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Association between conventional semen variables and sperm freezability in rams

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Abstract: Assessments of routine ejaculated samples were used to evaluate the conventional semen variables (sperm motility, semen volume, sperm concentration) as initial decision-making criteria to freeze or not to freeze. However, the association of these attributes to the cryotolerance of ram sperm has not been adequately studied yet. The aim of this study was therefore to analyse if the semen volume, sperm motility and concentration can be used to predict post-thaw ram sperm motility. Additionally, in the present study we tested if the order of ejaculate sampling (first versus second, within a single collection session) and the breed of rams influenced cryopreservation outcomes.

Keywords: breed; cryopreservation; ejaculate volume; order of ejaculate collection; sperm concentration; spermatozoa motility

Sperm freezing represents an effective method for preserving and transporting genetic material that eliminates geographical barriers and allows for increased sire use without risking their reproductive health. Nonetheless, the process of cryopreservation induces irreversible cryodamage, which leads to decreased fertilizing ability of sperm. Like many other species, ovine sires present their inter-individual variability that affects the results of semen storage and the freeze-thaw process, which in turn leads to their classification according to the freezability of their semen (Soleilhavoup et al. 2014; Pequeno et al. 2023). Several explanations for this phenomenon have previously been described, including the age of the ram and birth type (twins or singletons) (Malkova et al. 2024). It is also important to note that even ejaculates from the same sire exhibit varying cryo-resistance, but the responsible endogenous factors are not yet fully understood (Martinez-Fresneda et al. 2019). Therefore, it is not surprising that researchers across multiple industries are looking for an effective way to predict the freezability of sperm.

In previous years, both conventional (Chikhaliya et al. 2018; Asaduzzaman et al. 2021) and non-conventional (Casas et al. 2009) qualitative and quantitative variables of freshly obtained semen have been used as potential predictors of sperm freezability, including post-thaw variability parameters.

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In order to evaluate non-conventional semen variables, sophisticated approaches employing proteomics like Western blot, immunocytochemistry, and liquid chromatography-mass spectrometry, or even more advanced transcriptomic methods (microRNA and mRNA profiles of sperm), have previously been suggested by several authors (Pequeno et al. 2023; Ren et al. 2023; Sun et al. 2023). Qin et al. (2018) reported that aconitate 2 and pyruvate kinase M2 localised in the body and tail of spermatozoa could be used as good predictors of human sperm freezability. Recently, it was shown that ram sperm cryotolerance is related to aquaporin 3 expression, which suggests this protein could be used as a good freezability predictor (Pequeno et al. 2023). Furthermore, one study found that the ram spermatozoa with superior freezability contained specific proteins involved in regulating Ca^{2+} transport and maintaining the structure of flagella (S100A8, S100A9, S100A12, S100A14 and S100A16), providing antioxidant protection and preventing apoptosis (HYOU1, PRDX1), and maintaining cell motility and immune response (HSP90B1) (Ren et al. 2023).

It is obvious that such approaches are certainly useful not only in practical terms but also more importantly for enriching our theoretical knowledge of the mechanisms of spermatozoa cryodamage and developing effective methods to combat them. Unfortunately, the major drawbacks of such approaches are their high costs and time-consumption.

Key factors when choosing what sperm parameters to use as freezability predictors are time efficiency and cost-effectiveness. Previously, results from the hypo-osmotic swelling (HOS) test were considered a good indicator of sperm freezability, and despite its relatively low cost, the time required to take this test must be considered (Padrik et al. 2012; Prinosilova et al. 2014). In contrast, sperm motility is a conventional parameter that has long been considered an excellent predictor of sperm fertilizing ability (David et al. 2015). Due to its quick and low-cost analysis, many researchers are exploring its use as a freezability predictor of sperm from various species. Dorado et al. (2009) showed that sperm motility parameters of goats, including the overall percent of motile spermatozoa, were effective for predicting freezability. A similar conclusion was reached by Jiang et al. (2017), when they developed an effective model to predict post-thaw sperm motility based on the pre-freeze parameters of human sperm (progressive motility, straight line, average path velocity). This model helped the authors significantly improve the cryopreservation success at a human sperm bank from 67% to 94%. Apart from sperm motility, semen volume and sperm concentration are important conventional indicators of sperm quality. Sperm concentration is characterised by the number of spermatozoa per one millilitre of ejaculate, and based on this concentration, undiluted ram semen can be categorised as either spermatozoa rich (contains more than 2 billion sperm/ml) or spermatozoa poor (contains less than 1 billion sperm/ml). It is known that despite using optimised procedures, a significant proportion of ram spermatozoa die or are seriously damaged during freezing and thawing (Savvulidi et al. 2021), with the growth of intracellular ice crystals being one of the main causes (Savvulidi et al. 2023). Thus, in order to improve cryopreservation success, it is necessary to use not only high-volume ejaculates but also those with a high sperm concentration. This requirement allows for increased insemination dose production with an optimal number of spermatozoa in each dose.

Previously, Gil et al. (2005) reported a low association between conventional sperm parameters and freezability in some livestock species, but regardless of varying recommendations across species, the use of conventional semen parameters, including motility, concentration, and semen volume, as predictors of freezability is still of great interest to the sheep industry. In this study, we tested whether pre-freezing parameters of fresh ejaculate (sperm motility, semen volume, and concentration) are useful for predicting thawed ram sperm motility. Additionally, we tested if the order of ejaculate sampling and breed influenced cryopreservation outcomes, as these topics are still a focus area for researchers in the field of ovine reproduction (Ben Moula et al. 2022; Vozaf et al. 2022; Mujitaba et al. 2024).

MATERIAL AND METHODS

Animals

All rams used in the present study belonged to "Suleimenov" Farm in the Akmola region. For this study, semen collection, freezing-thawing procedures, and quality analysis were done at the Republican Centre for Breeding Livestock Asyl Tulik JSC, Kosshy city, Akmola region, Republic

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of Kazakhstan at 350 m above sea level (50.9897, 71.3636). This centre is nationally accredited for its equipment standards, technological processes, and animal husbandry. The climate in the Akmola region is continental and dry, with hot summers and cold winters. The highest amount of rain is in June, while the minimum is during February. Ram semen was obtained with the aid of an artificial vagina in different seasons throughout the years from 2021 to 2023. Semen collection was conducted 3 times a week (first and second ejaculation, within a single collection session, was collected) from healthy and sexually mature Edilbay (n = 10) and Hampshire Down (n = 2) rams. The rams were kept in individual enclosures located in a separated area and were already accustomed to an artificial vagina. A balanced diet was prepared daily with a feeding ration that consisted of 1.5 kg of hay (mixed grass), 1 kg of barley, 0.5 kg of carrots, 10 g of feed additive (premix), and 20 g of mineral licks. Furthermore, the rams were provided good-quality water *ad libitum*.

Analysis of collected semen quality

Semen was collected with the use of a standard artificial vagina optimised for ovine semen collection. Immediately after collection, the fresh semen was evaluated organoleptically for colour, odour, and density. Semen volume (VOL, ml) was measured using a 15 ml graduated Pyrex® test tube. Sperm concentration (CONC, 10^9/ml) was assessed using the "Accucell 783" photometer (IMV Technologies, L'Aigle, France). Pre-freeze sperm motility (FRESH) was evaluated in a prediluted sample with a coverslip, using a 10-point scale (where 1 point is equal to 10% and 10 points are equal to 100% motility) at 35–38 °C under a basic light microscope with 200 times magnification (Palmer 2016). The semen with a score of at least 8 points was further processed and eventually cryopreserved. However, in some cases, semen samples with scores lower than 8 points were also used.

Semen processing, cryopreservation, thawing, and analysis of thawed sperm quality

Semen was diluted with OptiXcell diluent (IMV Technologies, L'Aigle, France) to a concentration of 800 million sperm/ml and packed into 0.25 ml straws. An IS 4 packaging machine (IMV Technologies, L'Aigle, France) was used for straw processing. The straws were frozen using a Mini-Digitcool 1400 (IMV Technologies, L'Aigle, France) freezer with Win 3T software and they were frozen over 9 min from +4 °C to –140 °C. The temperature was gradually lowered to –10 °C over 4.5 min, then to –100 °C within 2.5 min, and finally to –140 °C in the last 2 minutes. After one month of storage, the sperm was thawed at 37 °C for 30 seconds. Post-thaw sperm motility was evaluated using a 10-point scale at 35–38 °C under a basic light microscope with 200 times magnification (Palmer 2016).

Statistical analyses

All statistical evaluations of frozen/thawed sperm were performed using SAS v9.3 (2011; Cary, NC, USA).

Freezability prediction using simple regression analysis

A simple regression analysis was performed using the REG procedure. Simple one-way (VOL, CONC, FRESH), multiple two-way (VOL × CONC; VOL × FRESH; CONC × FRESH), and multiple three-way (VOL × CONC × FRESH) linear regression equations were calculated to predict sperm freezability. The prediction value was estimated based on the coefficient of determination (R^2) and the statistical significance of each regression variable.

Freezability prediction using regression analysis corrected for systematic effects

In the next sequence, generalised linear models (GLM) and MIXED procedures were used to estimate frozen/thawed sperm freezability based on individual (VOL, CONC, FRESH) or multiple (VOL × CONC; VOL × FRESH; CONC × FRESH; VOL × CONC × FRESH) pre-freezing variables as covariates. Each relation was expressed after adjustment due to seasonal effect (from combining ejaculates taken in the course of the year), order of ejaculate sampling (1st or 2nd ejaculate within

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a single collection session), and breed variations. The prediction abilities of these statistical models were compared using the defined criteria: R^2 coefficient, *F*-value of particular factors, Akaike information criterion (AIC), corrected Akaike information criterion (AICC), and the Bayesian information criterion (BIC). The best model was the one that had the highest R^2 and the lowest AIC, AICC, and BIC. Significance was estimated at $P < 0.05$.

$$\text{THAWED}_{ijkl} = \mu + \text{YS}_i + \text{BREED}_j + \text{ORDER}_k + b1 \times \text{VOL} + e_{ijkl} \quad (1)$$

$$\text{THAWED}_{ijkl} = \mu + \text{YS}_i + \text{BREED}_j + \text{ORDER}_k + b2 \times \text{CONC} + e_{ijkl} \quad (2)$$

$$\text{THAWED}_{ijkl} = \mu + \text{YS}_i + \text{BREED}_j + \text{ORDER}_k + b3 \times \text{FRESH} + e_{ijkl} \quad (3)$$

$$\text{THAWED}_{ijkl} = \mu + \text{YS}_i + \text{BREED}_j + \text{ORDER}_k + b1 \times \text{VOL} + b2 \times \text{CONC} + e_{ijkl} \quad (4)$$

$$\text{THAWED}_{ijkl} = \mu + \text{YS}_i + \text{BREED}_j + \text{ORDER}_k + b1 \times \text{VOL} + b3 \times \text{FRESH} + e_{ijkl} \quad (5)$$

$$\text{THAWED}_{ijkl} = \mu + \text{YS}_i + \text{BREED}_j + \text{ORDER}_k + b2 \times \text{CONC} + b3 \times \text{FRESH} + e_{ijkl} \quad (6)$$

$$\text{THAWED}_{ijkl} = \mu + \text{YS}_i + \text{BREED}_j + \text{ORDER}_k + b1 \times \text{VOL} + b2 \times \text{CONC} + b3 \times \text{FRESH} + e_{ijkl} \quad (7)$$

where:

THAWED – frozen/thawed sperm motility (%);

μ – the mean of the trait evaluated;

YS_i – randomised combined year-seasonal effect;

BREED_j – fixed effect of ram breed (j – Edilbay, n – 396; j – Hampshire Down, n – 40);

ORDER_k – fixed effect of the order of ejaculate sampling (k – 1st ejaculate, n – 218; k – 2nd ejaculate, n – 218);

$b1 \times$ VOL – semen volume as a covariate;

$b2 \times$ CONC – sperm concentration as a covariate;

$b3 \times$ FRESH – sperm motility after collection as a covariate;

e_{ijkl} – the residual error.

RESULTS

Freezability prediction using simple regression analysis

Statistical models for the prediction of sperm freezability based on an initial evaluation of conventional semen variables (motility, semen volume, sperm concentration) using a simple linear regression analysis are reported in Table 1.

This table includes simple (one-way statistical models No. 1–3) as well as multiple (two- or three-way statistical models No. 4–7) linear regression.

As far as the simple (one-way) linear regressions are concerned, the statistical models for sperm CONC (No. 2) and FRESH (No. 3) showed a significant predictive character for THAWED. An increase of 1×10^9 sperm in fresh ejaculate or 0.1 point of fresh sperm motility corresponded with a significant increase of frozen/thawed sperm motility by 0.032 or 0.109 points, respectively. However, the coefficients of determination were low: 3.3% ($P > 0.05$) for CONC (statistical model No. 2) and 5.7% ($P < 0.05$) for FRESH (statistical model No. 3). When considering multiple covariates, the coefficient of determination increased, but by 7.4% at maximum ($P < 0.05$) (statistical model No. 7). Among the evaluated covariates, FRESH was a stronger predictor, where an increase of 0.1 point corresponded with a 0.117–0.187 point (depending on the statistical model) increase of THAWED. A weaker, but still significant predictive relationship was detected for VOL, when analysed using a two-way linear regression with CONC or FRESH.

Freezability prediction using regression analysis corrected for systematic effects

Statistical models to predict frozen/thawed sperm motility are reported in Table 2.

This table includes seven prediction model equations corrected for systematic effects with initial check attributes (VOL, CONC, FRESH) as covariates (independently, multiple: two or three simultaneously evaluated covariates). The predictive value for all the statistical models was similar when considering the coefficient of determination (R^2 = 26.0–29.3%, $P < 0.05$), Akaike information criterion (AIC = 596.5–604.6), corrected Akaike information criterion (AICC = 596.5–604.6), and the Bayesian information criterion (BIC = 594.7–602.8). Generally, all of these criteria indicate that the initially checked attributes had only a minor effect. Furthermore, this was confirmed by the significance of the effects and it was supported by the *F*-values.

Sperm motility after thawing was also largely influenced by the order of ejaculate sampling, when

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Table 1. Freezability prediction using simple regression analysis

Model No.	Linear regression equation	GLM procedure		Significance		
		R^2	P	VOL	CONC	FRESH
1.	THAWED = 3.335 – 0.162 × VOL	0.007	n.s.	n.s.	–	–
2.	THAWED = 3.227 + 0.032 × CONC	0.033	***	–	***	–
3.	THAWED = 2.728 + 0.109 × FRESH	0.057	****	–	–	****
4.	THAWED = 3.447 – 0.294 × VOL + 0.038 × CONC	0.049	***	*	***	-
5.	THAWED = 2.914 – 0.231 × VOL + 0.117 × FRESH	0.071	****	*	–	****
6.	THAWED = 2.462 – 0.033 × CONC + 0.187 × FRESH	0.063	****	–	n.s.	**
7.	THAWED = 2.709 – 0.205 × VOL – 0.023 × CONC + 0.170 × FRESH	0.074	****	n.s.	n.s.	**

CONC = sperm concentration after collection (10^9/ml); FRESH = sperm motility after collection (points); GLM = generalised linear models; n.s. = not significant; P-model = P-value of the statistical model; R^2 = coefficient of determination; THAWED = frozen-thawed sperm motility (points); VOL = semen volume after collection (ml)
*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.000 1

Table 2. Freezability prediction using regression analysis corrected for systematic effects

Model No.	Linear regression equation	GLM procedure		MIXED procedure			Significance (F-value)					
		R^2	P	AIC	AICC	BIC	YEAR*SEASON	BREED	ORDER	VOL	CONC	FRESH
1.	THAWED = 3.669 – 0.056 × VOL	0.260	****	604.6	604.6	602.8	**** (3.20)	**** (26.82)	*** (11.91)	0.614 (2.26)	–	–
2.	THAWED = 2.925 + 0.032 × CONC	0.288	****	597.6	597.7	595.8	**** (3.07)	**** (33.17)	*** (13.20)	–	*** (12.44)	–
3.	THAWED = 2.662 + 0.081 × FRESH	0.286	****	596.5	596.5	594.7	**** (2.80)	**** (29.68)	*** (12.06)	–	–	*** (11.67)
4.	THAWED = 3.177 – 0.170 × VOL + + 0.036 × CONC	0.293	****	597.9	597.9	596.1	**** (3.10)	**** (30.28)	** (10.73)	0.133 (2.26)	*** (14.49)	–
5.	THAWED = 2.800 – 0.131 × VOL + + 0.086 × FRESH	0.289	****	597.6	597.7	595.8	**** (2.80)	**** (26.90)	** (10.06)	0.242 (1.38)	–	*** (12.79)
6.	THAWED = 2.833 + 0.023 × CONC + + 0.025 × FRESH	0.288	****	601.1	601.2	599.3	**** (2.84)	**** (30.41)	*** (12.63)	–	n.s. (0.90)	n.s. (0.16)
7.	THAWED = 2.833 – 0.167 × VOL + + 0.033 × CONC + 0.010 × FRESH	0.293	****	601.5	601.6	599.7	**** (2.89)	**** (28.58)	** (10.53)	0.146 (2.12)	n.s. (1.65)	n.s. (0.02)

AIC = Akaike information criterion; AICC = corrected Akaike information criterion; BIC = Bayesian information criterion; BREED = fixed effect of breed; CONC = sperm concentration after collection (10^9/ml); FRESH = sperm motility after collection (points); GLM = generalised linear models; n.s. = not significant; ORDER = fixed effect of the order of ram semen collection; P-model = P-value of the statistical model; R^2 = coefficient of determination; THAWED = frozen-thawed sperm motility (points); VOL = semen volume after collection (ml); YEAR*SEASON = randomised combined year-seasonal effect
*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.000 1

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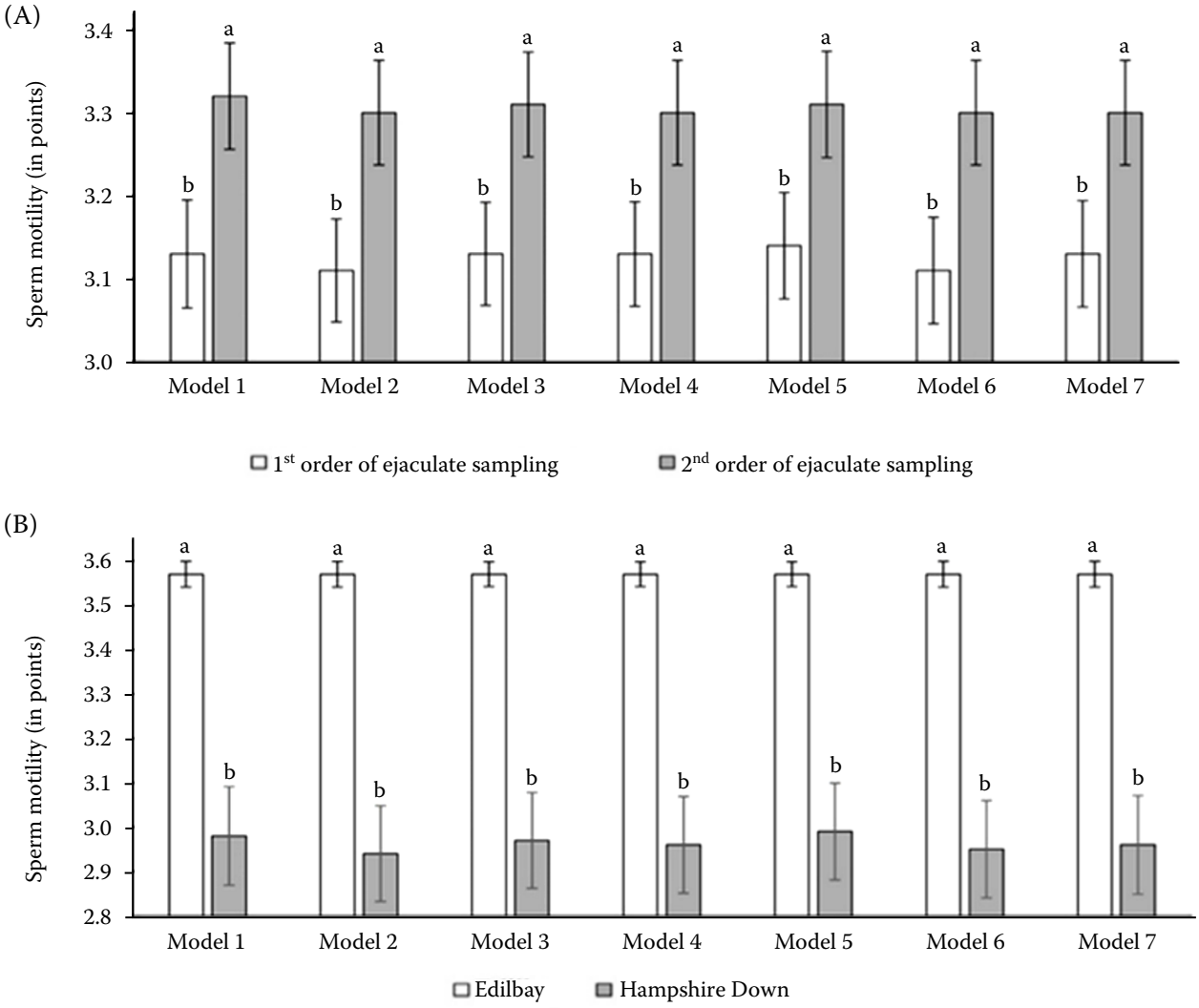

Figure 1. Fixed effects of the order of ejaculate sampling and breed as factors influencing frozen/thawed sperm motility

(A) Fixed effect of the order of ejaculate sampling. (B) Fixed effect of breed

[a,b]Different symbols mean statistically significant differences

the 2nd ejaculate reached higher freezability compared to the 1st one (Figure 1A).

The differences in frozen-thawed semen ranged from 0.172 points (statistical model 5: with sperm VOL and FRESH as covariates) to 0.192 points (statistical model 2: with sperm CONC as covariate), depending on the statistical model applied. The fixed effect of breed was a major driving factor influencing frozen/thawed sperm motility in the present study. Edilbay rams exhibited significantly higher sperm motility after thawing compared to Hampshire Down (Figure 1B) with the differences ranging from 0.58 to 0.63, depending on the statistical model used.

DISCUSSION

Effect of semen volume, sperm motility and concentration on frozen-thawed motility

Routine methods for predicting the freezability of ram sperm cells using conventional semen variables (motility, volume, concentration) are easy to implement. Our results revealed that both sperm concentration and motility have a significant effect on freezability. This is in line with the results previously reported by Dorado et al. (2009), who found a positive relationship between conventional semen variables (namely motility) and the prediction

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of sperm freezability in goats. However, it should be noted that in the present study, the coefficients of determination were low: 3.3% ($P < 0.05$) for concentration, and 5.7% ($P < 0.05$) for motility. In this sense, our results are in line with those of Gil et al. (2005), who demonstrated that motility parameters are insufficient for the determination of the freezability of boar sperm. Furthermore, in our study the semen volume was found to have a very low predictive character. Based on the obtained results, we concluded that the initial evaluation of sperm attributes is not a sufficient tool on its own. It is better to consider other methods such as proteomics, transcriptomics, or the HOS test (Padrik et al. 2012; Prinosilova et al. 2014; Qin et al. 2018; Pequeno et al. 2023; Ren et al. 2023; Sun et al. 2023) for now and deal with this topic in future.

From the practical point of view, since pre-freeze semen parameters are not a strong predictor of freezing success, it may be unnecessary to reject samples that do not meet the set minimum requirements before freezing. It is known that workers in most centres that produce insemination doses are guided by methodological recommendations, which describe the minimum requirements that the semen must meet to be included in the freezing process. Rejection based on these parameters leads to a decrease in the total number of insemination doses produced. We speculated about the possibility of producing acceptable insemination doses with the rejected semen, if the rejection was only based on the volume, concentration, and/or motility measurements before freezing. In the study by Nikitkina and Shapiev (2010), the bull semen was selected for freezing either according to the national methodological recommendation (with a concentration of $> 0.8 \times 10^9$/ml and with minimum motility of 80% before freezing) or with intentionally low concentration and motility ($< 0.8 \times 10^9$/ml and < 80%, respectively) before freezing. After thawing, it was found that the sperm from the two groups did not differ in their *in vitro* quality indicators. In addition, no differences were found in the *in vivo* experiments, after conducting experiments using artificial insemination. The authors concluded that if the semen that does not initially meet the specified parameters is allowed to be frozen, it would be possible to prepare 6% more doses for insemination. We believe that the results obtained in our study on rams support the previously reported conclusion made from the results of the study by Nikitkina and Shapiev (2010) conducted on bulls. Our practical recommendation is that the ram semen with relatively low concentration, volume, and/or motility can be included in the technological process of freezing and subsequent preparation of insemination doses, as we have found that these parameters are weakly associated with the outcome of the freezing procedure. However, further research, including a larger number of animals and different ovine breeds, is needed to confirm this recommendation.

Additionally, inter-individual differences in frozen sperm quality have previously been observed in small ruminants (Barbas and Mascarenhas 2009). There is not only significant variability between ejaculates of individuals of different breeds, but also among ejaculates of the same individual sires on different semen collection days (Yeste et al. 2015).

This unpredictable variability highlights the importance of pre-screening semen samples and of looking for new and robust strategies how to predict freezability more effectively.

Effect of the order of ejaculate sampling and breed on frozen-thawed motility

The order of ejaculate sampling played an important role. Sperm from the second ejaculation achieved demonstrably higher freezability ($P < 0.05$) than those from the first. This fact was previously confirmed by Nel-Themaat et al. (2006), who pointed out in their study that greater post-thaw progressive motility of cryopreserved sperm was observed in the ejaculate from the second collection. Similar results were also obtained by Ben Moula et al. (2022). Before ejaculation, mature sperm are usually stored in the *cauda epididymis*, and as more mature sperm enter the caudal region, the sperm that have not yet been ejaculated move into the *vas deferens*, where they begin to degrade. As a result, a large proportion of ejaculates from the first collection may contain these less viable sperm, which may also be one of the reasons why ejaculates from the second collection exhibit better freezability than those from the first.

The results of this study showed that the fixed effect of breed was the main driving factor influencing post-thaw sperm motility. Compared to the Hampshire Down breed, the Edilbay breed showed

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significantly higher sperm motility after thawing ($P < 0.05$). A statistically significant effect of breed on sperm motility was also confirmed by Tohura et al. (2019), yet several other authors found that the breed plays a very small role in these parameters (Barbas et al. 2023; Mujitaba et al. 2024). Vozaf et al. (2022) compared three ram breeds (Slovak Dairy, Native Slovak Wallachian, Improved Wallachian breeds) and observed no significant effect ($P > 0.05$) on standard motility parameters after thawing, but it should be noted that this case involved the use of three national Slovakian breeds, which are well adapted to local environmental conditions. In our study, two breeds were compared, only one of which is native to the area. It is well known that native purebred breeds are perfectly adapted to their environment and are more vital and healthy, which is also reflected in their reproductive traits (Kumaresan et al. 2021). Edilbay is a breed that was historically adapted to a nomadic lifestyle in the deserts and semi-deserts of Kazakhstan. We speculated that this may be one of the reasons why the sperm cell motility of this breed was higher after thawing compared to the imported British Hampshire Down breed of sheep.

CONCLUSION

Results from our study confirmed that sperm concentration and motility were significant predictive values for the outcome of the freezing procedure, but the coefficients of determination were very low: 3.3% for concentration, and 5.7% for motility. An even weaker, albeit significant predictive relationship was detected for the semen volume variable.

We concluded that the association between conventional semen variables (motility, volume, concentration) and sperm freezability in rams is low; analysing the measurements of ejaculated sperm before freezing is not a beneficial tool for predicting the outcome of ovine sperm freezing. The order of ejaculate sampling (first versus second, within a single collection session) played an important role in the outcome of sperm freezing. The sperm from the second ejaculation achieved higher freezability than those from the first. Furthermore, the results of this study showed that the effect of breed was the main driving factor influencing post-thaw ram sperm motility.

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Conflict of interest

The authors declare no conflict of interest.

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