Archiv Tierzucht 55 (2012) 3, 207-218, ISSN 0003-9438

Effect of sire and extender on sperm motility and share of live or dead sperm in bulls' fresh ejaculate and in AI doses after thawing

Jan Beran[1], Luděk Stádník[1], Jiří Bezdíček[2], František Louda[1], Jaroslav Čítek[1] and Jaromír Ducháček[1]

[1]Department of Animal Husbandry, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences, Prague, Czech Republic, [2]Agriresearch Rapotin Ltd., Rapotin, Czech Republic

Abstract

The objectives were to evaluate the effects of sire and content of fresh or ionised egg yolk in extenders on sperm motility and share of live and dead sperm in collected ejaculate, in thawed artificial insemination (AI) doses, and during thermodynamic testing compared to extenders not containing egg yolk. Ejaculates were collected once a week from 4 Holstein bulls. Each of the 20 ejaculate samples from each bull was diluted with 4 different extenders. AndroMed and Bioxcell (no egg yolk) and Triladyl and Optidyl (fresh, ionised egg yolk) were used. A total of 640 AI doses were analysed. The volume of samples, sperm concentration, and percentage of motile spermatozoa were evaluated after collection, as was sperm motility after thawing of AI doses and during thermodynamic testing. Percentages of live and dead sperm were also evaluated. The data set was analysed using SAS/STAT 9.1 (SAS Institute Inc., Cary, NC, USA). The results confirmed significant (P<0.05-0.01) between-sire differences in the volume, density, and activity of sperm as well as in share of live and dead sperm after collection; in decline of sperm motility in the fresh ejaculate, after thawing, and during the entire thermodynamic test, as well as in the share of live and dead sperm after thawing. The extenders ranked by sperm motility are: Optidyl, Triladyl, AndroMed, and Bioxcell, demonstrating the higher quality of AI doses produced using egg yolk extenders. Differences in sperm motility were significant (P<0.05-0.01) during the entirety of thermodynamic testing. Egg yolk extenders had a significantly (P<0.05-0.01) higher percentages of live sperm after thawing.

Keywords: bull semen, cryopreservation, egg yolk, sperm activity, AndroMed, Bioxcell, Triladyl, Optidyl

Introduction

Artificial insemination (AI) as the first biotechnology widely implemented in practice is important for selection and breeding of cattle (Gravance *et al.* 2009). However, negative changes in sperm membranes in relation to storage time and the extender used were demonstrated (e.g., Frydrychová *et al.* 2010). Worldwide application of AI was the main impulse for subsequent expansion of other procedures (Louda *et al.* 2008), e.g., management of oestrus (Stádník *et al.* 2008), freezing of sperm (Saragusty *et al.* 2009), sperm sexing (Andersson *et al.* 2006), embryo manipulation (Říha *et al.* 1998), and cloning (Liu *et al.* 2010).

The basic quantitative traits of sperm (volume, density, motility etc.) are evaluated across the animal species (Mocé & Graham 2008, Bozkurt *et al.* 2011). Every part of an ejaculate processing from its collection to production AI doses is very important and affects the final quality. Sperm quality is influenced by many factors, e.g., by breed, variation between individuals, and age of the sire (Bezdíček *et al.* 2007, Štolc *et al.* 2009, Bezdíček *et al.* 2010, Strapák *et al.* 2010, Hanuš *et al.* 2011), by feeding ration composition, content of specialised feeding supplements (Čeřovský *et al.* 2009, Jacyno *et al.* 2009), environmental conditions, frequency of ejaculate collection (Wolf & Smital 2009, Karoui *et al.* 2011), and by season of the year (Hajirezaee *et al.* 2010) or even calendar month, especially in animal species with seasonal sexual activity (Alavi *et al.* 2010, Król *et al.* 2011). Collection of ejaculate and its subsequent processing present further potential risk factors. The conventional procedure for evaluating ejaculate which is performed before and after cryopreservation includes determining the volume, density, activity, and sperm morphology (Bhoite *et al.* 2008, Karoui *et al.* 2011). These parameters mostly detect cases of significantly inadequate reproduction or infertility in bulls (Rodriguez-Martinez 1998). To assess the resistance and the fertilization ability of sperm have been developed biological tests of ejaculate, e.g., long-term cold test and short-term heat test of sperm survival, resistance of sperm against cold shock and determination the percentage of live and dead sperm by staining (Januskauskas *et al.* 2000, Gillan *et al.* 2008). Diluting of sperm, filling of straws, their cooling and freezing follow the macro- and micro-evaluation of ejaculate quality. These last 4 phases of producing AI doses have a significant effect on sperm motility, and the effect of the extender used is especially important (Siddique *et al.* 2006).

Egg yolk has been used as a basic component of extenders for bull ejaculate since 1939 (Amirat *et al.* 2004) and still remain popular, although the addition of egg yolk changes the composition of an extender. Use of these extenders is recommended because of their excellent protection of sperm cells (Celeghini *et al.* 2008). Nevertheless, the use of egg yolk as a cryoprotectant has recently been restricted in some countries for reasons of hygiene risk (Thun *et al.* 2002). Moreover, some researchers (e.g., Amirat *et al.* 2005) have demonstrated that extenders based on egg yolk can have negative effects on sperm respiration and motility due to other specific substances they contain. Pace & Graham (1974) purified egg yolk by ultracentrifugation and found that a fraction of egg yolk known as »low-density lipoprotein« (LDL) has a cryoprotective effect on the integrity of the plasma membrane, as well as on the percentage of normal spermatozoa and sperm motility (Bencharif *et al.* 2010) and preserves bull semen and maintains its fertility during freezing, storage, and thawing (Amirat *et al.* 2004). The motility of sperm was almost twice as high in LDL (54.4 %) compared to Optidyl (30.2 %, $P<0.05$).

Extenders containing industrially prepared LDL are not used in the Czech Republic during production of AI doses, and, due to the potential risk of bacterial contamination, the producers have ceased using extenders containing fresh egg yolk. We could hypothesise that significant differences in ejaculate quality traits before diluting (volume, density, activity, share of live and dead sperm) among individual sires will be detected, as well as between-sire differences in sperm motility after thawing and during thermodynamic testing in relationship also to the content of fresh or ionised egg yolk in extenders compared to extenders not containing egg yolk. We hypothesised that egg yolk in extenders will provide higher levels of sperm motility and a higher share of live sperm after thawing and that these facts will demonstrate the

higher quality of AI doses produced with egg yolk extenders. Further, one may assume that the destruction of fat globules in ionised egg yolk will enable detection of higher levels of qualitative traits for AI doses prepared with an extender based on ionised egg yolk.

The present study's objectives were to determine and evaluate the effects of an individual sire and the content of fresh or ionised egg yolk in extenders on sperm motility and share of live and dead sperm in freshly collected ejaculate, in thawed AI doses, and during thermodynamic testing compared to commonly used extenders not containing egg yolk.

Material and methods

Semen collecting and processing

The observations were made in a bull housing facility and laboratory at a single AI centre, where four Holstein bulls were selected for monitoring. During the period of observation, the bulls were fed an identical daily ration: hay (10 kg), straw (5 kg), soybean meal (0.5 kg), a mixture of cereals: 1/3 oats, 1/3 wheat, 1/3 barley (3 kg), plus the mineral mix Premin 22 Natural (0.1 kg; VVS Verměřovice s.r.o, Verměřovice, Czech Republic). This mineral feed contains 25 % of rye bran, 25 % of dihydrogen phosphate, 19 % of calcium carbonate, 13 % of sodium chloride, 9 % of magnesium oxide, 4 % of beet molasses and other minerals (content in one kilogram of the mixture): Ca (11.4 %), P (6 %), Na (5 %), Mg (5.2 %), Vitamin A (1 250 000 i.u.), Vitamin D3 (250 000 i.u.), Vitamin E (5 000 mg), Vitamin B1 (61 mg), $FeCO_3$ (8 200 mg), $CuSO_4$. $5H_2O$ (600 mg), MnO (3 000 mg), ZnO (5 500 mg), $Ca(IO_3)_2$ (45 mg), $Co(CH_3COO)_2$ (45 mg), Na_2SeO_3 (36.5 mg), Niacin (825 mg), and Beta Carotene (800 mg).

The bulls were of roughly similar age, and the frequency of semen collection from all was once weekly. Twenty ejaculates were obtained from each bull using an artificial vagina during the period from October 2008 to April 2009. Because climatic conditions during the observation period were similar, we did not detect significant differences during pre-evaluation of dataset, and did not include the effect of month in the statistical model. The volume of semen samples (VOL) was measured using an electronic scale (Scout Pro, Ohaus Corp., Parsippany, NJ, USA), sperm concentration (DEN) using a spectrophotometer (Genesys 10vis, Spectronic Unicam, Rochester, NY, USA), and the percentage of motile spermatozoa (ACT) subjectively by phase contrast microscopy (LP 3000, Arsenal, Praha, Czech Republic) immediately after collection. In addition, we evaluated the percentage of live and dead sperm by staining before diluting and freezing in accordance with standard methodology (Ball & Peters 2004): a drop of semen was mixed with eosin on a preheated microscope slide, spread, then examined under a phase contrast microscope at 1 000× magnification and with oil immersion. We classified a minimum of 100 spermatozoa as either dead (with red heads) or live (with white heads) and expressed as a percentage rate.

Only fresh semen with required quality (minimum progressive motility 70 % and sperm concentration 0.7×10^6 mm^{-3}) was used for the subsequent processing of samples for observation in accordance with common standards used for producing AI doses.

The fresh semen was divided into four equal fractions. The first was diluted with AndroMed (Minitüb GmbH, Tiefenbach, Germany) an extender containing soybean lecithin extract and antibiotics (Gentamycin, Spectinomycin, Lincomycin, and Tylosin. The second was Bioxcell (IMV, L'Aigle, France), an extender containing no product of animal origin and antibiotics

(Lincomycin, Spectinomycin, Gentamycin and Tylosin). The third fraction was diluted with Triladyl (Minitüb GmbH, Tiefenbach, Germany), containing 20 % (w/v) fresh egg yolk, TRIS, Citric acid, Sugar, Buffers, Glycerol and antibiotics (Tylosin, Gentamicine, Spectinomycine and Lincomycine). The fourth was diluted with Optidyl (Biovet, Fleurance, France), an extender which contains ionised egg yolk, glycerol, TRIS and antibiotics (Streptomycin, Penicillin, Lincomycine and Spectinomicyne).

Polyvinyl chloride (PVC) straws (0.25 cm^3; IMV, L'Aigle, France) were filled, cooled to 4 °C, and equilibrated for 90 min. Subsequently, they were frozen in a programmable freezing device (IMV-Digitcool, IMV, L'Aigle, France) then plunged into liquid nitrogen for storage.

Semen quality parameters after thawing

In vitro parameters were evaluated after thawing two pooled straws from each diluted sample of semen. In total, we sampled 4 sires, 20 samples of ejaculate from each sire, and subsequently diluted with 4 different extenders. Thus, we analysed 160 straws per bull as well as per extender (640 straws altogether).

We assessed sperm motility subjectively using phase contrast microscopy (LP 3000) at 200× magnification. The straws were thawed in a water bath at 38±1 °C for 45 s, and then put into preheated sterile tubes with sodium citrate (CIT) (2.98-3.28 %, pH 6.7-6.85) or a physiological solution (PHY) as a control. The motility values were determined at the beginning of the short-term heat test of sperm survival (CIT0 and PHY0) and then after 30, 60, 90, and 120 min (CIT30-120, and PHY30-120, respectively) of the test duration in a dry heater (Thermo-block, Falc Instruments, Treviglio, Italy) at a temperature of 38±1 °C. We also evaluated percentages of live and dead sperm with staining after freezing then thawing.

Statistical analysis

The data set was analysed using a generalised linear model in the statistical program SAS/STAT 9.1 (SAS Institute Inc., Cary, NC, USA). The following equation was used:

$$Y_{ijk} = \mu + A_i + B_j + e_{ijk}$$

where Y_{ijk} is the observed value of the dependent variable (volume of sperm in g, concentration of sperm in 10^6 mm^{-3}, motility of sperm before and after freezing then thawing in percent, live:dead sperm as a percentage rate), μ is the average value of the dependent variable, A_i is the fixed effect of the *i*-th bull (i=bull A, n=160; bull B, n=160; bull C, n=160; bull D, n=160), B_j is the fixed effect of the *j*-th extender (j=AndroMed, n=160; Bioxcell, n=160; Triladyl, n=160; Optidyl, n=160), and e_{ijk} is the residual effect.

The differences between the variables estimated were tested at the levels of significance $P<0.05$ and $P<0.01$.

Results and discussion

The effects of individual factors included in the statistical model are presented in Tables 1 and 2. The individual sire variable had a significant effect ($P<0.01$) on ejaculate quality before

freezing and on sperm motility after thawing. The effect of the extender used was significant ($P<0.05$) in relation to the percentage of live sperm after thawing, in accordance with sperm motility after thawing in sodium citrate at 0, 30, 60, 90, and 120 min of thermodynamic testing ($P<0.01$). No significant effect of the extender was detected in relation to the other traits evaluated ($P>0.05$).

Table 1
Effects of individual factors in a statistical model on quality traits of ejaculate after collection

Trait	Model		Bull	
	R^2	*P*	F-test	*P*
VOL	0.37	<0.01	14.52	<0.01
DEN	0.39	<0.01	13.08	<0.01
ACT	0.47	<0.01	21.81	<0.01
L_1	0.14	0.0248	3.14	0.0302
D_1	0.13	0.036	3.04	0.034

VOL: volume of ejaculate, DEN: density of sperm, ACT: activity of sperm, L_1 and D_1: percentage of live and dead sperm, respectively, after collection

Table 2
Effects of individual factors in a statistical model on sperm motility in AI doses after thawing and during thermodynamic testing, as well as share of live and dead sperm after thawing

Trait	Model		Bull		Extender	
	R^2	*P*	F-test	*P*	F-test	*P*
CIT0	0.11	<0.01	6.82	0.0002	3.95	0.0087
PHY0	0.14	<0.01	11.49	<0.01	2.54	0.0563
CIT30	0.13	<0.01	9.11	<0.01	3.86	0.01
PHY30	0.13	<0.01	11.38	<0.01	1.59	0.1906
CIT60	0.13	<0.01	9.88	<0.01	4.19	0.0063
PHY60	0.12	<0.01	11.73	<0.01	1.1	0.3497
CIT90	0.13	<0.01	10.53	<0.01	4.71	0.0031
PHY90	0.14	<0.01	14.39	<0.01	1	0.4
CIT120	0.15	<0.01	10.86	<0.01	7.18	0.0001
PHY120	0.15	<0.01	15.94	<0.01	1.14	0.333
L_2	0.12	<0.01	7.86	<0.01	3.25	0.0222
D_2	0.12	<0.01	7.05	0.001	3.96	0.0085

CIT: sodium citrate thawing solution, PHY: physiological thawing solution, CIT0 and PHY0: sperm motility after thawing, CIT30-120 and PHY30-120: sperm motility after 30, 60, 90, and 120 min of thermodynamic test, L_2 and D_2: percentage of live and dead sperm, respectively, after thawing

Sire effect on traits of ejaculate after collecting

The effect of the sire on the quality traits evaluated after collection is presented in Table 3. The volume of ejaculate ranged from 3.3 g in bull C to 6.9 g in bull D during the period observed, and differences among individual sires were determined to be significant ($P<0.05$-0.01). Ball & Peters (2004) reported range from 5 to 6 cm^3, while Karoui *et al.* (2011) assessed the overall mean volumes of bull ejaculates 5.49 cm^3. Hafez & Hafez (2000) stated the minimum volume of bull ejaculate suitable for long-term preservation 3 cm^3 in adult bulls and 2 cm^3 in young bulls.

Table 3
Sire effect on observed qualitative traits of fresh ejaculate after collection

Trait	A (n=160) LSM±SE	B (n=160) LSM±SE	C (n=160) LSM±SE	D (n=160) LSM±SE
VOL, g	5.2 ± 0.40^{CD}	4.4 ± 0.39^{D}	3.3 ± 0.39^{AD}	6.9 ± 0.39^{AB}
DEN, 10^6 mm^{-3}	1.1 ± 0.08^{Cd}	1.3 ± 0.08	1.5 ± 0.08^{AD}	0.9 ± 0.08^{aC}
ACT, %	89.3 ± 1.45^{BCD}	75.9 ± 1.44^{A}	78.9 ± 1.44^{Ad}	74.3 ± 1.44^{Ac}
L_1, %	76.7 ± 2.06^{dB}	68.9 ± 2.06^{ac}	74.7 ± 2.06^{b}	70.4 ± 2.05^{a}
D_1, %	22.8 ± 2.03^{B}	31.1 ± 2.03^{Ac}	25.3 ± 2.03^{b}	28.0 ± 2.02

$^{a,b,c,d}P<0.05$, $^{A,B,C,D}P<0.01$, different letters confirm statistical significance, VOL: volume of ejaculate, DEN: density of sperm, ACT: activity of sperm, L_1 and D_1: percentage of live and dead sperm, respectively, after collection

On the other hand, the lowest density of ejaculate was found in sire D (0.9×10^6 mm^{-3}) and the highest in sire C (1.5×10^6 mm^{-3}). A significant difference ($P<0.05$) was determined between sires A and D and simultaneously among sires A, C, and D ($P<0.01$). Louda *et al.* (2007), respectively Karoui *et al.* (2011) reported sperm density in bull ejaculates from 0.8 to 2.0×10^6 mm^{-3}, respectively from 0.3 to 3.815×10^6 mm^{-3}. The minimum requirement for sperm density in relation to subsequent processing is 0.7×10^6 mm^{-3} (Hafez & Hafez 2000).

Sperm activity in the collected ejaculates ranged from 74.3 % in sire D to 89.3 % in sire A. A statistically significant difference ($P<0.05$) was found in sperm motility of sires C and D. Significant differences among sire A and all other sires ($P<0.01$) were detected as well. Louda *et al.* (2007) reported sperm activity for bulls in a range of 45-75 % and even higher. Ball & Peters (2004) or Muino *et al.* (2007) mentioned that the lowest activity required for further sperm processing is 70 %.

The share of live sperm when analysed immediately after collection was the lowest in sire B (68.9 %) and the highest in sire A (76.7 %). Significant differences between sires B and C, as well as between A and D ($P<0.05$), and between A and B ($P<0.01$) were demonstrated during our observation. On the contrary, the percentage of dead sperm before extending was the lowest for sire A (22.8 %) and the highest for sire B (31.1 %). Significant differences between sires B and C ($P<0.05$) as well as between A and B ($P<0.01$) were also determined.

The complete results, presented in Table 3, indicate that the greatest level of sperm resistance was in sire A, while the lowest level for individual traits was for sire B ($P<0.01$). Result confirms effect of bull individuality as an important factor affecting initial, but final quality of AI dose as well.

Sire effect on traits of ejaculate after thawing

Table 4 shows the results of thermodynamic testing of sperm motility at 0, 30, 60, 90, and 120 min and the proportions of live and dead sperm after thawing in relation to AI doses for individual sires analysed in sodium citrate and in a physiological solution. Initial sperm activity at the beginning of the test was the highest in both solutions for sire A (53.2 and 54.3 %, respectively), and the lowest values were recorded for sire C (39.3 and 40 %). The sperm activity of all sires declined during the thermodynamic test by an average of 22.75 %. The highest level and lowest decline in sperm motility were for sire A. Its activity levels were 32.4 and 34.4 %, and its declines were 20.8 % in sodium citrate and 19.9 % in a physiological solution after 120 min. The greatest decline was observed for sire B, falling by 26.8 % in sodium

citrate and 24.5 % in a physiological solution. Differences detected among sires A, B, and C were significant ($P<0.01$) during the entirety of testing. Sperm activity can be expected to range from 30 to 50 % of sperm with progressive motion prior to freezing (Louda *et al.* 2008) and level of 30 % can be determined as a basic and necessary sperm activity after thawing of a dose before its application (Hafez & Hafez 2000). Only sire C did not reach this level already after 60 min of the test, but this result only documented its having the lowest fertility in comparison to the others. Ježková *et al.* (2008) described significant effect of individuality in reproduction of sires as well as dairy cows. Our findings confirmed effect of individuality as well as the initial quality of ejaculate on the final level of qualitative traits of AI doses thawed.

Table 4
Sire effect on observed qualitative traits of AI doses after thawing

Trait, %	A (n=160) LSM±SE	B (n=160) LSM±SE	C (n=160) LSM±SE	D (n=160) LSM±SE
CIT0	53.2 ± 2.12^{Cd}	52.7 ± 2.06^{C}	39.3 ± 2.06^{ABd}	46.3 ± 2.06^{ac}
PHY0	54.3 ± 2.07^{CD}	50.0 ± 2.12^{Cd}	40.0 ± 2.12^{AB}	46.2 ± 2.12^{Ab}
CIT30	49.4 ± 2.05^{Cd}	47.2 ± 2.04^{C}	35.4 ± 2.04^{ABD}	43.7 ± 2.04^{aC}
PHY30	51.1 ± 1.98^{CD}	48.9 ± 1.98^{C}	36.1 ± 1.98^{ABD}	43.7 ± 1.98^{AC}
CIT60	44.6 ± 1.97^{CD}	40.6 ± 1.96^{C}	30.1 ± 1.96^{ABD}	37.1 ± 1.96^{AC}
PHY60	44.8 ± 1.90^{CD}	41.2 ± 1.89^{C}	29.8 ± 1.89^{ABD}	36.5 ± 1.89^{AC}
CIT90	38.1 ± 1.92^{CD}	33.4 ± 1.91^{C}	23.3 ± 1.91^{ABD}	30.9 ± 1.91^{AC}
PHY90	39.2 ± 1.85^{CD}	34.4 ± 1.84^{Cd}	23.0 ± 1.84^{ABd}	29.0 ± 1.84^{Abc}
CIT120	32.4 ± 1.95^{bC}	25.9 ± 1.94^{aCD}	16.8 ± 1.94^{ABD}	24.0 ± 1.94^{BC}
PHY120	34.4 ± 1.96^{bCD}	28.3 ± 1.96^{aCD}	16.5 ± 1.96^{AB}	21.7 ± 1.96^{AB}
L_2	51.8 ± 1.66^{C}	48.8 ± 1.65^{Cd}	42.9 ± 1.65^{ABD}	53.3 ± 1.65^{bC}
D_2	47.7 ± 1.68^{C}	51.1 ± 1.67^{cd}	56.3 ± 1.67^{AbD}	46.4 ± 1.67^{bC}

$^{a,b,c,d}P<0.05$, $^{A,B,C,D}P<0.01$, different letters confirm statistical significance, CIT: sodium citrate thawing solution, PHY: physiological thawing solution, CIT0 and PHY0: sperm motility after thawing, CIT30-120 and PHY30-120: sperm motility after 30, 60, 90, and 120 min of thermodynamic test, L_2 and D_2: percentage of live and dead sperm, respectively, after thawing

Bull D presented the lowest share of live sperm, while the highest of dead sperm, with significant ($P<0.01$) differences to other bulls.

We can summarise that the highest viability of sperm was demonstrated in sire A in accordance with the highest level of, and the lowest decline in, sperm activity of its ejaculate and AI doses. The lowest viability was detected in sires B and D, which had the greatest decline in sperm activity during our observation. Lower level of R^2 in Table 2 compared to Table 1 and the significant individual differences in qualitative traits of bull ejaculate in Tables 3 and 4 emphasised importance of initial ejaculate quality for final quality of AI doses.

Extender effect on traits of sperm activity after thawing

The effect of the extender used on sperm motility is presented in Table 5. Sperm activity at the beginning of testing was the highest in Optidyl (52.1 % in PHY and 51.6 % in CIT) and the lowest in Bioxcell (44.1 % and 41.5 %). These differences were significant ($P<0.01$). Further significant differences ($P<0.05$) at the beginning of the test were determined among AndroMed (47.4 %), Triladyl (48.4 %), and Bioxcell (41.5 %). Extenders containing egg yolk

reached higher levels of sperm activity in both sodium citrate and a physiological solution at the beginning of the test (P<0.05-0.01).

Table 5
Extender effect on observed qualitative traits of AI doses after thawing

Trait, %	AndroMed (n=160) LSM±SE	Bioxcell (n=160) LSM±SE	Triladyl (n=160) LSM±SE	Optidyl (n=160) LSM±SE
CIT0	47.4±2.11^{b}	41.5±2.11^{cD}	48.4±2.11^{b}	51.6±2.11^{B}
PHY0	48.4±2.05	44.1±2.05^{D}	48.6±2.05	52.1±2.05^{B}
CIT30	44.1±2.04	38.5±2.04^{D}	44.9±2.04	48.1±2.04^{B}
PHY30	45.1±1.97	41.6±1.97^{d}	45.5±1.97	47.6±1.97^{b}
CIT60	37.2±1.96^{d}	33.4±1.96^{d}	38.7±1.96	43.0±1.96^{aB}
PHY60	38.5±1.89	35.2±1.89	38.7±1.89	39.9±1.89
CIT90	30.3±1.91^{d}	26.5±1.91^{cD}	32.6±1.91^{b}	36.3±1.91^{aB}
PHY90	31.9±1.84	28.7±1.84	32.3±1.84	32.7±1.84
CIT120	23.3±1.94^{D}	18.9±1.94^{cD}	25.5±1.94^{bd}	31.4±1.94^{ABc}
PHY120	25.9±1.95	22.2±1.95	26.7±1.95	26.3±1.95
L_2	47.8±1.65	45.8±1.65^{Cd}	52.6±1.65^{B}	50.4±1.65^{b}
D_2	52.1±1.67	54.2±1.67^{CD}	47.1±1.67^{B}	48.1±1.67^{B}

$^{a,b,c,d}P$<0.05, $^{A,B,C,D}P$<0.01, different letters confirm statistical significance, CIT: sodium citrate thawing solution, PHY: physiological thawing solution, CIT0 and PHY0: sperm motility after thawing, CIT30-120 and PHY30-120: sperm motility after 30, 60, 90, and 120 min of thermodynamic test, L_2 and D_2 percentage of live and dead sperm, respectively, after thawing

Declining sperm activity was observed in both solutions for all extenders during the entire test. The difference between Bioxcell (38.5 %) and Optidyl (48.1 %) in sodium citrate was significant at the P<0.01 level at 30 min of testing. In a physiological solution, at 47.6 % vs 41.6 %, the difference was significant only at the P<0.05 level.

The differences demonstrated in sodium citrate among Optidyl (43 %), Bioxcell (33.4 %), and AndroMed (37.2 %) after 60 min of testing were significant at the P<0.01 level. No significant differences were found between these in a physiological solution.

Significant differences in sodium citrate (P<0.05) were determined after 90 min of testing between AndroMed (30.3 %) and Optidyl (36.3 %) as well as between Bioxcell (26.5 %) and Triladyl (32.6 %). A higher level of significance (P<0.01) was found at this time between Bioxcell (26.5 %) and Optidyl (36.3 %). It was repeatedly true that no significant differences in sperm activity were recorded in a physiological solution (P>0.05).

Again, significant differences were determined after 120 min of testing in sodium citrate only. These differences were at the P<0.05 level among Bioxcell (18.9 %), Triladyl (25.5 %) and Optidyl (31.4 %), at the P<0.01 level between AndroMed (23.3 %) and Optidyl (31.4 %), and Bioxcell (18.9 %) and Optidyl (31.4 %).

Moreover, Table 5 demonstrates that the poorest results during the entirety of the thermodynamic test were found for the extender Bioxcell which does not contain egg yolk, while differences compared to the best results for the extender Optidyl containing egg yolk, were significant (P<0.01) after 30, 60, and 90 min. We can summarise that the greatest sperm activity during the entire test was for extenders containing egg yolk (P<0.05-0.01). Bioxcell was also used in the study of Celeghini *et al.* (2008), who reported the same conclusions – that the other extenders which they evaluated were significantly better than Bioxcell. A

contrary finding by Stradaioli *et al.* (2007) that Bioxcell achieved significantly ($P<0.05$) better results was not confirmed in our observation. At the same time, we cannot confirm the conclusions of Herold *et al.* (2000) and Janett *et al.* (2005), who reported AndroMed to be the most suitable extender for cryopreservation of bull semen.

Higher proportions of live sperm after thawing were detected in egg yolk extenders, specifically 52.6 % in Triladyl and 50.4 % in Optidyl, respectively. The differences between these two extenders compared to the 45.8 % in Bioxcell were significant at the $P<0.01$ and $P<0.05$ levels, respectively. The results repeatedly confirmed better levels of individual traits and of overall quality for the AI doses produced using egg yolk extenders.

Further, decline in sperm motility in individual extenders was evaluated. The results differed in relation to the solution used for thawing the AI dose. When using sodium citrate for dose preparation, the smallest decline in sperm motility was observed for Optidyl and the greatest decline for AndroMed. While the greatest decline in a physiological solution was detected for Optidyl ($P<0.05$), egg yolk extenders nevertheless achieved better final results due to their greater sperm motility already at the beginning of the test. This fact repeatedly confirms impact on final quality of used AI dose. The differences compared to extenders not containing egg yolk were significant in sodium citrate during the entire thermodynamic test, while in a physiological solution this was true only during the first 30 min of the test. Thus, our findings confirm the conclusions of Muino *et al.* (2007) that egg yolk extenders have a positive cryoprotective effect on sperm cells, and, because of this fact, that they are more valuable than extenders not containing egg yolk, and they can limit the risk of bacterial contamination.

In conclusion, the quality of AI doses is one of the factors relating to the ongoing decline in pregnancy rates of cows and heifers. Because of this decline, breeders, personnel at AI companies and breeder associations, as well as researchers, all seek possibilities for increasing reproductive performance in cattle. The quality traits of bull ejaculate – its volume, density, activity and live:dead sperm ratio – were evaluated in fresh ejaculate before subsequent processing, as were sperm motility after extending, freezing then thawing, and during thermodynamic testing in relation to the effects of sire and extender used.

The results confirmed significant ($P<0.05$-0.001) between-sire differences in the level of volume, density and sperm activity, and in decline of sperm motility in fresh ejaculate, after thawing, and during thermodynamic testing, as well as in the share of live and dead sperm. These findings provide basic information about bull´s individuality and its importance for quality of AI doses produced and selection of bulls for subsequent use in reproduction area.

The effect on sperm motility after thawing of the extender used in preparing the AI dose was significant ($P<0.01$). The ranking of extenders in accordance with sperm motility in all evaluated phases of the test was: Optidyl, Triladyl, AndroMed, and Bioxcell. This finding confirms the higher quality of AI doses manufactured using egg yolk extenders. Differences among the extenders were at all times significant when using sodium citrate for AI dose preparation ($P<0.05$-0.001), but this was true only after thawing and at 30 min of testing in a physiological solution ($P<0.05$-0.01). The results indicate higher sperm motility in the extender with ionised egg yolk, but the differences were not significant compared to the fresh egg yolk extender. AI doses diluted with egg yolk extenders presented significantly higher percentages of live sperm (+4.6-6.8 %, $P<0.05$-0.01) also after thawing.

Selection of a suitable extender for preparing AI doses is a very important part of the entire procedure. The use of fresh animal products (milk, egg yolk) as a component of extenders is restricted in the day-to-day operations of AI laboratories because of bacterial hazard. Nevertheless, egg yolk appears to be the most suitable cryoprotectant. In view of the facts that LDL extenders are not used in the Czech Republic and that there is a preference in the country for extenders not containing egg yolk when producing AI doses from bull semen. We can recommend, based on our findings, detailed evaluation of individual quality of bulls´ ejaculate and using an extender containing ionised egg yolk in order to produce AI doses of higher quality.

Acknowledgement

This research was funded by the Ministry of Education, Youth and Sports of the Czech Republic (Project No. MSM 6046070901) and by the Ministry of Agriculture (Projects No. NAZV QI91A061 and NAZV QH91270). We thank Natural Ltd. company for cooperation on this work. We thank Mrs. Lois Russell for editorial help with this manuscript.

References

Alavi SMH, Rodina M, Hatef A, Stejskal V, Policar T, Hámačková J, Linhart O (2010) Sperm motility and monthly variations of semen characteristics in Perca fluviatilis (Teleostei: Percidae). Czech J Anim Sci 55, 174-182

Amirat L, Tainturier D, Jeanneau L, Thorin C, Gérard O, Courtens JL, Anton M (2004) Bull semen *in vitro* fertility after cryopreservation using egg yolk LDL: a comparison with Optidyl®, a commercial egg yolk extender. Theriogenology 61, 895-907

Amirat L, Anton M, Tainturier D, Chatagnon G, Battut I, Courtens JL (2005) Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. Reproduction 129, 535-543

Andersson M, Taponen J, Kommeri M, Dahlbom M (2006) Pregnancy rates in lactating Holstein-Friesian cows after artificial insemination with sexed sperm. Reprod Domest Anim 41, 95-97

Ball PJH, Peters AR (2004) Reproduction in cattle. 3rd ed., Blackwell Publishing, Oxford, UK

Bencharif D, Amirat-Briand L, Garand A, Anton M, Schmitt E, Desherces S, Delhomme G, Langlois ML, Barrière P, Destrumelle S, Vera-Munoz O, Tainturier D (2010) Freezing canine sperm: Comparison of semen extenders containing Equex® and LDL (Low Density Lipoproteins). Anim Reprod Sci 119, 305-313

Bezdíček J, Říha J, Kučera J, Dufek A, Bjelka M, Šubrt J (2010) Relationships of sire breeding values and cutting parts of progeny in Czech Fleckvieh bulls. Arch Tierz 53, 415-425

Bezdíček J, Šubrt J, Filipčík R, Bjelka M, Dufek A (2007) The effects of inbreeding on service period and pregnancy length in Holsteins and Czech Fleckviehs after the first calving. Arch Tierz 50, 455-463

Bhoite UY, Sutar DA, Ulmek BR (2008) Studies on semen quality of crossbred bulls. Indian Vet J 85, 395-397

Bozkurt Y, Öğretmen F, Kökçü Ö, Erçin U (2011) Relationships between seminal plasma composition and sperm quality parameters of the Salmo trutta macrostigma (Dumeril, 1858) semen: with emphasis on sperm motility. Czech J Anim Sci 56, 355-364

Celeghini ECC, de Arruda RP, de Andrade AFC, Nascimento J, Raphael CF, Rodrigues PHM (2008) Effects that bovine sperm cryopreservation using two different extenders has on sperm membranes and chromatin. Anim Reprod Sci 104, 119-131

Čeřovský J, Frydrychová S, Lustyková A, Lipenský J, Rozkot M (2009) Semen characteristics of boars receiving control diet and control diet supplemented with L-carnitine. Czech J Anim Sci 54, 417-425

Frydrychová S, Čeřovský J, Lustyková A, Rozkot M (2010) Effects of long-term liquid commercial semen extender and storage time on the membrane quality of boar semen. Czech J Anim Sci 55, 160-166

Gillan L, Kroetsch T, Maxwell WMC, Evans G (2008) Assessment of *in vitro* sperm characteristics in relation to fertility in dairy bulls. Anim Reprod Sci 103, 201-214

Gravance CG, Casey ME, Casey PJ (2009) Pre-freeze bull sperm head morphometry related to post-thaw fertility. Anim Reprod Sci 114, 81-88

Hafez ESE, Hafez B (2000) Reproduction in Farm Animals. 7th ed. Wiley-Blackwell, Philadelphia, PA, USA

Hajirezaee S, Mojaziamizi B, Mirvaghefi A, Sheikh Ahmadi A (2010) Evaluation of semen quality of endangered Caspian brown trout (*Salmo trutta caspius*) in different times of spermiation during the spawning season. Czech J Anim Sci 55, 445-455

Hanuš O, Kučera J, Yong T, Chládek G, Holásek R, Třináctý J, Genčurová V, Sojková K (2011) Effect of sires on wide scale of milk indicators in first calving Czech Fleckvieh cows. Arch Tierz 54, 36-50

Herold FC, Gerber D, Aurich JE (2003) Influence of homologous seminal plasma on bovine epididymal semen frozen with TriladylTM or AndroMed®. Wien Tierärztl Monatsschr 90, 58-61

Jacyno E, Kołodziej A, Kawęcka M, Pietruzska A, Matysiak B, Kamyczek M (2009) The relationship between blood serum and seminal plasma cholesterol content in young boars and their semen qualitative traits and testes size. Arch Tierz 52, 161-168

Janett F, Keo S, Bollwein H, Hässig M, Thun R (2005) Comparison of AndroMed®, Bioxcell® and Triladyl® extender for cryopreservation of bull semen. Schweiz Arch Tierheilkd 147, 62

Januskauskas A, Johannisson A, Söderquist L, Rodriguez-Martinez H (2000) Assessment of sperm characteristics post-thaw and response to calcium ionophore in relation to fertility in Swedish dairy AI bulls. Theriogenology 53, 859-875

Ježková A, Stádník L, Vacek M, Louda F (2008) Factors affecting the cervical mucus crystallization, the sperm survival in cervical mucus, and pregnancy rates of Holstein cows. J Central Europ Agric, 9, 377-384

Karoui S, Díaz C, Serrano M, Cue R, Celorrio I, Carabaño MJ (2011) Time trends, environmental factors and genetic basis of semen traits collected in Holstein bulls under commercial conditions. Anim Reprod Sci 124, 28-38

Król J, Kowalski R, Demska-Zakęś K, Hliwa P, Glogowski J (2011) Proteolytic and anti-proteolytic activity in the seminal plasma of Eurasian perch (*Perca fluviatilis L.*) during the spawning period. Czech J Anim Sci 56, 390-397

Liu J, Westhusin M, Long C, Johnson G, Burghardt R, Kraemer D (2010) Embryo production and possible species preservation by nuclear transfer of somatic cells isolated from bovine semen. Theriogenology 74, 1629-1635

Louda F, Bjelka M, Ježková A, Pozdíšek J, Stádník L, Bezdíček J (2007) [Principles of breeding bulls´ use under natural breeding conditions]. Research Institute for Cattle Breeding Ltd., Rapotín, Czech Republic [in Czech]

Louda F, Vaněk D, Ježková A, Stádník L, Bjelka M, Bezdíček J, Pozdíšek J (2008) [The use of biological principles in management of female reproduction in cattle breeding]. Research Institute for Cattle Breeding Ltd., Rapotín, Czech Republic [in Czech]

Mocé E, Graham JK (2008) *In vitro* evaluation of sperm quality. Anim Reprod Sci 105, 104-118

Muiño R, Fernández M, Peña AI (2007) Post-thaw survival and longevity of bull spermatozoa frozen with an egg yolk-based or two egg yolk-free extenders after an equilibration period of 18 h. Reprod Domest Anim 42, 305-311

Pace MM, Graham EF (1974) Components in egg yolk which protect bovine spermatozoa during freezing. J Anim Sci 39, 1144-1149

Říha J, Frelich J, Golda J, Vaněk D (1998) Alternative methods utilizing embryotransfer (ET) for the formation of beef cattle herds. Arch Tierz 41, 345-357

Rodríguez-Martínez H (1998) Optimization of sperm quality in AI bulls. Reprod Domest Anim 33, 233-237

Saragusty J, Gacitua H, Zeron Y, Rozenboim I, Arav A (2009) Double freezing of bovine semen. Anim Reprod Sci 115, 10-17

Siddique M, Ali R, Raza A (2006) Effect of buffers on freezing of buffalo bull semen. J Agric Soc Sci 2, 117-119

Stádník L, Ježková A, Vacek M (2008) Factors affecting sperm survival in cervical mucus and pregnancy rates of OVSYNCH-treated Holstein cows. Sci Agric Bohem 39, 24-30

Štolc L, Stádník L, Ježková A, Louda F (2009) Relationships among herd, ram breeds, age of rams, sperm density before diluting and sperm motility during thermal survival test. Acta Univ Agric et Silvic Mendel Brun 57, 109-116

Stradaioli G, Noro T, Sylla L, Monaci M (2007) Decrease in glutathione (GSH) content in bovine sperm after cryopreservation: comparison between two extenders. Theriogenology 67, 1249-1255

Strapák P, Juhás P, Strapáková E, Halo M (2010) Relation of the length of productive life and the body conformation traits in Slovak Simmental breed. Arch Tierz 53, 393-402

Thun R, Hurtado M, Janett F (2002) Comparison of Biociphos-Plus® and TRIS-egg yolk extender for cryopreservation of bull semen. Theriogenology 57, 1087-1094

Wolf J, Smital J (2009) Effects in genetic evaluation for semen traits in Czech Large White and Czech Landrace boars. Czech J Anim Sci 54, 349-358

Received 18 July 2011, accepted 16 November 2011.

Corresponding author:

Jan Beran
email: beranj@af.czu.cz

Department of Animal Husbandry, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 21, Prague 6 – Suchdol, Czech Republic