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## RESEARCH ARTICLE



# The impact of retrieval method and breed on the motility and kinematic parameters of fresh and post-thaw ram epididymal spermatozoa

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## ABSTRACT

This study was conducted to develop ideal post-mortem gamete retrieval and conservation methods to establish a Hungarian *ex-situ in vitro* gene bank. Pairs of testes from German Mutton Merino ( $n = 7$ ) and Hungarian Black Racka ( $n = 7$ ) rams were collected at a slaughterhouse, transported to the laboratory and stored overnight (4–5 °C) before processing. Post mortem ram epididymal spermatozoa (REPS) were obtained from the cauda epididymidis by slice or incision methods. Fresh samples were extended to  $200 \times 10^6/\text{mL}$  cell concentration, filled into mini straws and equilibrated at 5 °C for 2 h. Freezing was performed manually in a Styrofoam box. The fresh and post-thaw total motility, progressive motility and kinematic parameters of REPS were assessed using the CASA technique. The collection method did not affect significantly the fresh and post-thaw motility and kinematic parameters. Merino had higher ( $P < 0.05$ ) testicular weight. Racka had significantly better fresh and post-thaw linear movement but had statistically the same ( $P > 0.05$ ) cryotolerance as Merino. In conclusion, both collection methods were found suitable for REPS retrieval. The REPS from Racka exhibited better linear movement values than those from the Merino breed. The cryotolerance of REPS of both breeds was comparable.

## KEYWORDS

Hungarian Racka, German Mutton Merino, ram, epididymal spermatozoa, collection methods, cryopreservation

## INTRODUCTION

Autochthonous sheep breeds are interesting because they are thrifty and hardier than commercial or modern sheep breeds. Unfortunately, most of them are rarely bred nowadays (<https://dengarden.com/agriculture/Rare-and-Endangered-Sheep-Breeds>). The Hungarian Racka, also called Hortobágyi Racka, is an important native sheep breed reared by farmers for meat, milk and wool production. It is a hardy, medium-sized sheep (males and females weigh average 60 and 40 kg, respectively), with heights of 61–76 cm at withers and having spiral,

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V-shaped horns in both sexes (60 and 30 cm for rams and ewes) ensuring an amazing appearance. Racka sheep are mostly of two distinct coat colours, solid black or white, with distinctive medium-fine spiral wool (Zsolnai et al., 2021; Anon et al., 2023). Racka rams have a seasonal reproductive cycle as indicated by changes in their scrotal circumference, sperm quality and quantity across the year. The highest scrotal circumference was observed in September, and the lowest in January. Total sperm count per ejaculate is highest in autumn and the lowest in spring, while the lowest sperm concentration and the highest percentage of abnormal spermatozoa have been recorded in winter (Sarlós et al., 2013).

German Mutton Merino (called Merinofleischschaf in German) is a large-framed white sheep reared for meat and wool. An old German Mutton Merino ram weighs 120–140 kg, while the ewes are of 70–80 kg (Mason, 1996). The sperm motility is better in the summer and autumn months, while it decreases during winter. In this breed, the sperm concentration is  $3,200\text{--}4,500 \times 10^6/\text{mL}$ , with the highest values recorded from April to October and the lowest ones between November and March at the climate of Israel (Amir and Volcani, 1965). In Spain, the breeding season is from October to February, when highest motility and progressive motility of the ejaculated spermatozoa have been observed (Arando et al., 2019).

According to the 2020 and 2021 local risk status reports DAD-IS (Domestic Animal Diversity Information System; Retrieved July 21, 2023, from: <https://www.fao.org/dad-is/sdg-252/en/>), both the population and the male to female ratio of certain Hungarian sheep breeds are declining. This leads to the extinction or decline of certain important genotypes including Alföldi suta racka (extinct; population:30/30 vs. 26/26 and male to female ratio 4/26 vs. 0/26); Cikta (endangered; population:826/826 vs 789/789 and male to female ratio 28/794 vs 22/767); and Cigája (at risk; population:3373/3373 vs. 3191/3191 and male to female ratio 163/3210 vs. 85/3106) for 2020 vs 2021, respectively. In the case of the Hungarian Racka breed, the population declined between 2019 and 2020 (8547/8547 vs. 5898/5898), while the male to female ratio increased in favour of males (270/8277 vs. 298/5600). A cryoconservation program exists for these breeds (DAD-IS, 2020). Similarly, *in situ* gene conservation programs of the breeds run in different national parks in Hungary. The main objective is to keep purebred animals in the colour variants (black and white). The main selection criteria include lamb weight gain, yearling weight and phenotypic characteristics such as wool length, colour and horn appearance. Both the *in situ* and *ex-situ* programs are supported by tenders entitled “*In situ* preservation of the genetic stock of protected native and endangered agricultural animal breeds” and “*Ex situ* or *in vitro* preservation of the genetic stock of protected native and endangered agricultural animal breeds, and support for advisory activities preventing genetic narrowing”. This led to the establishment of an *ex situ in vitro* gene bank consisting of ejaculated spermatozoa of the Hungarian Racka, Cikta and Cigája breeds in cooperation with the Hungarian Sheep and Goat Breeders’ Association and the Research Institute for Animal Breeding, Nutrition and Meat Science of the National

Agricultural Research and Innovation Centre, in Herceghalom during 2014–2017.

Epididymal spermatozoa (EPS) are considered the cheapest potential source of male gametes for assisted reproductive technology studies and genome resource banking of accidentally dead, severely injured, endangered or threatened elite males (Goovaerts et al., 2006). They can be retrieved differently depending on the species involved or epididymal size. The most used EPS retrieval methods include a) Slicing/mincing (Kaabi et al., 2003; Parra-Forero et al., 2015), b) Incision (Karja et al., 2010; Alvarez et al., 2012; Ahmed, 2019), c) Retrograde flushing via ductus deferens (resulting in less contamination) (Lorraine Leibfried-Rutledge et al., 1997; Bertol, 2016) and d) Floatation (in case of species with tiny testicles) (Bertol, 2016). Good pregnancy rates have been reported using EPS for artificial insemination in sheep (87.5%, 58.5%, and 55.0%) (Ehling et al., 2006; Rickard et al., 2014; Fernández Abella et al., 2015) and goats (61.2%) (Ocampo et al., 2021). Therefore, they can be used to preserve the genetic resources of elite rams. Our literature review, published recently, has revealed that collection of the epididymal spermatozoa is an ideal alternative for gene conservation because it has a recovery rate comparable to the use spermatozoa obtained by the use of artificial vagina (AV) and serves as the easiest and cheapest means of conserving livestock GnR (Mujitaba et al., 2022). Considering the limited published articles on factors affecting the quality of ram epididymal spermatozoa (REPS), we hypothesised that ram breed and collection methods might significantly affect the motility and kinematic parameters of the fresh sample and cryotolerance of REPS. Therefore, we conducted a pilot study out of season to develop an ideal post-mortem gamete retrieving and conservation method to establish a Hungarian *ex-situ in vitro* gene bank. The specific objectives of the study included to assess the effects of two REPS recovery methods (slicing vs. incision) and breed (German Mutton Merino vs. Hungarian Racka) on the fresh and post-thaw motility and kinematic parameters, and to compare the cryotolerance of the REPS obtained the above ways.

## MATERIALS AND METHODS

### Media, reagents, and materials

Andromed<sup>®</sup> semen extender (one-step, 200 mL, Minitube, Tiefenbach, Germany), 0.25 mL transparent semen straws (Minitube, Tiefenbach, Germany) and PBS tablets (Gibco, Lot:2565974) were used for the experiment. The extender was reconstituted according to the manufacturer’s guidelines, filled into sterilized 10 mL centrifuge tubes, and stored at frozen condition until required for use. All other plasticware was purchased from Falcon<sup>®</sup> (Corning, Inc., USA).

### Study location, duration and testicle collection

The study was conducted at the spermatology laboratory of the Department of Precision Livestock Farming and Animal



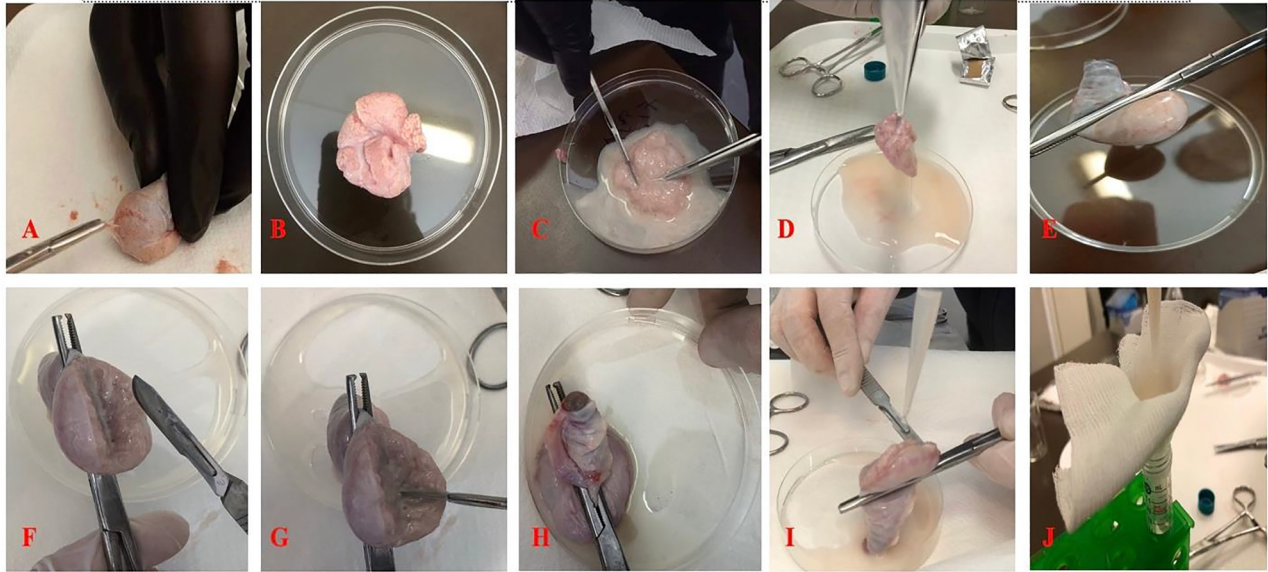


Fig. 1. Epididymal sperm collection by slicing (A, B, C, D and J) and incision (E, F, G, H, I and J) methods. A: stripping the cauda epididymis (CE) for slicing; B: the stripped CE ready for slicing; C: slicing the stripped CE; D: the engorged CE slice; E: the engorged CE ready for incision; F: incision of CE by a deep vertical cut; G: additional small horizontal incisions; H: pressing the incised CE against the bottom of the Petri dish for aiding the release of spermatozoa; I: rinsing the incised CE; H: filtering the spermatozoa

Biotechnics, Institute of Animal Sciences, Kaposvár Campus, Hungarian University of Agriculture and Life Sciences, Herceghalom, Hungary during the non-breeding season. Fourteen pairs of testes from seven 2–5-year-old healthy German Mutton Merino (weighing 80–100 kg) and seven black variants of Hungarian Racka rams (weighing 55–60 kg) were collected at a slaughterhouse in Hungary. The testes, left in the scrotum, were transported to the laboratory in an icepack (4–5 °C) within 2–3 h, stored overnight in a refrigerator (4–5 °C) to simulate field conditions, and processed the following day as described by Egerszegi et al. (2012).

### Epididymal sperm collection methods

After removing the scrotal sac and lamina parietalis of tunica vaginalis, the testis with epididymis was weighed using a digital scales. Each epididymis was carefully separated and spermatozoa from both caudae epididymidis (CE) of the same ram were retrieved randomly by either the slicing or incising method.

*The slicing method (SL).* The visceral layer of tunica vaginalis covering the CE is carefully removed to avoid blood contamination. The stripped CE was washed with PBS, then cut out and sliced with a scalpel in a Petri dish containing 3 mL of Andromed<sup>®</sup> semen extender. The sliced CE was left in the extender at room temperature for 10 min to enhance spermatozoa collection. It was then rinsed with 2 mL of the semen extender and filtered through gauze sheets (Fig. 1).

*The incision method (IN).* We adopted the procedure published by Ahmed et al. (2019) with little modifications. We used Andromed<sup>®</sup> semen extender to retrieve spermatozoa and a haemostatic forceps was used to engorge the CE to facilitate emptying the spermatozoa. Subsequently, a

single deep longitudinal incision at the ventral part of the CE with fewer blood vessels and 3–4 parallel incisions in the inner part were made using a sterile scalpel. It was then pressed against the bottom of the Petri dish containing 3 mL of Andromed<sup>®</sup> semen extender to aid in emptying the spermatozoa and then rinsed with 2 mL of the same extender. Finally, a sterile gauze was used to sieve the epididymal tissue debris (Fig. 1).

### Epididymal sperm dilution, equilibration, freezing and motility assessment

Sperm concentration was assessed with a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) using a phase contrast microscope at 200× magnification. Upon establishing the concentration, each sample was extended using Andromed<sup>®</sup> at room temperature to a final concentration of  $200 \times 10^6$  spermatozoa/mL and manually filled and sealed using PVA into well-labelled 0.25 mL French mini straws.

The filled and sealed straws were equilibrated in a refrigerator (5 °C for 2 h), and freezing was done manually in a Styrofoam box at 4 cm above liquid nitrogen for 8 min. Finally, the frozen straws were plunged into the liquid nitrogen container for permanent storage. After about 2 weeks, the frozen samples were thawed by warming at 37 °C for 30 s.

The motility and kinetic parameters of the fresh and frozen-thawed spermatozoa were assessed using a CASA AndroVision<sup>®</sup> system (Minitube, Tiefenbach, Germany). The samples were diluted to a concentration of  $50\text{--}60 \times 10^6$  spermatozoa/mL using the same extender. At least 10 random fields per sample or a total of 500 spermatozoa were analysed for standard motility [total motility (TM, %),

progressive motility (PM, %) and kinematic parameters such as average path velocity (VAP,  $\mu\text{m s}^{-1}$ ), straight line velocity (VSL  $\mu\text{m s}^{-1}$ ), curvilinear velocity (VCL,  $\mu\text{m s}^{-1}$ ), amplitude of lateral head displacement (ALH,  $\mu\text{m}$ ), beat cross frequency (BCF, Hz), straightness (STR =  $\text{VSL}/\text{VAP} \times 100$ , %), linearity (LIN =  $\text{VSL}/\text{VCL} \times 100$ , %) and wobble (WOB =  $\text{VAP}/\text{VCL} \times 100$ , %) as described previously (Goovaerts et al., 2006; Kang et al., 2018).

### Data analysis

Data regarding the fresh and frozen-thawed samples were collected and recorded. The data were tested for normality using Shapiro-Wilk test and were normally distributed. A two-way ANOVA was used to check the effects of the collection method and breed (pooled data of the slicing and incision methods) under the two conditions (fresh and post-thaw) separately at a level of significance set at  $P < 0.05$ .

The model for the experiment was  $Y_j = a + bx_1 + cx_2 + x_1 \cdot x_2$  where  $Y$  = dependant variable (e.g. TM),  $a$  = intercept,  $x_1$  = breed (Merino or Racka) and  $x_2$  = method (SL or IN).

The effect of freezing within breed was analysed using a paired Student's  $t$ -test as the ejaculate of a ram was separated into two doses. One dose was analysed fresh while the other dose was frozen. In contrast, the cryotolerance of the two breeds was calculated using the following formulae  $\text{CT} = \text{values after thawing}/\text{values before freezing} \times 100\%$  as described by Ehling et al. (2006). The differences between breeds (Merino vs. Racka) were analysed using an independent sample  $t$ -test and the significance was checked using a two-tailed test.

Results were presented as means  $\pm$  standard error (SE). The above-mentioned methods were carried out using IBM® SPSS® statistical software version 29.

## RESULTS

### Effects of collection method on the fresh and post-thaw ram epididymal sperm standard motility and kinematic parameters

Table 1 illustrates the effects of the collection methods (SL vs IN) on fresh and frozen-thawed REPS. Testicular weight did not differ between the collection methods (SL vs IN;  $P > 0.05$ ). Similarly, the collection methods did not have significant ( $P > 0.05$ ) effects on standard motility and any of the kinematic parameters in case of both the fresh and post-thaw REPS.

### Effects of breed on testicular weight, fresh and post-thaw ram epididymal spermatozoa motility and kinematic parameters

Considering that there was no significant difference ( $P > 0.05$ ) between the collection methods on any of the parameters, the data were pooled and analysed together to determine breed effects on testicular weight (TW; g), total motility (TM; %) and progressive motility (PM; %) and kinematic parameters of the fresh and frozen-thawed REPS. The results are summarized in Table 2. The TW was found significantly ( $P < 0.05$ ) higher in the Merino breed than in the Racka. In contrast, REPS from the Racka breed presented several, significantly ( $P < 0.05$ ) better kinematic parameters (LIN, STR, BCF and ALH) than the Merino breed. All other parameters were not significantly ( $P > 0.05$ ) different between the breeds.

The breed had no significant ( $P > 0.05$ ) effects on the post-thaw standard motility parameters and kinematic parameters except for LIN and BCF, which were significantly

Table 1. Effects of collection methods on testicular weight, standard motility and kinematic parameters of fresh and post-thaw ram epididymal spermatozoa

Parameters	Fresh		P-value	Post-thaw		P-value
	Collection methods (mean $\pm$ SE)			Collection methods (mean $\pm$ SE)		
	Slicing	Incision		Slicing	Incision	
TW (g)	213.45 $\pm$ 16.9	217.82 $\pm$ 16.3	0.789	–	–	–
TM (%)	82.07 $\pm$ 2.5	80.07 $\pm$ 2.8	0.598	45.83 $\pm$ 5.4	36.67 $\pm$ 4.9	0.324
PM (%)	73.00 $\pm$ 2.9	68.93 $\pm$ 3.4	0.376	31.58 $\pm$ 5.2	25.08 $\pm$ 4.9	0.504
VCL ( $\mu\text{m s}^{-1}$ )	132.61 $\pm$ 7.8	129.09 $\pm$ 9.8	0.781	102.73 $\pm$ 5.2	106.01 $\pm$ 5.9	0.776
VAP ( $\mu\text{m s}^{-1}$ )	66.24 $\pm$ 3.3	63.39 $\pm$ 4.1	0.591	50.41 $\pm$ 2.4	51.33 $\pm$ 2.9	0.880
VSL ( $\text{m s}^{-1}$ )	47.04 $\pm$ 2.3	44.70 $\pm$ 3.2	0.572	38.89 $\pm$ 1.9	39.72 $\pm$ 2.7	0.845
LIN (%)	35.57 $\pm$ 1.2	34.29 $\pm$ 1.0	0.368	37.42 $\pm$ 0.8	36.67 $\pm$ 0.8	0.587
STR (%)	70.86 $\pm$ 1.6	69.43 $\pm$ 1.4	0.483	76.50 $\pm$ 1.0	76.42 $\pm$ 1.4	0.913
WOB	49.93 $\pm$ 0.6	48.86 $\pm$ 1.1	0.389	44.75 $\pm$ 3.7	48.08 $\pm$ 0.4	0.463
BCF (Hz)	26.19 $\pm$ 0.6	25.60 $\pm$ 0.6	0.451	25.93 $\pm$ 0.7	25.92 $\pm$ 0.9	0.907
ALH ( $\mu\text{m}$ )	4.86 $\pm$ 0.3	4.7 $\pm$ 0.3	0.655	3.85 $\pm$ 0.2	3.83 $\pm$ 0.2	0.811

ALH: amplitude of the lateral head displacement; BCF: beat cross frequency; LIN: linearity, PM: progressive motility, SE: standard error; STR: straightness; TM: total motility, TW: testicular weight, VAP: average path velocity; VCL: curvilinear velocity; VSL: straight line velocity, WOB: wobble.

Testicle numbers: slicing ( $n = 14$ ), incision ( $n = 14$ ).



Table 2. Effects of breed on testicular weight, standard motility and kinematic parameters of fresh and post-thaw ram epididymal spermatozoa (mean  $\pm$  SE)

Parameters	Fresh		<i>P</i> -values	Post-thaw		<i>P</i> -values
	Breed			Breed		
	Merino	Racka		Merino	Racka	
TW (g)	260.50 $\pm$ 11.8 <sup>a</sup>	170.82 $\pm$ 10.1 <sup>b</sup>	0.001	–	–	
TM (%)	83.64 $\pm$ 2.2	78.50 $\pm$ 2.8	0.182	44.20 $\pm$ 6.47	39.14 $\pm$ 4.48	0.498
PM (%)	73.00 $\pm$ 3.1	68.93 $\pm$ 3.3	0.376	30.00 $\pm$ 6.97	27.14 $\pm$ 3.82	0.702
VCL ( $\mu\text{m s}^{-1}$ )	140.13 $\pm$ 8.5	121.58 $\pm$ 8.6	0.152	104.76 $\pm$ 4.69	104.09 $\pm$ 5.88	0.936
VAP ( $\mu\text{m s}^{-1}$ )	68.39 $\pm$ 3.3	61.24 $\pm$ 3.8	0.186	50.05 $\pm$ 2.02	51.45 $\pm$ 2.86	0.728
VSL ( $\text{m s}^{-1}$ )	46.78 $\pm$ 2.3	44.96 $\pm$ 3.2	0.659	37.75 $\pm$ 1.65	40.41 $\pm$ 2.56	0.456
LIN (%)	33.2 $\pm$ 1.0 <sup>b</sup>	36.6 $\pm$ 0.9 <sup>a</sup>	0.022	35.40 $\pm$ 0.7 <sup>b</sup>	38.21 $\pm$ 0.7 <sup>a</sup>	0.008
STR (%)	67.9 $\pm$ 1.6 <sup>b</sup>	72.43 $\pm$ 1.1 <sup>a</sup>	0.032	74.90 $\pm$ 1.3	77.57 $\pm$ 1.1	0.127
WOB (%)	48.43 $\pm$ 0.9	50.36 $\pm$ 0.8	0.127	47.30 $\pm$ 0.4	45.79 $\pm$ 3.2	0.698
BCF (Hz)	24.86 $\pm$ 0.5 <sup>b</sup>	26.92 $\pm$ 0.6 <sup>a</sup>	0.013	24.43 $\pm$ 0.72 <sup>b</sup>	26.99 $\pm$ 0.71 <sup>a</sup>	0.027
ALH ( $\mu\text{m}$ )	5.22 $\pm$ 0.2 <sup>b</sup>	4.35 $\pm$ 0.3 <sup>a</sup>	0.022	4.10 $\pm$ 0.16	3.66 $\pm$ 0.14	0.064

ALH: amplitude of the lateral head displacement; BCF: beat cross frequency; LIN: linearity, PM: progressive motility, SE: standard error of mean; STR: straightness; TM: total motility, TW: testicular weight, VAP: average path velocity; VCL: curvilinear velocity; VSL: straight line velocity, WOB: wobble.

Means in the same row with different superscript within a form (fresh or post-thaw) differ significantly.

Testicle numbers: Merino ( $n = 14$ ), Racka ( $n = 14$ ).

( $P < 0.01$  and  $P < 0.05$ , respectively) higher in the Racka breed.

### Comparison of the cryotolerance of epididymal spermatozoa from Merino or Racka rams

In Table 3, the comparison of the cryotolerance of REPS from Merino and Racka are presented. Freezing and thawing significantly ( $P < 0.05$ ) reduced the values of TM, PM, VCL, VAP and ALH but significantly ( $P > 0.05$ ) increased the STR. WOB and BCF were not affected significantly ( $P > 0.05$ ) in any of the breeds. The VSL of Merino declined significantly ( $P < 0.05$ ) following freezing and thawing, while that of Racka did not. The cryotolerance of the REPS from

the Merino or Racka breeds did not differ statistically ( $P > 0.05$ ).

### The interaction effects of the collection methods and breeds on motility and kinematic parameters of epididymal spermatozoa from Merino and Racka rams

Table 4 presents the interaction effects of the collection methods and breeds on the motility and kinematic parameters of Merino and Racka ram epididymal spermatozoa. There was no significant ( $P > 0.05$ ) collection methods and breed effects on the motility and all the kinematic parameters of Merino and Racka ram epididymal spermatozoa.

Table 3. Comparison of the cryotolerance of ram epididymal spermatozoa of Merino and Racka breeds (mean  $\pm$  SE)

Parameters	Merino		<i>P</i> -values	Racka		<i>P</i> -values	Cryotolerance		
	Fresh	Post-thaw		Fresh	Post-thaw		Merino CT (%)	Racka CT (%)	<i>P</i> -values
TM (%)	83.64 $\pm$ 2.2 <sup>a</sup>	44.20 $\pm$ 6.5 <sup>b</sup>	0.0001	78.50 $\pm$ 2.8 <sup>a</sup>	39.14 $\pm$ 4.5 <sup>b</sup>	0.0001	52.30 $\pm$ 6.9	49.91 $\pm$ 5.3	0.783
PM (%)	73.00 $\pm$ 3.1 <sup>a</sup>	30.00 $\pm$ 6.9 <sup>b</sup>	0.0001	68.93 $\pm$ 3.3 <sup>a</sup>	27.14 $\pm$ 3.8 <sup>b</sup>	0.0001	39.42 $\pm$ 8.7	39.67 $\pm$ 5.3	0.980
VCL ( $\mu\text{m s}^{-1}$ )	140.13 $\pm$ 8.5 <sup>a</sup>	104.76 $\pm$ 4.7 <sup>b</sup>	0.003	121.58 $\pm$ 8.6 <sup>a</sup>	104.09 $\pm$ 5.9 <sup>b</sup>	0.020	76.66 $\pm$ 5.2	88.44 $\pm$ 5.2	0.133
VAP ( $\mu\text{m s}^{-1}$ )	68.39 $\pm$ 3.3 <sup>a</sup>	50.05 $\pm$ 2.0 <sup>b</sup>	0.0001	61.24 $\pm$ 3.8 <sup>a</sup>	51.45 $\pm$ 2.9 <sup>b</sup>	0.004	73.60 $\pm$ 4.5	85.75 $\pm$ 4.2	0.067
VSL ( $\text{m s}^{-1}$ )	46.78 $\pm$ 2.3 <sup>a</sup>	37.75 $\pm$ 1.7 <sup>b</sup>	0.012	44.96 $\pm$ 3.2	40.41 $\pm$ 2.6	0.088	82.29 $\pm$ 5.7	92.39 $\pm$ 5.4	0.220
LIN (%)	33.2 $\pm$ 1.0	35.40 $\pm$ 0.7	0.247	36.6 $\pm$ 0.9	38.21 $\pm$ 0.7	0.074	108.76 $\pm$ 6.1	104.82 $\pm$ 2.3	0.505
STR (%)	67.9 $\pm$ 1.6 <sup>a</sup>	74.90 $\pm$ 1.3 <sup>b</sup>	0.039	72.43 $\pm$ 1.1 <sup>a</sup>	77.57 $\pm$ 1.1 <sup>b</sup>	0.001	113.22 $\pm$ 5.5	107.33 $\pm$ 1.8	0.261
WOB (%)	48.43 $\pm$ 0.9	47.30 $\pm$ 0.4	0.598	50.36 $\pm$ 0.8	45.79 $\pm$ 3.2	0.165	96.22 $\pm$ 0.9	90.97 $\pm$ 6.3	0.498
BCF (Hz)	24.86 $\pm$ 0.5	24.43 $\pm$ 0.7	0.740	26.92 $\pm$ 0.6	26.99 $\pm$ 0.7	0.915	100.45 $\pm$ 2.7	100.49 $\pm$ 2.5	0.792
ALH ( $\mu\text{m}$ )	5.22 $\pm$ 0.2 <sup>a</sup>	4.10 $\pm$ 0.16 <sup>b</sup>	0.005	4.35 $\pm$ 0.3 <sup>a</sup>	3.66 $\pm$ 0.1 <sup>b</sup>	0.001	79.13 $\pm$ 4.9	86.12 $\pm$ 3.4	0.241

ALH: amplitude of the lateral head displacement; BCF: beat cross frequency; CT: cryotolerance, LIN: linearity, PM: progressive motility, SE: standard error of mean; STR: straightness, TM: total motility, VAP: average path velocity; VCL: curvilinear velocity; VSL: straight line velocity, WOB: wobble.

Means in the same row with different superscript within a breed<sup>a,b</sup> (Merino or Racka) differ significantly.

Testicles number: Merino ( $n = 14$ ), Racka ( $n = 14$ ).



Table 4. Breed and collection method interaction effects on motility and kinematic parameters of Merino and Racka ram epididymal spermatozoa under fresh and post-thaw conditions

Parameters	Collection methods	Form (Mean $\pm$ SE)						
		Fresh			P-value	Post-thaw		P-value
		Merino	Racka	Merino		Racka		
TM (%)	SL	85.29 $\pm$ 3.7	78.86 $\pm$ 3.7	0.734	42.60 $\pm$ 7.9	48.14 $\pm$ 6.7	0.163	
	IN	82.00 $\pm$ 3.7	78.14 $\pm$ 3.7		45.80 $\pm$ 7.9	30.14 $\pm$ 6.7		
PM (%)	SL	77.14 $\pm$ 4.5	68.86 $\pm$ 4.5	0.360	28.00 $\pm$ 7.9	34.14 $\pm$ 6.7	0.235	
	IN	68.86 $\pm$ 4.5	69.00 $\pm$ 4.5		32.00 $\pm$ 7.9	20.14 $\pm$ 6.7		
VCL ( $\mu\text{m s}^{-1}$ )	SL	141.14 $\pm$ 12.5	124.09 $\pm$ 12.5	0.906	106.20 $\pm$ 8.9	100.26 $\pm$ 7.5	0.533	
	IN	139.14 $\pm$ 12.5	119.07 $\pm$ 12.5		103.33 $\pm$ 8.9	107.93 $\pm$ 7.5		
VAP ( $\mu\text{m s}^{-1}$ )	SL	70.03 $\pm$ 5.2	62.46 $\pm$ 5.2	0.936	50.70 $\pm$ 4.3	50.20 $\pm$ 3.6	0.637	
	IN	66.74 $\pm$ 5.2	60.03 $\pm$ 5.2		49.41 $\pm$ 4.3	52.70 $\pm$ 3.6		
VSL ( $\text{m s}^{-1}$ )	SL	47.90 $\pm$ 4.1	46.17 $\pm$ 4.1	0.982	37.80 $\pm$ 3.8	39.67 $\pm$ 3.2	0.824	
	IN	45.66 $\pm$ 4.1	43.74 $\pm$ 4.1		37.70 $\pm$ 3.8	41.16 $\pm$ 3.2		
LIN (%)	SL	33.86 $\pm$ 1.4	37.29 $\pm$ 1.4	1.000	35.00 $\pm$ 1.0	39.14 $\pm$ 0.9	0.181	
	IN	32.57 $\pm$ 1.4	36.00 $\pm$ 1.4		35.80 $\pm$ 1.0	37.29 $\pm$ 0.9		
STR (%)	SL	68.43 $\pm$ 2.0	73.29 $\pm$ 2.0	0.888	74.00 $\pm$ 1.8	78.29 $\pm$ 1.5	0.348	
	IN	67.29 $\pm$ 2.0	71.57 $\pm$ 2.0		75.80 $\pm$ 1.8	76.86 $\pm$ 1.5		
WOB (%)	SL	49.43 $\pm$ 1.2	50.43 $\pm$ 1.2	0.454	47.20 $\pm$ 4.2	43.00 $\pm$ 3.5	0.436	
	IN	47.43 $\pm$ 1.2	50.29 $\pm$ 1.2		47.40 $\pm$ 4.2	48.57 $\pm$ 3.5		
BCF (Hz)	SL	25.16 $\pm$ 0.8	27.21 $\pm$ 0.8	1.000	23.94 $\pm$ 1.2	27.36 $\pm$ 1.0	0.494	
	IN	24.57 $\pm$ 0.8	26.63 $\pm$ 0.8		24.92 $\pm$ 1.2	26.63 $\pm$ 1.0		
ALH ( $\mu\text{m}$ )	SL	5.33 $\pm$ 0.4	4.39 $\pm$ 0.4	0.832	4.23 $\pm$ 0.2	3.59 $\pm$ 0.2	0.393	
	IN	5.09 $\pm$ 0.4	4.30 $\pm$ 0.4		3.98 $\pm$ 0.2	3.73 $\pm$ 0.2		

ALH: amplitude of the lateral head displacement; BCF: beat cross frequency; LIN: linearity, PM: progressive motility, SE: standard error of mean; STR: straightness; TM: total motility, VAP: average path velocity; VCL: curvilinear velocity; VSL: straight line velocity, WOB: wobble.

## DISCUSSION

Epididymal spermatozoa (EPS) are an excellent alternative for collecting and freezing gametes from domestic and wild animals for gene conservation post mortem. The efficiency of retrieving high quality EPS is also of crucial importance in the case of late processing. The motility and concentration of spermatozoa are two main factors that determine sperm function (effective fertilization), with movement characteristics being the central component (Robayo et al., 2008). The purpose of the current study was to assess the effects of two EPS recovery methods (slicing vs incision) on the movement characteristics of fresh and frozen-thawed Merino and Racka REPS's and to compare the fresh semen quality and cryotolerance in the two breeds. The weight of the testicles between the collection methods was not significantly different, ensuring that any differences observed are not attributable to the variation in the testicular weight. The collection method did not significantly affect the standard motility and kinematic parameters of the fresh and post-thaw REPS. However, the IN method was faster as it does not require the removal of the tunica vaginalis and thus is more practical in field conditions. In contrast, we found it quite challenging to establish a total cell number with the SL method due to wide variations in the number of cells retrieved. This is in line with the report of Ehling et al. (2006). Our findings tallies with the findings of Martínez-Pastor et al. (2006) who have reported that the collection method (cuts vs flushing) did not significantly affect the

motility, total number and post-thaw results of EPS recovered from Iberian red deer. Similarly, Kang et al. (2018) have found no significant ( $P > 0.05$ ) difference between the post-thaw standard motility parameters of EPS obtained from Hanwoo bull by the flushing or mincing methods. In contrast, in rams, the IN method gave a sample with significantly ( $P > 0.05$ ) higher total motility than the mincing method (75.0 vs 59.2) (Lone et al., 2011). Our results are in agreement with the findings of Mogheiseh et al. (2022), who have reported that the EPS retrieval methods have no significant effects on the post-thaw standard motility and kinematic parameters of dog EPS.

The Merino breed presented significantly ( $P > 0.05$ ) higher testicular weight than the Racka breed (260.50  $\pm$  11.8 vs 170.82  $\pm$  10.1 g). This might be due to the testicular size/scrotal circumference difference or body weight between the breeds (Allaoui et al., 2014). Moreover, it was established that ram testicular diameter and scrotal circumferences were significantly ( $P < 0.01$ ) correlated with testicular weight (Endale et al., 2009). The lowest and the highest fresh TM and PM recorded in the current study (78.50  $\pm$  2.8 to 83.64  $\pm$  2.2%) and (68.93  $\pm$  3.3 to 73.00  $\pm$  3.1%) were slightly higher than that of Kaabi et al. (2003), 78.4 and 64.7% retrieved 24 h after animal death but lower than what was reported by Rahimizadeh et al. (2021), 95.89 and 90.40% (recovered few hours after the animals were slaughtered), respectively. This might be due to the differences in the REPS retrieval season and time past after slaughtering. Bergstein-Galan et al. (2018) reported that the PM and



viability of REPS declined significantly at 12 and 48 h after the animal death, respectively.

We found that the breed has no significant ( $P > 0.05$ ) effects on the fresh TM and PM. This is in contrast with the result of Vozaf et al. (2022) who have compared Slovak Dairy, Native Wallachian and Improved Wallachian breeds. Similarly, Kasimanickam et al. (2007) have reported that the progressive motility of chilled-stored electro-ejaculated ram spermatozoa were different in case of breeds Polled Dorset, Suffolk and Katahdin. The EPS of the Racka breed were moving straighter than the EPS of Merino, as indicated by their higher LIN, STR and lower ALH values. This contrasted with the results by Kasimanickam et al. (2007), according to which breed does not have significant effects on the kinematic parameters of chilled stored electro-ejaculated ram spermatozoa. Additionally, the spermatozoa of both breeds were rapid as sperm cells with a VAP value over  $50 \mu\text{m s}^{-1}$  have been categorized rapid-moving spermatozoa (Goovaerts et al., 2006).

After thawing, none of the standard motility parameters differed significantly ( $P > 0.05$ ) between the breeds as in the case of fresh sperm. The post-thaw TM of Merino ( $44.20 \pm 6.47\%$ ) was lower than that ( $50.9 \pm 3.1\%$ ) reported by Çoyan et al. (2011) for AV-collected spermatozoa of Merino. However, we recorded a higher PM of  $30.00 \pm 6.97\%$  vs  $19.6 \pm 1.1\%$ . In Racka, we obtained lower values for TM ( $39.14 \pm 4.48\%$ ) and PM ( $27.14 \pm 3.82\%$ ) than those ( $60.86 \pm 5.44\%$  and  $52.86 \pm 6.12\%$ , respectively) reported for the same breed previously (Egerszegi et al., 2012). This might be due to the difference in the collection seasons. Our results agree with those of Vozaf et al. (2022) who have compared three breeds and found that the ram breed does not have significant effects ( $P > 0.05$ ) on the post-thaw standard motility parameters. To the contrary, Tohura et al. (2019) have reported that the breed had significant effects ( $P < 0.05$ ) on the motility of bull spermatozoa. Similarly, most of the differences in the kinematic parameters of the two breeds remain the same as in the fresh except for STR, which was significantly higher in Racka in the fresh and not significantly different between the breed after thawing. In addition, the Racka breed presented significantly ( $P < 0.05$ ) higher LIN and BCF values in both fresh and post-thaw kinematic parameters than the Merino breed. This shows that EPS of the Racka breed had better linear movement than those of the Merino. Higher sperm swimming velocities determine male fertilizing ability (Malo et al., 2006), while higher BCF and lower ALH of the sperm head could facilitate the penetration of the zona pellucida (Goovaerts et al., 2006). Similarly, LIN was reported to impact positively the cleavage rate following IVF and is a significant predictor of cleavage rate (García-Alvarez et al., 2009).

Freezing and thawing affected the same parameters in both breeds except for VSL, which was not significantly affected in the Racka ( $P > 0.05$ ). Similarly, the REPS of both breeds had similar cryotolerance. Our results contradict those published by Andreeva et al. (2017), who have compared the Bulgarian dairy synthetic population with the

Ile de France breed and recorded a significant difference in the cryotolerance of the spermatozoa.

Our findings revealed that the collection method does not influence significantly the standard motility and kinematic parameters of the fresh or post-thaw ram epididymal spermatozoa. The incision method seems faster and more field-friendly than the slicing method. However, if the visceral layer of the tunica vaginalis communis contains larger blood vessels, removal of the tunica and the slicing method is recommended to avoid bloody sperm samples. We observed that the Racka EPS, either in fresh or post-thaw conditions have straighter movement than those of the Merinos. We found the cryotolerance of REPS from both breeds identical.

In literature, diverse motility parameters are described, however, it is well documented that the kinematic parameters of the sperm are insufficient to predict the fertilizing ability of the semen. Further investigation are needed for a more profound evaluation of REPS by the analysis of the possible effects of the side and position (right or left) of the epididymis of a given animal on the post-thaw quality of sperm. Similarly, the influence of the dilution rate and the freezing extenders should also be studied in more detail.

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