observe changes in cervical mucus following hormone treatment. Consequently, the use of frozen-thawed spermatozoa in the current study provided an insight on how the effect of these hormones on cervical mucus might reduce the fertility in sheep after cervical AI.

Further knowledge of the factors that influence fertility in sheep, particularly the effect of administrating exogenous hormones on cervical mucus, will be beneficial for the continued development and refinement of techniques to improve the success of cervical AI with frozen-thawed spermatozoa. Therefore, the aims of the current investigation were to 1) validate the preparation and storage methods for ovine cervical mucus to be used in CMTs and 2) investigate the effect of different exogenous hormones on the migration of spermatozoa through ovine cervical mucus *in vitro* and on the pH of mucus *in vivo*.

## Materials and Methods

Procedures herein were approved by The University of Sydney's Animal Ethics Committee.

### *Experiment 1: Validation of a Cervical Migration Test*

#### *Oestrus synchronisation and cervical mucus collection*

Oestrus was synchronised in mature Merino ewes (n=5), aged 24-36 months, during the breeding season (February-March) with progestagen sponges (Flugestone acetate; Ova-Gest®; 30mg; Bioniche Animal Health (A/Asia) Pty Ltd, Armidale, Australia) for 12 days, followed by an intramuscular injection (i.m.) of pregnant mare serum gonadotropin (PMSG; Pregnecol™; 400IU; Bioniche Animal Health (A/Asia) Pty Ltd, Armidale, Australia) at the time of sponge removal. Cervico-vaginal mucus was aspirated from each ewe during oestrus using a modified cervical insemination pipette and mucus samples were pooled into a single sterile tube.

#### *Preparation and storage treatments for cervical mucus*

Pooled cervical mucus was evenly divided into two components, with one component centrifuged (Spun; 1228 x *g*, 10 min) to remove cellular debris and the other component remaining at room temperature (Not Spun). The Spun and Not Spun samples were further divided and transferred into sterile tubes for storage at either room temperature (Fresh), 4°C (Chilled) or in liquid nitrogen after snap freezing (LN; Frozen). The Chilled and Frozen samples were further divided into three components and were stored under the specified conditions for several hours after mucus collection (D0), 7 days (D7) or 28 days (D28) after collection, while Fresh samples were only stored on D0. Artificial mucus (Control) was made on each day of assessment (D0, D7, D28) by diluting MAP-5

(Sodium hyaluronate; 10mg/mL; Bioniche Animal Health, Belleville, Ontario, Canada) with 33% warmed Androhep (Minitube Australia; Smythes Creek, Australia), supplemented with 1% w/v bovine serum albumin (BSA; Sigma-Aldrich, Australia). Three replicate CMTs were performed for each of the cervical mucus samples and control on each day of assessment (Fig. 1).

### *Cervical Migration Tests*

After storage, cervico-vaginal mucus samples and artificial mucus were warmed to 37°C and aspirated into flat, glass capillary tubes (0.3 x 3.0mm; Microslides; VitroCom, Mountain Lakes, NJ, USA) using a syringe, with care taken to avoid air bubbles. The tubes were sealed with Cristaseal (Hawksley, London, UK) at one end and kept at 37°C. Prior to this experiment, ejaculates from three Merino rams were pooled and diluted to 80 x $10^6$ spermatozoa/mL with Tris-citrate glucose (3.6 % Tris base, 0.5% Glucose, 15% egg yolk, 5% glycerol, 2% Citric acid, Penicillin 0.006%, Streptomycin 0.005%). The pooled sample was then chilled to 5°C and frozen in 200µl aliquots (250µL straws, IMV; L'Aigle Cedex, France), as described by Evans and Maxwell (1987). Straws were suspended in liquid nitrogen vapour for 6 minutes, then submerged in liquid nitrogen and stored until required. Cryopreserved semen for use in CMTs was thawed (37°C with agitation) and the progressive motility was assessed. Semen was diluted with warmed Androhep to 20 x $10^6$ motile spermatozoa/mL, stained 1:1 with Hoechst stain (semen : stain, v/v (H333342; 20µg/mL; Sigma-Aldrich, Australia)) and incubated (37°C, 5mins). Capillary tubes were inserted vertically into polyethylene capsules (BEEM; ProSciTech, Thuringawa, Australia) containing 50µL of the stained semen and were incubated (37°C, 1hr). After incubation, the open end of the capillary tube was sealed and spermatozoa were immobilised by rapid

heating (50°C; 30s) and cooling (-27°C, 1 min). The number of spermatozoa that travelled to 1 cm in mucus and the vanguard measurement (furthest travelled spermatozoon) were then assessed under fluorescent microscopy (200 X; 355-465nm; Olympus BX51).

## *Experiment 2: Effect of exogenous hormones on the penetrability and pH of ovine cervical mucus*

### *Hormone treatments*

The present study was performed over two periods (March-April and May-June) during the breeding season (February-June). Sixty-five mature Merino ewes, aged 24-36 months, were randomly assigned to one of the three following treatments: Natural oestrus (NAT; n=21), Synchronised oestrus (P4; n=22) and Superovulated (SOV; n=22). Oestrus was synchronised in all ewes using progestagen sponges (Flugestone acetate; Ova-Gest®; 30mg; Bioniche Animal Health (A/Asia) Pty Ltd, Armidale, NSW, Australia) for a period of 12 days and an i.m. injection of PMSG (Pregnecol™; 400IU; Bioniche Animal Health (A/Asia) Pty Ltd, Armidale, NSW, Australia) was administered at sponge removal. In NAT ewes, mucus collections were performed in the subsequent natural oestrus period following sponge removal, which was unaffected by hormone treatment. In both P4 and SOV ewes, mucus collections were performed in the synchronised oestrus period. All treatments were applied so as to ensure oestrus occurred at the same time for each treatment. SOV ewes were superovulated as described previously (de Graaf 2010). In brief, ewes were administered i.m. injections of Follicle Stimulating Hormone (FSH; Folltropin®-V; 22mg; Bioniche, Belleville, Ontario, Canada) following sponge insertion (day 0), with administration once on day 10 and 13 and twice on day 11 and 12.

### *Detection of oestrus and hormone concentration*

Wethers used to detect oestrus were initially treated with an i.m. injection of testosterone (Testosterone proprionate; Testoprop; 50mg/mL; Jurox, Rutherford, NSW, Australia) following sponge insertion in P4 and SOV ewes, with injections repeated every 7 days for two weeks. NAT, P4 and SOV ewes were then allowed to run with the crayon harnessed testosterone treated wethers following sponge removal and oestrus was detected by a crayon mark. In addition, blood samples were obtained daily by jugular venipuncture into lithium-heparinised tubes for a total of 11 days over each experimental period to measure the levels of circulating oestrogen, specifically oestradiol ($E_2$), and progesterone ($P_4$). The collected blood was centrifuged at room temperature (1228 x *g*, 15 min) and the supernatant was removed and stored at -20°C until hormone assays were performed. Wether blood was used as a control in the hormone assays. Serum samples were analysed using the validated radioimmunoassays (RIA) Coat-A Count Progesterone kit (Siemens Medical Solutions Diagnostics, Los Angeles, California, USA) for $P_4$ concentrations and Beckman Coulter Ultra-Sensitive Estradiol RIA (Bechman Coulter, Webster, Texas, USA) for $E_2$ concentrations. In collection period 1, the reference sera with mean $P_4$ concentrations of 0.55ng/mL and 10.5ng/mL obtained intra-assay CV of 13% and 1%, for low and high samples respectively. To improve the sensitivity of the $E_2$ assay, a parallelism between dilutions of a serum sample to the respective standard curve preparations was performed. For collection period 1, the reference sera with mean $E_2$ concentrations of 18.05pg/mL and 242.7pg/mL obtained inter-assay CV of 12% and 2%, respectively. For collection period 2, the reference sera with mean $E_2$ concentrations of 21.76pg/mL and 250pg/mL obtained intra-assay CV of 18% and 5% and inter-assay CV of 2% and 4%,

respectively. The sensitivity of the $P_4$ and $E_2$ assays was 0.02ng/mL and 1.5pg/mL respectively.

### *Measurement of cervical mucus pH in vivo*

During the second period of this experiment, the pH of cervico-vaginal and cervical os mucus was measured prior to mucus collection at oestrus and at the mid-luteal phase using a portable pH probe (Sentron; Roden, Netherlands). The probe tip was fully immersed in mucus before recording the pH value and the tip was cleaned thoroughly between each measurement to prevent cross contamination of samples. In addition, the probe was calibrated periodically to obtain the most reliable pH values.

### *Mucus collection*

Separate modified cervical insemination pipettes attached to 20mL syringes were prepared for each of the three hormone treatment groups as well as the two collection sites. Prior to collection, the vulva was cleaned to prevent sample contamination. Cervico-vaginal and cervical os mucus was aspirated from each ewe during oestrus and the mid-luteal phase. Samples were centrifuged (1228 x *g*, 10 min) to remove debris and were kept at room temperature until required for use in CMTs. All instruments were thoroughly cleaned to prevent cross contamination between mucus samples.

### *Cervical Migration Tests*

Three replicate flat, glass capillary tubes (0.3 x 3.0mm; Microslides; VitroCom, Mountain Lakes, NJ, USA) were filled with each mucus sample using a syringe and the CMTs were prepared and assessed as per the protocol in Experiment 1.

### *Statistical analysis*

Statistical analyses were performed using GENSTAT (16th Edition; VSN International, Hemel Hempstead, UK). Restricted maximum likelihood (REML) was used to determine the effect of the designated treatments on vanguard measurements in both Experiment 1 and 2 as well as the pH measurements in Experiment 2. For the analysis of sperm counts in both experiments, a Poisson general linear mixed model (GLMM) was tested and deemed unsuitable due to the large number of low sperm counts and over-dispersion of the data about the model. Consequently, the sperm counts were reallocated as the success or failure for spermatozoa to reach 1 cm in cervical mucus, where counts ≥1 were considered a success and a zero count was a failure. A Binomial GLMM was then used to analyse these observations to determine the effect of treatments and the results were interpreted as the probability for spermatozoa to reach 1 cm. A natural logarithm transformation was performed to attain normality where required. The results are presented as the mean ± s.e.m (standard error of the mean), except for the sperm counts analysis, which is presented as the mean probability ± s.e.m, and $P<0.05$ was considered significant.

## Results

### *Experiment 1: Validation of preparation and storage methods for cervical mucus to be used in CMTs*

#### *Migration of spermatozoa through treated and artificial cervical mucus*

The distance travelled by the vanguard spermatozoon was dependent on the interaction between the day of storage and storage method ($P<0.05$; Fig. 2). The vanguard spermatozoon travelled furthest in Fresh (D0) cervical mucus compared to Chilled mucus

on all storage days (P<0.05; D0, 7, 28). However, there was no significant difference in the vanguard distance on any storage day between Fresh cervical mucus and Frozen mucus, after thawing and warming to 37°C (P>0.05). The use of artificial mucus compared to all the other treatments on each day presented the greatest distances travelled by the vanguard, except on Day 7 (P>0.05).

In contrast to the vanguard measurements, the probability that spermatozoa would reach 1 cm in cervical mucus was dependent on the interaction between the day of storage and whether the mucus was centrifuged (P<0.05). The probability between Spun and Not Spun mucus samples was similar on Day 0 of storage (P>0.05). Although, as the storage day progressed, the probability that spermatozoa would reach 1 cm increased when cervical mucus was Not Spun (P<0.05), while in Spun mucus, this probability decreased (P<0.05; Fig. 3). It was additionally observed in mucus samples not centrifuged, that there were a large number of vaginal epithelial cells within CMTs, which created difficulties in assessing the number of spermatozoa that had reached 1 cm.

## *Experiment 2: The effect of exogenous hormones on the penetrability and pH of ovine cervical mucus*

### *Plasma progesterone and oestrogen concentration*

The concentrations of $P_4$ and $E_2$ over both collection periods are displayed in Fig. 4 and 5 respectively. The concentration of $P_4$ for each hormone treatment followed similar trends overall, with the concentration initially decreasing to plateau at levels <1 ng/mL between day 4 and 5 of blood collection, before rising again on days 6 to 11. There were clear differences in the $E_2$ concentration between hormone treatments, particularly

between SOV and P4 ewes during oestrus. The concentration of $E_2$ was highest in SOV ewes on day 4 of blood collection in comparison to the other treatments. In NAT ewes, although there was no distinct increase in $E_2$ during the timed oestrus period, there was evidence of a slight increase in the $E_2$ concentration on day 3 of blood collection before declining as the days progressed. These results confirm that hormone administration was successful for each treatment group and that the timing of treatment resulted in the occurrence of oestrus during the same time period for all treated groups.

### *Migration of spermatozoa through cervical mucus obtained from ewes in natural oestrus and hormone treated*

There was no significant effect of exogenous hormones on the distance travelled by the vanguard spermatozoon ($P>0.05$).

Conversely, the effect of treatment was significant for the probability that spermatozoa would reach 1 cm in cervical mucus ($P<0.05$), which is displayed in Fig. 6. It was more likely for spermatozoa to reach 1 cm in NAT ewes compared to SOV treated ewes ($P<0.05$; NAT: 79±15%; SOV: 44±21%). However, this probability was similar in NAT ewes and P4 ewes ($P>0.05$; NAT: 79±15%; P4: 63±20%).

### *Measurement of cervical mucus pH in vivo*

There was no significant effect of exogenous hormones or collection site on the pH of cervical mucus but the pH significantly differed between phases of the oestrous cycle ($P <0.05$; Fig. 7). During the follicular phase, cervical mucus was more acidic compared to the luteal phase ($P<0.05$; Follicular phase: 6.4±0.037; Luteal Phase: 6.8±0.045).

## Discussion

The results of this study demonstrate that exogenous hormones commonly used in breeding programs exhibits an effect on cervical mucus through changes in sperm migration, specifically following FSH treatment for superovulation. This suggests that administration of FSH may change the properties of cervical mucus, which could impede the transit of spermatozoa through the cervix. Hormone administration did not influence the pH of mucus, but rather the pH fluctuated over the oestrous cycle. Furthermore, this study has validated the preparation and storage methods for ovine cervical mucus used in CMTs. Cervical mucus that is not centrifuged and is either fresh or frozen-thawed is most appropriate for use in CMTs to ensure mucus quality is maintained and that sperm migration can be reliably assessed. These results are of importance, specifically the effect of exogenous hormones on cervical mucus, as this could be a potential factor leading to the reduced fertility observed following cervical AI with frozen-thawed ram spermatozoa.

Interestingly, the storage of cervical mucus under various conditions for up to 28 days presented clear differences in the sperm vanguard distance between fresh and chilled mucus. The significantly lower vanguard measurement obtained in chilled mucus on day 0, 7 and 28, indicates that the properties of cervical mucus were altered as a result of the type and duration of storage, thus causing the observed impediment to sperm migration. This finding challenges what has been considered an acceptable means of storing cervical mucus (Ulstein 1972), particularly in fertility diagnostics where in humans, it has been proposed as the preferred method of storage (WHO 2010). However, in a similar study investigating the effect of storage temperature and duration on bovine cervical mucus, a reduction in the migration of spermatozoa in mucus chilled to 4°C and stored for up to 21 days was also observed, although this effect only approached statistical significance

(Kummerfield *et al.* 1981). The reduction in sperm migration in chilled mucus as observed in this study, provides evidence that this storage method may compromise mucus quality and could confound results obtained in fertility investigations.

Whilst chilling mucus reduced sperm migration, the vanguard measurements in frozen-thawed mucus were similar to those obtained in fresh mucus. This suggests that freezing and thawing cervical mucus does not adversely impact its quality, the first known report of this finding in sheep. Interestingly, previous investigations utilising bovine cervical mucus verified the ability to freeze mucus for prolonged periods of time with no detrimental impact to sperm migration or mucus quality (Lee *et al.* 1981; Murase and Braun 1990). Therefore the finding in this current study presents the opportunity to freeze and store ovine cervical mucus for a period of time without affecting the quality, which is particularly useful for application in field studies and future investigations requiring the use of previously collected mucus.

Unexpectedly, the probability that spermatozoa would progress to 1 cm in cervical mucus was significantly reduced in centrifuged mucus in comparison to mucus that was not centrifuged, except on the first day of storage. Despite the lack of research investigating the negative effect of centrifugation on cervical mucus, it can be postulated that this method may distort the delicate microstructure within mucus that enables spermatozoa to migrate effectively. During the ovulatory period, this microstructure forms as a loose network of fibres or a mesh that permits transport through the cervix (Wergin 1979 [sheep]; Chretien 2003 [humans]), and therefore, alteration to these important structures through centrifugation, may be the cause for the reduced likelihood of sperm migration in Spun samples.

Although cervical mucus that was not centrifuged obtained a greater probability for sperm migration, it was interesting to note that there were a large number of vaginal epithelial cells within the CMTs. The presence of these cells is likely an artifact from scraping the vaginal wall during mucus collection and as a result, could be a source of variability in the results obtained due to difficulty associated with counting spermatozoa. Consequently, this could create additional problems for the standardisation of results, as the number of these cells may vary greatly between different mucus samples and also between different replicates of the same sample. Therefore under these circumstances, it may be recommended to centrifuge cervical mucus to remove cellular debris in order to reduce the variability associated with the presence of these epithelial cells, however this requires further investigation.

The effect of exogenous hormones on cervical mucus was clearly evident in this study through the reduced probability for spermatozoa to progress to 1 cm in mucus, particularly following the administration of FSH to superovulate ewes. The reduction in sperm migration following FSH administration has been previously shown by Hawk *et al.* (1987), where sperm numbers were significantly lower in the middle and anterior cervix of treated ewes in comparison to those naturally ovulating. In a related study, Evans and Armstrong (1984) similarly observed a reduction in sperm numbers recovered in the uterus and oviducts of ewes following superovulation with FSH, but there was no evidence of impairment to fertility. This previous research not only supports the results of this investigation, it additionally validates the use of a CMT in this study as a means of assessing sperm migration through mucus. This conclusion is verified by the comparable findings of the current study and other studies utilising *in vivo* methods of migration assessment, by the dissection or flushing of the ewe reproductive tract. Nonetheless, the

observed reduction in sperm migration after FSH treatment in this study is supported by the results of previous investigations, and therefore it is suggested that alterations in cervical mucus following hormone treatment, could attribute to changes in sperm transport.

With respect to the effect of FSH on cervical mucus, it is evident that the exposure to surging $E_2$ levels present in SOV ewes (Fig 5.), could contribute to alterations in the properties of cervical mucus, potentially leading to changes in sperm migration. The effect of increasing $E_2$ concentrations on mucus properties was demonstrated by Adams and Sanders (1988), who reported that the Spinnbarkheit of cervical mucus was significantly reduced in naturally ovulating ewes that were repeatedly stimulated with oestradiol-17β over a period of 40 months. Furthermore, it was also found that the number of spermatozoa within the anterior cervix was also lowered following $E_2$ treatment in comparison to naturally ovulating ewes (Adams and Sanders 1988). The effect of oestrogen on the properties of cervical mucus is further demonstrated by Rexroad and Barb (1977), where ewes treated with oestradiol-17β were found to have significantly higher concentrations of protein within mucus compared to non-treated ewes. Despite the fact that ewes in the current study were not directly administered $E_2$ over prolonged periods of time, the rising $E_2$ concentration in SOV ewes could have a similar mode of action on the properties of cervical mucus. Consequently, these changes to mucus properties could have detrimental impacts on the ability for spermatozoa to traverse the cervix.

Although there was a definite influence of FSH treatment on cervical mucus, there was no significant difference in the probability of spermatozoa reaching 1 cm in mucus between progestagen synchronized (P4) or naturally cycling (NAT) ewes. This suggests that

the administration of the progestagen treatment caused little or no changes in the properties of cervical mucus, as the likelihood of sperm migration was similar in P4 and NAT ewes. As a consequence, this reassures the routine use of this particular progestagen in breeding programs to synchronise oestrus. However, this finding does contradict the results of earlier studies, where the administration of progestagens to ewes resulted in a considerable decline in sperm numbers within each segment of the cervix and upper reproductive tract, suggesting a hindrance in efficient sperm transport through the cervix (Quinlivan and Robinson 1969; Hawk and Conley 1975; Pearce and Robinson 1985).

Despite the proposed effect of progestagens on sperm transport, the insignificant impact of this treatment in the current study could be related to the effect of different progestagen doses on mucus properties. This conclusion is supported by Rexroad and Barb (1977) who reported higher Spinnbarkheit measurements and lower protein concentrations in cervical mucus obtained in ewes that were treated with a low dose (30mg) of the progestagen, 17*α*-acetoxy-9*α*-fluoro-11*β*-hydroxypregn-4-ene-3,20-dione (Cronolone), compared to the high dose (60mg). In addition, a marked decline in sperm numbers was recovered in the oviducts of ewes treated with 0 or 90mg of Cronolone compared to 30mg, which further demonstrates the influence of progestagen dose on mucus properties and the effect on sperm migration (Allison and Robinson 1970). In the case of this study, the dose of progestagen administered must have been optimal to synchronise oestrus without causing the adverse effects associated with this treatment, which is confirmed by the comparable probabilities of sperm migration through mucus in both P4 and NAT ewes.

The effect of exogenous hormones on ovine cervical mucus was not reflected in the distance travelled by the vanguard spermatozoon, despite the significance of the

probability that spermatozoa would migrate to 1 cm in mucus. This conflicting result obtained by the two alternative measures of sperm transport can be explained in terms of how these methods quantify different attributes of migration within mucus. Whilst the vanguard distance reflects the maximum capabilities of spermatozoa, this may not necessarily apply to the whole population, in which case the vanguard measurement may only partially explain the differences observed in migration between samples of cervical mucus (Katz *et al.* 1982). In addition, it has been suggested that sperm counts, or in the case of this study, the probability of sperm migration, is a more accurate diagnostic criterion for sperm function than vanguard distance in CMTs (Ola *et al.* 2003). As these two measures evaluate sperm migration through cervical mucus differently, this justifies the significance observed for the probability that spermatozoa will migrate to 1cm following hormone administration.

Through the measurement of pH *in vivo,* it was possible to examine the influence of exogenous hormones on the biochemical properties of cervical mucus in ewes. While these measurements did not differ between ewes treated with exogenous hormones and those naturally cycling, there was a definite difference in the pH between the luteal and follicular phase of the oestrous cycle. As there are very few studies investigating the fluctuation in pH over the oestrous cycle in sheep, this study provides strong evidence that there is a cyclic variation in the pH of cervical mucus. However, the significance of changes in pH over the cycle with respect to cervical mucus and sperm migration is yet to be elucidated, despite the agreement that the pH is considered an important determinant of sperm-mucus interactions (Eggert-Kruse *et al.* 1993). The variation in pH over the cycle has been found to play an essential role in regulating the expansion of fundamental glycoproteins or mucins after exocytosis from the secretory epithelium in the cervix

(Espinosa *et al.* 2002). Espinosa *et al.* (2002) observed that under acidic conditions, mucins exocytosed from human cervical cells expanded slower and the hydration capacity additionally reduced, resulting in poorly hydrated, viscous cervical mucus. In contrast, Wang *et al.* (2013) illustrated that major changes in pH ranging from 1-2 through to 8-9 only resulted in minor alterations to the microstructure of mucus and its physical properties, which contradicts the notion that the pH has a strong bearing on changes in mucus properties. This current study additionally challenges the importance of fluctuations in pH, as significant differences in sperm migration following hormone treatment were observed despite the similar pH measurements of mucus within each treatment group. Therefore, this suggests that there must be changes to other properties within cervical mucus that have caused the alteration to sperm migration, particularly after FSH treatment. However, continued research is required to further understand the importance of cyclic variation in mucus pH and how this may influence additional changes in mucus properties.

## Conclusion

This study demonstrates that the effect of particular exogenous hormones on ovine cervical mucus is evident due to the changes observed in sperm migration within CMTs. Through the validation of different preparation and storage methods for cervical mucus, it was found that it could be used frozen-thawed in CMTs, offering the opportunity to preserve mucus for future investigations without compromising quality. Most importantly, following the administration of FSH for superovulation, sperm migration through cervical mucus was reduced, which provides compelling evidence that particular hormone treatments can cause detrimental changes to the properties of mucus. Moreover, although

there was no effect of hormone treatment on mucus pH, this study illustrated that the pH does vary over the oestrous cycle in sheep. Judging by the results of the present study, further investigation of the influence of hormone administration on the biochemical, structural and physical properties of cervical mucus is required. Through this research, it will provide a greater understanding of how these factors can affect the ability for spermatozoa to efficiently migrate through mucus, especially following cervical AI with frozen-thawed ram spermatozoa.

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## Figures

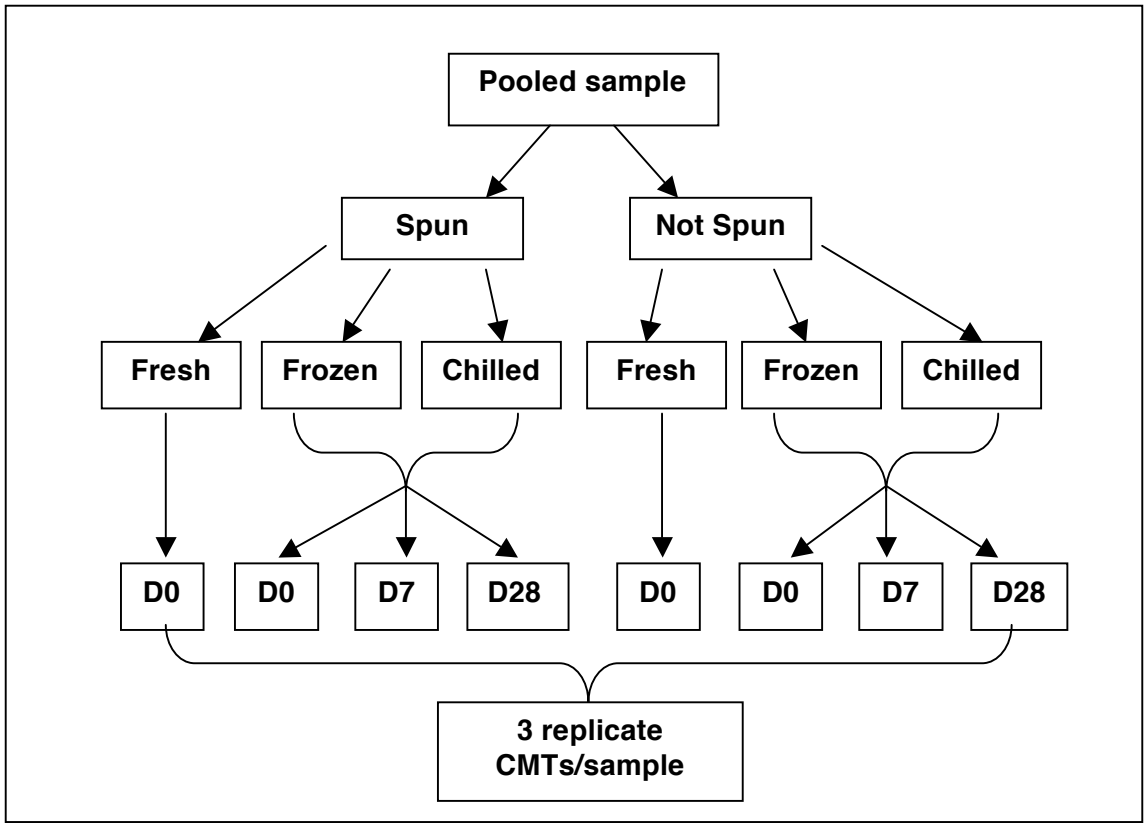


**Figure 1.** Diagram presenting the experimental design for the validation of preparation and storage methods for ovine cervical mucus used in Cervical Migration Tests (Experiment 1).

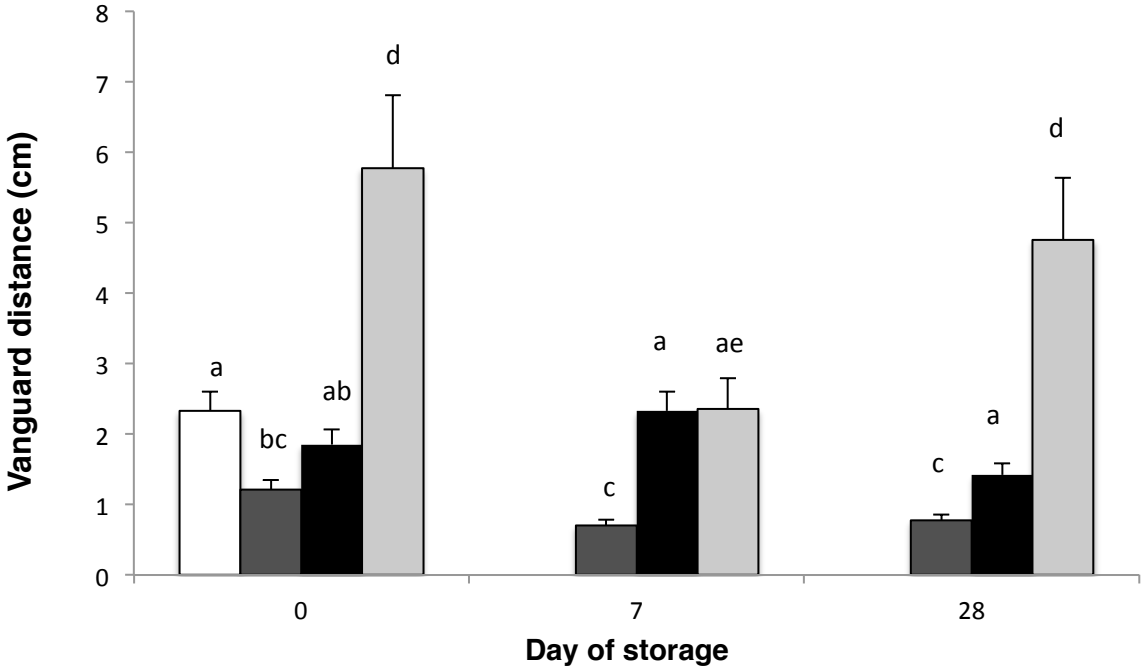


**Figure 2.** The mean vanguard distance (cm) in Fresh (□), Chilled (■), Frozen (■) and Artificial (□) mucus over each day of storage (D0, D7 and D28). Treatments with different letters denote significant differences (P<0.05). Data are geometric means ± s.e.m.

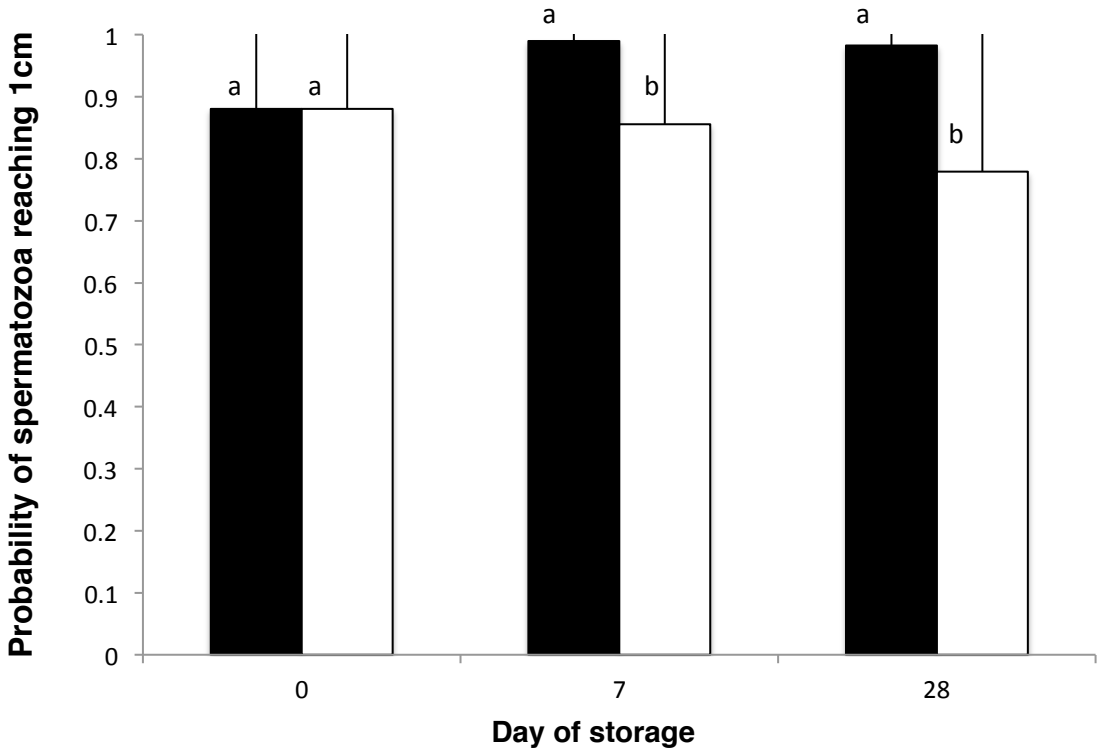


**Figure 3.** The probability of obtaining spermatozoa at 1 cm in cervical mucus that is Not Spun (black) or Spun (white) over each storage day (D0, D7 and D28). Within storage days, different letters denote significant differences (P<0.05). Data are probabilities ± s.e.m.

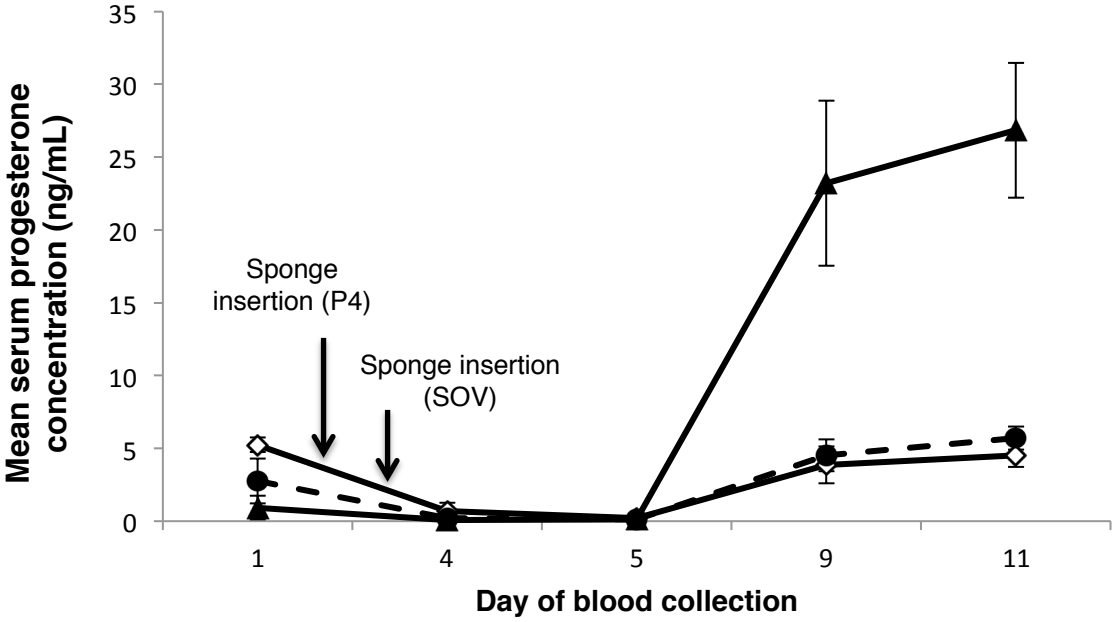


**Figure 4.** Progesterone concentrations for NAT (◊), P4 (-●-) and SOV (▲) ewes over 11 days of blood collection. The time of sponge insertion for P4 and SOV ewes is indicated. Data are means ± s.e.m.

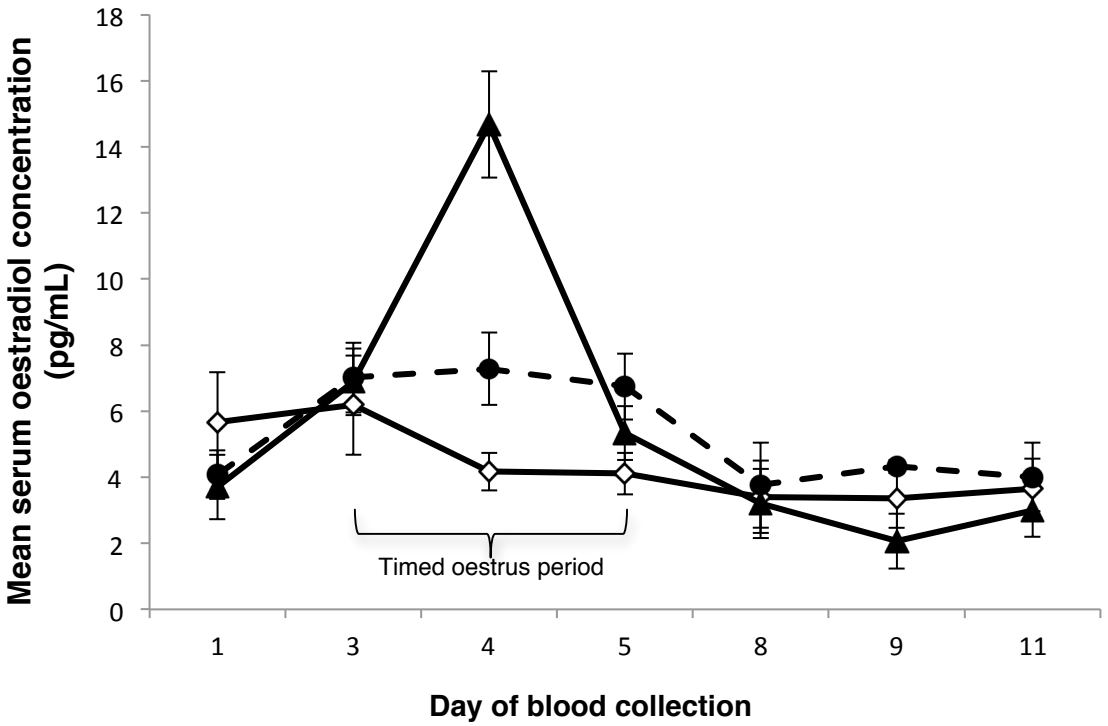


**Figure 5.** Oestradiol concentrations for NAT (◊), P4 (-●-) and SOV (▲) ewes over 11 days of blood collection. Timing of the oestrus period for all treatments is indicated. Data are means ± s.e.m.

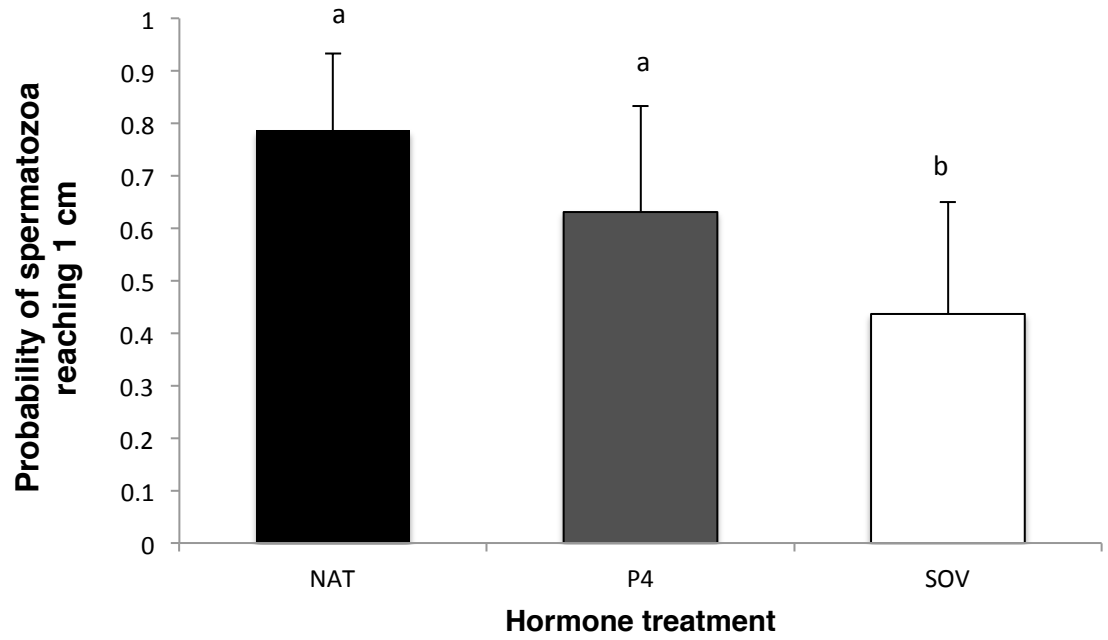


**Figure 6.** The probability that spermatozoa would reach 1 cm in cervical mucus obtained from ewes that are naturally cycling (NAT; black), synchronized (P4; grey) or superovulated (SOV; white). Hormone treatments with different letters denote significant differences ($P<0.05$). Data are probabilities ± s.e.m.

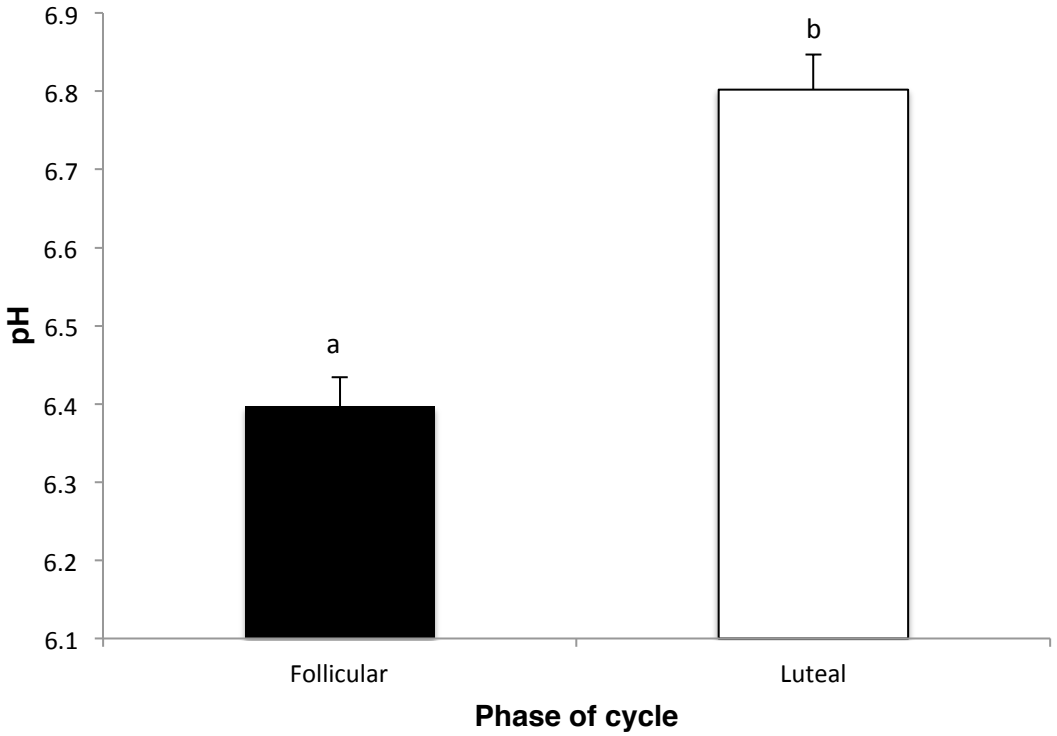


**Figure 7.** The pH of cervical mucus during the follicular (black) or luteal phase (white). Phases with different letters denote significant differences (P<0.05). Data are means ± s.e.m.

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