The effect of the freezing curve type on bull spermatozoa motility after thawing

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Received February 24, 2015
Accepted June 24, 2015

Abstract

The objective of this work was to determine the effect of selected freezing curves on spermatozoa survivability after thawing, defined by its motility. The ejaculates of nine selected sires of the same age, breed, and frequency of collecting, bred under the same breeding conditions including handling, stabling, feeding system and feeding ratio composition, were repeatedly collected and evaluated. Sperm samples of each sire were diluted using only one extender and divided into four parts. Selected four freezing curves – the standard, commercially recommended three-phase curve; a two-phase curve; a slow three-phase curve; and a fast three-phase curve, differing in the course of temperature vs time, were applied. The percentage rate of progressive motile spermatozoa above head was determined immediately after thawing, and after 30, 60, 90, and 120 min of the thermodynamic test (TDT). Moreover, average spermatozoa motility (AMOT) and spermatozoa motility decrease (MODE) throughout the entire TDT were evaluated. Insemination doses frozen using the simpler two-phase curve demonstrated the highest motility values (+2.97% to +10.37%; $P < 0.05$–0.01) immediately after thawing and during the entire TDT. Concurrently, the highest AMOT (+4.37% to +8.82%; $P < 0.01$) was determined. The highest spermatozoa motility values were detected after thawing doses frozen by the two-phase freezing curve in eight out of nine sires. Simultaneously, a significant effect of sire individuality was clearly confirmed. Inter-sire differences of spermatozoa motility during TDT as well as AMOT and MODE were significant ($P < 0.01$). The findings describing both factors of interaction indicate the necessity of individual cryopreservation of the ejaculate to increase its fertilization capability after thawing.

Reproduction, sire, ejaculate, cryopreservation, sperm survival, frost resistance

Evaluation of bull sperm quality during the entire course of processing as well as its subsequent fertilization ability are based on basic indicators such as spermatozoa concentration, morphology, and motility (Rodriguez-Martinez 2003), integrity of plasma membrane, acrosome, DNA, and traits of spermatozoa metabolism (Foote 2002). Spermatozoa are significantly affected by interactions of extracellular and intracellular fluid, sugars, proteins, salts of individual microelements and other substances whose concentration increases simultaneously with the osmotic pressure following ice crystal formation (Watson 2000). Cryopreservation is also associated with oxidative stress and increased formation of reactive oxidants reducing the fertilization ability of frozen spermatozoa (Wang et al. 1997; Piantadosi 2008). Cold shock caused by the action of low temperatures on the spermatozoa during the freezing process may damage mitochondria (Pena et al. 2009), plasma as well as the acrosomal membrane of spermatozoa (Meyers 2005), inducing changes in the lipid composition or in the integrity and permeability of plasma and membranes (Januskauskas et al. 2003). Furthermore, cold shock induces spermatozoa capacitation or acrosome reaction (Januskauskas et al. 2005) and influences the stability of spermatozoa chromatin structure (Gravance et al. 1998). These effects decrease fertilization capability up to 50% of spermatozoa during cryopreservation (Celeghini et al. 2008) and reduce subsequent spermatozoa viability and the ability to fertilize an oocyte (Defoin et al. 2008). The success of spermatozoa cryopreservation

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depends on many factors, including individuality (Härtlová et al. 2013), the interaction between cryoprotectants and the extender type (Špaleková et al. 2014; Stádník et al. 2015), the length of cooling and equilibration period (Andrabi 2007), and the speed of freezing or thawing (Clulow et al. 2008).

The freezing process causes the release of latent heat from the semen changing the course of temperature, leading to ice crystal formation and therefore, prolonging its duration (Bwanga et al. 1991). Kumar (2003) stated the range from -5 °C to -50 °C as the critical temperatures determining whether spermatozoa will remain in equilibrium with the extracellular plasma or will be gradually cooled, followed by a higher risk of intracellular ice crystal formation consequently threatening the survival of cells (Saragusty et al. 2007). Reducing the temperature is associated with a lower proportion of unfrozen fractions as well as an increase of osmotic pressure. This fact indicates that the freezing speed should be maximized as much as possible. However, fast freezing causes intracellular ice crystal formation. Therefore, freezing has to be slow enough for sufficient spermatozoa dehydration (Watson 2000).

Semen can be frozen by conventional methods without checking the ice crystal formation (Saragusty et al. 2007). On the other hand, freezing with a direct and precise control of temperature in the freezer box and with a precise control of the ice crystal formation can provide higher quality of frozen-thawed sperm (Stradaioli et al. 2007).

Based on the above mentioned statements, we can presume a dissimilar course of the freezing curve will significantly and differently affect the sperm characteristics after the thawing of insemination doses. Therefore, the objective of this work was to determine the effect of selected freezing curves on spermatozoa survivability after thawing defined by its motility.

Materials and Methods

Semen collection

The ejaculates of nine selected sires bred in a private sire insemination centre and ordinarily used for commercial purposes were repeatedly collected during the period from April 2013 to February 2014. Bulls were of the same age, breed (Czech Fleckvieh), and frequency of collecting, bred under the same breeding conditions including handling, stabling, feeding system, and feeding ratio composition. Samples of ejaculates were obtained using an artificial vagina and immediately evaluated in the centre lab according to the methodology applied. The volume of fresh semen (VOL, g), density of spermatozoa (DEN, $\times 10^6$ mm^{-3}), and the percentage rate of progressive motile spermatozoa above head (MOT, %) were evaluated by only one trained technician. Only ejaculates corresponding to the initial required values (minimum DEN 0.7 $\times 10^6$ mm^{-3} and MOT 70%) were subsequently processed. The observed average quality of fresh semen per sire is stated in Table 1. The average volume of fresh semen collected represented 9.97 g. The mean spermatozoa density of fresh ejaculate was 1.00 $\times 10^6$ mm^{-3}. The mean percentage rate of progressive motile spermatozoa above head in fresh ejaculate achieved 72.22%.

Table 1. Mean values of fresh ejaculate variables per sire.

Sire	VOL	DEN	MOT
1	16.1	0.9	70
2	10.9	1.3	70
3	1.9	1.0	80
4	4.7	1.2	70
5	16.3	1.1	70
6	3.6	0.8	70
7	18.2	0.8	70
8	2.3	0.9	80
9	15.7	1.0	70

VOL - volume of fresh semen in g; DEN - density of spermatozoa $\times 10^6$ mm^{-3}; MOT - percentage rate of progressive motile spermatozoa above head

Semen dilution and processing

Samples of fresh semen were ordinarily immediately diluted with the phospholipid extender Andromed® (Minitübe GmbH, Tiefenbach, Germany) to a spermatozoa concentration of 10×10^6 per one insemination dose. The required amount of extender was applied using sterile pipettes directly to the tubes with samples. Subsequently, the diluted semen was filled to 0.25 ml straws (IMV, L'Aigle, France) and equilibrated at 4 °C for 2 h. Diluted semen of each sire was divided into four parts frozen differently according to one of the 4 selected freezing curves. Freezing was performed using computer controlled freezing methodology Direct Freezing in

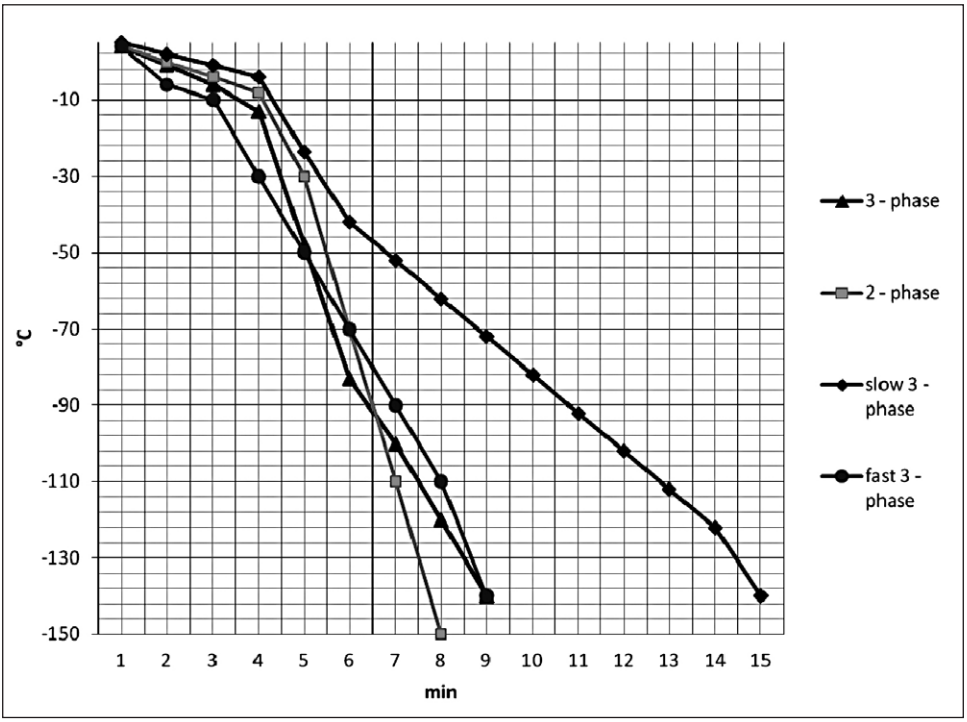

Fig. 1. Course of the freezing curves applied

the freezer box DigitCool (Digitcool®; IMV CryoBio-System, L'Aigle, France). In total, 15 insemination doses were analysed per sire and curve. Figure 1 describes the course of four selected freezing curves. As the first, the standard commercially recommended three-phase curve designed for the freezing of bull semen (Muiño et al. 2007) was applied. The second was the two-phase freezing curve according to the methodology described by Januskauskas et al. (1999) and Gil et al. (2000). Furthermore, a slower (Stradioli et al. 2007) as well as a faster freezing curve (Saragusty et al. 2007) based on the three-phase freezing curve were selected and used for freezing of doses. Thereafter, doses were handled into a liquid nitrogen container and stored at -196 °C.

Evaluation of spermatozoa motility

The straws were thawed in a water bath at 38 ± 1 °C for 30 s (Rubio-Guillén et al. 2007). The percentage rate of progressive motile spermatozoa above head was determined immediately after thawing (T0), while the ejaculate was heated at 38 ± 1 °C all the time. Spermatozoa motility was evaluated subjectively using phase contrast microscopy (LP 3000, Arsenal®, Prague, Czech Republic) at 200–300 × magnification when at least three fields of view per each straw were evaluated (Tuncer et al. 2011). Subsequently, thermodynamic test (TDT) of spermatozoa survivability was performed (Beran et al. 2012b). Thus, spermatozoa motility values (T30, T60, T90, and T120) using the same above mentioned methodology, were determined 30, 60, 90, and 120 min of the test duration in a dry heater (Thermo-block, FALC®, Treviglio, Italy) at a temperature of 38±1 °C.

Statistical analysis

Statistical evaluation was performed using SAS® 9.3 (SAS/STAT 2011) UNIVARIATE, and GLM procedures. Based on Akaike information criterion the values of average spermatozoa motility (AMOT – average of T0, T30, T60, T90, and T120 motility values) throughout the entire thermodynamic test (TDT) were corrected for the effect of the following fixed factors: sire, freezing curve, time of thermodynamic test, and interaction between sire and freezing curve. Simultaneously, spermatozoa motility decrease (MODE – T120 minus T0 motility values) as well as spermatozoa motility at individual phases of TDT (T0, T30, T60, T90, and T120) was evaluated by the same model without the effect of TDT duration. Comparison of details was performed by Tukey-Kramer test. The model equation for average spermatozoa motility was as follows:

$Y_{ijkl} = \mu + A_i + B_j + C_k + (AB)_{ij} + e_{ijkl}$

where:
Y_{ijkl} = dependent variable (spermatozoa motility at the beginning and after 30, 60, 90, and 120 min of the TDT; average spermatozoa motility and decrease of spermatozoa motility through the entire thermodynamic test);
μ = mean value of dependent variable;
A_i = fixed effect of i^{th} freezing curve (j = 1, n = 135; j = 2, n = 135; j = 3, n = 135; j = 4, n = 135);
B_j = fixed effect of j^{th} sire (i = 1, n = 60; i = 2, n = 60; i = 3, n = 60; i = 4, n = 60; i = 5, n = 60; i = 6, n = 60; i = 7, n = 60; i = 8, n = 60; i = 9, n = 60);
C_k = fixed effect of k^{th} time of thermodynamic test (k = 0 min, n = 108; k = 30 min, n = 108; k = 60 min, n = 108; k = 90 min, n = 108; k = 120 min, n = 108) in case of average spermatozoa motility evaluation;
AB_{ij} = interaction between fixed effect of sire and freezing curve (ij = always 36 groups, n = always 15);
e_{ijkl} = random error.

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

Results

The model repeatability ranged from $r^2 = 0.555$ to 0.740 during the evaluation of observed traits when the significance of all models used was $P < 0.001$ in all evaluations. The effect of freezing curve was significant ($P < 0.05$–0.001) with respect to spermatozoa motility in individual phases of TDT, significant ($P < 0.001$) in relation to the average spermatozoa motility throughout the entire TDT, and non-significant ($P > 0.05$) within evaluation of the spermatozoa motility decrease. The effect of sire was significant ($P < 0.001$) in relation

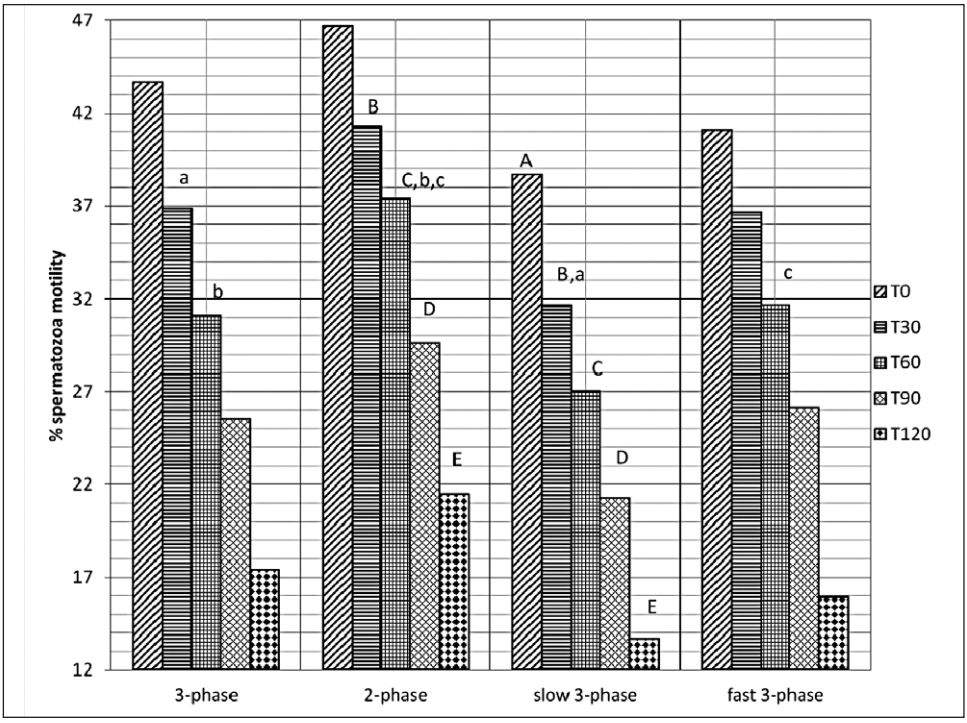

Fig. 2. Effect of the freezing curve on spermatozoa motility in individual phases of the thermodynamic test T0, T30, T60, T90, T120 – percentage spermatozoa motility evaluated immediately after thawing, and after 30, 60, 90, and 120 min of the thermodynamic test; the same upper-script letters confirm the significance of difference A, B, C, D, E ($P < 0.01$); a, b, c ($P < 0.05$)

Table 2. Effect of the freezing curve on average spermatozoa motility and its decrease throughout the entire thermodynamic test.

Freezing curve	AMOT LSM ± SE	MODE LSM ± SE
1st (3-phase)	30.93 ± 0.643^{A}	15.97 ± 0.757
2nd (2-phase)	35.30 ± 0.643^{A,B}	14.21 ± 0.757
3rd (slow 3-phase)	26.48 ± 0.643^{A,B,C}	15.28 ± 0.757
4th (fast 3-phase)	30.30 ± 0.643^{B,C}	13.52 ± 0.757

AMOT – average spermatozoa motility; MODE – spermatozoa motility decrease; the same upper-script letters confirm the significance of difference A, B, C ($P < 0.01$); SE – standard error.

to all the evaluated traits. The effect of the thermodynamic test time as well as sire and freezing curve interaction was significant ($P < 0.001$) in all evaluations.

Figure 2 shows TDT prolonging declined spermatozoa motility in individual phases of the test regardless of the curve type. However, the highest values of spermatozoa motility in all phases of TDT were detected using the two-phase freezing curve. The most frequent and significant differences ($P < 0.05$) were determined compared to the third, i.e. slower three-phase freezing curve representing the lowest spermatozoa motility during all phases of TDT. Comparing the first and fourth freezing curves, a similar trend was detected, although without significant differences.

Table 2 documents the second, i.e. two-phase freezing curve as the most appropriate when average spermatozoa motility throughout the entire TDT (AMOT) achieved 35.30%, and the differences in comparison with other curves were significant ($P < 0.01$). The motility decline detected throughout the entire TDT (MODE) was similar (13.52–15.97%) with non-significant differences among all 4 curves analysed.

Although the fresh ejaculate of all observed sires achieved spermatozoa motility higher than 70%, the courses of the TDT after thawing differed, as documented in Fig. 3. Sires

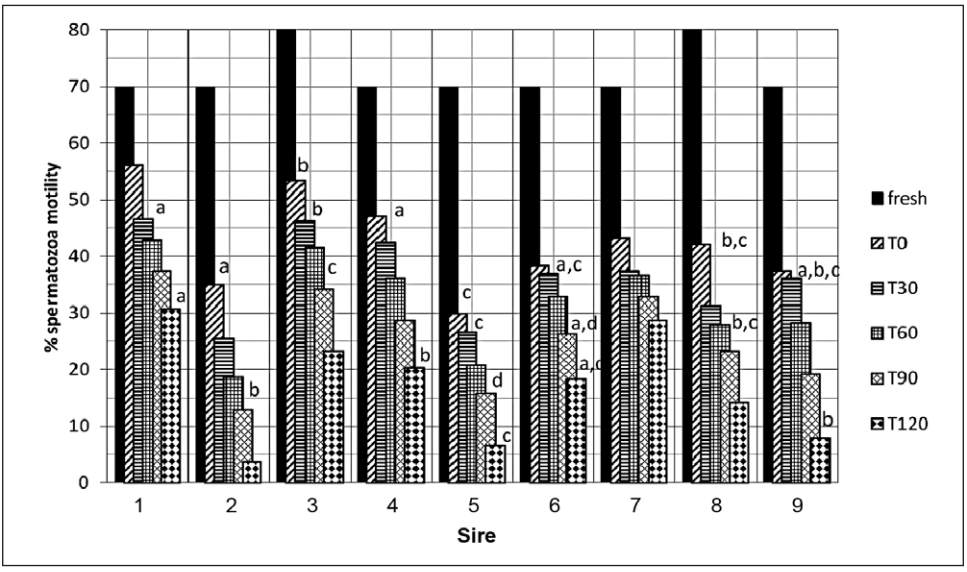

Fig. 3. Effect of sire individuality on spermatozoa motility in individual phases of the thermodynamic test

fresh, T0, T30, T60, T90, T120 – percentage spermatozoa motility evaluated immediately after collecting and thawing respectively, and after 30, 60, 90, and 120 min of the thermodynamic test; the same upper-script letters confirm the significance of difference a, b, c, d ($P < 0.05$)

Table 3. Effect of sire individuality on average spermatozoa motility and its decrease throughout the entire thermodynamic test.

Sire	AMOT LSM ± SE	MODE LSM ± SE
I	42.83 ± 0.965^{A}	16.77 ±1.136^{A}
II	19.17 ± 0.965^{A,B}	19.79 ±1.136^{B,a}
III	39.75 ± 0.965^{B,C,a}	16.98 ±1.136^{C}
IV	35.00 ± 0.965^{A,B,D,a,b}	15.10 ±1.136^{c}
V	20.00 ± 0.965^{A,C,D,E}	12.50 ±1.136^{B,d}
VI	30.58 ± 0.965^{A,B,C,D,E,F,b,c}	9.69 ±1.136^{A,B,C,D,a,c}
VII	35.83 ± 0.965^{A,B,E,F,G}	9.38 ±1.136^{A,B,C,E,c,e}
VIII	27.75 ± 0.965^{A,B,C,D,E,G}	17.92 ±1.136^{D,E,d}
IX	25.83 ± 0.965^{A,B,C,D,E,G,c}	14.58 ±1.136^{a,e}

Key: AMOT – average spermatozoa motility; MODE – spermatozoa motility decrease; the same upper-script letters confirm the significance of difference a, b, c, d, e ($P < 0.05$); A, B, C, D, E, F, G ($P < 0.01$); SE – standard error.

with higher spermatozoa motility of fresh semen did not achieve higher motility during phases of TDT concurrently.

Table 3 shows that the highest AMOT (42.83%; fresh semen motility 70%) was found in Sire 1, whereas the lowest one in Sire 2 (19.17%, fresh semen motility 70%). This difference as well as other differences between the evaluated sires were in most cases significant ($P < 0.05–0.01$). Simultaneously, Sire 2 presented significantly ($P < 0.05–0.01$) the highest value of MODE. On the other hand, the lowest MODE was detected in Sire 7 ($P < 0.05–0.01$).

Figure 4 documents that the highest spermatozoa motility values were detected after thawing doses frozen by the second, i.e. two-phase freezing curve in eight out of nine sires evaluated. Inter-sire differences of spermatozoa motility were significant ($P < 0.01$). However, Sire 6 presented the highest spermatozoa motility values of doses frozen by the first, standard freezing curve regularly applied in the sire insemination centre. Overall, the two-phase freezing curve was the most suitable for most sires.

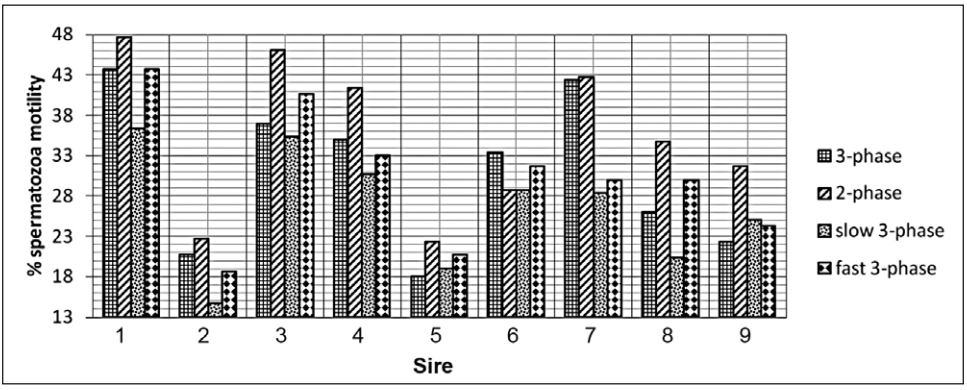

Fig. 4. Effect of sire individuality vs freezing curve interaction on average spermatozoa motility throughout the entire thermodynamic test.

The vast majority of differences was significant at $P < 0.05–0.01$

Discussion

Based on the overall findings (Table 2, Figs 2 and 4) the second, i.e. two-phase curve was the most appropriate using the extender AndroMed® in relation to subsequent spermatozoa motility after thawing. Figure 1 documents the two-phase curve's intermediate course of temperature vs time compared to different types of three-

phase curves. According to Watson (2000), a medium freezing rate supports optimal spermatozoa dehydration by the selected cryoprotective substance and minimizes the negative effect on frozen spermatozoa, especially on ice crystal formation. Our results also generally correspond (Figs 2 and 3) with the conclusion by Januskauskas et al. (2005) that freezing affects all spermatozoa motility indices and negatively influences the percent proportion of motile spermatozoa in total as well as progressive motile spermatozoa above head.

According to Defoin et al. (2008) it is possible to predict spermatozoa frost resistance already before freezing based on motility indices of fresh ejaculate directly during its processing. This fact was confirmed as Fig. 2 describes evaluating different freezing curves in individual phases of TDT. The highest spermatozoa motility at the beginning of TDT represented the highest spermatozoa motility during the entire TDT. Simultaneously, the spermatozoa motility level at the beginning comparatively determines the motility level in individual phases of TDT. The mentioned option allows to process only ejaculates with the required progressive motile spermatozoa above head after thawing and to dispose the substandard ones. This fact significantly improves the efficiency of insemination dose processing and production.

With regard to Table 3 and the report by Amann and Katz (2004), spermatozoa frost resistance differs significantly between sires and even between individual ejaculates of one sire. Also Defoin et al. (2008) determined different effects of freezing on subsequent spermatozoa motility after thawing in relation to individual ejaculates. Our results confirm the mentioned findings and validate the necessity of individual processing technology proposed for each specific sire.

Figure 3 documents that individual motility in TDT phases significantly differed despite the same values of spermatozoa motility in the fresh ejaculate. This fact confirms individual variability and is in agreement with results of Beran et al. (2012a). However, these findings partially contradict the results of the above mentioned Defoin et al. (2008) who stated significant correlations between spermatozoa motility before and after freezing, when ejaculates with high motility maintained high values after thawing as well. The authors stated that these correlations can be used to determine sperm quality after freezing and thawing. Our results emphasize more individual variables of the ejaculate after collecting and during processing. Furthermore, Thurston et al. (2002) indicated that spermatozoa resistance towards cryopreservation can also correlate with genetic factors explaining inter-species, breed as well as individual differences.

According to Muiño et al. (2008) sire individuality and cryopreservation procedure significantly contribute to the final spermatozoa motility as well. Evaluation of spermatozoa motility immediately after thawing (Fig. 2) as well as average spermatozoa motility throughout the entire TDT (Fig. 4) with the highest values detected in doses frozen using the two-phase curve corresponds with the mentioned statement. Our findings confirm previous results and innovatively demonstrate a significant effect of the interaction between sire individuality and the course of the freezing curve.

The effect of the freezing curve type on sire spermatozoa motility after thawing and during the thermodynamic test was observed and evaluated. The two-phase freezing curve, different from the standard, commercially recommended one, was detected as the most appropriate in relation to spermatozoa motility after thawing. Insemination doses frozen using the two-phase curve demonstrated the highest motility values immediately after thawing and during the entire thermodynamic test as well. Concurrently, a significant effect of sire individuality was conclusively confirmed. The findings describing both factors of interaction indicate the necessity of individual cryopreservation of each ejaculate with the potential to increase its fertilization capability after thawing and thus the efficiency of insemination dose production.

Acknowledgments

The work was funded by the "S" grant of the Ministry of Education, Youth and Sports of the Czech Republic and project NAZV QJ1210109. We thank the company Natural Ltd. for cooperation on this work and Mrs. Lois Russell for her editorial help with this manuscript.

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