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# Optimizing the conventional method of sperm freezing in liquid nitrogen vapour for Wallachian sheep conservation program

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**Abstract:** The aim of the present study was to optimize the conventional method of sperm freezing in liquid nitrogen ($LN_2$) vapour for successful cryopreservation of Wallachian ram sperm, the genetic resources of the Czech Republic. Sperm in straws were frozen using the conventional freezing method via a static exposure of sperm doses to $LN_2$ vapour, or by four different modified freezing methods. Under modified freezing, straws were frozen by a discontinuous, time-dependent decremental change in the distance between the straws and the surface of $LN_2$. The viability of sperm was evaluated by flow cytometry after sperm equilibration, and immediately after thawing. Besides the observed inter-sire and daily variation, the obtained results suggest the methodological weakness of the conventional freezing method via the static exposure of sperm doses to $LN_2$ vapour. With the use of the optimized freezing procedure, all parameters of thawed sperm were significantly ($P < 0.05$) improved in comparison with the conventional method: percentage of thawed sperm viability increased up to 48.3%, percentage of sperm with plasma membrane damage after thawing decreased to 6.58%, percentage of sperm with acrosome damage decreased to 24.4%, and percentage of sperm with deteriorated mitochondrial activity decreased to 6.28%. In conclusion, our results suggest that an optimized freezing procedure should be routinely used instead of the conventional method to cryopreserve Wallachian ram sperm.

**Keywords:** cryopreservation; flow cytometry; multicolour analysis; sperm doses; static exposure

The Wallachian sheep is an original Czech medium-sized horned breed with a triple production purpose (milk, meat, wool). The breed originated from the area of the Beskids Mountains, and is characterized by adaptability and resistance to harsh climatic conditions (Jandurova et al. 2005). At present, Wallachian sheep in the Czech Republic are not usually milked. However, it was shown that Wallachian ewes grazed on mountain pastures provide highly nutritional and health valuable milk (Ptacek et al. 2019a). An increase in the population size of this breed

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for wider use for milk production could be beneficial. The sheep is a typical seasonally polyoestrous breed (Busa et al. 2019). While outcrossing, Wallachian sheep are used in the maternal position as they have been historically adapted to breeding in very extensive conditions (Ptacek et al. 2019b). In the second half of the 20$^{th}$ century Wallachian sheep were widely crossed with imported sheep breeds (primarily to improve the wool quality). This crossing scheme caused that by the mid-1980s the population of original Wallachian sheep was completely suppressed, and only a few individuals of original Wallachian sheep survived. Since 1999 Wallachian sheep have been classified among the genetic resources of the Czech Republic (Ptacek et al. 2019a). In 2018, in total 1 148 purebred Wallachian ewes were registered in the animal performance recording in the Czech Republic. One of the key elements in the conservation program for Wallachian sheep is the cryopreservation of sperm collected from native Wallachian rams.

The optimal freezing method must guarantee satisfactory viability of thawed sperm in terms of undamaged plasma membrane, undamaged acrosome, and undeteriorated activity of mitochondria at least (Martinez-Pastor et al. 2010). The use of optimal freezing rate is crucial for successful sperm cryopreservation. According to the generally accepted theory, the best freezing rate must be slow enough to allow water to leave the sperm cells. This prevents intracellular water crystallization and subsequent cryodamage during freezing. On the other hand, optimal freezing rate must be fast enough to allow cells to pass quickly through the temperature critical range (–10 °C to –25 °C) during freezing (Salamon and Maxwell 2000). Additionally, it must be fast enough to avoid severe cell dehydration and to avoid the solution injury to cells. However, it is not a simple task to categorize what it is "slow enough", and what it is "fast enough" in terms of digits (Byrne et al. 2000).

In fieldwork, the freezing methodology is usually simplified. The simplification is well reasoned when a small amount of insemination doses is produced from the collected ejaculate (this especially applies to the production of ovine sperm doses). The sperm doses are packed into plastic cryostraws, and then exposed statically to liquid nitrogen vapour at the distance of 4–6 cm above the surface of liquid nitrogen for freezing (Barbas and Mascarenhas 2009). This conventional freezing method has already been previously applied for cryopreservation of sperm from rams and bucks of several breeds (Anel et al. 2003; Choe et al. 2006; Jha et al. 2019).

From the practical point of view, it is interesting to apply this well-established, simple freezing method in the Wallachian sheep conservation program to cryopreserve Wallachian ram sperm. However, considering previously reported interbreed (Beran et al. 2011; Olah et al. 2013), and even inter-sire (Ramon et al. 2013; Dolezalova et al. 2016; Jovicic et al. 2020) differences in livestock sperm freezability, we are aware of the fact that the freezing method must first be thoroughly optimized to use with the specific breed.

Therefore, the general aim of this study was to optimize the conventional freezing method via static exposure of sperm doses to liquid nitrogen vapour in the procedure of Wallachian ram sperm cryopreservation.

## MATERIAL AND METHODS

### Rams

It was demonstrated previously that mature Wallachian rams (> 3 years of age) are more suitable for the purpose of insemination dose production, as the sperm collected from older animals maintains the highest level of sperm motility during a thermal survival test (Stolc et al. 2009). Therefore, in the present study, two mature (4–5 years-old) original Wallachian rams were used. Rams with typical exterior signs of the breed and with an excellent breeding history were transported from the area of the Beskids Mountains. Afterwards, animals were kept at the Demonstration and Experimental Centre of Czech University of Life Sciences in Prague (50°07'47.6''N 14°22'07.0''E), at the nearest distance to the laboratory of flow cytometry. This was done with a special intention to reduce any variability due to possible delay between the collection of semen and its subsequent evaluation. Also, this enabled to use rams in the same health condition under strict veterinary inspection, in the same breeding conditions, under the same feeding ration, with the semen collected and processed in the same manner and in the same time duration. As we were aware of the use of a lim-

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ited number of animals, the repetition of semen collection from both rams was highly increased in the present study (as described in the section "Semen collection").

Both rams were kept in free-range paddocks with a possibility of housing in stables. All housing construction, minimal floor area, and technology equipment were in full accordance with legislative regulations of the Czech Republic. Also, procedures performed with the animals during semen collection were in accordance with Ethics Committee of Central Commission for Animal Welfare at the Ministry of Agriculture of the Czech Republic (Prague, Czech Republic), and carried out in accordance with Directive 2010/63/EU for animal experiments and Local Ethics Commission (No. 5/2020). Feed ration for rams was composed of grazing pasture and meadow hay of own production (*ad libitum*), supplemented with lucerne concentrates (1580 OVCE UNI; De Heus a.s., Bucovice, Czech Republic) at the amount of 0.75–1.50 kg per ram per day (regulated according to the animal body condition score). Mineral licks and drinking water were available in *ad libitum* amount during the whole trial. Body condition of animals ranged from 3.5 to 4.0 points (Russel et al. 1969) during the experimental period.

## Semen collection

After three weeks of adaptation at the Demonstration and Experimental Centre, semen collection from both rams began. Semen was naturally collected using a sheep/goat artificial vagina (Minitub GmbH, Tiefenbach, Germany) prepared according to the manufacturer's instruction manual, with all the necessary precautions to prevent cold shock of spermatozoa. Semen samples were collected regularly during a 2-month period (from 8$^{th}$ January to 27$^{th}$ May 2020) at 15 control days. Rams gave samples twice a week at most, with a 48-hour pause between any two adjacent semen collections. On the day of semen collection, mostly the semen of the 1$^{st}$ ejaculate was collected from each ram individually. In total, thirty ejaculates were collected from both rams. From these, 319 experimental samples were prepared for the purposes of the present study (232 samples from the semen of ram 1, and 87 samples from the semen of ram 2). The difference in the sample size between rams reflected the fact that ram 1 demonstrated high *libido sexualis* already at the beginning of semen collection. As so, ram 1 produced a higher number of ejaculates during the whole semen collection period.

## Semen extension

Immediately after collection (except the samples intended for mass motility evaluation), the semen was diluted 1 : 4 (vol/vol) with pre-warmed egg yolk-free glycerol supplemented ANDROMED (Minitub GmbH, Tiefenbach, Germany) commercial semen diluent. The exact composition of this commercial extender is proprietary, but most likely it contains 6–7% of glycerol (Akcay et al. 2012). Sperm samples were transferred in a thermo-box to the flow cytometry laboratory for further analyses.

## Sperm motility evaluation

The mass motility was evaluated subjectively in undiluted semen, using 0–5 scores scale according to David et al. (2015). Only the semen of good initial quality (scored 3 out of 5, or higher) was used for further processing.

## Final sperm dilution

The sperm concentration was assessed using a pre-calibrated Genesys™ 10vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Semen samples were diluted to a final concentration of 200 × $10^6$/ml (Alvarez et al. 2012) using ANDROMED (Minitub GmbH, Tiefenbach, Germany). Diluted semen samples were filled into 0.25 ml French straws (IMV Technologies, L'Aigle, France) under ambient temperature (25 °C) and were sealed with the sealing powder (IMV Technologies, L'Aigle, France).

## Semen cooling and equilibration

Sealed straws were transferred to the refrigerator for cooling (from 25 °C to 8 °C; cooling rate 1.0 °C per min, on average) and subsequent equilibration for 2–4 h (Lv et al. 2019).

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## Sperm freezing

After equilibration, the straws were frozen using a conventional freezing method via static exposure of sperm doses to liquid nitrogen vapour, or four modified freezing methods.

In the conventional freezing method (Curve 1 in the present study, Figure 1), the straws were frozen by static exposure to liquid nitrogen vapour for 10 min 4 cm above the $LN_2$ surface.

In all the modified freezing methods used in the present study, the straws were frozen in a special polystyrene freezing box (adapted from Animal Reproduction Systems, Inc., Chino, CA, USA; modified) as described in Ptacek et al. (2019c). The freezing box was specially designed to allow a discontinuous, time-dependent decremental change in the distance between the cryostraws and the surface of $LN_2$, and thus a precisely regulated freezing curve:

Curve 2: 15 cm above $LN_2$ surface for the duration of 3 min; 9.5 cm above $LN_2$ surface for the duration of 2 min; 5 cm above $LN_2$ surface for the duration of 1 min; 1.5 cm above $LN_2$ surface for the duration of 2 min;

Curve 3: 15 cm above $LN_2$ surface for the duration of 4.5 min, 9.5 cm above $LN_2$ surface for the duration of 3 min, 5 cm above $LN_2$ surface for the duration of 1.5 min, 1.5 cm above $LN_2$ surface for the duration of 3 min;

Curve 4: 15 cm above $LN_2$ surface for the duration of 6 min, 9.5 cm above $LN_2$ surface for the duration of 4 min, 5 cm above $LN_2$ surface for the duration of 2 min, 1.5 cm above $LN_2$ surface for the duration of 4 min;

Curve 5: 15 cm above $LN_2$ surface for the duration of 9 min, 9.5 cm above $LN_2$ surface for the duration of 6 min, 5 cm above $LN_2$ surface for the duration of 3 min, 1.5 cm above $LN_2$ surface for the duration of 6 minutes.

An HH506A digital thermometer, equipped with "K type" thermocouple (Omega Engineering Inc., Norwalk, CT, USA) was used to record temperature changes directly inside the straw during freezing (Figure 1).

## Sperm thawing

Frozen straws were stored in liquid nitrogen for at least 24 h before thawing. Straws were thawed in a water bath (38 °C/30 s).

## Sperm viability evaluation by flow cytometry

The viability of sperm samples was evaluated using the flow cytometry. Sperm plasma membrane damage, acrosome damage, and mitochondrial activity were assessed. These parameters were assessed either in combination (to assess the total sperm viability) or separately. Viable sperm were defined as cells with the intact plasma membrane, intact acrosome, and high mitochondrial activity. The initial viability analysis of samples was performed in sperm after equilibration, but before freezing. This was done to exclude any effects the equilibration might have on evaluated parameters. Subsequently, the viability of sperm immediately after thawing was also evaluated. For flow cytometry, the semen samples were diluted in Dulbecco's phosphate-buffered saline (–Ca/–Mg) to the final concentration of $20 \times 10^6$/ml, and they were stained for 10 min at 38 °C in the dark with the following assays (final concentra-

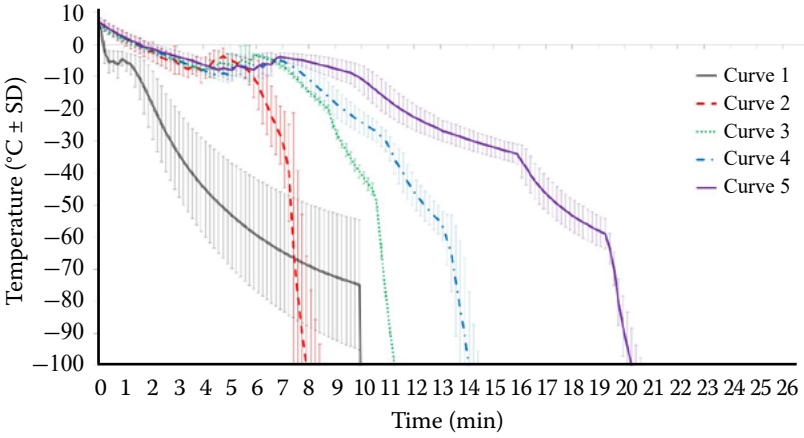


Figure 1. Temperature changes inside the straws during freezing

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tions): 10 µg/ml Hoechst-33342 (H-342) for DNA content identification; 8 µg/ml propidium iodide for assessing plasma membrane damage; 0.5 µg/ml lectin PNA from *Arachis hypogaea* (PNA-FITC) for assessing acrosome damage; and 80 nM Mito Tracker Deep Red (MTR DR) for assessing mitochondrial activity. Initial stock solutions of the assays were prepared by their dissolving in aqueous or organic solvents according to the manufacturer's instructions. To eliminate any possible negative effect of solvents used for the preparation of initial stocks on sperm cells, working stock solutions for all the assays used in the present study were prepared by the re-dissolving of initial stocks in Dulbecco's phosphate-buffered saline. H-342 and PI were purchased from Sigma Aldrich (St. Louis, MO, USA), and PNA-FITC and MTR DR were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Subsequently, sperm samples were analysed using a NovoCyte digital flow cytometer, model number 3000 (Acea Biosciences, part of Agilent, Santa Clara, CA, USA). The flow cytometer was equipped with violet (405 nm), blue (488 nm), and red (640 nm) lasers and appropriate optical filters for detection of emitted fluorescence signals. H-342 can be successfully excited with violet (405 nm) laser (Martinez-Pastor et al. 2010). The samples were run at low speed and at least 10 000 events were recorded for each sample. NovoExpress software, v1.3.0 (Acea Biosciences, part of Agilent, Santa Clara, CA, USA) was used for automated cytometer setup and performance tracking as well as data acquisition. The same software was also used to analyse acquired flow cytometry data. No compensation was required with the optical filter setup used. Gating strategy is presented in Figure 2.

## Statistical analyses

Statistical analyses were performed using the generalized linear model (GLM) procedure of SAS/STAT® v9.4. (SAS Institute Inc., Cary, NC, USA).

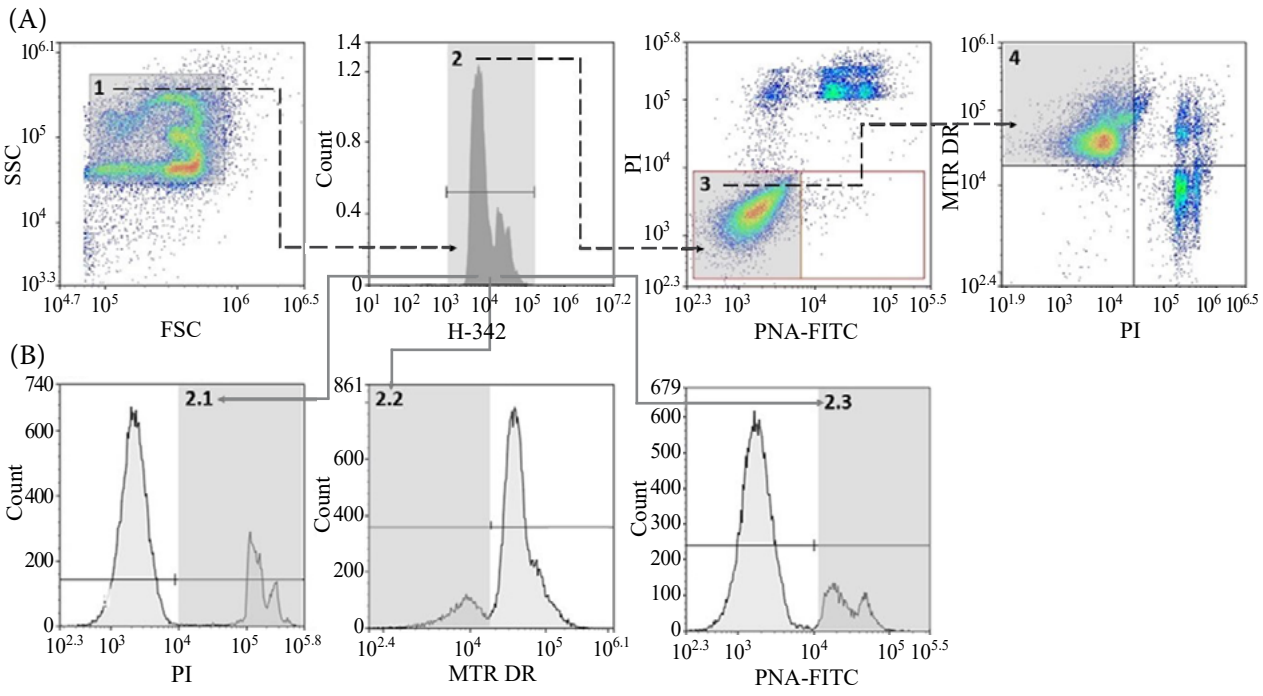


Figure 2. Flow cytometric gating strategy and hierarchy

(A) Cluster of events was initially identified using a side scatter (SSC) versus forward scatter (FSC) bivariate histogram plot (1). Spermatic events were identified based on the gating set with the Hoechst-33342 stain (H-342; DNA content) (2). Spermatic events with intact plasma membrane and acrosome were identified based on the propidium iodide (PI) and *Arachis hypogaea* lectin PNA (PNA-FITC) signal intensities (3). Finally, viable sperm were evaluated by the combination of the above-mentioned fluorescent markers with Mito Tracker Deep Red (MTR DR) positive cells ($H\text{-}342^{+}$/$PNA\text{-}FITC^{-}$/$PI^{-}$/$MTR\ DR^{+}$) (4). (B) Clusters of DNA-containing sperm with damaged plasma membrane (2.1), deteriorated mitochondrial activity (2.2), and damaged acrosome (2.3) are presented. Illustrative dot plots and histograms are shown

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For the analysis, the dependent variables percentage of viable sperm (Figure 2A), percentage of sperm with plasma membrane damage, percentage of sperm with acrosome damage, percentage of sperm with deteriorated mitochondrial activity (Figure 2B) were defined for the semen after thawing. These variables were corrected for fixed effects of semen collection day, sire, freezing curve, and initial percentages of flow cytometry sperm parameters after equilibration as covariates.

The description model equations for particular dependent variables are as follows:

$$THW_{ijkl} = \mu + DAY_i + SIRE_j + CURVE_k + b^*EQ + e_{ijkl} \quad (1)$$

$$THW(PM)_{ijkl} = \mu + DAY_i + SIRE_j + CURVE_k + b^*EQ(PM) + e_{ijkl} \quad (2)$$

$$THW(ACR)_{ijkl} = \mu + DAY_i + SIRE_j + CURVE_k + b^*EQ(ACR) + e_{ijkl} \quad (3)$$

$$THW(MTCH)_{ijkl} = \mu + DAY_i + SIRE_j + CURVE_k + b^*EQ(MTCH) + e_{ijkl} \quad (4)$$

where:

$THW_{ijkl}$ – percentage of viable sperm after thawing;

$THW(PM)_{ijkl}$ – percentage of sperm with plasma membrane damage after thawing;

$THW(ACR)_{ijkl}$ – percentage of sperm with acrosome damage after thawing;

$THW(MTCH)_{ijkl}$ – percentage of sperm with deteriorated mitochondrial activity after thawing;

$\mu$ – mean value of dependent variable;

$DAY_i$ – fixed effect of the control day of semen collection ($i$ = 1st control day, $n$ = 16; $i$ = 2nd control day, $n$ = 20; $i$ = 3rd control day, $n$ = 20; $i$ = 4th control day, $n$ = 20; $i$ = 5th control day, $n$ = 20; $i$ = 6th control day, $n$ = 28; $i$ = 7th control day, $n$ = 36; $i$ = 8th control day, $n$ = 24; $i$ = 9th control day, $n$ = 40; $i$ = 10th control day, $n$ = 10; $i$ = 11th control day, $n$ = 15; $i$ = 12th control day, $n$ = 16; $i$ = 13th control day, $n$ = 8; $i$ = 14th control day, $n$ = 8; $i$ = 15th control day, $n$ = 8);

$SIRE_j$ – fixed effect of $j$-th ram ($j$ = ram 1, $n$ = 232; $j$ = ram 2, $n$ = 87);

$CURVE_k$ – fixed effect of the freezing curve ($k$ = Curve 1, $n$ = 56; $k$ = Curve 2, $n$ = 56; $k$ = Curve 3, $n$ = 136; $k$ = Curve 4, $n$ = 28; $k$ = Curve 5, $n$ = 43);

b*EQ – initial percentage of viable sperm after equilibration as covariate (range = 51.94–80.36);

$e_{ijkl}$ – residual error;

b*EQ(PM) – initial percentage of sperm with plasma membrane damage after equilibration as covariate (range = 16.29–44.78);

b*EQ(ACR) – initial percentage of sperm with acrosome damage after equilibration as covariate (range = 4.96–28.01);

b*EQ(MTCH) – initial percentage of sperm with deteriorated mitochondrial activity after equilibration as covariate (range = 1.4–35.61).

There was a difference in the sample size between curves because it was not possible to freeze sperm by using all five curves during a single semen collection day and some curves were replicated less frequently during the experiment.

Significance level $P < 0.05$ was used to evaluate differences between groups.

## RESULTS AND DISCUSSION

### Cryopreservation decreases the viability of Wallachian ram sperm

The summary statistics of sperm viability in equilibrated and thawed Wallachian ram semen are presented in Table 1. The procedure of cryopreservation decreased the viability of sperm by as much as six and a half times. A decrease of thawed ram sperm viability was reported previously by Salamon and Maxwell (2000). These authors reported that only 20–30% of ram sperm remained viable and biologically undamaged after thawing. Lower results of sperm viability after thawing observed in the present study (10.8% on average) might be due to the use of ANDROMED commercial semen extender supplemented with glycerol. We used this semen extender throughout the whole procedure of semen processing. The sperm were exposed to glycerol from the beginning of semen collection until post-thaw sperm analysis. There exists a collective concern that equilibrating ram

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Table 1. Summary statistics of sperm viability after equilibration, and after thawing

| | *n* | AM | SD | Median | Mode | Min. | Max. | CV | Kurtosis | Skewness |
|---|---|---|---|---|---|---|---|---|---|---|
| EQ | 319 | 69.8 | 5.85 | 70.2 | 78.8 | 51.9 | 80.3 | 8.37 | 0.50 | –0.40 |
| EQ(PM) | 319 | 26.0 | 5.58 | 25.9 | 17.9 | 16.2 | 44.7 | 21.3 | 1.37 | 0.72 |
| EQ(ACR) | 319 | 17.6 | 5.11 | 18.1 | 14.2 | 4.96 | 28.0 | 28.9 | 0.47 | –0.38 |
| EQ(MTCH) | 319 | 19.2 | 6.45 | 20.3 | 23.8 | 1.40 | 35.6 | 33.5 | 2.06 | –0.82 |
| THW | 319 | 10.8 | 7.93 | 8.68 | – | 1.09 | 39.3 | 73.0 | 0.90 | 1.06 |
| THW(PM) | 319 | 85.8 | 9.43 | 88.3 | 77.4 | 57.9 | 98.0 | 10.9 | –0.30 | –0.76 |
| THW(ACR) | 319 | 58.4 | 15.3 | 59.2 | 44.2 | 18.9 | 90.6 | 26.2 | –0.67 | –0.23 |
| THW(MTCH) | 319 | 77.7 | 9.79 | 79.2 | 70.6 | 48.4 | 96.0 | 12.5 | –0.18 | –0.39 |

AM = arithmetic mean; CV = coefficient of variation; EQ = initial percentage of viable sperm after equilibration; EQ(ACR) = initial percentage of sperm with acrosome damage after equilibration; EQ(MTCH) = initial percentage of sperm with deteriorated mitochondrial activity after equilibration; EQ(PM) = initial percentage of sperm with plasma membrane damage after equilibration; THW = percentage of viable sperm after thawing; THW(ACR) = percentage of sperm with acrosome damage after thawing; THW(MTCH) = percentage of sperm with deteriorated mitochondrial activity after thawing; THW(PM) = percentage of sperm with plasma membrane damage after thawing

sperm at 5 °C for longer than 12 h in the presence of glycerol may have a damaging effect. Shorter equilibration (< 6 h) at 5 °C was reported to be safer for sperm cells because of good post-thaw motility and/or non-return rates observed (Purdy 2006). In our study, equilibration for the duration of 2–4 h was chosen. Such equilibration procedure was reported as a usual procedure for ram sperm (Lv et al. 2019). Nevertheless, there exists a recommendation to add glycerol immediately just before the sperm freezing (Jovicic et al. 2020). This recommendation might be well-reasoned because of the known glycerol toxicity, as Domingo et al. (2019) reviewed. To this matter, using the glycerol-free ANDROMED commercial semen extender for semen collection, processing and equilibration, and adding glycerol immediately just before freezing might be a good practice to follow.

In addition, the clear seasonality of reproduction can be observed in sheep breeds in the moderate latitudes. In the study of Ptacek et al. (2019c), ram semen was collected in August (before the beginning of the mating season), in October (during the mating season), and in December (after the mating season). Sperm samples collected in October had demonstrably better motility parameters after thawing. Importantly, D'Alessandro and Martemucci (2003) previously reported that ram insemination doses prepared during the mating season, and especially in autumn (September–November), showed better freezing properties. For the purpose of the present study, sperm samples were collected from January to May. This can be a reason for the observed overall decrease in the sperm quality.

## Inter-sire and daily variation impacted the viability of thawed Wallachian ram sperm

Significant impact of inter-sire (ram's individuality) effect on the viability of thawed Wallachian ram sperm was observed in the present study ($P < 0.001$, Table 2). Sperm viability in ram 1 was 38.3% higher (assuming ram 2 = 100%; $P < 0.005$, Table 2). Furthermore, the damage to the plasma membrane, acrosome damage, and the percentage of thawed sperm cells with deteriorated mitochondria in ram 1 were 5.77%, 23.57%, and 4.4% lower, respectively (assuming ram 2 = 100%; $P < 0.05$, Table 2). Inter-sire effect in the present study was observed in healthy mature animals of the same breed, in the same breeding conditions, with the semen collected and processed in the same manner. These results are supported by the previously reported inter-sire effect in livestock (Dolezalova et al. 2016; Jovicic et al. 2020) and in rams (D'Alessandro and Martemucci 2003; Ramon et al. 2013). To this matter, the general recommendation can be to select the Wallachian rams with the best sperm viability after thawing for the preparation of insemination doses as one of the criteria in the cryopreservation program.

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Table 2. The impact of freezing curve, intersire, and daily variation on the viability of thawed Wallachian ram sperm (least square means ± standard error)

| | THW | THW(PM) | THW(ACR) | THW(MTCH) |
|---|---|---|---|---|
| **Curve** | *** | *** | *** | *** |
| 1 ($n$ = 56) | 8.91 ± 0.36$^{a}$ | 87.4 ± 0.39$^{a}$ | 70.0 ± 0.56$^{c}$ | 78.2 ± 0.48$^{a}$ |
| 2 ($n$ = 56) | 12.7 ± 0.57$^{b}$ | 82.9 ± 0.62$^{b}$ | 52.8 ± 0.87$^{a}$ | 73.5 ± 0.76$^{b}$ |
| 3 ($n$ = 136) | 12.6 ± 0.80$^{b}$ | 83.2 ± 0.88$^{b}$ | 58.5 ± 1.24$^{b}$ | 73.3 ± 1.07$^{b}$ |
| 4 ($n$ = 28) | 13.2 ± 0.57$^{b}$ | 82.5 ± 0.62$^{b}$ | 57.8 ± 0.87$^{b}$ | 74.0 ± 0.76$^{b}$ |
| 5 ($n$ = 43) | 12.9 ± 0.65$^{b}$ | 81.7 ± 0.71$^{b}$ | 61.4 ± 1.00$^{b}$ | 75.9 ± 0.87$^{ab}$ |
| **Sire** | *** | *** | *** | *** |
| 1 ($n$ = 232) | 14.0 ± 0.33$^{a}$ | 81.1 ± 0.36$^{a}$ | 47.0 ± 0.59$^{a}$ | 73.3 ± 0.44$^{a}$ |
| 2 ($n$ = 87) | 10.1 ± 0.53$^{b}$ | 86.0 ± 0.58$^{b}$ | 61.5 ± 0.96$^{b}$ | 76.6 ± 0.71$^{b}$ |
| **Day** | *** | *** | *** | *** |
| b*EQ | n.s. | – | – | – |
| b*EQ(PM) | – | n.s. | – | – |
| b*EQ(ACR) | – | – | n.s. | – |
| b*EQ(MTCH) | – | – | – | *** |

b*EQ = initial percentage of viable sperm after equilibration as covariate; b*EQ(ACR) = initial percentage of sperm with acrosome damage after equilibration as covariate; b*EQ(MTCH) = initial percentage of sperm with deteriorated mitochondrial activity after equilibration as covariate; b*EQ(PM) = initial percentage of sperm with plasma membrane damage after equilibration as covariate; Curve = fixed effect of the freezing curve (1 = Curve 1; 2 = Curve 2; 3 = Curve 3; 4 = Curve 4; 5 = Curve 5); Day = fixed effect of the control day of semen collection; Sire = fixed effect of ram; THW = percentage of viable sperm after thawing; THW(ACR) = percentage of sperm with acrosome damage after thawing; THW(MTCH) = percentage of sperm with deteriorated mitochondrial activity after thawing; THW(PM) = percentage of sperm with plasma membrane damage after thawing

***Significance of particular effects in the model equation at $P < 0.001$; $^{a-c}$different superscripts within columns indicate that the means differ at $P < 0.05$ level of significance

Significant impact of daily variation on the viability of thawed Wallachian ram sperm was observed in the present study ($P < 0.001$, Table 2). Obtained results of thawed sperm viability vary in magnitude without any obvious tendency (coefficient of variation = 51.1; full data set and statistics for the impact of daily variation on thawed sperm viability and cell damage are available from the corresponding author on request). Thus, we were not able to cover this variation by means of optimization. Previously, David et al. (2007) reported on daily variation in sperm motility in Manech tête rousse and Lacaune rams. In their study, the daily variation was observed in ejaculates immediately after natural ejaculation in an artificial vagina. The authors hypothesized that the daily variation effect could be a result of human behaviour during semen collection. Daily variation in sperm viability observed immediately after ejaculation could theoretically be a reason for the daily variation observed after thawing. However, the observed daily variation in viability after thawing was not correlated with the sperm viability before freezing (Table 2). Therefore, further research is needed to identify the reason for the daily variation observed in Wallachian rams in the present study.

## Optimizing the conventional freezing method for Wallachian ram sperm cryopreservation

To freeze sperm collected from Wallachian rams, the conventional freezing method (Curve 1 in the present study) was used when the straws were frozen by static exposure to liquid nitrogen vapour for 10 min 4 cm above the $LN_2$ surface. However, the obtained results suggest the methodological weakness of this conventional freezing method for Wallachian sperm in terms of viability and cell damage after freezing. To this matter, an effort was made to optimize this conventional method

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with the use of modified freezing. Under modified freezing, the straws were frozen by a discontinuous, time-dependent decremental change in the distance between the cryostraws and the surface of $LN_2$, and thus, under a precisely regulated freezing curve (Curve 2, 3, 4, 5 in the present study). All the modified freezing methods were significantly ($P < 0.05$) superior to the conventional freezing method, as evaluated by flow cytometry (Table 2). The percentage of thawed sperm viability increased up to 48.3%, percentage of sperm with plasma membrane damage after thawing decreased to 6.58%, percentage of sperm with acrosome damage decreased to 24.4%, and percentage of sperm with deteriorated mitochondrial activity decreased to 6.28% (assuming the conventional method = 100%). Importantly, in terms of viability, plasma damage, and deteriorated mitochondrial activity after freezing, there was no significant difference between the freezing modifications used (Table 2). However, the lowest acrosomal damage was observed in thawed sperm frozen with the use of Curve 2. In general, we hypothesized that the superiority of the modified freezing is due to a moderate temperature drop at the initial phase of freezing. Moderate temperature drop through the temperature range of +8 °C to –10 °C was clearly demonstrated for Curve 2, 3, 4 and 5 (Figure 1). Importantly, in our experimental setup, Curve 2 demonstrates the fastest (approx. 15 °C/min) transition through the critical temperature range of –10 °C to –25 °C, which is the most dangerous for ram sperm (Salamon and Maxwell 2000). Our results are in accordance with Galarza et al. (2019), who demonstrated that the lowest ram sperm damage was associated with the fastest (60 °C/min) transition through the critical temperature range of –10 °C to –25 °C. Based on the above mentioned, sperm freezing with the use of Curve 2 should be recommended to cryopreserve Wallachian ram sperm.

## CONCLUSION

The flow cytometry results suggest methodological weakness of the conventional freezing method via the static exposure of sperm doses to $LN_2$ vapour to freeze Wallachian ram sperm. In general, any of the modified freezing methods established in the present study should be used alternatively to the conventional method to provide cryopreserved Wallachian ram sperm of superior quality. However, sperm freezing with the use of Curve 2 is recommended to obtain the best post-thaw results in terms of the lowest acrosomal damage.

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## Conflict of interest

The authors declare no conflict of interest.

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