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

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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, Strapáč 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), FeCO₃ (8 200 mg), CuSO₄. 5H₂O (600 mg), MnO
(3 000 mg), ZnO (5 500 mg), Ca(IO₃)₂ (45 mg), Co(CH₃COO)₂ (45 mg), Na₂SeO₃ (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 0.7×10⁶ 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
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(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 Spectinomycine).

Polyvinyl chloride (PVC) straws (0.25 cm³; 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 10⁶ mm⁻³, motility of sperm before and after freezing then thawing in percent, live:dead sperm as a percentage rate), \( \mu \) 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

<table>
<thead>
<tr>
<th>Trait</th>
<th>Model</th>
<th>R²</th>
<th>P</th>
<th>F-test</th>
<th>Bull</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>VOL</td>
<td></td>
<td>0.37</td>
<td>&lt;0.01</td>
<td>14.52</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>DEN</td>
<td></td>
<td>0.39</td>
<td>&lt;0.01</td>
<td>13.08</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>ACT</td>
<td></td>
<td>0.47</td>
<td>&lt;0.01</td>
<td>21.81</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>L₁</td>
<td></td>
<td>0.14</td>
<td>0.0248</td>
<td>3.14</td>
<td>0.0302</td>
<td></td>
</tr>
<tr>
<td>D₁</td>
<td></td>
<td>0.13</td>
<td>0.036</td>
<td>3.04</td>
<td>0.034</td>
<td></td>
</tr>
</tbody>
</table>

VOL: volume of ejaculate, DEN: density of sperm, ACT: activity of sperm, L₁ and D₁: 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

<table>
<thead>
<tr>
<th>Trait</th>
<th>Model</th>
<th>R²</th>
<th>P</th>
<th>F-test</th>
<th>Bull</th>
<th>Extender</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIT0</td>
<td></td>
<td>0.11</td>
<td>&lt;0.01</td>
<td>6.82</td>
<td>0.0002</td>
<td>3.95</td>
<td>0.0087</td>
</tr>
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<td>PHY0</td>
<td></td>
<td>0.14</td>
<td>&lt;0.01</td>
<td>11.49</td>
<td>&lt;0.01</td>
<td>2.54</td>
<td>0.0563</td>
</tr>
<tr>
<td>CIT30</td>
<td></td>
<td>0.13</td>
<td>&lt;0.01</td>
<td>9.11</td>
<td>&lt;0.01</td>
<td>3.86</td>
<td>0.01</td>
</tr>
<tr>
<td>PHY30</td>
<td></td>
<td>0.13</td>
<td>&lt;0.01</td>
<td>11.38</td>
<td>&lt;0.01</td>
<td>1.59</td>
<td>0.1906</td>
</tr>
<tr>
<td>CIT60</td>
<td></td>
<td>0.13</td>
<td>&lt;0.01</td>
<td>9.88</td>
<td>&lt;0.01</td>
<td>4.19</td>
<td>0.0063</td>
</tr>
<tr>
<td>PHY60</td>
<td></td>
<td>0.12</td>
<td>&lt;0.01</td>
<td>11.73</td>
<td>&lt;0.01</td>
<td>1.1</td>
<td>0.3497</td>
</tr>
<tr>
<td>CIT90</td>
<td></td>
<td>0.13</td>
<td>&lt;0.01</td>
<td>10.53</td>
<td>&lt;0.01</td>
<td>4.71</td>
<td>0.0031</td>
</tr>
<tr>
<td>PHY90</td>
<td></td>
<td>0.14</td>
<td>&lt;0.01</td>
<td>14.39</td>
<td>&lt;0.01</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>CIT120</td>
<td></td>
<td>0.15</td>
<td>&lt;0.01</td>
<td>10.86</td>
<td>&lt;0.01</td>
<td>7.18</td>
<td>0.0001</td>
</tr>
<tr>
<td>PHY120</td>
<td></td>
<td>0.15</td>
<td>&lt;0.01</td>
<td>15.94</td>
<td>&lt;0.01</td>
<td>1.14</td>
<td>0.333</td>
</tr>
<tr>
<td>L₂</td>
<td></td>
<td>0.12</td>
<td>&lt;0.01</td>
<td>7.86</td>
<td>&lt;0.01</td>
<td>3.25</td>
<td>0.0222</td>
</tr>
<tr>
<td>D₂</td>
<td></td>
<td>0.12</td>
<td>&lt;0.01</td>
<td>7.05</td>
<td>&lt;0.001</td>
<td>3.96</td>
<td>0.0085</td>
</tr>
</tbody>
</table>

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, L₂ and D₂: 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 cm³, while Karoui et al. (2011) assessed the overall mean volumes of bull ejaculates 5.49 cm³. Hafez & Hafez (2000) stated the minimum volume of bull ejaculate suitable for long-term preservation 3 cm³ in adult bulls and 2 cm³ in young bulls.
Table 3

<table>
<thead>
<tr>
<th>Trait</th>
<th>Trait A (n=160)</th>
<th>Trait B (n=160)</th>
<th>Trait C (n=160)</th>
<th>Trait D (n=160)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSM±SE</td>
<td>LSM±SE</td>
<td>LSM±SE</td>
<td>LSM±SE</td>
</tr>
<tr>
<td>VOL, g</td>
<td>5.2±0.40&lt;sup&gt;CD&lt;/sup&gt;</td>
<td>4.4±0.39&lt;sup&gt;CD&lt;/sup&gt;</td>
<td>3.3±0.39&lt;sup&gt;AD&lt;/sup&gt;</td>
<td>6.9±0.39&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN, 10&lt;sup&gt;6&lt;/sup&gt;mm&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>1.1±0.08&lt;sup&gt;CD&lt;/sup&gt;</td>
<td>1.3±0.08&lt;sup&gt;AD&lt;/sup&gt;</td>
<td>1.5±0.08&lt;sup&gt;AD&lt;/sup&gt;</td>
<td>0.9±0.08&lt;sup&gt;AC&lt;/sup&gt;</td>
</tr>
<tr>
<td>ACT, %</td>
<td>89.3±1.45&lt;sup&gt;BCD&lt;/sup&gt;</td>
<td>75.9±1.44&lt;sup&gt;A&lt;/sup&gt;</td>
<td>78.9±1.44&lt;sup&gt;AD&lt;/sup&gt;</td>
<td>74.3±1.44&lt;sup&gt;AC&lt;/sup&gt;</td>
</tr>
<tr>
<td>L&lt;sub&gt;1&lt;/sub&gt;, %</td>
<td>76.7±2.06&lt;sup&gt;AE&lt;/sup&gt;</td>
<td>68.9±2.06&lt;sup&gt;AC&lt;/sup&gt;</td>
<td>74.7±2.06&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>70.4±2.05&lt;sup&gt;AE&lt;/sup&gt;</td>
</tr>
<tr>
<td>D&lt;sub&gt;1&lt;/sub&gt;, %</td>
<td>22.8±2.03&lt;sup&gt;AE&lt;/sup&gt;</td>
<td>31.1±2.03&lt;sup&gt;AC&lt;/sup&gt;</td>
<td>25.3±2.03&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>28.0±2.02&lt;sup&gt;AE&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c,d</sup>P<0.05,  <sup>A,B,C,D</sup>P<0.01, different letters confirm statistical significance, VOL: volume of ejaculate, DEN: density of sperm, ACT: activity of sperm, L<sub>1</sub> and D<sub>1</sub>: 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<sup>6</sup>mm<sup>-3</sup>) and the highest in sire C (1.5×10<sup>6</sup>mm<sup>-3</sup>). 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<sup>6</sup>mm<sup>-3</sup>, respectively from 0.3 to 3.815×10<sup>6</sup>mm<sup>-3</sup>. The minimum requirement for sperm density in relation to subsequent processing is 0.7×10<sup>6</sup>mm<sup>-3</sup> (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 22.7 %.

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

<table>
<thead>
<tr>
<th>Trait, %</th>
<th>A (n=160)</th>
<th>B (n=160)</th>
<th>C (n=160)</th>
<th>D (n=160)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSM±SE</td>
<td>LSM±SE</td>
<td>LSM±SE</td>
<td>LSM±SE</td>
</tr>
<tr>
<td>CIT0</td>
<td>53.2±2.12cd</td>
<td>52.7±2.06c</td>
<td>39.3±2.06abd</td>
<td>46.3±2.06ac</td>
</tr>
<tr>
<td>PHY0</td>
<td>54.3±2.07cd</td>
<td>50.0±2.12cd</td>
<td>40.0±2.12ab</td>
<td>46.2±2.12ac</td>
</tr>
<tr>
<td>CIT30</td>
<td>49.4±2.05cd</td>
<td>47.2±2.04a</td>
<td>35.4±2.04bd</td>
<td>43.7±2.04ac</td>
</tr>
<tr>
<td>PHY30</td>
<td>51.1±1.98cd</td>
<td>48.9±1.98c</td>
<td>36.1±1.98bd</td>
<td>43.7±1.98ac</td>
</tr>
<tr>
<td>CIT60</td>
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<td>40.6±1.96c</td>
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<td>37.1±1.96ac</td>
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<tr>
<td>PHY60</td>
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<td>29.8±1.89bd</td>
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</tr>
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<td>38.1±1.92cd</td>
<td>33.4±1.91c</td>
<td>23.3±1.91bd</td>
<td>30.9±1.91ac</td>
</tr>
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<td>PHY90</td>
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</tr>
<tr>
<td>CIT120</td>
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<td>48.8±1.65cd</td>
<td>42.9±1.65bd</td>
<td>53.3±1.65pc</td>
</tr>
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<td>D2</td>
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<td>51.1±1.67cd</td>
<td>56.3±1.67bd</td>
<td>46.4±1.67bc</td>
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</tbody>
</table>

$A,B,C,D$ $P<0.05$, $A,B,C,D,p<0.01$, 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, L2 and D2: 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%), Tri Ladyl (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

<table>
<thead>
<tr>
<th>Trait, %</th>
<th>AndroMed (n=160)</th>
<th>Bioxcell (n=160)</th>
<th>Triladyl (n=160)</th>
<th>Optidyl (n=160)</th>
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</thead>
<tbody>
<tr>
<td>CIT0</td>
<td>47.4±2.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.5±2.11&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>48.4±2.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.6±2.11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
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<td>44.1±2.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>48.6±2.05</td>
<td>52.1±2.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
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<td>44.1±2.04</td>
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<td>48.1±2.04&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>41.6±1.97&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>47.6±1.97&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CIT60</td>
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<td>33.4±1.96&lt;sup&gt;d&lt;/sup&gt;</td>
<td>38.7±1.96</td>
<td>43.0±1.96&lt;sup&gt;cd&lt;/sup&gt;</td>
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<td>CIT90</td>
<td>30.3±1.91&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>36.3±1.91&lt;sup&gt;cd&lt;/sup&gt;</td>
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<td>CIT120</td>
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<td>45.8±1.65&lt;sup&gt;cd&lt;/sup&gt;</td>
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<td>50.4±1.65&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>D&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>54.2±1.65&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>47.1±1.67&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>48.1±1.67&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c,dP<0.05, A,B,C,D P<0.01</sup>, 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<sub>2</sub> and D<sub>2</sub> 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 Stradaioi 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.

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