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Ewe breed differences in cervical anatomy and cervicovaginal mucus properties: An international study

L. Abril-Parreño [a, b], A.K. Krogenæs [c], C.J. Byrne [d], A. Donovan [e], S. Stuen [f], E. Caldas [g], M. Diskin [e], X. Druart [g], S. Fair [a, *]

[a] Laboratory of Animal Reproduction, School of Natural Sciences, Biomaterials Research Cluster, Bernal Institute, Faculty of Science and Engineering. University of Limerick, Limerick, V94 T9PX, Ireland
[b] Animal & Bioscience Research Department, Animal & Grassland Research and Innovation Centre, Teagasc Grange, Dunsany, Co. Meath, C15 PW93, Ireland
[c] Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, P.O. Box 369, Sentrum, Oslo, 0102, Norway
[d] School of Veterinary and Life Sciences, Murdoch University, 90 South Street, Perth, WA, 6150, Australia
[e] Teagasc, Animal & Grassland Research and Innovation Centre, Mellows Campus, Athenry, Co. Galway, H65 R718, Ireland
[f] Norwegian University of Life Sciences, Faculty of Veterinary Medicine, Department of Production Animal Clinical Sciences, Sandnes, Norway
[g] UMR PR China, INRA 85, CNRS 7247, Université de Tours, IFCE, Physiologie de La Reproduction et des Comportments, Institut National de La Recherche Agronomique, Nouzilly, 37380, France

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ABSTRACT

In sheep, cervical artificial insemination (AI) involves depositing semen at the cervical opening, as it is not possible to traverse the cervix due to its complex anatomy. However, internationally this method yields low pregnancy rates when frozen-thawed semen is used. An exception to this is in Norway, in which vaginal deposition of frozen-thawed semen to a natural estrus yields pregnancy rates around 70%. As the cervix and its secretions are the principal factors influencing sperm transport to the site of fertilization the aim of this study was to characterise the differences in the cervical anatomy as well as the cervicovaginal mucus properties of six European ewe breeds across three countries known to have differences in pregnancy rates following cervical AI with frozen-thawed semen. These were Suffolk and Belclare in Ireland, Fur and Norwegian White Sheep (NWS) in Norway and Ile de France and Romanov in France (n = 28–30 ewes/breed). Cervicovaginal mucus was collected at the follicular and luteal phases of both a synchronized and natural cycle and assessed for mucus weight, viscosity and colour. The anatomical characteristics of the cervix (length of the cervix, number of cervical rings and the appearance of the external *os*) were assessed post-mortem. There was a type of the cycle by ewe breed interaction represented by no differences in mucus production between ewe breeds at the natural cycle for both the follicular and luteal phases of the cycle. However, there were differences between ewe breeds at the synchronized cycle ($P < 0.05$). Belclare had the lowest mucus production at the follicular phase while NWS had the lowest amount of mucus at the luteal phase of the synchronized cycle. Overall, across all ewe breeds, mucus production was higher at the follicular than at the luteal phase ($P < 0.05$). Despite reports of Suffolk and NWS having the most divergent pregnancy rates following cervical AI with frozen-thawed semen, both breeds had the lowest overall mucus viscosity at the follicular phase of both types of cycle with no differences between both ewe breeds ($P > 0.05$). The length of the cervix, number of cervical rings and the external os type were affected by ewe breed ($P < 0.05$). Suffolk ewes had longer cervices but lower number of cervical rings than NWS and Fur ewes (both with higher pregnancy rates). In conclusion, while mucus production and mucus viscosity was affected by breed, these changes are not consistent with the known differences between ewe breeds in their pregnancy rates following cervical AI with frozen-thawed semen.

1. Introduction

Artificial insemination (AI), when combined with the use of frozen-thawed semen, is probably the single most important tool for

* Corresponding author.
E-mail address: sean.fair@ul.ie (S. Fair).

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the genetic improvement in farm animals. This is particularly evident in cattle where there is an easy to use transcervical insemination method using frozen-thawed semen, which farmers/technicians can perform themselves on-farm and yields similar pregnancy rates/AI to natural mating. In cattle penetrating the cervix with an AI pipette and depositing the semen in the uterine common body or into the uterine horns is essential to achieve high pregnancy rates [1]. However, in sheep, it is not possible to penetrate the cervix and cervical or vaginal AI with frozen-thawed semen has consistently yielded unacceptable pregnancy rates of less than 30% worldwide (See review by Fair et al. [2]). The ovine cervix is a long fibrous organ composed of cartilaginous tissue, with a series of 4–7 cervical folds, the lumen of which, is not concentrically aligned [3,4] thus inhibiting the passage of an inseminating pipette. Therefore, the only effective method for AI in sheep using frozen-thawed semen is laparoscopic insemination, in which the semen is deposited into the uterine horns. However, this is an invasive surgical procedure and requires veterinary expertise, which limits its use to genetic improvement programmes involving high value animals.

An exception to this is in Norway, where vaginal (shot-in-the-dark) insemination, with frozen-thawed semen, at a natural estrus is performed by farmers and yields pregnancy rates/AI in excess of 70% in Norwegian White Sheep (NWS). The reason for the success in Norway has been the focus of a number of studies by our group. Earlier studies found no differences in ram/semen variables between countries but did identify significant ewe breed effects with pregnancy rates of 18, 28, 44 and 77% for Suffolk, Texel, Belclare and Finnish Landrace ewes respectively, following cervical AI using frozen-thawed semen [5,6]. These differences between breeds were not due to differences in hormonal profiles (luteinising hormone, oestrogen or progesterone) during the peri-ovulatory phase [7] or related to breed differences in the gross cervical anatomy [8]. Interestingly, while fertilization rates after laparoscopic AI were similar between Suffolk and Belclare ewes, both fertilization rates and accessory sperm number following cervical AI with frozen-thawed semen was higher in Belclare than the Suffolk ewes demonstrating that frozen-thawed spermatozoa can traverse the cervix in greater numbers in some ewe breeds (Belclare) than in other breeds (Suffolk).

In order to traverse the ovine cervix spermatozoa must battle against an outward flow of cervicovaginal mucus, which is a non-newtonian fluid secreted by the cervical epithelium and acts as a medium for protection, lubrication and transport. Richardson et al. [9] found no effect of ewe breed (Suffolk vs Belclare) on mucus pH or on ferning pattern (a measure of mucus hydration), but a higher number of spermatozoa penetrated the cervicovaginal mucus of Belclare than Suffolk ewes *in vitro*. Cervicovaginal mucus properties also change over the estrus cycle. It is well described that at the luteal phase, cervical mucus is less hydrated and more viscous [10], which is also accompanied by an increase in protein production [11–13], which results in cloudier mucus than that at the follicular phase [11].

Given the aforementioned ewe breed effects and the fact that AI is performed to a natural estrus in Norway, but to a synchronized estrus in other countries, we hypothesised that both ewe breed and synchronization could affect the mucus properties and the anatomical characteristics of the cervix. In order to address this hypothesis, we generated a novel experimental model of six ewe breeds across three countries: Ireland (Suffolk and Belclare), Norway (Fur and NWS) and France (Ile de France and Romanov). These ewe breeds have known divergent pregnancy rates following cervical AI with frozen-thawed semen. The objective was to assess (i) the properties (weight, viscosity and colour) of ovine cervicovaginal mucus collected at a synchronized and a natural estrus at both the follicular and luteal phase of the cycle, and (ii) the length of the cervix, the number of cervical rings and the appearance of the external *os*.

2. Material and methods

Ethical approval

Protocols were developed in accordance with the *Cruelty to Animals Act* (Ireland 1876, as amended by European Communities regulations 2002 and 2005) and the European Community Directive 86/609/EC. In Ireland, the study was approved by the Teagasc Animal Ethics Committee and all animal procedures performed were conducted under experimental license from the Health Products Regulatory Authority. In Norway, the study was approved by Norwegian Food safety Authority (FOTS ID 13168). In France, the study was approved by the ethics committee and the Ministry of Research.

2.1. Experimental design

The experiment was carried out during one season (September to February) across three countries in the northern hemisphere (Ireland, Norway and France) using six European ewe breeds: Suffolk (n = 29) and Belclare (n = 30) in Ireland; Fur (n = 28) and NWS (n = 28) in Norway and Ile de France (n = 30) and Romanov (n = 29) in France. The NWS breed are known to have the highest pregnancy rates reported worldwide following cervical/vaginal AI with frozen-thawed semen [14–16] while, Fur sheep in Norway have lower pregnancy rates (63% with over 10,000 vaginal inseminations across the 2013–2018 breeding seasons; Thor Blichfeldt, personal communication). In Ireland, Belclare ewes have been shown to have significantly higher pregnancy rates than Suffolk ewes following cervical insemination with frozen-thawed semen [17]. While there are no published pregnancy rate data following cervical AI with frozen-thawed semen in French ewe breeds, the Romanov and Ile de France are high and medium prolific breeds, respectively. Within each country, the breeds were managed under similar environmental and nutritional conditions. All ewes were maintained indoors for the duration of the experiment with ad libitum access to forage and clean water. In each replicate cervicovaginal mucus was collected from each ewe at the follicular (estrous) and luteal phases of a synchronized and then a natural cycle (Fig. 1). This was then replicated 3 times with the same animals over a period of approximately 6 months. All mucus collections were performed by trained personnel in each country. After completion of the third replicate of mucus collection, all ewes were slaughtered and their reproductive tracts recovered and sampled.

2.2. Synchronization of estrus and cervical mucus collection

Estrous cycles of multiparous ewes were synchronized using intravaginal progestagen vaginal sponges (20 mg Flugestone Acetate; Chronogest® vaginal sponges, Intervet, Boxmeer, The Netherlands). After 14 days, the sponges were removed and ewes were treated with equine chorionic gonadotropin (400 IU; Intervet, Boxmeer, The Netherlands). Cervicovaginal mucus was collected by aspiration at both the follicular (56 h post sponge removal = Day 0) and luteal phases (Day 9; Day −2 = day of sponge removal). At the natural cycle, all ewes were checked twice daily for signs of estrous over a 6 day period using a teaser ram with an apron fitted (no semen/seminal plasma was allowed to be deposited into the vagina of the ewe). The cervicovaginal mucus was collected by aspiration at both the follicular (12 h post detection of standing estrous, Day 0) and luteal phase (Day 9).

To collect the mucus from the cervix, the ewes were held in a standing position and a duckbilled speculum (IMV Technologies,

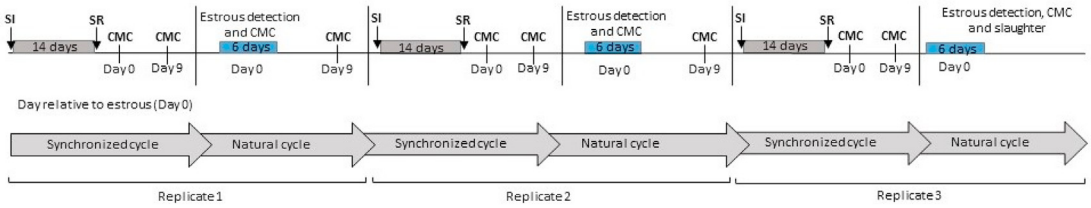

Fig. 1. Timeline of experimental model for mucus collection. Cervicovaginal mucus was collected from all ewes (n = 28 to 30 per ewe breed) at the follicular (Day 0) and luteal (Day 9) phases of a synchronized (14 day progestagen vaginal sponge + 400 IU equine chorionic gonadotropin at sponge removal) and a natural cycle (ewes were checked for estrous using teaser rams with aprons fitted). SI, sponge inserted; SR, sponge removal. CMC, cervicovaginal mucus collection. Day 0, mucus collection at the follicular phase. Day 9, mucus collection at the luteal phase.

L'Aigle, France) with an internal light source was inserted into the vagina to locate the external cervical *os*. All available mucus was collected from the cervical *os* as well as from the fornix of the vagina using an adapted suction pipette and suctioning was performed with a 20 mL syringe. Samples were transported to the laboratory (maximum time = 1 h) for assessment of cervicovaginal mucus properties.

2.3. Assessment of cervicovaginal mucus properties

All of the cervicovaginal mucus collected from each ewe was assessed for total weight (grams), viscosity and colour. All the mucus viscosity was assessed by recording the time in seconds for 4 μl of mucus to fill a single chamber of a Leja 20 μm, 4 Chamber slide® (IMV Technologies, L'Aigle, France). The more viscous mucus was, the longer it took to fill the chamber. A maximum of 420 s was allowed per sample for mucus to fill the chamber. Following an assessment of the distribution of the data it was then separated into two categories namely, samples that filled the Leja chamber in less than or greater than 200 s. This method has previously been used to assess semen viscosity [18], demonstrating that the Leja chamber slide is a simple method to assess the viscosity of fluids. The mucus colour was assessed (in Ireland and Norway only) via a scoring system from 1 to 7 (1: clear, 2: clear-cloudy, 3: cloudy, 4: cloudy-milky, 5: milky, 6: milky-creamy, 7: creamy) based on the classification described by Maddison et al. [11].

2.4. Characterisation of the cervical anatomy

After completion of the third replicate of mucus collection, ewes were slaughtered at either the (i) follicular phase of a natural cycle (n = 8–10 ewes per breed), (ii) follicular phase of a synchronized cycle (n = 8–10 ewes per breed) or (iii) luteal phase of a synchronized cycle (n = 8–10 ewes per breed).

Following slaughter, the ovaries were assessed for the presence of an active corpus luteum (luteal phase) or dominant follicles (follicular phase). The appearance of the external cervical *os* was classified as slit, papilla, duckbill, flap or rose as described by Kershaw et al. [4]. The cervix was then opened longitudinally in order to measure the length (centimetres) of the cervix and the number of cervical rings was recorded. The length was defined as the distance between the external *os* and the last cervical ring. Cervical tissue segments were biobanked for future studies.

2.5. Statistical analysis

Data were analysed using appropriate procedures of Statistical Analysis Software (SAS version 9.4, Cary, NC, USA). Data were tested for normality of distribution (UNIVARIATE procedure) and, where appropriate, transformed to the power of lambda (TRANSREG procedure). Mucus weight and cervical length were analysed using ANOVA (MIXED procedure). Breed, type of cycle (synchronized or natural), phase of cycle (follicular or luteal), replicate (replicate 1, 2 and 3 was at the beginning, middle and end of the experiment, respectively) and their interactions were included in the model. The interaction term, if not statistically significant ($P > 0.05$), was subsequently excluded from the final model. The covariance matrix was determined for each variable by examining the Bayesian Information Criteria (BIC) (smaller is better) value. Animal was the experimental unit. The replicate was included as a repeated term for mucus weight with ewe as the subject, since the same animals were used over the three replicates. Ewe was included as a random effect for cervical length. Colour and the number of cervical rings were analysed using the GLIMMIX procedure. Fixed effects were the same as for mucus weight and cervical length and replicate was included as a random effect. The model for number of cervical rings did not include the replicate and ewe was included as a random effect. Mucus viscosity was analysed as binomial data (0 = <200 s, 1 = >200 s to fill chamber) using the LOGISTIC procedure with a binary logit function. The odds ratio and 95% confidence intervals were used to determine significance. Country was included as a random effect for all variables, except viscosity. All values are presented as mean ± SEM.

3. Results

3.1. Cervicovaginal mucus properties

3.1.1. Mucus weight

There was a type of the cycle (synchronized vs natural) by ewe breed interaction ($P < 0.001$). There were no differences between ewe breeds at the natural cycle (at both the follicular and luteal phases) but there were significant differences between some ewe breeds at the synchronized cycle at both the luteal and follicular phase ($P < 0.05$; Fig. 2). At the synchronized estrus, Romanov ewes had more mucus than Fur, Suffolk and Belclare ewes at the follicular phase ($P < 0.001$). In addition, NWS had more mucus than Fur and Belclare ewes ($P < 0.001$) at the follicular phase of the synchronized cycle. At the luteal phase of the synchronized cycle, Romanov had more mucus than the other ewe breeds ($P < 0.05$). NWS had the lowest amount of mucus compared with all the other ewe breeds ($P < 0.05$). There was no phase of the cycle (follicular vs luteal) by ewe breed interaction ($P > 0.05$). In all ewe breeds, the mucus at the follicular phase was more abundant than at the luteal phase at both a synchronized and natural estrus ($P < 0.001$; Fig. 2).

3.1.2. Mucus viscosity

There was a ewe breed by type of the cycle (synchronized vs natural) interaction ($P < 0.05$). There was no difference in mucus viscosity in Belclare, Fur and NWS ewes between the synchronized and the natural cycle at the follicular phase ($P > 0.05$). However, at the follicular phase, Suffolk, Romanov and Ile de France had more

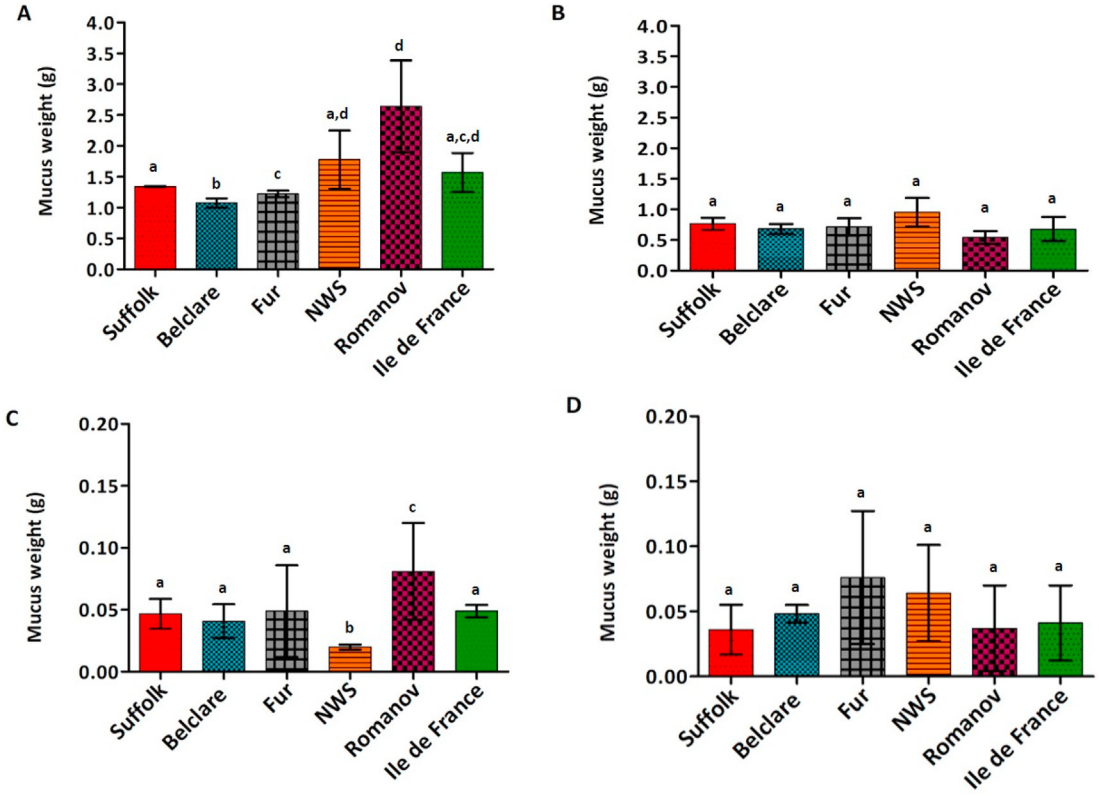

Fig. 2. Cervicovaginal mucus weight (mean ± SEM) for Suffolk, Belclare, Fur, Norwegian White Sheep (NWS), Romanov and Ile de France at the follicular phase of a synchronized (A) and a natural (B) cycle as well as, at the luteal phase of both a synchronized (C) and natural (D) cycle. ^{abcd} Different superscripts differ significantly ($P < 0.05$).

viscous mucus at the natural than at the synchronized cycle ($P < 0.05$). There was a ewe breed by type of the cycle by phase of the cycle interaction ($P < 0.001$). This was represented by Suffolk having lower viscous mucus than Belclare, Fur and Romanov at the follicular phase of the synchronized cycle, while, at natural cycle Suffolk had lower viscosity than Belclare, Fur, Romanov and Ile de France also ($P < 0.05$; Fig. 3). However, there was no difference between Suffolk and NWS ewes at the follicular phase for both types of cycle ($P > 0.05$). At the luteal phase of both types of cycle, Suffolk and Belclare ewes had higher mucus viscosity than Romanov and Ile the France ($P < 0.05$). At the luteal phase of the natural cycle, Fur had lower mucus viscosity that the other breeds but this was not evident at the synchronized cycle ($P < 0.05$; Fig. 3).

3.1.3. Mucus colour

There was a type of the cycle (synchronized vs natural) by ewe breed interaction ($P < 0.001$) for mucus colour. Fur ewes had cloudier mucus colour than NWS ewes at the synchronized ($P < 0.001$) but not at the natural cycle ($P > 0.05$). There was an interaction between ewe breed and phase of the cycle for mucus colour ($P < 0.001$) as luteal mucus was cloudier than follicular mucus in Suffolk and Belclare ewes but not in Fur and NWS ewes.

3.2. Cervical anatomy

3.2.1. Cervical length

Length of the cervix was not affected by type (natural vs synchronized) or phase (follicular vs luteal) of the cycle ($P > 0.05$). The mean length of the cervix was 6.50 ± 0.280 cm and was affected by ewe breed ($P < 0.001$; Table 1). Suffolk ewes had the longest cervix followed by Belclare and Ile de France and these were significantly longer than Fur, NWS and Romanov ($P < 0.001$; Table 1).

3.2.2. Number of cervical rings

There was an interaction between ewe breed and type of the cycle (synchronized vs natural; $P < 0.001$) and an interaction between ewe breed and phase of the cycle (follicular vs luteal; $P < 0.05$) on the number of cervical rings, however this was unlikely to be a true biological effect. The mean number of rings was 5.0 ± 0.25 and was affected by ewe breed ($P < 0.001$; Table 1). Fur ewes had the highest number of cervical rings followed by NWS and these had significantly more cervical rings than Suffolk, Belclare, Ile de France and Romanov ($P < 0.001$).

3.2.3. External os type

The appearance of external *os* type was affected by ewe breed ($P < 0.05$), but there was no effect of the type or phase of cycle ($P > 0.05$). The most frequent external *os* type was flap (42.9% of ewes) followed by duckbill (19.6%) and slit (16.6%). Papilla and rose were the least common (10.4% for both flap types; Fig. 4). Fur ewes had less flaps than Belclare and NWS ($P < 0.05$). Fur had less duckbill *os* type than Suffolk, Belclare and NWS ($P < 0.05$).

4. Discussion

This is the first comprehensive study of the mucus production, rheological properties of cervicovaginal mucus as well as cervical anatomy of six economically important ewe breeds across three European countries. The main aim of this study was to ascertain if the reason why cervical/vaginal AI works well in some ewe breeds but not in others is related to the cervicovaginal mucus properties and/or gross cervical anatomical parameters. The results of the

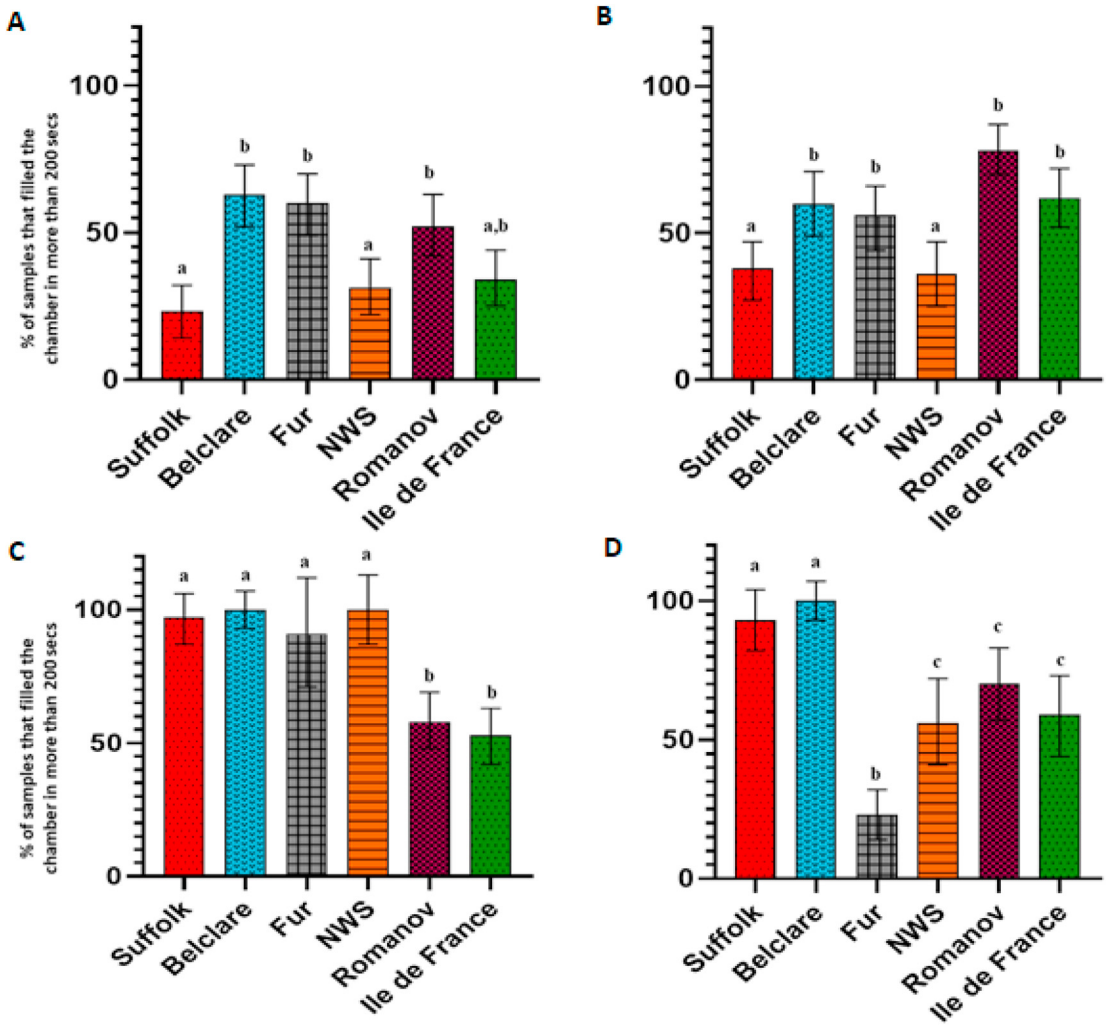

Fig. 3. Cervicovaginal mucus viscosity measured by % of samples that filled the Leja chamber in greater than 200 s (% of samples and 95% CI) at the follicular phase of a synchronized (A) and a natural cycle (B) for Suffolk, Belclare, Fur, Norwegian White Sheep (NWS), Romanov and Ile de France. As well as, at the luteal phase of both a synchronized (C) and natural (D) cycle. [abc] Different superscripts differ significantly ($P < 0.05$).

present study showed differences in mucus production between ewe breeds at the synchronized cycle while there were no differences at the natural cycle. In all ewe breeds, mucus production was higher at the follicular than at the luteal phase in both types of cycle. However, the effect of synchronization or phase of the cycle was different between ewe breeds. There were no differences in mucus viscosity between Suffolk and NWS, despite the differences in pregnancy rates following cervical AI with frozen-thawed semen. Despite AI in Norway being only performed to a natural estrus, there were no differences in mucus viscosity between synchronized and natural cycles in both Norwegian ewe breeds. The length of the cervix, the number of cervical rings and the appearance of the external os were affected by ewe breed. Suffolk ewes had a longer cervix but a lower number of cervical rings than NWS and Fur ewes (both with higher pregnancy rates). This demonstrates that the neither the gross mucus properties or cervical anatomical parameters explain the previously reported ewe breed differences in pregnancy rates following cervical AI with frozen-thawed semen.

There were no differences in mucus production between ewe breeds at the natural cycle but there were significant differences between some ewe breeds at the synchronized cycle at both the luteal and follicular phases. However, there was no clear biological pattern between previously reported ewe breed differences in pregnancy rates following cervical AI with frozen-thawed semen and mucus production in the present study. There was an increase in mucus production in the synchronized cycle compared to the natural cycle at the follicular phase in all ewe breeds.

There are contradictory reports regarding the effect of synchronization on mucus production. Rexroad and Barb [19] reported an increase of mucus production at estrus using sponges impregnated with 6-α- methyl-17α-acetoxyprogesterone or 9-fluoro-11β-17-dihydroxyprogesterone (FGA), while, Smith and Allison [20]

Table 1
Cervical length and number of rings in six ewe breeds (n = 28–30 ewes per breed). Cervices were collected post mortem. Values are mean ± SEM. [abc] Different superscripts differ significantly within each column ($P < 0.05$).

Ewe Breed	Cervix Length (cm)	Number of Cervical Rings
Suffolk	7.52 ± 0.169^{a}	5.0 ± 0.26^{a}
Belclare	7.42 ± 0.177^{a}	4.9 ± 0.18^{a}
Fur	5.54 ± 0.138^{b}	5.6 ± 0.15^{b}
NWS	5.52 ± 0.153^{b}	5.4 ± 0.16^{b}
Ile de France	7.11 ± 0.232^{a}	4.7 ± 0.15^{a}
Romanov	6.02 ± 0.173^{b}	4.2 ± 0.17^{a}

NWS = Norwegian White Sheep.

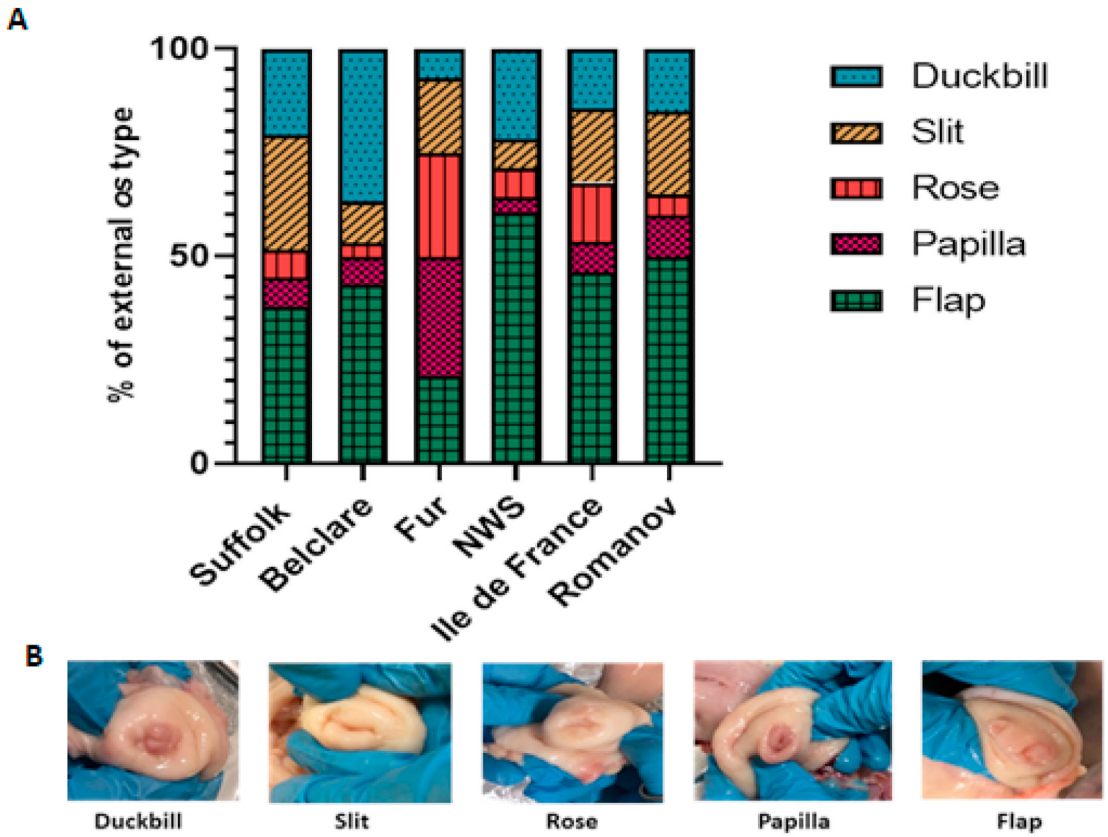

Fig. 4. (A) Percentage of external *os* type for the six ewe breeds (Suffolk, Belclare, Fur, NWS, Ile de France and Romanov). (B) Classification of the appearance of the external *os* type of the ewe.

found decreased levels of mucus produced following progesterone synchronization. Maddison et al. [11] reported similar volumes of mucus between synchronized and natural cycle at the follicular phase. These contradictory results could be due many reasons but the results of the current study would suggest that ewe breed differences play an important and significant role in the cervical response to exogenous hormones.

In the current study, there was a significant effect of phase of the cycle on mucus production, with more mucus at the follicular than at the luteal phase. The production of cervical mucus has been shown to increase under the influence of oestrogen in the lead up to ovulation [10,21]. This explains the higher mucus production at the follicular than at the luteal phase in our study. At the follicular phase, under the influence of oestrogen, the secretory activity of the uterine and cervical epithelium increases and mucus, produced by the goblet cells, is more hydrated. Gorodeski [22] demonstrated increased levels of mucus produced after the oestrogen supplementation, concluding that this may in part be due to increase paracellular permeability of the cervical cells. A recent study indicated an increase in size and number of cervical cells in response to E2 and P4 [23]. The increase in volume decreases the mucus viscosity enabling sperm penetration through the cervical mucus while flushing out pathogens around the time of ovulation [24].

In the present study, mucus viscosity was decreased under the effect of synchronization in Suffolk, Romanov and Ile de France. However, there were no differences in mucus viscosity between types of cycle (synchronized vs natural) in Belclare, Fur and NWS ewes at the follicular phase. These differences between ewe breeds under the effect of synchronization could indicate differences in mucus composition. It has been reported that calcium modifies the mucin charge and their expansion, which effects the mucus hydration, therefore the mucus viscosity [25]. Maddison et al. [11] working with Merino ewes found similar levels of calcium in mucus from natural and synchronized ewes. This may explain why there were no differences in mucus viscosity values between a natural and synchronized cycle in some ewe breeds (Belclare, Fur and NWS). In agreement with this, Rexroad and Barb [19] indicated that the spinnbarkeit values were similar between synchronization with progesterone and PGF2α and naturally cycling ewes. Our group previously studied the effect of synchronization in Suffolk and Beclare, there were no differences between these two ewe breeds in ferning patterns or on the viscous modulus of cervicovaginal mucus collected at a synchronized estrus [9]. Although, we demonstrated that the elastic and complex moduli of cervicovaginal mucus was lower in Belclare than in Suffolk ewes. This is despite Suffolk ewes having poorer sperm transport following cervical AI with frozen-thawed semen [17]. The variation in the effect of exogenous hormones for synchronization in previous studies could be due to a variation in the viscosity parameter analysed (spinnbarkeit, viscous modulus and elastic and complex moduli).

There was a ewe breed by phase of the cycle interaction for mucus viscosity, which was represented by Belclare, Fur and Romanov ewes having more viscous mucus than Suffolk ewes at the follicular phase at both types of the cycle as measured by the time to fill a Leja chamber. It is well established that the cervical mucus viscosity decreases during estrus due to increased hydration. This facilitates the penetration of sperm around the time of ovulation while preventing the influx of pathogens during the luteal phase of the cycle [24]. Mucus viscosity during estrus is partly due to the increase of secreted mucins, which are large glycosylated

glycoproteins [21]. In addition, biochemical modifications in cervical mucins increase the proportion of water in the cervical mucus, facilitating sperm penetration [26]. This supports our findings since there was an increase in mucus viscosity at the luteal phase in Suffolk and Belclare ewes at both types of cycle. Although, Romanov and Ile de France did not differ in mucus viscosity between the follicular and the luteal phases. Fur and NWS had higher mucus viscosity at the luteal than at the follicular phase of the synchronized cycle but not at the natural cycle. A possible explanation for this disparity could be due to the complex structural characteristics of mucus, as it is a non-Newtonian fluid and challenging to analyse.

However, we identified a clear pattern between high and low fertility ewe breeds in mucus colour, which could also indicate differences in mucus composition between ewe breeds across the estrus cycle. In the present study, NWS and Fur ewes had clearer mucus than Suffolk and Belclare ewes at the follicular phase of both types of cycle. These differences in mucus colour between high and low fertility ewe breeds could be key to elucidate molecular components in the cervical mucus related to impaired sperm transport in some ewe breeds. It has been previously reported that mucus colour is affected by mucus volume, hydration and protein concentration [10,11]. As outlined earlier, mucus structure changes by varying the molecular configuration and cross linkages between mucin proteins, which allow sperm migration through the mucus mesh [27,28]. Thus, cloudier mucus in the low fertility ewe breeds could indicate low mucus hydration and decreased sperm transport. Sperm migration through the cervical mucus is not fully understood, however, cervical mucins and their molecular modifications are likely to be fruitful areas of investigation.

Differences in mucus colour may also be due to an increase of pathogens in the cervical mucus resulting in an increase of proteins involved in pathogen recognition and immune response. It has been previously described that the microflora of the cervix can also modify mucin proteins [29–31] affecting mucus hydration and colour. Overall, the changes in mucus structure, increase of the bacterial load, including inflammatory mediators and immune cells, could explain the relationship between more opaque mucus and reduced sperm transport in the low fertility ewe breeds.

Mucins are the most common proteins around the time of ovulation while proteins associated with the immune system are increased during the luteal phase [13,32]. Our results indicate that the luteal mucus was cloudier than follicular mucus in Suffolk and Belclare ewes only. This is supported by previous studies [10,33] who reported that mucus from the follicular phase was less cloudy than that of the luteal phase, and it is in part due to the protein concentration. Maddison et al. [11] reported an increase of protein concentration, which was correlated with cloudier mucus at the luteal phase. In a more recent analysis of the cervicovaginal mucus proteome of the ewe, Maddison et al. [34] indicated that 127 proteins were more abundant during the luteal phase compared to the follicular phase. This could suggest that there are more proteins involved in immune response and preventing ascending pathogens from the vagina to the uterus at the luteal phase. Thus, the cervix and its secretions are dynamic components that respond to internal signals such as circulating hormones as well as the cervical microbiome which can have knock on downstream effects on the cervical transcriptome, proteome, metabolome and/or glycome.

Cervical anatomical parameters, such as the cervical length, number of cervical rings and the appearance of external *os* were affected by ewe breed. NWS had the shortest cervix (5.52 cm) but higher number of cervical rings (5.4 rings) compared to the other ewe breeds. In contrast, Suffolk ewes had longer cervix (7.52 cm) with 5 cervical rings. Previous studies have investigated the effect of ewe breed on these parameters. Halbert et al. [3] previously reported an average cervical length of 6.3 cm and average number cervical rings of 4 for Clun-Forest ewes, whereas, the Cheviot breed had a longer cervix (7.3 cm) with more cervical rings (5.6). Kaabi et al. [35] obtained variations in the anatomy of the cervix of four ewe breeds (Churra, Assaf, Merino and Castellana). The same study reported that the cervical length was not correlated to pregnancy rates following cervical AI with frozen-thawed semen. However, the number of cervical rings was correlated to pregnancy rates.

In our study, Suffolk ewes had a lower number of cervical rings than Fur and NWS, both with higher pregnancy rates than Suffolk. Therefore, higher number of cervical rings was correlated to ewe breeds with higher pregnancy rates. However, the complexity of the cervix is not only described by the length or the number of rings, other factors such as alignment of the cervical folds along the lumen and the diameter could be important as previous described by Wulster-Radcliffe et al. [36]. As expected, there were no significant differences in the cervical anatomical parameters between the follicular and luteal phases.

Another important anatomic factor is the appearance of external *os* as this could affect sperm entrance into the cervix. There was no relationship between the number of rings and the appearance of external *os*. Overall, the most frequent external *os* type was flap followed by duckbill in line with the work of Kershaw et al. [4]. Our results indicate that the external *os* type was determined by breed. Fur ewes had less number of duckbills *os* type that Suffolk, Belclare and NWS. Fur ewes had less flaps than Belclare and NWS. However, there was no clear pattern between breeds with high and low pregnancy rates.

In conclusion, this study provides the first quantification of mucus properties and anatomic characteristics of six European ewe breeds under similar conditions. However, there was no clear biological pattern between these ewe breeds in mucus properties or gross cervical anatomy despite previous reports by our group that these breeds vary widely in pregnancy rates following cervical AI with frozen-thawed semen. In the present study, the use of exogenous hormones for synchronization produced differences in mucus production between ewe breeds, although, these differences did not affect mucus viscosity. Therefore, more detailed biochemical and molecular characterisation of the cervix and its secretions is required to elucidate why frozen-thawed spermatozoa can traverse the cervix in some ewe breeds but not in others.

Conflicts of interest

Authors have declared no conflict of interest.

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Author's contribution

L. Abril-Parreño, X. Druart, E. Caldas, A. Donovan and A. Krogenaes collected the samples from the ewes. L. Abril-Parreño drafted the manuscript. S. Fair, A. Krogenaes and X. Druart conceived and designed the experiments, secured funding and overseen the work. C J Byrne performed the statistical analysis. M. Diskin and S. Stuen facilitated the animal work. L. Abril-Parreño drafted the manuscript and all other authors edited and proof read it.

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