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Equilibration and freezing interactions affecting bull sperm characteristics after thawing

M. Doležalová, L. Stádník, Z. Biniová, J. Ducháček, R. Stupka

Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Animal Husbandry, Prague, Czech Republic

ABSTRACT: The objective was to determine effects of equilibration length and freezing curve type as well as their interactions on motility and live spermatozoa proportion in bull sperm after thawing. The ejaculates of 6 sires were repeatedly collected. Fresh semen was diluted with one extender and divided into 3 groups equilibrated for 30, 120, and 240 min. Subsequently, half straws of each group were frozen using standard 3-phase or 2-phase freezing curve differing in the rate of temperature decrease. The spermatozoa motility (M) was evaluated immediately after thawing and at 30, 60, 90, and 120 min of thermodynamic test (TDT). Live spermatozoa proportion was evaluated after thawing and at the end of TDT. Average of spermatozoa motility (AM), decrease of spermatozoa motility (MD), average proportion of live spermatozoa (ALS), and decrease of live spermatozoa proportion (DLS) through the TDT were calculated. Significant inter-sire differences in AM (0.45–17.0%; $P < 0.05$–0.01), MD (0.76–12.57%; $P < 0.05$–0.01), and ALS (0.99–23.8%; $P < 0.01$) were detected. The longest equilibration ensured the highest M during TDT and AM (+2.72 and +4.58%; $P < 0.05$–0.01), however higher MD (+4.06%; $P < 0.01$) compared to standard length as well. Straws freezed using 2-phase curve achieved higher M through TDT, AM (+7.3%; $P < 0.01$) as well as ALS (+11.77%; $P < 0.01$). The 2-phase curve presented higher M compared to the 3-phase freezing curve within all equilibration lengths. Significant differences in AM, MD, and ALS (0.45–6.78%, 0.62–5.35%, and 20.79–21.11%; $P < 0.05$–0.01) between equilibration length vs freezing curve interactions were determined. Results document the importance of equilibration length, freezing curve, and their interaction effect on live spermatozoa proportion and sperm motility after thawing as well as necessity of individual conditions for bulls semen processing and insemination doses production.

Keywords: sire; spermatozoa; cryopreservation; insemination dose; fertilization capability

INTRODUCTION

Growing interest in improving the quality of frozen bull semen for the needs of the market in recent years has been determined by the declined fertility of dairy cows (Beran et al. 2013a, Stadnik et al. 2015a) and world-wide practical usage of artificial insemination (Shahverdi et al. 2014). Therefore, it is necessary to ensure optimal genetic sires selection (Zhang et al. 2015) as well as semen processing including semen collection (Dzyuba et al. 2015), dilution (Meamar et al. 2016), slow cooling to 4–6°C (Kristan et al. 2014), and equilibration (Beran et al. 2013b) as well as cryopreservation maintaining spermatozoa fertilization capability (Andrabi 2007) evaluated objectively if possible (Simonik et al. 2015).

Equilibration as the total period of spermatozoa contact with a cryoprotectant prior to freezing helps keep spermatozoa membrane integrity as well

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as their survival (Leite et al. 2010). Extenders used serve as protection during cooling and freezing (Linhartova et al. 2014) and preserve spermatozoa motility after subsequent thawing (Muchlisin et al. 2015). Nevertheless, the most suitable interactions of extender vs equilibration length still are not determined sufficiently (Shahverdi et al. 2014). Leite et al. (2010) recorded only limited interactions between the extender used and the equilibration length. Dhami et al. (1992) recommended 30 min to 24 h equilibration prior to freezing, while Muino et al. (2007) determined optimal length of equilibration from 4 to 18 h. Selected studies indicate that 18 h or overnight equilibrations prior to freezing are the most appropriate equilibration lengths in order to increase quality of the insemination doses produced (Shahverdi et al. 2014). However, from the doses production point of view, effort to shorten equilibration or to eliminate it completely and thereby to accelerate sperm processing and cryopreservation is definitely desirable. On the other hand, efficiency of doses production contradicts the fact that equilibration is essential for the spermatozoa characteristics after subsequent thawing (Gao et al. 1997).

The success of cryopreservation depends on the course of cooling and freezing (Clulow et al. 2008). Interaction of temperature vs extender used affects spermatozoa cold shock resistance (Stadnik et al. 2015b). The extent of spermatozoa damage caused by cold shock depends on temperature, however on the rate of temperature decrease as well. Generally, the higher the cooling rate, the more serious the sperm damage (Lemma 2011) determining the subsequent post-thaw spermatozoa motility (Andrabi 2007). Thus the result of sperm cryopreservation is also affected by the type and course of freezing curve (Dolezalova et al. 2015).

Summarily, the length of equilibration as well as the freezing curve type are responsible for many important physicochemical changes leading to different degrees of spermatozoa structure damage (Forero-Gonzalez et al. 2012) deteriorating spermatozoa characteristics after thawing (Spalekova et al. 2014). Despite this fact, interactions mentioned have not been sufficiently known yet. Therefore, the objective of study was to evaluate the effect of different equilibration lengths and freezing curve type interactions on motility and proportion of live spermatozoa in bull sperm after insemination doses thawing.

MATERIAL AND METHODS

Bulls and semen collecting. The ejaculates of pre-selected bulls (n = 6) ordinarily used for commercial purposes were repeatedly collected during April and September. Sires of the same age, breed, and frequency of sperm collecting were stabled in a private Sire insemination centre under the same breeding conditions as handling, stabling, feeding system, and feeding ratio composition. Samples of ejaculate were obtained using an artificial vagina and immediately evaluated in the lab of the Sire insemination centre according to methodology applied. Volume of fresh semen (VOL, g), density of spermatozoa (DEN, $\times 10^6$ mm^{-3}), and percentage rate of progressive motile spermatozoa above head (MOT, %) were evaluated by only one trained technician. Only ejaculates corresponding to the initial parameters required (minimum DEN 0.7 $\times$ 10^6 mm^{-3} and MOT 70%) were subsequently processed. Average quality of fresh semen collected is presented in Table 2. The average values of VOL, DEN, and MOT achieved in all sires observed were 6.15 g, 0.93 $\times$ 10^6 mm^{-3}, and 74.17%, respectively.

Semen dilution and processing. Fresh semen was ordinarily diluted with phospholipid extender Andromed® (Minitübe GmbH, Tiefenbach, Germany) to a spermatozoa concentration 10 $\times$ 10^6 per one insemination dose immediately after lab evaluation of fresh sperm quality. Diluted ejaculate was mixed up at room temperature (25°C) for 10 min and automatically loaded into 0.25 ml differently coloured straws using a computer-controlled filler (IMV Technologies, L'Aigle, France). After filling, straws were divided into 3 groups (n = 20 insemination doses per sire at least), immediately placed into a cooling box, cooled at an average speed of 0.2°C per min to 4–5°C, and equilibrated (Camara et al. 2011). According to pre-defined methodologies, equilibration lengths of 30 min (Leite et al. 2010), 120 min (Shahverdi et al. 2014), and 240 min (Januskauskas et al. 1999) were applied and tested. Subsequently, equilibrated straws were divided into 2 groups (n = 10 insemination doses per sire per equilibration length at least) and freezed using 2 freezing curves providing the highest spermatozoa motility after thawing (Dolezalova et al. 2015). Freezing was performed using the controlled freezing methodology Direct Freezing in a freezer box Digitcool® (IMV Cryo Bio System, L'Aigle, France). Figure 1 documents

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the course of the freezing curves. The standard, commercially recommended 3-phase curve (according to Muino et al. 2007) and the 2-phase freezing curve described by Januskauskas et al. (1999) and Gil et al. (2000) were applied.

Evaluation of semen characteristics. The straws cryopreserved were subsequently thawed in a water bath at 38 ± 1°C for 30 s according to methodology recommended by Rubio-Guillen et al. (2007). The percentage rate of progressive motile spermatozoa above head (MOT) was evaluated and recorded using CASA system (SCA® Production v. 5.3.; MICROPTIC S.L., Barcelona, Spain) with a phase contrast microscope Eclipse E200 (Nikon, Tokyo, Japan) at 200–300× magnification when five fields of view per each straw were evaluated at least (Tuncer et al. 2011). MOT was determined immediately after thawing (M0) as well as during the subsequent thermodynamic test (TDT) of spermatozoa survivability after 30, 60, 90, and 120 min (M30, M60, M90, and M120) of the test duration in a dry heater Thermoblock (Falc Instruments, Treviglio, Italy) at a temperature of 38 ± 1°C.

The proportion of live sperm was also evaluated immediately after straw thawing (L0) and at the end of TDT (L120). Sample volume of 20 µl was gently mixed on preheated hour glass (37 ± 1°C) with 20 µl of eosin by circular motion for 30 s and then 40 µl of nigrosine were added. A volume of 20 µl of the resulting suspension was pipetted on preheated glass slide and smear was done. The rest of the thawed semen was stored in a water bath at 38 ± 1°C for 120 min. Subsequently, the second smear was prepared according to the above mentioned procedure. A phase contrast microscope with oil immersion (Eclipse E200, Nikon) at 1000× magnification was used by only one evaluator to examine all smears acquired. Minimally 100 spermatozoa were classified as either dead (with red heads) or live (with white heads), and expressed as a percentage rate of live sperm (Beran et al. 2012).

Data handling and statistical analysis. The data were analyzed by SAS software (Statistical Analysis System, Version 9.3., 2011) using the UNIVARIATE and GLM procedures. The procedures MEANS and UNIVARIATE were used to calculate basic statistics. The REG procedure (STEPWISE option) was used to develop a final model. Average of spermatozoa motility (AM) and decrease of spermatozoa motility (DM) through the entire TDT were calculated according to MOT values during individual phases of TDT. Average proportion of live spermatozoa (ALS) and decrease of live spermatozoa (LSD) through the entire TDT was determined corresponding to L0 and L120 values as well. Based on Akaike information criterion, the values of M0, M30, M60, M90, and M120, DM as well as ALS and LSD were evaluated using a model corrected for the fixed effects of bull, length of equilibration, type of freezing curve, and interaction of the equilibration length vs the type of freezing curve. For evaluation of average sperm motility the fixed effects of thermodynamic test duration as correction of MOT values in individual phases of TDT were added into the model. Detail comparison was performed by Tukey-Kramer test. The model equation for spermatozoa motility, proportion of live spermatozoa, and decrease of both these parameters during TDT was as follows:

$$Y_{ijkl} = \mu + BULL_i + EQI_j + CUR_k + EQICUR_{jk} + e_{ijkl}$$

where:

Y_{ijkl} = dependent variable (spermatozoa motility at the beginning and after 30, 60, 90, and 120 min of the TDT; average spermatozoa motility, average proportion of live spermatozoa, and decrease of parameters during TDT)

μ = mean value of dependent variable

$BULL_i$ = fixed effect of i^{th} bull individuality (i = 1, n = 36; i = 2, n = 36; i = 3, n = 36; i = 4, n = 36; i = 5, n = 36; i = 6, n = 36)

EQI_j = fixed effect of j^{th} length of equilibration (j = 30 min, n = 72; j = 120 min, n = 72; j = 240 min, n = 72)

CUR_k = fixed effect of k^{th} type of freezing curve (k = 1, n = 108; k = 2, n = 108)

$EQICUR_{jk}$ = interaction between length of equilibration and freezing curve (jk = always 6 groups)

e_{ijkl} = random error

Within the evaluation of average spermatozoa motility through the entire TDT, the model applied was supplemented with fixed effect:

DUR_l = fixed effect of l^{th} thermodynamic test duration (l = 0 min, n = 216; l = 30 min, n = 216; l = 60 min, n = 216; l = 90 min, n = 216; l = 120 min, n = 216)

Significance levels $P < 0.05$, $P < 0.01$, and $P < 0.001$ were used to evaluate the differences between groups.

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Table 1. Reliability of the model

Index	Model		FC		Sire		E		Time		FC * E	
	r^2	*P*	*F*-test	*P*	*F*-test	*P*	*F*-test	*P*	*F*-test	*P*	*F*-test	*P*
ALS	0.37	< 0.001	14.07	< 0.001	7.5	< 0.001	2.84	0.06	63.69	< 0.001	3.35	0.048
AM	0.65	< 0.001	10.16	0.002	84.95	< 0.001	17.67	< 0.001	65.96	< 0.001	4.18	0.016

FC = freezing curve, E = length of equilibration, ALS = average proportion of live spermatozoa through the entire thermodynamic test, AM = average spermatozoa motility

RESULTS

In Table 1 the model repeatability ranged from $r^2 = 0.37$ to 0.65 during the evaluation of observed traits when statistical significance of all models used was $P < 0.001$ in all evaluations. Effect of sire was significant ($P < 0.01$ to 0.001) in relation to all evaluated traits. Significance of the freezing curve effect differed ($P < 0.05$ to 0.001) according to individual dependent characteristics. The effect of equilibration length represented significance level $P < 0.05$ at average spermatozoa motility. The effect of equilibration length vs freezing curve interaction represented significance level $P < 0.05$.

Table 2 documents the initial parameters of fresh semen. The volume ranged from 3.0 to 9.4 g, density ranged from 0.8 to 1.5 × 10^6 mm^{-3}, and initial motility of processed semen was 70–80%.

Figure 2 documents different course of spermatozoa motility in individual TDT phases corresponding to sires observed. The highest value of spermatozoa motility for all bulls was evaluated immediately after thawing (33.89–55.83%). During the entire TDT motility declined gradually and finally reached values of 21.94–40.83%. However, sire individuality describing higher motility after thawing does not determine the higher motility during the entire TDT.

Table 3 shows the highest average spermatozoa motility through the entire TDT in sire V (46.33%) significantly ($P < 0.05$–0.01) differing in comparison with all other sires (–3.33% to –17.00%). On the contrary, sire II achieved the lowest value (29.33%) significantly ($P < 0.01$) differing from levels detected in sires I, V, and VI (+12.5% to +17.00%). Spermatozoa motility decrease through the TDT differed significantly ($P < 0.05$–0.01) among sires as well. The highest value of average proportion of live spermatozoa through the TDT (37.18%; $P < 0.01$) was determined in sire V, the sire with the highest average spermatozoa motility. On the other hand, the lowest proportion (13.38%) was detected in sire III. Table 3 documents other significant inter-sire differences as well. Although different decreases of live spermatozoa proportion were determined, no differences were statistically significant.

Evaluating the effect of equilibration length demonstrated in Figure 3, the longest equilibration (240 min) ensured the highest values of spermatozoa motility in all TDT phases. This fact corresponds to the highest average spermatozoa

Table 2. Average values of fresh ejaculate parameters per sire

Sire	VOL (g)	DEN (× 10^6 mm^{-3})	MOT (%)
I	6.9	0.8	80
II	9.4	1.1	80
III	6.4	1.5	70
IV	6.3	0.8	70
V	3.0	0.7	75
VI	4.9	0.7	70
Average	6.15	0.93	74.17

VOL = volume of fresh semen, DEN = density of spermatozoa, MOT = percentage rate of progressive motile spermatozoa above head

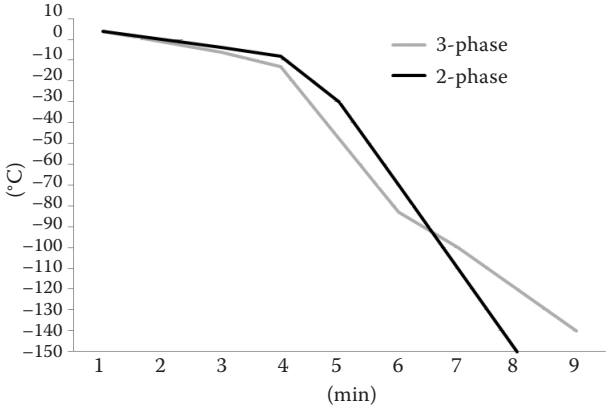

Figure 1. The course of 3-phase and 2-phase freezing curves applied

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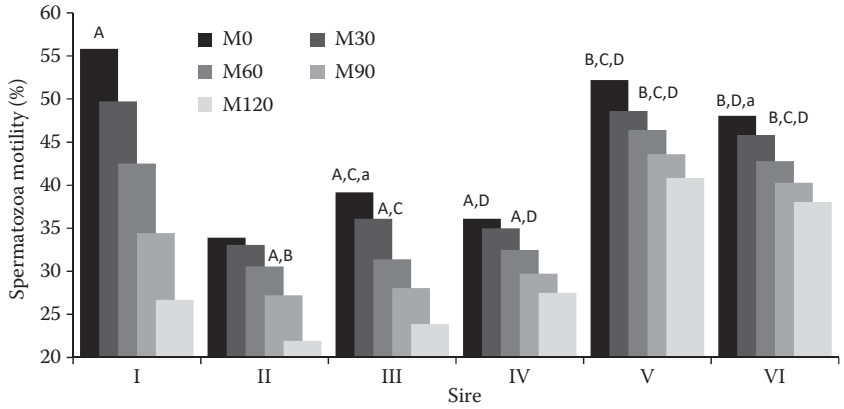

Figure 2. Effect of sire individuality on the percentage rate of progressive motile spermatozoa above head in individual phases of thermodynamic test M0, M30, M60, M90, M120 = percentage rate of progressive motile spermatozoa above head evaluated immediately after thawing, at 30, 60, 90, and 120 min of thermodynamic test, respectively

motility through the entire TDT (39.83%) significantly ($P < 0.05–0.01$) differing compared to equilibration of 30 min (–4.58%) and 120 min (–2.72%). The highest decline of spermatozoa motility during the entire TDT was recorded at 30 min equilibration (10.45%), while the smallest decline of motility was reached at 120 min equilibration (5.52%). Differences were significant ($P < 0.01$), and are presented in Table 3.

Figure 3 shows that the highest values of spermatozoa motility during TDT (0, 30, 60, 90, and 120 min) were reached at 240 min equilibration (47.5, 43.89, 40, 35.69, and 32.8%) in comparison to 120 min equilibration (–5.97, –3.33, –2.36, –1.38, –0.55%) and 30 min equilibration (–3.89, –4.17, –4.58, –4.02, –6.25%). On the other hand, 30 min equilibration presented higher spermatozoa motility after thawing compared to 120 min

Table 3. Effect of bull, equilibration length, freezing curve, and equilibration length vs freezing curve interaction on the decrease and average of sperm motility (%) and proportion of live spermatozoa (%) through the thermodynamic test

		Motility		Live spermatozoa	
		MD	AM	DLS	ALS
Sire	I	17.50 ± 0.938^{A}	41.83 ± 0.776^{A}	33.02 ± 7.332	33.46 ± 3.838^{A}
	II	5.69 ± 0.938^{A}	29.33 ± 0.776^{A,B}	11.37 ± 7.332	14.37 ± 3.838^{A,B}
	III	9.31 ± 0.938^{A,a}	31.72 ± 3.839^{A,C}	19.39 ± 7.332	13.38 ± 3.838^{A,C}
	IV	4.93 ± 0.938^{A,a}	32.17 ± 0.776^{A,D}	27.97 ± 7332	34.84 ± 3.838^{B,C}
	V	7.36 ± 0.938^{A}	46.33 ± 0.776^{A,B,C,D,a}	22.68 ± 7.332	37.18 ± 3.838^{B,C}
	VI	6.32 ± 0.938^{A}	43.00 ± 0.776^{B,C,D,a}	35.68 ± .7332	25.14 ± 3.838
Equilibration (min)	30	10.45 ± 0.663^{A}	35.25 ± 0.548^{A,a}	22.44 ± 5.184	22.90 ± 2.715
	120	5.52 ± 0.663^{A,B}	37.11 ± 0.548^{B,a}	29.68 ± 5.184	31.58 ± 2.715
	240	9.58 ± 0.663^{B}	39.83 ± 0.548^{A,B}	22.93 ± 5.184	24.71 ± 2.715
Freezing curve	3-phase	8.80 ± 0.542	36.39 ± 0.448^{A}	21.37 ± 4.233	20.51 ± 2.217^{A}
	2-phase	8.24 ± 0.542	38.41 ± 0.448^{A}	28.67 ± 4.233	32.28 ± 2.217^{A}
Equilibration length vs freezing curve interaction	30 vs 3	11.18 ± 1.133^{A,a}	33.28 ± 1.018^{A}	20.62 ± 7.306	19.18 ± 4.389^{a}
	120 vs 3	5.21 ± 1.133^{A,b}	35.83 ± 1.018^{a}	19.31 ± 7.306	22.87 ± 4.389
	240 vs 3	10.00 ± 1.133^{b}	40.06 ± 1.018^{A,a}	24.17 ± 7.306	19.50 ± 4.389^{b}
	30 vs 2	9.72 ± 1.133	37.22 ± 1.018	24.27 ± 7.306	26.62 ± 4.389
	120 vs 2	5.83 ± 1.133^{a}	38.39 ± 1.018^{A}	40.04 ± 7.306	40.29 ± 4.389^{a,b}
	240 vs 2	9.17 ± 1.133	39.61 ± 1.018^{A}	21.69 ± 7.306	29.91 ± 4.389

MD = spermatozoa motility decrease, AM = average spermatozoa motility, DLS = decrease of live spermatozoa proportion, ALS = average proportion of live spermatozoa through the entire thermodynamic test

the same superscript letters confirm statistical significance of differences $^{A–D}(P < 0.01)$, $^{a,b}(P < 0.05)$

values are given as Least Squares Means + standard error

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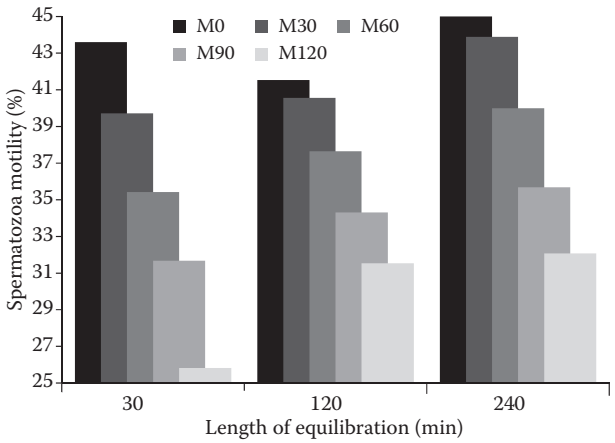

Figure 3. Effect of equilibration length on the percentage rate of progressive motile spermatozoa above head in individual phases of thermodynamic test

M0, M30, M60, M90, M120 = percentage rate of progressive motile spermatozoa above head evaluated immediately after thawing, at 30, 60, 90, and 120 min of thermodynamic test, respectively

equilibration. However, this motility significantly decreased after 30, 60, 90, and 120 min of TDT. Therefore, using 30 min equilibration, the highest decrease of spermatozoa motility significantly ($P < 0.01$) differing compared to 120 min equilibration was determined. On the other hand, Table 3 documented that the highest average proportion of live sperm was detected in straws equilibrated for 120 min (31.58%), although differences (from –6.87 to –8.68%) from other equilibrations were

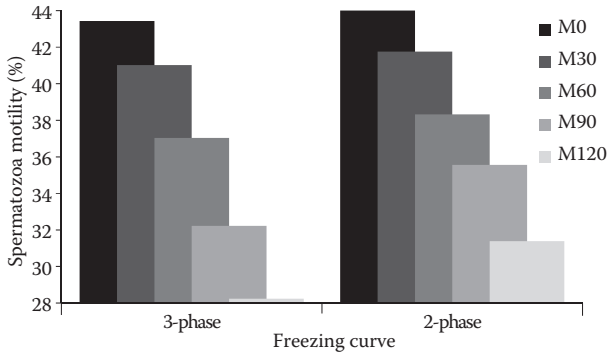

Figure 4. Effect of freezing curve on the percentage rate of progressive motile spermatozoa above head in individual phases of thermodynamic test

M0, M30, M60, M90, M120 = percentage rate of progressive motile spermatozoa above head evaluated immediately after thawing, at 30, 60, 90, and 120 min of thermodynamic test, respectively

insignificant, probably due to the non-significantly highest decrease of live spermatozoa (29.68%) during the TDT. Our results describe the 30 min equilibration as the least appropriate from this point of view.

The direct effect of freezing curve type is demonstrated in Figure 4. The 2-phase freezing curve was less threatening to sperm as documented by spermatozoa motility level in all individual phases of TDT after thawing (45, 41.76, 38.33, 35.56, 31.39%) in comparison to 3-phase freezing curve (–1.57, –0.76, –1.29, –3.34, –3.15%). This fact is confirmed by higher average spermatozoa motility (+2.02%; $P < 0.01$) as well as higher average proportion of live sperm (+11.77; $P < 0.01$) through the entire test as shown in Table 3. However, differences in the decrease of spermatozoa motility and live spermatozoa proportion were insignificant.

Based on the evaluation of individual effects and interactions described in Table 3, the most appropriate is 240 min equilibration combined with 2-phase freezing curve. Compared to 120 and 240 min equilibration, 30 min equilibration using both 3-phase (–2.55% and –6.78%) and 2-phase (–1.17% and –2.39%) curves presented the lowest values of motility. In evaluating the average proportion of live spermatozoa, 30 min equilibration using both 3-phase (–3.69% and –0.32%) and 2-phase (–13.67% and –3.29%) curves presented the lowest values compared to 120 and 240 min equilibration. The highest proportion of live spermatozoa in comparison to the other variants was reached using 120 min equilibration and 2-phase freezing curve (+21.11, +17.42, +20.79, +13.67, +10.38%). Significant differences ($P < 0.05$) between this variant and variants using 3-phase freezing curve and equilibration length 30 and 240 min were found as well. The highest decrease of live spermatozoa proportion during TDT using 120 min equilibration and 2-phase freezing curve was found (40.04%), conversely, the lowest decline (19.31%) was reached using 120 min equilibration and 3-phase freezing.

Figure 5 shows the course of motility in individual phases of TDT in relation to freezing curve and equilibration length interactions. The 2-phase curve presented higher motility values compared to 3-phase curve within all lengths of equilibration, except motility immediately after thawing at 240 min equilibration (–1.12%). The longer the equilibration, the higher the motility levels

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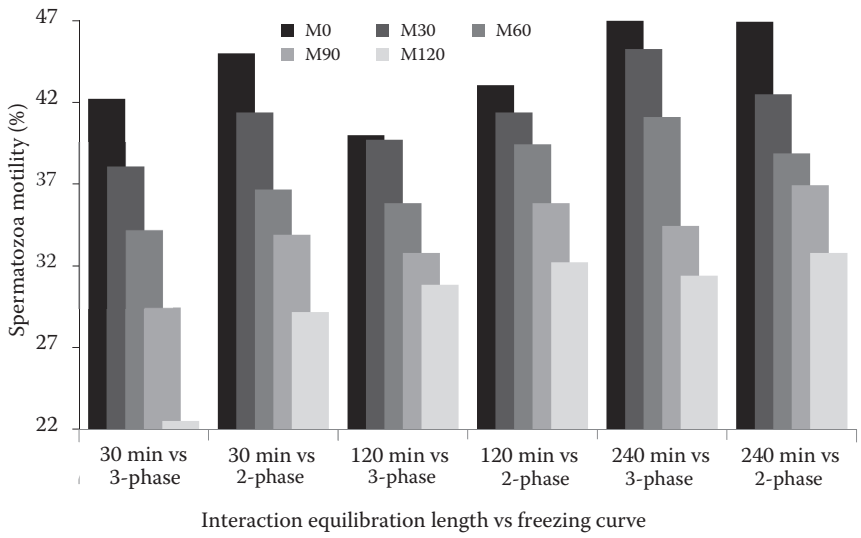

Figure 5. Effect of equilibration length and freezing curve interaction on the percentage rate of progressive motile spermatozoa above head in individual phases of thermodynamic test
M0, M30, M60, M90, M120 = percentage rate of progressive motile spermatozoa above head evaluated immediately after thawing, at 30, 60, 90, and 120 min of thermodynamic test, respectively

determined. However, 30 min equilibration vs 2-phase curve interaction achieved higher motility levels compared to standard interaction of 120 min equilibration vs 3-phase curve. These facts are supported by average spermatozoa motility presented in Table 3. Applying the 2-phase curve ensured higher motility irrespective of equilibration length, compared to standard combination of 120 min equilibration and 3-phase curve. However, the highest average spermatozoa motility (40.06%) was determined within the interaction of 240 min equilibration vs 3-phase curve compared to 30 min (–6.78%) and 120 min (–4.23%) equilibration. Similar motility results were detected within the 2-phase curve. No trend was described in decrease of spermatozoa motility during the TDT. On the other hand, average proportion of live spermatozoa through the entire TDT was significantly ($P < 0.05$) the highest under 120 min equilibration vs 2-phase curve, especially compared to the lowest values of 30 min vs 3-phase and 240 min vs 3-phase interactions. A similar trend in the decrease of live spermatozoa proportion was not statistically significant.

DISCUSSION

The present study showed significant interactions between equilibration times and freezing curves affecting sperm motility and live spermatozoa proportions. Simultaneously inter-sire individuality was confirmed. Evaluation of semen quality is based on several parameters related to concentration, morphology, and motility (Defoin et al. 2008).

Initial parameters of observed fresh semen agreed with those of Ball and Peters (2004).

Sperm quality is influenced by many factors, e.g. internal factors like breed, variation between individuals, and age of sire (Beran et al. 2011). The values of spermatozoa motility during TDT declined; this finding is in accordance with the results of previous studies (Defoin et al. 2008; Beran et al. 2011). The results reported by Stadnik et al. (2002) and Beran et al. (2013a) indicated individual differences in resistance of sperm to freezing as well as to environmental conditions after thawing, theoretically including the conditions in cow vagina and uterus after insemination. From this point of view, results of TDT should be very interesting and important. Also according to Dolezalova et al. (2015), sperm frost resistance largely differs among bulls. Differences in average proportion of live spermatozoa as well as in its decrease during the TDT confirm the effect of sire individuality on final fertilization capability of sperm and correspond to Thurston et al. (2002) finding spermatozoa resistance towards cryopreservation can correlate also with genetic factors explaining inter-species, breed, as well as individual differences. The results of Beran et al. (2011) documented these relationships according to significant decline of spermatozoa motility after thawing. Evaluating interbreed differences, spermatozoa motility after collection and dilution was higher in Holstein bulls but spermatozoa motility after freezing/thawing was higher in Czech Fleckvieh bulls.

Our findings are in agreement with Shahverdi et al. (2014) who stated the length of contact be-

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tween sperm and cryoprotectant is essential for maintaining motility and integrity of sperm membranes and directly affects its post-thawed characteristics. With respect to average spermatozoa motility, Leite et al. (2010) determined 240 min as the most appropriate length of equilibration period in comparison to non-equilibration and 120 min equilibration, in accord with our study. The non-equilibration gave the lowest values of total and progressive sperm motility, and higher percentage proportion of sperm with intact plasma and acrosomal membranes, with no significant differences between different extenders used. In a similar study focusing on the cryopreservation of bull semen, Dhami et al. (1992) reported that 2 h of equilibration at 5°C compared with 0 h improved the post-thaw recovery, incubation survival, and fertility rates of buffalo frozen semen. Herold et al. (2006) found that 120–540 min equilibration was suitable for spermatozoa freezing, however that specific length did not affect straw characteristics after thawing. We can agree with this statement in accordance with spermatozoa motility at the end of TDT in straws equilibrated for 120 and 240 min. Equilibration length exceeding 2 h resulted in the greatest preservation of total and progressive motility, as well as the integrity of plasma and acrosomal membranes during cryopreservation. The use of 4 h equilibration period can be safe with soybean lecithin extender for cryopreservation of buffalo spermatozoa (Shahverdi et al. 2014). Shahverdi et al. (2014) tested equilibration period of 2, 4, 8, and 16 h and two extenders (tris or Bioxcell®) on cryopreservation of buffalo semen. The post-thaw sperm motility and the percentage of progressive motile spermatozoa after 2 h equilibration in both extenders were lower than for the other equilibration times. On the other hand, post-thaw sperm motility for equilibration times of 4, 8, and 16 h did not show significant differences ($P < 0.05$) in either extender (Shahverdi et al. 2014). Contrary to these results, in the cryopreservation of bull semen some studies have detected higher conception rates for semen frozen following 12–18 h of equilibration compared with 4–6 h (Foote and Kaprotht 2002). Although equilibration time significantly affected total and progressive motility in the present study, there was no significant effect on detailed characteristics of sperm movement (Shahverdi et al. 2014). According to Leite et al. (2010), optimum equilibration time for semen storage in straws ranged from 2 to 10 h and was dependent on cooling time, and slowly cooled semen seemed to require less equilibration for optimal final motility. Equilibration for 4 h resulted in the greatest preservation of total and progressive motility, as well as the integrity of plasma and acrosomal membranes during cryopreservation.

Freezing of AI doses, storage for long period, and thawing is a demanding process for sperm membranes and sperm viability (Frydrychova et al. 2010). The freezing and thawing process can adversely affect the nucleus, plasma, acrosome and mitochondrial membranes of spermatozoa and is associated with production of reactive oxygen species which can generally lead to increased permeability of membranes, release of soluble inter-membrane mitochondrial regulator proteins and apoptotic factors that might activate apoptogenic metabolic pathways (Mohan et al. 2014). Recent studies suggest that the sensitivity of sperm to cryopreservation might be partly related to genetic factors, which could explain variations between species, breeds, and individuals (Thurston et al. 2002). Indeed, one obvious characteristic of cryopreserved spermatozoa is the decline in motility (Watson 2000). Alterations in sperm motility and structure occur simultaneously at different stages of freezing and thawing. It has been reported that fast cooling induces lethal stress in some cells in the semen, and this stress is proportional to freezing curve and rate of temperature decline (Forero-Gonzalez et al. 2012). Our finding corresponds to that of Leite et al. (2010) who stated longer equilibration is absolutely necessary for maintaining spermatozoa motility during freezing. According to Watson (2000), the medium freezing rate presented in our observation by the 2-phase curve, supports optimal spermatozoa dehydration by the selected cryoprotective substance and minimizes negative effect on spermatozoa freezed, especially on ice crystal formation. Also Dolezalova et al. (2015) in previous study confirmed these results. However, Chen et al. (1993) obtained a successful protocol for freezing bull semen commercially cooling sperm by the rate of 15°C/min from +5 to –100°C, followed by transfer to liquid nitrogen. In general, semen cryopreservation protocols use freezing curves ranging from 10 to 100°C/min, resulting in good survival rates after thawing. Moreover, the freezing rate depends on the method of processing as well as of storage (Lemma 2011). The rate of temperature drop was found

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to be most critical over the specific temperature range of 0–5°C when motility was evaluated later. In general, the faster the rate of cooling, the more severe the damage. There is further evidence which suggests that the rate of temperature drop also determines the subsequent active life of spermatozoa (Andrabi 2007). The type of extender used and the speed of temperature drop are known to affect susceptibility of spermatozoa to cold shock and the success rate of freezing semen (Shahverdi et al. 2014). Interaction of equilibration length and freezing curve documented positive effect of prolonged equilibration combined with less demanding slower temperature decline of the 2-phase curve. Mentioned results have to be taken into account within the adjustment of AI doses processing.

CONCLUSION

Effects of the equilibration length and freezing curve type on motility and live spermatozoa proportion in bull sperm after thawing were observed and evaluated. Significant inter-sire differences in observed spermatozoa characteristics were confirmed. The longer the equilibration, the higher was the spermatozoa motility during the entire thermodynamic test. The 2-phase freezing curve ensured higher spermatozoa motility and higher proportion of live spermatozoa compared to the standard 3-phase curve. Within interactions of equilibration length vs freezing curve analyzed, similar trends were detected. The significant effect of the selected interactions documents the importance of appropriate length of equilibration and freezing curve for the proportion of live spermatozoa and their motility after thawing. Results emphasize the necessity to detect and subsequently apply the optimum combination of equilibration length and freezing curve for each individual sire in order to optimize efficiency of its ejaculate processing as well as insemination doses processing.

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Corresponding Author

doc. Ing. Luděk Stádník, Ph.D., Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Animal Husbandry, Kamýcká 129, 165 21 Prague 6-Suchdol, Czech Republic
Phone: +420 224 383 057, e-mail: stadnik@af.czu.cz