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Development of bovine embryos after intracytoplasmic sperm injection (ICSI): effect of gamete donors, sperm chromatin structure and activation treatment

Vývoj bovinních embryí po intracytoplasmatické injikaci spermatu (ICSI): vliv dárce gamet, chromatinové struktury spermatu a aktivačního ošetření

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ABSTRACT: This work was undertaken to study the effect of gamete donors, sperm chromatin structure and activation procedure on ICSI outcome. Four experiments were carried out. In Experiment 1, oocytes recovered from six donor cows by the ovum pick-up method (OPU) were matured and injected with the spermatozoa of one bull. The cleavage rate between individual donor cows ranged from 15.4 to 43.7%. Development of embryos to the M/B stages was observed in the case of three donor cows (4.3%, 6.7% and 21.4%, respectively). Embryos from the remaining three donor-cows did not reach this developmental stage. In Experiment 2, the oocytes recovered by OPU or from slaughtered animals were injected with spermatozoa from one of two bulls (A or B). The cleavage ratio was 15/28 (53.6%) and 7/53 (13.2%) for bulls A and B, respectively. The M/B stages were reached by 3/15 embryos (10.7%) and 1/7 embryo (1.9%). In Experiment 3, the IVM oocytes derived from abattoir ovaries were injected with the spermatozoa of two bulls (C or D) differing in their chromatin structure. The cleavage ratio was 31/98 (31.8%) and 36/83 (43.4%), respectively for oocytes injected with spermatozoa from bulls C and D. The M/B stages were reached by 8/98 embryos (8.2%) and 11/83 (13.2%), respectively for cleaved embryos derived from oocytes injected with sperms of bulls C and D. No relationship between developmental potential of embryos and sperm chromatin structure was observed. In Experiment 4, two different agents, either ethanol or calcium ionophore A23187, were used for the activation treatment of injected oocytes. The cleavage ratio for oocytes treated with A23187 and ethanol was relatively high in comparison with the control group (45.2% and 23.1% vs. 5.2%, respectively). The rate of development to the M/B stages was the highest for those activated with A23187 (11.6%), followed by ethanol (3.8%) and non-activated oocytes (0.0%). These results indicate that there are individual variations between bovine gametes from both male and female donors, regarding their development after ICSI. The quality of sperm chromatin structure did not affect the ICSI outcome. Moreover, though the artificial activation treatment of injected oocytes increased the developmental competence of embryos, however, the cleavage ratio and blastocyst yield still remain low.

Keywords: cattle; oocyte; sperm; ICSI; activation; gamete effect; chromatin structure

ABSTRAKT: V této práci jsme sledovali vliv dárců gamet, chromatinové struktury spermatu a metody aktivace na výsledek ICSI. Uskutečnili jsme čtyři pokusy. V 1. pokusu oocyty získané metodou OPU od šesti dojnic dárkyň byly po dozrání injikovány spermiemi jednoho býka. Poměr štěpení se pohyboval u jednotlivých dárkyň od 15.4 do 43.7%. Vývoj embryí do stadia M/B jsme zjistili u tří dárkyň (4,3 %, 6,7 % resp. 21,4 %). Embrya ostatních tří dárkyň tohoto vývojového stadia nedosáhla. Ve 2. pokusu jsme oocyty získané metodou OPU nebo z poražených zvířat injikovali spermiemi jednoho ze dvou býků (A nebo B). Poměr štěpení činil 15/28 (53,6 %) a 7/15 (13,2 %) u býka A resp. B. Stadia M/B

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dosáhlo 3/15 embryí (10,7 %) a 1/7 embryí (1,9%). Ve 3. pokuse jsme injikovali oocyty IVM získané z vaječníků poražených zvířat spermiemi dvou býků (C nebo D), které se lišily svou chromatinovou strukturou. Poměr štěpení činil 31/98 (31,8 %) resp. 36/83 (43,4%) u oocytů injikovaných spermiemi býka C a D. Stadia M/B dosáhlo 8/98 embryí (8,2 %) resp. 11/83 embryí (13.2 %) u štěpených embryí získaných z oocytů injikovaných spermiemi býka C a D. Nezjistili jsme žádnou závislost mezi vývojovým potenciálem embryí a chromatinovou strukturou spermií. Ve 4. pokusu jsme pro aktivační ošetření injikovaných oocytů použili dvě různá činidla, etanol nebo iontofor kalcia A23187. Poměr štěpení u oocytů ošetřených činidlem A23187 a etanolem byl ve srovnání s kontrolní skupinou relativně vysoký (45.2 % a 23.1 % versus 5,2 %). Rychlost vývoje do stádia M/B byla nejvyšší u oocytů aktivovaných A23187 (11,6 %), následovaly oocyty ošetřené etanolem (3,8 %) a neaktivované oocyty (0,0 %). Výsledky naznačují, že existují individuální rozdíly mezi bovinními gametami od samčích a samičích dárců, pokud se jedná o jejich vývoj po ICSI. Kvalita chromatinové struktury spermií neovlivnila výsledek ICSI. Ačkoliv umělé aktivační ošetření injikovaných oocyt zvýšilo schopnost vývoje embryí, poměr štěpení a výtěžnost blastocyst jsou stále nízké.

Klíčová slova: skot; oocyt; sperma; ICSI; aktivace; vliv gamet; chromatinová struktura

The technique of direct injection of single spermatozoa into cytoplasm of matured oocytes (ICSI) bypasses important events that occur during normal fertilization. The process of sperm binding to the *zona pellucida* and the subsequent penetration of the *zona pellucida* and vitelline membrane are eliminated in the case of ICSI. In humans, the procedure of ICSI is increasingly applied in *in vitro* technology because no special treatment of sperm is necessary for achieving a moderately high rate of fertilization after injection. Therefore, this technique can be useful in male factor infertility (Van Steirteghem *et al.*, 1993; Palermo *et al.*, 1995; Payne and Matthews, 1995). By contrast, the effectiveness of ICSI in cattle remains unsatisfactory, with a relatively low cleavage ratio and limited developmental competences. Although the first ICSI calf was born by 1990 (Goto *et al.*, 1990), some problems involving ICSI in cattle have not been resolved over the last decade. However, recent results (Horiuchi *et al.*, 2000, 2001) promise to be developed. The purpose of this study was to determine the developmental competence of bovine embryos resulting from ICSI, considering the effect of male and female gamete donors, as well as chromatin structure and activation treatment on ICSI outcome.

MATERIAL AND METHODS

Collection and maturation of oocytes

The oocytes were collected using the ovum pick-up (OPU) method from non-stimulated cows or standard techniques from ovaries of slaughtered heifers and cows. The procedure of transvaginal follicular aspiration was conducted on 6 non-stimulated cows once or twice a week. The puncturing of the follicles was performed by the use of a specific aspiration probe (Model Mariensee), a 6.5 MHz fingertip probe (Picker International), and the Picker CS9000 ultrasound machine, allowing for a resolution of approximately 2 mm.

The ovaries of slaughtered heifers and cows were transported to the laboratory within two hours of recovery. The ovaries were washed three times in warm PBS (BioMed, Lublin, Poland) solution supplemented with kanamycin (Sigma, St. Louis, USA). Cumulus-oocyte complexes (COC's) were recovered from ovaries following the isolation and subsequent rupture of antral follicles > 2 mm in diameter in TCM199 (Sigma, St. Louis, USA) manipulation medium (Earle's salt with 25mM HEPES) containing 10% fetal bovine serum (FBS). Oocytes with compact and dense *cumulus oophorus* and evenly granulated cytoplasm were cultured in 2 ml TCM199 (Earle's salt, buffered with 26.2 mM sodium bicarbonate), supplemented with estrous cow serum and additional 3 to 5 × 10^6/ml granulosa cells. Oocytes were cultured for 23 to 24 hours at 39°C under 5% CO_2 in air and at maximum humidity (Kątska *et al.*, 1995). After maturation COCs were washed three times with washing HEPES-buffered TCM-199 medium. Next, COCs were treated with hyaluronidase (1 mg/ml; Sigma) for about 60 seconds followed by a rinsing of HEPES-buffered TCM199 supplemented with 10% of FBS and mechanically denuded from surrounding cumulus cells with a fine bore hand-pulled glass pipette.

Sperm preparation

For the ICSI experiments, frozen-thawed cauda-epididymal spermatozoa obtained from two bulls (A and B) and ejaculated semen obtained from two bulls (C and D) were used. Straws of frozen semen were thawed in a 35°C water bath. Motile spermatozoa were obtained by discontinuous density gradient centrifugation (90% : 45% Percoll; Lazzari and Gali, 1994). Spermatozoa were layered on the top of a 45% Percoll solution in a TALP capacitation medium (Parrish *et al.*, 1988) in a centrifuge tube. The samples were centrifuged for 30 minutes at 250 × g and the supernatant was removed.

Sperm chromatin analysis

Ejaculated sperm was derived from two selected bulls (C and D) that varied according to the percentage of chromatin damage (1.62 and 23.8% respectively for C and D bull). The semen was thawed in a water bath at 37°C and expelled from straws into 1 ml PBS solution at 37°C. To remove the extender the samples were centrifuged at 600 × G for 10 min. After extracting the supernatant, PBS was added up to 1 ml of sample volume. After determining the sperm concentration in Bürker chamber, the samples were diluted in PBS to contain 1×10^6/ml spermatozoa. The two-steps SCSA protocol was followed for chromatin denaturation and staining (Evenson *et al.*, 1999). 0.4 ml of solution I (0.1 ml Triton X-100 + 8.0 ml HCl 1.0 N + 0.877 g NaCl + H_2O up to 100 ml) and, after 30 sec, 1.2 ml of solution II (0.6 ml acridine orange + 34.0 mg EDTA-Na_2 + 0.877 g NaCl + 100 ml citric-phosphate buffer) was added to 0.2 ml of prepared semen. After 3 min this mixture was proceeded to the cytometric analysis. Staining procedure was carried out at 4°C.

Flow Cytometer

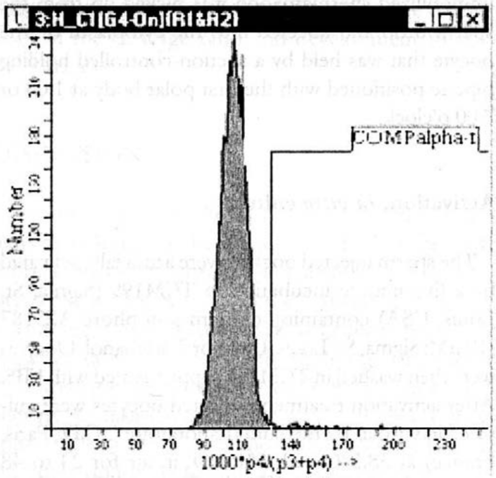
A Coulter Epics Elite (Coulter, Miami, USA) flow cytometer was used. Acridine orange was excited at 488 nm and 35 mW laser beam. Fluorescence was read out through 550DL and 525BP filters in the green band and through 675BP filter in the red band. The parameters measured were forward scatter, side scatter and fluorescence in the green and red bands (linear scales). To standardize all analyses, the same frozen/thawed ejaculate was used to calibrate the cytometer, and the signal of its fluorescence was set on the same level. Calibration was checked every 30 min.

Semen sample analyses were saved in listmode files as measurements of the fluorescence of 5 000 spermatozoa.

Data Analysis

Listmode files were analysed using WinList32 (Verity SH, Topsham, USA) software. Debris and cellular contaminations were gated out on a red vs. green fluorescence cytogram. The number of spermatozoa with structurally abnormal chromatin was analysed on the basis of an artificial parameter α_t calculated for each spermatozoon, where α_t = red/(green + red) fluorescence (Figure 1).

Bull C; COMPat = 1.62%



Bull D; COMPat = 23.8%

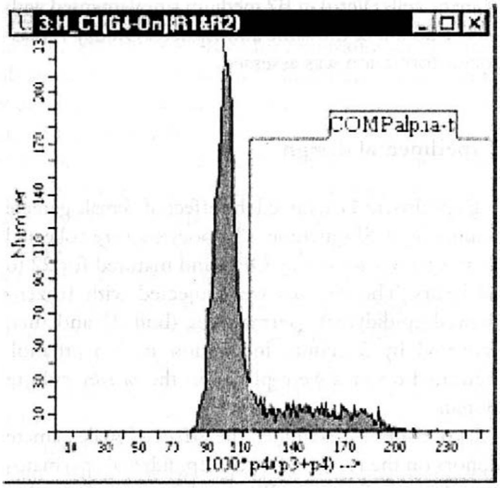


Figure 1.

ICSI procedure

The microinjection procedure was carried out using the inverted microscope (Olympus IMT-2, Tokyo, Japan) equipped with Nomarski interference contrast optics and fitted with two arms of the Leitz micromanipulators (Leitz, Wetzlar, Germany) and microsyringe systems. Oocytes were placed in a drop (20 μl) of injection medium (HEPES-buffered TCM199 supplemented with 10% of FBS) covered with paraffin oil. A small volume (1–2 μl) of sperm suspension was added to another drop containing 10% polyvinylpyrrolidone (PVP-360: Sigma, St. Louis, USA). Single immobilized spermatozoon was picked up from the sperm drop and injected into the cytoplasm of the oocyte that was held by a suction-controlled holding pipette positioned with the first polar body at 1:00 or 7:00 o'clock.

Activation, *in vitro* culture

The sperm injected oocytes were artificially activated by a five minute incubation in TCM199 (Sigma, St. Louis, USA) containing calcium ionophore A23187 (10 μM; Sigma, St. Louis, USA) or 7% ethanol. Oocytes were then washed in TCM199 supplemented with FBS. After activation treatment, injected oocytes were cultured in vitro in B2 medium (Laboratoire C.C.D., Paris, France) at 38.5°C with 5% CO_2 in air for 24 to 48 hours. The cleaved embryos were co-cultured with somatic cells (Vero) in B2 medium supplemented with 10% FBS under the same atmosphere. On day 7, blastocyst formation was assessed.

Experimental design

Experiment 1 examined the effect of female gamete donors on ICSI outcome. The oocytes were collected from six donor-cows by OPU and matured for 22 to 24 hours. The oocytes were injected with frozen-thawed epididymal spermatozoa (bull A) and then activated by 5-minute incubation in 7% ethanol. Activated oocytes were placed in the *in vitro* culture system.

Experiment 2 examined the effect of male gamete donors on the results of ICSI. Epididymal spermatozoa of two bulls (A and B) with differing IVF fertilizability were used for injection. The oocytes collected both from donor cows by OPU (as a mix population) and from slaughtered cows were used after 22 to 24 hours of *in vitro* maturation. Injected oocytes were activated by 5-minute incubation in 7% ethanol and then placed in the *in vitro* culture system.

Experiment 3 investigated the effect of chromatin damage of injected spermatozoa on the results of ICSI. For the experiment, oocytes were collected from slaughtered heifers/cows and ejaculated sperm with 1.62% (bull C) and with 23.8% (bull D) of chromatin damage was used. Injected oocytes were activated by 5-minute incubation in calcium ionophore (10 μM A23187) and then placed in the *in vitro* culture system.

Experiment 4 investigated the effect of an artificial activation treatment of oocytes after ICSI. The oocytes were collected from slaughtered heifers or cows and, after 22 to 24 hour maturation *in vitro* were used for injection with epididymal spermatozoa. Epididymal spermatozoa were obtained from the *caudae epididymides* of bull A. Injected oocytes were activated by 5-minute of incubation in 7% ethanol or calcium ionophore (10 μM A23187) and then placed in the *in vitro* culture system. Unactivated oocytes served as controls.

RESULTS

Experiment 1. The results of sperm injection using epididymal spermatozoa of bull A and oocytes derived by OPU from six individual donor cows are shown in Table 1.

The cleavage ratio ranged from 15.4% to 43.7% between the donor-cows. The differences were not statistically significant. In the case of three donor cows, the obtained embryos developed to the morula/blastocyst stages.

Experiment 2. The results of ICSI using the epididymal sperm from two different bulls (A and B) are shown in Table 2. Statistically significant differences

Table 1. The *in vitro* development of embryos after ICSI using oocytes derived by OPU

Donor-cow	No. injected oocytes/No.repl.	No. cleaved (%)	No. M/B (%)
168	23/7	10 (43.5)	1 (4.3)
336	16/3	7 (43.7)	0
337	45/8	10 (22.2)	3 (6.7)
338	13/6	2 (15.4)	0
378	10/4	2 (20.0)	0
384	14/5	6 (42.8)	3 (21.4)

The values within column do not differ ($P > 0.05$, test χ^2)

Table 2. The *in vitro* development of embryos after ICSI or IVF using spermatozoa from two bulls with differing IVF fertility

Fertilization technique	No. injected oocytes/No. repl.	Bull	No. cleaved (%)	No. M/B (%)
ICSI	28/3	A	15 (53.6)^{a}	3 (10.7)^{e}
	53/3	B	7 (13.2)^{b}	1 (1.9)^{e}
IVF (control)	355/5	A	300 (84.5)^{c}	102 (28.7)^{f}
	239/4	B	67 (28.1)^{d}	0

Statistical comparisons were done within treatment groups between bulls and for the same bull between fertilization technique. The values within a column with different superscripts differ as follows: ab, ac, cd ($P < 0.001$); bd, ef ($P < 0.05$)

Experiment 3. The results of ICSI using ejaculated sperm with the cytometrically evaluated chromatin of two bulls (C and D) are shown in Table 3. The spermatozoa of bull C were characterized by 1.62% chromatin damage and those of bull D by 23.8% chromatin damage. No statistically significant difference was found between the two bulls in either the cleavage ratio or development to the M/B stage.

Table 3. The *in vitro* development of embryos after ICSI using spermatozoa from two bulls with different chromatin damage

Bull	No. injected oocytes/No repl.	No. cleaved (%)	No. M/B (%)
C	83/3	36 (43.4)^{a}	11 (13.2)^{a}
D	98/3	31 (31.6)^{a}	8 (8.2)^{a}

The values within columns with the same superscript do not differ ($P > 0.05$)

Experiment 4. The results of ICSI after activation of injected oocytes with two activation agents are shown in Table 4. The cleavage ratio of embryos after activation with A23187 and ethanol were higher than that of unactivated oocytes (45.2% and 23.1% vs. 5.2%). Statistically significant differences were found both in the cleavage ratio and development to the M/B stages.

Table 4. The effect of the activation method on the developmental competence of embryos after ICSI

Activation agent	No. injected oocytes/No repl.	No. cleaved (%)	No. M/B (%)
7% ethanol	77/3	13 (16.9)^{a}	3 (3.9)^{d}
10 μM A23187	43/3	19 (44.2)^{b}	5 (11.6)^{d}
Unactivated	58/4	3 (5.2)^{c}	0

The values within a column with different superscripts differ as follows: a, b ($P < 0.01$); a, c ($P < 0.05$); b, c ($P < 0.001$)

DISCUSSION

The present study shows the effect of individual gamete donors on the frequency of cleavage and morula/blastocyst formation in the population of embryos obtained after ICSI. In the first experiment, the injected oocytes derived from all six heifers/cows used as gamete donors cleaved but the percentage of cleaved embryos varied between the cows (Table 1). The development to morula/blastocyst stages, however, was found only in the case of the embryos derived from the three donor cows. This suggests that there may exist an individual developmental competence after fertilization, depending on the oocyte donors. It may limit the application of ICSI for fertilization of oocytes obtained by OPU. However, this limitation was not found by the other authors. Gibbson *et al.* (1995) believed that, in comparison with commercially slaughtered oocytes, transvaginal follicular aspiration can provide a more consistent and synchronous population of oocytes and can be used with the *in vitro* system. According to Oikawa (2001), the combination of ICSI with OPU can be an excellent method for *in vitro* embryo production. In Experiment 2, the sperm used for ICSI was derived from two different bulls and was tested earlier by the standard IVF method (Table 2). The outcome of IVF with spermatozoa of bull A or B found corroboration with the results after ICSI. Contrary to these conclusions, the findings of Wei and Fukui (1999) indicate that the fertilizability of sperm in ICSI is not coincident with their performance in IVF, so the authors suggest that the fertilization rate in IVF is not a useful parameter for bull fertilizability in ICSI. According to Horiuchi *et al.* (2000), the cleavage and blastocyst ratios following ICSI were not different from those following IVF in individual bulls. In Experiment 3, the sperm used for ICSI was derived from two bulls differing in the quality of their sperm chromatin. The results obtained were rather surprising (Table 3). In spite of significant differences in the percentage of chromatin damage between the bulls, developmental competence of embryos after ICSI with both categories of sperm was statistically non-significant. However, cleavage and blastocyst ratios were slightly lower after the injection of poorer sperm. This suggests that the negative effect of damaged chromatin structure is not yet observable during preimplantation development. However, this does not rule out the possibility of developmental anomalies at a later time after implantation, as indicated by the results of human semen studies which demonstrated that the presence of more than 30% of spermatozoa with damaged chromatin results in infertility while damage in the 15 to 30% range is responsible for lower fertility (Evenson, 1990; Larson *et al.*, 2000). Such studies were not performed in cattle. Cytometric analysis of bull sperm chromatin structure makes it possible to eliminate those ejaculates that have a high rate of sperm with damaged chromatin (Bochenek *et al.*, 2001). In Experiment 4, a relationship was found between the artificial activation of injected oocytes and the developmental competence of embryos after ICSI. Out of the two activation agents (A23187 and 7% ethanol) activation with Ca^{++} ionophore seems more effective (Table 4). The differences were especially significant with reference to the non-activated batch of oocytes. According to Rho *et al.* (1998), a satisfactory rate of oocyte activation was achieved using ionomycin plus DMAP protocol, though often resulting in abnormal embryos. The recent investigation of Horiuchi *et al.* (2001) indicated that a later period of activation after microinjection enhanced the ICSI results. Nevertheless, in other species such as rabbits (Deng and Yang, 2001), monkeys (Fujinami *et al.*, 2001), mice (Yanagimachi, 1998) and humans (Palermo *et al.*, 1995), additional activation treatment was unnecessary to obtain satisfactory results after ICSI. It is clear, however, that in cattle artificial oocyte activation, along with special pretreatment of sperm before injection (Rho *et al.*, 1998), is necessary to achieve better ICSI results.

We concluded from this study that:

1. There are individual variations between bovine gametes from both male and female donors, regarding their development.

2. The quality of sperm chromatin structure did not affect the ICSI outcome.

3. The artificial activation treatment of injected oocytes increased the developmental competence of embryos though the cleavage ratio and blastocyst yield remain still low.

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Production of embryos from repeated superovulations of cows during one calving interval

Produkce embryí z opakovaných superovulací krav v průběhu jednoho mezidobí

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ABSTRACT: The objective of the present study was to acquire information about quantitative and qualitative embryo production from cows of milked cattle breeds during multiple superovulations during one calving interval. For this purpose an experiment was established in three herds of Czech Pied cattle and two Holstein herds. The cows with above-average milk efficiency, the potential donors of embryos, were temporarily excluded from the natural reproduction process and controlled superovulation was performed in 2 to 4-month intervals. A total of 292 repeated superovulations were performed in 82 cows of both breeds, obtaining 2 444 embryos, of which 1 645 were transferable. The number of superovulations was not the same for all the selected cows; 82 1st and 2nd superovulations were conducted, 54 3rd superovulations, 40 4th superovulations, 21 5th superovulations and 8 6th superovulations. From 6 superovulations an average of 6.82 embryos was obtained, of which 4.59 were transferable. Most of the transferable embryos (6.16) were obtained in the 1st superovulation but also in the first four superovulations (5.16–6.16 embryos). The entire yield of embryos from all the superovulations was very variable. When we evaluated the effects influencing the production of transferable embryos we discovered that the combined effect of the herd-year-season – HYS ($P \leq 0.01$) was significant. The effect of the number of lactation, number of superovulation and the effect of milk efficiency in the previous lactation expressed by the herd index were all statistically insignificant. We discovered that the yield of transferable embryos from the first superovulation was largely affected by the individuality of the cow (the yield of embryos was variable and ranged between 0 and 27). The period between 56 to 115 days after calving is considered to be the most suitable interval for the first superovulation after calving, and the next superovulations in the interval 2 to 3.5 months. The results of the first superovulations can be used to predict the success of the following superovulations, because the correlation coefficient $r = 0.710$ ($P \leq 0.01$) was found between the n and the $n + 1$ superovulation. The correlation coefficient between the first and the following superovulations was statistically significant until the 3rd flushing (1–2, $r = 0.669$, $P \leq 0.01$; 1–3, $r = 0.701$; $P \leq 0.01$).

Keywords: cattle; Holstein; Czech Pied; repeated superovulation; embryo production; factors affecting embryo production

ABSTRAKT: Cílem předložené práce bylo získat poznatky o kvantitativní a kvalitativní produkci embryí od krav dojených plemen skotu při opakovaných superovulacích v průběhu jednoho mezidobí. Za tím účelem byl založen experiment ve třech stádech s chovem českého strakatého plemene a dvou stádech holštýnského plemene skotu. Potenciální dárkyně embryí – podle úrovně mléčné užitkovosti nadprůměrné plemenice – byly dočasně vyřazeny z přirozeného reprodukčního procesu a řízeně superovulovány ve dvou- až čtyřměsíčních intervalech. U 82 krav obou plemen bylo provedeno celkem 292 opakovaných superovulací při nichž bylo získáno celkem 2 444 embryí, z toho 1 645 přenosuschopných. U vybraných krav nebyl počet všech superovulací stejný. Prvních a druhých superovulací bylo provedeno 82, třetích 54, čtvrtých 40, pátých 21 a šestých 8. Ze šesti superovulací bylo v průměru získáno 6,82 embryí, z toho 4,59 přenosuschopných. Největší počet přenosuschopných embryí (6,16) byl získán v první superovulaci, ale také

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v prvních čtyřech superovulacích (5,16–6,16 ks). Celý soubor se vyznačoval velkou variabilitou zisku a kvality embryí ve všech superovulacích. Při posouzení vlivů působících na produkci přenosuschopných embryí byl zjištěn průkazný vliv sloučeného efektu stádo-rok-období otelení – HYS ($P \leq 0,01$). Statisticky neprůkazný se projevil vliv pořadí laktace, pořadí superovulace a vliv mléčné užitkovosti v předcházející laktaci vyjádřené indexem stáda. Zisk přenosuschopných embryí z první superovulace je ovlivněn individualitou dárkyně (variabilita rozpětí zisku embryí 0–27). Za nejvhodnější interval pro první superovulaci lze považovat období 56–115 dní po otelení a pro další superovulace intervaly 2 až 3,5 měsíců. Výsledky prvních superovulací je možné využít k předpovědi úspěchu v následujících superovulacích. Mezi n a $n + 1$ superovulací byl zjištěn korelační koeficient $r = 0,710$ ($P \leq 0,01$). Korelační koeficient mezi první a dalšími superovulacemi byl statisticky průkazný až po třetí výplachy (1–2, $r = 0,669$, $P \leq 0,01$; 1–3, $r = 0,701$; $P \leq 0,01$).

Klíčová slova: skot; holštýnské plemeno; české strakaté plemeno; opakovaná superovulace; produkce embryí; vlivy působící na produkci embryí

Superovulation of cows followed by embryo transfer (ET) is used, in the first place, for the production of top quality bullocks of potential sires that would have a positive influence on the production and genetic standard of whole cattle populations. Nowadays this method of using ET after insemination is well elaborated, described and is used across the globe in all countries with advanced cattle breeding where it has become a component of cattle breeding programmes. Embryo transfer can also be used for a more intensive production of female calves from selected dams and sires. Intentional formation of families enables targeted production of cattle herds of cow progeny of high breeding value. This way of using ET is important for the improvement of herds. In scientific literature and in practical breeding it is less known.

The focus of the present study is on multiple superovulations and the implementation of this method of ET for production and genetic improvement of cattle herds. The basic condition for implementing ET in cattle herds is to obtain as many high-quality embryos as possible from individual intentionally reproduced cows. This target can be reached through induced superovulation and particularly through induced multiple superovulations. The authors differ in their estimations of the average yield of embryos from one superovulation. In most cases it ranges between 6 and 11 embryos from one flushing, or 4 to 7 fertilisable embryos (Molina and Saturnino, 1993; Chagas *et al.*, 1993; Saner, 1994; Říha, 1996). One of the requirements for superovulation response in cows is the efficiency of the respective FSH preparations. Sergejev *et al.* (1991) compared the preparations used in Europe and found that the Czech Folicotropin, Spofa Praha is comparable with the other preparations. A higher number of embryos can be obtained from cows after repeated superovulation (Maslev *et al.*, 1990; Wichmann, 1990) but the cow must be temporarily excluded from the natural reproduction process. Maslev *et al.* (1990) recommended 5 superovulations in 60-day intervals that provide an average of 4.8–6.1 fertilisable embryos from one flushing. The highest number of embryos was obtained from the 1st and 4th flushing. Wichmann (1990) discovered that a number of other factors have an effect on whether the cow is suitable as a donor for embryo production; for instance, the interval between insemination and flushing, the time of inducing superovulation after calving, milk efficiency of the cows, age of the cows, number of flushings, effect of the herd and individuality of the cow. The author reported that the repeatability between the 1st to the 4th flushing was 0.35, 0.23 and 0.18. Říha (1996) obtained the best results in terms of the stimulation of yields and quality of embryos from donors inseminated during superovulation oestrus in the period between days 61 and 150 after calving. Říha (1997a,b) discovered that the best-quality embryos were obtained from donors of the Czech Pied cattle and from the Czech Pied × Ayrshire crossbreds. Žižlavský *et al.* (1997) concluded that planned formation of cattle herds using embryo transfer is possible.

MATERIAL AND METHODS

The objective of the present investigations was to consider whether quantitative and qualitative recovery of embryos from selected cows used for further production of heifer calves to produce families in cattle herds was possible. For this purpose we established an experiment with 82 cows from two Holstein herds and three herds of Czech Pied cattle. The selection of donors, i.e. potential embryo donors, was based on the milk efficiency, as a matter of fact on protein production in the maximal standardised lactation. The group of selected cows consisted of dairy cows after the 2nd to 7th calving. The average milk efficiency of 40 Holstein cows was 8 924 kg of milk per lactation (ranging between 7 122 and 11 526 kg) and average protein production was 296 kg (ranging between 238 and 350 kg). The average milk efficiency of the 42 Czech Pied cows was 6 854 kg of milk per lactation (ranging between 6 015 and 7 912 kg) and average protein production was 232 kg (ranging between 203 and 264 kg).

The cows were withdrawn from the conventional process of reproduction and controlled superovulation was conducted in 2 to 4 month intervals. The fertilisable embryos were transferred to the prepared recipient heifers of the same breed.

The first treatment of cows before superovulation was the examination of the reproductive organs. The donors were synchronised using two applications of a prostaglandin F_2 analogue (Chlorprostenol) in the preparations Oestrophan or Remophan produced by Léčiva, Prague, CR, in a dose corresponding to 500 μg of effective substance and administered in an interval of 11 days. Oestrus checking on the next day after the second treatment (3rd day) was indicated as day 0. The actual superovulation treatment began on day 9 of the sexual cycle when the condition and development of the *corpus luteum* was controlled. Folicotropin (Léčiva, Prague) in FSH was applied twice a day (7 and 19 h), the total dose was 560 IU in doses (morning, evening) 2.3 : 3.2 : 1.1 : 1.1 ampoules (1 ampoule = 40 IU of FSH). On the 3rd day of the regimen treatment, i.e. on day 12 of the sexual cycle, 500 and 250 μg of prostaglandin F_2 was applied together with FSH. The donors were inseminated and reinseminated on days 14 and 15 of the sexual cycle with one insemination dose from a selected sire. On the 7th day after the first insemination the non-surgical method of flushing with 300 ml of Krebs-Ringer phosphate with added BSA was used to recover the embryos. In the following morphological assessment the embryos unfit for transfer were discarded (unfertilised oocytes, degenerated embryos and embryos retarded in their development). Embryos in the morula, early blastocysts and expanded blastocysts stage were classified as suitable for transfer.

Basic statistical values were applied to evaluate the production of all embryos and of the fertilisable embryos. In order to assess other effects influencing the yields of transferable embryos during the superovulations the results of the representative part were interpreted by means of the multi-factorial analysis of variance using the GLM procedure of the SAS 6.12 statistical programme. The effects of the following factors were evaluated: herd, year, calving period; number of lactation, number of flushing and regression on the herd index in milk efficiency (fat and protein production). The following equation was used to estimate the above effects:

$$y_{ijkl} = \mu + A_i + B_j + C_k + \beta\,(x - x) + e_{ijkl}$$

where: y_{ijkl} = measured value
μ = mean value
A_i = fixed effect of herd – year – season (HYS)
B_j = fixed effect of number of lactation
C_k = fixed effect of number of flushing
$\beta\,(x - x)$ = regression on herd index
e_{ijkl} = residue

RESULTS AND DISCUSSION

In 82 selected cows a total of 292 repeated superovulations was performed when 2 444 embryos were recovered; the quality of 1 645 embryos was good and they were transferred. Table 1 gives the results of the superovulations.

Table 1. Embryo production from multiple superovulation

Number of super ovulation	Number of cows	Number of recovered embryos				Number of transferable embryos				Proportion of transferable embryos (%)
		x	*s*	*v*	max.	*x*	*s*	*v*	variation range	
1	82	10.94	6.58	60.15	0–31	6.16	0.67	67.86	0–27	56.3
2	82	8.32	7.88	94.71	0–30	5.51	0.91	114.34	0–25	66.2
3	59	7.96	6.53	82.04	0–27	5.70	1.12	87.02	0–16	71.6
4	40	7.35	6.27	85.31	0–22	5.16	1.21	86.24	0–13	70.2
5	21	3.56	3.52	98.88	0–9	2.98	1.20	107.38	0–8	83.7
6	8	2.82	2.72	96.45	0–7	2.01	1.02	142.29	0–6	71.3
Total	–	6.82	5.58	86.27	0–31	4.59	1.02	100.86	0–25	67.3

Table 1 presents the number of cows and number of superovulation after calving and the average and statistical values of the total number of recovered embryos and transferable embryos in the respective superovulations. The results show that 6.82 embryos were recovered on average from 6 superovulations, of which 4.59 were transferable, i.e. 67.3% suitable embryos. Most of the embryos, and the highest proportion of transferable embryos, were obtained in the first superovulation. The complete collection of embryos in the 6 repeated superovulations was very variable. The relatively lowest variability of recovered and transferable embryos was observed in the first superovulation. The maximal yield of fertilisable embryos, i.e. 27, was obtained from the first superovulation of the cow. The present results of embryo production from multiple superovulations are comparable with the conclusions of Saner (1994) in Switzerland and better than the results of Molina and Saturnino (1993), Chagas *et al.* (1993); indirectly they confirm the finding of Sergejev *et al.* (1991) that in terms of the superovulation response the preparation Folicotropin, Spofa Prague of Czech provenance is comparable with other world preparations. The observation of Maslev *et al.* (1990) that 5 superovulations in 60-day intervals were sufficient to obtain an average of 4.8 to 6.1 fertilisable embryos from one flushing has been confirmed only partly. It is true that our investigations proved that this number could be obtained, however, the yields from the individual flushings were more variable. The results of the first three superovulations are comparable with the results of Říha (1996). The results from the other superovulations are different.

The effects influencing the production of fertilisable embryos were estimated by means of the GLM (SAS 6.12) procedure. Table 2 presents the results of the *F*-test and the statistical significance of the analysed effects. Of all the studied effects on the total production of embryos in multiple superovulations the combined effect of the herd-ear-season – HYS ($P \leq 0.01$) and the number of lactation ($P \leq 0.05$) was statistically significant. The number of flushing was immediately beyond the level of significance ($P \leq 0.05$). The effect of the level of milk efficiency of the cows evaluated by the herd index was insignificant.

The production of good quality transferable embryos affects the actual result of embryo transfer more than the total production of embryos. From this point of view, compared with previous evaluations, only the combined effect of the herd, year and season was repeated. The other effects were found to be insignificant. Table 2 also evaluates the success of embryo

Table 2. Values of the *F*-test and statistical significance of the analysed effects

Effect	DF	Total embryos		Transferred embryos		Success	
		F-test	significance	*F*-test	significance	*F*-test	significance
HYS	29	3.16	0.0001^{++}	1.95	0.0070^{++}	0.97	0.5223
Number of lactation	5	2.34	0.0446^{++}	1.21	0.3093	1.01	0.4154
Number of flushing	5	2.25	0.0533	1.62	0.1598	2.81	0.0190^{+}
Herd index	1	0.30	0.5822	0.93	0.3379	0.32	0.5755

$^{+}P \leq 0.05$; $^{++}P \leq 0.01$

Table 3. Mean values of the parameters based on the effect of the number of flushing

Parameters		Number of flushing					
		1	2	3	4	5	≥6
	n	50	50	20	14	7	14
Flushing – flushing (days)	$\mu + a_i$	–	114.3	137.5	114.3	99.2	116.8
	$s_{\mu + ai}$	–	10.45	15.49	17.38	22.86	21.38
Total embryos (*n*)	$\mu + a_i$	10.7	8.1	7.9	7.0	4.2	5.5
	$s_{\mu + ai}$	1.13	1.13	1.71	1.95	2.64	2.31
Transferable embryos (*n*)	$\mu + a_i$	6.1	4.7	4.7	3.3	1.7	2.7
	$s_{\mu + ai}$	0.89	0.89	1.34	1.54	2.08	1.82
Success (%)	$\mu + a_i$	70.7	57.7	64.0	42.7	33.0	75.5
	$s_{\mu + ai}$	6.42	6.42	9.67	11.07	15.0	13.2

Table 4. Mean values and statistical significance of the production and use of embryos based on the period between flushings

Parameter	Class	1	2	3	Significance	
	n interval	17 < 55	62 56–115	26 > 115	F-test	significance for F
Flushing – flushing (days)	$\mu + a_i$	50.8	73.3	165.6	38.96	0.0001^{++}
	$s_{\mu+ai}$	14.99	8.91	10.89		
Total embryos (n)	$\mu + a_i$	4.8	7.1	5.9	0.61	0.5468
	$s_{\mu+ai}$	2.77	1.65	2.01		
Transferable embryos (n)	$\mu + a_i$	0.3	3.6	3.7	1.59	0.2124
	$s_{\mu+ai}$	2.33	1.38	1.69		
Success (%)	$\mu + a_i$	14.6	41.9	62.3	3.43	0.0386^{++}
	$s_{\mu+ai}$	17.88	10.63	12.99		

$^{+}P \le 0.05$; $^{++}P \le 0.01$

Table 5. Pearson's correlation coefficients for the number of transferable embryos from the individual superovulations

Number of superovulation		1	2	3	4	Sum of embryos
2	r	0.669^{++}		0.547^{+}	0.234	0.810^{+++}
	n	50		20	14	50
3	r	0.701^{++}	0.547^{+}		0.594^{+}	0.616^{++}
	n	20	20		14	20
4	r	0.407	0.234	0.594^{+}		0.362
	n	14	14	14		14
5	r	0.185	0.001	0.406	–0.327	–0.339
	n	7	7	7	7	7
Sum of embryos	r	0.717^{++}	0.810^{++}	0.616^{++}	0.362	
	n	50	50	20	14	

$^{+}P \le 0.05$; $^{++}P \le 0.01$; $^{+++}P \le 0.001$

transfer although only the number of flushing was statistically significant ($P \le 0.05$). However, this effect is more complicated because it includes the individuality of the recipient and its ability to conceive after ET.

Table 3 presents the mean values of production and application of embryos based on the number of flushing documenting a declining tendency of the estimated mean values in total production, production of transferable embryos and success of the used embryos in the following flushings observing similar intervals between the respective flushings. These data confirm the results of Říha *et al.* (1999) that top-quality embryos can be obtained from donors treated from day 60 to day 100 after calving, and/or in the 1st to 3rd superovulation.

Superovulation performed in the intervals of 2 to 4 months made it possible to estimate the most convenient interval between flushings for the recovery and transfer of embryos (Table 4). We observed that as the period between flushings was prolonged (in intervals < 55, 56–115 and > 115 days), the success of the flushing (superovulation) increased, i.e. the percentage of transferable embryos. In the present study, however,

Table 6. Pearson's correlation coefficients for the n and $n + 1$ superovulation

n superovulation	$n + 1$ superovulation		
	total embryos	transferable embryos	success
Total embryos	0.790^{+++}	0.594^{+++}	-0.318^{++}
Transferable embryos	0.580^{+++}	0.710^{+++}	–0.076
Success	–0.029	0.161	0.250^{+}

$^{+}P \le 0.05$; $^{++}P \le 0.01$; $^{+++}P \le 0.001$

this tendency in total production and in the production of transferable embryos did not appear.

In order to use repeated superovulations for embryo production it is also important to have some knowledge of the prognosis of embryo recovery based on the preceding flushing; in other words the repeatability of embryo production in the individual successive superovulations. The results in Table 5 give an answer to these problems. These results confirmed by the statistical significance of correlation coefficients show that prognosis is possible, i.e. the repeatability of embryo production, particularly in the first four superovulations. Its was also confirmed by the correlation coefficients between the *n* and *n* + 1 superovulation shown in Table 6.

The present results partly confirmed the conclusions of Wichman (1990) that the success of donors for embryo production is based on the period of conducting superovulation after calving, the age of the cow, number of flushings and the effect of the herd. The results of the repeatability between the 1st to 4th flushing were also confirmed in part while the effect of the age and efficiency of the cow on embryo yields during superovulation was not proved. The conclusion of Maslev *et al.* (1990) that the result of the first superovulation can be used as a prognosis for embryo recovery in the following repeated superovulations was confirmed too.

As for the possible yield of embryos from the respective multiple superovulations the conclusions of Žižlavský *et al.* (1997) were confirmed. At the same time our conclusions about the applicability of the first superovulations for prognoses of the results of successive superovulations were different.

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The use of blood protein polymorphism for determining the genetic distance between the Moravian warm-blooded horse and the Czech warm-blooded and Trakehner horses

Stanovení rozdílnosti genofondu moravského teplokrevníka od českého teplokrevníka a trakénského koně na základě genetického polymorfismu

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ABSTRACT: In the present study the genetic relationship between 3 breeds was investigated in 3 groups of stallions (Czech warm-blooded horse, $n = 48$; Trakehner horse, $n = 13$; Moravian warm-blooded horse, $n = 25$). A total of 17 genetic polymorphism markers (10 protein and enzyme loci, 7 blood groups) were investigated in 62 alleles. Based on the estimated allele frequencies the populations were characterised for their genetic variability on the basis of heterozygosity (H), number of alleles (n_a) and genetic distances (D) between the breeds. The degree of heterozygosity was as follows: Czech warm-blooded horse 0.367, Trakehner horse 0.319, Moravian warm-blood horse 0.353. The closest genetic relationship was found between the Czech warm-blooded and the Trakehner horses $D = 0.027$; between the Czech warm-blooded and Moravian warm-blooded horse the genetic relationship was more distant $D = 0.076$ while the most distant genetic relationship was observed between the Trakehner and Moravian warm-blooded horses $D = 0.102$.

Keywords: horses; Moravian warm-blooded horse; Trakehner horse; Czech warm-blooded horse; genetic polymorphism; allele frequencies

ABSTRAKT: Pro genetickou charakteristiku a srovnání tří plemen koní v České republice (český teplokrevník, $n = 48$; trakénský kůň, $n = 13$; moravský teplokrevník, $n = 25$) byly vytvořeny tři skupiny hřebců. Byly sledovány biochemické polymorfní znaky na 17 genetických polymorfních systémech (10 lokusů bílkovin a enzymatických bílkovin krevního séra, sedm krevních skupin) s 62 alelami. Sledované populace byly charakterizovány na základě frekvence alel, stupně heretozygotnosti (H), počtu alel (n_a) a genetické vzdálenosti (D). Pro sledovaná plemena byl vypočítán následující stupeň heterozygotnosti: český teplokrevník 0,367, trakénský kůň 0,319, moravský teplokrevník 0,353. Nejmenší hodnota genetické vzdálenosti byla zjištěna mezi českým teplokrevníkem a trakénským koněm ($D = 0,027$), větší mezi českým teplokrevníkem a moravským teplokrevníkem ($D = 0,076$) a nejvyšší mezi trakénským koněm a moravským teplokrevníkem ($D = 0,102$).

Klíčová slova: koně; moravský teplokrevník; trakénský kůň; český teplokrevník; genetický polymorfismus; frekvence alel

The Moravian warm-blooded horse is a breed of warm-blooded horses of the saddle horse type whose breeding was based on the former Austrian-Hungarian half-bred Furioso and Przedwit stock. The Anglo-Arab Gidran stock and the former Arabian half-bred Shagya contributed to its further development. At the present

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time it is an endangered species because there is no interest in breeding this horse. The joint effort of Moravian breeders is to use the last quality sources of the Moravian warm-blooded horse for the regeneration of the breed and its registration as a genetic source of horses in the Czech Republic. To achieve this target it is necessary to characterise the differences of the Moravian warm-blooded horse from the present breed of the Czech warm-blooded horse (Misař, 2001).

In Slovakia and Hungary the English half-bred Furioso stock was registered as a particular breed. The Slovakian Regulations of the Furioso Stud Book (Hučko *et al.*, 1998) give a set of breeding measures for the preservation of this breed as a gene reserve.

Glasnák (1996) defines genetic polymorphism as the occurrence of two or more alleles of the same gene in the population in more or less medium frequencies. The author calculated the frequencies of alleles of biochemical polymorphism in ca 400 English Thoroughbred horses from the stud farm at Napajedla and compared them with the contrasting breed of trotters and with data on the frequency of alleles of biochemical polymorphism in the English Thoroughbred from authors from foreign countries.

Pikula *et al.* (1997, 1998) compared the biochemical polymorphism markers of 3 different Polish warm-blooded breeds – the Malopolski (Small-Polish) horse, the Wielkopolski (Great-Polish) horse and the high grade half-bred. The authors analysed blood samples of 683 stallions and drew a conclusion that the genetically most consolidated breed was the Malopolski horse and that the Malopolski horse and the high grade half-bred were genetically the most distant.

Bowling (1996) compared 13 breeds improved in the USA with the English Thoroughbred, the Arab and Andalusian horse on the basis of the following parameters: n_a (number of alleles), H (degree of heterozygosity) and D (genetic distance). In the English Thoroughbred, the Arab and Andalusian horses the author found that n_a and H were reduced and these are the reasons she gives for their purebred breeding within the closed stud book.

Müller-Eckert *et al.* (1999) characterised 6 heavy warm-blooded horses on the basis of the Oldenburger and East-Friesian horse and 3 German sport horses and they carried out comparisons based on analyses of genetic polymorphism systems. They estimated the genetic distance D and arrived at a conclusion that the sport horse and Groningen horse were genetically most distant; on the other hand, between the sport horses and the East-Friesian horse the genetic distance was the closest.

In the Czech Republic the genetic distance was determined in the Old Kladrub horse between the individual pairs of paternal groups (Jakubec *et al.*, 1995). The authors proved a very close relationship between the high values of genetic distance and low coefficients of affinity.

The first experiment characterising the Moravian warm-blooded horse on the basis of polymorphism markers was the paper of Misař *et al.* (2000). The result of the analysis was only informative; due to economic reasons the authors had to confine to a smaller group of 19 mares and one stallion that had a consolidated origin for factors of breeding the Moravian warm-blooded horse (the English half-bred lines Furioso, Pryedswit, North Star, the English Thoroughbred, the Anglo-Arab horse and Arab half-bred lines Shagya and Dahoman). Blood samples were analysed for the incidence of polymorphism markers in the systems of albumin (*A1B*), carboxyl esterase (*ES*), protein binding vitamin D (*GC*), glycoprotein *A1B* (*A1B*) and transferrin (*TF*).

Applying a larger number of polymorphism systems we therefore attempted to characterise the Moravian warm-blooded horse on the basis of genetic polymorphism and to formulate how it differs from, or is similar to, the Czech warm-blooded horse and the Trakehner horse.

MATERIAL AND METHODS

The source materials for the analyses were blood samples of selected breeds of stallions qualified for breeding in the Czech Republic. The population was divided as follows:

1. stallions of the Czech warm-blooded breed with a proportion of migrating genes of imported breeds; these stallions were subjected to a 100-day test and they were incorporated into the Czech warm-blooded horse breeding in 1991–2000,

2. Trakehner stallions included in Czech (Czechoslovakian) breeds since 1982,

3. stallions suitable for inclusion in the Moravian warm-blooded breed due to their origin.

The numbers of stallions in the respective groups were based on the extent of the database of blood sample analyses of the immunological laboratory of CMSCH at Hradištko pod Medníkem.

Genetic polymorphism was analysed in the following 10 protein polymorphism systems and 7 blood groups:

System	Symbol	Alleles
Protein polymorphism systems:		
Transferrin	*TF*	$D, D_2, F_1, F_2, H, M, O, R$
Vitamin D binding protein	*GC*	F, S
A1B glycoprotein	*A1B*	F, K, S
Carboxyl esterase	*ES*	F, I, S
Albumin	*ALB*	A, B
6-phospho gluconate dehydrogenase	*PGD*	D, F, S
Glucose-phosphate isomerase	*GPI*	F, I, S
Haemoglobin-alpha	*HBA*	*A*I, *A*II, *B*I, *B*II
Phosphoglucomutase	*PGM*	F, S, V
Carbon anhydrase	*CA*	F, I, L, O, S
Blood groups:		
A		*a, b, c, f, g, –*
C		*a, –*
D		*a, b, c, d, e, f, g, h, i, k, l, m, n, o, –*
K		*a, –*
P		*a, b, c, –*
Q		*a, b, c, –*
U		*a, –*

In the respective polymorphism systems the frequency of alleles for the breeds was determined and heterozygosis (*H*) and genetic distance (*D*) between the breeds was calculated.

The homozygosis of the ith locus (g_i) is expressed as:

$$g_i = \sum_{j=1}^{n_i} X_{ij}^2$$

where: X_{ij} = frequency of the jth allele of the ith locus
X_{ij}^2 = frequency of homozygotes of the *i*th locus
n_i = number of alleles of the ith locus

The heterozygosis of the ith locus is given as:

$$h_i = 1 - g_i$$

The average heterozygosis (*H*) for the studied breed is expressed as:

$$H = \frac{\sum_{i=1}^{r} h_i}{r}$$

where: r = number of loci

The genetic distance (*D*) between the studied breeds is based on the following formula:

$$D = -\ln \frac{\sum i \sum j X_{ij} Y_{ij}}{\sqrt{(\sum i \sum j X_{ij}^2)\ (\sum i \sum j Y_{ij}^2)}}$$

The Excel programme was used to set up the database and calculations of the studied parameters.

The individual breeds were compared on the basis of the calculated data.

RESULTS AND DISCUSSION

The investigations covered 48 Czech warm-blooded stallions, 13 Trakehner stallions and 25 Moravian warm-blooded stallions.

The Czech warm-blooded horse representing the control group is a breed of non-homogeneous origin and type. In the last decade the breeding of the Czech warm-blooded horse was focused on its sport performance. The prevailing method of breeding is improvement or grading up crossing with imported breeds improved for the required sport performance, particularly jumping. The criterion for the selection of stallions into the respective group was a performance test of young stallions (100-day test) that was carried out in 1991–2000. This group includes stallions with a different proportion of migrating genes of imported improved breeds.

The group of stallions of the Moravian warm-blooded horse comprises sires with gene pool of the Austrian-Hungarian half-bred lines Furioso, Przedswit, North Star, Star of Hannover, Gidran, Nonius and Shagya Arabic with improvement proportion of the English Thoroughbred, including proportions of genes of Arab and Anglo-Arab horses. The criterion for selection is the condition that the father and fathers of the mother in the direct maternal line pertained to the above given factors of breeding of the Moravian warm-blooded at least as far back as to the 3rd generation of ancestors.

The Trakehner horse was chosen for the purpose of comparison with the Moravian warm-blooded horse because it has the same original primary use as the Moravian warm-blooded horse. Available blood samples of all Trakehner sires used in Czech (Czechoslovakian) breeding, i.e. stallions from domestic and foreign breeds, were used for the investigations. The number of animals in this group is lowest because the number of Trakehner sires used in Czech breeding has recently been reduced in favour of stallions of foreign breeds bred for jumping performance.

Table 1. Frequency of alleles of stallions of the studied horse breeds in 10 polymorphism systems

Locus	Alleles	Czech warm-blooded horse (n = 48)	Trakehner horse (n = 13)	Moravian warm-blooded horse (n = 25)
TF	*D*	0.302	0.115	0.400
	D_2	0.010	0.000	0.000
	F_1	0.188	0.269	0.060
	F_2	0.240	0.308	0.320
	H	0.104	0.000	0.060
	O	0.083	0.077	0.120
	R	0.073	0.231	0.040
GC	*F*	0.948	0.769	0.880
	S	0.052	0.231	0.120
A1B	*F*	0.052	0.000	0.000
	K	0.927	1.000	1.000
	S	0.021	0.000	0.000
ES	*F*	0.083	0.231	0.160
	I	0.896	0.769	0.840
	S	0.021	0.000	0.000
ALB	*A*	0.323	0.192	0.340
	B	0.677	0.808	0.660
PGD	*F*	0.823	0.808	0.780
	S	0.177	0.192	0.220
GPI	*F*	0.000	0.038	0.000
	I	0.990	0.962	0.960
	S	0.010	0.000	0.040
HBA	*A*I	0.042	0.000	0.000
	*A*II	0.031	0.000	0.000
	*B*I	0.313	0.308	0.380
	*B*II	0.614	0.692	0.620
PGM	*F*	0.083	0.038	0.100
	S	0.917	0.962	0.900
CA	*F*	0.073	0.125	0.080
	I	0.865	0.875	0.920
	L	0.031	0.000	0.000
	M	0.031	0.000	0.000

Table 2. Frequency of alleles of stallions of the studied groups of horses in 7 blood groups

Blood group	Alleles	Czech warm-blooded horse ($n = 48$)	Trakehner horse ($n = 13$)	Moravian warm-blooded horse ($n = 25$)
A	*a*	0.042	0.000	0.120
	ac	0.000	0.000	0.040
	ag	0.020	0.000	0.060
	af	0.677	0.885	0.720
	b	0.094	0.115	0.020
	c	0.052	0.000	0.040
	–	0.115	0.000	0.000
C	*a*	0.937	1.000	1.000
	–	0.063	0.000	0.000
D	*adl*	0.063	0.000	0.020
	bcm	0.104	0.193	0.180
	cgm	0.229	0.077	0.040
	cegimn	0.021	0.038	0.060
	del	0.031	0.000	0.020
	delo	0.052	0.193	0.240
	dfkl	0.063	0.038	0.020
	dghm	0.125	0.038	0.020
	dkl	0.302	0.423	0.360
	dln	0.010	0.000	0.040
K	*a*	0.063	0.000	0.080
	–	0.937	1.000	0.920
P	*a*	0.302	0.308	0.320
	ac	0.208	0.308	0.260
	b	0.073	0.076	0.060
	–	0.417	0.308	0.360
Q	*abc*	0.406	0.615	0.400
	c	0.177	0.270	0.080
	–	0.417	0.115	0.520
U	*a*	0.365	0.231	0.460
	–	0.635	0.769	0.540

Tables 1 and 2 show the frequencies of alleles of biochemical polymorphism markers of the studied group of sires.

In the transferrin system seven alleles of locus *TF* occur in the population of the Czech warm-blooded horse. The allele TF^{D2} did not occur in the group of the Moravian warm-blooded horse and in the Trakehner stallions where, in addition, the allele TF^{H} was not present either. In the Czech and Moravian warm-blooded horse the frequency of allele TF^{D} was highest. In contrast, the allele TF^{F2} showed the highest frequency in the group of Trakehner stallions. The frequency of allele TF^{D} was relatively low in the Trakehner horse.

The frequency of alleles $A1B^F$ and $A1B^S$ in locus $A1B$ is low only in the Czech warm-blooded horse. These alleles did not occur in the groups of Moravian warm-blooded or Trakehner horses.

It is similar in the *ES*, *HBA* and *CA* systems where only variants ES^F, ES^I in locus *ES*, HBA^{BI}, HBA^{BII} in locus *HBA* and CA^F, CA^I in locus *CA* occur in the Moravian warm-blooded and Trakehner horses.

We discovered smaller differences between the Moravian and Czech warm-blooded horses in the blood group systems than in the protein polymorphism systems.

In comparison with the Moravian warm-blooded horse, in blood group A the allele A^{ac} did not exist in the Czech warm-blooded horse; the group of Trakehner stallions had only alleles A^{af} and A^b in blood group A. It was the same with blood group D where alleles D^{adl}, D^{del} and D^{dln} are absent in the Trakehner horses unlike the Czech and Moravian warm-blooded horses, and allele K^a in blood group K. It can be concluded that the Trakehner population is more homogeneous, but this result may be influenced to some extent by the fact that this group was small.

We can conclude from these results and from data given in Table 3 that in comparison with the Moravian warm-blooded and Trakehner horses the Czech warm-blooded horse has a higher number of alleles. Although the differences in heterozygosity of the groups of stallions under study were statistically insignificant, it can be stated that the Czech warm-blooded horse is less consolidated than the other two breeds. The reason for the lower homogeneity is the process of its breeding. The Czech warm-blooded horse is a result of the administrative combination of the former heterogeneous types – former Czech warm-blooded horse resulting from the improvement of warm-blooded horses with the Oldenburger horse, Moravian warm-blooded horse, Moravian and former Czech warm-blooded horses improved with Trakehner and Hannover horses, offering a basis for breeding; priority was given to mating the imported individuals of breeds bred for sport performance. Improvement breeding increased the genetic variability of this population. It was possible to reduce the variability by selection in a relatively short period of breeding.

Bowling (1996) arrived at similar conclusions in American horse breeds that originated and developed recently or originated by crossing of various breeds. In comparison with these breeds the n_a and H parameters of the purebred English Thoroughbred, Arab and Andalusian horses were lower.

The average heterozygosity H was lowest in the Trakehner stallions, and highest in the Czech warm-blooded horse. This conclusion confirms the fact that the Trakehner horse, which is a thoroughbred breed, is the most consolidated horse in terms of genetics. The Trakehner horse was used as a corrector of properties required for the breeding of the present horse breeds (Hannover horse, KWPN, Czech warm-blooded horse). From this point of view the opinions that the Trakehner horse should be used as the carrier of strong constitution and correct body conformation in the breeding of the Moravian warm-blooded horse are fully substantiated.

Pikula *et al.* (1998) from Poland discovered that in the Wielkopolski horse $H = 0.338$. This breed comes from the Trakehner horse and is under its influence to a large extent. On the basis of genetic polymorphism markers the authors also analysed other breeds, the Malopolski horse ($H = 0.254$) and the high grade half-bred ($H = 0.314$). Although the high grade half-bred is a horse upgraded with imported sport breeds, the degree of heterozygosity is lower than in the Wielkopolski horse. On the other hand, according to our findings the population of the Czech warm-blooded horse, which is very heterogeneous due to the influence of imported breeds, is genetically the least consolidated breed ($H = 0.367$).

The H parameter of the Moravian warm-blooded horse is 0.353. Compared with the results of German authors (Müller-Eckert *et al.*, 1999) who defined the average homozygosity in German sport breeds as

Table 3. Genetic variability of the studied horse breeds based on the number of alleles (n_a) and degree of heterozygosity (H)

Population	Number of alleles			H
	proteins	blood groups	n_a	
Czech warm-blooded	31	29	60	0.367
Trakehner horse	21	20	41	0.319
Moravian warm-blooded	22	28	50	0.353

Table 4. Genetic distance (*D*) between the studied horse breeds

D	Czech warm-blooded horse	Trakehner horse
Trakehner horse	0.027	
Moravian warm-blooded horse	0.076	0.102

$H = 0.397$ (the Oldenburger sport horse) the Moravian and Czech warm-blooded horses appear to be more consolidated breeds. In the Moravian warm-blooded horse it is based on its uniform breeding. This horse comes from half-bred stock bred on stud farms. In Moravia by the 1950s high-grade uniform saddle horses were incorporated into breeding, particularly due to the intensive influence of the stud farm at Pohořelice near Napajedla. After the stud farm at Pohořelice was shut down, the Moravian breed was gradually incorporated into the single Czech warm-blooded breed. A small difference in the *H* values between the Moravian and Czech warm-blooded horses could result from the fact that in the origins of some of the Moravian warm-blooded stallions the genes of upgrading breeds occurred in several generations of mothers (e.g. the influence of the Hannover stallion Alarm).

Table 4 presents the quantified values of genetic distance between the studied populations.

We could consider the genetic consolidation of the Trakehner horse (Table 3) as a recommendation to use it for breeding the Moravian warm-blooded horse. However, the opinion that the Trakehner horse is close to breeds based on the English half-breds (Hučko *et al.*, 1998) was not confirmed. Of all the three breeds compared in the present investigations the Trakehner horse and the Moravian warm-blooded were genetically the most distant ($D = 0.102$). The commercial trend of the Trakehner horse was the same as that of the Austrian-Hungarian English half-bred lines, the basis of the Moravian warm-blooded horse. Both breeds were aimed at the production of thoroughbred and sturdy saddle horses for elite army service. Another thing they also had in common was the use of the English Thoroughbred for breeding and the proportion of the Arab horse genes. On the other hand, the history of the breeding of Trakehner and Moravian warm-blooded horses in the 20th century was different. While the horses of English Thoroughbred lines were bred as sturdy and in constitution strong saddle horses, the Trakehner horse was steered for sport performance.

The genetic distance between the Czech warm-blooded and Trakehner horses is smaller than between the Czech and Moravian warm-blooded horses. This conclusion is based on the fact that the Trakehner horse was one of the factors of breeding when the Czech warm-blooded breed was created.

The genetic differences between the Czech and Moravian warm-blooded horses ($D = 0.076$) are based on the diversity of effects of upgrading breeds in the course of production of the Czech warm-blooded horse during the 20th century. The Oldenburger horse (turn of the 19th and 20th centuries and the period between the two world wars), the Hannover horse (since the beginning of World War II) and breeds bred for sport performance (second half of the 20th century) were gradually used in the Czech warm-blooded breed. On the other hand, the Moravian warm-blooded horse, thanks to the existence of the integrated breeding doctrine (stud farm at Pohořelice), avoided these influences. The effort to preserve the Moravian warm-blooded breed without the influence of imported breeds was the cause of higher *D* value between the groups of Czech and Moravian warm-blooded stallions. It shows that the efforts of the breeders of the Moravian warm-blooded horse for an independent studbook and for the preservation of this breed as a gene resource in the Czech Republic were well founded.

The conclusions drawn from the present results confirm the intention to use the imported stallions of the Furioso breed (particularly from Slovakia and Hungary) to extend the "blood" pool of the Moravian warm-blooded breed and to incorporate the Trakehner sires only exceptionally.

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Effect of plant oils and malate on rumen fermentation *in vitro*

Vplyv rastlinných olejov a malátu na bachorovú fermentáciu *in vitro*

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ABSTRACT: The objective of this study was to determine the effect of plant oils (rapeseed – RO; sunflower – SO; linseed – LO; 10% wt/wt) and maleic acid, disodium salt (MAL; 8 mM) on the rumen fermentation of diet (250 mg) consisting of hay, barley and sugar beet molasses (60 : 30 : 10). Rumen fluid was collected from two sheep (Slovak Merino) fed with the same diet twice daily. Mixed ruminal microorganisms were incubated in fermentation fluid (40 ml) which contained 20% vol/vol rumen fluid and McDougall's buffer. Incubations were carried out in batch cultures at 39°C for 72 h two times within 3-weeks intervals. Compared to control, all supplemented diets (RO, SO, LO, MAL) significantly increased pH ($P < 0.001$), mol% propionate (LO + 8.7% ($P < 0.001$), SO + 10.12% ($P < 0.001$), RO + 8.65% ($P < 0.01$), MAL + 5.8% ($P < 0.05$, acetate to propionate ratio ($P < 0.001$) and numerically decreased methane production (SO – 32.8%, LO, RO – 30.08%, MAL – 20.53%). The additives – plant oils, not malate, significantly decreased lactate and *in vitro* dry matter digestibility – IVDMD ($P < 0.001$). Incorporation of plant oils into malate – treated incubations negated the decrease of *n*-butyrate, lactate and the increase of pH and ammonia associated with malate treatment. The effect of combined additives (RO + MAL, SO + MAL, LO + MAL) on methane production (LO + MAL – 13.84%, SO + MAL – 17.66%, RO + MAL – 15.03%) and mol% propionate (LO + MAL + 4.4%, SO + MAL, RO + MAL + 3.2%) was less effective. All combined additives caused significant decrease of feed digestibility. It seems, the combination of the additives did not show an additive effect on rumen fermentation.

Keywords: plant oils; malate; rumen fermentation

ABSTRAKT: Cieľom tejto práce bolo skúmať vplyv rastlinných olejov (repkového – RO; slnečnicového – SO; ľanového – LO (10 hmot.j/hmot.j.) a kyseliny maleovej (sodná soľ, MAL; 8 mM) na fermentáciu diéty (250 mg) pozostávajúcej zo sena, jačmeňa a repnej melasy (60 : 30 : 10). Bachorová tekutina bola získaná od 2 oviec plemena slovenské merino kŕmených tou istou diétou 2× denne. Bachorové mikroorganizmy boli inkubované vo fermentačnej tekutine (40 ml), ktorá obsahovala zmes bachorovej šťavy a McDougallovho pufru (1 : 4). Inkubácie boli uskutočnené vo fermentačných nádobách po dobu 72 hodín pri teplote 39°C. Experiment bol uskutočnený dvakrát v priebehu troch týždňov. V porovnaní ku kontrole, všetky supplementované diéty (RO, SO, LO, MAL) signifikantne zvýšili pH ($P < 0,001$), mol% propionátu (LO + 8,7 %, SO + 10,12 %, RO + 8,65 %, MAL + 5,8 % ($P < 0,05$)), pomer acetát : propionát ($P < 0,001$) a numericky znížili produkciu metánu (SO – 32,8 %, LO, RO – 30,08 %, MAL – 20,53 %). Aditíva (rastlinné oleje, nie však malát), signifikantne znížili laktát a stráviteľnosť sušiny *in vitro* – IVDMD ($P < 0,001$). Zahrnutie rastlinných olejov do diét obsahujúcich malát negovalo pokles n-butyrátu, laktátu a zvýšenie pH a amoniaku spojeného s prídavkom malátu. Vplyv kombinovaných aditív (RO + MAL, SO + MAL, LO + MAL) na produkciu metánu (LO + MAL – 13,84 %, SO + MAL – 17,66 %, RO + MAL – 15,03 %) a mol% propionátu (LO + MAL + 4,4 %, SO + MAL, RO + MAL + 3,2 %) bol menej efektívny. Všetky kombinované aditíva zapríčinili signifikantný pokles stráviteľnosti krmiva. Zdá sa, že kombinácia aditív nemala aditívny efekt na bachorovú fermentáciu.

Kľúčová slová: rastlinné oleje; malát; bachorová fermentácia

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Anti-microbial compounds are routinely incorporated into ruminant diet to improve production efficiency (Callaway and Martin, 1996). Some authors (Martin and Streeter, 1995) have suggested that organic acids (aspartate, fumarate, malate) can provide an alternative to use anti-microbial compounds mainly ionophores. Organic acids can stimulate the growth of the prominent Gram-negative ruminal bacterium *Selenomonas ruminantium*, they can favourably alter the mixed ruminal microorganism fermentation (Martin, 1998; Martin *et al.*, 1999). And, they can decrease the methane production in the rumen (Lopez *et al.*, 1999). Certain plant oils have also been described as methane reducing feed ingredients for ruminants (Jouany, 1994; Van Nevel and Demeyer, 1996; Wettstein *et al.*, 2000). The extent of this inhibition was related to the incorporation level of lipids (Czerkawski *et al.*, 1966), but also to their degree of unsaturation. A direct toxic effect of unsaturated fatty acids on methanogens has also been reported for mixed rumen bacteria *in vitro* (Demeyer *et al.*, 1967). Fatty acids are especially toxic to Gram-positive bacteria, whereas Gram-negative bacteria are less sensitive to fatty acids at the same concentration (Maczulak *et al.*, 1981). Three types of fat were used in this experiment: rapeseed oil, rich in oleic acid ($C_{18:1}$ cis), sunflower oil, rich in linoleic acid ($C_{18:2}$) and linseed oil, rich in both linoleic and linolenic acid ($C_{18:3}$), respectively. To determine whether organic acids and plant oils have an additive effect on ruminal fermentation, the effect of the organic acid – maleic acid and rapeseed, sunflower and linseed oils on the *in vitro* mixed ruminal microorganisms fermentation were evaluated.

MATERIAL AND METHODS

Diet and additives

Meadow hay, crushed barley and sugar beet molasses were used as the components of diet for *in vitro* incubation. The composition of dietary components is recorded in Table 1. The individual compounds were obtained from commercial supplier. The dicarboxylic organic acid – maleic acid (disodium salt) was purchased from Fluka Chemie AG (Buchs, Switzerland). Weighed amount of malate was added to achieve final concentration 8 mM to the diet. Plant oils – rapeseed, linseed and sunflower were obtained from commercial sources and were also used as the additives (10% wt/wt) in this experiment.

Table 1. Chemical composition of the diet (% of DM)

	Meadow hay	Crushed barley	Sugar beet molasses
Organic matter	94.5	97.8	89.4
Total lipids	2.1	1.9	0
Crude protein	8.8	12.9	9.7
NDF	64.9	49.8	0
ADF	39.9	3.6	0
ADL	10.9	0.7	0

The composition of the diet (250 mg) – meadow hay 150 mg, barley 75 mg, sugar beet molasses 25 mg (60 : 30 : 10)
NDF – neutral detergent fiber, ADF – acid detergent fiber, ADL – acid detergent lignin

In vitro batch fermentations

Short term *in vitro* incubations were carried out with rumen fluid which was obtained from two rumen cannulated sheep (Slovak Merino).The sheep (40.6 ± 1.2 kg) received 1.3 kg/day of mixed diet of meadow hay, barley and sugar beet molasses (600, 300 and 100 g/kg dry matter), respectively in two equal meals. Rumen fluid was withdrawn via the cannula 3 h after morning feeding and after mixing the samples from two sheep, it was strained through 3 layer of cheesecloth and maintained at 39°C under oxygen free CO_2. Rumen fluid was mixed (1 : 4) with buffer containing (per l): 9.24 g $NaHCO_3$; 7.12 g $Na_2HPO_4.12\ H_2O$; 0.47 g NaCl; 0.45 g KCl; 0.055 g $CaCl_2$; 0.047 g $MgCl_2$ (McDougall, 1948). After mixing, 40 ml buffered rumen fluid was dispensed into 120 ml serum bottles containing 250 mg of the diet described previously (Table 1), ground to pass through a 1 mm mesh screen. Weighed amounts of the additives – malate (MAL), rapeseed oil (RO), sunflower oil (SO) and linseed oil (LO) were added individually or in the combination. To ensure that the diet contained 11% crude protein (CP) 0.38 mg of nitrogen (N) was added in the form of urea into all fermentation bottles. In addition, sulfur – S ($ZnSO_4$, $CoSO_4$, $MgSO_4$) as mineral solution was added to the fermentation fluid to achieve a N : S ratio 10 : 1. The bottles were sealed (CO_2 atmosphere) with rubber stoppers and aluminium caps and were placed in a thermostat at 39°C. The bottles without additives were control samples and the bottles with additives – LO, SO, RO, MAL, LO + MAL, SO + MAL, RO +

MAL were experimental samples. Each group contained four bottles, consequently the fermentation was realized in 32 fermentation bottles. The next 4 bottles served as a blank (rumen fluid + buffer). After 72 hours, fermentation was stopped and the bottles were put into an ice bath. The experiment was repeated two times within 3-weeks interval. The cellulose degradation as the indicator of cellulolytic activity was determined by fermentation of 250 mg filter paper after 24 h of incubation in all fermentation bottles with and without additives (3 parallels in each group). After incubation, the filter paper residues were washed with distilled water and dried at 60°C for 48 hours.

Chemical analyses

After cooling, the fermentation fluid was discarded from bottles for ammonia nitrogen, volatile fatty acids (VFA), lactic acid analyses. For the determination of digestibility of dry matter, the contents of the bottles were transferred into a tube and centrifuged at 3 500 × g for 10 min. The residues were washed twice with distilled water, centrifuged and dried to the constant weight at 105°C. The concentrations of VFA in fermentation fluid were determined by gas chromatography (Cottyn and Boucque, 1968) using crotonic acid as the internal standard with the Perkin-Elmer 8500 gas chromatograph. Ammonia nitrogen concentration was measured by microdiffusion method (Conway, 1962). L-lactate was determined by gas chromatography procedure using methylation reaction with sulfuric acid (50%) 0.1 ml and methanol 1.0 ml (Bricknell *et al.*, 1979). Methane production was calculated stoichiometrically from VFA analysis (Blümmel *et al.*, 1999).

Statistical analysis

The differences in total and individual volatile fatty acids, L-lactate, NH_3-N production as well as *in vitro* dry matter digestibility (IVDMD) and other parameters were analyzed by analysis of variance using ANOVA procedure of the Statistical Analysis Systems Institute (SAS, 1989). The treatment means were statistically compared with the Tukey-Kramer multiple comparison test. The tables give the group means and standard error of mean (SEM).

RESULTS AND DISCUSSION

Effect of plant oils on rumen fermentation *in vitro*

The oils contain unsaturated fatty acids which great part is hydrogenated in the rumen (Fellner *et al.*, 1995), when freely accessible to the microbes after lipolysis. Rumen fluid pH was significantly lower ($P < 0.001$) in the unsupplemented control diets than in plant oils diets. The diets with sunflower and rapeseed oil showed numerically higher ammonia concentration in rumen fluids (about 1.2–3.0 mg/100 ml). Concerning the diet with linseed oil ammonia concentration was significantly ($P < 0.001$) higher in comparison to control. In

Table 2. Effect of plant oils and malate on VFA production of rumen fermentation (72 hours) in batch cultures *in vitro*

Treatments	Acetate	Propionate	*n*-butyrate	Isobutyrate (mmol)	*n*-valerate	Isovalerate	Total VFA	A/P
Control	0.99	0.55	0.23	0.06	0.04	0.04	1.75	1.81
LO	0.91	0.74***	0.20	0.02***	0.04	0.04	1.84	1.22***
SO	0.92	0.79***	0.20	0.02***	0.04	0.04	1.91*	1.15***
RO	0.91	0.73**	0.19	0.02***	0.04	0.04	1.83	1.23***
MAL	0.98	0.70*	0.20	0.02***	0.05	0.04	1.88	1.40***
LO + MAL	0.89	0.67	0.31***	0.02***	0.06*	0.04	1.88	1.34***
SO + MAL	0.86	0.59	0.29**	0.02***	0.03	0.04	1.71	1.44***
RO + MAL	0.86	0.62	0.32***	0.02***	0.03	0.04	1.80	1.38***
± SEM	0.03	0.03	0.01	0.003	0.004	0.003	0.02	0.02

VFA – volatile fatty acids; SEM – standard error mean; LO, SO, RO – linseed, sunflower, rapeseed oil; MAL – maleic acid, disodium salt; A/P – acetate: propionate ratio

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

the present study, the oils – rapeseed, sunflower and linseed affected rumen fermentation pattern with higher molar proportion of propionate (+8.65, +10.12, +8.7%) and smaller acetate to propionate ratio (Table 2). Canola oil supplementation (30 g/kg fatty acids) also led to reduction of acetate to propionate ratio due to higher production followed by higher mol% of propionate (Wettstein *et al.*, 2000). Jenkins (1990) and Doreau *et al.* (1993) reported reduction in acetate production after oil supplementation into diets. While according to our results, the oil supplementation only slightly reduced the acetate production (Table 2). Production of *n*-butyrate was also slightly reduced after oil supplementation (Table 2). In turn, canola oil (Dong *et al.*, 1997; Wettststein *et al.*, 2000) or rapeseed, sunflower and linseed seed supplementation (Machmüller *et al.*, 1998) significantly reduced mol% of *n*-butyrate *in vitro*. Garnsworthy (1997) suggested that if added dietary fat interfere with normal fiber digestion in the rumen, acetate and butyrate will be reduced. Reduced protozoa counts were observed using each kinds of plant oils (Doreau and Ferlay, 1995). Prins *et al.* (1972) also showed that $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ acids inhibit growth of rumen methanogens in pure culture media. Probably for adversing effect against protozoa by plant oils, the methane production was numerically reduced (about 30%) in this experiment (Table 3). Intraruminal infusions of 60 g per day of $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ respectively, (which are predominant fatty acids in the oils used in this study), depressed methane in sheep by 31%, 24% and 38% respectively (Czerkawski *et al.*, 1966). Our results confirmed that oil-mediated depression in methane production was not directly associated with the degree of unsaturation of the fatty acids. The ruminal degradation of dry matter (determined by the percentage of DM disappearing from the fermentation bottles during 72 h of incubation) in the fermentation fluid was significantly ($P < 0.001$) reduced by each type of oil supplementation. The depression of cell wall degradation by the oils (rich in polyunsaturated fatty acids) is caused by physical coating of fibers by lipids by inhibition of rumen microbial activity and by modification of the microbial population due to intoxication (Devendra and Lewis, 1974). The differences in the effectiveness of oils in depressing feed digestion (Table 3) was reflective of the observed changes in microbial population. The plant oils showed the different effect on cellulolytic activity of fermentation fluid. The disappereance of filter paper after 24 h of incubation was higher (LO 33.66%, RO 36.10%) or lower (SO 27.95%, $P < 0.01$), respectively in comparison to control (33.13%). In the other experiment, canola oil and cod liver oil caused increase in the number of cellulolytic bacteria (Dong *et al.*, 1997). But coconut oil reduced the cellulolytic bacterial population in hay diet.

The effect of malate on rumen fermentation *in vitro*

Maleic acid is a key intermediate in the production of succinate or propionate in some ruminal bacteria. Therefore, it could stimulate propionate production. In the fact, propionate (mol%) has been increased (+5.8%) by adding of malate (8 mM) in this experiment. Carro *et al.* (1999) also found increased propionate

Table 3. Effect of plant oils and malate on some parameters of rumen fermentation (72 hours) in batch cultures *in vitro*

Treatment	PH	IVDMD (%)	Lactate (mmol)	NH_3N (mg/100 ml)	Methane (µmol)
Control	6.67	64.88	0.156	12.7	523.6
LO	6.79***	53.46***	0.144	17.54***	366.3
SO	6.82***	59.14***	0.101***	15.73	351.7
RO	6.83***	44.30***	0.122***	13.91	366.4
MAL	6.80	64.70	0.155	19.36***	416.0
LO + MAL	6.74**	53.26***	0.182*	12.10	451.1
SO + MAL	6.66	48.45***	0.186**	12.10***	431.1
RO + MAL	6.67	57.20***	0.186**	14.52	444.9
± SEM	0.01	0.31	0.01	1.72	41.83

IVDMD – *in vitro* dry matter digestibility; SEM – standard error mean; LO, SO, RO – linseed, sunflower, rapeseed oil; MAL – maleic acid, disodium salt

$*P < 0.05$, $** P < 0.01$, $*** P < 0.001$

production (+3.84 %) by adding of malate (5.62 mM). The formation of propionate from malate would result in a lower availability of H_2 for other biochemical processes, particularly for the methanogenesis. The total methane production was numerically reduced (–20.55%) by the addition of malate when compared with the control (Table 3). The methane production was not significantly reduced by the addition of 4, 8, 12 mM of malate in fermentation of cracked corn (Callaway and Martin, 1996); 5.26 mM of malate in fermentation of hay and concentrate (50 : 50%; Carro *et al.*, 1999). Nisbet and Martin (1993) reported that adding of malate to *in vitro* cultures stimulated the growth of the predominant ruminal bacterium *Selenomonas ruminantium,* which can form up to 51% of the total viable bacteria in the rumen. These authors reported that malate increased lactate utilization by *S. ruminantium* (causing a reduction of lactate levels). In this experiment, lactate production was slightly reduced (Table 3). There were not significant differences between control and malate treatment in the production of acetate, *n*-butyrate, *n*-valerate, isovalerate and total VFA production. Malate addition (5.26 mM) also haven't done a significant effect on the production of these VFA in fermentation of hay and concentrate (Carro *et al.*, 1999).

In the agreement with previous work (Callaway and Martin, 1996) incubation with malate lead to higher ammonia concentration in fermentation fluid (Table 3). It is known, malate supplementation in ruminant diet has been shown to increase nitrogen retention in sheep (Stallcup, 1979). The ruminal degradation of DM after 72 h of incubation was not affected by malate supplementation probably for positive effect of malate on bacterial cellulolytic population (disappereance of filter paper was higher about 4 units – 37.52% vs. 33.13% – control). Newbold *et al.* (1996) showed that feeding of 100 mg of malate per day by sheep caused an increase in the number of total bacteria and tended to increase the population of cellulolytic bacteria.

The effect of malate and plant oils on rumen fermentation *in vitro*

The combination of the additives caused certain changes in the production of some parameters of rumen fermentation. Incorporation of plant oils into the diets treated by malate negated the decrease of *n*-butyrate, lactate and the increase of pH and ammonia associated with malate treatment. Lower pH probably resulted from the lower ammonia content at the constant concentration of VFA (Table 2, 3). Additive effect was not observed on rumen fermentation in the diet containing malate and plant oils. Namely, the influence of plant oils and malate on methane production RO + MAL (–15.03%), SO + MAL (–17.66%) LO + MAL (–13.84%) and mol% propionate LO + MAL (+4.4%), SO + MAL, RO + MAL (+3.2%) was less effective in comparison to the additives supplemented separately. The effects of both additives (RO + MAL, SO + MAL, LO + MAL) on the other fermentation parameters (acetate, propionate, isobutyrate, *n*-valerate, isovalerate, total VFA) were similar to the effects with plant oils, malate. All combined additives caused the depression of feed digestion. Thus, malate did not eliminate depressive effect of plant oils on feed digestion. This fact was probably caused by changes in microbial population during fermentative process. Indeed, plant oils and malate had similar effect on bacterial cellulolytic population as plant oils alone (disappereance of filter paper after 24 h of incubation was 31.44% LO + MAL, 27.53% SO + MAL, 37.18% RO + MAL, 33.13% control).

In conclusion we can state:

- the supplementation of plant oils and malate separately had similar effect on rumen fermentation (increase mol% propionate, decrease lactate and methane) as other antimicrobial compounds, i.e. ionophores
- all oils decreased feed digestion (lower IVDMD), while malate did not affect it
- the combination of the additives (plant oils and malate) did not have an additive effect on rumen fermentation
- cellulolytic activity was affected by the supplementation of additives (decrease by SO, LO + MAL, SO + MAL, increase by RO, RO + MAL, MAL, LO).

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Analysis of the commercial grades for beef carcasses

Analýza obchodních tříd jatečně upravených těl skotu

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ABSTRACT: Based on a statistical analysis of data acquired in the process of instrument testing (video image analysis, bioelectrical impedance analysis) for objective classification, the objective character of the criteria for classification of conformation and fatness was also identified. During the tests, inspectors subjectively classified 118 young bulls and carried out their detailed dissection. The analysis identified significant correlations between conformation and weight of beef carcass (correlation coefficient $r = 0.82$) and its cuts. In addition, the analysis revealed almost a zero correlation between conformation and the relative (percentage) proportion of meat in beef carcass. On the contrary, the estimated fatness proved a close dependence on the proportion of fat in the whole carcass and in its separate cuts. During the analysis of a group of animals with similar fatness values, significant correlations were identified between conformation and the relative proportion of saleable meat and meat I – large cuts ($r = 0.758$). It is evident from the data ascertained in this way that an estimate of fatness is very important for the actual quality of the carcass and proportion of its lean meat. The analysis also revealed that the fatness as an autonomous criterion offers a relatively precise estimate of the fat content in carcass.

Keywords: conformation; fatness; beef carcass composition; estimation of composition; commercial grading

ABSTRAKT: Na základě statistického rozboru dat získaných při zkouškách přístrojů (video image analysis, bioelectrical impedance analysis) pro objektivní klasifikaci byl také zjišťován objektivní charakter kritérií třídění: zmasilosti, protučnělosti. Během zkoušek bylo inspektory subjektivně klasifikováno 118 kusů mladých býků a současně byla prováděna jejich detailní disekce. Analýzou bylo zjištěny významné korelace mezi zmasilostí a hmotností jatečně upraveného těla – JUT (korelační koeficient $r = 0{,}82$) a jeho částmi. Byla zjištěna téměř nulová korelační závislost zmasilosti na relativním (procentickém) podílu masa v JUT. Odhadnutá protučnělost, vykazovala naopak velmi těsnou závislost na podílu tuku v celém JUT i v jeho jednotlivých částech. Při analýze na skupinách zvířat se stejnou protučnělostí byly nalezeny významné korelační závislosti zmasilosti na relativním podílu prodejného masa i masa I – velké kusy ($r = 0{,}758$). Z této skutečnosti vyplývá důležitost správného odhadu protučnělosti pro přesné ohodnocení kvality jatečně upraveného těla, resp. odhadnutí procentického podílu libové svaloviny. Analýza rovněž odhalila, že protučnělost vykazuje, i jako samostatné kritérium, velmi přesný odhad obsahu tuku v JUT.

Klíčová slova: zmasilost; protučnělost; jatečně upravené tělo skotu; odhad složení; obchodní třídy

According to the EU standards, abattoirs in the Czech Republic have also introduced quality classification of the half-carcasses, serving for the purposes of correct commercial classification and assessment. The adopted classification system is based on two criteria: conformation and fatness. The estimation of the conformation or fatness level is carried out rather subjectively; however, a more objective instrument classification has been used lately. The purpose of this paper is to identify the character of the given criteria, based on a statistical analysis of dependence between the said criteria and the composition of the half-carcass. The objective is to find out to what extent the given criteria describe the composition of the carcass and how these

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characteristics correspond to the definition based on national standards (ČSN – Czech National Standard). The standard ČSN 466120(21), amended with respect to the standards and regulations of the EU, is described in detail by Vrchlabský and Golda (2000). Both the EUROP classification grid and ČSN use 5-point scales for conformation (E,U,R,O,P) and fat (1, 2, 3, 4, 5) classes. Currently, the classification according to conformation is extended by the highest point (class S), but the principle of classification remains the same. Due to this modification the final commercial classification provides thirty combinations of conformation and fat classes. Because the 5-point scale for tests of new instrumentation for objective classification is inaccurate, every class is divided into 3 subclasses and the original EUROP classification grid has 15-point scales. The results of the research and practical application of the Video Image Analysis (VIA) for objective carcass classification according to Madsen (1996) and Branscheid *et al.* (1999a) and detailed coverage of this topic in the Czech agricultural literature were published by Bohuslávek (1997a,b, 2000).

An evaluation of the conformation and fatness characteristics was provided by Kempster *et al.* (1982), who referred to the fact that only 30% of the variations in the percentage of meat in carcass are explained in terms of conformation classes. According to Sack and Scholz (1987) and Bach *et al.* (1986), conformation is sometimes easier to correlate via the proportion of fat in carcass. Schneijdenberg (1991) referred to a significant relation between conformation and weight of saleable meat ($r = 0.58$) in carcass. Low correlations between conformation and the proportion of meat in carcass were reported by Augustini *et al.* (1993) and in latest publication by Branscheid *et al.* (1999a).

MATERIAL AND METHODS

The analysis of the conformation and fatness characteristics was carried out on the basis of an analysis of statistical data acquired during co-operation with the Federal Meat Research Institute (BAFF Kulmbach) in Germany, during research on the use of video image analysis and bioelectrical impedance analysis for the classification of beef carcasses. The instructors of the BAFF training centre provided the estimation of conformation and fatness subjectively. The components of the carcass composition were ascertained objectively based on the detailed dissection. A set of animals subjected to analyses comprised 118 young bulls of the Deutsches Fleckvieh breed. See Table 1 for the characteristics of the set of animals, concerning the basic statistical evaluation for the estimate of conformation and fatness and selected data from the detailed dissection. Classification and detailed dissection were carried out according to the procedure described by Augustini *et al.* (1997). The carcasses were classified in accordance with 15-point scales (EUROP grid with subclasses – 15 conformation subclasses ($^{+}E^{-}$, $^{+}U^{-}$, …$^{+}P^{-}$) and 15 fat subclasses ($^{+}1^{-}$, $^{+}2^{-}$, …$^{+}5^{-}$). Similar classification was reported by Allen (1999), Branscheid *et al.* (1999a, b), Madsen (1996). Detailed dissection was carried out pursuant to the German standard DLG, which is used with small differences in the EU and CR as well. The yield of saleable meat was divided according to the size into three groups: meat I – large cuts – suitable for roasting, meat II – small cuts – suitable for goulash, meat III – other cuts – suitable for technological processing.

During the first stage, the analysis was focussed on the dependence of conformation and fatness on the

Table 1. Characteristics of the set of evaluated animals ($n = 118$)

	Conformation* (15)	Fatness* (15)	Carcass weight (kg)	Saleable meat (%)	Half-carcass weight (kg)	Half-carcass meat I (kg)	Half-carcass meat I (%)	Half-carcass fat (%)
Max	14	14	510.5	77.4	252.1	91.08	38.9	23.39
Min	2	2	156.5	61.9	76.9	23.4	29.1	7.29
Mean (x)	8	6.5	337.5	69.8	166.9	56.875	336.6	13.57
s	3.05	2.71	70.71	3.2	35.3	12.45	2.015	3.99
s/x	0.381	0.417	0.209	0.046	0.211	0.218	0.06	0.293

*EUROP grid with subclasses – 15 conformation subclasses ($^{+}E^{-}$. $^{+}U^{-}$.…$^{+}P^{-}$) a 15 fat subclasses ($^{+}1^{-}$, $^{+}2^{-}$, …$^{+}5^{-}$)
s = standard deviation

dissection components, based on the correlation analysis of the whole set of examined animals. To illustrate how close the identified correlation was, a simple regression analysis was carried out with respect to selected variables. Considering the fact that the classification sorting criteria (commercial conformation and fat classes) are not used separately for commercial classification, an analysis of correlations between the conformation classes was carried out, together with examination of the best regressions, only in the group of animals with similar fat class. Particularly in the group of animals with fat class "1" and fat class "3" according to the EU, i.e. in subclasses (1, 2, 3) and (7, 8, 9) in the 15-grade classification scale. Fat class "1" comprised a group of 24 animals and fat class "3" included 43 animals. Similarly, correlation analysis of the fat class was carried out with respect to the group of animals with conformation class "R", i.e. subclasses (7, 8, 9) in the 15-grade classification scale, comprising 38 animals. An examination of simple regressions was carried out in order to ascertain variables with higher correlation. The statistical analysis was carried out using the Winstat programme.

RESULTS AND DISCUSSION

According to the methodology, the correlation analysis of conformation and fat classes was carried out, based on objectively ascertained parameters of beef carcasses.

The analysis was carried out with respect to the range of absolute and relative data based on detailed dissection, and only selected data are presented in Table 2a,b as linear variables. Table 2a shows the correlations between the commercial grades for beef and the absolute values of weight of the whole carcass, its half, or the weight of separate cuts and parts, e.g. leg, saleable meat, meat I (large cuts) or fat. Table 2b comprises the correlations between the commercial grades for beef and relative variables that characterise the carcass quality much better as they reflect the percentage of the demanded and well-marketable parts, e.g. proportion of saleable meat or meat I. The correlations in Table 2a show a significant relationship to the conformation and all weight gains of the carcass while the relationship to the total weight is most significant. This fact corresponds to the principle of conformation determination, i.e. evaluation of the convexity of carcass profiles. However, further correlations in Table 2b show a zero relationship of conformation to the relative (percentage) proportion of meat and a relatively low relationship to the percentage of fat in beef carcass. These findings were also presented by Kempster *et al.* (1982), Augustini *et al.* (1993), and Branscheid (1999a,b). From this point of view, the estimated conformation class *per se* provides no information on the estimated yield or quality of the carcass,

Table 2a. Correlations between the commercial grades for beef and physical characteristics (kg), $n = 118$

			Carcass weight (kg)	Half-carcass			Round	Round
				weight (kg)	meat I (kg)	fat (kg)	weight (kg)	meat I (kg)
Conformation	(15)	1	-0.822^{+++}	-0.807^{+++}	-0.790^{+++}	-0.663^{+++}	-0.801^{+++}	-0.781^{+++}
Fatness	(15)	1	0.538^{+++}	0.537^{+++}	0.335^{+++}	0.831^{+++}	0.441^{+++}	0.292^{+++}

1 = correlation coefficient

$^{+++}P < 0.001$

Table 2b. Correlations between the commercial grades for beef and physical characteristics (%), $n = 118$

			Saleable meat (%)	Half-carcass meat I (%)	Hind quarter s. meat (%)	Round s. meat (%)	Half-carcass fat (%)	Hind quarter fat (%)
Conformation	(15)	1	0.052	–0.083	0.026	0.052	-0.437^{+++}	-0.442^{+++}
		2	0.287	0.186	0.392	0.290	3.84E–07	2.81E–07
Fatness	(15)	1	-0.711^{+++}	-0.614^{+++}	-0.661^{+++}	-0.707^{+++}	0.866^{+++}	0.866^{+++}

1 = correlation coefficient, 2 = significance level

s. meat = saleable meat

$^{+++}P < 0.001$

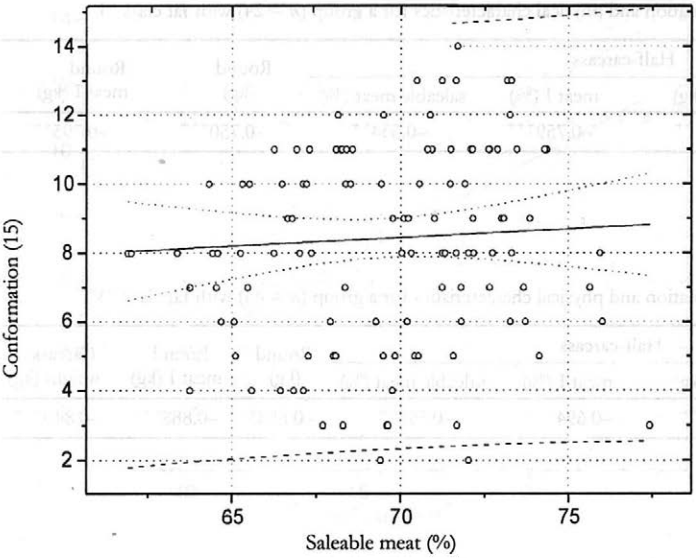

Figure 1. Simple regression of conformation on the percentage of saleable meat ($r = 0.0523$)

as regards the percentage of lean meat. The nearly zero relationship between conformation and the percentage of saleable meat is also illustrated in the circular correlation field of variables in the simple regression relationship as described in Figure 1.

The situation is different for the estimated fatness when a high dependence on the fat percentage in the whole carcass and in its separate cuts is reported. It is evident that the fatness itself (without any additional criteria) defines the carcass quality very well, concerning the fat percentage. For the illustration of the high relationship see the diagram in Figure 2.

A more detailed analysis of correlations between conformation classes was carried out for selected groups of animals with identical commercial fatness grade, according to the EU grid with 5-point scales. For the analysis of results see Table 3 and Table 4. Table 3 shows commercial grade "1", and Table 4 refers to the animals of grade "3". In both cases, we can find significant correlations between conformation and the

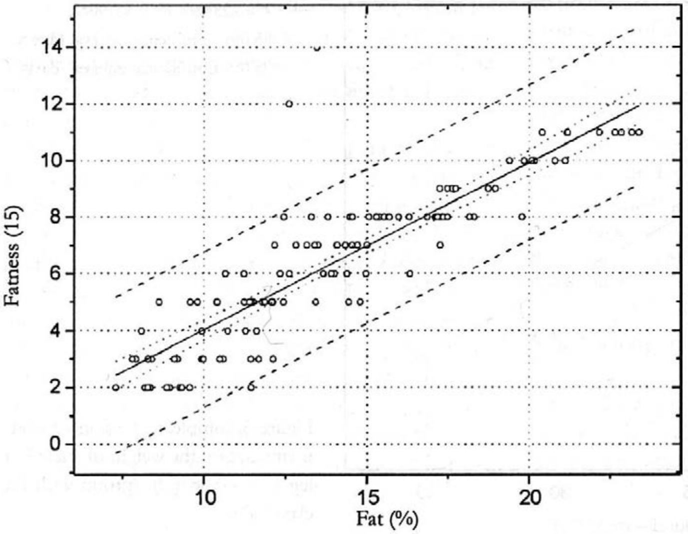

Figure 2. Simple regression of fatness on the percentage of fat in half-carcass ($r = 0.8658$)

Table 3. Correlations between conformation and physical characteristics for a group (n = 24) with fat class "1"

	Half-carcass				Round	Round
	weight (kg)	meat I (kg)	meat I (%)	saleable meat (%)	(kg)	meat I (kg)
1	–0.709^{+++}	–0.740^{+++}	–0.759^{+++}	–0.534^{++}	–0.750^{+++}	–0.795^{+++}

1 = correlation coefficient
$^{++}P < 0.01$, $^{+++}P < 0.001$

Table 4. Correlations between conformation and physical characteristics for a group (n = 43) with fat class "3"

	Half-carcass				Round	Round	Carcass
	weight (kg)	meat I (kg)	meat I (%)	saleable meat (%)	(kg)	meat I (kg)	weight (kg)
1	–0.833^{+++}	–0.858^{+++}	–0.694^{+++}	–0.595^{+++}	–0.858^{+++}	–0.888^{+++}	–0.840^{+++}

1 = correlation coefficient
$^{+++}P < 0.001$

Table 5. Correlations between fatness and physical characteristics for a group (n = 38) with conformation class "R"

	Half carcass					Carcass weight (kg)
	weight (kg)	fat (kg)	meat I (%)	fat (%)	sale. meat (%)	
1	0.435^{++}	0.912^{+++}	–0.852^{+++}	0.933^{+++}	–0.879^{+++}	0.447^{++}

1 = correlation coefficient
$^{++}P < 0.01$, $^{+++}P < 0.001$

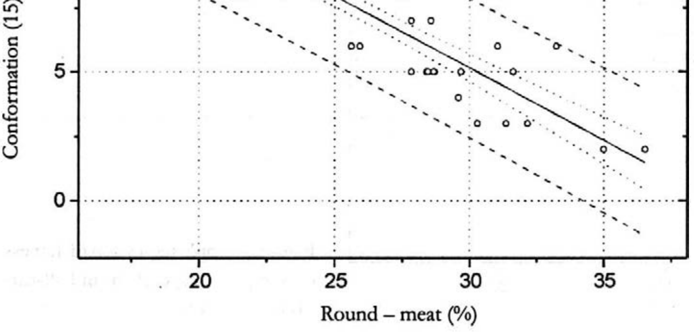

Figure 3. Simple regression of conformation on the weight of meat I in leg (r = –0.88762), (group with fat class "3")

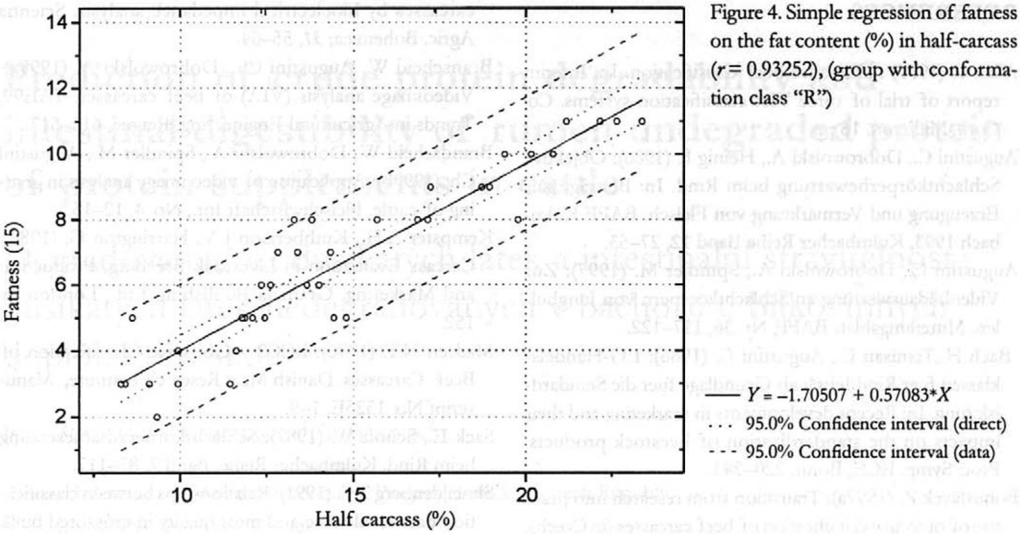

Figure 4. Simple regression of fatness on the fat content (%) in half-carcass (r = 0.93252), (group with conformation class "R")

absolute weight of carcass, saleable meat or meat I. In the analysis of groups with identical fatness we can see a significant correlation between the conformation and the relative (percentage) proportion of saleable meat and meat I. The relevance is not very high, and therefore the estimate of an additional criterion – fatness – is important in order to adequately assess the carcass quality with respect to the proportion of lean meat. Excellent results were reported with respect to the correlation between conformation and the weight of saleable meat, particularly meat I in leg. This fact reveals the information concerning the yield of the best marketable cuts of the carcass, which is very important for meat processors and which can serve as another available criterion for the evaluation of carcass quality. This relationship is depicted in a graph of linear regression equation (Figure 3).

In order to ensure more detailed characteristics of the classification by fatness, correlation analysis was carried out in a group of animals belonging to identical conformation class "3" according to the EU. The results of the correlation relationship of fatness are shown in Table 5 and in Figure 4. Less significant is the relationship of the estimated fatness to the weight of carcass or half-carcass. On the other hand, the relationship to both the absolute and relative proportion of fat indicates considerable closeness, i.e. the subjective fatness estimate is quite precise with respect to the objectively ascertained values of fat content in carcass.

CONCLUSION

The results of analysis reveal that the conformation as a classification criterion according to the EU estimates – on the basis of evaluation of the carcass profile convexity grades – the actual development of the overall mass and that it does not depend on the percentage of meat in carcass. This conclusion also refers to the fact that the Czech translation of conformation (zmasilost) does not correspond to the original term. In combination with the additional criterion, fatness, the animal evaluation spectrum narrows, and therefore the estimated conformation depends on the proportion of lean meat in carcass. The precision of the carcass quality estimate depends on the estimate of its fatness to a large extent. The knowledge acquired in this way will be used for searching for new objective methods of classification, particularly for research into the use of the bioelectrical impedance analysis method. This particularly concerns the selection of adequate measurements of current frequencies and analyses of the real and imaginary impedance component.

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Prediction of crude protein degradability and intestinal digestibility of rumen undegraded protein of protein supplements in cattle

Degradovatelnost dusíkatých látek a intestinální stravitelnost dusíkatých látek nedegradovaných v bachoru u bílkovinných doplňků pro skot

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ABSTRACT: Crude protein degradability and intestinal digestibility of rumen undegraded protein were predicted for Vitex fodder yeast, soybean meal, meat and bone meal and fish meal. The evaluations were performed *in vitro* using enzymatic methods. In order to determine crude protein degradability, the enzymatic method with bromelain was used. To determine the intestinal digestibility of rumen undegraded protein, the combined enzymatic method with bromelain and pancreatin was applied. The results were adjusted using regression equations for cereals and protein concentrates. The crude protein degradability was 79% (adjusted by regression equation 72%) for Vitex yeast, 77% (70%) for soybean meal, 65% (59%) for meat and bone meal, and 55% (50%) for fish meal. The intestinal digestibility of rumen undegraded protein was 79% (adjusted by regression equation 87%) for Vitex yeast, 90% (98%) for soybean meal, 61% (70%) for meat and bone meal, and 85% (94%) for fish meal.

Keywords: cattle; protein supplement; crude protein degradability; intestinal digestibility

ABSTRAKT: U kvasnic Vitex, sójového extrahovaného šrotu, masokostní moučky a rybí moučky byla predikována degradovatelnost dusíkatých látek a intestinální stravitelnost dusíkatých látek nedegradovaných v bachoru skotu. Stanovení byla provedena *in vitro* pomocí enzymatických metod. Pro stanovení degradovatelnosti dusíkatých látek byla použita enzymatická metoda s bromelainem a pro stanovení střevní stravitelnosti dusíkatých látek nedegradovaných v bachoru kombinovaná enzymatická metoda s bromelainem a pankreatinem. Získané výsledky byly korigovány regresními rovnicemi pro jadrná krmiva a bílkovinné koncentráty. Stanovená degradovatelnost dusíkatých látek činila 79 % (korigovaná regresní rovnicí 72 %) u kvasnic Vitex, 77 % (70 %) u sójového extrahovaného šrotu, 65 % (59 %) u masokostní moučky a 55 % (50 %) u rybí moučky. Intestinální stravitelnost dusíkatých látek nedegradovaných v bachoru činila 79 % (korigovaná regresní rovnicí 87 %) u kvasnic Vitex, 90 % (98 %) u sójového extrahovaného šrotu, 61 % (70 %) u masokostní moučky a 85 % (94 %) u rybí moučky.

Klíčová slova: skot; bílkovinné doplňky; degradovatelnost N-látek; intestinální stravitelnost

The precise feed quality evaluation is one of the main assumptions for an increase in milk production in dairy cows and for the efficient utilisation of feeds. Constantly increasing milk production of cows requires the development of more accurate systems of protein evaluation and the necessary pressure on scientific research focused on this area was applied. Based on the current knowledge of ruminant physiology of nutrition, new systems of protein evaluation in feeds for ruminants have been introduced and accepted in

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most European countries and in the USA. These systems determine how adequately the requirements of the organism for amino acid intake are met according to the quantity of protein actually entering the small intestine. All these systems are based on the same principles:

1. Separate evaluation of protein used by a host animal and by micro-organisms in the rumen.
2. The use of protein degradability (the most important criterion) and intestinal digestibility of rumen undegraded protein.

Protein supplements are the essential part of diets for high-production dairy cows. As a consequence of the BSE (bovine spongiform encephalopathy) disease in cattle, the EU legislation (Commission Dec. 2001/25/EC) prohibited to feed animal protein including fish meal to ruminants. In the Czech Republic feeding animal protein to ruminants was banned by Regulation of the Ministry of Agriculture of the Czech Republic No. 451/2000. Since the end of 2001 feeding fish meal to ruminants has been banned as well (Regulation of the Ministry of Agriculture of the Czech Republic No. 343/2001). These limitations disqualified the time-proven sources of animal proteins. The advantage of animal protein was in its lower degradability ensuring its sufficient supply of the protein to the small intestine. More attention is therefore paid to the sources of vegetable or microbial protein.

The objective of this study was to determine crude protein degradability and intestinal digestibility of protein undegraded in the rumen for Vitex fodder yeast, soybean meal, meat and bone meal and fish meal. Dried Vitex yeast is produced by Biocel Paskov, Inc., which operates the largest fermentation capacity in Central Europe. It is a by-product arising during the process of sulphite pulp production. Vitex yeast is a quality component used in feeding mixtures for all the species and categories of farm animals, poultry, dogs, cats, fur-bearing animals and fish. The main customers used to be pig and poultry producers but nowadays Vitex yeast is also used for cattle feeding. The contents of yeast in feed mixtures recommended by the producer are up to 3% for broilers, 1–5% for piglets, 3–6% for fattening pigs and up to 5% for cattle (Torula yeast Vitex, 2002). Production of Vitex yeast has to comply with the Company Standard (PN 38-065-01, 2001). This standard declares colour, consistence, appearance, smell, moisture max. 8%, nitrogen min. 70.4 g/kg, protein min. 352 g/kg and ash max. 80 g/kg. Soybean is a quality and traditional source of protein in feeding mixtures. Formerly used fish meal and meat and bone meal were included into evaluation for comparison.

MATERIAL AND METHODS

The original dry matter values of the samples of Vitex yeast, soybean meal, meat and bone meal and fish meal were 94.39, 90.81, 94.35 and 91.83%, respectively. The samples originated from the Biocel Paskov company.

Dry matter and nutrient analyses were performed according to Regulation of the Ministry of Agriculture of the Czech Republic No. 222/1996. Nitrogen content was determined according to Kjeldahl. Heat energy (gross energy) was determined using the adiabatic calorimeter IKA C-400 according to the methodology provided by the producer.

The feeds were evaluated in the Research Institute of Animal Production in Prague using enzymatic *in vitro* methods as an alternative to the methods *in sacco* and mobile bag performed on cannulated animals. It was impossible to employ these standard methods as the bags used for feed incubation were made of UHELON 130 T fabric with the pore size of 42 μm. However, Vitex yeast size ranged from 15 to 80 μm and the results would be biased due to the partial sifting of yeast through the fabric.

Crude protein degradation was predicted using the enzymatic method based on the incubation of feed samples with bromelain (Tománková and Kopečný, 1995). The results were adjusted using the following regression equation (derived on the basis of comparison of the values obtained by enzymatic and in situ methods, $n = 40$) for feeding mixtures and protein concentrates (Tománková and Kopečný, 1995):

$$y = -0.46 + 0.914x \; (r = 0.839; \mathrm{RSD} = 7.072)$$

Similarly like other *in vitro* methods, this method does not provide any real values comparable to the values observed in animals using the methods *in sacco* or mobile bag. The use of regression equations describing the relationship between the values determined in animals and the values obtained by the enzymatic method is a necessary assumption for the application of this enzymatic method.

The intestinal digestibility of rumen undegraded protein was determined by the combined enzymatic method (Tománková and Homolka, 1995). This method consists of two parts. The first part simulating rumen degradation is based on the adaptation of our own method (Tománková and Kopečný, 1995). The second part, determining the intestinal digestibility, is based on the modification of the method described by Antoniewicz *et al.* (1992). The modification of the traditional enzymatic method involves the substitution of the first phase (16 h incubation in cow's rumen) by the incubation of feed in so called final incubation solution for 1 hour. The results were recalculated using the regression equation for cereals and protein concentrates (Tománková and Homolka, 2002):

$y = 7.5 + 1.013x$ ($r = 0.867$; RSD = 4.47)

RESULTS AND DISCUSSION

Nutrient and gross energy contents in absolute dry matter of particular feeds are given in Table1. Laboratory values and adjusted final values of degradability and digestibility of crude protein in particular feeds are presented in Table 2.

The adjusted crude protein degradability was 72% for Vitex yeast, 70% for soybean meal, 59% for meat and bone meal and 50% for fish meal. The degradability of Vitex yeast was similar to that of soybean meal and higher than that of meat and bone and fish meal. The results of higher degradability of vegetable protein in comparison with animal protein are in agreement with findings of other authors whose results are shown in Table 3. We suppose that the partial differences in degradability and intestinal digestibility reported by individual authors were caused by the different origin of feeds and technological treatments applied during their production, and by small differences in the used experimental methods. Our values correspond with the findings of these authors that were obtained using the method *in sacco* in cannulated animals. A high consistency of results was obtained particularly for soybean and fish meal. Our values for meat and bone meal were slightly higher in comparison with the literature data. The literature data related to Vitex yeast were not available.

The intestinal digestibility of rumen undegraded protein adjusted by the regression equation was 87% for Vitex yeast, 98% for soybean meal, 70% for meat and bone meal and 94% for fish meal. The digestibility of Vitex yeast was 11% lower than for soybean meal, 7% lower than for fish meal and 17% higher than for meat and bone meal. The results of other authors are given in Table 3. There is a high consistency of our results with those of the mentioned authors that were obtained using the mobile bag method in cannulated ruminants. The literature data related to the protein digestibility of Vitex yeast were not available.

The determination of degradability and intestinal digestibility for Vitex fodder yeast and three other protein supplements – soybean meal, meat and bone meal and fish meal – allowed us to develop the value series and to compare Vitex yeast with the other protein supplements. The lowest crude protein degradability was found in fish meal followed by meat and bone meal, soybean meal and Vitex yeast. The highest intestinal digestibility of protein was observed in soybean meal followed by fish meal, Vitex yeast and meat and bone meal.

Table 1. Nutrient and energy contents of protein supplements

Feed	Dry matter	Organic matter	Crude protein (g/kg)	Ether extract	Crude fibre	Nitrogen-free extract	Ash	Gross energy (MJ/kg)
Vitex fodder yeast	1 000	923.3	551.5	55.8	2.8	313.2	76.7	20.370
Soybean meal	1 000	926.9	525.3	26.8	48.9	325.9	73.1	19.551
Meat and bone meal	1 000	723.8	524.0	174.1	25.7	0	276.2	19.724
Fish meal	1 000	820.9	672.8	94.2	3.2	50.7	179.1	20.849

Table 2. Crude protein degradability and intestinal digestibility of rumen undegraded protein of protein supplements

Feed	Degradability (%)		Intestinal digestibility (%)	
	Enzymatic method	Expressed using linear regression	Enzymatic method	Expressed using linear regression
Vitex fodder yeast	79	72	79	87
Soybean meal	77	70	90	98
Meat and bone meal	65	59	61	70
Fish meal	55	50	85	94

Table 3. The survey of literature data on protein degradability and intestinal digestibility of undegraded feed protein

	Degradability (%)							Intestinal digestibility (%)									
	A	B	C	D	E	F	G	A	B	C	D	E	H	I	J
Soybean meal	64	69	58.2	62	58.62	57.9	76	99	96	93.9	90	97.56	95.8	99.00	97.2
		75			63.34			98	97			95.71		97.33	
		76												97.20	
Meat and bone meal	56				49.45			65				91.69	65.1	72.40	
Fish meal	43	44	48	45	29.07	24.4		92	90	93.9	85	87.45	91.8	94.53	93.0
				52.10						92.42					
					60.88							95.42			

A – Van Straalen and Tamminaga (1990); B – Hvelplund (1985); C – Volden and Harstad (1995); D – Demarquilly *et al.* (1989); E – Masoero *et al.* (1994); F – Várhegyi *et al.* (1998); G – Arieli *et al.* (1993); H – De Boer *et al.* (1987); I – Frydrych (1992); J – Voight *et al.* (1985)

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