https://doi.org/10.17221/32/2024-CJAS

Effects of age and litter-of-origin on cryopreserved spermatozoa in Sumava rams

Anežka Málková[1]*, *Martin Ptáček*[1], *Filipp Georgijevič Savvulidi*[1], *Szabolcs Tamás Nagy*[2], *Luděk Stádník*[1]

[1]*Department of Animal Science, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences, Prague, Prague – Suchdol, Czech Republic*
[2]*Hungarian University of Agriculture and Life Sciences, Institute of Animal Sciences, Georgikon Campus, Keszthely, Hungary*

**Corresponding author: malkovaa@af.czu.cz, manezka209@gmail.com*

Citation: Málková A., Ptáček M., Savvulidi FG., Nagy ST., Stádník L. (2024): Effects of age and litter-of-origin on cryopreserved spermatozoa in Sumava rams. Czech J. Anim. Sci, 69: 129–138.

Abstract: This study aimed to evaluate the influence of the internal factors of ram age and litter-of-origin on semen quality during the cryopreservation process in Sumava sheep rams. This breed is included in the protected genetic resources of the Czech Republic. The sires were systematically divided into four groups according to age (under 1.5 years, 1.5 to 2.5 years, 2.5 to 3.5 years, and over 4.5 years) and according to litter frequency (singletons vs twins). Semen was evaluated after equilibration, and after cryopreservation using iSperm® mCASA and flow cytometry. During cryopreservation, there was a significant decrease in total sperm motility by 53.5%, progressive motility by 38%, and cells with intact plasma membrane and acrosome by 47%. Frozen-thawed sperm kinematic parameters showed significant age-related variations, with rams aged 4.5 and older displaying notably higher total and progressive motility (16.2% and 6.24%, respectively). Rams born as twins exhibited 3.77% ($P < 0.05$) higher progressive motility and 5.5% ($P < 0.05$) higher total motility compared to those born as singles. The sperm of older rams (> 4.5 years) exhibited higher viability (10.1%) and lower damage to the plasma membrane after freeze-thawing (23.6%), ($P < 0.05$). Twins showed significantly higher sperm viability (4.98%, $P < 0.05$) than singletons. These rams produce a larger quantity of higher-quality insemination doses after cryopreservation. For Sumava rams, in particular, broadening the sire selection base helps to select suitable rams for breeding. As the sire ages, his genetic value within the production herd may decline with time. This contrasts with genetic resource protection, where the aim is to preserve and store as many high-quality semen samples as possible.

Keywords: internal factors; flow cytometry; motility evaluation; sperm quality; sperm freezing

Sumava sheep is a breed native to the Sumava mountains in the southwestern Czech Republic. This small to medium-sized breed is robust and adaptable to unfavourable climate conditions, and is included in the national conservation programme. Therefore, this breed, together with Wallachian sheep, is an important part of the landscape management of the Carpathian breeding

Supported by the "S" grant of Ministry of Education Youth and Sports of the Czech Republic; and by the project of SGS grant (Grant No. SV 23-7-21320).

https://doi.org/10.17221/32/2024-CJAS

system in the Czech Republic (Ptacek et al. 2023). An effective reservoir of cryopreserved insemination doses can increase effective population size (N_e). Cryopreservation of semen is also very important for this breed to preserve sufficient genetic variability in a small population for long-term viability, as well as for rescue in the event of health problems or sudden predator attacks (Taberlet et al. 2008; Mattiello et al. 2012; Smith et al. 2014).

To maintain the highest possible viability of ram sperm after cryopreservation, it is important to consider internal and environmental factors that can significantly affect the process, including hormonal levels (Courot and Ortavant 1981; El-Alamy et al. 2001), protein levels (Li et al. 2020), genetic predisposition (Pimenta et al. 2012; Barbas et al. 2023), health status (Ridler et al. 2012), seasonal variation (Azawi and Ismaeel 2011), and the age of the animal (Ntemka et al. 2019), among others. In the context of semen cryopreservation, the impact of litter size on the process has not been systematically investigated in sheep. Factors such as age and litter size are generally considered for reproductive capacity. However, its impact on sperm preservation and evaluation of cryopreserved insemination doses has not yet been explained. The maturation of the rams over time can result in better sperm quality and ram age appears to be correlated with the efficacy of semen cryopreservation. Although female reproductive capacity is often assessed based on litter size, the effect of this factor on semen cryopreservation in rams has not been thoroughly studied. It is essential to understand the complex relationship between age, litter size, and semen quality to optimise assisted reproductive technologies in livestock breeding programmes (Lymberopoulos et al. 2010; Ntemka et al. 2019; Flowers 2023). Genetic control of litter size is a complex process that poses challenges for study and remains less than fully understood. Identifying improvements in sperm quality that correspond to increased conception rates has the potential to simplify the selection of rams based on the size of their litter-of-origin at birth. Foxcroft et al. (2009) and Flowers (2023) used the term "litter-of-origin" to convey how the prenatal environment collectively shapes the characteristics of an entire litter. The aforementioned concept highlights that circumstances and stimuli experienced throughout gestation can affect the attributes and progress of all individuals within a given litter.

This approach could prove helpful in breeding programs, as it enables the identification of males with a greater probability of producing superior sperm and achieving improved reproductive outcomes (Wang et al. 2020; Flowers 2023). However, it is essential to consider the limitations of sire selection and the use of individually tailored procedures when attempting to cryopreserve every individual in the population of rare breeds such as Sumava sheep. This work aimed to study the influence of selected internal factors, namely the age of the ram and the size of its litter-of-origin, on the quality of the sperm during different phases of cryopreservation.

MATERIAL AND METHODS

Animal management

The animals were kept at a commercial sheep farm in Vimperk County, South Bohemian Region, Czech Republic, at an altitude of 945 metres above sea level. The rams had access to a barn shelter, free-range areas, and a permanent fenced grazing area, where they could roam freely. The 21 rams were kept together in individual pens in a sheepfold. The rams were divided into groups according to age; five rams were included in the group up to 1.5 years of age, nine rams were included in the group aged up to 2.5 years, three rams in the group aged up to 3.5 years, and finally four rams in the group aged over 4.5 years. In addition, eight rams were included in the group of rams born as singletons and 13 rams were included in the group of rams born as twins. The rams' nutrition is provided through a year-round supply of hay available to them *ad libitum*. Additionally, they received haylage and grain (specifically oats) as supplementary feed, which was mixed with additional minerals. The rams had *ad libitum* access to water and mineral salt licks. As the facility was an organic farm, all feed must also be certified organic. The health of the animals was continuously monitored. The rams were used for breeding and were accustomed to frequent contact with their keepers, as they were used for hand mating. The procedures for the collection of semen were carried out in accordance by regulatory standards. The study solely involved the collection of semen using an artificial vagina. Pharmacological or surgical

https://doi.org/10.17221/32/2024-CJAS

procedures, animal pathogen infections, or euthanasia were not employed. The study only implemented non-experimental agricultural practices according to Czech Republic Laws No. 166/1999 (the Veterinary Medicine Act) and 154/2000 (the Animal Breeding Act). Therefore, this research can be classified as not harmful to animal welfare.

Semen collection, processing and equilibration

The semen was collected using an artificial vagina (Minitub GmbH, Tiefenbach, Germany) prepared according to the manufacturer's instructions. The rams were stimulated by a dummy, representing a heat-identified ewe, to simulate natural mating conditions and facilitate semen collection. Semen was collected from all rams (n = 21) over a single day in June. After collection, semen was diluted 1 : 1 (vol/vol) with the OptiXcell semen extender at body temperature and then cooled to 7 °C for transportation. Semen ejaculates (n = 21) were transported to the laboratory in a portable automatic cooling box.

At the laboratory, the sperm concentration of each extended sperm sample was assessed using a precalibrated Genesys™ 10vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). After determining the concentration, the sperm was diluted to a concentration of 200 × 10^6 sperm cells/ml. Semen was filled into 0.25 ml French straws (IMV Technologies, L'Aigle, France). Straws were then sealed with polyvinyl alcohol (PVA) sealing powder (IMV Technologies, L'Aigle, France). After sealing, the straws were equilibrated in a refrigerator at 4 °C for approximately 12 hours.

Before the freezing procedure was started, all samples were assessed for their kinematic para meters using the mCASA iSperm® instrument (for a detailed methodology description, see "Sperm Motility Evaluation by iSperm® mCASA" below). Table 1 provides the average kinematic parameters of the spermatozoa before equilibration.

Reagents and solutions

Dulbecco's phosphate-buffered saline (PBS, without Ca and Mg) solution was acquired from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The freezing extender used was OptiXcell (IMV Technologies, L'Aigle, France). For flow cytometry analysis, Hoechst-33342 (H-342) and propidium iodide (PI) were purchased from Sigma Aldrich (St. Louis, MO, USA), while lectin PNA from Arachis hypogaea (PNA-FITC) and MitoTracker™ Deep Red (MTR DR) were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Table 1. Basic statistical parameters for CASA criteria before equilibration (n = 84)

Parameter	$\bar{x}$	σ	Minimum	Maximum
MOT (%)	67.6	13.6	23.6	92.8
PROG (%)	45.8	13.3	10.1	78.3
VAP (μm/s)	116.4	16.0	73.9	155.3
VCL (μm/s)	147.3	14.9	95.4	177.1
VSL (μm/s)	95.6	19.8	49.3	144.5
LIN (%)	77.8	7.6	51.7	95.4
STR (%)	63.3	10.7	35.5	91.8

LIN = linearity; MOT = total motility; PROG = progressive motility; STR = straightness; VAP = average path velocity; VCL = curvilinear velocity; VSL = straight line velocity; $\bar{x}$ = arithmetic mean; σ = standard deviation

Sperm freezing and thawing

The straws were frozen by suspending them above liquid nitrogen vapour in the polystyrene mobile freezing box (Animal Reproduction Systems, Inc., Chino, CA, USA). Straws on the rack were placed 5 cm above the liquid nitrogen for 10 minutes. Subsequently, the straws were fully immersed in liquid nitrogen to achieve complete freezing. Frozen doses were then transferred to goblets and stored in a liquid nitrogen container for a minimum period of 15 days. Thawing was carried out by immersing the straws in a water bath set at a temperature of 38 °C for 60 seconds. No thawing diluent was required. After the thawing process, the doses were placed in a thermostat and incubated at a temperature of 38 °C for 1 hour.

Sperm motility evaluation by iSperm® mCASA

Sperm samples (n = 84; four replicates for each ejaculate) were evaluated at three-time points: before equilibration (BE), after equilibration (AE)

https://doi.org/10.17221/32/2024-CJAS

and after thawing (THW). The mobile computer-assisted sperm analyser iSperm® mCASA (Aidmics Biotechnology, Taipei, Taiwan) was used to assess motility and kinematic parameters following the manufacturer's instructions for software use. At least three fields of each iSperm sample chip were recorded at a frame rate of 22 frames per second, with an analysis rate of 30 frames per second.

The following settings were applied for the kinetic parameters: a minimum average path velocity (VAP) of 50 µm/s and a minimum straightness (STR) of 70% were considered progressive motile, while a minimum VAP of 20 µm/s and a minimum straight-line velocity (VSL) of 0 µm/s were considered motile. Additional properties measured included curvilinear velocity (VCL, µm/s) and linearity (LIN, %).

Flow cytometry assessment

Sperm samples (n = 84; four replicates for each ejaculate) were subjected to flow cytometry analysis both after equilibration (EQ) and after thawing (THW). For THW analysis, the sperm samples were kept at a temperature of 38 °C prior to the flow cytometry procedure.

Before evaluation in a 96-well plate, the samples were diluted in PBS containing appropriate dyes to achieve a final concentration of 20 × 10^6 spermatozoa/ml. Subsequently, the samples were incubated in the dark at 38 °C for a duration of 10 minutes. Fluorescent dyes were prepared on the same day of evaluation. Sperm samples were stained with the following fluorescent dyes, each at their respective final concentrations: 10 µg/ml H-342 for identification of DNA (deoxyribonucleic acid) content, 8 µg/ml PI to detect damage to the plasma membrane, 0.5 µg/ml PNA-FITC to assess acrosome damage, and 80 nM MTR DR to assess mitochondrial activity, specifically mitochondrial membrane potential (MMP).

The evaluation was performed using a digital flow cytometer, NovoCyte 3000 (Acea Biosciences, Agilent, Santa Clara, CA, USA). This flow cytometer was equipped with a set of optimal bandpass filters and solid-state lasers: a violet laser (405 nm, 50 mW) for exciting H-342, a blue laser (488 nm, 60 mW) for exciting PI and PNA-FITC, and a red laser (640 nm, 40 mW) for exciting MTR-DR. Specifically, H-342 was successfully excited using the violet (405 nm) laser (Martinez-Pastor et al. 2010). Prior to sample analysis, the instrument was calibrated using calibration beads (NovoCyte, QC Particles, Agilent Technologies, Santa Clara, CA, USA).

The samples were analyzed at a low speed, and a minimum of 10 000 events identified as sperm cells were evaluated from each sample. For automated cytometer setup, performance tracking, and data acquisition, NovoExpress software, v1.3.0 (Acea Biosciences, Agilent, Santa Clara, CA, USA) was utilized.

The data were saved and subsequently analysed using the same software. No compensation was required for the optical filter settings used. The gating strategy of Savvulidi et al. (2021) was followed.

Statistical analysis

The statistical analyses were performed using the GLM procedure in SAS/STAT® v9.4 (SAS Institute Inc., Cary, NC, USA).

Separate statistical models were created for each evaluated trait, as follows:

$$Y_{ijk} = \mu + age_i + LS_j + b*conc + e_{ijk} \quad (1)$$

where:

Y_{ijk} – dependent variable, which includes measurements of mCASA and flow cytometry (MOT, PROG, VAP, STR, VSL, LIN, and VCL measured before, after cell equilibration, and after thawing; total cell viability and mitochondrial membrane potential measured in equilibrated cells and 1 h after thawing);

μ – the mean of the evaluated trait;

age_i – fixed effect of the age of rams (i = 1.5 years, n = 20; i = 2.5 years, n = 36; i = 3.5 years, n = 12; i = > 4.5 years, n = 16);

LS_j – fixed effect of the litter size of rams (j = rams from single-born lambs, n = 32; j = rams from twin-born lambs, n = 52);

$b*conc$ – linear regression on the sperm concentration in the insemination dose (250–750 million cells);

e_{ijk} – residual error.

Statistical significance was determined using Tukey-Kramer adjustment at a significance level of 0.05.

https://doi.org/10.17221/32/2024-CJAS

RESULTS

CASA assessments

Statistical models were significant for all sperm kinematic parameters except total motility, progressive motility, and VSL after equilibration. The summary of these results, along with the corresponding R^2 values and significance of particular factors, is reported in Table 2.

Tables 3 and 4 report the results of the equilibrated sperm kinematic parameters related to the age of the ram and litter-of-origin. No significant differences were found for the total progressive motility related to the age of the ram.

Contrarily, significant differences were observed for the kinematic parameters of VAP, VCL, VSL, LIN, and STR of equilibrated spermatozoa. These tendencies exhibited a fluctuating pattern without any discernible age-related trend. Additionally, no significant differences were detected across different rams' litters-of-origin for all evaluated CASA parameters.

Significantly highest total and progressive motility of frozen-thawed sperm were observed for rams aged 4.5 years and above, compared with rams aged 1.5 and 2.5 years. The maximal differences reached 16.2% for total motility (4.5 vs 2.5-year-old rams) and 6.24% for progressive motility (4.5 vs 2.5 year old rams).

Rams born as twins reached 3.77% ($P < 0.05$) higher progressive motility and 5.50% ($P < 0.05$) higher total motility compared to those born as singles. No significant differences were found for other ki nematic parameters in frozen-thawed sperm related to the age of the rams or litter-of-origin factors. More detailed information on the observed differences is given in Table 3.

Flow cytometry assessments

The significance of all models and particular factors in the model equations in explaining the variability of flow cytometry parameters are reported in Table 4.

Table 5 shows the flow cytometry results of the equilibrated and frozen-thawed semen of the original Sumava sheep in relation to the age of the rams or the litter-of-origin.

After equilibration, sperm viability was significantly higher in the 3.5 and 4.5-year-old ram groups compared to the 1.5 or 2.5-year-old groups. The maximum difference was 18.1% (between the > 4.5 and 1.5 age groups).

Rams aged > 4.5 years had 7.51% lower ($P < 0.05$) plasma membrane and acrosomal damage compared to 1.5-year-old rams. No significant differences were found in any of the flow cytometric parameters evaluated in relation to the rams' litter-of-origin.

Significantly higher viability and less damage to the plasma membrane of frozen-thawed sperm were found in rams aged 3.5 years and 4.5 years and older compared to those at 1.5 or 2.5 years of age. Maximum differences reached 10.1% for viability

Table 2. Significance of factors used in statistical models for CASA parameters

Parameter	R^2	P	Age	Litter	b*conc	R^2	P	Age	Litter	b*conc
	after equilibration					after frozen-thawing				
MOT	0.058	NS	NS	NS	NS	0.352	***	***	*	NS
PROG	0.111	NS	NS	NS	NS	0.177	**	*	*	NS
VAP	0.145	*	NS	NS	*	0.187	**	NS	NS	***
VCL	0.153	*	*	NS	NS	0.173	*	NS	NS	***
VSL	0.112	NS	*	NS	NS	0.245	***	NS	NS	***
LIN	0.138	*	*	NS	NS	0.147	*	NS	NS	**
STR	0.262	***	***	NS	NS	0.149	*	NS	NS	**

Age = age of rams in each individual age group; b*conc = linear regression on the sperm concentration in the insemination dose; LIN = linearity; litter = litter size of rams at birth; MOT = total motility; NS nonsignificant; P = significance of model equations; PROG = progressive motility; R^2 = coefficient of determination; STR = straightness; VAP = average path velocity; VCL = curvilinear velocity; VSL = straight line velocity

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

https://doi.org/10.17221/32/2024-CJAS

Table 3. The effect of ram age on CASA parameters for equilibrated and frozen-thawed spermatozoa

	Age group				Litter-of-origin	
Equilibrated spermatozoa						
Parameter	1.5 (n = 20)	2.5 (n = 36)	3.5 (n = 12)	> 4.5 (n = 16)	1 (n = 32)	2 (n = 52)
MOT (%)	40.1 ± 4.04	42.8 ± 3.02	35.4 ± 5.00	41.4 ± 4.55	41.5 ± 3.23	38.3 ± 2.68
PROG (%)	17.1 ± 2.68	24.5 ± 2.02	16.0 ± 3.32	20.0 ± 3.02	19.8 ± 2.14	18.9 ± 1.79
VAP (µm/s)	89.6 ± 2.99^{a}	94.7 ± 2.24^{ab}	102.2 ± 3.71^{b}	93.5 ± 3.37^{ab}	93.3 ± 2.39	96.6 ± 1.99
VCL (µm/s)	142.8 ± 5.17^{ab}	133.7 ± 3.86^{a}	158.9 ± 6.41^{b}	143.7 ± 5.83^{ab}	146.1 ± 4.13	143.5 ± 3.44
VSL (µm/s)	57.5 ± 3.02^{a}	67.8 ± 2.26^{b}	66.4 ± 3.74^{ab}	62.6 ± 3.40^{ab}	62.3 ± 2.41	64.8 ± 2.01
LIN (%)	63.4 ± 2.12^{a}	70.9 ± 1.58^{b}	64.5 ± 2.63^{ab}	65.0 ± 2.39^{ab}	65.6 ± 1.69	66.3 ± 1.41
STR (%)	43.7 ± 2.28^{a}	55.5 ± 1.70^{b}	44.8 ± 3.83^{a}	45.0 ± 2.57^{a}	46.3 ± 1.82	48.2 ± 1.52
Frozen-thawed spermatozoa						
Parameter	1.5 (n = 20)	2.5 (n = 36)	3.5 (n = 12)	> 4.5 (n = 16)	1 (n = 32)	2 (n = 52)
MOT (%)	13.0 ± 2.17^{ac}	7.27 ± 1.62^{c}	18.9 ± 2.68^{ab}	23.5 ± 2.44^{b}	12.9 ± 1.73^{a}	18.4 ± 1.44^{b}
PROG (%)	6.45 ± 1.48^{ab}	5.01 ± 1.10^{a}	10.6 ± 1.92^{ab}	11.3 ± 1.66^{b}	6.45 ± 1.21^{a}	10.2 ± 0.98^{b}
VAP (µm/s)	88.1 ± 5.92	78.3 ± 4.42	84.9 ± 7.34	84.6 ± 6.67	82.8 ± 4.73	85.1 ± 3.93
VCL (µm/s)	112.1 ± 7.09	103.8 ± 5.30	112.3 ± 8.79	123.0 ± 7.99	109.2 ± 5.67	116.4 ± 4.71
VSL (µm/s)	69.3 ± 5.86	62.5 ± 4.38	63.4 ± 7.26	61.5 ± 6.61	62.8 ± 4.68	65.5 ± 3.90
LIN (%)	76.3 ± 4.35	68.0 ± 3.25	74.7 ± 5.39	70.1 ± 4.90	73.7 ± 3.47	70.9 ± 2.89
STR (%)	61.6 ± 4.06	54.0 ± 3.03	58.4 ± 5.03	51.1 ± 4.57	58.5 ± 3.24	54.1 ± 2.70

Age group = age of rams in each individual age group; LIN = linearity; litter-of-origin = litter size of rams at birth; MOT = total motility; PROG = progressive motility; STR = straightness; VAP = average path velocity; VCL = curvilinear velocity; VSL = straight line velocity

$^{a–c}$Different letters within particular parameters indicate significant differences at a $P < 0.05$ level of significance

Table 4. Significance of factors used in statistical models for flow cytometry parameters

Parameter	R^2	P	Age	Litter	b*conc	R^2	P	Age	Litter	b*conc
	after equilibration					after frozen-thawing				
PAI	0.304	**	**	NS	NS	0.486	***	***	*	NS
PM	0.404	***	*	NS	***	0.624	***	***	NS	***
PMA	0.329	***	*	NS	***	0.584	***	***	NS	***
PA	0.336	***	NS	NS	***	0.363	**	**	NS	NS
MMP	0.412	***	*	NS	***	0.541	***	***	*	NS

Age = age of rams in each individual age group; b*conc = linear regression on the sperm concentration in the insemination dose; litter = litter size of rams at birth; MMP = mitochondrial membrane potential; NS nonsignificant; P = significance of model equations; PA = acrosome damage; PAI = viable sperm (cells with plasma membrane and acrosome intactness); PM = plasma membrane damage; PMA = plasma membrane and acrosome damage; R^2 = coefficient of determination

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

and 23.6% for plasma membrane damage. Other flow cytometry parameters also indicated lower cryodamage in older rams, as reported in Table 5. Furthermore, rams born as twins had a significantly higher sperm viability (4.98%, $P < 0.05$) than those born as singletons, although these rams had a significantly lower mitochondrial membrane potential.

https://doi.org/10.17221/32/2024-CJAS

Table 5. The effect of ram age and rams litter-of-origin on flow cytometry parameters for equilibrated and frozen-thawed spermatozoa

	Age group				Litter-of-origin	
Equilibrated spermatozoa						
Parameter	1.5 (n = 15)	2.5 (n = 27)	3.5 (n = 9)	> 4.5 (n = 12)	1 (n = 24)	2 (n = 39)
PAI (%)	48.5 ± 2.87^{a}	57.0 ± 2.21^{a}	61.2 ± 3.54^{b}	66.6 ± 3.21^{b}	56.4 ± 2.30	60.3 ± 1.94
PM (%)	32.7 ± 2.58	28.4 ± 1.99	22.7 ± 3.18	22.1 ± 2.89	28.5 ± 2.07	24.4 ± 1.75
MA (%)	18.5 ± 1.69^{a}	14.3 ± 1.30^{ab}	15.7 ± 2.09^{ab}	11.0 ± 1.90^{b}	14.8 ± 1.36	14.9 ± 1.15
PA (%)	0.33 ± 0.04	0.35 ± 0.03	0.42 ± 0.05	0.26 ± 0.05	0.30 ± 0.03	0.38 ± 0.03
MMP (%)	90.4 ± 2.07	90.0 ± 1.59	97.9 ± 2.55	94.3 ± 2.32	95.2 ± 1.66	91.1 ± 1.40
Frozen-thawed spermatozoa						
Parameter	1.5 (n = 10)	2.5 (n = 18)	3.5 (n = 6)	> 4.5 (n = 8)	1 (n = 16)	2 (n = 26)
PAI (%)	6.71 ± 1.71^{a}	7.70 ± 1.32^{a}	14.7 ± 2.11^{b}	16.8 ± 1.92^{b}	8.99 ± 1.38^{a}	14.0 ± 1.16^{b}
PM (%)	59.6 ± 3.58^{a}	74.5 ± 2.76^{b}	58.1 ± 4.42^{a}	51.0 ± 4.01^{a}	63.7 ± 2.88	57.9 ± 2.43
PMA (%)	33.6 ± 2.92^{a}	17.6 ± 2.25^{b}	26.9 ± 3.61^{ab}	31.9 ± 3.28^{a}	27.1 ± 2.35	27.9 ± 1.98
PA (%)	0.10 ± 0.05^{a}	0.14 ± 0.04^{a}	0.34 ± 0.06^{b}	0.29 ± 0.06^{ab}	0.18 ± 0.04	0.25 ± 0.03
MMP (%)	98.3 ± 2.02^{a}	84.8 ± 1.56^{b}	93.8 ± 2.50^{a}	94.3 ± 2.27^{a}	95.1 ± 1.63^{a}	90.5 ± 1.37^{b}

Age group = age of rams in each individual age group; litter-of-origin = litter size of rams at birth; MMP = mitochondrial membrane potential; PA = acrosome damage after equilibration; PAI = viable sperm (cells with plasma membrane and acrosome intactness); PM = plasma membrane damage; PMA = plasma membrane and acrosome damage

^{a,b}Different letters within particular parameters indicate significant differences at a $P < 0.05$ level of significance

DISCUSSION

Our study aims to achieve a better understanding of age, litter size, and sperm quality in rams. A major challenge for sperm preservation in small ruminants is the low survival rate of the sperm after cooling or cryopreservation, which can hinder the successful preservation of genetic material. Furthermore, the anatomical shape of the sheep's cervix represents an obstacle during the insemination process, which further complicates the preservation of ram semen in terms of pressure on the quality of cryopreserved semen (Masoudi et al. 2017). Sperm preservation techniques are still evolving. However, obtaining a high-quality native ejaculate is the key to successful preservation. Therefore, the study aimed to assess the internal factors that affect the ejaculate quality of Sumava rams and to evaluate their impact on sperm quality parameters during the process of preserving sperm cells. The effect of age and litter-of-origin size on ram sperm quality was a subject of interest in the study. Within our research, these factors influenced the cytometric and CASA parameters of ram sperm after equilibration and after thawing. The effects of male ageing on semen quality have been extensively studied, with sperm production reaching a peak at a certain age. However, the effect of the male's age is often not considered in the selection or adaptation of the system for individuals and should be contextualised with other factors involved (Abah et al. 2023).

Tabbaa et al. (2006) found no significant differences in semen quality between Awassi rams of different age categories. However, Ntemka et al. (2019) found that the quality of semen was better in older Chios rams (> 5 years). It is important to emphasise that the study by Chella et al. (2017) did not include rams older than 5 years in their research. These older rams, as suggested by Ntemka et al. (2019), have above-average semen quality. Furthermore, Ntemka et al. (2019) found that older rams had better semen quality than younger rams (< 12 months), which is supported by Marti et al. (2011) on Rasa Aragonesa rams. Therefore, while Chella et al. (2017) described a decrease in semen quality after the Zulu rams reached three years of age, it is important to consider the range of ram ages examined in the studies and possible differences in semen

https://doi.org/10.17221/32/2024-CJAS

quality between different age groups. Sperm quality remained stable up to three years of age, during which semen quality was three times better (Hassan et al. 2009; Marti et al. 2011). In our study, rams were between two and five years old. Age is a significant factor in livestock breeding and performance, as it can increase profits per animal. However, high selection can also increase the risk of inbreeding. Therefore, the combination of reproductive technology and genomic selection can lead to greater genetic gain (Granleese et al. 2015).

In our study, significant differences due to age were observed for almost all CASA parameters after equilibration, but after thawing, only the MOT and PROG differences remained significant ($P < 0.05$). Rams aged 3.5 and over 4.5 years had a demonstrably higher percentage of sperm cells with an intact plasma membrane and acrosome after equilibration. The flow cytometric parameters after thawing differed significantly, showing that ram age is relevant in the sperm cryopreservation process. Additionally, litter size has been suggested as a potential factor influencing sperm quality (Flowers 2023). It is hypothesised that rams from different-sized litters may display variations in sperm characteristics due to factors such as the intrauterine environment, maternal nutrition, or genetic factors associated with litter size (Wang et al. 2020; Flowers 2023). According to Koyuncu et al. (2005), the testicular diameter was significantly larger in lambs from multiple litters, which may support the theory of higher semen quality from these individuals. Flowers (2023) also observed that litter-of-origin traits had an impact on lifetime productivity accounting for more than 50% of the variation for sows and boars. Our results partially support this hypothesis for the Sumava rams, as we found significant differences in certain cytometric parameters after thawing. Specifically, we observed significant differences in the PAI and MMP parameters between rams from singleton and twin litters after thawing ($P < 0.05$). Sires from twin litters exhibited significantly higher PAI values, indicating improved sperm quality. On the other hand, sires from singleton litters had significantly higher MMP values, suggesting better mitochondrial function in their sperm ($P < 0.05$). The same applies to rams from a twin litter for the MOT and PROG parameters after thawing ($P < 0.05$). These findings suggest that the size of the litter may influence sperm quality in rams, particularly after thawing.

However, the underlying mechanisms and biological significance of these differences require further research. For example, how does the decline in testosterone in rams relate to semen quality, does DNA fragmentation increase, does the proportion of morphologically abnormal sperm cells increase with age, or does the sex of the twins have any effect on reproductive outcomes? Preserving sperm from older rams through cryoconservation could help maintain genetic diversity in small populations of endangered breeds, and also reintroduce lost diversity to strongly selected breeds. After fertility testing, using this semen could significantly improve conception rates in certain breeds and less utilised species through artificial insemination; however small populations and breeds require cryopreservation regardless of semen quality (Makarevich et al. 2022; Jacques et al. 2023; Pesan et al. 2023).

CONCLUSION

Age and litter-of-origin can affect the quality of ram semen, and our results warrant further research into the relationship between the quality of ram semen and litter-of-origin or the age of the ram. We observed changes in sperm characteristics during the cryopreservation phases, specifically after equilibration and after freeze-thawing. The results of this research could improve the effectiveness of assisted reproductive techniques in sheep breeding.

It is recommended to select sires based on the obtained results. When dealing with endangered breeds or species, it is important to consider the genetic value of individuals that require preservation regardless of the outcome.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Abah KO, Fontbonne A, Partyka A, Nizanski W. Effect of male age on semen quality in domestic animals: Potential for advanced functional and translational research?. Vet Res Commun. 2023 Jul 11;47(3):1125-37.

https://doi.org/10.17221/32/2024-CJAS

Azawi OI, Ismaeel MA. Effects of seasons on some semen parameters and bacterial contamination of Awassi ram semen. Reprod Domest Anim. 2011 Aug 29;47(3): 403-6.

Barbas JP, Pimenta J, Baptista MC, Marques CC, Pereira RMLN, Carolino N, Simoes J. Ram semen cryopreservation for Portuguese native breeds: Season and breed effects on semen quality variation. Animals. 2023 Feb 7; 13(4):579.

Chella L, Kunene N, Lehloenya K. A comparative study on the quality of semen from Zulu rams at various ages and during different seasons in KwaZulu-Natal, South Africa. Small Rumin Res. 2017 Apr 10;151:104-9.

Courot M, Ortavant R. Endocrine control of spermatogenesis in the ram. J Reprod Fertil Suppl. 1981 Jan 1;30:47-60.

El-Alamy MA, Foote RH, Hare E. Sperm output and hormone concentrations in Finn and Dorset rams exposed to long-and short-day lighting. Theriogenology. 2001 Sep 15;56(5):839-54.

Flowers WL. Litter-of-origin traits and their association with lifetime productivity in sows and boars. Mol Reprod Dev. 2023 Jul;90(7):585-93.

Foxcroft GR, Dixon WT, Dyck MK, Novak S, Harding JCS, Almeida FCRL. Prenatal programming of postnatal development in the pig. Soc Reprod Fertil Suppl. 2009 Jan 1;66:213-31.

Granleese T, Clark SA, Swan AA. van der Werf JHJ. Increased genetic gains in sheep, beef and dairy breeding programs from using female reproductive technologies combined with optimal contribution selection and genomic breeding values. Genet Sel Evol. 2015 Sep 14; 47:70.

Hassan MR, Pervage S, Ershaduzzaman M, Talukder MAI. Influence of age on the spermiogramic parameters of native sheep. J Bangladesh Agril Univ. 2009;7(2):301-4.

Jacques A, Duclos D, Danchin-Burge C, Mercat MJ, Tixier-Boichard M, Restoux G. Assessing the potential of germplasm collections for the management of genetic diversity: The case of the French National Cryobank. Peer Community Journal. 2024 Feb 4; e13.

Koyuncu M. Kara Uzun S, Ozis S, Duru S. Development of testicular dimensions and size, and their relationship to age and body weight in growing Kivircik (Western Thrace) ram lambs. Czech J Anim Sci. 2005 Jun 30; 50(6):243-8.

Li C, Liu Q, Wang X, Hu W, Han D, Mwacharo JM, Wei C, Chu M, Di R. Expression and localization of PIWI proteins in testis and ovary of domestic sheep. Czech J Anim Sci. 2020 Mar 31;65(3):86-96.

Lymberopoulos AG, Tsakmakidis IA, Khalifa TAA. Effect of ram age on structural and functional competence of frozen-thawed spermatozoa in dairy sheep. Reprod Domest Anim. 2010 Jul 11;45(4):572-8.

Makarevich A, Spalekova E, Kubovicova E, Bezdicek J, Chrenek P. Cooling storage of ram sperm in presence of antioxidant glutathione. Czech J Anim Sci. 2022 Sep 30;67(9):356-64.

Marti JI, Aparicio IM, Garcia-Herreros M. Sperm morphometric subpopulations are differentially distributed in rams with different maturity age in cryopreserved ejaculates. Theriogenology. 2011 Jul 1;76(1):97-109.

Martinez-Pastor F, Mata-Campuzano M, Alvarez-Rodriguez M, Alvarez M, Anel L, de Paz P. Probes and techniques for sperm evaluation by flow cytometry. Reprod Domest Anim. 2010 Jun;45(S2):67-78

Masoudi R, Shahneh AZ, Towhidi A, Kohram H, Akbarisharif A, Sharafi M. Fertility response of artificial insemination methods in sheep with fresh and frozen-thawed semen. Cryobiology. 2017 Nov 27;74:77-80.

Mattiello S, Bresciani T, Gaggero S, Russo C, Mazzarone V. Sheep predation: Characteristics and risk factors. Small Rumin Res. 2012 Jun;105(1-3):315-20.

Ntemka A, Kiossis E, Boscos C, Theodoridis A, Kourousekos G, Tsakmakidis I. Impact of old age and season on Chios ram semen quality. Small Rumin Res. 2019 Sep; 178:15-17.

Pesan V, Reckova Z, Hosek M, Filipcík R, Souskova K, Kopec T, Tesarova MP. Evaluation of crystallisation structures of cervical mucus in Zwartbles sheep with previous oestrus synchronisation. Czech J Anim Sci. 2023 Sep 26; 68(9):383-90.

Pimenta J, Domingos A, Santos P, Marques CC, Cantante C, Santos A, Barbas JP, Baptista MC, Horta AE, Viegas A, Mesquita P, Gonçalves J, Fontes CA, Prates JA, Pereira RM. Is prnt a pseudogene? Identification of ram Prt in testis and ejaculated spermatozoa. PLoS One. 2012 Aug 24;7(8):e42957.

Ptacek M, Milerski M, Michlova T, Duchacek J, Tancin V, Uhrincat M, Schmidova J, Savvulidi FG, Stadnik L. Monitoring of milk performance of Wallachian sheep grazed under traditional Carpathian management in Western Beskids location. Czech J Anim Sci. 2023 Dec 1;68(11):460-8.

Ridler AL, Smith SL, West DM. Ram and buck management. Anim Reprod Sci. 2012 Aug 24;130(3-4):180-3.

Savvulidi FG, Ptacek M, Malkova A, Beranek J, Stadnik L. Optimizing the conventional method of sperm freezing in liquid nitrogen vapour for Wallachian sheep conservation program. Czech J Anim Sci. 2021 Dec 4;66(2): 55-64.

Smith JB, Jenks JA, Grovenburg TW, Klaver RW. Disease and predation: sorting out causes of a bighorn sheep (Ovis canadensis) decline. PLoS One. 2014 Feb 7;9(2):e88271.

https://doi.org/10.17221/32/2024-CJAS

Tabbaa MJ, Kridli RT, Amashe MG, Barakeh FS. Factors affecting scrotal circumference and semen characteristics of Awassi rams. Jordan J Biol Sci. 2006 Sep 4;2(3):243-50.

Taberlet P, Valentini A, Rezaei HR, Naderi S, Pompanon F, Negrini R, Ajmone-Marsan P. Are cattle, sheep, and goats endangered species?. Mol ecol. 2008 Jan;17(1):275-84.

Wang K, Kang Z, Jiang E, Yan H, Zhu H, Liu J, Qu L, Lan X, Pan C. Genetic effects of DSCAML1 identified in genome-wide association study revealing strong associations with litter size and semen quality in goat (Capra hircus). Theriogenology. 2020 Apr 1;146:20-5.

Received: February 28, 2024
Accepted: April 9, 2024
Published online: April 30, 2024