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REVIEW

Systematic review and meta-analysis of fertility outcome following in vivo insemination with sex-sorted semen in sheep

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Background: Sex-sorted semen (SS) offers economic benefits to sheep producers, but lower fertility outcome (FO), defined as probability of pregnancy, pregnancy rate or lambing rate, than that following conventional semen (CS) artificial insemination (AI) may limit its use.

Objectives: To systematically review the literature, and analyse factors associated with FO following AI in sheep using SS vs CS.

Methods: Available literature was searched using the PRISMA guidelines, resulting in 11 studies with 14 experiments that were reviewed. From these, information from 70 study cohorts representing 13 experiments was used to conduct a meta-analysis which confirmed that FOs for SS and CS AI were 37% (95% CI = 31–43%) and 52% (95% CI = 45–59%), respectively ($p < 0.01$).

Results: FO improved over time, with FO following SS and CS AI improving at the same rate over the period investigated (1997–2022). In a subgroup analysis, five factors were identified that potentially decrease the FO after SS AI disproportionately. These were sperm dose ≤ 4×10^6 spermatozoa, semen preservation (fresh vs frozen-thawed), oestrus synchronisation using an intravaginal sponge (vs a controlled internal drug release device), presence of gonadotropin-releasing hormone in the synchronisation protocol, and absence of biostimulation using a teaser ram. In a random-effects model analysis, an interaction between sperm dose and SS vs CS, and semen preservation were independent predictors of FO after adjusting for the effect of timing of insemination.

Conclusion: FO following insemination with SS is 15% lower than that of CS, which can be narrowed by increasing the semen dose for SS inseminations and improving the synchronisation of ovulation with the timing of insemination.

Keywords: sex-sorted, sexed, semen, spermatozoa, sheep fertility, systematic review, meta-analysis

Introduction

Use of sex-sorted semen (SS) is reported to maximise efficiency by increasing production of either male or female animals from genetically superior animals (González-Marín et al. 2021; Rath et al. 2013). Different methods of sex-sorting include an albumin gradient, a centrifugal counter with an aqueous two-phase system, flow cytometry (Yotov et al. 2021) or immuno-sexing (Brito et al. 2021). Flow cytometry is a reliable and validated technology to separate X- and Y-chromosome-bearing sperm based on their difference in DNA content (De Graaf et al. 2009; González-Marín et al. 2021). Artificial insemination (AI) using X-sorted or Y-sorted semen is therefore possible to produce either predominantly female or male progeny, respectively.

The process of sex-sorting using flow cytometry is advanced and involves several intricate processes (Johnson 1995, Rath et al. 2013). Since production of the first lambs using SS more than 25 years ago, sperm sexing techniques have been modified to increase efficiency and spermatozoa separation purity (Rath et al. 2013). Considerable time and resources have been expended on improving fertility of sex-sorted spermatozoa (De Graaf et al. 2009).

Studies have evaluated the fertility outcome (FO) using SS compared with conventional semen (CS) and have demonstrated that SS can be used successfully to inseminate sheep ewes and result in the birth of live offspring (Cran et al. 1997; Hollinshead et al. 2002; Cattaneo et al. 2004; Hollinshead et al. 2003; De Graaf et al. 2007a; De Graaf et al. 2007b; Beilby et al. 2009; Leahy et al. 2010; González-Marín et al. 2021; Milovanović et al. 2022). However, many confounding factors make comparisons between the results using SS and CS challenging.

In contrast to sheep, many articles have been published that evaluated the use of SS in cattle, as reported in a recent meta-analysis (Reese et al. 2021) that evaluated reproductive success of bovine SS and found that pregnancy and calving rate ratios following insemination using SS were reduced by 23% and 24%, respectively, compared with CS. Several publications and a recent review demonstrated that multiple factors, including ram, semen, ewe and environmental factors, may affect FO in sheep after AI using CS (Hill et al. 1998; Anel et al. 2005; Fukui et al. 2010; McCappin & Murray 2011; Diaz & Emsen 2012; Ake-Villanueva et al. 2017; Spanner et al. 2024a; El Amiri & Rahim 2024; Spanner et al. 2024b).

Identifying factors that may influence FO using SS in sheep are lacking, and further research is needed. Several unanswered questions remain regarding the optimal dose of sex-sorted spermatozoa, time of insemination, and hormonal synchronisation protocol to achieve the highest possible FO when using SS in sheep. To the knowledge of the authors, no studies have reviewed the potential factors affecting the FO differential between the use of SS and CS to inseminate sheep, and no meta-analysis has been performed with the data available from the literature.

The objectives of the present study were:

- To systematically review the current literature on AI of sheep with SS
- To evaluate the FO (probability of pregnancy, pregnancy rates or lambing rates) after AI using SS vs CS in sheep
- To identify potential factors that influence the FO of AI with SS and CS in sheep.

Methods

Information sources

A systematic literature search was conducted using Web of Science All Databases, PubMed and Scopus. Google Scholar was used to search for additional studies. The PRISMA 2020 guidelines were followed to perform the systematic review and meta-analysis (Page et al. 2021).

Search strategy

The following search term was used: ("sheep" OR "ovine" OR "ewe" OR "ewes" OR "ram" OR "rams") AND ("sex-sorted semen" OR "sex-sorted spermatozoa" OR "sexed semen" OR "sexed spermatozoa" OR "sorted semen" OR "sorted spermatozoa" OR "sperm sexing" OR "flow cytometrically sorted" OR "sex preselection" OR "sex pre-selection" OR "sex sorting" OR "sperm sorting"). No filters were included, and no refinements were made to the search. The reference lists of selected studies were screened to identify additional studies not included in the search results.

Eligibility criteria

Inclusion criteria for the systematic review were the following:

- Research conducted using domestic sheep
- Ewes inseminated intra-uterine (laparoscopically) or cervically using fresh or frozen-thawed semen
- Ewes inseminated using SS (sex-sorted using any method) compared with CS
- Data on FO reported
- Peer-reviewed articles including theses and dissertations and congress/conference abstracts were included.

Exclusion criteria for the systematic review were the following:

- Studies evaluating in vitro fertilisation or embryo transfer
- Duplicate manuscripts or results of the same experiments repeated in different manuscripts.

Inclusion criteria exceptions applied for the meta-analysis were as follows:

- Only studies where sheep were inseminated laparoscopically (intra-uterine or utero-tubular) were included
- Only data from cohorts exposed to X-sorted semen and conventional semen within the reviewed experiments were included.

Data collection process

Information extraction for the systematic review

A systematic approach was followed to extract the following information for each cohort studied:

1. author and year of publication of the various trials;
2. breed and number of animals included;
3. reproductive management strategy, including synchronisation protocol and hormones used, and use of teaser rams or androgenised wethers for biostimulation;
4. route of insemination (laparoscopic – intra-uterine or utero-tubular or cervical);
5. time of insemination relative to progestagen device removal in hours;
6. dose of spermatozoa used to inseminate ewes (total or total motile spermatozoa);
7. semen preservation (fresh or frozen-thawed [pellets or straws]);
8. semen type (SS or CS);
9. FO.

Additional information reviewed for some studies included pregnancy rate determined by measuring progesterone concentration on day 18 and number of lambs born per ewe inseminated (litter size), semen preservation and in vitro semen characteristics, and differences between rams (FO and semen characteristics).

Data extraction for meta-analysis

A systematic approach was followed to extract categorical and numerical data from each cohort studied, for the meta-analysis:

1. author and year of publication;
2. reproductive management strategy (insemination after synchronisation of ovulation or insemination based on natural heat);
3. the progestagen source for synchronisation (none, fluorogestone acetate [FGA] 30 mg, FGA 40 mg or controlled internal drug release [CIDR] device 0.3 g for the random-effects model; or CIDR 0.3 g or sponge [30 mg and 40 mg FGA combined] for the subgroup analysis);
4. the addition of equine chorionic gonadotropin (eCG) and the dose of eCG (0, 350–360 IU or 400–500 IU for the random-effects model) to the synchronisation protocol;
5. the addition of gonadotropin-releasing hormone (GnRH) to the synchronisation protocol or not;
6. the additional use of a teaser as biostimulation;
7. the time of insemination relative to progestagen device removal (≤ 56 hours or ≥ 58 hours);
8. semen preservation (fresh, frozen-thawed pellets or frozen-thawed straws for the random-effects model or fresh or frozen-thawed for the subgroup analysis);
9. sperm dose as total or total motile spermatozoa (≤ 1×10^6, 2–10×10^6, > 10×10^6 for the random-effects model, or ≤ 4×10^6 or ≥ 5×10^6 for the subgroup analysis);
10. semen type (SS or CS);
11. FO.

Additionally, authors from reviewed studies (Beilby et al. 2009; González-Marín et al. 2021; Milovanović et al. 2022) were

contacted, and through personal communication, specific information was clarified or additional information was obtained.

Statistical analysis

The meta-analysis was performed using Stata 18 (StataCorp, USA). To include studies in the meta-analysis, FO was defined as either the pregnancy rate (proportion of inseminated ewes confirmed pregnant by ultrasound diagnosis 30–62 days later), the probability of pregnancy (using a linear mixed model), or the lambing rate (proportion of ewes that lambed within a normal gestational period following insemination) (Cran et al. 1997; Hollinshead et al. 2002; Cattaneo et al. 2004; Hollinshead et al. 2003; De Graaf et al. 2007a; De Graaf et al. 2007b; González-Marín 2018; González-Marín et al. 2021; Milovanović et al. 2022; Beilby et al. 2009).

Most studies included in the meta-analysis investigated more than two experimental cohorts simultaneously (under the same conditions). Each cohort within a study was therefore considered an experimental unit, which made it impossible to estimate a rate ratio. The study was included as a fixed effect, with the main predictor of interest being spermatozoa sexing (yes vs no). The outcome (FO) was modelled as a Freeman-Tukey transformed proportion within a random-effects model (Freeman & Tukey 1950). Forest plots were generated, and heterogeneity was assessed by evaluating I^2. Publication bias was investigated by analysing the asymmetry of the funnel plot.

After categorising the linear variables, a random effects meta-regression model was performed with all the available potential covariates. Variables were removed where collinearity existed, whereafter the remaining variables were removed one by one based on the highest Wald *p*-value until all remaining variables were significant ($p < 0.1$).

A subgroup analysis was performed to demonstrate the impact within and between SS and CS, and the various categories as described above, on FO. Mean FOs within SS inseminations, within CS inseminations and between SS and CS inseminations were considered different if $p < 0.05$ (two-sided t-test).

This systematic review and meta-analysis are part of a project that has been evaluated and approved by the Research and Animal Ethics Committee of the University of Pretoria (REC127-23 & HUM013/1223).

Results

Systematic search, selection and data extraction for the systematic review and meta-analysis

A total of 11 studies, consisting of 14 trials, were included in the systematic review and a total of 10 studies with 13 trials were included in the meta-analysis. Figure 1 outlines the results of the systematic search, selection and data extraction strategy for the systematic review and meta-analysis. All the studies that met the specific inclusion criteria used flow cytometry as a method for sex-sorting of spermatozoa.

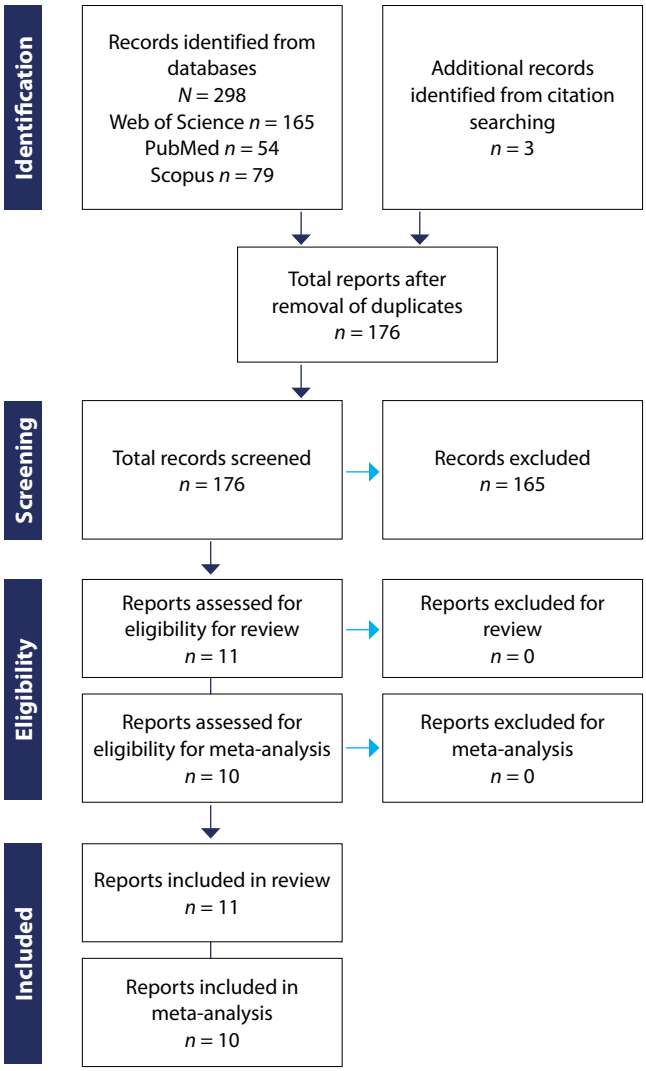

Figure 1. Flow diagram of the search and selection strategy in the systematic review and meta-analysis of factors affecting fertility outcome when using sex-sorted semen to inseminate sheep ewes

Systematic literature review

Table I presents a summary of the extracted information from the reviewed literature.

Meta-analysis

The FO was evaluated in 68 observations (two observations were excluded from the model where the time to insemination was missing [Cattaneo et al. 2004]). The FO for SS was significantly lower (37.0%) than that for CS (52.0%) ($p = 0.001$) (Figure 2). The overall residual heterogeneity (I^2) was 86.73% (Figure 3).

Results for the final meta-regression model are summarised in Table II.

After adjusting for the study, a number of variables had independent effects on the FO (Table II).

Subgroup analysis to evaluate fertility outcome

Results of a subgroup analysis in the meta-analysis of FO are summarised in Table III.

Table I: Summary of information evaluating the potential factors that affected fertility outcome included in the systematic review

Publications	Breed & number of animals included (*n*)	Reproductive management strategy: synchronisation protocol or natural heat	Method of insemination	Time of insemination (hours after progestagen removal)	Dose of spermatozoa (T = total spermatozoa or M = total motile spermatozoa)	Semen preservation (fresh or frozen semen)	Sex-sorted or conventional spermatozoa	Pregnancy percentage determined by ultrasound (d30–62) [pregnancy percentage determined by progesterone (d18)]	Lambing percentage (and proportion) [mean no of lambs born/ewe lambing]
Cran et al. 1997	Trial 1: Blue-faced Leicester x Scottish Blackface ewes (*n* = 30) Suffolk ram (*n* = 1)	30 mg FGA for 12 d; 400 IU eCG (d12)	Laparoscopic	51.5–53 h	T = 1 × 10^5	Fresh	X-sorted		0% (0/18)
							Conventional		42% (5/12) [1.4]
	Trial 2: Scottish Blackface ewes (*n*=60) Suffolk ram (*n* = 1)	30 mg FGA for 12 d; 400 IU eCG (d12)	Laparoscopic	53.5–56.5 h	T = 1 × 10^5	Fresh	Y-sorted		0% (0/5)
							X-sorted		16% (4/25) [1.5]
							Conventional		7% (2/30) [1.0]
Hollinshead et al. 2002	Merino ewes (*n* = 144) Merino rams (*n* = 2)	40 mg FGA for 12 d; 400 IU eCG (d12)	Laparoscopic	54–57 h	T = 2–4 × 10^6	Frozen thawed pellet	Y-sorted	15%[b] (7/48) [31%[b] (15/48)]	15%[b] (7/48) [1.1 ± 0.14[a]]
					T = 2–4 × 10^6	Frozen thawed pellet	X-sorted	25%[b] (12/48) [44%[b] (21/48)]	25%[b] (12/48) [1.5 ± 0.19[a]]
					T = 140 × 10^6	Frozen thawed pellet	Conventional	54%[a] (26/48) [71%[a] (34/48)]	54%[a] (26/48) [1.5 ± 0.11[a]]
Hollinshead et al. 2003	Trial 1: Merino ewes (*n* = 375) Merino rams (*n* = 3)	40 mg FGA for 12 d; 400 IU eCG (d12)	Laparoscopic	54–58 h	T = 100 × 10^6	Frozen thawed pellet	Conventional	40%[a] (6/15)	
		40 mg FGA for 12 d; 400 IU eCG (d12); GnRH (36 h later)	Laparoscopic	54 h	T = 1 × 10^6	Frozen thawed pellet	X-sorted	17%[a] (5/29)	
					T = 4 × 10^6		X-sorted	27%[a] (8/30)	
					T = 16 x 10^6		X-sorted	37%[a] (11/30)	
					T = 100 x 10^6		Conventional	41%[a] (12/29)	
				58 h	T = 1 × 10^6	Frozen thawed pellet	X-sorted	17%[a] (5/30)	
					T = 4 × 10^6		X-sorted	23%[a] (7/30)	
					T = 16 × 10^6		X-sorted	40%[a] (12/30)	
					T = 100 × 10^6		Conventional	70%[a] (21/30)	
				62 h	T = 1 × 10^6	Frozen thawed pellet	X-sorted	17%[a] (5/30)	
					T = 4 × 10^6		X-sorted	23%[a] (7/30)	
					T = 16 × 10^6		X-sorted	17%[a] (5/30)	
					T = 100 × 10^6		Conventional	63%[a] (19/30)	
	Trial 2: Merino ewes (*n* = 144) Merino rams (*n* = 2)	40 mg FGA for 12 d; 400 IU eCG (d12)	Laparoscopic	57–58 h	T = 100 × 10^6	Frozen thawed pellet	Conventional	54%[a,b,c] (7/13)	
Hollinshead et al. 2003	Trial 2: Merino ewes (*n* = 144) Merino rams (*n* = 2)	40 mg FGA for 12 d; 400 IU eCG (d12); GnRH (36 h later)	Laparoscopic	57–60 h	T = 5 × 10^6	Frozen thawed pellet	X-sorted	20%[a] (3/15)	
							Conventional	47%[a,b,c] (7/15)	

Table I: *Continued*

Hollinshead et al. 2003	Trial 2: Merino ewes ($n = 144$) Merino rams ($n = 2$)	40 mg FGA for 12 d; 400 IU eCG (d12); GnRH (36 h later)	Laparoscopic	57–60 h	$T = 10 \times 10^6$	Frozen thawed pellet	X-sorted	43%^{a,b,c} (6/14)	
							Conventional	71%^{c} (10/14)	
					$T = 20 \times 10^6$	Frozen thawed pellet	X-sorted	31%^{a,b} (5/16)	
							Conventional	39%^{a,b} (5/13)	
					$T = 40 \times 10^6$	Frozen thawed pellet	X-sorted	73%^{c} (8/11)	
							Conventional	67%^{b,c} (8/12)	
Cattaneo et al. 2004	Ewes and hoggets ($n = 183$) Merino rams ($n = 2$)	Natural heat	Laparoscopic	14 h after heat detection	$T = 8 - 10 \times 10^6$	Frozen thawed straw	X-sorted	38%^{a} (51/138)	
							Conventional	60%^{b} (29/48)	
De Graaf et al. 2007a	Merino ewes ($n = 292$) Merino rams ($n = 3$)	40 mg FGA for 12 d; 400 IU eCG (d12); Androgenised wethers introduced (d12); GnRH (36 h later)	Laparoscopic	57–59 h	$M = 15 \times 10^6$	Frozen thawed (following sex sorting of fresh semen)	X-sorted	46%^{a,b} (35/76) [51%^{a,b} (39/76)]	46%^{a,b} (35/76) [1.6 ± 0.08^{a}]
					$M = 15 \times 10^6$	Frozen thawed (following sex-sorting of previously frozen thawed pellet)	X-sorted	36%^{b} (26/72) [39%^{b} (28/72)]	36%^{b} (26/72) [1.7 ± 0.10^{a}]
					$M = 15 \times 10^6$	Frozen thawed pellet	Conventional	49%^{a,b} (36/74) [60%^{a,b} (44/74)]	49%^{a,b} (36/74) [1.6 ± 0.09^{a}]
					$M = 50 \times 10^6$	Frozen thawed pellet	Conventional	54%^{a} (38/70) [63%^{a} (44/70)]	54%^{a} (38/70) [1.5 ± 0.08^{a}]
De Graaf et al. 2007b	Merino ewes ($n = 360$) Merino rams ($n = 3$)	40 mg FGA for 12 d; 400 IU eCG (d12); Androgenised wethers introduced (d12); GnRH (36 h later)	Laparoscopic	57–59 h	$M = 1 \times 10^6$	Frozen thawed pellet	X-sorted	62%^{a} (32/52)	62%^{a} (32/52) [1.5 ± 0.09^{a}]
					$M = 5 \times 10^6$			68%^{a} (38/56)	66%^{a} (37/56) [1.7 ± 0.08^{a}]
					$M = 15 \times 10^6$			67%^{a} (38/57)	67%^{a} (38/57) [1.6 ± 0.08^{a}]
					$M = 1 \times 10^6$	Frozen thawed pellet	Conventional	35%^{b} (19/55)	35%^{b} (19/55) [1.7 ± 0.11^{a}]
					$M = 5 \times 10^6$			40%^{b} (19/48)	40%^{b} (19/48) [1.6 ± 0.11^{a}]
					$M = 15 \times 10^6$			69%^{a} (37/54)	69%^{a} (37/54) [1.6 ± 0.08^{a}]
					$M = 50 \times 10^6$			63%^{a} (24/38)	63%^{a} (24/38) [1.6 ± 0.10^{a}]
Beilby et al. 2009	Merino ewes ($n = 732$) Merino rams ($n = 3$)	30 mg FGA for 12 d; 400 IU eCG (d12); GnRH (36 h later)	Laparoscopic	50 h	$M = 1 \times 10^6$	Frozen thawed pellet	X-sorted	15%^{a,b}	
					$M = 15 \times 10^6$			36%^{c,d}	
				54 h	$M = 1 \times 10^6$	Frozen thawed pellet	X-sorted	14%^{a}	
					$M = 15 \times 10^6$			55%^{c}	
				58 h	$M = 1 \times 10^6$	Frozen thawed pellet	X-sorted	46%^{c,d}	
					$M = 15 \times 10^6$			43%^{c,d}	

Table I: *Continued*

Beilby et al. 2009	Merino ewes ($n = 732$) Merino rams ($n = 3$)	30 mg FGA for 12 d; 400 IU eCG (d12); GnRH (36 h later)	Laparoscopic	62 h	M = 1×10^6 M = 15×10^6	Frozen thawed pellet	X-sorted	33%[d] 54%[c]	
				66 h	M = 1×10^6 M = 15×10^6	Frozen thawed pellet	X-sorted	29%[b,d] 56%[c]	
Leahy et al. 2010*	Merino ewes ($n = 288$) Merino ram ($n = 3$)	30 mg FGA 12–13 d; 400 IU eCG (d12/d13)	Cervical	50 & 57 h	M = 3.5×10^6	Frozen thawed pellet	X-sorted X-sorted – SPP supplemented before freezing	14% (4/28) 3% (1/28)	
			Cervical	55 h	M = 100×10^6	Frozen thawed pellet	Conventional Conventional – SPP supplemented before freezing Conventional – SPP supplemented after thawing	28% (17/61) 17% (9/60) 38% (23/64)	
			Laparoscopic	57 h	M = 20×10^6	Frozen thawed pellet	Conventional	60% (29/47)	
González-Marin 2018	Trial 1: East Friesian ewes ($n = 285$) East Friesian rams ($n = 2$)	CIDR (0.3 g progesterone) for 12 d; 360 IU eCG (d10)	Laparoscopic	54–56 h	T = 1×10^6	Fresh	X-sorted		36%[a] (57) [1.4[a]]
					T = 2×10^6	Fresh	X-sorted		44%[a] (56) [1.4[a]]
					T = 3×10^6	Frozen pellet	X-sorted		33%[a] (62) [1.4[a]]
					T = 6×10^6	Frozen straw	X-sorted		56%[a] (51) [1.3[a]]
					T = 60×10^6	Frozen	Conventional		42%[a] (60) [1.6[a]]
González-Marin et al. 2021	Trial 2: Merino ewes ($n = 293$) Merino ram ($n = 1$)	CIDR (0.3 g progesterone) for 12 d; 360 IU eCG (d12)	Laparoscopic	49–55 h	T = 2×10^6	Fresh	X-sorted	51%[b] (69)	[1.6[a]]
					T = 10×10^6		X-sorted	65%[a] (62)	[1.7[a]]
					T = 2×10^6		Conventional	69%[a] (64)	[1.6[a]]
					T = 10×10^6		Conventional	69%[a] (62)	[1.7[a]]
					T = 25×10^6		Conventional	78%[a] (36)	[1.6[a]]
	Trial 3: East Friesian ewes ($n = 210$) East Friesian ram ($n = 1$)	CIDR (0.3 g progesterone) for 12 d; 350 IU eCG (d12)	Laparoscopic	53–54 h	T = 2×10^6 T = 10×10^6	Fresh	X-sorted Conventional	86%[a] (96/112) 83%[a] (81/98)	[1.5[a]] [1.7[a]]
Milovanović et al. 2022	Dorper ewes ($n = 205$) Dorper rams ($n = 3$)	40 mg FGA 9 d Prostaglandin (d8) 500 IU eCG (d9) GnRH (36 h) Vasectomised rams introduced before AI	Laparoscopic	58–62 h	T = 3×10^6 & 6×10^6	Fresh	X-sorted	31% (24/77)	
					T = $40–50 \times 10^6$		Conventional	51% (65/128)	

*Not included in the meta-analysis

CIDR – controlled internal drug release, eCG – equine chorionic gonadotropin, FGA – fluorogestone acetate, GnRH – gonadotropin-releasing hormone, SPP – seminal plasma protein

Proportions with different superscripts ([a,b,c,d]) indicate statistically significant differences between experimental groups within the same trial

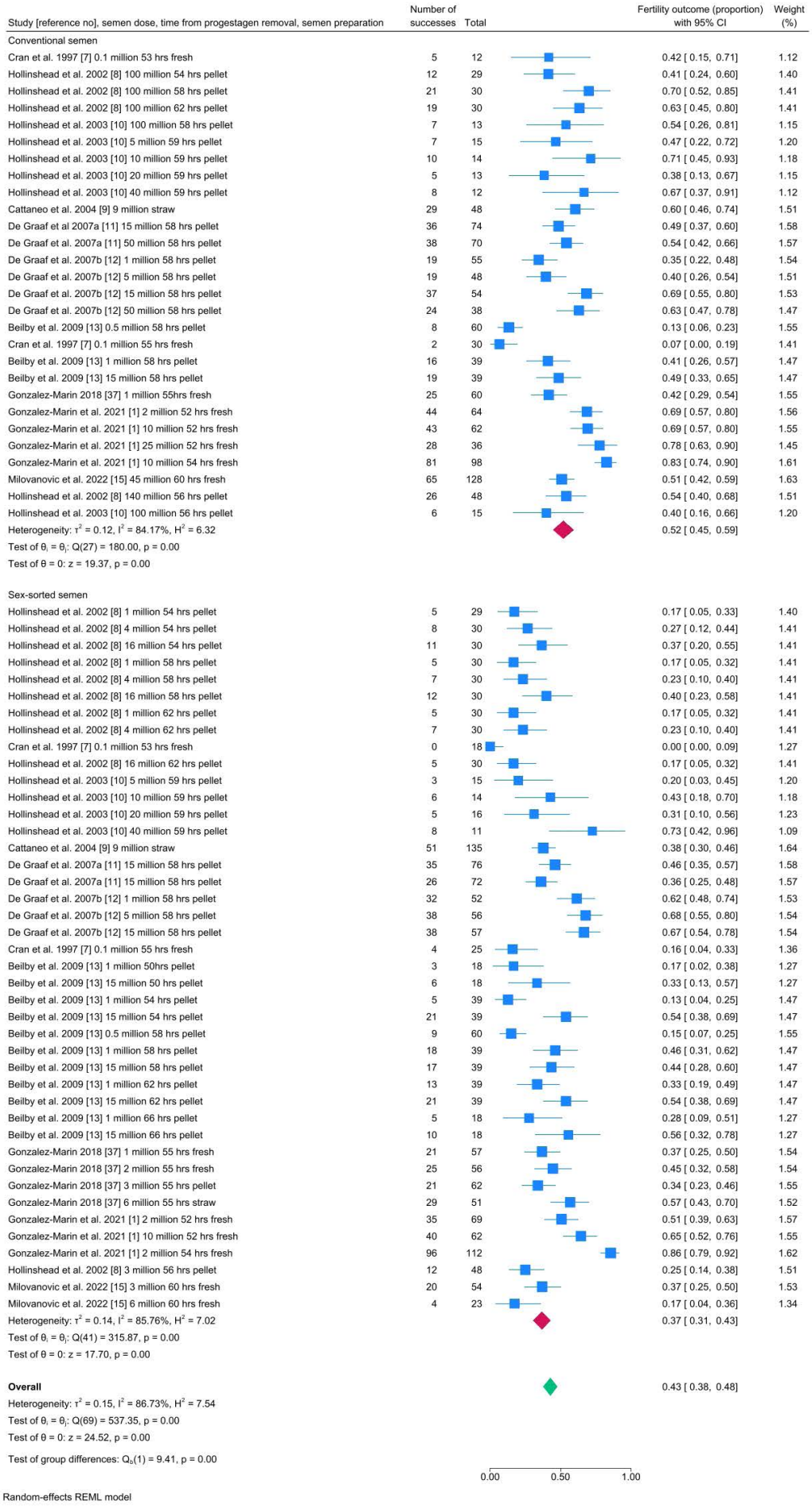

Figure 2. Forest plot of the meta-analysis of fertility outcome after the use of sex-sorted semen vs conventional semen

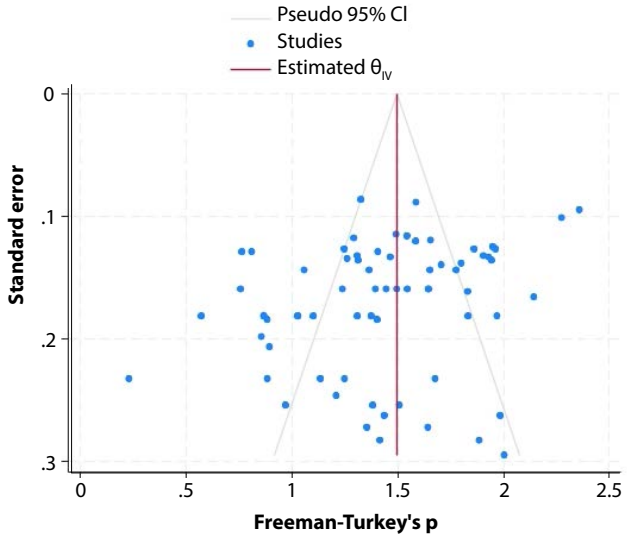

Figure 3. Funnel plot evaluating publication bias of the studies included in the meta-analysis.

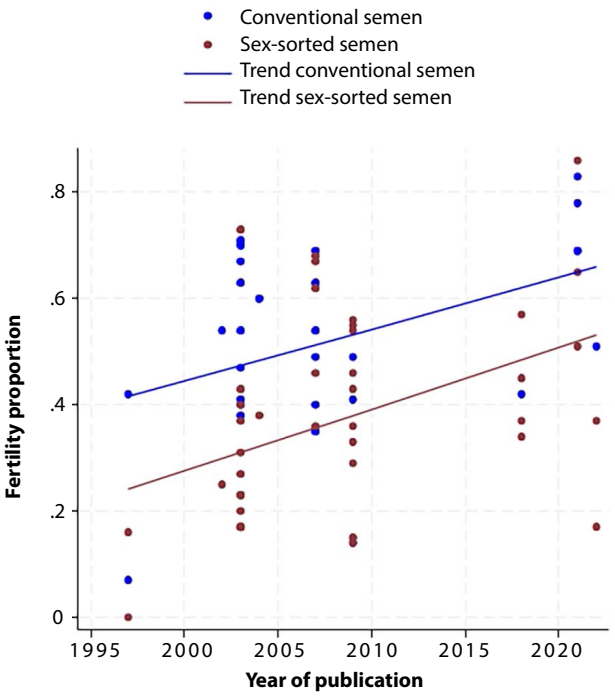

Figure 4: Scatterplot demonstrating the trends in published fertility outcome following artificial insemination with conventional and sex-sorted semen in sheep over the past 2½ decades

Discussion

FO following artificial insemination in sheep varies, and confounding factors make it difficult to draw conclusions (Figure 2). A meta-analysis was performed to place the data from this review in perspective and to identify potential factors that affect FO in sheep inseminated with SS.

Defining fertility outcome

Throughout the reviewed literature, researchers used different measures of FO after insemination, which had to be considered one outcome in our analysis. In the studies that evaluated both pregnancy and lambing rate, no significant difference existed between the two measures (Hollinshead et al. 2002; De Graaf et al. 2007a).

Table II: Meta-regression model of factors associated with fertility outcome in studies investigating sex-sorted vs conventional semen insemination in sheep ewes

Variable	Level	Coefficient	95% CI		*p*-value
Paper [experiment]	Cran et al. 1997		Referent		-
	Beilby et al. 2009	0.605	0.042	1.169	0.035
	De Graaf et al. 2007a	0.496	-0.114	1.106	0.111
	De Graaf et al. 2007b	0.930	0.344	1.517	0.002
	González-Marin et al. 2018	0.555	0.118	0.993	0.013
	González-Marin et al. 2021 [1]	0.852	0.448	1.256	< 0.001
	González-Marin et al. 2021 [2]	1.338	0.880	1.796	< 0.001
	Hollinshead et al. 2002	0.584	-0.033	1.200	0.064
	Hollinshead et al. 2003 [1]	0.450	-0.113	1.013	0.117
	Hollinshead et al. 2003 [2]	0.646	0.034	1.257	0.038
	Milovanovic et al. 2022	0.116	-0.367	0.598	0.639
Timing of insemination (from progestagen removal)	≤ 56 hours		Referent		-
	≥ 58 hours	0.121	-0.088	0.329	0.256
Semen preservation	Frozen-thawed pellet		Referent		-
	Frozen-thawed straw	0.576	0.036	1.116	0.037
	Fresh	0.354	-0.087	0.796	0.116
Spermatozoa dose per insemination by sex-sorting (interaction)	≤ 1×10^6 Conventional		Referent		-
	≤ 1×10^6 Sex-sorted	0.023	-0.242	0.288	0.864
	2–10×10^6 Conventional	0.318	-0.036	0.672	0.079
	2–10×10^6 Sex-sorted	0.218	-0.089	0.524	0.164
	> 10×10^6 Conventional	0.612	0.321	0.904	< 0.001
	> 10×10^6 Sex-sorted	0.433	0.149	0.716	0.003

Table III: Subgroup analysis demonstrating the impact within and between sex-sorted and conventional semen, and various categories, on fertility outcome

Level	Subgroups	Sex-sorted semen			Conventional semen			p^3
		n	Mean fertility proportion (95% CI)	p^1	*n*	Mean fertility proportion (95% CI)	p^2	
Year of publication	Before 2015	33	0.35 (0.28–0.41)	0.069	22	0.49 (0.41–0.56)	0.037	0.006
	After 2015	9	0.48 (0.32–0.63)		6	0.65 (0.49–0.82)		0.094
Spermatozoa dose per insemination	≤ 4×10^6 spermatozoa	22	0.30 (0.22–0.39)	0.013	6	0.35 (0.11–0.58)	0.004	0.643
	≥ 5×10^6 spermatozoa	20	0.45 (0.37–0.53)		22	0.57 (0.51–0.63)		0.014
Semen preservation	Fresh	9	0.37 (0.31–0.43)	0.730	7	0.57 (0.33–0.81)	0.412	0.004
	Frozen thawed*	33	0.39 (0.19–0.60)		21	0.51 (0.44–0.57)		0.206
Timing of insemination (from progestagen removal)	≤ 56 hours	17	0.36 (0.25–0.48)	0.781	10	0.53 (0.36–0.69)	0.889	0.079
	≥ 58 hours	24	0.38 (0.30–0.46)		17	0.51 (0.44–0.59)		0.017
Synchronisation protocol	CIDR**	7	0.54 (0.37–0.70)	0.013	5	0.68 (0.49–0.88)	0.021	0.175
	Sponge***	34	0.34 (0.28–0.40)		22	0.48 (0.41–0.55)		0.005
Gonadotropin stimulation	GnRH	32	0.36 (0.29–0.41)	0.301	18	0.51 (0.43–0.58)	0.624	0.003
	None	10	0.43 (0.26–0.60)		10	0.54 (0.38–0.71)		0.287
Biostimulation	Teaser ram or androgenised wether	8	0.46 (0.31–0.61)	0.142	8	0.53 (0.43–0.62)	0.929	0.421
	None	34	0.35 (0.29–0.42)		20	0.52 (0.43–0.61)		0.004

p^1 – p-value comparing the mean fertility proportion within sex-sorted semen inseminations, p^2 – p-value comparing the mean fertility proportion within conventional semen inseminations, p^3 – p-value comparing the mean fertility proportion between sex-sorted and conventional semen inseminations, GnRH – gonadotropin-releasing hormone, eCG – equine chorionic gonadotropin
*Frozen thawed semen was preserved either in pellets (n = 51) or in straws (n = 3)
**CIDR – progesterone-impregnated continuous intravaginal drug release (CIDR) device and 350–360 IU eCG
***Sponge – intravaginal sponge impregnated with either 30 mg or 40 mg fluorogestone acetate and 400–500 IU eCG

The FO differential between SS and CS insemination

Sex-sorting using flow cytometry may have various effects on in vitro semen characteristics, and after processing and preparation of frozen-thawed SS, motility, and in some studies acrosome integrity, were evaluated and comparisons between SS and CS were reported (Hollinshead et al. 2002; Hollinshead et al. 2003; De Graaf et al. 2007a; De Graaf et al. 2007b; Beilby et al. 2009).

Through the flow cytometric sex-sorting process, in vitro characteristics of semen including motility and acrosome integrity can either be improved or be comparable to those of CS (Hollinshead et al. 2002; Hollinshead et al. 2003; De Graaf et al. 2007a; De Graaf et al. 2007b; Beilby et al. 2009). However, sex-sorting could be detrimental with respect to other semen characteristics, including capacitation-like changes, reduced velocity characteristics and reduced ability to migrate through artificial cervical mucus, and affect the binding and release from an oviduct epithelial cell monolayer (Hollinshead et al. 2003; de Graaf et al. 2006).

From the reviewed studies, reported FO following insemination with SS vs CS was either lower (Cran et al. 1997; Hollinshead et al. 2002; Hollinshead et al. 2003; Cattaneo et al. 2004; Leahy et al. 2010; Milovanović et al. 2022), similar or higher (De Graaf et al. 2007a; De Graaf et al. 2007b; Beilby et al. 2009; González-Marín et al. 2021).

The results of our meta-analysis, however, confirmed that sex-sorting of semen had a significant negative impact on FO, after adjusting for other independent predictors (37.0% [95% CI = 31.0–43.0%] vs 52.0% [95% CI = 45.0–59.0%]; p = 0.001). Hence, the FO difference between insemination with SS vs CS was 15% lower. A meta-analysis in cattle also reported a similar differential for pregnancy rate and calving rate (43.9% vs 56.1% and 41.3% vs 54.6%, respectively) (Reese et al. 2021).

Improvements over time in FO

Through more recent advances in flow cytometric sperm sex-sorting technology such as the sexedULTRA™ methodology, it is attempted to maintain sex-sorted sperm in a benign environment with a balanced pH and low levels of reactive oxygen species, resulting in improved sperm characteristics and increased longevity (González-Marín et al. 2021). It has been argued that sex sorting by flow cytometry may select a superior subpopulation of spermatozoa that are highly motile and capable of enhanced longevity, with intact acrosomes and more fertile compared with CS even when using a lower spermatozoa concentration per dose (Hollinshead et al. 2002; Hollinshead et al. 2003; Hollinshead et al. 2004; De Graaf et al. 2006; De Graaf et al. 2007a; De Graaf et al. 2007b; De Graaf et al. 2009; González-Marín 2018; González-Marín et al. 2021). However, this meta-analysis shows that overall, FO following the use of SS and CS to inseminate sheep improved at the same rate between 1997 and 2022 (Figure 4).

The differential in FO following insemination with SS and CS has remained consistent over time (Table III). Advancements in other assisted reproductive technologies that led to consistent FO following insemination with CS, around 65% in recent times (Spanner et al. 2024b), are therefore more likely to be responsible for the recent gains in FO following insemination with SS. These include improved synchronisation of ovulation (Menchaca &

Rubianes 2004; Menchaca et al. 2017) and advancements in cryopreservation of ram semen (Ntemka et al. 2018).

Factors that may reduce the FO differential between SS and CS

Method and site of insemination

Higher FO is achieved following laparoscopic insemination compared with cervical insemination (Anel et al. 2005; Dos Santos-Neto et al. 2015), and also when performing insemination closer to the site of fertilisation compared with the lower reproductive tract (Hollinshead et al. 2002). In the only study that evaluated the cervical route to inseminate with frozen-thawed SS, the success rate was low, but the dose was only 7% of the recommended dose when cervical insemination with frozen-thawed CS is performed (Leahy et al. 2010). Considering the lifespan of frozen-thawed SS and the time needed to traverse the genital tract of the ewe (Hollinshead et al. 2003), laparoscopic insemination ensures efficient delivery of SS as close to the fertilisation site as possible (Beilby et al. 2009), requiring a lower dose of spermatozoa inseminated in comparison with cervical insemination (Salamon and Maxwell 1995b). This is important considering the cost of SS and the commercial viability of this technology.

Insemination using CS into both uterine horns vs only the horn ipsilateral to the ovulating ovary results in higher lambing rates (Maxwell 1986). Insemination at various sites of the uterine horn, including the utero-tubular junction (UTJ), does not seem to affect lambing rates, but it may affect fecundity (Maxwell 1986). FO after insemination using SS into the UTJ or intra-uterine did not differ (Hollinshead et al. 2002).

Semen preparation and preservation (frozen vs fresh, pellet vs straw)

The preparation and preservation of semen involves a series of processes from collection to thawing just prior to insemination. The viability and fertility of sex-sorted spermatozoa may be influenced by preparing and handling methods before, during and after sorting, since the spermatozoa are subjected to various damaging processes that may negatively impact on the percentage of live and motile spermatozoa (De Graaf et al. 2009; Rath et al. 2013; Maxwell et al. 2004). Insemination using frozen-thawed, sex-sorted then refrozen-thawed spermatozoa has been successful, albeit with lower FO (De Graaf et al. 2007a).

FO can either be higher (Langford et al. 1979; Hill et al. 1998; Donovan et al. 2004) or lower (McCappin & Murray 2011) with the use of fresh compared with frozen CS. With frozen semen, cooling and freezing causes physical, biochemical and functional damage to the spermatozoa (Salamon & Maxwell 1995b), which is likely to likely result in lower FO. Frozen-thawed spermatozoa have more capacitation-like changes compared with fresh spermatozoa, which affects the interactions of the spermatozoa with the oviducal cells and fertilisation rates (Hollinshead et al. 2003).

In comparison with CS, SS has reduced motility with more acrosome reactions and capacitation-like changes (De Graaf et al. 2009; Hollinshead et al. 2003). Longevity of SS is shorter than that of CS and more accurate synchronisation of insemination with the time of ovulation is required to increase FO, especially using low-dose frozen SS (Hollinshead et al. 2003). Further technological improvements are required to improve the longevity of SS, after thawing and insemination, to improve FO.

The subgroup analysis failed to demonstrate a difference in FO following insemination with fresh vs frozen-thawed SS (Table III), although it is widely accepted that fresh semen has better motility and longevity (González-Marín et al. 2021). The availability of frozen-thawed semen has the benefit of reducing the risk of insemination programmes failing as a result of lack of available semen, which is a risk when fresh semen is used. More studies are needed to compare FO between frozen and fresh SS.

The random-effects model demonstrated that, in comparison with semen frozen as a pellet, semen frozen in a straw resulted in a better FO. This is in contrast to studies that demonstrated that higher FO (Maxwell et al. 1995) or similar FO was obtained when CS was frozen as a pellet vs straws (Hill et al. 1998). Straws might be considered a more commercially viable option (González-Marín et al. 2021). Available data are limited, and more studies are necessary to compare FO between frozen SS prepared as pellets vs straws.

The effect of the ram

Variation in FO following insemination using CS from different rams is well known (Anel et al. 2005; Spanner et al. 2024b). From the reviewed studies, this variation was evident for both CS and SS (Hollinshead et al. 2002; Cattaneo et al. 2004; Hollinshead et al. 2003; De Graaf et al. 2007a; De Graaf et al. 2007b; Leahy et al. 2010; Milovanović et al. 2022).

Semen characteristics, including post-thaw motility, were similar between rams (Hollinshead et al. 2002; Hollinshead et al. 2003; De Graaf et al. 2007a; De Graaf et al. 2007b), and acrosome integrity was either similar (Hollinshead et al. 2003; De Graaf et al. 2007b) or differed between rams (Hollinshead et al. 2002). Differences have been demonstrated between rams in the distance of migration, but not in the number of SS that migrate through artificial cervical mucus (Hollinshead et al. 2003). Differences in FO and in vitro semen characteristics should be considered together, and more studies are necessary to investigate the ram effect when SS is used, including the use of molecular biomarkers (Hitit et al. 2021; Hitit et al. 2024). Individual rams that produce semen with optimal characteristics and proven FO after preparation of SS should be identified and used to ensure maximum success of this technology.

Synchronisation of ovulation

Synchronisation of ovulation can be achieved by using various synthetic or natural hormones including progestagens, prostaglandins and gonadotropins during the breeding and non-breeding season to facilitate fixed-time artificial insemination in sheep. Various commercial progestagen intravaginal devices are available for use in ewes, including polyurethane devices (sponges) impregnated with FGA (20 mg, 30 mg or 40 mg) or medroxyprogesterone acetate (MPA), and silicone devices impregnated with 0.3 g progesterone (e.g. CIDR 0.3 g), which can

affect FO when CS is used (Hill et al. 1998; McCappin & Murray 2011; Ake-Villanueva et al. 2017; Greyling et al. 1988; Dos Santos-Neto et al. 2015).

The subgroup analysis demonstrated that the use of a CIDR 0.3 g device significantly improved FO in SS as well as CS inseminations compared with using any of the sponge options (Table III). The FO differential between SS and CS inseminations can therefore be decreased when using a CIDR device. A possible reason for this is that the use of a CIDR 0.3 g device maintains adequate serum progesterone levels for longer, resulting in better synchronisation of ovulation and therefore improved timing of insemination, which may benefit insemination with SS more than with CS.

The time of ovulation relative to progestagen removal has been evaluated for variations of long and short progestagen-based synchronisation protocols, in conjunction with the addition of eCG and GnRH, and varies between 52.5 and 73.8 hours (Vilariño et al. 2010; Martemucci & D'Alessandro 2011; Vilariño et al. 2013; Silva et al. 2015; Martinez-Ros et al. 2018a; Martinez-Ros & Gonzalez-Bulnes 2019; Vilariño et al. 2010). Closer synchronisation of ovulation may be more advantageous when inseminating with SS (Hollinshead et al. 2003; Maxwell et al. 2004). Short protocols may result in the ovulation of a more viable oocyte which may lead to improved FO, and synchronisation of ovulation can be improved by the addition of technologies such as prostaglandin, eCG and GnRH (Viñoles et al. 2001; Ungerfeld & Rubianes 2002; Menchaca et al. 2010; Vilariño et al. 2010; Martemucci & D'Alessandro 2011; Vilariño et al. 2013; Dos Santos-Neto et al. 2015; Martinez-Ros et al. 2018b; Martinez-Ros & Gonzalez-Bulnes 2019; Doğan et al. 2023; Menchaca & Rubianes 2004; Menchaca et al. 2017).

Equine chorionic gonadotropin, also known as pregnant mare serum gonadotropin, is a gonadotropin with both follicle-stimulating hormone (FSH) and luteinising hormone (LH) properties, used to improve the synchronisation of oestrus and ovulation, as well as to increase ovulation and pregnancy rates (Murphy 2012). Breed, age, animal weight and time of the year are considered when the dosage of eCG is determined, and it may range between 200 IU and 600 IU, although current evidence is unclear or contradictory (Hill et al. 1998; McCappin & Murray 2011; Ake-Villanueva et al. 2017; Greyling et al. 1988; Gardón et al. 2015). The dose of eCG could not be evaluated in the random-effects model in this meta-analysis owing to collinearity with other variables.

The addition of GnRH to the synchronisation protocol is used to stimulate ovulation, decrease the interval from progestagen intravaginal device removal to ovulation, increase the proportion of ewes that ovulate close to the time of insemination, reduce the variation in time of ovulation and improve synchronisation of ovulation (Quirke et al. 1979; Cavalcanti et al. 2012; Silva et al. 2015; Martinez-Ros & Gonzalez-Bulnes 2019). This has the potential to increase FO after insemination, when SS is used (Biehl et al. 2017). Different GnRH sources have different stimulatory effects on LH secretion, and the administration of buserelin as a GnRH source at the time of insemination using CS increased the pregnancy rate to timed cervical insemination (Pereira et al. 2024). In contrast, the addition of GnRH in a synchronisation protocol may have a negative effect on the pregnancy rate in ewes inseminated cervically, possibly by affecting metabolites related to endometrial collagen and prostaglandin synthesis, which hampers embryo implantation (Zhang et al. 2024).

The subgroup analysis demonstrated that the use of GnRH significantly increased FO with CS inseminations compared with SS inseminations. However, the use of GnRH did not affect FO within SS or within CS inseminations (Table III). The effect of GnRH was not significant in the random-effects model. Further studies are required to investigate interactions between various synchronisation protocols (progestagen source, long vs short protocol, the addition of eCG or GnRH) and the use of SS, to optimise synchronisation programmes for the use of SS in sheep.

The use of biostimulation

Biostimulation, in the form of androgenised wethers, vasectomised ('teaser') rams or exposure to intact rams, is used in reproductive programmes to enhance the LH surge and thereby stimulate the expression of oestrus and the rate of ovulation in ewes, with beneficial effects on reproductive efficiency when using laparoscopic AI (Lucidi et al. 2001; McCappin & Murray 2011). In contrast, lower FO after cervical insemination using fresh semen has been reported in biostimulated ewes (Priskas et al. 2019).

The effect of biostimulation had no significant effect on FO in our study and was removed from the random-effects model. The subgroup analysis, however, demonstrated that the use of biostimulation had a slight tendency to improve FO with SS, but not with CS inseminations (Table III). Conclusive data are lacking on the effect of biostimulation on the FO differential between SS and CS, although it is feasible that biostimulation may improve FO and narrow the differential if the timing of insemination can be optimised.

Timing of insemination

The timing of ovulation and insemination should be considered together. Variations in FO may be explained by the differences in the median time of ovulation in different breeds and at different locations (Salamon & Maxwell 1995b). Since freezing and sex-sorting may result in physical, biochemical and functional damage to ram spermatozoa with resultant decreased longevity (Salamon & Maxwell 1995a), timing of insemination using frozen-thawed SS may be especially important (Hollinshead et al. 2002).

Timing of ovulation varies between 54.5 and 71.4 hours after progestogen removal using variations of long progestagen-based synchronisation protocols (Vilariño et al. 2010; Martemucci & D'Alessandro 2011; Silva et al. 2015). Insemination time periods ≤56 hours or ≥58 hours were evaluated in the random-effects model and subgroup analysis, with ≥58 hours corresponding to a time theoretically closer to ovulation of 60 hours (Menchaca et al. 2018); however, no differences were evident in either of our analyses.

Significant interactions may occur between the time of insemination, the synchronisation protocol and the semen dose. Conclusive data on the optimal time of insemination using

SS in sheep ewes are lacking, and more research is needed to determine the optimal time of insemination relative to the time of ovulation.

Semen dose

Semen dose can be considered as a major factor that influences FO when SS is used to inseminate sheep, especially when compared with the use of CS. In initial studies, functional comparison between SS and CS is confounded by the semen dose, since the CS doses contained up to 100 times more spermatozoa (Hollinshead et al 2002; Hollinshead et al. 2003).

Several studies suggested that SS is highly fertile in low doses and that in rams in particular, the sorting process probably selects for a functional population of spermatozoa with increased fertility (De Graaf et al. 2007a; De Graaf et al. 2007b; Beilby et al. 2009; González-Marín et al. 2021; González-Marín 2018). Initially it was suggested that the minimum dose of total frozen-thawed SS should be 40×10^6 or 20×10^6 motile spermatozoa (Hollinshead et al. 2003). However, to make the use of SS commercially feasible, very low doses in comparison with those applied to CS are used. Over time, and in recent studies, it was demonstrated by various authors that low doses of SS may yield acceptable, similar or better FO under experimental conditions compared with CS (De Graaf et al. 2007a; De Graaf et al. 2007b; Beilby et al. 2009; González-Marín et al. 2021; Gonzalez-Marin 2018).

However, the random-effects model in our meta-analysis demonstrated an interaction between spermatozoa dose per insemination and semen type; increasing spermatozoa dose had a positive effect for both SS and CS, but the effect was greater for CS than for SS. The subgroup analysis demonstrated that within SS inseminations, the FO can be significantly increased by using a dose of $\geq 5 \times 10^6$ spermatozoa compared with a dose of $\leq 4 \times 10^6$ spermatozoa (Table III).

The FO is similar between SS and CS inseminations when using a dose of $\leq 4 \times 10^6$ spermatozoa, but is significantly higher in CS inseminations compared with SS inseminations when a dose of $\geq 5 \times 10^6$ spermatozoa is used (Table III). The results demonstrate that although the FO differential may be reduced when using lower semen dosages, higher dosages remain beneficial for both SS and CS inseminations and should therefore be preferred if financially viable.

Limitations of the study

Research evaluating the FO following insemination with SS in sheep is limited in comparison with that in cattle (Reese et al. 2021). Available data from sheep inseminated using Y-sorted semen were excluded from this meta-analysis, because only two relatively small cohorts of data were available from early research (Cran et al. 1997; Hollinshead et al. 2002).

Conclusion

The FO after intra-uterine insemination in sheep using sex-sorted semen is 15% lower compared with that using conventional semen, despite advancements in artificial reproductive technologies over time. This differential can potentially be mitigated by increasing the spermatozoa dose to $\geq 5 \times 10^6$ when SS is used, and by improving the synchronisation of ovulation with the timing of insemination. Evaluating variations of progestogen-based synchronisation protocols, differences between rams and in vitro semen characteristics on FO using sex-sorted semen merits further investigation.

Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

The study forms part of a project that was approved by the Animal Ethics Committee of the Faculty of Veterinary Science, University of Pretoria, South Africa, in terms of the SANS 10386:2021 (Edition 2): The care and use of animals for scientific purposes (South African National Standard) (REC127-23), and by the Research Ethics Committee of the Faculty of Humanities, University of Pretoria, South Africa (HUM013/1223).

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References

Ake-Villanueva, J.R., Ake-Lopez, J.R., Segura-Correa, J.C., 2017, Factors affecting conception rate of hair ewes after laparoscopic insemination with chilled semen under tropical conditions, *Small Ruminant Research* 153, 114-117. https://doi.org/10.1016/j.smallrumres.2017.06.006.

Anel, L., Kaabi, M., Abroug, B., et al., 2005, Factors influencing the success of vaginal and laparoscopic artificial insemination in churra ewes: a field assay, *Theriogenology* 63(4), 1235-1247. https://doi.org/10.1016/j.theriogenology.2004.07.001.

Beilby, K.H., Grupen, C.G., Thomson, P.C., et al., 2009, The effect of insemination time and sperm dose on pregnancy rate using sex-sorted ram sperm, *Theriogenology* 71(5), 829-835. https://doi.org/10.1016/j.theriogenology.2008.10.005.

Biehl, M.V., Ferraz, M.V.C., Ferreira, E.M., et al., 2017, Effect of reproductive methods and GnRH administration on long-term protocol in Santa Ines ewes, *Tropical Animal Health and Production* 49(6), 1303-1308. https://doi.org/10.1007/s11250-017-1329-1.

Brito, B.F., Bandeira Santos, B.M., Rodrigues Cabral, L.A., et al., 2021, Ram and goat semen immunosexed and diluted in powdered coconut water-based preservation medium (ACP101/102c), *Acta Scientiae Veterinariae* 49. https://doi.org/10.22456/1679-9216.114066.

Cattaneo, L., Martin, P., Caballero, J., et al., 2004, Pregnancy rate in ewes inseminated with sexed semen in Argentina, *Reproduction, Fertility and Development* 16, 284-284. https://doi.org/10.1071/RDv16n1Ab329.

Cavalcanti, A.D., Brandao, F.Z., Nogueira, L.A.G., et al., 2012, Effects of GnRH administration on ovulation and fertility in ewes subjected to estrous synchronization, *Revista Brasileira de Zootecnia-Brazilian Journal of Animal Science* 41(6), 1412-1418. https://doi.org/10.1590/S1516-35982012000600014.

Cran, D., Mckelvey, W., King, M., et al., 1997, Production of lambs by low dose intrauterine insemination with flow cytometrically sorted and unsorted semen, *Theriogenology* 47(1), 267. https://doi.org/10.1016/S0093-691X(97)82394-1.

De Graaf, S., Beilby, K., Underwood, S., et al., 2009, Sperm sexing in sheep and cattle: the exception and the rule, *Theriogenology* 71(1), 89-97. https://doi.org/10.1016/j.theriogenology.2008.09.014.

De Graaf, S., Evans, G., Maxwell, W., et al., 2007a, Birth of offspring of pre-determined sex after artificial insemination of frozen–thawed, sex-sorted and re-frozen–thawed ram spermatozoa, *Theriogenology* 67(2), 391-398. https://doi.org/10.1016/j.theriogenology.2006.08.005.

De Graaf, S., Evans, G., Maxwell, W., et al., 2007b, Successful low dose insemination of flow cytometrically sorted ram spermatozoa in sheep, *Reproduction in Domestic Animals* 42(6), 648-653. https://doi.org/10.1111/j.1439-0531.2006.00837.x.

De Graaf, S.P., Evans, G., Maxwell, W.M., et al., 2006, In vitro characteristics of fresh and frozen-thawed ram spermatozoa after sex sorting and re-freezing, *Reproduction, Fertility and Development* 18(8), 867-874. https://doi.org/10.1071/rd06061.

Diaz, C.A.G., Emsen, E., 2012, Factors affecting pregnancy rates following laparoscopic insemination in Turkish native sheep, *Reproduction in Domestic Animals* 47, 93-94. https://doi.org/10.1111/j.1439-0531.2012.02044.x.

Doğan, İ., Toker, M., Aktar, A., et al., 2023, Effect of GnRH and hCG treatment following a short-term estrus synchronization protocol on ovulation and the fertility in merino ewes, during the breeding season, *Journal of the Hellenic Veterinary Medical Society* 74(4), 6523-6530. https://doi.org/10.12681/jhvms.31541.

Donovan, A., Hanrahan, J., Kummen, E., et al., 2004, Fertility in the ewe following cervical insemination with fresh or frozen–thawed semen at a natural or synchronised oestrus, *Animal Reproduction Science* 84(3-4), 359-368. https://doi.org/10.1016/j.anireprosci.2003.12.014.

Dos Santos-Neto, P.C., Garcia-Pintos, C., Pinczak, A., et al., 2015, Fertility obtained with different progestogen intravaginal devices using Short-term protocol for fixed-time artificial insemination (FTAI) in sheep, *Livestock Science* 182, 125-128. https://doi.org/10.1016/j.livsci.2015.11.005.

El Amiri, B., Rahim, A., 2024, Exploring endogenous and exogenous factors for successful artificial insemination in sheep: a global overview, *Veterinary Sciences* 11(2), 86. https://doi.org/10.3390/vetsci11020086.

Freeman, M.F., Tukey, J.W., 1950, Transformations related to the angular and the square root, *The Annals of Mathematical Statistics* 21(4), 607-611. https://doi.org/10.1214/aoms/1177729756.

Fukui, Y., Kohno, H., Okabe, K., et al., 2010, Factors affecting the fertility of ewes after intrauterine insemination with frozen-thawed semen during the non-breeding season, *Journal of Reproduction and Development* 56(4), 460-466. https://doi.org/10.1262/jrd.10-015t.

Gardón, J.C., Escribano, B., Astiz, S.M., et al., 2015, Synchronization protocols in Spanish Merino sheep: reduction in time to estrus by the addition of eCG to a progesterone-based estrus synchronization protocol, *Annals of Animal Science* 15(2), 409-418. https://doi.org/10.1515/aoas-2015-0003.

González-Marín, C., 2018, Evolution of cervine, caprine and ovine sex-sorted semen processing, *Clinical Theriogenology* 10(3), 205-209. https://doi.org/10.58292/ct.v10.9914.

González-Marín, C., Góngora, C., Moreno, J., 2021, Small ruminant SexedULTRA™ sperm sex-sorting: status report and recent developments, *Theriogenology* 162, 67-73. https://doi.org/10.1016/j.theriogenology.2020.12.028.

Greyling, J., Greeff, J., Brink, W., et al., 1988, Synchronization of oestrus in sheep of low-normal mass under range conditions: the use of different progestagens and PMSG. *South African Journal of Animal Science* 18(4), 164-167.

Hill, J.R., Thompson, J.A., Perkins, N.R., 1998, Factors affecting pregnancy rates following laparoscopic insemination of 28,447 Merino ewes under commercial conditions: A survey, *Theriogenology* 49(4), 697-709. https://doi.org/10.1016/S0093-691X(98)00019-3.

Hitit, M., Kaya, A., Memili, E., 2024, Sperm long non-coding RNAs as markers for ram fertility, *Frontiers in Veterinary Science* 11, 1337939. https://doi.org/10.3389/fvets.2024.1337939.

Hitit, M., Özbek, M., Ayaz-Guner S., et al., 2021, Proteomic fertility markers in ram sperm, *Animal Reproduction Science* 235, 106882. https://doi.org/10.1016/j.anireprosci.2021.106882.

Hollinshead, F., O'Brien, J., Maxwell, W., et al., 2002, Production of lambs of predetermined sex after the insemination of ewes with low numbers of frozen–thawed sorted X-or Y-chromosome-bearing spermatozoa, *Reproduction, Fertility and Development* 14, 503-508. https://doi.org/ 10.1071/rd02034.

Hollinshead, F.K., Evans, G., Evans, K.M., et al., 2004, Birth of lambs of a pre-determined sex after in vitro production of embryos using frozen-thawed sex-sorted and re-frozen-thawed ram spermatozoa, *Reproduction* 127(5), 557-568. https://doi.org/10.1530/rep.1.00049

Hollinshead, F.K., Gillan, L., O'Brien, J.K., et al., 2003, In vitro and in vivo assessment of functional capacity of flow cytometrically sorted ram spermatozoa after freezing and thawing, *Reproduction, Fertility and Development* 15(6), 351-359. https://doi.org/10.1071/RD03060.

Johnson, L.A., 1995, Sex preselection by flow cytometric separation of X and Y chromosome-bearing sperm based on DNA difference: a review, *Reproduction, Fertility and Development* 7(4), 893-903. https://doi.org/10.1071/rd9950893.

Langford, G., Marcus, G., Hackett, A., et al., 1979, A comparison of fresh and frozen semen in the insemination of confined sheep, *Canadian Journal of Animal Science* 59(4), 685-691. https://doi.org/10.4141/CJAS79-088.

Leahy, T., Evans, G., Maxwell, W.M.C., et al., 2010, Seminal plasma proteins do not consistently improve fertility after cervical insemination of ewes with non-sorted or sex-sorted frozen-thawed ram spermatozoa, *Reproduction, Fertility and Development* 22(4), 606-612. https://doi.org/10.1071/RD09207.

Lucidi, P., Barboni, B., Mattioli, M., 2001, Ram-induced ovulation to improve artificial insemination efficiency with frozen semen in sheep, *Theriogenology* 55(9), 1797-1805. https://doi.org/10.1016/s0093-691x(01)00522-2.

Martemucci, G, D'Alessandro, A., 2011, Synchronization of oestrus and ovulation by short time combined FGA, PGF2α, GnRH, eCG treatments for natural service or AI fixed-time, *Animal Reproduction Science* 123(1-2), 32-39. https://doi.org/10.1016/j.anireprosci.2010.11.007.

Martinez-Ros, P., Astiz, S., Garcia-Rosello, E., et al., 2018a, Effects of short-term intravaginal progestagens on the onset and features of estrus, preovulatory LH surge and ovulation in sheep, *Animal Reproduction Science* 197, 317-323. https://doi.org/10.1016/j.anireprosci.2018.08.046.

Martinez-Ros, P., Rios-Abellan, A., Gonzalez-Bulnes, A, 2018b, Influence of progesterone-treatment length and eCG administration on appearance of estrus behavior, ovulatory success and fertility in sheep. *Animals (Basel)* 9(1), 9. https://doi.org/10.3390/ani9010009.

Martinez-Ros, P., Gonzalez-Bulnes, A., 2019, Efficiency of CIDR-based protocols including GnRH instead of eCG for estrus synchronization in sheep, *Animals (Basel)* 9(4), 146. https://doi.org/10.3390/ani9040146.

Maxwell, W., 1986, Artificial insemination of ewes with frozen-thawed semen at a synchronised oestrus. 2. Effect of dose of spermatozoa and site of intrauterine insemination on fertility, *Animal Reproduction Science* 10(4), 309-316. https://doi.org/10.1016/0378-4320(86)90006-0.

Maxwell, W., Evans, G., Hollinshead, F., et al., 2004, Integration of sperm sexing technology into the ART toolbox, *Animal Reproduction Science* 82, 79-95. https://doi.org/10.1016/j.anireprosci.2004.04.013.

Maxwell, W., Landers, A., Evans, G., 1995, Survival and fertility of ram spermatozoa frozen in pellets, straws and minitubes, *Theriogenology* 43(7), 1201-1210. https://doi.org/10.1016/0093-691x(95)00092-m.

McCappin, N., Murray, R.D., 2011, Some factors affecting pregnancy rate in ewes following laparoscopic artificial insemination, *Veterinary Record: Journal of the British Veterinary Association* 168(4), 99-99. https://doi.org/10.1136/vr.c5979.

Menchaca, A., Dos Santos-Neto, P.C., Cuardro, F., et al., 2018, From reproductive technologies to genome editing in small ruminants: an embryo's journey, *Animal Reproduction* 15(Suppl 1), 984-995. https://doi.org/10.21451/1984-3143-AR2018-0022.

Menchaca, A., Dos Santos-Neto, P.C., Cuardro, F., 2017, Estrous synchronization treatments in sheep: brief update, *Revista Brasileira de Reproducao Animal* 41(1), 340-344.

Menchaca, A., Rubianes, E., 2004, New treatments associated with timed artificial insemination in small ruminants, *Reproduction, Fertility and Development* 16(4), 403-413. https://doi.org/10.10371/RD04037.

Menchaca, A., Vilariño, M., Rubianes, E., 2010, 29 Short-term v. long-term progesterone protocol using cervical or intrauterine fixed-time insemination in sheep, *Reproduction, Fertility and Development,* 22(1), 172-172. https://doi.org/10.1071/RDv22n1Ab29.

Milovanović, A., Apić, J., Barna, T., 2022, Laparoscopic insemination (LAI) with fresh sex-sorted semen in sheep: first results, *Reproduction in Domestic Animals*, 57, 105-106.

Murphy, B., 2012, Equine chorionic gonadotropin: an enigmatic but essential tool, *Animal Reproduction* 9(3), 223-230.

Ntemka, A., Tsakmakidis, I., Kiossis, E., et al., 2018, Current status and advances in ram semen cryopreservation, *Journal of the Hellenic Veterinary Medical Society*, 69(2), 911-924. https://doi.org/10.12681/jhvms.18014.

Page, M.J., McKenzie, J.E., Bossouyt, P.M., et al., 2021, The PRISMA 2020 statement: an updated guideline for reporting systematic reviews, *BMJ* 372, n71. https://doi.org/10.1136/bmj.n71.

Pereira, R.G., Pires, O.S., Hoefle, C.A., et al., 2024, GnRH analogs induce a LH peak and increase pregnancy per timed-AI in ewes, *Theriogenology* 215, 132-137. https://doi.org/10.1016/j.theriogenology.2023.12.003.

Priskas, S., Termatzidou, S., Gargani, S., et al., 2019, Factors affecting pregnancy rate after cervical insemination in dairy sheep flocks, *ICAR Technical Series* 24, 349-356.

Quirke, J.F., Jennings, J.J., Hanrahan, J.P., et al., 1979, Oestrus, time of ovulation, ovulation rate and conception in progestagen-treated ewes given GN-RH, GN-RH analogues and gonadotrophins, *Journal of Reproduction and Fertility* 56(2), 479-488. https://doi.org/10.1530/jrf.0.0560479.

Rath, D., Bardikowski, S., De Graaf, S., et al., 2013, Sex selection of sperm in farm animals: status report and developmental prospects, *Reproduction* 145(1), R15-R30. https://doi.org/10.1530/REP-12-0151.

Reese, S., Pirez, M.C., Steele, H., et al., 2021, The reproductive success of bovine sperm after sex-sorting: A meta-analysis, *Scientific Reports* 11(1), 17366. https://doi.org/10.1038/s41598-021-96834-2.

Salamon, S., Maxwell, W., 1995a, Frozen storage of ram semen I. Processing, freezing, thawing and fertility after cervical insemination, *Animal Reproduction Science* 37, 185-249. https://doi.org/10.1016/0378-4320(94)01327-I.

Salamon, S., Maxwell, W., 1995b, Frozen storage of ram semen II. Causes of low fertility after cervical insemination and methods of improvement, *Animal Reproduction Science* 38, 1-36. https://doi.org/10.1016/0378-4320(94)01328-J.

Silva, B.D.M., Silva, T., Moreira, N.H., et al., 2015, Ovulation induction in ewes using GnRH in long and short-term synchronization protocols, *Animal Reproduction* 12(2), 312-315.

Spanner, E., De Graaf, S., Rickard, J., 2024a, Factors affecting the success of laparoscopic artificial insemination in sheep, *Animal Reproduction Science* 264, 107453. https://doi.org/10.1016/j.anireprosci.2024.107453.

Spanner, E., De Graaf, S., Rickard, J., 2024b, Uterine tone influences fertility of Merino ewes following laparoscopic artificial insemination, *Theriogenology* 222, 45-53. https://doi.org/10.1016/j.theriogenology.2024.04.002.

Ungerfeld, R., Rubianes, E., 2002, Short term primings with different progestogen intravaginal devices (MAP, FGA and CIDR) for eCG-estrous induction in anestrus ewes, *Small Ruminant Research* 46(1), 63-66. https://doi.org/10.1016/S0921-4488(02)00105-0.

Vilariño, M., Rubianes, E., Menchaca, A., 2013. Ovarian responses and pregnancy rate with previously used intravaginal progesterone releasing devices for fixed-time

artificial insemination in sheep. *Theriogenology* 79(1), 206-210. https://doi.org/10.1016/j.theriogenology.2012.10.007.

Vilariño, M., Rubianes, E., Van Lier, E., et al., 2010, Serum progesterone concentrations, follicular development and time of ovulation using a new progesterone releasing device (DICO®) in sheep, *Small Ruminant Research* 91(2-3), 219-224. https://doi.org/10.1016/j.smallrumres.2010.02.014.

Viñoles, C., Forsberg, M., Banchero, G., et al., 2001, Effect of long-term and short-term progestagen treatment on follicular development and pregnancy rate in cyclic ewes, *Theriogenology* 55(4), 993-1004. https://doi.org/10.1016/s0093-691x(01)00460-5.

Yotov, S., Kistanova, E., Abadjieva, D., et al., 2021, Comparative analysis of motility characteristics and kinematic parameters of fresh, chilled and sexed ram semen – preliminary study, *Veterinarija ir Zootechnika* 79(2), 37-42.

Zhang J., Sun, S., Bai, X., et al., 2024, Metabolomics analysis of the effect of GnRH on the pregnancy rate of ewes with estrus synchronization scheme based on progesterone, *Frontiers in Veterinary Science* 11, 1442931. https://doi.org/10.3389/fvets.2024.1442931.