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POKYNY PRO AUTORY

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Effect of activation treatments on the *in vitro* developmental potential of porcine nuclear transfer embryos

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ABSTRACT: In this study the effect of either chemical or physical activating agents was examined as exerted on the *in vitro* developmental potential of porcine embryos reconstructed by direct microinjection of cumulus cell nuclei into the cytoplasm of enucleated oocytes. In group A reconstituted oocytes were exposed to ionomycin and then to cycloheximide (CHXM), and in group B the oocytes were treated with electrical pulses. The reconstituted oocytes activated electrically showed a higher survival rate than those activated by ionomycin + CHXM (82.2% vs. 71.5%; $P \leq 0.01$). Cleavage activity and blastocyst formation rates tended to be slightly higher in the chemical activation group than in the electroporation group (61.4% vs. 58.4% and 11.3% vs. 6.2%, respectively), but the differences were not statistically significant ($P > 0.05$). However, the *in vitro* developmental rate of nuclear transferred embryos to the morula stage was significantly higher in group A than in group B (57.7% vs. 43.1%; $P \leq 0.05$). The blastocyst cell numbers in both experimental groups were comparable (approximately 26.0). In conclusion, the results of the present study indicate that both artificial activation protocols were efficient for the production of porcine nuclear transferred blastocysts to a similar extent.

Keywords: nuclear microinjection; ionomycin; cycloheximide; electroporation; *in vitro* culture

Nuclear transfer (NT) techniques have advanced in the last five years, and cloned offspring have been obtained from somatic cells in several species of farm animals including pig (Wilmut *et al.*, 1997; Baguisi *et al.*, 1999; Wells *et al.*, 1999; Yin *et al.*, 2002). However, the efficiency of somatic cloning technology in pigs remains still lower than in other livestock species.

Development of the studies on pig somatic cloning has been inspired especially in recent years not only by the necessity of quick improvement of nuclear transfer technique efficiency in this species but also above all by the possibility of its practical application to multiplication of transgenic piglets, on the grounds of important implications for transplantation medicine and immunology and also pharmacy as well as animal breeding (Betthauser *et al.*, 2000; Polejaeva *et al.*, 2000). The shortage of organs for human allotransplantation became a stimulus to the search for new, alternative sources of grafts. Therefore the possibility of using transgenic pig organs in xenotransplantation is a particularly attractive aspect of pig somatic cloning (Polejaeva *et al.*, 2000; Dai *et al.*, 2002; Lai *et al.*, 2002).

An extremely important factor influencing the developmental potential of reconstructed porcine oocytes is their activation procedure. In pig somatic cloning technology, the most often used activating stimuli are physical agents such as DC pulses (Onishi *et al.*, 2000; Polejaeva *et al.*, 2000; Kuhholzer *et al.*, 2001; Lai *et al.*, 2001; Park *et al.*, 2001; De Sousa *et al.*, 2002; Yin *et al.*, 2002, 2003; Samiec *et al.*, 2003) or chemical agents such as ionomycin (Betthauser *et al.*, 2000; Boquest *et*

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al., 2002; Yin *et al.*, 2002; Samiec *et al.*, 2003) or thimerosal in combination with dithiothreitol (Tao *et al.*, 1999a,b; Kuhholzer *et al.*, 2000). The current intensive studies on improving artificial activation methods of porcine cytoplasmic hybrids are chiefly aimed at optimising technical parameters of electrical field (strength, duration of DC pulses, number of pulses and time interval between them; Koo *et al.*, 2000; Onishi *et al.*, 2000; Verma *et al.*, 2000; De Sousa *et al.*, 2002; Martinez Diaz *et al.*, 2002; Zhu *et al.*, 2002) or, more frequently, at associating an activating stimulus (most often ionomycin or Ca^{2+} ionophore A23187 or DC pulses) with agents blocking the activity of cyclin-dependent protein kinases (CDK) such as 6-dimethylaminopurine (6-DMAP; Betthauser *et al.*, 2000; Boquest *et al.*, 2002; Roh and Hwang, 2002; Yin *et al.*, 2002), butyrolactone (Yin *et al.*, 2002) or agents inhibiting protein synthesis (translation), for example cycloheximide (CHXM; Cheong *et al.*, 2000; Martinez Diaz *et al.*, 2002; Yin *et al.*, 2002; Samiec *et al.*, 2003).

The basic aim of our study was to investigate the effect of activation protocols (with chemical or physical agents) on the *in vitro* developmental potential of porcine embryos reconstructed by the intraooplasmic injection of cumulus cell nuclei.

MATERIAL AND METHODS

Oocyte collection and maturation

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO).

Slaughterhouse ovaries were collected from prepubertal and postpubertal gilts as well as sows. Cumulus-oocyte complexes (COC's) were recovered by aspiration of follicular fluid from 2- to 6-mm antral follicles using an 18-gauge needle attached to a 10-ml disposable syringe. COC's were collected into HEPES-buffered Tissue Culture Medium 199 (TCM 199-HEPES; Gibco BRL, Life Technologies Inc., Grand Island, NY) supplemented with 50 IU/10 ml heparin, maintained at 39°C. COC's with evenly granulated ooplasm and several uniform layers of compact cumulus cells were selected for *in vitro* maturation. Ten to fifteen COC's were transferred in 10 µl of this medium and placed into 40 µl-droplets of maturation medium in 35 × 10 mm^2 Petri dishes (Falcon, Becton, Dickinson and Co, Lincoln Park, NY) that had been overlaid with paraffin oil and equilibrated at 38.5°C in the atmosphere of 5% CO_2 in air for 1 to 3 hours. The maturation medium comprised 25 mM HEPES and 26 mM sodium bicarbonate-buffered Tissue Culture Medium 199 (Gibco BRL, Grand Island, NY) supplemented with 20% heat-inactivated estrus porcine serum (EPS), 10% porcine follicular fluid (pFF), 1 mM L-glutamine and 75 µg/ml kanamycin monosulphate (kanamycin A). The oocytes were cultured for 42 to 44 h at 38.5°C in a 100% water-saturated atmosphere of 5% CO_2 in air. After maturation, expanded cumulus cells and corona cells were completely removed by vigorous pipetting COC's without or in the presence of 0.1% hyaluronidase in 500 µl of HEPES-buffered TCM-199 for 1 to 2 minutes. *In vitro* matured, metaphase II oocytes, selected on the basis of accepted morphological criteria, and first of all with evenly granulated, dark cytoplasm and intact plasma membrane, and with distinctly expelled first polar bodies provided a source of recipient cells for exogenous cell nuclei in the somatic cloning procedure.

Preparation of donor cells

To prepare karyoplasts, the cells were dissociated from COC's by vortexing matured COC's for 1 min in TCM 199-HEPES with addition of 4 mg/ml BSA (fraction V) or by centrifugation for 10 min at 300 × g in the same medium. After centrifugation, the supernatant was removed and the cell pellet was resuspended in 500 µl of TCM 199-HEPES/BSA until use. Immediately before microinjection, a single cell suspension of the cumulus cells was prepared by vigorous pipetting.

Nuclear transfer

Cumulus-free oocytes were stained with 5 µg per ml DNA fluorochrome – bisbenzimide (Hoechst 33342) for 15 to 20 min in TCM 199-HEPES/BSA medium supplemented with 7.5 µg/ml cytochalasin B (CB), and then transferred into a glass micromanipulator chamber filled with TCM-199 containing 4 mg/ml BSA, and 7.5 µg/ml CB. Enucleation was accomplished by gently aspirating the first polar body and the metaphase II plate in a small amount (10–20% of the volume of the

oocyte) of surrounding cytoplasm with the aid of a bevelled micropipette of 20- to 25-μm external diameter. Completeness of enucleation was visually ascertained in every case by UV light identification of blue fluorescence emitted by both labelled chromatin of the polar body and metaphase II plate chromosomes of the removed karyoplast, while still inside the enucleation pipette, keeping exposure to a minimum. Following enucleation, the resulting cytoplasts were washed extensively in TCM 199-HEPES + 0.4% BSA and held in this cytochalasin B-free medium until microinjection of donor nuclei. Intracytoplasmic injection of cumulus cells was performed in TCM 199-HEPES/BSA containing 2.5 μg/ml CB. Donor cells were selected under differential interference contrast (DIC) optics according to their size and shape. Small cells (10–15 μm diameter) with the smooth, intact plasma membrane surface were chosen as donor nuclei to be injected directly into enucleated oocytes. By using an injection pipette whose sharp bevelled tip had an external diameter about half smaller than the diameter of the selected cumulus cells (7–8 μm), the plasma membrane was broken by gentle repeated aspiration of the entire cells into and out of the pipette. Then the injection pipette was introduced mechanically through the *zona pellucida* (using the same slit as made during enucleation) and up to halfway into the ooplasm of each cytoplast and the tiny karyoplast was quickly deposited with the cytoplasm. Cell nuclei were transplanted between 47 and 49 h after the initiation of oocyte maturation, and the reconstructed preembryos were incubated in NCSU-23 medium (Petters and Wells, 1993) with 4 mg/ml BSA (fraction V) at 38.5°C in a 100% humidified atmosphere of 5% CO_2 in air for 1 to 1.5 h before activation.

Activation protocol

After a preincubation period, cytoplasmic clonal hybrids were artificially activated with two different activating agents: either chemical or physical ones. For two-grade chemical postactivation, clonal cybridic zygotes were exposed to 15 μM ionomycin in NCSU-23/BSA medium for 5 to 6 min, then washed three times in 500 μl of NCSU-23 medium containing 0.4% BSA, and incubated in 10 μg/ml cycloheximide (CHXM) and 5 μg/ml cytochalasin B for 1.5 to 2 h in NCSU-23 medium with 4 mg/ml BSA at 38.5°C in humidified air with 5% CO_2. Afterwards reconstituted zygotes were transferred to the same medium supplemented only with 10 μg/ml CHXM for additional 2 to 2.5 hours.

For electrical poststimulation, reconstructed preembryos were equilibrated in 500 μl of electroporation medium for 5 min at 39°C and then transferred to an electroactivation chamber with two wire electrodes 0.5 mm apart overlaid with 10 ml of the same medium. Activation was induced with two consecutive DC pulses of 1.2 kV/cm for 60 μs each, delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA). Immediately after electrostimulation, cybridic clonal zygotes were left once more in the electroporation medium for 5 min at 39°C, before being transferred to the NCSU-23/BSA medium supplemented with 5 μg per ml CB for 2 hours. The electroporation medium was an isotonic dielectric solution consisting of 0.3 M D-mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.2 mg/ml fatty acid free BSA (FAF-BSA).

In vitro culture of embryos

After activation treatments, surviving reconstructed embryos (with intact plasma membrane) were cultured in 50-μl droplets of NCSU-23 medium containing 4 mg/ml BSA (fraction V) at 38.5°C, in a 100% water-saturated atmosphere of 5% CO_2 in air. After 72 to 96 h of *in vitro* culture, cleavage-stage embryos were selected. Twenty to forty cleaved embryos were cultured together in a 50-μl drop of NCSU-23 medium supplemented with 10% FBS at 38.5°C in an incubator with 100% humidity and 5% CO_2 in air for additional 72 hours. At the end of the *in vitro* culture period (Days 6–7), embryos were evaluated morphologically for blastocyst formation and subsequently stained with 5 μg/ml bisbenzimide (Hoechst 33342) to analyse the number of nuclei on an epi-fluorescent microscope (Olympus IMT-2, Tokyo, Japan).

Experimental design

In the first series of experiments, the effect of cumulus cell nuclei microsurgical transfer operation on the viability of reconstructed embryos depending on the applied postactivation procedure was estimated. Reconstituted oocytes were divided into two experimental groups according to the type of

activating stimulus: group A (two-grade chemical activation) and group B (electroactivation).

In the second series of experiments, developmental competences of *in vitro* cultured embryos reconstructed with cumulus cell nuclei, but exposed to two different activating agents: either chemical (ionomycin and cycloheximide; group A) or physical ones (electric pulses; group B) were compared. After 6 to 7 days of *in vitro* culture, the quality of blastocysts obtained in both experimental groups was compared, taking the cell number in blastocysts as the estimation criterion.

Statistical analysis

In order to compare the number of embryos surviving the cell nuclei microinjection operation, the number of cleaved embryos and the number of embryos at morula and blastocyst stages between both groups of reconstructed oocytes, exposed to two different activating stimuli: either physical (electrical pulses) or chemical ones (two-grade activation with the use of ionomycin and CHXM combination), Chi-square test was used.

RESULTS

The qualitative and quantitative composition of the medium prepared in our studies for *in vitro* maturation of porcine oocytes turned out to be optimal and in the established culture conditions allowed reaching nuclear and cytoplasmic maturity for approximately 50 to 70% of recipient oocytes.

The efficiency of enucleation operation verified under UV light after staining the oocytes with bisbenzimide (Hoechst 33342) was 87.7% (221/252 oocytes) and 88.1% (304/345 oocytes) in groups A and B, respectively.

In group A, 221 enucleated oocytes were subjected to direct intracytoplasmic microinjection of cumulus cell nuclei. After chemical activation, 158 reconstructed embryos (71.5%; Table 1) kept the integrity of plasmolemma intact and were classified into *in vitro* culture.

In group B, microsurgical nuclear transfer was performed on a total of 304 enucleated oocytes. From this initial number of micromanipulated cytoplasts, after electrical activation viability was maintained by 250 reconstructed embryos (82.2%; Table 1), which were then cultured *in vitro*.

The data in Table 1 clearly show that significant differences in percentages of favourably reconstructed oocytes were found ($P \leq 0.01$) according to the artificial activation protocol used. Cleavage and blastocyst formation (Figure 1) rates tended to be higher in the ionomycin/CHXM group than in electroactivation group, but the difference was not significant ($P > 0.05$). However, the *in vitro* developmental competences to the morula stage were significantly higher in group A than in group B ($P \leq 0.05$).

The results given in Table 1 show comparable cell numbers in blastocysts produced from oocytes reconstructed by the microsurgical transfer technique of cumulus cell nuclei, but treated with two different activating agents: either chemical (25.5 cells on average) or physical ones (26.7 cells on average).

Table 1. Comparison of *in vitro* developmental competences of porcine NT embryos reconstructed with cumulus cells, exposed to two different activating agents

Activation treatment	No. injected/ No. repl.	No. cultured (%)**	No. cleaved (%)	No. morulae (%)*	No. blastocysts (%)	Blastocyst mean nuclear number
Ionomycin + CHXM	221/5	158/221	97/158	56/97	11/97	25.5
		(71.5)[a]	(61.4)[a]	(57.7)[a]	(11.3)[a]	
Electrical pulses	304/6	250/304	146/250	63/146	9/146	26.7
		(82.2)[b]	(58.4)[a]	(43.1)[b]	(6.2)[a]	

*, a, b the values with different superscript letters within columns are significantly different ($P \leq 0.05$)
**, a, b the values with different superscript letters within columns are significantly different ($P \leq 0.01$)

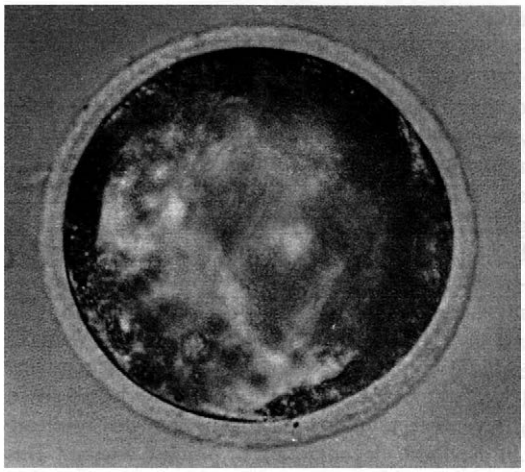

Figure 1. A day 7 expanding blastocyst reconstructed with cumulus cell nucleus

DISCUSSION

The results of the present studies show conclusively that it is possible to obtain compact morulae and blastocysts, and even hatching blastocysts from *in vitro* cultured porcine oocytes reconstructed with cumulus cell nuclei and postactivated chemically or physically.

In our study, two protocols of reconstructed porcine oocyte artificial activation were used. The first of the used activating agents were electrical pulses. Although the derivation of Ca^{2+} ions during electroactivation is generally considered to be extracellular, it is suggested that electroporation also stimulates Ca^{2+} release from intracellular stores (Ozil, 1990). The application of single DC pulses leads to a single mobilization of intracellular calcium reservoirs. On the contrary, the strategy of electric pulse multiplication allows for generation of long series of oscillatory Ca^{2+} peaks, many times raising the concentration of these cations in ooplasm (cit. according to Fissore *et al.*, 1999). That is why the protocol based on multiplication of electrical stimulation partially imitates physiological activation caused by single sperm penetration into oocyte. In the studies on pig somatic cloning (Verma *et al.*, 2000; De Sousa *et al.*, 2002; Zhu *et al.*, 2002), multiplication of electrical pulse was positively correlated with higher developmental ability of reconstructed oocytes to the morula stage and higher percentage of formed blastocysts. In our experiments, the application of two successive DC pulses of 1.2 kV/cm for 60 μs each led to obtaining 43.1% morulae and 6.2% blastocysts. Similar results in the way of *in vitro* developmental potential of reconstructed oocytes were obtained by Onishi *et al.* (2000) as well as by De Sousa *et al.* (2002), Martinez Diaz *et al.* (2002) and Yin *et al.* (2002). However, in the studies directed by Onishi (2000), optimisation of electrical field parameters led to adumbrating of an inverse tendency in the *in vitro* developmental competences of porcine clonal zygotes. The higher developmental abilities were characteristic of oocytes reconstructed by microinjection of foetal fibroblast nuclei that were activated with a single electrical pulse compared to those stimulated with three DC pulses. However, a decrease in DC pulse number in the electroactivation protocol from three to one was accompanied by a prolongation of their duration from 60 to 100 μs and an increase in the electrical field strength from 1.3 to 1.5 kV/cm. Thus elaborated physical parameters of electroporation led to obtaining 2.4% blastocysts in the same *in vitro* culture conditions, which were used in our own experiments. However, we suppose that in the experiments by Onishi *et al.* (2000) only a single DC pulse proved sufficient for stimulating the developmental program of reconstructed oocytes and for maintaining it to the blastocyst stage. Microsurgical transfer of somatic cell nuclei directly into cytoplasm might provide reconstituted oocytes with sufficiently strong signals stimulating intracellular calcium deposits, supporting appropriate activating stimuli, applied during electroporation. Another reason for premature mobilization of endogenous Ca^{2+} ion release channels in the calcium intracellular stores can be the aging process of reconstructed porcine oocytes as a result of their overlong (3- to 4-h) incubation in the medium of low ionic strength before electrical activation (Onishi *et al.*, 2000; Park *et al.*, 2001). The high intensity of ionic signals in reconstructed porcine oocytes brought about by interference of Ca^{2+} oscillation effects induced by two different factors: spontaneous autoactivation by aging of oocytes and electroporation led to an increase in developmental abilities of *in vitro* cultured embryos to the blastocyst stage. In turn, in our own studies (Samiec *et al.*, 2003) the incubation time of reconstructed porcine zygotes before the activation procedure was shortened to approximately 1 to

1.5 h in order to avoid the negative side effects of premature autoactivation of aging cloned zygotes.

The second activating factor, which was used in our studies, was ionomycin. The incubation of reconstructed porcine oocytes with ionomycin generates a prolonged rise of intracellular calcium ion concentration ($[Ca^{2+}]_i$), which partially assimilates this artificial chemical activation strategy with the physiological pattern of calcium concentration changes in cytosol of porcine oocytes, observed during fertilization. In the case of exposure of porcine reconstituted oocytes to ionomycin, the source of Ca^{2+} cations present in cytoplasm probably has both endogenous and extracellular derivation (Fissore *et al.*, 1999). The mechanism of stimulation of reconstructed oocytes to further development consists in inducing two-phase changes in the cytoplasmic concentration of calcium ions. Instead of direct action upon the plasmolemma ultrastructure and facilitation of transmembrane Ca^{2+} transport, ionomycin first mobilizes intraoocytic calcium deposits, which in turn, by virtue of positive feedback stimulates diffusion of exogenous calcium cations into reconstituted porcine oocytes (probably, generation of two waves of $[Ca^{2+}]_i$ increase; cit. according to Prather *et al.*, 1999; Morgan and Jacob, 1994)

In comparison with the standard chemical activation systems commonly used for cattle somatic cloning (Dominko *et al.*, 1999; Wells *et al.*, 1999) in which the incubation of reconstructed zygotes in 5 μM ionomycin solution for 4 to 5 min is recommended, in pig cloning the strategy consisting in an increasing of ionomycin concentration to 15 μM (Betthauser *et al.*, 2000; Yin *et al.*, 2002) yields considerably better results in terms of both the increase in developmental potential of *in vitro* cultured cloned porcine embryos and the enhancement of morphological quality of obtained blastocysts. We suppose that such an advantageous influence of high ionomycin concentration on further embryonic development of reconstituted porcine oocytes probably results from the fact that the increase in this activating agent concentration from 5 to 15 μM induces rapid appearance of two interference $[Ca^{2+}]_i$ rise waves of increasing amplitude or outpouring of single progressive (translational) wave of Ca^{2+} increase enhanced with extracellular Ca^{2+} signal transduction (wave with two amplitudes of deflections from $[Ca^{2+}]_i$ homeostasis state, probably). However, it results from our previous unpublished observations that prolongation to 20 min of exposure time of reconstituted porcine oocytes to increased ionomycin concentration causes a considerable decrease in embryo viability. But a slight modification of this experimental system led to improvements of *in vitro* developmental competences of reconstructed embryos. In our experimental protocol the shortening of exposure time to 5 to 6 min while simultaneously preserving the same concentration (15 μM) of activating agent allowed us to increase the viability of reconstructed embryos (Samiec *et al.*, 2003). We suggest that 20-min incubation in the ionomycin solution of such a high molar concentration could lead to a rapid increase in intracellular Ca^{2+} cation release (Ca^{2+} excytotoxicity phenomenon), which caused irreversible cellular metabolism changes, resulting in inevitable degeneration of porcine oocytes. This hypothetical hyperactivation of oocytes as an effect of excessive mobilization of intracellular calcium deposits of oocytes in combination with partial loss of cell membrane integrity and drastic displacements of cytoskeleton and membrane skeleton microfilaments during nuclei microinjection could impair the viability of reconstructed embryos to a higher extent. The results of the present studies also show that the two-step activation procedure with ionomycin and CHXM is a more invasive method than double electrostimulation because above 80.0% of reconstructed cybrids preserved viability after microsurgical nuclear transfer and treatment with DC pulses compared to approximately 70.0% of microinjected oocytes treated with chemical activating agents.

Betthauser *et al.* (2000) showed that using the combination of ionomycin (in the variant of increased concentration in prolonged incubation time) with 6-DMAP was positively correlated with the development rate of porcine nuclear transferred zygotes to the blastocyst stage, and also with cell number in the obtained blastocysts. However, in our previous studies (unpublished data) we did not observe any similar relationships. Accurate repetition of these chemical activation parameters was limited to the minimum percentage of *in vitro* developing embryos. In this connection we undertook tests to modify the original variant of this two-grade activation (proposed by Betthauser *et al.*, 2000) through replacement of 6-DMAP by a protein synthesis (translation) inhibitor – cycloheximide (Samiec *et al.*, 2003). Only such an experimental system allowed us to obtain satisfactory results as for the *in vitro* developmental potential of porcine cloned zygotes. Therefore it cannot be ruled out that it

was not the increase in ionomycin concentration to 15 μM that contributed to obtaining approximately 11% blastocysts, but above all the combination of the action of this activating agent with 4-h incubation of oocytes with cycloheximide. In turn, Boquest *et al.* (2002) received a considerably higher blastocyst percentage (23.0%) after decreasing the ionomycin concentration to 5 μM and maintaining the same reconstructed oocyte incubation period as in our experiments. The results of both studies carried out by Boquest *et al.* (2002) and our experiments (Samiec *et al.*, 2003) suggest that the increase in ionomycin concentration may also have an inhibiting effect on the *in vitro* developmental potential of clonal embryos. However, the use of the high ionomycin concentration–induced activation method as an alternative protocol to artificial stimulation by a low concentration of ionomycin will require further comparative investigations into factors responsible for the generation of calcium oscillations/transient elevations in the cytosol of pig nuclear transferred oocytes.

In conclusion, the present study has demonstrated that reconstructed porcine oocytes exposed to the two-grade chemical activation treatment with ionomycin and CHXM showed significantly higher *in vitro* developmental competences to the morula stage and only slightly higher ones to the blastocyst stage, compared to those exposed to electroporation. In contradistinction to these results, Yin *et al.* (2002) reported that neither developmental ability of nuclear transferred zygotes to the blastocyst stage nor morphological quality of reconstructed blastocysts was different in different methods of activation (physical, chemical and combined, physicochemical). In our experiments, the cell numbers of the blastocysts obtained from reconstituted oocytes treated with either activating agents were relatively low. That is why we suggest that improvements of *in vitro* culture conditions may result in blastocysts with higher cell numbers, thus being competent for further *in vivo* development after transfer into surrogate recipient sows. Future experiments are also needed to establish whether the use of artificial activation protocols elaborated in our laboratory can lead to producing viable cloned piglets.

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ABSTRAKT

Vliv způsobu aktivace na vývojový potenciál *in vitro* prasečích embryí z nukleárního transferu

Byl sledován vliv chemických a fyzikálních aktivačních stimulů na *in vitro* vývojový potenciál prasečích embryí, získaných přímou mikroinjekcí jader kumulárních buněk do cytoplasmy enukleovaných oocytů. Ve skupině A byly

rekonstruované oocyty vystaveny působení ionomycinu a cykloheximidu (CHXM), ve skupině B působení elektrických impulsů. Elektricky aktivované rekonstruované oocyty vykazovaly vyšší procento přežití než ty, které byly aktivovány kombinací ionomycinu a cykloheximidu (82,2 % vs. 71,5 %; $P \leq 0,01$). Aktivita dělení a rychlost tvorby blastocyst byly o něco vyšší ve skupině s chemickou aktivací než ve skupině s elektroporací (61,4 % vs. 58,4 % resp. 11,3 % vs. 6,2 %); rozdíly nebyly statisticky významné ($P > 0,05$). Vývojová rychlost *in vitro* embryí z nukleárního transferu do stadia moruly byla významně vyšší ve skupině A než ve skupině B (57,7 % vs. 43,1 %; $P \leq 0,05$). Počty buněk blastocyst byly v obou pokusných skupinách srovnatelné (přibližně 26,0). Výsledky této studie ukazují, že oba aktivační protokoly, zaměřené na produkci prasečích blastocyst po přenosu jader, byly srovnatelně účinné.

Klíčová slova: mikroinjekce jader; ionomycin; cykloheximid; elektoporace; kultivace *in vitro*

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Laparoscopic and non-surgical transfer of fresh and frozen porcine embryos

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ABSTRACT: This paper is aimed at studying factors affecting the success rate of porcine embryo transfers. The results of experiments conducted until now allow to evaluate effects of transfer methods (laparoscopic vs. non-surgical), effects of recipient parity (sows vs. gilts) and effects of embryo types (fresh vs. frozen-vitrified). Embryos in the stage of expanded, hatching and hatched blastocyst were recovered *post mortem* from superovulated donors – selected breeding gilts in 5.5 to 6.0 days after oestrus and artificial insemination with homospermic doses. Recipients were synchronized either by feeding Regumate and treatment with PMSG and HCG (gilts) or by piglet weaning (sows – 1st and 2nd parity). Recipients showing standing reflex at the time of donor insemination were used for laparoscopic and non-surgical ET on day 5.5–6.0 of the cycle. The vitrification method of putting embryos into liquid nitrogen in a medium containing 30% v/v of glycerol, 20% v/v of foetal calf serum and 50% v/v of 2M sucrose in tridistilled water (max. 1.5 min) was used for cryopreservation of porcine embryos; H-MEMD medium with 10% v/v of glycerol (10 min) was used for equilibration. Thawing and washing were carried out in H-MEMD medium with 0.8M sucrose (8 min). Laparoscopic transfers were realised in anaesthetised recipients by three insertions (Wolf laparoscope, Germany). An insemination probe was used for non-surgical transfers. A silicone (adequately rigid) catheter with an injection syringe containing approximately 5 ml of culture medium was passed through the AI catheter. In total, 36 ET were evaluated, 19 recipients were pregnant (55%), 4 of them aborted. No significant differences ($P > 0.05$) were found between gilts and sows in success rates of the laparoscopic transfer of fresh and frozen embryos, differences in birth rate were statistically significant in some experimental groups ($P < 0.01$). ET of fresh embryos and ET of vitrified embryos resulted in 48.0% (12/25) and 63.6% (7/11) pregnancy rate, resp. ($P > 0.05$). The birth rate resulting from vitrified embryo transfers was significantly higher ($P > 0.01$), but on average a higher number of thawed embryos was transferred (10.8 ± 2.10 fresh and 13.1 ± 4.22 thawed embryos per pregnant recipient). Pregnancy rate in recipients of non-surgically transferred embryos ranged from 25.0% to 50.0%. The rate of surviving embryos out of transferred embryos ranged from 36.1% to 69.2% and amounted to approximately 50%, the rate of weaned piglets oscillated at 95% level in all experimental groups. The evaluated set is adequately representative, therefore the results of the analysis are valuable and convincing.

Keywords: pig; embryo; transfer; cryopreservation; recipient; gilt; sow

Nowadays embryo transfer is applied routinely in the reproduction process and selection schemes in cattle, sheep and goats.

The application of this progressive method in pigs is associated with many problems resulting from specific physiological and morphological functions and specific build of porcine reproductive organs and from characteristics of porcine embryos.

Embryo recovery

In most cases, a surgical method is used for embryo recovery – total anaesthesia induced with barbiturates or with halothane, laparotomy at the *linea alba* (back position). The mentioned procedure cannot be carried out under practical conditions. Methodical principles for the surgi-

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cal embryo recovery published by Hancock and Hovell (1962) and Dzuik *et al.* (1964), König *et al.* (1989), Brussow and König (1992), Modl (1994) were modified – Oberfranc *et al.* (1992), Oberfranc (1994), Niemann (1991, 1995), Říha *et al.* (1997a), Gordon (1997) and others. In embryos recovered on Day 5.5–6 after insemination the recovery rate amounts to 80–90% (Gordon, 1997). The length and structure of the cervix and uterine horns complicate development and ensuing application of the non-surgical recovery method. Hazeleger *et al.* (1989) shortened uterine horns to 20 cm approximately. Embryos were recovered from 4 sows. These sows were not, however, utilisable in the normal routine reproductive system. In general, porcine embryos for experimentation are recovered *post mortem*.

Embryo cryopreservation

Brussow and König (1992), Niemann (1991) specified problems associated with cryopreservation of porcine embryos and characterised the actual situation as an experimental stage of embryotechnology application. Problems associated with cryopreservation and long-term storage of porcine embryos, developmental competency of vitrified embryos, and success of laparoscopic transfers of fresh and vitrified embryos were published and discussed in papers of Říha *et al.* (1996, 1997a,b), Říha and Vejnar (2000). In spite of positive and favourable results numerous problems still characterise cryopreservation of porcine embryos.

Ceullo *et al.* (2002) published the results of experiments studying procedures of vitrification and non-surgical transfer of porcine embryos. Expanded blastocysts recovered on Day 5–5.5 after the 1st insemination were used for vitrification. The population issuing from 20 vitrified embryos recovered from three donors was transferred asynchronously (–24 h) with a catheter used for deep intracornual insemination into 10 cyclic sows (Meishan breed). Pregnancy was diagnosed with an ultrasonic probe on Day 23 post ET in five recipients. Dinnyes *et al.* (2003) also tested cryopreservation of porcine embryos. They used expanded or expanding blastocysts and two types of vitrification media – they put drops of media containing embryos on the metal cooled to –180°C. Thawing was made by inclusion of microdrops into the culture medium containing trehalose. The testing of embryo development in culture medium (24 h) documented a reduction in the embryo survival rate of frozen embryos as compared to unpreserved ones: 15 and 16% vs. 41% (expanding blastocysts) and 24 and 25% vs. 58% (expanded blastocysts). According to Esaki *et al.* (2003) the success of vitrification could be improved by pre-vitrification delipidisation of IVP porcine embryos. Fujino *et al.* (2003) studied the cryotoleration of expanded blastocysts and hatched blastocysts recovered on Day 6 after the 1st insemination under a slow freezing regimen (by 0.4°C from –7°C to –30°C before their transfer into liquid nitrogen). The embryos were divided into three groups (low, medium and high tolerance to low temperature). Survival rates found in specific groups (after *in vitro* cultivation as well as after ET) differed. Conception rate amounted to 78%, 22%, and 0%, respectively. In total, 9 recipients were pregnant and 46 piglets were born, i.e. 15.3% survival rate and 3.2% survival rate characterised highly tolerant and medium tolerant groups, respectively. Kobayashi *et al.* (2003) used two methods for vitrification of blastocysts (B), expanded blastocysts (EB) and hatched blastocysts (HB) by OPS (open pulled straw) procedure. According to their results, a minimum concentration of cryoprotective agent is necessary for successful vitrification. Vitrification of EB and HB was more successful than vitrification of B. In total, 87 embryos (EB and HB) were transferred after 20–23 days of storage. Three of 4 recipients were pregnant – 8 and 4 piglets were born (one pregnant recipient aborted). Ushijima *et al.* (2003) froze 5-day embryos (morula stage). Development of morulae (delipidised before freezing) after *in vitro* culture was comparable to development of fresh morulae. The mentioned authors recommended to use the smallest possible quantity of medium.

Embryo transfer

Embryos are transferred predominantly by surgical methods – the laparotomic one or the laparoscopic one (less invasive procedure). The latter method described by Oberfranc (1994) was applied in our experiments mentioned above. First surgical transfers of fresh embryos were characterised by a very low and variable pregnancy rate and by a very small litter size. These results were summarised by Gordon (1997).

With regard to the mentioned problems ET has been applied only in U.S. clinical experimentation and in curative programs. Nowadays, an approximately 75% pregnancy rate characterises surgical transfers of 3 to 7-day embryos (Day 0 = day of the 1st insemination). This rate is reduced significantly in embryos transferred on Day 8 and Day 9. Embryos are transferred in a small pipette into the lumen of the uterine horn incised slightly at the utero-tubal junction. The trauma of endometrium and haemorrhage affect significantly the success of transfers. Říha *et al.* (1997a,b) reported relatively favourable pregnancy rate and litter size in the case of fresh and vitrified embryos transferred by the laparoscopic procedure.

Similarly like in sheep and goat, surgical procedures of embryo recovery and embryo transfer are limiting factors reducing the development and commercial utilization of ET in pig production. A very low success rate (3%) was mentioned in the case of non-surgical transfer of porcine embryos (Polge and Day, 1968).

The anatomic structure of the cervix and the small orifice of the cervical channel complicate the penetration of catheter into the lumen. Sims and First (1987) used a non-surgical method without any success. Reichenbach *et al.* (1993), Galvin *et al.* (1994), Modl (1994), Hazeleger *et al.* (1995a,b), Wallenhorst and Holz (1995) successfully used non-surgical methods for transcervical transfers of fresh porcine embryos. Li *et al.* (1995), Gordon (1997) mentioned the necessary construction of the catheter. Development of a complex method for porcine ET is still in the experimental phase. Cameron and van der Lende (1998) presented a more successful non-surgical procedure ET program in spite of some achievements in the transfer of fresh porcine embryos. According to Yonemura *et al.* (2003) the application of a routinely used insemination catheter and 15 ml of transfer medium for non-surgical transfer to a standing anaesthetised recipient was associated with the highest pregnancy rate of recipients. Cuello *et al.* (2002) used a catheter for deep intrauterine insemination for successful transfer of vitrified embryos – five of 8 recipients were pregnant. Caamano *et al.* (2003) studied the effects of the day of sexual cycle at recovery and transfer on success rates of transfers. Differences in pregnancy rate and in embryo survival were insignificant.

The evaluation of factors affecting the success rate of porcine embryo transfer is the principal objective of this study. The results of conducted experiments enable to evaluate ET type (laparoscopic vs. non-surgical), parity of recipients (sows vs. gilts) and types of transferred embryos (frozen vs. fresh).

MATERIAL AND METHODS

Animals

Donors. Selected puberal breeding gilts (Czech Super Meat and Landrace) superovulated according to the schemes described by Říha and Vejnar (2000) were used as embryo donors (feeding of Regumate for 15 days and treatment with 750 I.U. PMSG and 300 I.U. HCG). Homospermic doses were used for insemination.

Recipients. Weaning of piglets was used for synchronization of recipients – sows after the 1st and 2nd weaning, between Day 28 to Day 32 *post partum* (5.5 days after the controlled oestrus). Sexual cycles of puberal gilts-recipients were synchronized by feeding Regumate (active agent: altrenogest, producer: Hoechst, doses recommended by the producer) for 15 days and treatment with 500 I.U. PMSG (Sergon, ad us. vet., Bioveta Ivanovice in Haná, C.R.) and 300 I.U. HCG (Praedyn, Léčiva Prague, C.R., or Werfachor, Austria). Recipients showing standing reflex at the time of donor insemination were used for ET only.

Embryo recovery, cultivation and transfer

Procedures of *post mortem* embryo recovery, cultivation, morphological classification, vitrification, thawing and laparoscopic transfer were specified completely in our previous studies (Říha *et al.*, 1996, 1997a,b; Říha and Vejnar, 2000). The non-surgical intubation (transfer) was made with an A.I. probe – a silicone (adequately rigid) catheter completed with a syringe containing culture medium (5 ml approx.) was drawn through the probe. Sows of different colour were mostly used as donors and recipients.

Transferred embryos

Fresh embryos stored in complete conditioned medium MEMD completed with 20% foetal calf serum for 0.5–2.0 h at 22°C min and cryopre-

served embryos (vitrified according to procedures described by Říha *et al.*, 1997a) were used for transfers.

Analysis and evaluation of data

The results of ET carried out in the period 1998–2001 were compiled and analysed. The following criteria were used for analyses: pregnancy characteristics of recipients (ultrasonic probe – 2nd month after oestrus and ET, born piglets in total, piglets born alive, weaned piglets) and parameters characterising the embryo survival rate (born piglets from transferred embryos, born alive piglets/born piglets in total, weaned piglets/born alive piglets). Routine statistical methods (chi-square test, Student's *t*-test) were used for analysis and evaluation of data (Unistat, version 6.0).

RESULTS AND DISCUSSION

Summarised ET results

In total, 577 embryos were transferred into 36 recipients (14.36 ± 3.58 embryos per recipient on average). Pregnancy was diagnosed (ultrasonic

Table 1. General results of porcine embryo transfers

Item	*n*	$\bar{x}$ ± sd	%
ET	36		
Transferred embryos in total	577	14.36 ± 3.58	
Pregnant recipients	19*	–	55.0
Embryos transferred to pregnant recipients	222	14.8 ± 3.26	38.5
Born piglets	112	7.47 ± 3.18	50.0
Born alive piglets	105	7.00 ± 2.80	93.8
Weaned piglets	100	6.67 ± 2.10	95.2

*4 recipients aborted

Table 2. Results of ET – comparison of parity of embryo recipients (sows vs. gilts)

Item	Parity of recipients			
	sows		gilts	
ET (*n*)	16		18	
Transferred embryos (*n*/$\bar{x}$ ± sd)	258	16.13 ± 4.75	259	14.39 ± 5.57
Pregnant recipients (*n*/%)	9	56.3	10	55.6
Birth rate (*n*)	9		6*	
Embryos transferred to pregnant recipients (*n*/$\bar{x}$ ± sd)	130	14.4 ± 4.37	92	15.33 ± 5.6
Born piglets				
total (*n*/$\bar{x}$ ± sd)	70	7.78 ± 1.82	42	7.00 ± 1.83
percentage of transferred embryos	53.8		45.7	
born alive (*n*/$\bar{x}$ ± sd)	65	7.22 ± 2.16	40	6.67 ± 1.67
percentage of all piglets	92.8		95.3	
weaned piglets (*n*/$\bar{x}$ ± sd)	60	6.67 ± 1.67	40	6.67 ± 1.67
percentage of born alive piglets	92.3		100	

*4 recipients aborted, N.S., $P > 0.05$

Table 3. Results of ET – laparoscopic method. Effect of recipient parity

Item	Parity of recipients			
	gilts		sows	
ET (*n*)	2		6	
Transferred embryos ($n/\bar{x}$ ± sd)	36	18.0 ± 9.00	79	13.20 ± 3.62
Pregnant recipients (*n*/%)	2	100	5	83
Birth rate				
Embryos transferred to pregnant recipients ($n/\bar{x}$ ± sd)	36	18.00 ± 9.00	65	13.00 ± 3.58
Born piglets				
total ($n/\bar{x}$ ± sd)	13	6.50 ± 0.50**	45	9.00 ± 0.89**
percentage of transferred	36.1		69.2	
born alive ($n/\bar{x}$ ± sd)	12	6.00 ± 0.00**	42	8.40 ± 1.62**
percentage of all piglets	92.3		93.3	
weaned piglets ($n/\bar{x}$ ± sd)	12	6.00 ± 0.00**	37	7.40 ± 1.02**
percentage of born alive piglets	100		88.1	

$P > 0.05$, $^{**}P < 0.01$

Table 4. Results of non-surgical transcervical method. Effect of recipient parity

Item	Parity of recipients			
	sows		gilts	
ET (*n*)	12		16	
Transferred embryos ($n/\bar{x}$ ± sd)	179	14.9 ± 5.64	223	13.9 ± 4.86
Pregnant recipients (*n*/%)	4	33	8; 4*	50; 25*
Birth rate: (*n*)	4		4	
Embryos transferred to pregnant recipients ($n/\bar{x}$ ± sd)	65	16.3 ± 5.86	56	16.0 ± 5.20
Born piglets				
total ($n/\bar{x}$ ± sd)	25	6.3 ± 1.65	29	7.3 ± 3.60
percentage of transferred embryos	38.5		51.8	
born alive ($n/\bar{x}$ ± sd)	23	5.8 ± 1.27	28	7.0 ± 3.10
percentage of all piglets	92		97	
weaned piglets ($n/\bar{x}$ ± sd)	23	5.8 ± 1.27	28	7.0 ± 3.10
percentage of born alive piglets	100		100	

*4 recipients aborted, N.S., $P > 0.05$

probe during the 2nd month after ET) in 19 recipients (55%, Table 1). The mentioned result is better or comparable with data recorded by Reichenbach *et al.* (1993), Galvin *et al.* (1994), Modl (1994), Hazeleger *et al.* (1995a,b), Wallenhorst and Holtz (1995), Gordon (1997), Ceullo *et al.* (2002), Fujino *et al.* (2003). Similar results were also found in our previous studies (Říha *et al.*, 1996, 1997a,b; Říha and Vejnar, 2000). Four recipients aborted during the third trimester of pregnancy.

Utilisation of sows and gilts as embryo recipients

Table 2 presents the results of ET as related to the parity of recipients – sows (synchronised by piglet weaning, n = 16) and gilts (synchronised by feeding Regumate, n = 18). Differences between the mentioned groups (pregnancy rate, embryo survival, birth rate, percentage of weaned piglets) are minimal and statistically insignificant ($P > 0.05$) regardless of the type of transferred embryos (preserved or fresh ones). The mentioned results are interesting – the above-cited sources present data on recipients – synchronised gilts in most cases. It is evident that the two categories are utilisable – satisfactory success rate of ET is conditioned by the precise control of oestrus (onset and intensity) following piglet weaning or synchronization treatment (Gordon, 1997, and our results not published until now). Tables 3 and 4 present the results of ET as related to the parity of recipients after laparoscopic and non-surgical ET methods. Pregnancy rates after laparoscopic ET are high (100 and 83%, gilts and sows, resp.) in relation to the results presented in the introductory part of this paper. Birth rate in the sow group is statistically higher in relation to birth rate in the gilt group ($P < 0.01$, Tables 5 and 7).

Utilisation of fresh and frozen embryos

General results. Data illustrating the transfer of vitrified and fresh embryos are presented in Table 5.

In total, 11 transfers of frozen-thawed embryos recovered post mortem on Day 5.5–6 after oestrus and 1st insemination into recipients were made (Table 5). Seven recipients were pregnant (63.6%), in total 92 embryos were transferred to all pregnant recipients. In total, 57 piglets (61.9% of transferred embryos) were born, i.e. 8.14 ± 2.03 piglets on average; 53, i.e. 7.57 ± 1.78 piglets (93.0% of born piglets) were born alive, 49 piglets, i.e. 7.00 ± 1.28 piglets on average (92.5% of born piglets) were weaned. The same data on ET fresh embryos were: 25 transfers, 12 pregnant recipients (48%), 130 embryos transferred to the pregnant recipients, 55 born piglets (42.3%), 52 born alive (94.5%) and 52 weaned piglets (100%). Differences between the experimental groups were not statistically significant ($P > 0.05$, Table 5).

The first successful non-surgical transfer of vitrified embryos in our laboratory was reported in the paper of Říha and Vejnar (2000). Two transfers of vitrified embryos were carried out. The transfer of 19 embryos to a gilt synchronized with Regumate

Table 5. Results of ET – survival rate of fresh and frozen porcine embryos

Item	embryos			
	fresh		frozen	
ET (n)	25		11	
Transferred embryos ($n/\bar{x}$ ± sd)	380	15.20 ± 4.73	137	12.45 ± 3.42
Pregnant recipients (n/%)	12*	48.0	7	63.6
Birth rate				
Embryos transferred to pregnant recipients ($n/\bar{x}$ ± sd))	130	10.8 ± 2.12	92	13.1 ± 4.22
Born piglets				
total ($n/\bar{x}$ ± sd)	55	6.88 ± 1.78	57	8.14 ± 2.03
percentage of transferred embryos	42.3		61.9	
born alive ($n/\bar{x}$ ± sd)	52	6.50 ± 1.57	53	7.57 ± 1.78
percentage of all piglets	94.5		93.0	
weaned piglets ($n/\bar{x}$ ± sd)	52	6.50 ± 1.57	49	7.00 ± 1.28
percentage of born alive piglets	100		92.5	

*4 recipients aborted, N.S., $P > 0.05$

and hormonal preparations mentioned above was not successful. The transfer of 11 thawed embryos (expanded blastocysts) to a sow synchronized by weaning (1st parity) was successful. The sow gave birth to 6 piglets born alive, all the piglets were weaned. The available literature sources do not mention any similar success, so it can be considered as the 1st successful non-surgical transfer of cryopreserved porcine embryos. In total, 25 transfers of fresh embryos and 11 transfers of vitrified embryos were made in the evaluated experiments – no significant difference was found in survival rates. Pregnancy rate amounted to 48.0% and to 63.3%, resp. – 4 abortions were recorded in recipients of fresh embryos. A somewhat higher birth rate was found in recipients of vitrified embryos (Table 3). As for survival rates (surviving embryos out of the transferred ones), an insignificantly higher rate was found in vitrified embryos (61.9% vs. 42.3%). The mentioned results are surprising with regard to problems associated with cryopreservation of porcine embryos and to literature data. The evaluated set is representative (as compared to the above-cited sources). It is possible to support trends outlined by Caamano *et al.* (2003) – it is advisable to go on with experimentation leading to commercialisation of porcine ET. In spite of indisputable significance of these experimental results it is evident that the studies of porcine ET realised until now characterise the phase of fundamental research.

Laparoscopic transfer. In total, 5 laparoscopic transfers of frozen embryos recovered post mortem on Day 5.5–6 after oestrus and 1st insemination into recipients were made (Table 6). Five recipients were pregnant (100%), in total 65 embryos were transferred to all pregnant recipients. In total, 45 piglets (69.2% of transferred embryos) were born, i.e. 9.00 ± 0.89 piglets on average (10, 10, 9, 8, 8 in individual recipients), 42, i.e. 8.40 ± 1.62 piglets (64.6% of transferred embryos, 93.3% of born piglets) were born alive (10, 10, 9, 8, 7), 37 piglets, i.e. 7.40 ± 1.02 piglets on average (56.9% of transferred embryos, 82.2% of born piglets, and 88.1% of piglets born alive) were weaned (9, 8, 7, 7, 6 in individual recipients) – Table 6. The laparoscopic method (3 ET, 50 fresh embryos) was also used for the transfer of 36 fresh embryos to 2 pregnant recipients. The two recipients were pregnant and gave birth to 6 and 7 piglets, resp. One of the 7 piglets was born dead. All piglets born alive were weaned. Gordon (1997) reported higher pregnancy rate (75%) in the case of surgical transfers. As compared to small ruminants, the laparoscopic procedure in pigs is complicated by the recipient's body weight and by different characteristics of the uterine wall and musculature. Therefore, literature

Table 6. Results of laparoscopic ET method. Effect of the type of transferred embryos

Item	Type of transferred embryos			
	fresh		frozen	
ET (*n*)	3		5	
Transferred embryos ($n/\bar{x}$ ± sd)	50	16.7 ± 6.71	65	13.0 ± 3.58
Pregnant recipients (*n*/%)	2	67	5	100
Natality				
Embryos transferred to pregnant recipients ($n/\bar{x}$ ± sd)	36	18.00 ± 9.00	65	13.00 ± 3.58
Born piglets				
total ($n/\bar{x}$ ± sd)	13	6.50 ± 0.50**	45	9.00 ± 0.89**
percentage of transferred	36.1		69.2	
born alive (n/x ± sd)	12	6.00 ± 0.00**	42	8.40 ± 1.62**
percentage of all piglets	92.3		93.3	
weaned piglets ($n/\bar{x}$ ± sd)	12	6.00 ± 0.00**	37	7.40 ± 1.02**
percentage of born alive piglets	100		88.1	

$P > 0.05$, $^{**}P < 0.01$

sources dealing with the application of laparoscopic ET in pigs are less numerous. The above-mentioned rates are, however, substantially higher than those found by Říha *et al.* (1997b), Říha and Vejnar (2000) in smaller sets of recipients (10%–50%). Birth and weaning rates are comparable to the rates found by Gordon (1997) in the case of fresh embryos transferred surgically. The application of laparoscopic procedures in porcine ET schemes has not been mentioned in available literature sources. Thirteen laparoscopic transfers of vitrified embryos (the method specified by Říha *et al.*, 1996, 1997a,b) to the recipients – sows inseminated by a phenotypically different (different colour) boar during oestrus following weaning – were made in previous experiments. Seven recipients (54%) were pregnant, 4 sows gave birth to own piglets only (produced by insemination). In total 37 piglets were born, 35 piglets were born alive, 35 piglets were weaned. One sow gave birth to 10 piglets – 5 of them were produced by insemination, five piglets were born after the transfer of 12 vitrified embryos. All piglets were weaned. The second and the third recipient gave birth to 9 and 10 piglets, or produced by ET of 16 and 11 vitrified embryos, respectively. In total, three recipients were pregnant after ET of 39 vitrified embryos, 24 piglets were born alive and weaned, the embryo survival rate amounted to 62% (Říha and Vejnar, 2000). Our previous paper (Říha *et al.*, 1997a) reported the birth of first piglets produced by ET of cryopreserved embryos. Cryopreservation of porcine embryos is characterised by many problems (Niemann, 1995; Gordon, 1997, our previous studies). The mentioned experiment can therefore be considered as a very successful one.

Non-surgical transfer. A more detailed analysis of non-surgical transfer (fresh and frozen embryos) made by intubation with an A.I. intra-cervical probe is illustrated in Table 7. As for frozen embryos, on average 12.0 ± 2.84 were transferred to recipients. In the case of fresh embryos, on average 15.0 ± 5.72 were transferred to recipients. Pregnancy rate ranged from 33.0 to 45%. A high abortion rate was found in the group of recipients of fresh embryos – 30% recipients gave birth to live piglets. The parameters of birth rate were statistically different between the experimental groups ($P < 0.05$, Table 7). In our previous study (Říha and Vejnar, 2000) four non-surgical transfers of fresh embryos to gilts (age 8 months) treated according to the analogical scheme (Regumate, PMSG, HCG regimen) were made. Pregnancy was diagnosed in two gilts on Day 28 after oestrus. Four and 10 viable embryos (of 10 and 12 transferred embryos, resp.) were found in gilts slaughtered on Day 30. A sow (1st and 2nd parity) was synchronized by weaning – 19 embryos were transferred non-sur-

Table 7. Results of non-surgical transcervical method. Effect of the type of transferred embryos

Item	Type of embryos			
	fresh		frozen	
E (*n*)	22		6	
Transferred embryos ($n/\bar{x}$ ± sd)	330	15.0 ± 5.72	72	12.00 ± 2.84
Pregnant recipients (*n*/%)	10; 6*	45; 30*	2	33
Natality	6		2	
Embryos transferred to pregnant recipients ($n/\bar{x}$ ± sd)	94	15.7 ± 5.42	27	13.5 ± 3.54
Born piglets				
total ($n/\bar{x}$ ± sd))	42	7.0 ± 2.00	12	6.0 ± 0.00
percentage of transferred embryos	44.7		44.0	
born alive (n/x ± sd)	40	6.7 ± 1.93*	11	5.5 ± 0.50*
percentage of all piglets	95.2		91.7	
weaned piglets ($n/\bar{x}$ ± sd)	40	6.7 ± 1.93*	11	5.5 ± 0.50*
percentage of born alive piglets	100		100	

*4 recipients aborted, $P > 0.05$, *$P < 0.05$

gically to this recipient. Pregnancy was diagnosed by a sonographic control on Day 26. The abortion of 10 piglets terminated this pregnancy at the end of the 2nd month. Non-surgical transfers did not include sedation of recipients. The results of this experiment are relatively favourable as compared to the data published in the above-cited sources. Polge and Day (1968) published the first results (very low, approx. 3%) of non-surgical transfers. Sims and First (1987) used a non-surgical method without any success. Reichenbach *et al.* (1993), Gavin *et al.* (1994), Modl (1994), Hazeleger *et al.* (1995a,b), Wallenhorst and Holz (1995) successfully used non-surgical methods for transcervical transfers of fresh porcine embryos using a catheter similar to the catheter in our experiment. Li *et al.* (1995), Gordon (1997) reported the importance of catheter construction. Development of a complex method for porcine ET is still in the phase of experimentation. Cameron and van der Lende (1998) presented a more successful non-surgical procedure ET program in spite of some achievements for the transfer of fresh porcine embryos. Yonemura *et al.* (2003) found the highest pregnancy rate after non-surgical transfer using a conventional catheter and 15 ml transfer medium (to standing anaesthetised recipients). Cuello *et al.* (2002) transferred vitrified embryos with a catheter used for deep intrauterine insemination. This method was successful – five of eight recipients were pregnant. The use of the mentioned catheter could be effective (results of our experiment not published until now).

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ABSTRAKT

Laparoskopický a nechirurgický přenos čerstvých a zmrazených embryí prasat

Cílem práce bylo vyhodnocení vlivu faktorů na úspěšnost přenosu embryí u prasat. Na základě zatím provedených experimentů bylo možné vyhodnotit vliv způsobu přenosu (laparoskopický vs. nechirurgický), vliv parity příjemkyň (použití prasnic na 1. resp 2. vrhu a prasniček jako recipientek) a typ embryí (použití mražených vitrifikovaných a čerstvých embryí). Embrya ve stadiu expandované blastocysty v období okolo opuštění zony pellucidy byla získávána *post mortem* od superovulovaných dárkyň – vyřazených plemenných prasniček plemene landrace a české výrazně masné 5,5 až 6 dní po říji a inseminaci homospermními inseminačními dávkami. Ošetření dárkyň zahrnovalo zkrmování přípravku Regumate (Hoechst) podle návodu výrobce a ošetření PMSG (Sergon, Bioveta Ivanovice na Hané) a HCG (Praedyn, Léčiva Praha nebo Werfachor, Rakousko). Recipientky – prasničky byly synchronizovány zkrmováním prípravku Regumate a ošetřeny preparaty PMSG a HCG; recipientky – prasnice po 1. a 2. vrhu byly synchronizovány odstavem selat mezi 28. a 32. dnem po porodu. Pouze recipientky, které vykázaly říji a reflex nehybnosti v čase inseminace dárkyň, byly použity k ET. Pro kryokonzervaci embryí byla použita metoda vitrifikace nakapáním embryí do tekutého dusíku v médiu složeném ze 30 % glycerolu, 20 % fetálního telecího séra a 50 % 2M sacharózy v tridestilované vodě (max. 1,5 min); k ekvilibraci embryí bylo použito médium H-MEMD s 10 % glycerolu po dobu 10 minut. Rozmrazování embryí bylo prováděno v kondiciovaném médiu H-MEMD s 0,8M obsahem sacharózy po dobu 8 minut. Laparoskopické přenosy byly prováděny u recipientek v anestezii třemi vpichy (Laparoskop Wolf); pro nechirurgický přenos byl použit katetr pro intracervikální inseminaci, kterým byla provlečna silikonová přiměřeně rigidní hadička naplněná embryi a napojená na injekční stříkačku s cca 5 ml média. Celkem bylo provedeno 36 přenosů, zabřezlo 19 recipientek (55 %) a u čtyř pak došlo v průběhu posledního trimestru k abortu. Byly zjištěny statisticky neprůkazné rozdíly ($P > 0,05$) při použití prasniček a prasnic jako recipientek při celkovém hodnocení i po laparoskopickém a nechirurgickém přenosu. Parametry natality byly v některých experimentálních skupinách statisticky průkazné ($P < 0,01$). Přenos čerstvých a konzervovaných embryí byl úspěšný

u 48,0 % (12/25) a 63,3 % (7/11) recipientek. Byla dosažena vyšší natalita po přenosu vitrifikovaných embryí ($P < 0,01$), protože byl přenášen vyšší počet rozmražených embryí (10,8 ± 2,10 čerstvých a 13,1 ± 4,22 rozmražených embryí) na jednu zabřezlou recipientku. Zabřezávání recipientek po nechirurgickém přenosu se pohybovalo od 33 do 50 %. Podíl přežívajících embryí z přenesených zabřezlým recipientkám se pohyboval od 36,1 do 69,2 % a v průměru dosahoval okolo 50 %; podíl odstavených selat z živě narozených se pohyboval v experimentálních skupinách na úrovni okolo 95 až 100 %. Rozsah hodnoceného souboru je v porovnání s ostatními citovanými autory více než reprezentativní, ale dosažené výsledky překvapivé. Je možné souhlasit s názorem uvedeným v práci Caamano *et al.* (2003), kde autoři doporučují, i přes značný zájem o ET u prasat, pokračovat v experimentech, aby se i tato metoda dala komercializovat.

Klíčová slova: prase; embryo; transfer; kryokonzervace; příjemkyně; prasnička; prasnice

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Multiple-breed, multiple-traits evaluation of beef cattle in the Czech Republic

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ABSTRACT: The objective of this study was to estimate breeding values for 12 beef breeds (Aberdeen Angus, Belgian Blue-White, Blond d'Aquitaine, Charolais, Galloway, Gasconne, Hereford, Highland, Limousin, Piemontese, Salers and Simmental) and their crosses with Czech Pied (dual-purpose) and dairy breeds. Data collected in the last 12 years consisted of 125 482 records on calving ease and birth weight, 57 863 records on weight at 120 days of age, 56 947 records on weight at 210 days of age, and 22 410 records on yearling weight. The complete pedigree included 183 754 animals. Evaluation was made by Multiple-breed Multi-traits Animal model with maternal effect. Fixed effects included in the model were sex, dam age and regressions on calf and maternal heterosis. Random effects were herd × year × season herd-mate group, direct and maternal genetic and maternal environmental effects. Direct effects were highest for all traits in Charolais followed by Simmental. Maternal effects were highest for weight at 120 and 210 days of age in Czech Pied followed by Salers and Simmental, and for yearling weight in Salers followed by Simmental. The lowest effects were determined for all traits in Highland followed by Galloway.

Keywords: beef; breeding value; Animal model; Maternal effect; breeds

Beef breeds in the Czech Republic is a new industry that has expanded especially since 1990. Herds of beef cattle were constituted by imports of pure-bred animals and by absorptive crossing on dual-purpose inland cattle (Czech Pied cattle) and some culled cows of dairy breeds (Black-Pied cattle). Twelve beef breeds in total and their crosses are kept. The number of animals included in performance testing has increased every year, currently about 25 000 cows and their offspring are tested. Out of this number, approximately a half of the animals is pure-bred ones or with the genetic share of beef breed above 88%. The other cows under performance testing are products of crossing with the other higher genetic share of beef breeds. These elite breeding herds produce sires for the needs of elite and commercial herds. Only pure-bred sires of beef breeds are used for breeding.

The objective of this paper is an estimation of breeding value for animal growth.

Maternal effect influences the expression of many production traits. A general genetic model for maternal effect estimation was presented by Willham (1980). Diop *et al.* (1999) reported that maternal effect in the growth of beef cattle was still important at the age of 18 months. Boldman *et al.* (1991) calculated the estimates of direct and maternal effect on the growth of beef cattle in crossbred and pure-bred animals. Heritability of direct and maternal effect was estimated by Waldron *et al.* (1993). Their model comprised correlations between direct and maternal effect, and permanent maternal environment, and they developed an algorithm to determine the components of variance. They stated that animal model ignoring maternal effect tended to overvalue direct heritability. Janss *et al.* (1994)

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used a similar model for sheep. A similar model was also applied by Muniz *et al.* (2002), who assumed that direct genetic and maternal effects were not correlated. Dodenhoff *et al.* (1998, 1999), Diop *et al.* (1999) and Choi *et al.* (2000) included grandmaternal effect in the model in addition to the above-mentioned effects. The correlation between maternal and grandmaternal effect is usually opposite (Alenda *et al.*, 1980), which accentuates the theory that the environment of dam rearing influences their own mothering abilities. Quintanilla *et al.* (1999) stated that the correlation between non-genetic maternal environments of related dams was important for the estimation of the components of variance. Lee and Pollak (1997) included a year × sire interaction in the model considering it an important factor to calculate the correlation between direct genetic and maternal effect. Hagger (1998), who used a sire × herd interaction, drew a similar conclusion.

Crump *et al.* (1994) described BLUP evaluation of beef cattle for several mutually correlated traits with maternal effect. They used direct effect for birth weight, 200 and 400 days weight, backfat thickness, lean meat content and maternal effects for birth and 200 days weight. The model comprised the effects of the group of herd mates, sex, course of calving, month of birth, embryo transfer, dam age, mother at birth and at weaning and the level of crossing. Similar effects used for multi-traits evaluation Hoon and Chesnais (1994). Klei *et al.* (1996) included the curve of dam age in the model and they composed the groups of herd mates on the basis of sex and management system. Robinson (1996) compared several methods of beef cattle evaluation. He used fixed effects – number of calves (single calves, twins), year, sex, group of herd mates, dam age, calf age at weighing, and random effects – direct genetic, maternal effects and permanent maternal environment. A similar method was used by Groeneveld *et al.* (1997), who employed fixed effects – herd × year × season, sex, dam age, calf age at weighing, and the same three random effects as the preceding author. Hagger (1998) compared twelve models for the processing of data on maternal effect in a flock of sheep with meat production. Nombre *et al.* (2002) compared multi-traits model and random regression model to evaluate the growth of beef cattle.

It is often necessary to compare animals of different breeds including crosses in the breeding process in practice. Arnold *et al.* (1992) evaluated multi-breed data taking into account interbreed differences and heterosis effects. They categorised heterosis into a fixed and random component and derived a system of equations for animal model. Klei *et al.* (1996) applied specific heterosis for direct and maternal effect for each pair of breeds in multi-breed evaluation. Estimation of breeding value with exploitation of crosses information was described by Cantet and Fernando (1995) through heterogeneous additive genetic variability. Přibyl *et al.* (2000) included the effect of crossing in the model as the effect of breed cross combination. When Jakobsen *et al.* (1996) evaluated the growth of bulls on the basis of dairy breeds, they included the effect of regression on the share of genes of original breeds and regression on general heterozygosity directly in the model. As a part of multi-breed evaluation Elzo and Wakeman (1998) used a sire-maternal grandsire model for two parallelly evaluated traits (birth weight and weaning weight) where they estimated direct and maternal effects for additive and non-additive genetic effects. Multi-breed genetic evaluation for weights in Gelbvieh cattle with inclusion of maternal effects is described by Legarra *et al.* (2003). Different lines are included in the calculation through genetic groups, external information is also involved.

Renand *et al.* (2003) and Bullock *et al.* (2003) described system of international beef evaluation.

In general, the models used for estimation of breeding value in beef cattle are identical in the cited authors. They mostly take into account fixed effects – groups of herd mates, calf sex, dam age, genetic groups, heterosis effects during crossing, and random effects – correlated direct and maternal effects and permanent maternal environment. Multi-traits models are applied, increasing the reliability of estimation of breeding values for particular traits and coping with problems due to missing data.

Evaluated traits in the above-cited authors are birth weight, weaning weight, yearling weight or weight at older age and weight gains. Another trait is calving ease for which a threshold model is used (King *et al.*, 1993; Wiggans *et al.*, 2002). The reliability of evaluating the calving ease will increase significantly with parallel multi-traits evaluation with birth weight (Ramirez-Valverde *et al.*, 2001).

Besides the above-mentioned authors, other authors (Meyer, 1994; Ferraz *et al.*, 2002; Ortiz Peña *et al.*, 2002; Oyama *et al.*, 2002; Rosales-Alday *et al.*, 2002) determined population-genetic parameters necessary for breeding value estimation in beef

cattle. The results are considerably influenced by the choice of statistical model and evaluated population. The range of published values is large, correlations of identical parameters assume the values from negative to positive ones. It documents how difficult is to determine these parameters, particularly if the action of direct and maternal effects is parallel.

MATERIAL AND METHODS

Production records since 1990 are summarised in Table 1. The evaluated traits are calving ease (ce), birth weight (birth), weight at the age of 120 days (120), 210 days (210) and at one year of age (365). Production recording was carried out in 12 beef breeds and crosses with dairy breeds (HI – Highland, GA – Galloway, PI – Piemontese, HE – Hereford, GS – Gasconne, BB – Belgian Blue-White, CP – Czech Pied (dual-purpose breed), LI – Limousin, SA – Salers, BA – Blonde d'Aquitaine, AA – Aberdeen Angus, SI – Simmental, CH – Charolais, Others – dairy breeds). As the breeds are very different, the range of the maximum and minimum values of measured data is large.

The evaluated set including the generations of ancestors comprises 183 754 evaluated animals in total. Out of this number, 1 142 sires are currently used for breeding. Besides other crossbred cows the number of living cows of particular breeds (above 88% of the breed concerned) is 12 119 animals. The total number of evaluated animals belonging to the particular breeds except crosses is 104 838 individuals.

Fixed effects included in the evaluation are tested by the least-squares method (GLM/SAS). Breeding value is determined by MT-AM, programme BLUP90IOD (Tsuruta *et al.*, 2001) according a model equation:

$$Y = HYS + CS + DAG + BVD + BVM + PE + HEC + HED + e$$

where: Y = measured performance (Ce, Birth, 120, 210, 365)

HYS = group of herd mates within herd-year-season. Pure-bred animals, sometimes several breeds, and crosses encounter each other within HYS

CS = calf sex – bullocks, heifers/single calves, twins

DAG = dam's age at calving – below three years, four-years, five- to seven-years old dams, above seven years

BVD = an individual, breeding value for direct effect

BVM = an individual, breeding value for maternal effect. Direct effect and maternal effect are correlated with each other, with common matrix of relationship and genetic groups (28 groups according to breeds and genetic shares of crossing)

PE = permanent maternal environment for cows

HEC = heterosis of calves – regression according to calf heterozygosity

HED = heterosis of dams – regression according to dam heterozygosity

e = random uncontrollable environment

The population-genetic input parameters from Tables 2–5 were used for the calculation of breeding values. Data are based on the breakdown of the values of variabilities in Table 6 into particular effects and on our own other calculations (Přibyl *et al.*, 2000) while literary data were also taken into account. Direct and maternal effects of an individual and permanent maternal environment were used as random effects. As the number of offspring

Table 1. Production recording of beef cattle

	Number	Mean	s	Minimum	Maximum
Calving ease (points)	125 482	1.10	0.40	1.00	4.00
Birth weight (kg)	125 482	34.58	6.04	10.00	99.00
Weight at 120 days (kg)	57 863	161.79	31.11	65.00	293.00
Weight at 210 days (kg)	56 947	244.95	49.49	90.00	464.00
Weight at 365 days (kg)	22 410	361.90	87.84	150.00	749.00

Table 2. Standard deviations substituted in the calculation

	e	HYS	PE	BVD	BVM
Calving ease (points)	0.29	0.17	0.07	0.11	0.07
Birth weight (kg)	2.28	4.87	0.88	1.70	1.25
Weight at 120 days (kg)	14.13	19.90	6.24	12.20	9.75
Weight at 210 days (kg)	20.39	35.60	8.99	17.60	14.05
Weight at 365 days (kg)	35.80	63.90	9.27	26.31	10.34

Table 3. Residual correlations in % substituted in the calculation

	Ce	Birth	120	210	365
Calving ease (points)		25	5	2	0
Birth weight (kg)			20	15	6
Weight at 120 days (kg)				80	55
Weight at 210 days (kg)					65
Weight at 365 days (kg)					

Table 4. Permanent maternal environment, correlations in % substituted in the calculation

	Ce	Birth	120	210	365
Calving ease (points)		30	5	2	2
Birth weight (kg)			24	20	11
Weight at 120 days (kg)				76	60
Weight at 210 days (kg)					75
Weight at 365 days (kg)					

Table 5. Genetic correlations in % substituted in the calculation

	Direct effect					Maternal effect				
	Ce	Birth	120	210	365	Ce	Birth	120	210	365
Direct effect										
Calving ease (points)		30	15	15	10	–17	–10	–7	–7	–5
Birth weight (kg)			33	29	28	–9	–14	–4	–5	–3
Weight at 120 days (kg)				70	63	–4	–9	–18	–15	–10
Weight at 210 days (kg)					72	–5	–4	–15	–18	–14
Weight at 365 days (kg)						–4	–5	–14	–13	–18
Maternal effect										
Calving ease (points)							30	15	13	8
Birth weight (kg)								29	17	3
Weight at 120 days (kg)									81	45
Weight at 210 days (kg)										62
Weight at 365 days (kg)										

is low in some herds within HYS, this effect was also used as a random one.

RESULTS AND DISCUSSION

Performance level

Table 1 shows the results of performance testing for the period of observations. The achieved performance documents a high average growth rate. The recorded average values are in accordance with those reported by Woodward *et al.* (1992), Crump *et al.* (1994), Quintanilla *et al.* (1999) and Rossales-Alday *et al.* (2002). Lower values were given by Alenda *et al.* (1980), Waldron *et al.* (1993), Meyer (1994) and Ferraz *et al.* (2002).

The variability of the data is high, with a wide range of maximum and minimum values for the particular animals; it is related with the individuality of animals and with the fact that extreme breeds from highly intensive (Charolais, Simmental) to extensive (Highland) ones are kept. Very contrast breeds were also used in intentional experiments by Gregory *et al.* (1991). Moreover, the recorded data are influenced by sex, litter size and other systematic effects of the external environment. The lower level of achieved performance is in agreement with literary data (Diop *et al.*, 1999).

Effects in the model

Linear model with fixed effects (LSM) was used to test the effects included in the evaluation. The results are shown in Table 6. All effects – HYS, CS, DAG, breed and cross combination were statistically highly significant. The effects jointly explained 26% of variability for the calving ease. For growth they explained a higher portion of variability from 56% at 120 days of age to 78% at the age of one year. It is evident that the importance of the effects of external environment increases with animal age (cumulative growth). The high effects of external environment and breed were also observed for birth weight where the coefficient of determination was 75%.

It is evident from a more detailed analysis that the effect of HYS is the most important of all because it explains a major portion of variability and increases the percentage of explained variability in comparison with the separately used effects herd, year and season (Přibyl *et al.*, 2000). Residual standard deviations and standard deviations of recorded performance are consistent with the presented data. The importance of particular effects of external environment that should consequently be included on the model of breeding value estimation was tested also by Szabo *et al.* (2002).

The results presented in tables below were obtained by animal model.

In Table 7 the effect of calf sex is presented in comparison with single bullocks. The expression of the effect of sex as well as of litter size was observed. Dystocia and highest weights were recorded in single bullocks. Twin bullocks had lower weights than single heifers until weaning. Twin bullocks exceeded these heifers as late as by yearling weight. Szabo *et al.* (2002) reported considerably smaller differences in weaning weight between bullocks and heifers than were our values.

Table 8 shows the effect of dam age. Dystocia and slowest growth were recorded in calves of the youngest dams. Calves delivered by five- to seven-year dams had the fastest growth. Calves of the oldest dams had the highest birth weight, but the differences were small.

The results are analogical to those of Klei *et al.* (1996), who reported the highest weaning weight

Table 6. Variability explained by systematic factors of the environment HYS, CS, DAG, breed and cross combination (all fixed effects)

	S		R^2 explained variability (%)	
	recorded	adjusted	total	HYS
Calving ease (points)	0.40	0.35	26	23
Birth weight (kg)	6.04	3.16	75	66
Weight at 120 days (kg)	31.11	21.61	56	48
Weight at 210 days (kg)	49.49	31.02	64	57
Weight at 365 days (kg)	87.84	43.10	78	69

Table 7. The effect of calf sex, comparison with single bullocks

	Twin bullocks	Single heifers	Twin heifers
Calving ease (points)	–0.06	–0.08	–0.07
Birth weight (kg)	–6.30	–2.98	–8.02
Weight at 120 days (kg)	–24.96	–11.36	–33.37
Weight at 210 days (kg)	–29.37	–19.87	–45.69
Weight at 365 days (kg)	–30.97	–67.66	–94.89

Table 8. The effect of dam age, comparison with dams at three years of age and younger

	Four years	Five to seven years	Eight years and older
Calving ease (points)	–0.05	–0.09	–0.09
Birth weight (kg)	0.39	0.60	0.64
Weight at 120 days (kg)	9.28	16.24	13.63
Weight at 210 days (kg)	12.35	21.12	18.15
Weight at 365 days (kg)	13.05	21.76	16.89

in seven years old dams. The numerical values of differences correspond to our data. Klei *et al.* (1996) also reported the highest birth weight in dams at 7 to 10 years of age, in relation to breed. Szabo *et al.* (2002) found out the highest weaning weight in Simmental breed in dams on the 3rd and 4th parturition, which corresponds to our data.

Dams at the age above 5 years had the easiest parturitions. King *et al.* (1993) described a relationship between the calving ease and calf weight.

Heterosis

Breeds and groups of crossbreds are included in the model directly as genetic groups in the relationship matrix that also involve a portion of non-additive genetic variability. Nevertheless, heterosis effects are still expressed in calves and dams. They are considered for the model as average direct (calf) and average maternal heterosis. The calculated values are given in Table 9. The average heterosis effect for the calving ease is negative –0.03 points, which is approximately –2 to –3% of point evaluation. This effect is –0.29 kg for birth weight (less than –1% of average weight). Heterosis for maternal effect is highest at calf age of 210 days, +5.33 kg. For direct effect, heterosis increases with age absolutely up to 5 kg at yearling weight. Heterosis effects for weights at 120 days, 210 days and 365 days range about +2% of average performance.

Table 9. Heterosis effects

	Ce	Birth	120	210	365
Direct	–0.03	–0.29	1.79	2.18	5.00
Maternal	–0.02	0.05	3.36	5.33	1.85

These heterosis effects are applied mainly to the adjustment of breeding values and they are not the main objective of evaluation. Measured performance is evaluated in pure-bred animals and products of absorptive crossing, so it does not provide the best data for the evaluation of hybridisation effects (Klei *et al.*, 1996). For the combinations of British, Continental and Zebu breeds these authors reported maximum direct heterosis effects for birth weight from 0 to about +2 kg and maximum maternal heterosis effects for weaning weight from +6 to +17 kg. In an intentional experiment of breed crossing Alenda *et al.* (1980) determined higher values for maternal heterosis than for direct heterosis. Heterosis effects for birth weight were in the range from about +0.5 to +1.0 kg and for weaning weight from about –12.0 to +15.0 kg. Also in an intentional experiment Gregory *et al.* (1991) recorded heterosis effects of about 2.0 kg for birth weight, +15.0 to +20.0 kg for weaning weight and +23.0 to +29.0 kg for yearling weight. All these values are considerably higher than our "in the model residual" results.

Improvement of the results could be by applying the specific heterosis for each combination of breeds, but with disadvantage of decreasing number of observation for each regression coefficient according heterozygosity. Various crossbreds, and at a high number, can be present in herds in practical conditions, so the evaluation is difficult. Therefore Elzo and Wakeman (1998) recommended to include the breeds in groups according to their combining abilities.

Model

Models of breeding value estimation tend to have too many parameters, and it makes the interpretation of results difficult. Therefore the goal is to include important effects in the model, but their lowest number possible. The above-mentioned effects were used in the model of breeding value estimation.

In accordance with literary data (e.g. Crump *et al.*, 1994; Hoon and Chesnais, 1994) the evaluation was made by multi-traits model that enables to make a more exact prediction of breeding value especially for traits with a lower number of measurements. The evaluated traits are calving ease and weights at different age of animals. The number of recorded data decreases with age in a herd in practical conditions due to transfers and sale of animals (Table 1). The reliability of breeding value for weight at older age that is very important for breeders can be increased by the use of considerably larger sets of data on previous weights determined at a lower age of animals.

Different crossbreds are present as herd mates in herds, therefore multiple-breed evaluation is used. Klei *et al.* (1996) believed that this method facilitated easier formation of herd mate groups within herds and that it was not necessary to take into account different cross combinations separately. Currently, crossbreds are generally included in the evaluation as documented by the cited authors (Arnold *et al.*, 1992; Woodward *et al.*, 1992; Klei *et al.*, 1992; Elzo and Wakeman, 1998; Legarra *et al.*, 2003). In our study the cross combinations were included in genetic groups through the relationship matrix. These were 12 beef breeds + 2 original dairy breeds and products of absorptive crossing. Pure-bred sires of beef breed were always used, groups of crossbreds were therefore in the range of 50–87% of the given breed. A total of 28 genetic groups was used. As the time of performance testing was relatively short, time was not considered in the genetic groups.

Maternal effect is very important in beef cattle. It is an indicator of dams' milking capacity. Its influence in cumulative growth outlasts to a very old age (Diop *et al.*, 1999). The importance of maternal effect in the evaluation of weaning weight was accentuated by Lee and Pollak (1997). Therefore maternal effect was used in all evaluated traits.

In practical conditions breeding values must be determined for all tested animals although there need not be a sufficient number of herd mates in some small herds. This is the reason why the groups of herd mates in a herd (HYS) were used as random effect; it allowed to determine nonzero breeding values for individuals in small groups of herd mates on the basis of variance ratios. Random HYS selected like suitable effect in the model Grotheer (1996) for beef and Wolf *et al.* (2001) for pig population.

Přibyl *et al.* (2002) evaluated the stability of breeding values (rank of sires within breeds) using the applied model. Stability was evaluated on the basis of repeated evaluations in a six-month interval, after further data on performance testing were received. Changes in the rank occurred among the adjacent animals within the breed, without any leaps. Genetic gain for the studied, relatively short period showed a positive trend of direct effects in the particular breeds, in maternal effects the genetic level did not change systematically and was practically stable with random fluctuation (Šeba, 2002).

Breed differences

On the basis of inclusion of animals in genetic groups average breeding values were determined for direct and maternal effect in each breed. Comparisons were made with HE breed, which had the highest number of animals in the evaluated database (Table 10). Contrast breeds were evaluated, therefore the differences are large. Breeds are permanently in the process of improvement. Most important are therefore results for the youngest generation of living animals.

Table 11 shows average breeding values according to the breeds for sires currently used for breeding. The breeds are arranged in an ascending order according to the breeding value of direct effect for yearling weight. The results of yearling weight are

Table 10. Standard deviations of breeding values within breed

Breed	No.	Direct effect					Maternal effect				
		Ce	Birth	120	210	365	Ce	Birth	120	210	365
HI	679	0.022	0.95	4.79	6.61	11.16	0.010	0.55	3.00	4.42	1.86
GA	1 281	0.024	1.41	6.08	9.24	12.71	0.013	0.56	3.34	4.93	2.56
PI	2 226	0.100	1.39	5.58	8.32	12.31	0.060	0.72	3.90	5.66	3.19
HE	22 827	0.028	0.91	7.72	11.79	14.36	0.015	0.37	3.59	5.34	3.70
GS	414	0.046	1.08	7.06	9.68	12.04	0.037	0.46	3.88	4.54	2.74
BB	205	0.086	1.47	4.66	6.83	9.46	0.066	0.86	2.98	3.48	1.30
CP	14 777	0.026	0.59	3.39	5.79	7.98	0.019	0.30	3.17	4.48	2.42
LI	4 902	0.053	1.06	7.34	10.09	14.32	0.029	0.68	4.54	6.16	3.67
SA	266	0.025	0.85	5.94	6.68	9.45	0.012	0.31	2.17	2.79	1.64
BA	2 899	0.095	1.13	6.39	8.71	12.66	0.059	0.71	3.79	5.73	3.01
AA	12 337	0.050	1.03	6.76	10.44	15.27	0.025	0.53	4.25	6.19	3.61
SI	9 330	0.043	0.75	5.34	7.66	11.90	0.026	0.45	3.87	5.68	2.88
CH	17 656	0.074	1.41	7.75	11.09	16.69	0.036	0.71	4.32	6.16	3.23
Others	15 039	0.049	0.37	3.06	4.02	4.65	0.038	0.37	3.03	4.24	1.96
Total	104 838	0.051	0.99	6.38	9.33	13.12	0.029	0.50	3.79	5.42	3.16

HI – Highland, GA – Galloway, PI – Piemontese, HE – Hereford, GS – Gasconne, BB – Belgian Blue-White, CP – Czech Pied, LI – Limousin, SA – Salers, BA – Blonde d'Aquitaine, AA – Aberdeen Angus, SI – Simmental, CH – Charolais, Others – dairy breeds

represented in an ascending order according to the breeds also in Figure 1. For direct genetic effect the breeds Charolais and Simmental achieved the highest values for weights at the age of 120 days, 210 days and 365 days. Belgian Blue-White and Charolais had the highest values for birth weight. Dystocia was recorded in Belgian Blue-White and Piedmontese breeds.

For maternal effects, Salers breed (dairy breed until recently) had the highest yearling weight; it was followed by Charolais. The highest weights at 120 and 210 days were recorded in Czech Pied breed (dual-purpose breed) while Simmental and Salers ranked as the second. The breeds Belgian Blue-White and Piedmontese had the highest birth weight and dystocia at the same time. For maternal effect, Galloway breed had relatively high values of birth weight.

The lowest values of growth traits were recorded in extensive breeds Highland and Galloway.

Differences between breeds are not necessary similar for different sex due the different selection intensity of parents. The category of living cows (Table 12) comprises a group of cows of dairy breeds (Others), but it does not include Belgian Blue-White breed. Figure 2 shows the data on yearling weight of cows. The order of breeds in tables

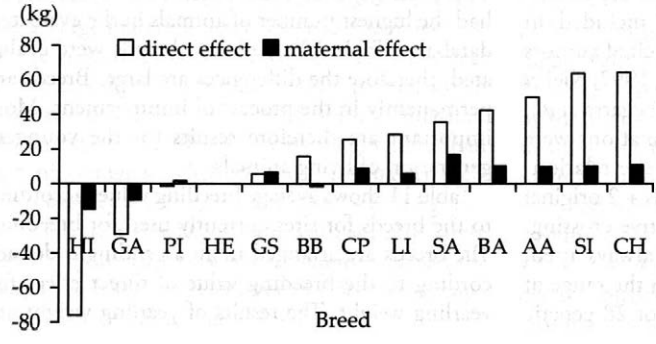

Figure 1. Bulls-direct and maternal effects, yearling weight, comparison with HE breed

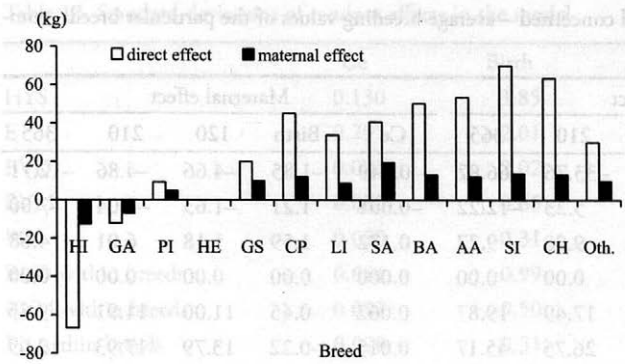

Figure 2. Living cows above 88% of the breed concerned – direct and maternal effects, yearling weights, comparison with HR breed

and graphs is the same as in Table 11. Differences between the breeds are similar to those between the bulls. The highest growth traits for direct effect were recorded in Simmental and Charolais. If compared with the table for bulls, the order within this pair changed. As the cows of Belgian Blue-White breed are not included here, its position in birth weight and calving ease was taken by a successive breed, Blonde d'Aquitaine. For direct effect CP breed ascended to a higher position within the breeds in weaning and yearling weight.

For maternal effect the highest yearling weight was found out in Salers and Simmental, 120 and 210 days weight was highest in Czech Pied cattle, followed by Simmental and Salers.

The differences between the breeds are similar to those reported by Alenda *et al.* (1980) for weaning weight in breeds AA, CH and HE in an intentional hybridisation experiment. In experiment HE breed was better than AA breed. Our differences in birth weight between the breeds are smaller than in the cited author. Our data on weaning and yearling weight show the same trends of breed comparison as the data of Gregory *et al.* (1991). Klei *et al.* (1996) reported almost a twofold difference for weaning weight between AA and CH breeds in

Table 11. Bulls – average breeding values of the particular breeds, comparison with HE breed

Breed	No.	Direct effect					Maternal effect				
		Ce	Birth	120	210	365	Ce	Birth	120	210	365
HI	19	–0.008	–4.49	–23.54	–41.50	–76.01	–0.053	–1.56	–4.28	–3.69	–14.70
GA	29	–0.021	–1.63	–5.67	–5.89	–29.00	0.005	1.31	–0.88	–1.05	–9.32
PI	57	0.204	2.48	4.08	0.98	-1.37	0.171	1.52	3.88	5.27	1.69
HE	155	0.000	0.00	0.00	0.00	0.00	0.000	0.00	0.00	0.00	0.00
GS	16	–0.006	3.68	5.82	8.03	5.86	0.058	–0.46	10.38	10.55	6.73
BB	20	0.237	5.30	5.78	17.72	15.92	1.52	2.94	6.92	–2.52	–1.52
CP	24	–0.004	2.11	7.61	11.48	25.60	0.023	–0.05	12.48	16.40	8.87
LI	95	0.070	2.53	13.35	16.37	28.44	0.011	–0.74	3.60	8.17	5.95
SA	15	–0.059	2.59	14.08	26.38	35.70	0.022	0.29	10.79	15.68	16.83
BA	63	0.175	4.47	14.96	24.67	42.55	0.076	–0.15	10.98	11.57	10.26
AA	218	0.019	1.18	12.57	25.39	50.02	–0.002	–0.68	8.56	12.47	9.90
SI	154	0.046	2.42	17.95	32.46	63.78	0.004	–0.11	11.05	13.78	10.18
CH	277	0.139	4.70	22.24	35.58	64.27	0.050	–0.63	8.35	13.87	10.89
Total	1 142										

HI – Highland, GA – Galloway, PI – Piemontese, HE – Hereford, GS – Gasconne, BB – Belgian Blue-White, CP – Czech Pied, LI – Limousin, SA – Salers, BA – Blonde d'Aquitaine, AA – Aberdeen Angus, SI – Simmental, CH – Charolais

Table 12. Living cows above 88% of the breed concerned – average breeding values of the particular breeds, comparison with HE breed

Breed	No.	Direct effect					Maternal effect				
		Ce	Birth	120	210	365	Ce	Birth	120	210	365
HI	122	0.010	–4.94	–18.24	–33.76	–66.82	–0.049	–1.85	–4.66	–4.86	–12.71
GA	204	–0.003	–4.25	2.16	5.23	–12.22	–0.001	1.21	–1.63	–2.04	–7.00
PI	280	0.197	3.69	9.45	9.88	9.27	0.172	1.59	5.18	6.01	4.68
HE	2 238	0.000	0.00	0.00	0.00	0.00	0.000	0.00	0.00	0.00	0.00
GS	77	–0.011	4.70	10.52	17.49	19.87	0.062	–0.45	11.00	11.91	9.85
CP	723	0.003	3.09	16.50	26.75	45.17	0.015	–0.22	13.79	17.93	12.09
LI	503	0.061	3.17	17.66	21.08	33.85	0.004	–0.90	4.46	8.63	8.69
SA	51	–0.045	2.90	17.94	32.61	40.85	0.030	0.27	11.21	16.37	19.64
BA	339	0.157	5.07	20.80	32.89	50.44	0.096	–0.19	11.42	12.53	13.06
AA	1 912	0.020	2.01	16.20	30.32	53.66	–0.001	–0.75	8.93	12.23	12.11
SI	1 099	0.042	3.10	22.22	38.92	70.28	0.008	–0.06	12.82	15.60	13.74
CH	2 325	0.130	5.27	24.32	38.15	63.68	0.051	–0.58	9.35	14.52	13.18
Others	2 246	0.089	1.98	14.96	23.24	30.13	–0.006	0.33	11.18	14.18	9.87
Total	12 119										

HI – Highland, GA – Galloway, PI – Piemontese, HE – Hereford, GS – Gasconne, CP – Czech Pied, LI – Limousin, SA – Salers, BA – Blonde d'Aquitaine, AA – Aberdeen Angus, SI – Simmental, CH – Charolais, Others – dairy breeds

comparison with our results. Jakubec *et al.* (2003) evaluated a part of the population kept in the Czech Republic to compare breed differences on the basis of pure-bred animals. Breeds were not compared in the same herds, but each were kept in the wide range of farm conditions and authors expected, that results for breeds are comparable. Their results are consistent with ours except of BA breed, which had the highest value.

On the contrast of cited authors, there are in our results the differences between breeds split into direct and maternal components. The differences between the breeds are in agreement with expected values. They agree with expectations during the whole evaluated period of growth. In our evaluation AA breed took a relatively high position in the order of breeds (a smaller deviation from the breeding values of intensive breeds with large body frame SI and CH). It can be explained by the genetic value of used sires that can be significantly different from the mean of the same breed kept in other countries due to a low number of animals and careful selection. This breed is young in the conditions of the CR, so the individuals from old genetic groups are not represented. Breeds develop continually and a selection aim of all breeds is basically identical. It is to expect that the differences between the breeds will diminish.

Variabilities of breeding values

Table 13 shows standard deviations of random effects calculated by animal model. The highest variability was determined for HYS effect and random residual from the model. The standard deviation of direct genetic effect in all traits except for the calving ease was several times higher than that of maternal effect, which documents its higher importance. The standard deviation of permanent maternal environment is approximately 1/3 compared to maternal effect.

Breed differences play an important role in estimated random effects. A linear model (GLM method) was used to determine the variability of estimated constants within breeds. The effect of breed was statistically highly significant for all traits, with the exception of PE for weight at 120, 210 and

Table 13. Standard deviations of random effects in the model

	Ce	Birth	120	210	365
HYS	0.130	3.85	14.45	23.94	44.82
E	0.295	2.01	11.88	16.99	28.75
BVD	0.071	2.02	10.07	16.00	26.42
BVM	0.080	0.65	5.45	7.44	5.73
PE	0.020	0.31	1.97	2.82	2.33
BVD within breeds	0.051	0.99	6.38	9.33	13.12
BVM within breeds	0.029	0.50	3.79	5.42	3.16
PE within breeds	0.020	0.31	1.97	2.82	2.33
R^2 – BVD* (%)	49	76	60	66	75
R^2 – BVM** (%)	87	43	52	47	70
R^2 – PE*** (%)	5	1	0	0	0

*variability of breeding values for direct effect explained by breed
**variability of breeding values for maternal effect explained by breed
***variability of permanent maternal environment explained by breed

365 days. In the breeding value of direct effect the breed explains 49% of variability for the calving ease and from 60% to 76% of variability for weights. On the contrary, in maternal effect breed differences explain the highest portion of variability for the calving ease –87%, and from 43% to 70% for weights. In the permanent maternal environment the interbreed differences hardly play any role (from 0 to 5% of variability) because it is a residual effect associated with the individual within families.

Standard deviations of random effects within breeds correspond with data on explained variability.

Table 10 shows standard deviations of breeding values for each breed. Intrabreed standard deviations calculated by a linear model (repetition of the values from Table 13) are indicated on the last line, they roughly correspond to the mean of standard deviations for the particular breeds. There exist differences in standard deviations within breeds, but the trend of higher variability of breeding values in breeds with higher performance was not observed. Jakubec *et al.* (2003) observed different genetic variability in dependency on body size and growth intensity for different breeds.

CONCLUSION

Multiple-breed, multi-traits animal model allows to make an objective evaluation of animals in an active population of relatively small size and decreasing amount of records with age of animals. The highest portion of variability of recorded growth traits is explained by systematic factors of farm environment. Direct genetic effects are more significant than maternal ones and genetic maternal effects are more significant than the permanent maternal environment. The interbreed differences determined on the basis of the mean of breeding values are an objective indicator for breed comparison in field conditions.

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ABSTRAKT

Společné hodnocení masného skotu pro více plemen a více vlastností v České republice

Předmětem práce je odhad plemenné hodnoty pro 12 masných plemen (angus, belgické modro-bílé, blond d'aquitaine, charolais, galloway, gasconne, hereford, highland, limousin, piemontese, salers a masný simental) a jejich křížence s českým strakatým (kombinovaná užitkovost) a dojnými plemeny. Databáze zahrnuje 12 let kontroly užitkovosti a obsahuje 125 482 údajů o průběhu porodu a hmotnosti telat při narození, 57 863 údajů o hmotnosti ve věku 120 dnů, 56 947 ve věku 210 dnů a 22 410 údajů o hmotnosti v roce. Počet hodnocených jedinců včetně rodokmenu je 183 754. Plemenná hodnota je stanovena Multitraits Animal modelem s maternálním efektem. Model výpočtu zahrnuje efekty: pohlaví telete (pevný), věk matky (pevný), heterózu telat (regrese), heterózu matek (regrese), skupiny vrstevníků – HYS (náhodný), přímý genetický (náhodný s maticí příbuzností a genetickými skupinami podle plemen a druhu křížení), maternální genetický (náhodný s maticí příbuzností a genetickými skupinami), trvalé mateřské prostředí (náhodný). Přímý genetický a maternální efekt jsou navzájem korelovány. Řešení programem BLUP90IOD na osobním počítači s frekvencí 1,4 GHz trvá přibližně jednu hodinu. Systematické faktory chova-

telského prostředí vysvětlují pro průběh porodu 26 %, pro hmotnosti od 56 do 78 % proměnlivosti. Nejvyšší hodnoty v přímých efektech u všech vlastností dosahují plemena charolais a masný simental. U maternálních vlastností dosahuje ve věku 120 a 210 dnů nejvyšších hodnot český strakatý (kombinované plemeno) následovaný plemenem salers a masný simental, ve věku jeden rok salerský skot, následován masným simentalem. Nejnižší hodnoty pro všechny vlastnosti byly u plemen highland a galloway.

Klíčová slova: masný skot; plemenná hodnota; animal model; maternální efekt; plemena

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Analysis of genetic variation of eight candidate genes in two wild boar subspecies

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ABSTRACT: Genetic variability and diversity of two subspecies of wild boar (*Sus scrofa scrofa* [*SSS*] $n = 67$ and *Sus scrofa attila* [*SSA*] $n = 42$) were studied by the PCR-RFLP method. Eight candidate genes with two-allele polymorphisms were analysed. No polymorphism in genes encoding ryanodine (*RYR1* = *CRC*) and oestrogen receptors (*ESR*) was found in either of the subspecies. In *SSS*, the frequencies of alleles of individual genes were as follows (frequency of one allele for each gene/locus is given): MYC protooncogene protein (*MYC*) $A = 0.49$; growth hormone (locus *GH-Hae*II)+ = 0.57 and (locus *GH-Msp*I)+ = 0.43; leptin (*LEP*) $T = 0.79$; heart fatty acid-binding protein (*H-FABP*) $H = 1.00$; prolactin receptor (*PRLR*) $A = 0.22$ and follicle-stimulating hormone – beta polypeptide (*FSHB*) $A = 0.40$. In *SSS*, average heterozygosity of genotypes was 0.295. The *SSA* subspecies showed the following allele frequencies of individual genes: *MYC* $A = 0.01$; *GH-Hae*II+ = 0.65 and *GH-Msp*I+ = 0.35; *LEP* $T = 1.00$; *H-FABP* $H = 0.98$; *PRLR* $A = 0.33$; *FSHB* $A = 0.49$. In *SSA*, the average heterozygosity of genotypes was 0.216. Highly significant differences in genotype frequencies between these two subspecies were found in *MYC* and *LEP*. PIC values of the polymorphic loci ranged from 0.023 to 0.375 in both subspecies. Differences between both subspecies of wild boar on the one hand and compared to some breeds of domestic pigs were found in the polymorphism of genes *RYR1*, *ESR*, and *MYC*.

Keywords: wild boar; candidate genes; *RYR1*; *MYC*; *GH*; *LEP*; *H-FABP*; *ESR*; *PRLR*; *FSHB*; heterozygosity; subspecies diversity

Genetic polymorphism of candidate genes and their association with performance traits of breeding pigs is intensively studied in many laboratories all over the world. The knowledge of polymorphism of the same candidate genes in wild boars is only marginal and has not been systematically studied yet. The estimation of genetic diversity existing between different wild boar subspecies and comparison of their polymorphisms of candidate genes with that of domestic pigs is very important with regard to taxonomic studies and potential use of candidate genes in pig breeding.

Hartl *et al.* (1993) studied the diversity of isoenzymes within a population of 155 wild boars in South-western and Central Europe. Altogether 11.6% of 40 evaluated isoenzymes were polymorphic and genetic distances helped to reveal some differences between wild boar populations.

Tikhonov and Bobovich (1997) compared blood group systems in five wild boar subspecies in Eurasia and found very marked differences within and between individual subspecies and populations of wild and domestic pigs. Results of the analysis enabled to differentiate between those existing subspecies that could not be identified exactly on the basis of their phenotypic traits.

Based on the values of creatine phosphokinase activity and testing of *RYR1* genotypes, Müller *et al.* (2000) presented data about homozygous stress resistance of wild boars of European type as measured in 7 males and 3 females. Okumura *et al.* (2001) analysed genetic relationships between wild

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boars originating from East Asia and some breeds of domesticated pigs on the basis of major non-coding regions of mtDNA.

Because of their higher degree of polymorphism, microsatellite markers (MS) are often used to estimate genetic distances between different populations of pigs and their wild ancestors (Laval *et al.*, 2000; Rajeev *et al.*, 2001; Behl *et al.*, 2002).

The aim of this study was to describe intra-population and inter-population genetic polymorphism of wild boar subspecies *Sus scrofa scrofa* and *Sus scrofa attila*. Genetic analysis of wild boars was performed on the basis of estimation of the polymorphism of some known candidate genes associated with performance traits of breeding pigs. The second objective was to compare frequencies of genes found in populations of wild boars with those occurring in different breeds of domestic pigs. An evaluation of genetic distances existing between the two wild boar subspecies under study by means of MS will be presented in another paper.

MATERIAL AND METHODS

Animals

The study involved 67 individuals of *Sus scrofa scrofa* (*SSS*) originating from Northern Germany, Northern Bohemia, Southern Moravia and Central Slovakia and 42 individuals of *Sus scrofa attila* (*SSA*) from the game preserve Sedlice in Southern Bohemia and from Eastern Slovakia. In *SSS*, blood was sampled immediately after shooting (or within 30 minutes at the latest) from the chest after an incision of the diaphragm. If the animal was shot into the digestive and excretory tract and there was a risk of contamination, blood was sampled from the neck after cutting carotids and jugular veins in the region of clavicles. In *SSA*, peripheral blood was sampled from jugular veins of living animals. Blood was always sampled into test tubes containing an anticoagulant solution (150 µl 0.5 M EDTA/5 ml of blood).

Genotyping

Genomic DNA was isolated by means of a modified proteinase method (Nebola *et al.*, 1994). For amplification of target DNA sequence involving a potential polymorphic site, the polymerase chain reaction (PCR) was used. Synthetic primers of oligonucleotides were used for the identification of variants of individual genes. Genotypes were detected by means of the RFLP method. A total of 10–15 µl PCR product was digested with 5U of restriction enzyme at 37°C for 12 hours. Fragments were detected by electrophoresis in 1 × TAE buffer (40 mM Tris-acetate; 1 mM EDTA, pH 8), voltage max. 5 V/cm, on agarose gel containing ethidium bromide.

The gene of ryanodine receptor 1 (*RYR1*) was detected by using restriction endonucleases *Hin*PI and *Hgi*AI (Fujii *et al.*, 1991) (in our study isoschizomers *Hin*6I and *Alw*21I). The gene *MYC*, which is usually digested with *Hpa*II (Reiner *et al.*, 2000), was cut with isoschizomer *Msp*I in our experiment. The polymorphism of growth hormone gene (*GH-Hae*II), usually revealed with *Hae*II, was analysed with isoschizomer *Bsp*143II and the second polymorphism of *GH* with *Msp*I (Kirkpatrick *et al.*, 1992). The gene of heart fatty acid-binding protein (*H-FABP*) was cut with *Hin*fI enzyme (Gerbens *et al.*, 1997) as well as leptin gene (*LEP*) (Stratil *et al.*, 1997). The *ESR* gene was digested with *Pvu*II (Rothschild *et al.*, 1991). Prolactin receptor gene (*PRLR*) was cut with *Alu*I (Vincent *et al.*, 1998; Van Rens, 2001) and the gene of follicle stimulating hormone, beta polypeptide (*FSHB*) with *Bsu*RI (Zhao *et al.*, 1998; Li *et al.*, 1998).

Statistical methods

Frequencies of genes and genotypes were estimated in both populations of wild boars under study. Differences between genotype frequencies of both wild boar subspecies were estimated by χ^2 test. The rate of genetic variance was determined on the basis of non-biased degree of heterozygosity (Nei, 1978) and polymorphic information content (PIC; Botstein *et al.*, 1980).

RESULTS AND DISCUSSION

This study presents the first application of DNA tests within a larger population of wild boars. Frequencies of genotypes and alleles of candidate genes found in both subspecies of wild boars and in the whole population are presented in Table 1. Out of the eight genes studied, two were monomorphic in both subspecies (*RYR1* and *ESR*) and

Table 1. Genotype and allele frequencies of candidate genes from *SSS* (n = 67), *SSA* (n = 42) and whole population (109) of wild boar and differences between subspecies

Genes and populations	Genotype frequencies			χ^2 *SSS/SSA*	Allele frequencies	
RYR1	*CC*	*CT*	*TT*		*C*	*T*
SSS	1.00	0.00	0.00	NS	1.00	0.00
SSA	1.00	0.00	0.00		1.00	0.00
WB	1.00	0.00	0.00		1.00	0.00
MYC	*AA*	*AB*	*BB*		*A*	*B*
SSS	0.19	0.60	0.21	***	0.49 ± 0.04	0.51 ± 0.04
SSA	0.00	0.02	0.98		0.01 ± 0.01	0.99 ± 0.01
WB	0.12	0.38	0.50		0.31 ± 0.03	0.69 ± 0.03
*GH-Hae*II	+/+	+/–	–/–		+	–
SSS	0.33	0.49	0.18	NS	0.57 ± 0.04	0.43 ± 0.04
SSA	0.38	0.55	0.07		0.65 ± 0.05	0.35 ± 0.05
WB	0.35	0.51	0.14		0.61 ± 0.03	0.39 ± 0.03
*GH-Msp*I	+/+	+/–	–/–		+	–
SSS	0.18	0.49	0.33	NS	0.43 ± 0.04	0.57 ± 0.04
SSA	0.07	0.55	0.38		0.35 ± 0.05	0.65 ± 0.05
WB	0.14	0.51	0.35		0.39 ± 0.03	0.61 ± 0.03
LEP	*TT*	*TC*	*CC*		*T*	*C*
SSS	0.60	0.39	0.01	***	0.79 ± 0.04	0.21 ± 0.04
SSA	1.00	0.00	0.00		1.00	0.00
WB	0.75	0.24	0.01		0.87 ± 0.02	0.13 ± 0.02
H-FABP	*HH*	*Hh*	*hh*		*H*	*h*
SSS	1.00	0.00	0.00	NS	1.00	0.00
SSA	0.98	0.00	0.02		0.98 ± 0.01	0.02 ± 0.01
WB	0.99	0.00	0.01		0.99 ± 0.01	0.01 ± 0.01
ESR	*CC*	*CD*	*DD*		*C*	*D*
SSS	1.00	0.00	0.00	NS	1.00	0.00
SSA	1.00	0.00	0.00		1.00	0.00
WB	1.00	0.00	0.00		1.00	0.00
PRLR	*AA*	*AB*	*BB*		*A*	*B*
SSS	0.21	0.03	0.76	NS	0.22 ± 0.04	0.78 ± 0.04
SSA	0.33	0.00	0.67		0.33 ± 0.05	0.67 ± 0.05
WB	0.26	0.02	0.72		0.27 ± 0.03	0.73 ± 0.03
FSHB	*AA*	*AB*	*BB*		*A*	*B*
SSS	0.10	0.60	0.30	NS	0.40 ± 0.04	0.60 ± 0.04
SSA	0.24	0.50	0.26		0.49 ± 0.05	0.51 ± 0.05
WB	0.16	0.56	0.28		0.44 ± 0.03	0.56 ± 0.03

Note: *SSS* – *Sus scrofa scrofa*; *SSA* – *Sus scrofa attila*; WB – wild boar; NS – non significant

***$P \leq 0.001$

Table 2. Genetic variability (heterozygosity, average heterozygosity and PIC) in *SSS* and *SSA* subspecies of wild boar

Locus	Heterozygosity		PIC	
	SSS	*SSA*	*SSS*	*SSA*
RYR1	0.000	0.000	0.000	0.000
MYC	0.504	0.024	0.375	0.023
*GH-Hae*II	0.493	0.458	0.369	0.350
*GH-Msp*I	0.493	0.458	0.369	0.350
LEP	0.333	0.000	0.276	0.000
H-FABP	0.000	0.047	0.000	0.045
ESR	0.000	0.000	0.000	0.000
PRLR	0.350	0.450	0.287	0.346
FSHB	0.485	0.506	0.365	0.375
Average	0.295	0.216		

Note: *SSS* – *Sus scrofa scrofa*; *SSA* – *Sus scrofa attila*

the remaining six were polymorphic. In contradistinction to the heterozygous genotype of one wild boar described by Andersson-Eklund *et al.* (1998) *T* allele in *RYR1* gene was not detected among the animals (n = 109) included in our study. Results presented by Müller *et al.* (2000) in 10 wild boars of European type (all *CC* homozygotes) were thus corroborated. Interestingly, Ciobanu *et al.* (2001) also observed only *RYR1 CC* homozygotes in two local pig breeds in Romania. In our study the polymorphism of *MYC* gene showed a high frequency of *B* allele. Reiner *et al.* (2000) also found a higher frequency of *B* allele (0.73) in wild boar (n = 21). The gene of growth hormone (*GH*) has not been described in wild boars in available literature. As far as the polymorphism of GH-*Hae*II was concerned, the highest frequency was found in + allele (0.61) while in the case of *GH-Msp*I the frequency of + allele was 0.39. In the case of *LEP* gene, the frequency of *T* allele was 0.87. This result could not be compared with any findings in literature. When evaluating the variability of *H-FABP* gene, the frequency of *H* allele was 0.99. Gerbens *et al.* (1997) found one heterozygous animal (frequency of *H* allele = 0.90) in wild boars (n = 5). In our study only homozygous genotype *CC* was demonstrated in *ESR* gene. *PRLR* in our study was polymorphic, the frequency of *B* allele being 0.27, while Putnová *et al.* (2002) observed only *B* allele in 10 wild boars studied. The frequency of *A* allele of *FSHB* in our study could not be compared with results published by other authors.

Polymorphic systems of individual subspecies were compared on the basis of differences in genotype frequencies (Table 1). The highest frequency of *B* allele of *MYC* gene was found in *SSA*; in *SSS* its value was not so high. There was a highly significant difference between both subspecies in genotype frequencies. For *GH-Hae*II locus, the frequencies of + allele were similar in *SSS* and *SSA*, similarly like for *GH-Msp*I. No significant differences were found between genotypes for both subspecies. When evaluating the genotypes of *LEP* gene, only the occurrence of *T* allele was detected in the homozygous genotype *TT* in *SSA* subspecies. The difference between frequencies of genotypes in both subspecies was highly significant. A non-significant difference between both subspecies was detected in *H-FABP*, *PRLR* and *FSHB* genotypes.

Values of heterozygosity, average heterozygosity and PIC of individual genes are presented in Table 2. The respective values of heterozygosity and PIC ranged in a lower interval in *SSS* subspecies than in *SSA*. A lower diversity in *SSA* corresponded with lower numbers of animals kept in enclosures. On the other hand, a higher diversity in *SSS* resulted from the fact that the samples were obtained from animals living in open landscape.

A comparison of allele frequencies in wild boars (WB) and some pig breeds is presented in Table 3. *RYR1* and *ESR* were monomorphic in wild boars, while they are polymorphic in commercial breeds of pigs. For *MYC* gene Urban *et al.* (2002a) found a considerably lower frequency of *B* allele (0.18)

Table 3. Comparison of allele frequencies in wild boar populations under study with those of other wild boar and domestic pig populations

Genes	Pigs	*n*	Allele frequencies		References
			C	T	
RYR1	WB	109	1.00	0.00	present results
	WB	10	1.00	0.00	Müller *et al.* (2000)
			A	*B*	
MYC	WB	109	0.31 ± 0.03	0.69 ± 0.03	present results
	LA	6	0.27 ± 0.12	0.73 ± 0.12	Reiner *et al.* (2000)
	DU	117	0.82 ± 0.02	0.18 ± 0.02	Urban *et al.* (2002a)
			+	–	
GH-HaeII	WB	109	0.61 ± 0.03	0.39 ± 0.03	present results
	LW	77	0.53 ± 0.04	0.47 ± 0.04	Handler *et al.* (1996)
	LA	269	0.16 ± 0.02	0.84 ± 0.02	Handler *et al.* (1996)
	DU	117	0.54 ± 0.03	0.46 ± 0.03	Urban *et al.* (2002a)
			+	–	
GH-MspI	WB	109	0.39 ± 0.03	0.61 ± 0.03	present results
	LW	77	0.71 ± 0.04	0.29 ± 0.04	Handler *et al.* (1996)
	LA	269	0.98 ± 0.01	0.02 ± 0.01	Handler *et al.* (1996)
	DU	117	0.85 ± 0.02	0.15 ± 0.02	Urban *et al.* (2002a)
			T	*C*	
LEP	WB	109	0.87 ± 0.02	0.13 ± 0.02	present results
	DU	117	0.65 ± 0.03	0.35 ± 0.03	Urban *et al.* (2002a)
			H	*h*	
H-FABP	WB	109	0.99 ± 0.01	0.01 ± 0.01	present results
	WB	5	0.90 ± 0.09	0.10 ± 0.09	Gerbens *et al.* (1997)
	LW	60	0.67 ± 0.04	0.33 ± 0.04	Urban *et al.* (2002b)
	LA	35	0.87 ± 0.04	0.13 ± 0.04	Urban *et al.* (2002b)
	DU	10	0.70 ± 0.10	0.30 ± 0.10	Gerbens *et al.* (1997)
			C	*D*	
ESR	WB	109	1.00	0.00	present results
	LW	539	0.65 ± 0.02	0.35 ± 0.02	Vrtková and Dvořák (2001)
	LA	284	0.91 ± 0.01	0.09 ± 0.01	Vrtková and Dvořák (2001)
			A	*B*	
PRLR	WB	109	0.27 ± 0.03	0.73 ± 0.03	present results
	$LW^{(A)}$	75	0.52 ± 0.04	0.48 ± 0.04	Putnová *et al.* (2002)
	$LW^{(B)}$	86	0.47 ± 0.04	0.53 ± 0.04	Putnová *et al.* (2002)
	LA	83	0.27 ± 0.04	0.73 ± 0.04	Putnová *et al.* (2002)
			A	*B*	
FSHB	WB	109	0.44 ± 0.03	0.56 ± 0.03	present results
	ER	75	0.62 ± 0.05	0.38 ± 0.05	Jiang *et al.* (2002)
	LA	113	0.27 ± 0.03	0.73 ± 0.03	Jiang *et al.* (2002)

Note: DU – Duroc; ER – Erhualian; LA – Landrace; LW – Large White; WB – wild boar

in Duroc breed (DU). In WB our observations of *B* allele corroborated results published by Reiner *et al.* (2000). Some differences were also observed in allele frequencies of other genes in wild boar and some pig breeds.

FAO recommended microsatellite loci to be used for the study of genetic relationships and/or distances between breeds (Barker *et al.*, 1998; Laval *et al.*, 2000). Ciobanu *et al.* (2001) suggested that the analysis based on microsatellites might provide more information if it is complemented with type 1 (gene) markers.

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ABSTRAKT

Analýza genetické variability osmi kandidátních genů dvou poddruhů divokých prasat

Metodou PCR-RFLP jsme vyhodnotili genotypové a alelové frekvence osmi strukturních kandidátních genů a analyzovali genetickou variabilitu a diverzitu mezi dvěma poddruhy divokých prasat *Sus scrofa scrofa* (*SSS*) při $n = 67$ a *Sus scrofa attila* (*SSA*) při $n = 42$. Polymorfismus nebyl zjištěn u genů ryanodinového (*RYR1* = *CRC*) a estrogenového receptoru (*ESR*) u obou poddruhů. Frekvence alel jednotlivých genů (pro každý gen/lokus je uvedena frekvence jedné ze dvou alel) u *SSS* byla: protein MYC protoonkogenu (*MYC*) $A = 0,49$; růstový hormon (*GH-Hae*II)+ = 0,57 a (*GH-Msp*I)+ = 0,43; leptin (*LEP*) $T = 0,79$; protein vázající mastné kyseliny (*H-FABP*) $H = 1,00$; prolaktinový receptor (*PRLR*) $A = 0,22$ a beta polypeptid folikuly stimulujícího hormonu (*FSHB*) $A = 0,40$. Průměrná heterozygotnost genotypů u *SSS* byla 0,295. Poddruh *SSA* měl frekvence alel jednotlivých genů: *MYC* $A = 0,01$; *GH-Hae*II+ = 0,65 a *GH-Msp*I+ = 0,35; *LEP* $T = 1,00$; *H-FABP* $H = 0,98$; *PRLR* $A = 0,33$; *FSHB* $A = 0,49$. Průměrná heterozygotnost genotypů u *SSA* byla 0,216. Vysoce průkazné rozdíly byly zjištěny mezi dvěma poddruhy ve frekvencích genotypů *MYC* a *LEP*. PIC hodnoty se pohybovaly v polymorfních lokusech u obou poddruhů v rozmezí 0,023 až 0,375. Rozdíly byly zjištěny v polymorfismu genů *RYR1*, *ESR* a *MYC* mezi divokými prasaty ve srovnání s některými plemeny prasat.

Klíčová slova: prase divoké; kandidátní geny; *RYR1*; *MYC*; *GH*; *LEP*; *H-FABP*; *ESR*; *PRLR*; *FSHB*; heterozygotnost; PIC; genetická diverzita

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Composition and properties of milk in White Short-haired goats on the third lactation

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ABSTRACT: Evaluation of changes in milk composition and properties in the course of the third lactation was carried out on the basis of analyses of milk samples obtained from nine goats of the White Short-haired breed. Milk was sampled in the morning on the 35th, 68th, 100th, 135th, 163rd, 194th, 226th and 258th day of lactation. Goats were milked by hand. Significant changes were found in contents of all milk components under study. These changes were dependent on the day and/or stage of lactation and only milk fat gradually increased from 3.38 to 4.07%. Within the time interval from the 35th to the 163rd day of lactation average contents of protein and casein were relatively stable (2.60–2.82% and 1.87–2.02%, resp.) but thereafter a marked increase was observed in both cases. Average contents of dry matter (DM), SNF, fat, protein, casein, calcium, lactose and urea nitrogen for the whole lactation were 11.95%; 8.33%; 3.62%; 2.90%; 2.10%; 0.93 g/l, 4.47% and 23.62 mg/100 ml, respectively. The values of titratable acidity and milk pH were relatively stable during the whole lactation; the only exception was the value of milk pH on the 135th day of lactation (6.91). The shortest clotting interval was observed at the beginning of lactation (58 and 50 s on the 35th and 68th day, resp.). The clotting interval increased highly significantly ($P \leq 0.01$) from the 68th to the 135th day of lactation (up to 143 s). Thereafter, however, the rennetability of milk stabilized and the clotting interval ranged from 125 to 135 seconds. Rennet curdling quality (RCQ) was highly uniform within the whole experimental period (score 2.56–3.11). Correlations between all basic components of milk (DM, SNF, fat, protein and casein) were positive in all cases ($P \leq 0.001$). As far as the individual milk properties were concerned, it was found out that correlations between pH and titratable acidity and/or milk density were very highly negative ($P \leq 0.001$) while that between milk pH and its rennetability was very highly positive ($P \leq 0.001$). As far as the relationships between individual milk properties and RCQ were concerned, a significant positive correlation ($P \leq 0.05$) was found only between milk rennetability and RCQ. The evaluation of relationships between the contents of individual milk components and properties revealed a very highly positive correlation ($P \leq 0.001$) between protein and titratable acidity and a very highly negative correlation ($P \leq 0.001$) between urea nitrogen and milk density.

Keywords: goat milk; milk composition; milk properties; rennet curdling quality; White Short-haired breed

Currently, Czech consumers have an increasing interest in non-traditional foodstuffs. In contradistinction to e.g. France and Italy, in the Czech Republic goat cheese is a non-traditional commodity and is produced directly on farms. The White Short-haired (WSH) goat is the most important Czech breed used for production of milk processed to goat cheese. Milk composition, its properties and rennet curdling quality (RCQ) belong to the most important factors that influence the final quality of goat cheese. Boroš *et al.* (1985) studied changes in goat milk composition in the course of lactation. Rennetability of raw goat milk and its interactions with titratable acidity, curd temperature and supplements of calcium chloride in relation to the optimum retention of pasteurised goat milk were

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evaluated in a study by Boroš and Ševonková (1987). Somatic cell counts in goat milk and their relationship to the composition and properties of milk were investigated by Gajdůšek *et al.* (1996). Effects of non-genetic factors on the production and composition of milk in WSH goats were studied by Margetín and Milerski (2000). Khaled *et al.* (1999) evaluated relationships between nutrition on the one hand and metabolic blood profile and milk composition of WSH goats on the other. Abroad, Zeng *et al.* (1997) studied the composition of milk produced by goats of the Alpine breed, Chornobai *et al.* (1999) in Saanen goats and their crosses, Antunac *et al.* (2001b) in Saanen and Alpine breed, and Prasad and Sengar (2002) in Barbari goats and their crosses. Hadjipanayitou (1995) and Jandal (1996) compared the composition of goat and sheep milk and Clark and Sherbon (2000) studied coagulation properties of goat milk.

The main objective of our study was to evaluate changes in the composition and properties of goat milk and to analyse their mutual relationships in WSH goats in the course of the third lactation. Attention was also paid to changes in RCQ and in milk performance in the course of lactation. The main reason why the third lactation was chosen is that milk yield usually culminates in this stage of life so that this lactation is one of the most important as far as the impact of milk composition and properties on final quality of goat cheese are concerned.

MATERIAL AND METHODS

The evaluation of changes in milk composition and properties was performed using milk samples obtained from nine goats of WSH breed reared on a farm in Šošůvka in Moravian Karst. All goats were on the third lactation and the following parameters were investigated: contents of basic milk components (dry matter – DM, solids non-fat – SNF, fat and lactose), N-compounds (protein, casein and urea nitrogen) and calcium (Ca). These milk properties were evaluated: pH, titratable acidity, specific density and rennetability. The evaluation of RCQ and milk performance in the course of lactation was an integral part of this study. Parturitions of all experimental goats took place in the last decade of February (i.e. from 19th to 27th February 2000). At the time of the first milk sampling, the live body weight of goats ranged from 47 to 56 kg and the birth rate was absolutely uniform (200%). After the birth all kids remained with their mothers and were weaned at the average age of 21 days. Milk was always sampled during the morning milking that took place from 07.00 to 08.00 hours. Milk performance was recorded not only in the morning but also in the evening. The evening milking took place regularly from 19.00 to 20.00 hours. In the evening before sampling and on the day of sampling, all experimental goats were milked manually. Milk sampling was performed on the 35th, 68th, 100th, 135th, 163rd, 194th, 226th and 258th day of lactation. Altogether 72 milk samples were obtained. Milk samples were not conserved, but after milking, all samples were cooled to 5 to 8°C and transported in a thermo-box to a specialised milk laboratory at Mendel University of Agriculture and Forestry (MUAF) in Brno. Analyses always started within an interval of 4 hours after sampling.

During the whole period of study, all experimental goats were reared in one group under identical housing conditions and without any principal differences in nutrition and management. All goats were in a good condition and clinically healthy (including their udders) during the whole experiment. Their daily feed ration from the parturition to the average 57th day of lactation consisted of 2.5 kg grass haylage, 1.5 kg sugar-beet silage, meadow hay (*ad libitum*), straw (*ad libitum*), 1 kg of concentrate mixture (30% wheat, 40% barley and 30% triticale) and mineral lick (*ad libitum*). From the average 58th day of lactation to the end of experiment, the main part of daily feed ration was *ad libitum* grazing on a clover-grass stand supplemented with meadow hay (*ad libitum*), mineral lick (*ad libitum*) and 1 kg of the aforementioned concentrate mixture.

Milk analyses were performed in a specialised laboratory at MUAF Brno. DM content (%) was estimated by a weighing method without sand application at the temperature of 102 ± 1°C (Gajdůšek, 1999). SNF content (%) was calculated on the basis of total DM and fat content (Gajdůšek, 1999). Fat content (%) was determined by Gerber's acidobutyrometric method (Czech Standard CSN 57 0530). Contents of protein and casein were determined using a PRO-MILK apparatus (manufactured by the Danish Co. Foss Electric). Ca content in milk (g/l) was determined by complexometric titration with fluorexone as an indicator (CSN 57 0530). Lactose content (%) was determined polarimetrically according

Table 1. Evaluation of RCQ

Category	Curd and whey appearance
1	Curd very good and hard, keeping its shape after its removal from the container. Whey is clear, of yellow-greenish colour.
2	Curd good but a little softer, not keeping its shape quite perfectly. Excretion of whey not perfect. Whey is greenish.
3	Curd not good, soft, partly not keeping its shape. Whey milky white.
4	Curd very bad, not keeping its shape. Whey milky white.
5	Flocculation of casein very weak or not visible.

to CSN 57 0530. Urea nitrogen (mg/100 ml) was measured spectrophotometrically using the method with *p*-dimethylaminobenzaldehyde (Gajdůšek *et al.*, 1996). Active acidity (pH) was measured with the pH-meter WTW 95 with the electrode WTW SenTix 97 (CSN 57 0530). Milk acidity (°SH) was determined by titration using the Soxhlet-Henkel method (Gajdůšek, 1999). Milk density was measured lactodensimetrically (g/cm^3) (CSN 57 0530). Rennetability (in seconds) was expressed as an interval between the application of the liquid rennet Fromáza and the moment of milk clotting. Clotting was performed at the temperature of 35°C. Milk samples were clotted with stabilised rennet that was stored in a concentrated form at the temperature of 0–5°C. RCQ was evaluated after the incubation of renneted milk (Gajdůšek, 1999) in a thermostat at 35°C for 1 hour. Subsequently RCQ was evaluated by a score description of curd and whey appearance (Table 1). Milk yield was weighed to the nearest 0.1 kg.

Statistical analysis was preformed on the basis of the results of laboratory analyses. Dynamics of changes occurring between two subsequent samplings was evaluated by means of one-factor analysis of variance followed by multiple comparisons. Correlation coefficients were calculated by Pearson's method. The program UNISTAT version 5.1 was used for the statistical analysis.

RESULTS AND DISCUSSION

The evaluation of changes in contents of some goat milk components in the course of the third lactation (Table 2) revealed that there were significant differences in these values in dependence on the day and/or stage of lactation. This finding corresponded with data published by Zeng *et al.* (1997) and Prasad and Sengar (2002). On the other hand, Agnihotri *et al.* (2002) reported that they did not find any significant effect of stage of lactation on the parameters they investigated. As one can see in Table 2, a stable increasing tendency in the course of lactation was found only in fat content (from 3.38 to 4.07%), while the difference between average fat contents recorded on the 35th and the 258th day of lactation was ($P \leq 0.01$). This increasing tendency of fat content in dependence on the day of lactation corresponds with data mentioned by Prasad and Sengar (2002). On the contrary, Antunac *et al.* (2001b) observed a relatively marked decrease from the 50th to the 100th day of lactation (from 3.60 to 3.23%) in goats on the 3rd lactation, followed by an increase from 3.26 to 3.68% between the 150th and 200th day of lactation. The average content of fat for the whole lactation was 3.62% and this value corresponded with data published by Margetin and Milerski (2000) but it was higher than that mentioned by Zeng *et al.* (1997). On the other hand, Boroš *et al.* (1985), Khaled *et al.* (1999) and Agnihotri *et al.* (2002) found higher values of average fat content. As far as DM and SNF were concerned, their contents gradually increased till the 100th day of lactation. On the 135th day of lactation, however, a non-significant decrease to 11.59%, and 8.07%, resp., was observed. Thereafter their contents gradually increased till the end of the experiment. Within the whole experimental period, average contents of DM and SNF were 11.95% and 8.33%. These values were higher than those reported by Zeng *et al.* (1997) and comparable with data published by Clark and Sherbon (2000) for goats of Oberhasli and Toggenburg breeds. Chornobai *et al.* (1999) and Agnihotri *et al.* (2002), however, mentioned

Table 2. Evaluation of changes in some components of goat milk in the course of the 3rd lactation

Characteristic		Average day of lactation								Average	*F*-test
		35 (A)	68 (B)	100 (C)	135 (D)	163 (E)	194 (F)	226 (G)	258 (H)		
Dry matter (%)	$\bar{x}$	11.41^{GH}	11.54^{gH}	11.70^{gH}	11.59^{gH}	11.77^{gH}	12.09^{H}	12.45^{Abcdeh}	$13.06^{ABCDEFg}$	11.95	8.61**
	$s_{\bar{x}}$	0.435	0.421	0.413	0.453	0.575	0.664	0.673	0.815	0.556	
SNF (%)	$\bar{x}$	8.02^{GH}	8.11^{gH}	8.22^{gH}	8.07^{gH}	8.19^{gH}	8.39^{H}	8.66^{Abcde}	9.00^{ABCDEF}	8.33	7.96**
	$s_{\bar{x}}$	0.262	0.220	0.396	0.374	0.373	0.419	0.434	0.331	0.351	
Fat (%)	$\bar{x}$	3.38^{H}	3.43^{h}	3.48^{h}	3.52^{h}	3.58^{h}	3.70	3.79	4.07^{Abcde}	3.62	3.28**
	$s_{\bar{x}}$	0.374	0.399	0.285	0.287	0.242	0.389	0.388	0.557	0.365	
Protein (%)	$\bar{x}$	2.60^{FGH}	2.72^{FGH}	2.67^{FGH}	2.70^{FGH}	2.82^{fGH}	3.08^{ABCDeh}	3.18^{ABCDEh}	$3.43^{ABCDEfg}$	2.90	12.58**
	$s_{\bar{x}}$	0.123	0.180	0.217	0.191	0.322	0.279	0.280	0.326	0.240	
Casein (%)	$\bar{x}$	1.87^{FGH}	2.00^{gH}	1.97^{gH}	1.97^{gH}	2.02^{fgH}	2.24^{Ae}	2.30^{Abcde}	2.43^{ABCDE}	2.10	7.41**
	$s_{\bar{x}}$	0.132	0.145	0.206	0.141	0.368	0.249	0.191	0.205	0.205	
Ca (g/l)	$\bar{x}$	0.93	0.97	1.13^{FGH}	1.13^{FGH}	0.95	0.79^{CD}	0.76^{CD}	0.78^{CD}	0.93	4.75**
	$s_{\bar{x}}$	0.328	0.273	0.205	0.152	0.134	0.143	0.147	0.152	0.192	
Lactose (%)	$\bar{x}$	4.56	4.48	4.63^{f}	4.40	4.42	4.36^{c}	4.41	4.48	4.47	2.36*
	$s_{\bar{x}}$	0.212	0.108	0.213	0.193	0.174	0.162	0.176	0.173	0.176	
Urea nitrogen (mg/100 ml)	$\bar{x}$	24.18^{DGh}	26.69^{dFGH}	26.68^{deFGH}	$30.92^{AbcEFGH}$	22.63^{cDg}	21.06^{BCD}	17.62^{ABCDe}	19.16^{aBCD}	23.62	14.71**
	$s_{\bar{x}}$	4.601	3.033	4.012	4.257	2.497	2.672	2.190	3.495	3.345	

* a b c d e f g h $P \leq 0.05$

** A B C D E F G H $P \leq 0.01$

slightly higher average contents of DM. Average contents of protein and casein were relatively balanced from the 35th to the 163rd day of lactation and thereafter they gradually increased till the end of the experiment. Boroš *et al.* (1985) observed a similar tendency in protein content. But Antunac *et al.* (2001b) found the highest levels of protein on the 50th and the 200th day of lactation (i.e. 2.86 and 2.90%, resp.) in goats on the third lactation; the lowest content of protein was observed on the 150th day of lactation (2.71%). In our experiment, the average content of casein was 2.10%; this value was higher than that mentioned by Khaled *et al.* (1999) but comparable with data published by Gajdůšek *et al.* (1996). Clark and Sherbon (2000), however, reported higher total levels of casein (between 2.16% and 2.77%) in all goat breeds under study.

The evaluation of the dependence of changes in the content of Ca on the day of lactation indicated that there was a gradual but insignificant increase in the content of this mineral in milk from the 35th to 100th day of lactation (i.e. from 0.93 to 1.13 g/l). Thereafter, on the 135th day of lactation, the same content of Ca was found out as on the 100th day of lactation. Thereafter, a gradual decrease was recorded till the 194th day of lactation (to 0.79 g/l), followed by a period of the relatively stable content of this mineral that lasted till the end of the experiment. The average content of Ca for the whole period of study was 0.93 g/l; this value was lower than those mentioned by Ben Goumi *et al.* (1996), Antunac *et al.* (2001b) and Hejtmánková *et al.* (2002). On the contrary, Hošek (2001) published the values that were comparable with Ca contents recorded in our experiment. The highest and the lowest contents of lactose (viz. 4.63% and 4.36%, resp.) were found out on the 100th and the 194th day of lactation, resp., and the difference between them was ($P \leq 0.05$). Boroš *et al.* (1985) observed a similar tendency while Zeng *et al.* (1997) and Antunac *et al.* (2001a) recorded the highest levels of lactose at the beginning of lactation, followed by a gradual decrease till the end of lactation. In our experiment, the average content of lactose for the whole period of study was 4.48%. This value was comparable with data published by Gajdůšek *et al.* (1996), Zeng and Escobar (1996), Khaled *et al.* (1999) and Antunac *et al.* (2001b). Boroš *et al.* (1985) and Prasad and Sengar (2002), however, found higher contents of lactose while Jandal (1996) reported an average content of lactose only 4.08%. The evaluation of the dependence of changes in the content of urea nitrogen on the day of lactation revealed that there was a gradual increase in this parameter between the 35th and the 135th day of lactation (from 24.18 to 30.92 mg/100 ml). Thereafter, a gradual decrease was observed till the 226th day of lactation. In our experiment, the average content of urea nitrogen in goat milk was 23.62 mg/100 ml and this value was higher than that reported by Gajdůšek *et al.* (1996).

The evaluation of the dependence of changes in milk pH and titratable acidity on the day of lactation indicated that the average values of milk pH and titratable acidity were relatively stable within the whole period of study (Table 3). The only exception was pH value recorded on the 135th day of lactation (6.91). The average value of milk pH for the whole experimental period was 6.77 and this corresponded with data published by Gajdůšek *et al.* (1996), Chornobai *et al.* (1999) and Hošek (2001). On the other hand, Galina *et al.* (1996) and Agnihotri *et al.* (2002) mentioned lower average values of milk pH. As far as the titratable acidity was concerned, the highest values of this parameter were recorded at the beginning of lactation, i.e. on the 35th day (5.97 °SH) and at the end of lactation, i.e. on the 226th and 258th day (6.06 and 6.24 °SH, resp.). The lowest, but insignificantly different, value of titratable acidity was recorded on the 100th day of lactation (5.14 °SH). This tendency was very similar to that described by Boroš *et al.* (1985) and Antunac *et al.* (2001a). The highest value of milk density was recorded on the 258th day of lactation (1.028 g/cm^3) while the lowest one on the 135th day (1.025 g/cm^3). The average milk density for the whole experimental period was 1.026 g/cm^3. This value was lower than those mentioned by Gajdůšek *et al.* (1996) and Chornobai *et al.* (1999).

When evaluating milk rennetability (Table 3), the shortest clotting period was observed at the beginning of lactation, on the 35th and the 68th day of lactation (58 and 50 s, resp.). After the 68th day, the clotting interval increased highly significantly ($P \leq 0.01$) till the 135th day of lactation (143 s). Thereafter, this time interval was relatively uniform and ranged from 125 to 135 seconds. The overall average length of clotting period was 109 s and it was shorter than the time interval reported by Gajdůšek *et al.* (1996). Boroš and Ševonková (1987) and Clark and Sherbon (2000) published much longer clotting intervals but it should be

Table 3. Evaluation of changes in some milk properties, RCQ and milk yield in goats on the 3rd lactation

Characteristic		Average day of lactation								Average	*F* test
		35 (A)	68 (B)	100 (C)	135 (D)	163 (E)	194 (F)	226 (G)	258 (H)		
pH	$\bar{x}$	6.73^{d}	6.73^{d}	6.84	6.91^{abeh}	6.72^{d}	6.76	6.78	6.68^{d}	6.77	3.34**
	$s_{\bar{x}}$	0.099	0.100	0.135	0.090	0.088	0.148	0.160	0.155	0.122	
Titr. acidity (°SH)	$\bar{x}$	5.97	5.29	5.14	5.34	5.77	5.85	6.06	6.24	5.71	2.25
	$s_{\bar{x}}$	0.772	0.743	0.724	0.834	1.007	0.962	0.556	0.753	0.794	
Density (g/cm^3)	$\bar{x}$	1.026^{h}	1.026^{dh}	1.026^{H}	1.025^{befGH}	1.027^{dh}	1.026^{dh}	1.027^{Dh}	$1.028^{abCDefg}$	1.026	5.63**
	$s_{\bar{x}}$	0.002	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	
Rennetability (s)	$\bar{x}$	58^{CDEFGH}	50^{CDEFGH}	107^{AB}	143^{AB}	125^{AB}	129^{AB}	135^{AB}	125^{AB}	109	8.49**
	$s_{\bar{x}}$	37.495	12.004	32.396	48.925	54.741	27.487	35.343	21.949	33.793	
Rennet curdling quality	$\bar{x}$	2.67	2.56	2.89	3.11	2.89	2.78	2.89	3.00	2.85	0.46
	$s_{\bar{x}}$	0.500	0.727	0.928	0.782	0.928	0.667	0.782	0.866	0.675	
Milk yield (kg)	$\bar{x}$	4.12^{dEFGH}	4.18^{dEFGH}	3.84^{EFGH}	3.60^{abeFGH}	$3.13^{ABCdfGH}$	$2.68^{ABCDeGH}$	$1.99^{ABCDEFH}$	$1.27^{ABCDEFH}$	3.10	56.81**
	$s_{\bar{x}}$	0.291	0.264	0.416	0.592	0.490	0.380	0.483	0.339	0.407	

* a b c d e f g h $P \leq 0.05$

** A B C D E F G H $P \leq 0.01$

RCQ = rennet curdling quality

Table 4. Correlation coefficients of all goat milk parameters under study within the whole lactation

Characteristic	SNF	Fat	Protein	Casein	Ca	Lactose	Urea nitrogen	pH	Titr. acidity	Density	Rennetability	RCQ	M. yield
Dry matter	0.882***	0.842***	0.841***	0.799***	–0.188	0.169	–0.272*	0.083	0.182	0.156	0.223	0.055	–0.583***
SNF		0.499***	0.905***	0.868***	–0.146	0.318**	–0.231	–0.061	0.295*	0.288*	0.119	0.004	–0.584***
Fat			0.523***	0.496***	–0.199	–0.081	–0.243*	0.219	0.008	–0.066	0.266*	0.092	–0.406***
Protein				0.914***	–0.277*	–0.038	–0.350**	–0.155	0.402***	0.292*	0.135	0.018	–0.674***
Casein					–0.175	0.024	–0.283*	–0.099	0.322**	0.162	0.161	–0.045	–0.614***
Ca						0.331**	0.549***	0.374**	–0.375**	–0.317**	0.030	–0.048	0.338**
Lactose							0.267*	0.089	–0.107	0.129	–0.133	–0.050	0.204
Urea nitrogen								0.337**	–0.303**	–0.396***	–0.112	–0.048	0.620***
pH									–0.623***	–0.522***	0.487***	0.133	0.156
Titr. acidity										0.406	–0.281*	–0.044	–0.261*
Density											–0.078	–0.080	–0.399***
Rennetability												0.241*	–0.470***
RCQ													–0.052
Milk yield													

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$
RCQ = rennet curdling quality

reminded that they used different types of rennet. RCQ was relatively highly uniform within the whole period of study. The best score was recorded on the 68th day of lactation (2.56) while the insignificantly worst RCQ was found on the 135th day (score 3.11). The highest average milk yield (4.18 l per day) was recorded during the second sampling, i.e. on the 68th day of lactation. Thereafter, there was a relatively uniform decrease in milk yield till the end of experiment. The average milk yield for the whole lactation was 3.10 l/day. This value was significantly higher than the values mentioned by Zeng *et al.* (1997), Hošek (2001), Prasad and Sengar (2002) but comparable with results obtained by Khaled *et al.* (1999) in experiments with WSH breed. As to the data presented above, it is necessary to emphasise that there were no significant differences in the results obtained for the first and the second sampling although there was a change from winter to summer feed ration.

The evaluation of correlations between all parameters of goat milk under study for the whole lactation is presented in Table 4. The evaluation of relationships between contents of individual milk components indicated that there were statistically very high positive correlations ($P \leq 0.001$) between DM content on the one hand and contents of SNF, fat and protein on the other ($r = 0.882$; $r = 0.842$ and $r = 0.841$, resp.). This observation corresponds with data published by Zeng *et al.* (1997), Clark and Sherbon (2000) and Antunac *et al.* (2001a). On the contrary, Prasad and Sengar (2002) reported highly significant negative correlations between DM content and those of fat and protein in both cases. Further very high positive correlations ($P \leq 0.001$) were found out between contents of DM and casein ($r = 0.799$); content of SNF and that of fat ($r = 0.499$), protein ($r = 0.905$) and casein ($r = 0.868$); content of fat and that of protein ($r = 0.523$) and casein ($r = 0.496$) and between contents of protein and casein ($r = 0.914$). Clark and Sherbon (2000) found similar correlations between SNF and contents of protein and casein as well as between contents of protein and casein. Antunac *et al.* (2001a) also found a very high significant correlation ($P \leq 0.001$) between contents of SNF and protein. Negative correlations existed between Ca and contents of DM, SNF, fat, protein and casein. A negative correlation between contents of Ca and protein ($r = -0.277$) was statistically significant ($P \leq 0.05$). On the other hand, correlations between the content of Ca and that of lactose ($r = 0.331$; $P \leq 0.01$) and urea nitrogen ($r = 0.549$; $P \leq 0.001$) were both positive.

When evaluating the relationships between individual milk properties it was found out that correlations between pH and titratable acidity and/or specific density were both highly significant and negative ($P \leq 0.001$). Negative correlations, but not significant, between milk pH and its titratable acidity were also reported by Galina *et al.* (1996) and Khaled *et al.* (1999). The correlation between pH and rennetability was, however, highly significant and positive ($r = 0.487$; $P \leq 0.001$). This finding corresponded with data on rennetability of cow milk published by Gajdůšek (1989) and Hanuš *et al.* (1995). As far as the relationships between individual milk properties on the one hand and RCQ on the other were concerned, there was only one significant positive correlation, viz. between rennetability and RCQ ($P \leq 0.05$; $r = 0.241$).

The evaluation of correlations between milk properties on the one hand and its individual components on the other revealed a very highly significant positive ($P \leq 0.001$) correlation between protein content and titratable acidity ($r = 0.402$) and a very highly significant negative ($P \leq 0.001$) correlation between urea nitrogen content and milk density ($r = -0.396$). When evaluating the effect of individual milk components on its rennetability it was found out that a significant correlation ($P \leq 0.05$) existed only between fat content and milk rennetability ($r = 0.226$). On the other hand, Clark and Sherbon (2000) calculated only an insignificant correlation between these two variables. As far as the effects of individual milk components on RCQ are concerned, it is possible to conclude that no significant correlations were found out in our experiments. When evaluating the correlations presented in Table 4, it is necessary to add that very high negative relationships were found between milk yield on the one hand and contents of DM, SNF, fat, protein and casein on the other. Zeng *et al.* (1997) also reported a negative correlation between milk yield and fat content. Nevertheless, these authors found a significant positive correlation between milk yield and protein content.

CONCLUSIONS

The evaluation of changes in milk composition in the course of the third lactation showed that there were significant differences in contents of all com-

ponents and that these changes were dependent on the day and/or stage of lactation. A tendency of gradual increase was found only in fat. As far as the stability of contents of basic milk components was concerned, it was found out that the contents of DM and SNF were relatively stable from the 35th to the 194th day of lactation and those of protein, casein, calcium and lactose within the interval of the 35th and 163rd day. Thereafter, a significant increase was observed in contents of DM, SNF, protein and casein while contents of Ca were significantly lower in the period to come. When evaluating changes in individual milk properties it was found out that the values of titratable acidity and pH were relatively stable during the whole lactation. The only exception was pH on the 135th day of lactation. The shortest clotting interval was recorded during the first two samplings; thereafter, the time of clotting was markedly longer. But it was concluded in general that the clotting interval ranged within adequate time limits.

Correlations between basic milk components (DM, SNF, fat, protein and casein) were all positive and highly significant. As far as the relationship between the contents of individual milk components and its properties were concerned, a highly significant positive correlation was found only between protein content and titratable acidity. Correlations between pH and titratable acidity and between pH and milk density were very negative and highly significant in both cases while the correlation between pH and rennetability was very highly positive. As far as the relationships between RCQ on the one hand and milk properties on the other were concerned, a significant positive correlation was found only between rennetability and RCQ.

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ABSTRAKT

Složení a vlastnosti mléka u bílých krátkosrstých koz v průběhu třetí laktace

Složení a vlastnosti mléka v průběhu třetí laktace byly hodnoceny na základě analýz vzorků mléka získaných od devíti koz plemene bílá krátkosrstá koza. Byly sledovány tyto ukazatelé: složení mléka – základní složky mléka (sušina (S), tukuprostá sušina (TPS), tuk (T) a laktóza (L)), dusíkaté látky v mléce (bílkoviny (B), kazeín (K) a močovinový dusík (MN)) a obsah vápníku (Ca); vlastnosti mléka – pH, titrační kyselost (TK), hustota mléka (HM) a syřitelnost (SYŘ). Nedílnou součástí sledování bylo i hodnocení jakosti sýřeniny (JS) a dojivosti. Odběry vzorků mléka byly realizovány v průměrném 35., 68., 100., 135., 163., 194., 226. a 258. dni laktace z ranního dojení, které bylo prováděno ručně. Všechny kozy byly po celou dobu sledování chovány v rámci jedné skupiny v identických podmínkách a mezi jednotlivými kusy nebyl rozdíl v úrovni péče a výživy. Analýzy mléka byly prováděny ve specializované laboratoři pro rozbory mléka na MZLU v Brně. Z hodnocení změn základních složek kozího mléka vyplývá, že ve všech případech byly zjištěny průkazné rozdíly v obsahu těchto látek v závislosti na dni, respektive období laktace, pouze u tuku byla zjištěna tendence postupného zvyšování jeho průměrných obsahů (od 3,38 do 4,07 %). U S a TPS bylo zjištěno postupné zvyšování jejich obsahů do 100. dne laktace, ve 135. dni byl zaznamenán neprůkazný pokles obou těchto látek na 11,59 % a 8,07 %. Poté však u obou těchto ukazatelů bylo opětovně registrováno postupné zvyšování jejich obsahů, a to až do konce sledování. Nejvyšší (4,63 %) a nejnižší (4,36 %) obsah L byl zjištěn ve 100. a 194. dni laktace, když rozdíl mezi těmito hodnotami byl ($P \leq 0,05$). Průměrné obsahy B a K byly v intervalu od 35. do 163. dne laktace poměrně vyrovnané, avšak v následném období došlo k postupnému zvyšování jejich obsahů až do konce sledování. Z hodnocení změn obsahů Ca vyplývá, že v období od 35. do 100. dne laktace byl zaznamenán neprůkazný nárůst obsahů Ca (od 0,93 do 1,13 g/l), přičemž ve 135. dni laktace byl zjištěn shodný obsah Ca jako ve 100. dni laktace. Poté však došlo k postupnému snižování obsahů Ca, a to až do 194. dne laktace (0,79 g/l); od 194. dne byly obsahy Ca až do konce sledování stabilní. Z hodnocení změn obsahů močovinového dusíku v závislosti na dni laktace vyplývá postupné zvyšování jeho obsahu od 35. (24,18 mg/100 ml) do 135. dne laktace (30,92 mg/100 ml), když rozdíl mezi těmito hodnotami byl ($P \leq 0,01$). Následně byl však zjištěn postupný pokles jeho obsahu, a to až do 226. dne. Průměrné obsahy S, TPS, T, L, B, K, Ca a MN za celou laktaci byly: 11,95 %, 8,33 %, 3,62 %, 4,47 %, 2,90 %, 2,10 %, 0,93 g/l, a 23,62 mg/100 ml. Ze zhodnocení změn vybraných vlastností mléka v závislosti na dni laktace zejména vyplývá, že hodnoty pH a TK byly v průběhu celého sledování poměrně vyrovnané, s výjimkou pH mléka ve 135. dni, kdy došlo jednorázově ke zvýšení na 6,91. Statisticky průkazně ($P \leq 0,05$), respektive vysoce průkazně ($P \leq 0,01$) nejvyšší HM byla zjištěna u posledního odběru (1,028 g/cm^3), když nejnižší hustota byla stanovena ve 135. dni laktace (1,025 g/cm^3). Nejkratší doba SYŘ byla zjištěna na počátku laktace, v 35. (58 s) a v 68. (50 s) dni. Od 68. dne se však doba SYŘ statisticky vysoce průkazně ($P \leq 0,01$) prodlužovala, a to až do 135. dne laktace (143 s). V následném období však byla doba srážení poměrně stabilní. Průměrné hodnoty

pH, TK, HM a SYŘ za celé sledované období byly: 6,77 a 5,71 °SH, 1,026 g/cm^3 a 109 sekund. Jakost sýřeniny byla po celou dobu sledování vysoce vyrovnaná (2,56–3,11). Ani u jednoho ukazatele nebyl zaznamenán průkazný rozdíl mezi prvním a druhým odběrem, když mezi těmito odběry došlo k přechodu ze zimní na letní krmnou dávku. Vzájemné korelační vztahy mezi základními složkami mléka (S, TPS, T, B a K) byly ve všech případech pozitivní ($P \leq 0{,}001$). Z hodnocení závislostí mezi jednotlivými vlastnostmi mléka zejména vyplývá, že mezi pH a TK, respektive HM byly zjištěny velmi vysoce negativní závislosti ($P \leq 0{,}001$). Naproti tomu však mezi pH a SYŘ byla zjištěna velmi vysoká pozitivní závislost ($P \leq 0{,}001$). U závislostí mezi jednotlivými vlastnostmi mléka a JS byla zjištěna průkazná pozitivní závislost ($P \leq 0{,}05$) pouze mezi SYŘ a JS. Z hodnocení závislostí mezi jednotlivými složkami mléka a jednotlivými vlastnostmi mléka především vyplývá velmi vysoce pozitivní závislost ($P \leq 0{,}001$) mezi obsahem B a TK a velmi vysoce negativní závislost ($P \leq 0{,}001$) mezi obsahem MN a HM.

Klíčová slova: kozí mléko; složení; vlastnosti; jakost sýřeniny; bílá krátkosrstá koza

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Hydroxymethylfurfural in Czech honeys

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ABSTRACT: In this study the method of HPLC for the determination of hydroxymethylfurfural (HMF) in honey was introduced and validated. The comparison of the HPLC method with classical Winkler's method indicated significant differences in the results determined by both methods ($P < 0.05$). It was also found out that the HPLC method provided more accurate results than classical Winkler's method. Employing the established HPLC method we determined the content of HMF in the package of 56 samples of the honeys campaigned between the years 1999 and 2002 by Czech beekeepers. The ranges of the recorded values of HMF were: 24.8–66.1 mg/kg in the samples from the year 1999, 15.2–38.7 mg/kg in the samples from the year 2000 and the samples from the year 2001 contained 5.7–38.4 mg/kg of HMF. HMF in the honey samples campaigned in 2002 ranged between the values lower than the limit of detection up to the concentrations of 24.2 mg/kg. There were significant differences ($P < 0.01$) in the concentrations of HMF in the individual years, with the insignificant differences between the years 2001 and 2002. The limit of 40 mg/kg determined by the Czech legislation for the HMF content in honey was exceeded in 55.6% of the samples campaigned in 1999. The obtained results demonstrate that the limitation of the minimal shelf life by three years is legitimate.

Keywords: honey; HMF; storage; HPLC; Winkler's method

Hydroxymethylfurfural (HMF; 5-hydroxymethyl-2-furankarbaldehyde) ranks among the substances with mutagenic activity. However, from the human health point of view, the opinions on its possible cytotoxic, genotoxic and carcinogenic effects are not unified. However, it is obvious that being able to enter other reactions, this highly reactive compound can make the nutritional value of honey lower by the degradation of its unstable components, or possibly affect its colour if present in higher concentrations (Surh *et al.*, 1994; Kubiš and Ingr, 1998; Gangolli, 1999; Janzowski *et al.*, 2000).

This cyclic aldehyde comes into the existence either by the dehydration of hexoses in an acidic medium or as a result of Maillard's reaction. Honey offers friendly conditions for the origin of HMF: high concentration of saccharides (mainly hexoses), lower pH value, presence of organic acids and low water activity. The HMF content in fresh honey is very low, almost reaching zero. However, it grows up during the heating or storage processes. Considering all the facts mentioned above, the content of HMF is an important parameter in the evaluation of honey quality. It also enables to detect the damage of honey or its adulteration by invert sugar or starch syrup (Kubiš and Ingr, 1998; Wunderlin *et al.*, 1998; Nozal *et al.*, 2000).

In the Czech legislation Government Decree No. 76/2003 limits the content of HMF in honey to 40 mg/kg. In the international legislation this limit

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value is defined by the European Honey Directive (2002) and Codex Alimentarius (2001).

There are three methods of HMF determination in honey cited in Harmonized Methods of the European Honey Commission. Two spectrophotometric ones – Winkler's and White's methods (AOAC method) are used in dependence on the type of spectrophotometer at various workplaces. The determination of the HMF content using HPLC with UV detection is the third possibility (Bogdanov *et al.*, 1997).

The aim of this study was to introduce and validate the method of HMF determination in honey using HPLC and consecutively to compare this method with classical Winkler's one. Another aim was to determine the HMF content in 56 honey samples campaigned by Czech beekeepers in the period of 1999–2002 using the newly introduced HPLC method and to utilize the obtained results to find out the way HMF increased its value during 4 years of storage.

MATERIAL AND METHODS

Material. We analysed 56 samples of Czech honeys campaigned by the beekeepers during the period of 1999–2002 (1999 – 9 samples, 2000 – 18 samples, 2001 – 19 samples, 2002 – 10 samples). Starting HMF concentrations of the samples ranged from 0 to 5.1 mg/kg. Before the analysis the honey samples coming from the individual years were stored in shaded jars at a laboratory temperature of 20 ± 2°C.

10 samples of honeys with the wide concentration ranges (0–132.6 mg/kg) were selected to compare the HPLC and Winkler's methods. The samples largely exceeding 40 mg/kg were also used to cover the wide range of concentrations.

Methods. The HPLC method for HMF determination in honey on the ground of the method cited in Harmonized Methods of the European Honey Commission was used for validation (Bogdanov *et al.*, 1997): 10 g of the sample were diluted in water and quantitatively transferred into a 50 ml volumetric flask. The solution was filtered through the membrane filter for HPLC (0.45 μm). Two concurrent determinations were carried out for each sample.

The HMF concentration was determined using an Alliance 2695 liquid chromatograph from Waters (USA) with PDA 2996 detector. Separation was run on the Nova-Pak C_{18} column with the reverse phase of 3.9 × 150 mm, granularity of the stationary phases 4 μm, at the temperature of 25°C. Water (purity for HPLC) and methanol mixed at the ratio of 90 : 10 and the rate of flow of 1.0 ml/min in an isocratic regime constituted the mobile phase. Detection was carried out in UV range at the wavelength of 285 nm. Measurements were evaluated using Empower PDA software (Waters, USA).

Concurrently the HPLC method was compared with classical spectrophotometric Winkler's method (Bogdanov *et al.*, 1997). After the reaction with p-toluidine agent and barbituric acid the absorbance of the diluted sample of honey was measured at 550 nm on a single beam UV/VIS Lambda 11 spectrophotometer, from Perkin Elmer (USA). The HMF concentrations were obtained using calibration line and linear regression. The comparison of both methods was carried out in 10 selected samples of honeys.

The results were processed by STAT Plus statistic program (Matoušková *et al.*, 1992). The comparison of HMF concentrations in the honey samples campaigned in four different years was performed using unpaired *T*-test. The HMF contents meas-

Table 1. Summarisation of HMF concentrations in the analysed samples of honeys ($\bar{x}$ = average, SD = standard deviation)

Year of campaign	Number of samples	HMF (mg/kg)				Number of samples (%)
		$\bar{x}$	min	max	SD	HMF > 40 mg/kg
1999	9	44.2	24.8	66.1	11.9	55.6
2000	18	25.0	15.2	38.7	7.4	0.0
2001	19	12.7	5.7	38.4	8.1	0.0
2002	10	10.3	below the detection limit	24.2	9.0	0.0

ured by both methods were compared by pair *T*-test.

RESULTS AND DISCUSSION

Validation parameters of HPLC method

The linearity of the used HPLC method was tested by the HMF standards in the concentration range of 0.01–100 mg/l HMF. The detection limit of this method was 0.034 mg/kg HMF. To mention the parameters of the method repeatability, the coefficient of variation and the relative coefficient of variation were 1.4 mg/kg and 6.8%, respectively at the average HMF value of 20.8 mg/kg.

The results of the analysis of a package of 56 honey samples

The results of HMF determination by the HPLC method in the honey samples campaigned in the period of 1999–2002 are shown in Figure 1. The basic quantitative statistical characteristics of the obtained results are given in Table 1. The individual samples are divided into four groups depending on the year of the campaign. The results indicate the older the analysed samples, the higher the values of HMF concentrations. This corresponds with the fact that HMF concentration increases not only by honey heating but also during its storage.

We miss literary data describing the relation between the HMF content and long-term storage. We can compare our HMF values in the samples from 2002, the age of which is 1 year and less, with the results cited by Kubiš and Ingr (1998), who stored honey samples from their own hives for 1 year at the temperature of 18°C. Their HMF values of 5.36; 5.85 and 7.66 mg/kg are the average values of 10 samples of the same type of honey and are comparable with our average concentration of 10 samples of 10.3 mg/kg from 2002.

Sancho *et al.* (1992) stored honey samples from Spanish beekeepers at the average laboratory temperature of 20°C for 5 years. The average HMF concentrations of the samples were 4.7 mg/kg after four and 13.1 mg/kg after 16 months of storage. These results can be compared with our average HMF content of 10.3 mg/kg in the samples from 2002, the age of which is 11 months and less. The value 10.3 lies between the values of 4.7 and 13.1, which corresponds with the sample age that is more than 6 and not more than 11 months. Our samples from 2001, the age of which was between 18 and 23 months, showed the average HMF value of 12.7 mg/kg. This value is insignificantly lower than 13.1 mg/kg in Spanish honeys after 16 months of storage. This fact confirms the comparability of our measurements with those of Sancho *et al.*

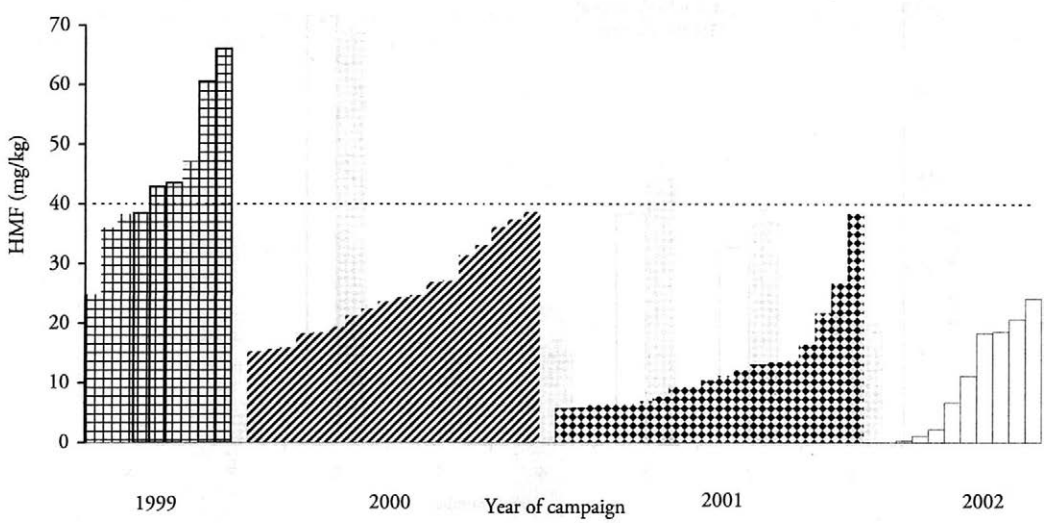

Figure 1. HMF content in honey samples from the individual years of campaign

(1992). After 28-month storage of the Spanish honeys Sancho *et al.* (1992) determined the value of 33.2 mg/kg as the average HMF concentration in these samples. Our samples from 2000 of the age from 30 to 35 months reached the average values of 25.0 mg/kg. This value is only by a few units lower than 33.2 mg/kg in Spanish honeys after 28 months of storage. A comparison of these values supports the speculation expecting a faster increase in HMF content in Spanish honeys. This hypothesis should be tested by the determination of HMF content at least after one more year of storage but Sancho *et al.* (1992) did not carry out such an experiment.

White *et al.* (1964) gave the HMF contents in three honey samples with different initial concentrations of HMF at the temperature of 21°C during 274, 371 and 538 days of storage. The first sample with the starting HMF concentration of 0.6 mg/kg contained 6.6 mg/kg after 274 days and 9.6 mg/kg of HMF after 371 days. These values are comparable with the average HMF value of 10.3 mg/kg in our samples from 2002. After 538 days of storage (1.5 year) the sample contained 14.3 mg/kg of HMF, which is again comparable with the amount of 12.7 mg/kg of HMF in the samples from 2001. The second sample with the starting amount of 2.2 mg/kg contained more HMF, namely 14.9 after 274, 21.9 after 371 and 32.2 mg/kg after 538 days. The increase was even more significant in the third sample whose starting content increased from 3.5 mg/kg to 16.2 after 274 days and to 22.4 after 371 days and to 35.7 mg/kg of HMF after 538 days. These results suggest that after certain storage time the HMF concentration is also influenced by the starting amount of HMF in honey.

Considering that the limit value of 40 mg/kg was exceeded only by some samples from 1999, the age of which was approximately from 3.7 to 4 years in the time of the analysis, we can declare the period of 3 years a legitimate minimal shelf life of honey.

Our numbers of above-limit samples are comparable with those reported by Sancho *et al.* (1992). None of their 115 analysed samples of Spanish honey of the age below 1 year exceeded the limit value of HMF 40 mg/kg. The same can be said about our samples from the year 2002. There is a slight difference between our honey samples from the year 2001 where none exceeded the limit value and the value 5.2% for the Spanish honeys that exceeded this limit when being stored from 1 to 2 years. However, there is a great difference between our samples from the year 2000 where none of them exceeded the limit value again and the Spanish samples where the limit value was exceeded by 89.6% of those stored for three years. A similar although not so striking difference can be found between the number of the above-limit Spanish honeys stored for four years that amounted

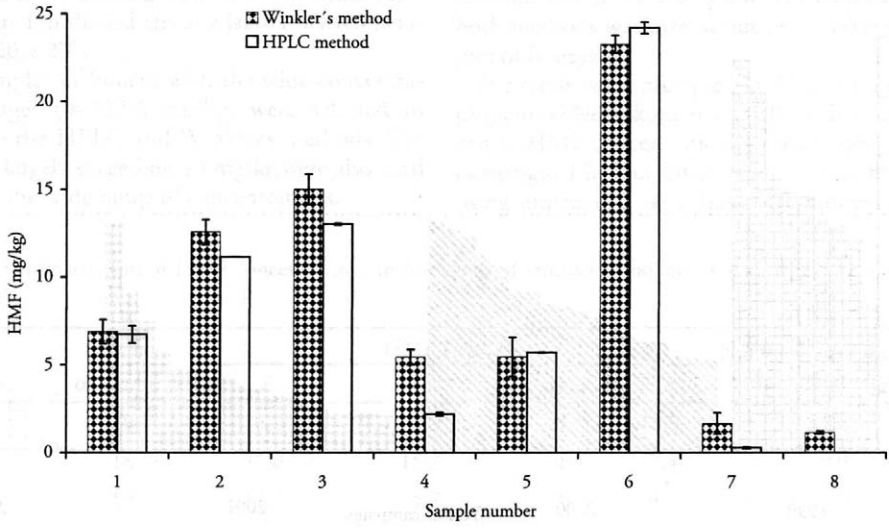

Figure 2. Comparison of HPLC and Winkler's method in honey samples with HMF concentrations up to 25 mg/kg

to 98.3% of the total of 115 analysed samples and the value 55.6% of our samples from 1999 that exceeded the limit of 40 mg/kg. These differences support the above-mentioned hypothesis that there is a comparable increase in the HMF content in both our and Spanish honeys if they are stored for 2 years. However, when this period is over, the HMF concentration grows faster in Spanish than in Czech honeys with Spanish honeys exceeding the limit value of HMF of 40 mg/kg not later than during the third year of storage (probably thanks to hotter climate) whereas in Czech honeys this happens not sooner than during the fourth year of their storage.

Table 2 summarizes the statistical evaluations of HMF concentrations in the samples from individual years of their campaigns. The HMF content determined in the samples from 1999 shows a highly significant statistical difference ($P < 0.01$) from the amounts of HMF in honeys from the years 2000, 2001 and 2002. The concentration of HMF in the samples from the years 2001 and 2002 is equally significantly different ($P < 0.01$) from the HMF content in the honeys from 2000. On the contrary, the differences between the concentrations of the samples from the years 2001 and 2002 are insignificant.

It is obvious from the results that honey will not reach the limit value of HMF within the period of its minimal shelf life if it is not damaged by mishandling. Currently it can be assumed that during proper storage conditions of large amounts of honey it is realistic to observe the stricter internal standard for honey with the registered mark "Czech Honey" that sets down the limiting value of HMF content at 20 mg/kg (Guideline, 1999).

Table 2. Statistical evaluation of HMF concentrations in honey samples from the individual years of campaign

Year	2000	2001	2002
1999	$P < 0.01$	$P < 0.01$	$P < 0.01$
2000	x	$P < 0.01$	$P < 0.01$
2001	x	x	x

Comparison of HPLC and Winkler's method

Using both methods we determined the HMF content in ten selected samples of honey. The obtained values are summarised in Figures 2 and 3. Figure 4 shows the correlations between the concentrations obtained by HPLC and Winkler's method. We found out by pair T-test that the differences between the results determined by both methods were significant ($P < 0.05$). The coefficients of variations show that the HPLC method offers more accurate results compared to classical Winkler's method.

Considering the significant differences ($P < 0.05$) of the results given by the two methods (HPLC and Winkler's method), the comparison of our results and results obtained by other authors who used other methods than HPLC to determine HMF is

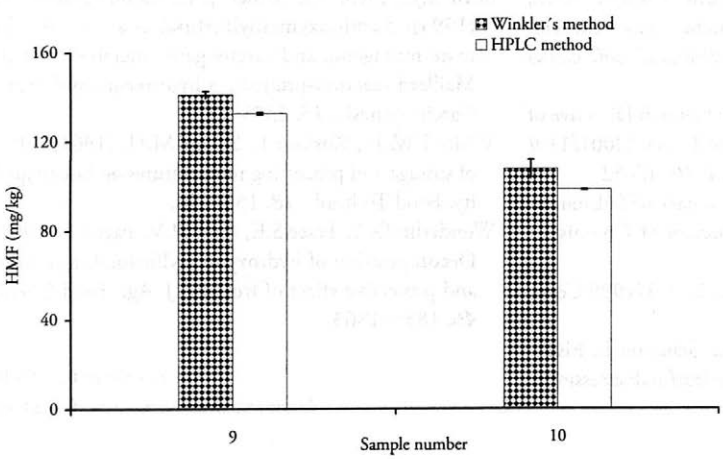

Figure 3. Comparison of HPLC and Winkler's method in honey samples with HMF concentrations above 90 mg/kg

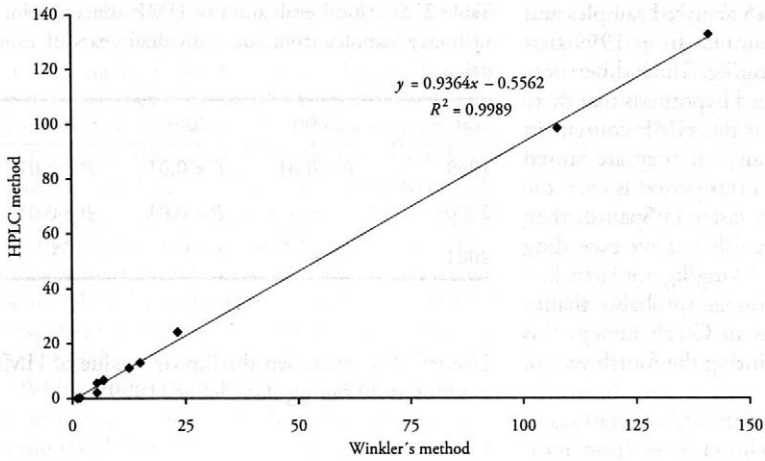

Figure 4. The correlation between HPLC and Winkler's method

more or less informative. It has nothing to do with the fact that the HMF content in honey increases gradually during its storage to exceed the values laid down by law. This is supported not only by our results but also by the results of other authors whose values were compared with ours.

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ABSTRAKT

Hydroxymethylfurfural v českých medech

V této studii byla zavedena a validována metoda HPLC pro stanovení hydroxymethylfurfuralu (HMF) v medu. Porovnáním HPLC metody s klasickou metodou Winklerovou bylo zjištěno, že rozdíly mezi výsledky stanovenými oběma metodami jsou statisticky významné ($P < 0,05$) a že metoda HPLC poskytuje přesnější výsledky oproti klasické metodě Winklerově. Využitím zavedené metody HPLC byl stanoven obsah HMF v souboru 56 vzorků medů vytáčených v letech 1999 až 2002 pocházejících přímo od včelařů z ČR. Naměřené hodnoty HMF se u vzorků medů pocházejících z roku 1999 pohybovaly v rozmezí 24,8–66,1 mg/kg, z roku 2000 v rozmezí 15,2–38,7 mg/kg a vzorky z roku 2001 obsahovaly 5,7–38,4 mg/kg. HMF v medech vytáčených v roce 2002 se pohyboval v hodnotách menších než mez detekce až po koncentrace 24,2 mg/kg. Koncentrace HMF se v jednotlivých letech statisticky signifikantně ($P < 0,01$) lišily. Statisticky nevýznamné rozdíly byly nalezeny mezi roky 2001/2002. Limit pro obsah HMF v medu 40 mg/kg stanovený českou legislativou byl překročen u medů z roku 1999 u 55,6 % vzorků. Ze získaných výsledků je zřejmé, že ohraničení doby minimální trvanlivosti třemi roky je opodstatněné vzhledem ke zvyšování obsahu HMF během skladování.

Klíčová slova: HMF; skladování; HPLC; Winklerova metoda; med

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