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Occurrence of aberrant secretion in bovine virgin mammary glands and effect on phagocytic activities of neutrophils

Výskyt aberantního sekretu v mléčných žlázách virginních jalovic a jeho vliv na fagocytární aktivity neutrofilních granulocytů

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ABSTRACT: A clinical examination of 224 virgin mammary glands in 56 heifers revealed aberrant secretion (AS) in 7 cases. The occurrence of AS was confirmed by light and transmission electron microscopy techniques. A cytological examination showed that total somatic cell counts in standardized volume lavages of mammary glands with aberrant secretion (AMG) were statistically significantly higher than in normal gland (NMG) lavages ($2.713 \pm 1.514.10^6$/ml; $0.985 \pm 0.882.10^6$/ml; $P < 0.05$). In differential cell counts, macrophages were dominant in lavages from both types of glands (NMG, AMG). AMG lavages, however, contained significantly more neutrophilic granulocytes (NG) ($P < 0.05$) and significantly fewer macrophages ($P < 0.05$) than NMG lavages. An examination of the phagocytic function of NG showed a statistically significant difference in relative numbers of NG that were phagocytizing bacterial cells (*Staphylococcus aureus*) (NMG 67.6%; AMG 34.7%; $P < 0.05$). Differences in phagocytic index values were not statistically significant (NMG 3.16 ± 0.50; AMNG 2.74 ± 0.27; $P > 0.05$). Aberrant secretion in virgin mammary glands of heifers therefore seems to be a factor that weakens the resistance of mammary glands to bacterial infections.

Keywords: aberrant secretion; virgin bovine mammary gland; neutrophil granulocytes; phagocytosis; *Staphylococcus aureus*

ABSTRAKT: Klinickým vyšetřením 224 virginních mléčných žláz 56 jalovic byl zaznamenán výskyt aberantního sekretu (AS) v sedmi žlázách. Výskyt AS byl doložen světelnou a transmisní elektronovou mikroskopií. Cytologické vyšetření ukázalo, že celkový počet somatických buněk ve standardizovaném objemu výplachu žláz s aberantním sekretem (AMG) byl statisticky významně vyšší než ve výplaších normálních žláz (NMG) ($2{,}713 \pm 1{,}514.10^6$/ml; $0{,}985 \pm 0{,}882.10^6$/ml; $P < 0.05$). V diferenciálním počtu buněk dominovaly v lavážích z obou žláz (NMG, AMG) makrofágy. V lavážích AMG však bylo statisticky významně vyšší zastoupení neutrofilních granulocytů (NG) ($P < 0{,}05$) a statisticky významně nižší zastoupení makrofágů ($P < 0{,}05$), než v lavážích NMG. Vyšetřením fagocytární funkce NG byl zaznamenán statisticky významný rozdíl v relativním počtu NG fagocytujících bakteriální buňky (*Staphylococcus aureus*) (NMG 67,6 %; AMG 34,7 %; $P < 0{,}05$). Rozdíl v hodnotách fagocytárního indexu nebyl statisticky významný (NMG $3{,}16 \pm 0{,}50$; AMNG $2{,}74 \pm 0{,}27$; $P > 0{,}05$). Aberantní sekret virginních mléčných žláz jalovic se tedy jeví jako činitel snižující obranyschopnost mléčných žláz k bakteriální infekci.

Klíčová slova: aberantní sekret; virginní mléčná žláza skotu; neutrofilní granulocyty; fagocytóza; *Staphylococcus aureus*

INTRODUCTION

In the course of their research into mammary glands of unbred heifers as the focal point for clinical diagnostics and prediction of resistance to infection (Ryšánek *et al.*, 1999, 2001), the authors observed the occurrence of milk-like secretion in several animals. They hypothesized that these were cases of aberrant secretion in virgin heifers described in goats by Cowie *et al.* (1968). According to Zaks (1964), the development of mammary glands in calves

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is sometimes accompanied by secretion and changes in the epithelium of mammary gland ducts (Hammond, 1927). Incidence of secretion in calves at the beginning of the oestrus when mammary gland ducts are developing and udder quarters are being formed was reported by Turevskij (1959).

The positive effect of mechanical stimulation of mammary glands to mammogenesis has been known for many years. Since Hans Selye's discovery in 1934 that the very act of sucking has a stimulating effect on prolactin production, there have been hundreds of experimental studies dealing with the issue of hormonal control of mammogenesis. Although it is acknowledged that oestrogens (stimulating the growth of mammary gland ducts), progesterone (stimulating the development of lobulo-alveolar structures) and especially prolactin and the somatotropic hormone (which mediate mammogenic effects of both oestrogens and progesterone) play the crucial role in mammogenesis, the role of prolactin in bovine mammogenesis is still largely unexplained (Kaskous *et al.*, 1995; Ball *et al.*, 2000; Tucker, 2000). There is, for instance, no doubt that prolactin plays an essential role in the initiation of secretion in the peripartal period, and that similar mechanisms with the same effect are also active in the initiation of mammogenesis. The question what mechanism is at work in the interaction between prolactin, oestrogens and progesterone in the mammogenesis control remains nevertheless unanswered (Tucker, 2000).

The mechanism of aberrant secretion in virgin bovine mammary glands is therefore difficult to explain. Nevertheless, it can be fairly safely assumed that the effects of mechanical stimulation of heifers sucking each other come into play, which, in the first oestrus period, will undoubtedly lead to a premature onset of milk secretion. This empirically well-known fact supports the previous finding by Akers and Lefcourt (1983) that prolactin levels of pregnant cows showed an increasingly intensive response to teat stimulation with progressing pregnancy.

The issue of the aberrant secretion triggering mechanism in virgin bovine mammary glands deserves a systematic attention. The aim of this paper is to report on the occurrence of aberrant secretion in unbred heifers, to investigate its effect on the phagocytic function of neutrophilic leucocytes and also its effect on the defence potential of the mammary gland.

MATERIAL AND METHODS

Animals

The study was based on a clinical examination of 224 mammary glands of 56 unbred heifers, crosses between Holstein and Czech Pied cattle, with no clinical symptoms of any disease. The heifers, 16 to 18 months old, came from a production herd. Mammary gland examinations were made in an experimental bedding barn with stanchions, and the animals were given standard feed rations consisting of hay and feed concentrate mixes with mineral additives.

Experimental design

Mammary glands of the heifers were first clinically examined by a procedure described in our previous paper (Ryšánek *et al.*, 1999) and then lavaged. Briefly, mammary glands were examined visually and by palpation, then lavages were obtained and visually evaluated. Standard cytological techniques were used to examine the lavage fluid for total and differential counts of cells and to determine their morphology. The glands were stimulated in order to obtain a population of NG for *in vitro* examinations of phagocytic functions of these cells. Bacteriological tests of the lavage fluid were also made.

Mammary gland lavages

The technique used for mammary gland lavages is described elsewhere (Ryšánek *et al.*, 1999). Samples of fluids obtained by mammary gland lavages (ML) were taken for a bacteriological examination that was performed by cultivation on blood agar with 5% washed sheep erythrocytes and aerobic cultivation at 37°C for 24 hrs (Quinn *et al.*, 1994). Only samples from healthy and uninfected mammary glands were used for further processing. Immediately after lavage, mammary glands of the heifers were stimulated by an intramammary application of 500 μg of a synthetic muramyl dipeptide analogue (nor MurANc-L-Abu-D-IzoGln, supplied by the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague) dissolved in 10 ml PBS. After 24 hours, the stimulated mammary glands were lavaged with PBS, and lavage fluids obtained both before and after stimulation were examined cytologically.

Total counts of somatic cells

Total counts of cells were determined using Fossomatic 90 (A/C N. Foss Electric, Hillerød, Denmark) and the procedure recommended by the International Dairy Federation (IDF, 1995).

Cell processing

To assess their vitality, cells were stained with trypan blue. The trypan blue dye exclusion test demonstrated viability in at least 97% of cells in each ML. The cell suspensions were centrifuged at 4°C and 200 g for 10 minutes. One ml of the supernatant was removed and retained to be used for resuspension of the pellet and the remaining supernatant was decanted.

Light microscopy (LM) and transmission electron microscopy (TEM)

One smear of each processed cell suspension was prepared by the conventional haematological procedure and stained panoptically by the Papanicolau method (Bessis, 1973). Differential leukocyte counts were assessed by enumeration of 200 cells in each smear (Sládek and Ryšánek, 1999a). For TEM, the cells were prepared according to the procedure described elsewhere (Sládek and Ryšánek, 1999b). The cells from stained smears and the electronograms were analysed using the supportive image analysis programme Lucia G (Laboratory Imaging, Prague, Czech Republic).

In vitro phagocytosis assay

After centrifugation of lavage fluid, cells were adjusted to about 10^6/ml by counting vital cells in Bürker's chamber. Broth culture of the Newbould 305 (CCM 6275) strain of *Staphylococcus aureus* obtained after 18 h incubation at 37°C was washed with PBS by centrifugation at 1000 × g and its density was adjusted with HBSS to 10^6 bacterial cells per 1 ml. The suspensions were opsonised with heterologous non-immunised bovine blood serum (Bovine adult serum, SIGMA, St. Louis, USA) at a final concentration of 10% v/v. Equal parts of the cell and the bacterial suspensions were mixed at the final cell ratio of 1:5. The mixture was incubated for 30 min at 37°C in a thermostat and stained panoptically by the Papanicolau method. To assess the phagocytic activity of neutrophilic granulocytes (NG), the percentage of phagocytizing cells and the phagocytic index (the number of phagocytized *Staphylococcus aureus* in one phagocytizing cell) were calculated.

Statistics

Basic statistics and Student's *t*-test were performed using the STAT Plus software (Matoušková *et al*., 1992).

RESULTS

Functional status

Aberrant secretions were observed in 7 out of 224 examined mammary glands. Palpation revealed an enlargement of the glands. Lavage fluids containing secretion were denser and with milk-like fogging.

Evidence of aberrant secretion

On cell count adjustments in Bürker's chamber, AMG lavage fluids exhibited typical fat globules. In a short while, almost all of them floated up to a higher optical level (Figure 1a), and then they were recorded only sporadically at the cell optical level. Macrophages and NG contained numerous intracytoplasmic vacuoles of phagocytized fat globules (Figure 1b).

The electronograms made by transmission electron microscopy showed vacuolisation with fat globules. Enlarged detail views of the globule walls showed a typical double membrane (Figure 1c).

Cytology of aberrant secretion

Mean total counts of cells were (0.985 ± 0.882 and 2.713 ± 1.514) . 10^6/ml for the NMG and the AMG, respectively. The difference was significant at $P < 0.05$.

Macrophages, lymphocytes, NG and other cells including epithelia and non-differentiated cellular elements were determined in cell suspensions of ML. In the differential cell count picture, mammary glands of heifers were characterized by the dominance of macrophages over all other cell types (Figure 2). AMG differential cell counts showed a statistically significant higher number of NG and a significantly lower level of macrophages ($P < 0.05$).

Besides differences in total and differential counts, AMG and NMG showed differences in the cell morphology, which was reported elsewhere (for details see Sládek and Ryšánek, 1999a,b). The intracytoplasmic vacuoles in the cytoplasm of NG and macrophages were observed (Figure 1c). The vacuoles were phagocytized fat globules. Free-floating fat globules were also found in the aberrant secretion. Fat globules present in cells changed their shape and size, making them rounder and larger.

Table 1. Phagocytic activities of NG from non-secreting and aberrantly secreting mammary glands

Mammary gland	Number of examination	Phagocytosing neutrophilic granulocytes (%) (min.–max.)	Phagocytary index (means ± SD) (min.–max.)
NMG	9	67.6 (60.1–74.2)	3.16 ± 0.50 (2.61–3.84)
AMG	7	34.7 (22.3–38.7)	2.74 ± 0.27 (2.28–3.03)
Statistical significance		$P < 0.05$	$P > 0.05$

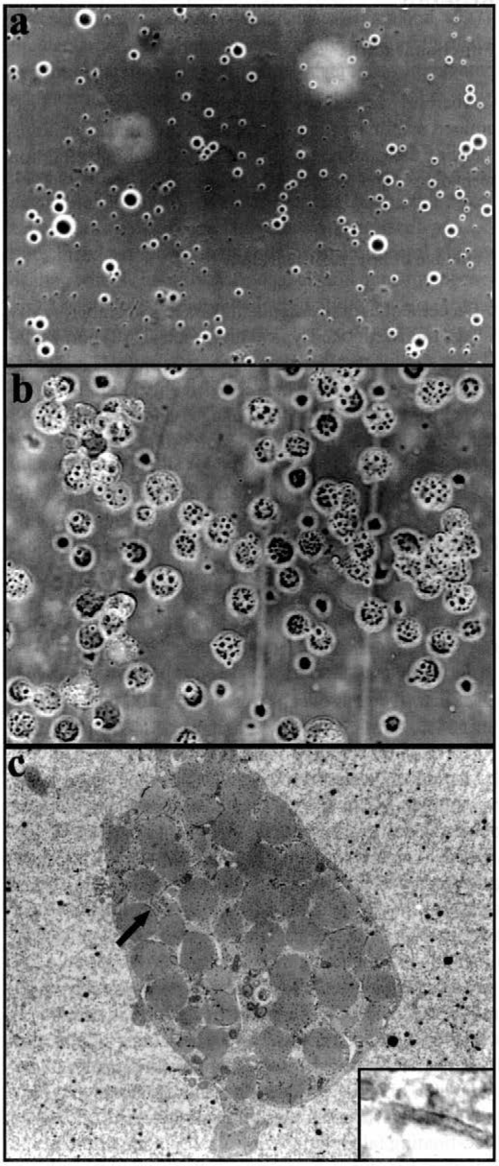

Figure 1. Light microscopy of aberrant secretion with free fat globules floating at the highest level (a) and fat globules phagocytized by macrophages on the bottom (b). Colour inverted image. Original magnification 450 ×

Transmission electron microscopy of a macrophage with phagocytized fat globules in cytoplasm (c). The insert shows a detail of the double membrane of phagocytized fat globule. Arrow shows the location of the insert. Original magnification 8 000 × (insert 50 000 ×)

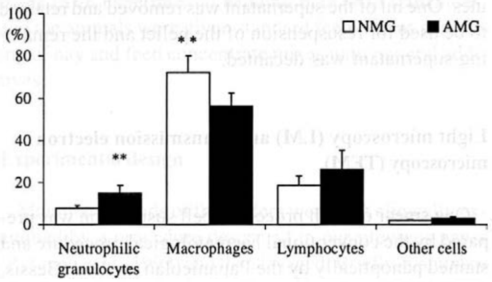

Figure 2. A comparison of differential leukocyte counts in mammary lavage fluids of normal mammary glands (open columns) or aberrantly secreting mammary glands (black columns). The differences are significant (** $P < 0.05$)

In vitro phagocytosis assay

The stimulation of virgin mammary glands induced an influx of cells within 24 hours. The total cell counts following stimulation were 37.8×10^6/ml and 35.3×10^6/ml in NMG and AMG, respectively. The relative proportions of NG were 92.9% and 92.7% in NMG and AMG, respectively. This means that no statistically significant differences were demonstrated in total and differential cell counts.

The phagocytic activity of NMG NG following their incubation with *Staphylococcus aureus* (SA) was higher than that observed in NMG. The number of phagocytizing NG in NMG was almost twice as high as in AMG, which was a statistically significant difference ($P < 0.05$). The difference between phagocytic indices of NMG and AMG NG was not statistically significant (Table 1).

The assessment of phagocytic activity of NG in AMG showed cells containing phagocytized fat globules in their cytoplasm. In these NG with phagocytized fat globules, phagocytized SA bacteria were observed only very rarely (data not shown).

DISCUSSION

The aim of the present study was to demonstrate the incidence of aberrant secretion in unbred heifers and to assess its influence on the phagocytic function of NG, in other words the influence of aberrant secretion on the mammary gland defence mechanisms.

The cavities of healthy virgin bovine mammary glands contain small volumes of clear fluid, which is a suspension of several types of cells. Wardley *et al.* (1976) classified virgin mammary gland cells as monocyte-like macrophages, macrophages, lymphocytes and neutrophils. In our earlier experimental studies of mammary glands of unbred heifers (Ryšánek *et al.*, 1999, 2001) we observed milk-like secretion in the cavities of some of the mammary glands studied.

In this study the cytological examination of aberrant secretion lavage fluids showed higher numbers of somatic cells than NMG lavage fluids. Moreover, different volumes of cell types and, at the same time, differences in their morphologies were observed. It was ascertained that aberrant secretion contained globule-like particles of milk fat morphologically identical with particles contained in the milk plasma of lactating dairy cows. The particles were also contained in the cytoplasm of some NG and macrophages, giving a foam-like appearance to those cells. Cells of the same morphology were observed in mammary glands of dairy cows during lactation (Schalm *et al.*, 1971), dry periods (Lintner and Eberhart, 1990) and in involution (Lee *et al.*, 1969). They are fat globules, as confirmed by an earlier electron microscopy examination (Sládek and Ryšánek, 1999b), which, according to Wooding (1971), is a convincing technique for the assay of phagocytized fat globules.

Besides the above, aberrant secretion also attenuated the phagocytic activity of NG: *Staphylococcus aureus* bacteria were phagocytized by one third of AMG NG compared to almost twice as many phagocytizing cells in NMG. The phagocytic index was also lower in AMG than in NMG although the difference was not statistically significant. It indicates that phagocytic functions of AMG NG are impaired, as demonstrated in the hypothetical calculation (based on relative numbers of phagocytizing NG and their phagocytic indices) according to which the phagocytization of 100 SA bacteria would require 45 NMG NG or 110 AMG NG.

The lower number of NG phagocytizing SA cells observed in AMG is probably related to the presence of fat globules in aberrant secretions. The fact that the presence of milk fat may markedly reduce the bacterial phagocytic functions of NG has been demonstrated in milk of dairy cows. The negative influence is mainly ascribed to the presence of corpuscular milk components, fat globules and casein micelles because they are phagocytized and ingested by NG, which reduces the effects of bacterial cell phagocytosis (Paape *et al.*, 1975; Russell *et al.*, 1976, 1977; Paape and Wergin, 1977; Reinitz *et al.*, 1982). This is the result of energy depletion of cells and their loss of pseudopodia in the course of fat globule ingestion (Paape and Wergin, 1977), inability to achieve lower pH for an optimum operation of lysosomal enzymes (Reinitz *et al.*, 1982), degranulation and the subsequent absence of lysosomes (Russell *et al.*, 1977), and the resulting reduction of the oxygen-independent intracellular killing mechanism (Paape and Wergin, 1977). We assume that the above factors that considerably reduce the phagocytic function of NG in milk of dairy cows will play an important role in the attenuation of the phagocytic activity of NG in aberrant secretions from virgin bovine mammary glands.

CONCLUSION

Virgin bovine mammary glands may produce some aberrant secretion. The milk-like secretion is characterized by higher total cell counts and by higher proportions of NG than the fluid of physiologically normal mammary glands of unbred heifers. The aberrant secretion plasma contains milk fat, which is phagocytized by NG and macrophages. The phagocytosis of the milk fat causes an impairment of the phagocytic function of NG, which corroborates the assumption of a negative effect of aberrant secretion on the defence system of the mammary gland. The above facts only emphasize the need for further study of aberrant secretion of unbred heifers.

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Biochemical studies of blood in hens during the laying period

Biochemické studie krve nosnic v průběhu snáškového období

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ABSTRACT: The aim of the present paper is to determine changes in selected biochemical parameters in the blood plasma of hens during the egg-laying period. Thirty clinically healthy hens were regularly examined every five weeks between the 5th and 50th week of the egg-laying period. Blood was taken from *vena basilica* and stabilized with heparin. Biochemical studies showed significant changes in layers during the egg-laying period. The mean values of the biochemical parameters in blood plasma varied within the following limits: 47.43–60.45 g/l for protein, 13.36–14.97 mmol/l for glucose, 2.73–6.18 mmol/l for cholesterol, 5.26–7.19 mmol/l for calcium, 1.25–1.90 mmol/l for phosphorus, 1.08–1.42 mmol/l for magnesium, 141.03–148.30 mmol/l for sodium and 3.40–5.00 mmol/l for potassium. Moreover, the dynamics of changes demonstrated that the plasma levels of some biochemical parameters reflected variations in the laying capacity. For example, in the first half of the laying period the protein and magnesium concentrations in plasma tended to decrease with an increase in the laying capacity while a decreasing tendency of the calcium concentration persisted towards the end of the laying period. On the contrary, plasma concentrations of glucose, cholesterol and phosphorus decreased with an increase in the laying capacity. Concentrations of sodium and potassium in plasma, however, were independent of the laying capacity. This paper provides new information on the dynamic changes that occur in clinically healthy layers during egg laying and that are likely to be connected with the laying capacity.

Keywords: domestic fowl; layer; egg-laying period; biochemistry of blood plasma

ABSTRAKT: Cílem práce je poznání změn u vybraných biochemických ukazatelů krevní plazmy nosnic v průběhu snáškového období. Vyšetření nosnic bylo prováděno v období od 5. do 50. týdne snášky na souboru 30 klinicky zdravých nosnic v pravidelných pětitýdenních intervalech. Krev byla získána punkcí *vena basilica* a stabilizována heparinem. Biochemické studie dokumentují významné změny, ke kterým dochází u nosnic v průběhu snášky. Z dosažených výsledků je zřejmé, že v průběhu snáškového období kolísají průměrné hodnoty sledovaných ukazatelů v krevní plazmě nosnic v určitých hranicích, a to u bílkoviny 47.43 až 60.45 g/l, glukózy 13.36 až 14.97 mmol/l, cholesterolu 2.73 až 6.18 mmol/l, vápníku 5.26 až 7.19 mmol/l, fosforu 1.25 až 1.90 mmol/l, hořčíku 1.08 až 1.42 mmol/l, sodíku 141.03 až 148.30 mmol/l a draslíku 3.40 až 5.00 mmol/l. Výsledky studia dynamiky změn uvedených biochemických ukazatelů dokládají, že v průběhu snáškového období, se změnou snáškové intenzity, dochází i k charakteristickým změnám v hladinách u některých ukazatelů. U plazmatického proteinu a hořčíku byla pozorována tendence jejich poklesu v prvé polovině snášky, kdy docházelo ke zvyšování snáškové intenzity, zatímco u vápníku tato tendence přetrvávala téměř až do konce snášky. Naopak u plazmatické glukózy, cholesterolu a fosforu byla zaznamenána tendence poklesu jejich hladin v krevní plazmě se vzrůstající snáškovou intenzitou. U sodíku a draslíku výše uvedené závislosti v krevní plazmě nosnic prokázány nebyly. Přínosem práce jsou nové poznatky o dynamických změnách, ke kterým dochází v průběhu snášky i u klinicky zdravých nosnic a které s velkou pravděpodobností souvisejí s intenzitou snášky.

Klíčová slova: kur domácí; nosnice; snáška; biochemie krevní plazmy

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This work is a part of research project no. 162700005 "The research on current hygienic aspects in the production of food and raw materials from animals in relation to their safety".

INTRODUCTION

The paper deals with dynamic changes of biochemical parameters in the blood plasma of hens during the egg-laying period. No data have been published on this subject until now in spite of a large number of papers concerning blood biochemistry in poultry.

Suchý *et al.* (1988) studied a relationship between maturity of gonads and haematological and biochemical parameters of blood in roosters and reported plasma concentrations of 43.31 g/l and 44.20 g/l for total protein and 14.67 mmol/l and 13.75 mmol/l for glucose. Furthermore, Suchý *et al.* (1989) also published a more detailed biochemical analysis of blood in breeding roosters during the sexual maturation (the 10th–25th week of age), showing that the mean values of biochemical parameters in blood serum ranged as follows: 30.86–36.40 g/l for total protein, 13.71–14.56 mmol/l for glucose, 3.75 mmol/l for cholesterol, 2.69–3.06 mmol/l for Ca, 1.13–1.86 mmol/l for P, 0.88–1.18 mmol/l for Mg, 142–145 mmol/l for Na and 4.84–5.27 mmol/l for K.

Straková *et al.* (1993) reported that the mean concentrations ranged from 37.42 to 38.07 g/l for total protein and from 3.01 to 3.56 mmol/l for cholesterol in the plasma of broiler chickens during breeding. One of the few papers on biochemical monitoring of hens during egg laying published by Jeřábek *et al.* (1993) provided the following concentration ranges of elements: 6.73–7.08 mmol per l for Ca, 1.84–2.08 mmol/l for P and 1.29–1.36 mmol/l for Mg. Another gallinaceous species such as Japanese quail was subjected to biochemical studies by Straková *et al.* (1994), who showed the plasma concentrations of some biochemical parameters to vary during the laying period as follows: 49.38–53.97 g/l for total protein, 16.13–16.44 mmol/l for glucose, 5.61–7.80 mmol/l for Ca, 2.63–2.81 mmol/l for P, 1.48–1.80 mmol/l for Mg, 140–155 mmol/l for Na and 1.41–2.46 mmol/l for K. Müller *et al.* (1993) studied blood biochemistry in pheasant and found that the mean values for calcium and phosphorus in the blood serum of adult birds were 1.9 mmol/l and 1.98 mmol/l, respectively. For exotic breeds such as guinea fowls, Oyewale *et al.* (1988) reported the following mean values in blood plasma: 4.95 g/dl (49.5 g/l) for total protein, 7.26 mmol per l for Ca, 3.81 mmol/l for P, 133.61 mmol/l for Na and 3.88 mmol/l for K.

MATERIAL AND METHODS

The aim of this work was to study the dynamics of selected biochemical parameters in the blood plasma of hens.

Thirty clinically healthy hens (Moravia BSL) aged 20 weeks and weighing 1.89 (± 0.230) kg on average were subjected to the experiment. Layers were reared separately in cages in a certified laboratory equipped with controlled air-conditioning, in the Institute for Nutrition, Dietetics, Zoohygiene and Vegetable Food Technology at the University of Veterinary and Pharmaceutical Sciences in Brno. Layers were supplied by Integra Ltd. Žabčice, Czech Republic, together with the corresponding technological procedure that was maintained during the whole breeding. The temperature was kept between 13 and 18°C; the relative humidity ranged between 70 and 75% in the experiment. The light-day was gradually extended during the laying period to a maximum of 14 hrs of light per day in the 42nd week.

Until the 30th week the layers received *ad libitum* a powdery feeding mixture containing 162.5 g/kg of nitrogen ingredients, 52.4 g/kg of fat, 31.7 g/kg of fibre, 11.2 MJ per g of metabolic energy, 36.3 g/kg of Ca and 6.3 g/kg of P. A mixture containing 149.3 g/kg of nitrogen ingredients, 48.9 g/kg of fat, 35.4 g/kg of fibre, 10.6 MJ/kg of energy, 34.8 g/kg of Ca and 5.2 g/kg of P was used from the 30th week.

Biochemical examinations of the blood of layers during the laying period were performed regularly in five-week intervals between 7 a.m. and 9 a.m. Blood samples (8 cm^3) taken from *vena basilica* were placed in test tubes coated with heparin and were immediately centrifuged. Plasma concentrations of total protein, glucose, cholesterol, calcium, phosphorus, magnesium, sodium and potassium were determined.

Some of the parameters were determined photometrically using commercially available Bio-La-test diagnostic kits. Phosphorus, sodium and potassium were determined by atomic absorption spectrophotometry. The results were processed by statistical methods using a programme Statgraphic.

RESULTS

The present paper provides new experimental data on the biochemical profile of blood plasma in hens and points out the changes in biochemical parameters that occur during the laying period.

Before discussing particular results of our investigations we wish to emphasize that the weight of hens gradually increased during the laying period from 1.89 kg (in the 5th week) to 2.24 kg (in the 50th week) on average. Monitoring the weight proved that the nutrition was adequate as the variations in weight were insignificant over five-week intervals. The biochemical parameters in blood were monitored in relation to the egg-laying capacity that increased from 40.77% in the 5th week to 90.57% in the 25th week of the laying period. However, it dropped from 80.29 to 56.08% between the 30th and 50th week.

The results of the biochemical investigation, including statistic evaluation, are summarized in Table 1.

It was observed during the laying period that the mean values of total protein in blood plasma ranged from 47.43

Table 1. The mean values of biochemical parameters in blood plasma of hens during the laying period (5th –50th week)

Age (weeks)	T P (g/l)	Gl (mmo/l)	Chol (mmo/l)	Ca (mmo/l)	P (mmo/l)	Mg (mmo/l)	Na (mmo/l)	K (mmo/l)
5	47.43	14.13	2.73	5.35	1.86	1.08	146.37	5.00
	(5.624)	(0.904)	(0.595)	(1.050)	(0.649)	(0.130)	(4.263)	(0.402)
10	57.49**	14.88**	3.57**	5.81	1.68	1.15	148.30	4.70**
	(5.689)	(1.224)	(0.836)	(0.838)	(0.297)	(0.150)	(5.046)	(0.451)
15	58.59	14.33	3.07	5.26*	1.46**	1.15	145.70*	4.18**
	(6.558)	(1.184)	(1.125)	(0.871)	(0.289)	(0.172)	(4.865)	(0.378)
20	59.77	13.95	3.66 *	6.18**	1.28*	1.20	145.87	4.66**
	(4.845)	(0.922)	(0.804)	(0.763)	(0.274)	(0.113)	(5.277)	(0.324)
25	60.45	13.80	3.13*	6.29	1.25	1.42**	145.83	3.93**
	(8.478)	(0.880)	(1.045)	(0.997)	(0.291)	(0.183)	(5.954)	(0.245)
30	56.82	13.36*	5.45**	6.30	1.43	1.32	141.03**	4.01
	(5.293)	(0.675)	(1.615)	(0.685)	(0.311)	(0.153)	(4.597)	(0.336)
35	58.58	13.59	6.18	6.72*	1.90*	1.35	141.87	3.40**
	(5.641)	(0.866)	(2.004)	(0.884)	(1.169)	(0.144)	(4.321)	(0.271)
40	57.77	13.81	5.69	7.19*	1.79	1.35	144.10*	3.41
	(5.878)	(1.345)	(1.688)	(0.778)	(0.431)	(0.128)	(3.916)	(0.302)
45	56.03	14.97**	5.76	6.67*	1.37*	1.37	142.70	3.56
	(6.889)	(0.805)	(1.974)	(1.091)	(0.691)	(0.211)	(3.426)	(0.287)
50	56.09	14.65	5.80	5.92**	1.76*	1.38	144.17	3.46
	(5.072)	(0.776)	(2.251)	(0.952)	(0.784)	(0.192)	(2.640)	(0.420)

T P = total protein
Gl = glucose
Chol = cholesterol
Ca = calcium

P = phosphorus
Mg = magnesium
Na = sodium
K = potassium

( ) = (standard deviation)
* $P \leq 0.05$
**$P \leq 0.01$

to 60.45 g/l between the 5th and 25th week, respectively. A statistically significant increase ($P \leq 0.01$) in plasma protein from 47.43 to 57.49 g/l occurred between the 5th and 10th week when the laying capacity increased from 40.77% to 85.71%. Subsequently, the level of plasma protein insignificantly increased to 60.45 g/l (in the 25th week) as the maximum laying capacity (90.75%) was reached. The second half of the laying period was characterized by a reduction of the laying capacity and by an insignificant increase in plasma protein from 56.03 to 58.58 g/l.

Plasma concentrations of glucose that ranged from 13.36 to 14.97 mmol/l varied significantly over the laying period. The concentration of glucose gradually decreased until the 30th week reaching significantly the lowest ($P \leq 0.05$) mean value of 13.36 mmol/l. From the 30th week it increased to the maximum value of 14.97 mmol/l in the 45th week.

Similarly, plasma concentrations of cholesterol that ranged from 2.73 to 6.18 mmol/l on average during the laying period showed significant ($P \leq 0.05$) or highly significant ($P \leq 0.01$) changes.

The dynamics of plasma cholesterol showed that its concentration rose significantly ($P \leq 0.01$) from 2.73 mmol/l (5th week) to 3.57 mmol/l (10th week); then followed its decrease to a minimum value of 3.13 mmol/l in the 25th week of the laying period at the highest laying capacity (90.57%). The increase in plasma cholesterol was significant ($P \leq 0.01$) from the 30th week to the end of the laying period ranging from 5.45 mmol/l to 6.18 mmol/l.

Interestingly, dynamic changes were also detected for mineral elements in the blood plasma of hens during the laying period.

Concentrations of calcium in plasma ranged from 5.26 to 7.19 mmol/l. The dynamics for calcium was characterized by a gradual increase in its concentration over the laying period till the 45th and 50th week when its concentration significantly dropped to 6.67 mmol/l ($P \leq 0.05$) and 5.92 mmol/l ($P \leq 0.01$).

Distinct dynamics during the laying period was observed in phosphorus concentrations ranging from 1.25 to 1.90 mmol/l. In contrast to calcium, its mean concentrations considerably declined to 1.25 mmol/l in the 25th week while they increased ($P \leq 0.05$) in the second half of the laying period and were maintained between 1.37 mmol/l and 1.90 mmol/l. Magnesium concentration in plasma gradually increased till the 25th week, reaching the maximum average value 1.42 mmol/l. In the second half of the laying period it decreased to a narrow range 1.32–1.38 mmol/l.

The biochemical analysis of blood plasma in hens included also assays for sodium and potassium. Mean concentrations of sodium ranged from 141.03 to 148.30 mmol per l. During the laying period, no relationship between the laying capacity and plasma concentrations of sodium was observed. Analogously, potassium concentrations ranging from 3.40 to 5.00 mmol/l over the laying period exhibited no dependence on the laying capacity.

DISCUSSION

The results presented in Table 1 show that significant changes in the biochemical profile of blood plasma occurred in clinically healthy layers during the laying period.

Protein concentrations ranged from 47.43 to 60.45 g/l. These values are significantly higher than those reported in breeder roosters by Suchý *et al.* (1988, 1989) or in broiler chickens by Straková *et al.* (1993) or in guinea hens by Oyewale *et al.* (1988). We assume that the high protein concentrations in blood plasma are closely related to higher proteosynthesis which is a prerequisite to high egg production. This conclusion is in agreement with the findings in quail by Straková *et al.* (1994). Our results also confirmed that plasma protein increased with an increase in the laying capacity reaching the maximum of 60.45 g/l in the 25th week.

Glucose concentrations in plasma ranging between 13.36 mmol/l and 14.97 mmol/l were found to be in good agreement with those reported for breeder roosters by Suchý *et al.* (1988, 1989). Higher values in quail probably caused by more intensive metabolism were reported by Straková *et al.* (1994). The dynamics of plasma glucose showed that its concentration increased significantly from 14.13 to 14.88 mmol/l in the early stages of the laying period as the laying capacity increased from 40.77 to 85.71% between the 5th and 10th week. Furthermore, the glucose concentration gradually decreased to a mean value of 13.36 mmol/l in 30th week of the laying period while it tended to rise in the later stages. These changes are likely to be connected with the laying capacity and increasing demands for energy.

Significant changes were also detected for cholesterol concentrations that ranged from 2.73 to 6.18 mmol/l during the laying period. Suchý *et al.* (1989) reported cholesterol concentrations around the average of the above limits while the values in layers published by Jeřábek *et al.* (1993) were close to the upper limit of the above range. The dynamics of plasma cholesterol showed that its significant ($P \leq 0.01$) increase from 2.73 to 3.57 mmol/l between the 5th and 10th week of the laying period was followed by its decrease to 3.13 mmol/l in the 25th week when the laying capacity was at its maximum. We assumed that the initial increase in cholesterol level was caused by its higher biosynthesis and accumulation in yolk. Subsequently, high demands for cholesterol due to the increasing laying capacity could not be satisfied by its biosynthesis. As a result, its concentration in blood gradually declined till the 25th week. This conclusion was also supported by the results obtained during the second half of the laying period when a reduction of the laying capacity in the 30th week was attended by a significant increase in cholesterol concentration to 5.45 mmol/l ($P \leq 0.01$); this increasing tendency persisted till the end of the laying period.

Dynamic changes in the concentration of calcium in blood plasma, ranging from 5.26 to 7.19 mmol/l, were characterized by a gradual increase from 5.35 mmol/l in the early stages to 7.19 mmol/l in the 40th week of the laying period followed by a decrease to 5.92 mmol/l in the 50th week. These values are significantly higher than those in roosters reported by Suchý *et al.* (1989), however, they are in agreement with those published by Jeřábek *et al.* (1993) in the laying hens. An increasing release of calcium into blood in hens is closely related to its accumulation in the shell. As a result, a sharp decrease in its concentration in the 45th and 50th week of the laying period corresponded to a reduced demand for calcium as the egg production decreased from 62.20% (45th week) to 56.08% (50th week).

Surprisingly, a completely opposite trend was observed for phosphorus. Its average levels ranging from 1.25 to 1.90 mmol/l during the laying period were in agreement with those reported by Suchý *et al.* (1989), Jeřábek *et al.* (1993) and Müller *et al.* (1993), though higher values were found in quail by Straková *et al.* (1994) and in guinea-hens by Oyewale *et al.* (1988). The plasma concentrations of phosphorus decreased to 1.25 mmol/l in the 25th week when the laying capacity reached its maximum. However, its concentration rose in the second half of the laying period again.

Similarly like calcium, the magnesium concentration also gradually increased but only till the 25th week when it reached the maximum value 1.42 mmol/l. It dropped significantly ($P \leq 0.05$) to 1.32 mmol/l in the second half of the laying period (30th week) as the laying capacity decreased, and then varied negligibly till the end of the laying period. The mean values of magnesium during the laying period ranged from 1.08 to 1.42 mmol/l, which is in agreement with those reported by the above authors.

Plasma concentrations of sodium and potassium ions were in the range of 141.03–148.30 mmol/l and 3.40–5.00 mmol per l, respectively. Similar results were reported by Suchý *et al.* (1989), Straková *et al.* (1994) and Oyewale *et al.* (1988). This implies that due to the electrolytic equilibrium the concentrations of both univalent ions in avian blood do not differ significantly either within one species or between the species. Furthermore, the plasma levels of both ions showed no direct dependence on the laying period or capacity.

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Red and white blood-cell analysis in hens during the laying period

Červený a bílý krevní obraz nosnic v průběhu snáškového období

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ABSTRACT: The aim of the present paper is to describe changes of haematological parameters in hens during the laying period. Thirty hens were subjected to haematological analysis of blood taken from *vena basilica* in regular five-week intervals between the 5th and 50th week of the egg-laying period. The results of haematological studies showed significant changes in hens during egg laying. A highly significant ($P \leq 0.01$) decrease was found for the following parameters between the 5th and 25th week of the laying period: the erythrocyte count decreased from 2.51 to 2.16 T/l, haematocrit values from 0.29 to 0.26 1/1 and haemoglobin content from 92.52 to 75.29 g/l. No variations in MCHC, MCH and MCV were observed. Furthermore, the total white-cell count decreased very significantly ($P \leq 0.01$) from 36.37 to 17.86 G/l particularly between the 5th and 10th week of the laying period. The decrease in total leukocyte count was simultaneously attended by a highly significant ($P \leq 0.01$) decrease in single types of leukocytes such as Ba, Eo, Ne, Ly and Mo. In the next stages of the laying period the total leukocyte count ranged between 12.85 G/l and 18.68 G/l reaching its minimum value 12.85 G/l in the 30th week. In the same week the lowest counts were also detected for basophilic granulocytes (0.14 G/l), eosinophilic granulocytes (0.14 G/l), neutrophilic granulocytes (3.94 G/l) and monocytes (0.13 G/l). The lowest mean value 8.15 G/l was determined for lymphocytes in the 40th week of the egg-laying period. This paper provides new facts on the dynamic changes in red- and white-cell counts in layers during an extremely demanding period of their life. The results can also be used for diagnostic purposes.

Keywords: domestic fowl; layer; laying period; haematology

ABSTRAKT: Cílem práce je poznání změn v hematologických ukazatelích u nosnic v průběhu snáškového období. V průběhu pokusného období od 5. do 50. týdne snášky byla u souboru 30 nosnic v pravidelných pětitýdenních intervalech odebírána punkcí *vena basilica* krev pro hematologická vyšetření. Výsledky hematologických studií odhalují významné změny, ke kterým dochází u nosnic v průběhu snášky. Tyto změny byly charakterizovány zejména vysoce průkazným ($P \leq 0,01$) poklesem (od 5. do 25. týdne snášky) celkového počtu erytrocytů z 2,51 na 2,16 T/l, hematokritové hodnoty z 0,29 na 0,26 1/1 a obsahu hemoglobinu z 92,52 na 75,29 g/l. U ostatních ukazatelů červeného krevního obrazu MCHC, MCH a MCV tyto změny pozorovány nebyly. U bílého krevního obrazu, a to u celkového počtu leukocytů, nastal v průběhu snášky vysoce průkazný ($P \leq 0,01$) pokles jejich průměrné hodnoty zejména mezi 5. a 10. týdnem snášky z 36,37 na 17,86 G/l. Pokles hladiny leukocytů byl současně provázen vysoce průkazným ($P \leq 0,01$) poklesem i jednotlivých typů leukocytů (Ba, Eo, Ne, Ly a Mo). V následujícím období snášky se hladiny celkového počtu leukocytů pohybovaly v rozmezí od 12,85 do 18,68 G/l, s nejnižší průměrnou hodnotou 12,85 G/l (ve 30. týdnu snášky). Ve 30. týdnu snášky byly diagnostikovány i nejnižší průměrné hodnoty bazofilních granulocytů 0,14 G/l, eozinofilních granulocytů 0,14 G/l, neutrofilních granulocytů 3,94 G/l a monocytů 0,13 G/l. U lymfocytů byla nejnižší průměrná hodnota 8,15 G/l stanovena až ve 40. týdnu snášky. Přínosem práce jsou nové poznatky o dynamických změnách v červeném a bílém krevním obraze v jednom z nejnáročnějších období života nosnic. Dosažené výsledky jsou využitelné i pro diagnostické účely.

Klíčová slova: kur domácí; nosnice; snáška; hematologie

This work is a part of research project no. 162700005 "The research on current hygienic aspects in the production of food and raw materials from animals in relation to their safety".

INTRODUCTION

The paper focuses on the dynamics of changes in haematological parameters in the blood of hens during the laying period. A large number of papers on haematological examination of domestic birds have been published. However, the majority of these papers deal with haematological parameters in relation to fowl diseases or to surgical procedures. In fact, there is a lack of knowledge of the basic haematological parameters and dynamic changes in layers during the laying period. Consequently, the interpretation of results obtained by haematological research on birds is often difficult. In the present paper we attempt to make a contribution to haematology in layers in order to fulfil a gap of knowledge of this subject. Not only academic researchers but also breeders in farming operations have called for publishing results of this research.

In spite of the fact that layers represent the most important domestic birds, scientific literature does not provide complete data on haematological characteristics of this species during the laying period. The only paper on a similar topic was published for *Coturnix coturnix japonica* by Straková *et al.* (1994). Oyewale (1990) studied haematology in guinea-hens during the laying period and compared differences in haematological parameters of laying and non-laying hens. Other literature sources concerning haematology in birds mainly deal with the relationship between the reproduction and haemopoiesis in males, for example in breeding roosters reported by Suchý *et al.* (1997) and in breeding turkey-cocks by Straková *et al.* (1996). The important haematological studies in various breeds of Galliformes were described in papers on haematology in gui-nea fowl by Brijesh *et al.* (1998), Uko and Ataja (1996), Kundu *et al.* (1993), and in red-legged partridge (Alector rufa) by Robles *et al.* (1998). Furthermore, Awotwi and Boohene (1992) published interesting comparative haematological studies of guinea-hen, duck and turkey.

MATERIAL AND METHODS

The aim of this work was to study the dynamics of haematological parameters in blood plasma of hens during the laying period.

Thirty clinically healthy hens (Moravia BSL) aged 20 weeks and weighing 1.89 (± 0.230) kg on average were included in the experiment. Layers were reared separately in cages in a certified laboratory equipped with controlled air-conditioning, in the Institute for Nutrition, Dietetics, Zoohygiene and Vegetable Food Technology at the University of Veterinary and Pharmaceutical Sciences in Brno. Layers were supplied by Integra Ltd., Žabčice, Czech Republic, together with the corresponding technological procedure that was maintained during the whole breeding. The temperature was kept between 13 and 18°C; the relative humidity ranged between 70 and 75% in the experiment. During the laying period the light day was gradually extended to a maximum of 14 hrs of light per day in the 42nd week.

Until the 30th week of the laying period the layers received *ad libitum* a powdery feeding mixture containing 162.5 g/kg of nitrogen ingredients, 52.4 g/kg of fat, 31.7 g per kg of fibre, 11.2 MJ/kg of energy, 36.3 g/kg of Ca and 6.3 g/kg of P. From the 30th week a mixture containing 149.3 g/kg of nitrogen ingredients, 48.9 g/kg of fat, 35.4 g per kg of fibre, 10.6 MJ/kg of energy, 34.8 g/kg of Ca and 5.2 g/kg of P was used.

Haematological examinations of blood in layers during the laying period were performed regularly in five-week intervals between 7 a.m. and 9 a.m. Blood samples (1 cm^3) taken from *vena basilica* were placed in test tubes coated with heparin.

Blood smear was examined immediately, other haematological analyses were performed within 2 hours after the blood taking.

The haematological parameters were determined as follows: erythrocyte count (Er) and leukocyte count (Le) were determined using a dilution method and Bürker chamber; the content of haemoglobin (Hb) was estimated photometrically at 540 nm in Drabkin solution using a photometer Specol-11. The haematocrit value was determined by capillary microhaematocrit method according to Janetzki. Individual leukocytes were determined from a blood smear on panoptic staining by Pappenheim method using May-Grünwald and Giemsa-Romanovski staining solutions. After the experimental values of Er, Hk and Hb became available, mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin – MCH and mean corpuscular volume (MCV) were calculated. The results were processed by statistical methods using a programme Statgrafphic.

RESULTS

The results of haematological studies demonstrated changes in hens during the egg-laying period.

The mean values of erythrocyte count in the blood of layers ranged between 2.16 T/l and 2.54 T/l (Table 1). A gradual decrease in erythrocyte count from 2.51 to 2.16 T/l was observed from the 5th week to the 25th week of the laying period. From the 30th week it increased significantly ($P \leq 0.05$) to 2.40 T/l. In the next stages of the laying period (Table 1) it ranged from 2.26 to 2.54 T/l.

Similar dynamic changes were detected for haematocrit values. Its mean values gradually decreased from 0.29 to 0.26 1/1 from the 5th to 25th week (Table 1). From the 30th week haematocrit values significantly increased ($P \leq 0.05$) to 0.28 1/1 followed by a highly significant ($P \leq 0.01$) increase to 0.31 1/1 in the 35th week and to 0.36 1/1 in the 40th week of the laying period. From the 40th week the haematocrit values remained unchanged.

Table 1. The mean values of haematological parameters in the red blood-cell analysis

Week	Erythrocytes (T/l)	Haematocrit (1/1)	Haemoglobin (g/l)	MCHC (%)	MCH (pg)	MCV (fl)
5	2.51 (0.353)	0.29 (0.026)	92.52 (12.325)	31.38 (2.749)	37.22 (4.395)	118.92 (13.369)
10	2.39 (0.399)	0.30 (0.022)	88.36 (6.545)	29.45** (2.760)	37.86 (6.457)	125.70 (20.807)
15	2.32 (0.529)	0.27** (0.026)	82.35** (8.125)	30.45 (1.410)	36.56 (5.578)	120.36 (19.707)
20	2.17 (0.344)	0.27 (0.022)	76.91** (5.739)	28.44** (1.626)	35.34 (3.617)	120.98 (13.718)
25	2.16 (0.402)	0.26 (0.028)	75.29 (7.760)	29.77** (1.986)	35.33 (6.190)	120.91 (18.928)
30	2.40* (0.400)	0.28* (0.032)	84.28** (7.772)	29.30 (2.070)	35.36 (7.392)	120.59 (24.994)
35	2.26** (0.404)	0.31** (0.020)	105.30** (12.009)	34.26** (3.651)	49.25** (10.557)	144.25** (24.25)
40	2.54** (0.332)	0.36** (0.030)	101.61 (11.112)	28.73** (2.169)	40.64** (6.595)	144.13 (21.305)
45	2.28** (0.297)	0.36 (0.023)	100.18 (13.136)	27.95 (3.433)	44.09* (3.768)	159.42** (19.617)
50	2.52* (0.501)	0.36 (0.043)	100.20 (6.772)	27.83 (3.917)	39.76** (6.987)	142.86** (20.743)

MCHC = mean corpuscular haemoglobin concentration
MCH = mean corpuscular haemoglobin
MCV = mean corpuscular volume determined during the 5th–50th week of the laying period

( ) = (standard deviation)
* $P \leq 0.05$
** $P \leq 0.01$

The above variations were even more pronounced in a haemoglobin content which decreased mostly highly significantly ($P \leq 0.01$) from 92.52 to 75.29 g/l during the 5th and 25th week of the laying period. However, from the 30th week its mean values increased significantly ($P \leq 0.01$) to 84.28 g/l and 105.30 g/l (the 35th week). Then its content ranged from 101.61 to 100.18 g/l (Table 1) till the end of the laying period.

The calculated parameters such as MCHC (mean corpuscular haemoglobin concentration), MCH (mean corpuscular haemoglobin) and MCV (mean corpuscular volume) did not reflect the above dynamic changes of the laying capacity. The MCHC values varied in rather a narrow range from 27.83 to 34.26% over the laying period. Though the differences in the mean values of MCHC in the 10th, 20th, 25th, 35th and 40th weeks of the laying period were found highly significant ($P \leq 0.01$), as seen in Table 1, they cannot be directly related to the laying period.

The mean values of MCH ranged in a narrow interval 35.33–37.86 pg between the 5th and 30th week of the laying period. From the 5th week to the end of the laying period (50th week) a significant increase of MCH to 39.76–49.25 pg was observed.

Similarly like MCH, the mean values of MCV also ranged in a narrow interval 118.92–125.70 fl between the 5th and 30th week (Table 1). From the 35th week to the end of the laying period (the 50th week), MCV considerably increased to 142.86–159.42 fl.

The variations of particular parameters in the white blood-cell analysis during the laying period were not as significant as those in red blood cells. The total leukocyte count dropped highly significantly ($P \leq 0.01$) from 36.37 to 17.86 G/l between the 5th and 10th week. Table 2 shows that this decrease was accompanied by a highly significant ($P \leq 0.01$) decrease in leukocyte counts. Basophilic granulocytes dropped from 1.12 to 0.60 G/l, eosinophilic granulocytes from 0.55 to 0.19 G/l, neutrophilic granulocytes from 13.57 to 8.17 G/l, lymphocytes from 20.82 to 8.74 G/l and monocytes from 0.30 to 0.16 G/l.

In the next stages of the laying period the mean values of white blood-cell counts ranged as follows: total leukocyte count 12.85–18.68 G/l, basophilic granulocytes 0.14–0.60 G/l, eosinophilic granulocytes 0.14–0.24 G/l, neutrophilic granulocytes 3.94–10.24 G/l, lymphocytes 3.94–11.70 G/l and monocytes 0.13–0.24 G/l. The lowest mean value of total count for a particular type of leukocytes,

Table 2. The mean values of haematological parameters in the white blood-cell count determined during the 5th–50th week of the laying period

Week	Leukocytes (G/l)	Basophiles (G/l)	Eosinophiles (G/l)	Neutrophiles (G/l)	Lymphocytes (G/l)	Monocytes (G/l)
5	36.37 (8.502)	1.12 (0.622)	0.55 (0.308)	13.57 (5.164)	20.82 (5.872)	0.30 (0.218)
10	17.86** (4.747)	0.60** (0.288)	0.19** (0.144)	8.17** (3.120)	8.74** (2.919)	0.16** (0.129)
15	18.66 (7.291)	0.40** (0.268)	0.24 (0.206)	10.24 (6.354)	8.99 (5.141)	0.24* (0.137)
20	18.68 (6.555)	0.32 (0.119)	0.18 (0.099)	9.72 (2.854)	10.13 (4.223)	0.21 (0.063)
25	17.65 (5.660)	0.20** (0.116)	0.19 (0.116)	5.38** (3.134)	11.70 (4.509)	0.18 (0.057)
30	12.85** (4.754)	0.14* (0.078)	0.14 (0.075)	3.94* (1.832)	8.40** (3.835)	0.13** (0.048)
35	17.13** (5.358)	0.26** (0.178)	0.20** (0.074)	5.00 (2.849)	11.50** (4.435)	0.17** (0.054)
40	14.32 (5.669)	0.19 (0.092)	0.18 (0.088)	5.66 (2.420)	8.15** (3.902)	0.14* (0.057)
45	16.69 (4.345)	0.23 (0.198)	0.17 (0.043)	4.51 (2.287)	11.62** (3.856)	0.16 (0.043)
50	18.19 (6.229)	0.25 (0.127)	0.19 (0.072)	7.28** (3.728)	10.14 (4.162)	0.18 (0.062)

( ) = standard deviation

* $P \leq 0.05$

** $P \leq 0.01$

except for lymphocytes (8.15 G/l in the 40th week), was observed in the 30th week of the laying period: total leukocyte count 12.85 G/l, basophilic granulocytes 0.14 G/l, eosinophilic granulocytes 0.14 G/l, neutrophilic granulocytes 3.94 G/l and monocytes 0.13 G/l.

DISCUSSION

The results of haematological studies in clinically healthy layers confirm that dynamic changes of haematological parameters occur during the laying period in relation to egg production. These changes are supposed to be linked to the laying capacity. It was found that the red-blood cell count, haematocrit values and the content of haemoglobin decreased with an increase in laying capacity. The lowest mean values of total erythrocyte count (Er) 2.16 T/l, haematocrit (Hk) 0.26 1/1 and haemoglobin content (Hb) 75.29 g/l were determined in the 25th week of the laying period as the laying capacity reached its maximum of 90.75%. From the 30th week to 50th week the laying capacity gradually decreased from 80.29 to 56.08%. This decrease was accompanied with a highly significant ($P \leq 0.01$) increase in the above haematological parameters (Table 1). We assume that the laying in hens represents an enormous metabolic stress which is manifested by decreasing erythropoiesis. A highly significant increase in the mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) occurred in the 35th week when the laying capacity dropped to 73.71%. The mean corpuscular haemoglobin concentration (MCHC) significantly ($P \leq 0.01$) increased to 34.26% in the 35th week. It gradually decreased to 27.83% in later stages (in the 50th week) due to increasing MCV and decreasing MCH.

The mean values of Er, Hk and Hb during the laying period ranged as follows: 2.16–2.54 T/l for Er, 0.26–0.36 1/1 for Hk and 75.29–105.30 g/l for Hb. These values are lower than those in *Coturnix coturnix japonica* reported by Straková *et al.* (1994), which might be caused by increased metabolism, and thus by a higher level of erythropoiesis. Suchý *et al.* (1997) published significantly higher values of Er, Hk and Hb in breeding roosters during the reproduction period. Variations in relation to gender were also reported by Oyewale and Ogwuegbu (1986) in *Numida meleagris*.

Similar changes, though less pronounced, were observed in single parameters of the white blood-cell count in relation to the laying capacity (Table 2).

The white blood-cell count also tended to decrease with an increase in laying capacity. In contrast to the red blood-cell count, the decrease in leukocyte count persisted till the 30th week of the laying period when it reached the lowest mean values not only for total leukocyte count but also for single leukocytes except for lymphocytes. Thus, the following values were determined: total leukocyte count 12.85 G/l, basophilic granulocytes 0.14 G/l, eosinophilic granulocytes 0.14 G/l, neutrophilic granulocytes 3.94 G/l, monocytes 0.13 G/l. The lowest mean value for lymphocytes (8.15 G/l) was detected in the 40th week of the laying period. In the second half of the laying period the leukocyte count tended to increase. Interestingly, the ratio of granulocytes to agranulocytes changed during the laying period. In the early stages of the laying period it was approximately 1:1. Later a number of granulocytes dropped whereas that of agranulocytes increased. The range for the mean values of leukocyte counts (total and single) roughly corresponded to that reported by Straková *et al.* (1994) and Suchý *et al.* (1989). The differential count was in the physiological range reported also for other species of poultry (e.g. Brijesh *et al.*, 1998). Higher values of total leukocyte count in domestic guinea-hens in comparison with our findings were reported by Uko and Ataja (1996). The cited authors reported the total leukocyte count of 25.2 G/l in females and concluded that its increased values were due to a permanent exposition of birds to different pathological organisms.

It can be concluded that high egg production, especially in the peak of the laying period, entails an enormous metabolic stress in layers that results in the lowering of haemopoiesis. Therefore, the laying capacity should also be considered when the results of haematological investigations are evaluated. The above results also demonstrate that the peak of egg laying is an extremely demanding period in the life of hens with respect to liability to infectious agents.

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Evaluation of the genetic diversity in cattle using microsatellites and protein markers

Genetická diverzita skotu hodnocená pomocí mikrosatelitů a proteinových markerů

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ABSTRACT: The aim of the study was to evaluate the degree of the genetic diversity in cattle breeds computed from microsatellites and protein markers. The study was carried out in Czech Pied (Simmental) cattle, Black and White cattle, German Black and White cattle, and in Czech Red, Polish Red, and German Red cattle, which are original cattle breeds endangered by extinction. The highest degree of genetic diversity as measured by heterozygosity and probability of genotypic identity of two individuals has been found in Czech Pied cattle. The total diversity of microsatellites and protein markers in Czech Red cattle was not reduced, even though its population is several dozen of heads only, but the tendency towards reduction was found in microsatellites. The lowest total diversity was found in German Red cattle. Three patterns of exclusion probability are discussed.

Keywords: cattle; diversity; heterozygosity; genotypic identity; exclusion probability; microsatellites; protein markers

ABSTRAKT: Cílem studie bylo posoudit stupeň genetické diverzity u některých plemen skotu na základě alelických frekvencí mikrosatelitů i polymorfních proteinů u českého strakatého, černostrakatého a německého černostrakatého skotu, dále u českých červinek, polského a německého červeného skotu, která patří k původním plemenům ohrožených vymizením. Nejvyšší stupeň genetické diverzity měřený heterozygotností a pravděpodobností genetické identity byl zjištěn u českého strakatého skotu. Celková diverzita u českých červinek nebyla nižší přes to, že se jedná o početně silně omezenou genovou rezervu, avšak tendence ke snížení byla zjištěna u mikrosatelitních lokusů. Nejnižší celková diverzita byla zjištěna u německého červeného skotu. Jsou diskutovány tři různé modely pravděpodobnosti vyloučení nesprávného rodiče.

Klíčová slova: skot; diverzita; heterozygotnost; genotypová identita; pravděpodobnost vyloučení; mikrosatelity; proteinové markery

INTRODUCTION

The genetic diversity and evolution are mutually connected. In farm animals, many breeds well accommodated to different conditions have been bred. On the other hand, man has decreased the genetic diversity lately.

Due to the industrialization of agriculture in developed countries, the preservation of diversity in domestic animals has become an important problem in recent years. The inbreeding and genetic drift, respectively, restrict the genetic variability in small populations. Also in cattle breeding, original breeds are endangered by extinction and highly efficient breeds have expanded, especially Holstein Friesian. The inbreeding in the industrially exploited breeds is also increasing due to their reduced effective population size.

Different methods are used to evaluate genetic diversity. Differences in body structure or the coefficients of inbreeding are studied, polymorphisms of blood groups, milk and blood proteins, and molecular markers, especially microsatellites are evaluated.

The latter are popular above all due to their high polymorphism and extension, they occur in the eucaryote genome every 10 kb (Tautz, 1989). Microsatellites have been defined as length variations within tandem arrays of short nucleotide motifs and localised by specific sequences of primers in PCR. Many bovine microsatellite loci are listed in literature (Moore *et al.*, 1992; Bishop *et al.*, 1994; Grosse *et al.*, 2000).

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A number of authors have used microsatellites to evaluate genetic diversity within and between populations. Edwards *et al.* (2000) gave the average heterozygosity of 20 microsatellite loci from 0.58 in Simmental to 0.71 in Pinzgauer, the mean number of alleles ranging between 5.2–6.0. The degree of heterozygosity was also given by Hirano *et al.* (1996). Peelman *et al.* (1998) reported the heterozygosity of a four-allelic system 0.27–0.42, and of a fourteen-allelic system 0.75–0.82. The other indices of intrapopulation genetic diversity incl. the exclusion probability or the probability of identity of two individuals were influenced by the number of alleles too.

Jamieson and Taylor (1997) described three formulae for the use of marker loci in parentage exclusion. Peelman *et al.* (1998) compared the exclusion probability in Belgian breeds.

The microsatellite multilocus genotype is particularly efficient in evaluating the between- and within-breeds genetic similarities. Hanslik *et al.* (2000) revealed the genetic differences between American and European Holstein populations by microsatellite analysis.

The marker microsatellite loci are widely used to construct gene maps. Thomsen *et al.* (2000) included 265 markers in a male linkage map, 248 of which were microsatellites, the map length over all chromosomes was 3135.1 cM. Maps are prepared primarily as a tool for scanning the genome for QTL (Thomsen *et al.*, 2000; Lien *et al.*, 2000).

The polymorphisms of proteins have become of some interest in the last decades because of the possible influence on physiological and production traits, they are also often incorporated into gene maps. Besides these motives, the polymorphisms of proteins are studied to evaluate genetic diversity (Medjugorac *et al.*, 1994).

In the casein kappa (*CSN10*, *CASK*), many alleles have been described (Prinzenberg *et al.*, 1996), *A* and *B* prevail. The genetic polymorphisms of lactoglobulin beta (*LGB*) has been known since the fifties, *A* and *B* are predominant. In the growth hormone 1 (*GH1*, *bGH*), the polymorphism on the 127th amino acid position (leucine-valine) is often studied. In prolactin (*PRL*), an A-G mutation at the codon for amino acid 103 in exon III causes a polymorphic restriction site revealed by *Rsa*I (Lewin *et al.*, 1992). The polymorphism of pituitary growth factor (*PIT1*) was described by Woolard *et al.* (1994).

MATERIAL AND METHODS

The aim of this study was to evaluate the degree of genetic diversity computed from the allelic frequencies of microsatellites and protein markers.

The study was carried out in Czech Pied cattle ($n = 48$), Black and White cattle ($n = 42$), and German Black and

Table 1. Allele frequencies

Locus	Allele	Czech Red	Czech Pied	Black and White	German Black and White	Polish Red	German Red
BM6438	*1*	0.119	0.179	0.222	0.321	0.073	0.104
	2	0.355	0.282	0.292	0.286	0.355	0.271
	3	0.421	0.372	0.458	0.357	0.161	0.344
	4	0.105	0.167	0.028	0.036	0.411	0.281
CSSM004	*1*	0.558	0.346	0.500	0.440	0.421	0.432
	2	0.154	0.269	0.214	0.155	0.254	0.307
	3	0.288	0.385	0.286	0.405	0.325	0.261
IDVGA9	*1*	0.079	0.250	0.113	0.263	0.057	0.039
	2	0.921	0.750	0.887	0.737	0.943	0.962
M6117	*1*	0.218	0.014	0.183	0.238	0.203	0.193
	2	0.487	0.432	0.450	0.400	0.458	0.466
	3	0.295	0.554	0.367	0.362	0.339	0.341
BM148	*1*	0.040	0.122	0.250	0.171	0.028	0.012
	2	0.081	0.049	0.013	0.012	0.370	0.500
	3	0.757	0.756	0.724	0.805	0.185	0.179
	4	0.122	0.073	0.013	0.012	0.417	0.309
RM012	*1*	0.515	0.317	0.351	0.300	0.014	0.559
	2	0.394	0.585	0.581	0.586	0.843	0.440
	3	0.091	0.098	0.068	0.114	0.143	0
BOVCASK35	*1*	0.053	0.183	0.469	0.197	0.214	0.200
	2	0.405	0.402	0.234	0.500	0.357	0.425
	3	0.237	0.232	0.219	0.212	0.286	0.238
	4	0.303	0.183	0.078	0.091	0.143	0.138
BOVIRBP	*1*	0	0.113	0.057	0	0.063	0
	2	0.088	0.350	0.271	0.139	0.054	0.034
	3	0.912	0.538	0.671	0.861	0.884	0.966

White cattle ($n = 42$). Non-related individuals were chosen at random in each of these groups. Another population was Czech Red cattle ($n = 54$). Because it is an original Czech cattle breed endangered by extinction, we tried to genotype maximum of its members. The group was composed of partially related offspring of 8 sires. German Red and Polish Red breeds are also endangered because they have survived in a very restricted number. There are about 100 000 cows in Poland (crossbreds and purebreds), but only 900 cows are under milk recording. The preservation programme covers less than 100 cows. Many herds have been crossed with Angler cattle, which is related to the breeds mentioned, therefore the original Polish genotypes are dwindling. The study was carried out in 65 Polish Red cattle offspring of 39 sires coming from two herds, and 28 German Red cattle coming from 4 herds. Especially the German Red is very low-numbered, therefore the group was made of partially related animals, offspring of twelve dams and nine sires; the sires of nine animals were unknown.

DNA was isolated from whole blood. Microsatellites were amplified in PCR and separated by electrophoresis on acrylamide sequencing gels, or in sequencer ALF ExpressII (Pharmacia Biotech, Uppsala, Sweden). The fragments were visualised by silver staining. The microsatellite markers used in the analysis were *BM6438* (4 alleles, 256–272 bp), *CSSM004* (3 alleles, 183 bp), *IDVGA9* (2 alleles, 201–203 bp), *BM6117* (3 alleles, 110–114 bp), *BM148* (4 alleles, 97–105 bp), *BM4621* (3 alleles, 137–145 bp), *RM012* (3 alleles, 107–111 bp), *SRC97* (3 alleles, 118–124 bp), *BOVCASK35* (4 alleles, 234–238 bp), *BOVIRBP* (3 alleles, 176–186 bp), *BTOBCAM* (3 alleles, 180–186 bp), *BOVPAI1MR* (2 alleles, 217–219 bp), *BOVSEMRN* (3 alleles, 202–223 bp). In Table 1, the longest allele is marked as 1. The found number and length of alleles corresponded to the literature. The primer sequences were as given by Bishop *et al.* (1994), Moore *et al.* (1992, 1994), Ferreti *et al.* (1994), Kossarek *et al.* (1994), Lang and Plante (1994), the PCR conditions (annealing temperature, magnesium chloride concentration) were optimised individually for every microsatellite.

The polymorphisms of genes for prolactin (*PRL*; alleles *A, B*), casein kappa (*CSN10*; alleles *A, B, E*), lactoglobulin beta (*LGB*; alleles *A, B*), growth hormone 1 (*GH1*; alleles *L, V*), and pituitary growth factor (*PIT1* alleles *A, B*) were also included in computations. Genotyping of protein loci was carried out by PCR/RFLP method according to Schlee *et al.* (1992) for casein kappa, Medrano and Aguilar-Cordove (1990) for lactoglobulin beta, Mitra *et al.* (1995) for growth hormone 1 and prolactin, and Woollard *et al.* (1994) for pituitary growth factor.

Table 1. Allele frequencies – continuation

Locus	Allele	Czech Red	Czech Pied	Black and White	German Black and White	Polish Red	German Red
BTOBCAM	*1*	0	0	0.041	0	0.051	0.068
	2	0.027	0.476	0.041	0.209	0.286	0.875
	3	0.973	0.524	0.919	0.790	0.663	0.057
BOVPAI1MR	*1*	0.065	0.095	0.115	0.029	0.016	0.093
	2	0.935	0.905	0.885	0.971	0.984	0.907
BM4621	*1*	0.184	0.385	0.155	0.256	0.063	0.023
	2	0.684	0.243	0.759	0.573	0.828	0.733
	3	0.132	0.372	0.086	0.171	0.109	0.244
BOVSEMRN	*1*	0.224	0.269	0.027	0.077	0.214	0.300
	2	0.434	0.679	0.932	0.821	0.777	0.620
	3	0.342	0.051	0.041	0.102	0.009	0.080
SRC97	*1*	1	0.925	0.878	0.925	0.873	0.936
	2	0	0.063	0.122	0.050	0.063	0.043
	3	0	0.012	0	0.025	0.063	0.022
PRL	*A*	0.559	0.826	0.905	0.808	0.869	0.909
	B	0.441	0.174	0.095	0.192	0.131	0.091
CSN10	*A*	0.559	0.514	0.756	0.713	0.685	0.814
	B	0.364	0.414	0.220	0.225	0.315	0.174
	E	0.076	0.071	0.024	0.063	0	0.012
LGB	*A*	0.441	0.472	0.405	0.303	0.192	0.321
	B	0.559	0.529	0.595	0.697	0.808	0.679
GH1	*L*	0.490	0.558	0.700	0.833	0.769	0.849
	V	0.510	0.442	0.300	0.167	0.231	0.151
PIT1	*A*	0.049	0.109	0.329	0.263	0.137	0.079
	B	0.951	0.891	0.671	0.737	0.863	0.921

Allele frequencies and heterozygosity were estimated:

$$H = 1 - \sum_{i=1}^{n_i} p_i^2$$

where: p_i = frequency of *i*-allele

n_i = number of alleles

The effective number of alleles, a reciprocal value of the homozygosity was calculated according to the formula:

$$EA = \frac{1}{\sum_{j=1}^{n} p_{ij}^2}$$

where: p_{ij} = frequency of *j*-allele on the *i*-locus

The probability of genotypic identity of two individuals chosen at random in a population (G1), and the random probability of two individuals' genotypic identity both belonging to a different breed (G2) were calculated (Peelman *et al.*, 1998):

$$G1 = \prod_{i=1}^{r} \left(\sum_{j=1}^{n_i} a_{ij}^4 + 4 \sum_{j=i}^{n_i-1} \sum_{k=j+1}^{n_i} a_{ij}^2 . a_{ik}^2 \right)$$

where: r = number of loci

n_i = number of alleles on the *i*-locus

a_{ij}, a_{ik} = frequency of *j*-allele on the *i*-locus

$$G2 = \prod_{i=1}^{r} \left(\sum_{j=1}^{n_i} a_{ij}^2 . b_{ij}^2 + 4 \sum_{j=1}^{n_i-1} \sum_{k=j+1}^{n_i} a_{ij} . b_{ij} . a_{ik} . b_{ik} \right)$$

where: r = number of loci

n_i = number of alleles on the *i*-locus

a_{ij}, a_{ik} = frequency of *j*- and *k*-allele on the *i*-locus in the 1st population

b_{ij}, b_{ik} = frequency in the 2nd population

The probabilities of exclusion of an alleged parent were calculated using a formula according to Jamieson and Taylor (1997) if the genotypes of a mother, its offspring and a putative sire are known:

$$P1 = 1 - 2\sum_{i=1}^{n} p_i^2 + \sum_{i=1}^{n} p_i^3 + 2\sum_{i=1}^{n} p_i^4 -$$

$$-3\sum_{i=1}^{n} p_i^5 - 2\left(\sum_{i=1}^{n} p_i^2\right)^2 + 3\sum_{i=1}^{n} p_i^2 \sum_{i=1}^{n} p_i^3$$

The formula for the exclusion probability if the genotypes of only an offspring and one parent are known:

$$P2 = 1 - 4\sum_{i=1}^{n} p_i^2 + 2\left(\sum_{i=1}^{n} p_i^2\right)^2 + 4\sum_{i=1}^{n} p_i^3 - 3\sum_{i=1}^{n} p_i^4$$

Finally, given two parents and one offspring, excluding both parents (substituted offspring):

$$P3 = 1 + 4\sum_{i=1}^{n} p_i^4 - 4\sum_{i=1}^{n} p_i^5 - 3\sum_{i=1}^{n} p_i^6 - 8\left(\sum_{i=1}^{n} p_i^2\right)^2 +$$

$$+ 8\left(\sum_{i=1}^{n} p_i^2\right)\left(\sum_{i=1}^{n} p_i^3\right) + 2\left(\sum_{i=1}^{n} p_i^3\right)^2$$

where: p_i = allele frequencies

The combined exclusion probability of *k* loci is:

$$P = 1 - (1 - P_1)(1 - P_2) \dots (1 - P_k)$$

RESULTS AND DISCUSSION

The internal variability within the breeds has developed being influenced by the selection for different goals in various regional, climatic and nutritional conditions, genetic drift has also contributed to these processes. The aim of this paper was to evaluate the genetic diversity in some cattle breeds including the Central European Red cattle breeds Czech Red, Polish Red, and German Red cattle, which were studied to estimate the risk of loss in diversity in the gene pools. The allelic frequencies included in the computations are given in Table 1.

The highest heterozygosity was found in Czech Pied cattle (Table 2). It corresponds with the lowest probability of the genotypic identity of two individuals chosen at random in one population (Table 3). The degree of internal variability depends mainly on the breeding history of the respective population. The Czech Pied breed came into being by crossing of the autochthonous Czech Red breed with many breeds in the 19th century. Successively, Simmental cattle dominated especially due to its high production performance. The unification of the Czech Pied cattle population continued, the gene flow of Simmental went on, and moreover, other breeds were used to improve the population. Owing to these facts, the high degree of genetic variability could be expected. Our results are not contradictory to this hypothesis. The diversity computed from frequencies in microsatellite loci was also highest in Czech Pied breed.

In Czech Red cattle, the total degree of polymorphism was relatively high in spite of the fact that its number as restricted, the variability in microsatellite loci was lower, namely the monomorphism of *SRC97* should be pointed out. The results showed a tendency to reduced diversity in German Red cattle caused mainly by protein markers. Indeed, the frequencies especially in prolactin, casein kappa or growth hormone 1 deviated towards one allele more than in other populations, the variability in microsatellites was not lower compared to the other breeds. The total variability in Polish Red cattle also suggested a tendency towards restriction. The random genetic drift and inbreeding contribute to these processes in small populations of the old original cattle breeds. Based on erythrocyte antigen systems and transferrin polymorphisms, Kantanen *et al.* (1999) report on the maintenance of reasonable genetic variability in North European native breeds, but some low-frequency alleles became extinct. Due to their low effective size they consider the loss of genetic variation in the future as probable.

Table 2. Heterozygosities (H) and the numbers of effective alleles (E.A.)

Locus		Czech Red	Czech Pied	Black and White	German Black and White	Polish Red	German Red
BM6438	H	0.672	0.722	0.655	0.686	0.674	0.719
	E.A.	3.049	3.597	2.899	3.185	3.067	3.559
CSSM004	H	0.582	0.659	0.622	0.618	0.653	0.651
	E.A.	2.392	2.933	2.646	2.618	2.882	2.865
IDVGA9	H	0.146	0.375	0.200	0.388	0.108	0.074
	E.A.	1.171	1.600	1.250	1.634	1.121	1.080
BM6117	H	0.628	0.506	0.629	0.652	0.634	0.629
	E.A.	2.688	2.024	2.695	2.874	2.732	2.695
BM148	H	0.404	0.406	0.413	0.322	0.654	0.622
	E.A.	1.678	1.684	1.704	1.475	2.890	2.645
RM012	H	0.571	0.548	0.535	0.554	0.269	0.493
	E.A.	2.331	2.212	2.151	2.242	1.368	1.972
BOVCASK35	H	0.685	0.718	0.671	0.658	0.725	0.704
	E.A.	3.175	3.546	3.040	2.924	3.636	3.378
BOVIRBP	H	0.159	0.575	0.473	0.231	0.212	0.066
	E.A.	1.189	2.353	1.898	1.300	1.269	1.071
BTOBCAM	H	0.053	0.499	0.152	0.331	0.476	0.227
	E.A.	1.056	1.996	1.179	1.495	1.908	1.294
BOVPAIIMR	H	0.122	0.172	0.204	0.056	0.032	0.169
	E.A.	1.139	1.208	1.256	1.059	1.033	1.203
BM4621	H	0.481	0.654	0.392	0.577	0.298	0.403
	E.A.	1.927	2.890	1.645	2.364	1.424	1.675
SRC97	H	0	0.140	0.214	0.141	0.230	0.121
	E.A.	1	1.163	1.272	1.164	1.299	1.138
BOVSEMRN	H	0.644	0.463	0.128	0.310	0.351	0.519
	E.A.	2.809	1.862	1.147	1.449	1.541	2.079
Average H microsat.	H	0.396	0.495	0.407	0.425	0.409	0.415
PRL	H	0.493	0.287	0.172	0.311	0.227	0.165
	E.A.	1.972	1.403	1.208	1.451	1.294	1.198
CSN10	H	0.549	0.559	0.379	0.438	0.432	0.307
	E.A.	2.217	2.268	1.610	1.779	1.760	1.443
LGB	H	0.493	0.498	0.482	0.422	0.311	0.436
	E.A.	1.972	1.992	1.931	1.730	1.451	1.773
GH1	H	0.500	0.493	0.420	0.278	0.358	0.257
	E.A.	2.000	1.972	1.724	1.385	1.558	1.346
PIT1	H	0.093	0.194	0.441	0.387	0.237	0.146
	E.A.	1.102	1.241	1.789	1.631	1.310	1.171
Average H Cod. loci	H	0.426	0.406	0.379	0.367	0.313	0.262
Average H Total	H	0.404	0.470	0.399	0.409	0.382	0.373

Table 3. The random probability of genotypic identity of two individuals' within one breed (G1)

Locus	Czech Red	Czech Pied	Black and White	German Black and White	Polish Red	German Red
BM6438	0.1681	0.1271	0.1845	0.1631	0.1676	0.1327
CSSM004	0.2451	0.1901	0.2139	0.2264	0.1944	0.1951
IDVGA9	0.7407	0.4609	0.6594	0.4501	0.8011	0.8621
BM6117	0.2103	0.3585	0.2145	0.1958	0.2085	0.2126
BM148	0.3820	0.3791	0.4105	0.4974	0.1889	0.2127
RM012	0.2731	0.2819	0.3040	0.2723	0.5634	0.3784
BOVCASK35	0.1593	0.1283	0.1623	0.1679	0.1262	0.1373
BOVIRBP	0.7189	0.2610	0.3484	0.6074	0.6316	0.8747
BTOBCAM	0.8989	0.3756	0.7241	0.5011	0.3491	0.6103
BOVPAI1MR	0.7784	0.6999	0.6542	0.8935	0.9370	0.7049
BM4621	0.3186	0.1943	0.4056	0.2450	0.5140	0.4206
BOVSEMRN	0.2011	0.3577	0.7642	0.4978	0.4769	0.3064
SRC97	1	0.7460	0.6403	0.7428	0.6054	0.7758
Microsatellite loci	**3.437×10^{-6}**	**1.947×10^{-7}**	**3.703×10^{-6}**	**1.823×10^{-6}**	**2.241×10^{-6}**	**1.973×10^{-6}**
PRL	0.3786	0.5492	0.7000	0.5235	0.6228	0.7104
CSN10	0.2921	0.2891	0.4411	0.3718	0.4160	0.5209
LGB	0.3786	0.3758	0.3844	0.4231	0.5234	0.4134
GH1	0.3751	0.3784	0.4246	0.5597	0.4790	0.5855
PIT1	0.8262	0.6687	0.4095	0.4504	0.6107	0.7394
Proteins	**1.297×10^{-2}**	**1.510×10^{-2}**	**2.064×10^{-2}**	**2.076×10^{-2}**	**3.967×10^{-2}**	**6.623×10^{-2}**
Total	**4.460×10^{-8}**	**2.940×10^{-9}**	**7.641×10^{-8}**	**3.785×10^{-8}**	**8.889×10^{-8}**	**1.307×10^{-7}**

Except for Czech Red cattle, there were higher heterozygosity and lower probability of identity, respectively, in microsatellite loci compared to the protein markers. Similarly Arranz *et al.* (1996) estimated higher heterozygosity from microsatellites than from proteins. E.g. the mean probability of two individuals' genotypic identity in Czech Pied cattle was 0.351 in microsatellite loci, 0.452 in proteins, in German Red cattle 0.448 and 0.594, respectively. The figures followed the number of alleles, the highest mean of heterozygosity across all breeds was found in a 4-allelic system of *BOVCASK35* (0.694), but there were also exceptions, *BM148* with 4 alleles possessed lower heterozygosity 0.470 than 3-allelic loci *CSSM004* (0.631), *BM6117* (0.613), and *RM012* (0.495). Something like exceptions was also observed by Hirano *et al.* (1996) or Moazami-Goudarzi *et al.* (1994). Moore *et al.* (1992) gave higher heterozygosity of 0.737 in 19 animals from the reference family for *BOVCASK35*, similarly for *BOVIRBP* 0.684, our average 0.286, and *BOVPAI1MR* 0.632, our figure 0.126. On the other hand, our heterozygosities in *BTOBCAM* and *BOVSEMRN* were higher (0.290 and 0.263; 0.403 and 0.158).

Table 4. The random probability of genotypic identity of two individuals belonging to two breeds (G2)

Breed combination	Probability
Czech Red and Czech Pied	4.343×10^{-10}
Czech Red and Black and White	5.216×10^{-9}
Czech Red and German Black and White	3.974×10^{-9}
Czech Red and Polish Red	5.096×10^{-10}
Czech Red and German Red	3.072×10^{-11}
Czech Pied and Black and White	7.223×10^{-10}
Czech Pied and German Black and White	1.354×10^{-9}
Czech Pied and Polish Red	1.228×10^{-10}
Czech Pied and German Red	1.019×10^{-10}
Black and White and German Black and White	1.871×10^{-8}
Black and White and Polish Red	6.219×10^{-10}
Black and White and German Red	2.481×10^{-11}
German Black and White and Polish Red	7.679×10^{-10}
German Black and White and German Red	1.574×10^{-10}
Polish Red and German Red	4.313×10^{-9}

Table 5. The probabilities of exclusion: summary

	Czech Red	Czech Pied	Black and White	German Black and White	Polish Red	German Red
P1 Microsatellites	0.9665	0.9833	0.9686	0.9615	0.9705	0.9709
P1 Proteins	0.6211	0.6110	0.5775	0.5691	0.5036	0.4523
P1 Total	0.9873	0.9935	0.9867	0.9834	0.9854	0.9841
P2 Microsatellites	0.8058	0.8782	0.8057	0.7773	0.8099	0.8442
P2 Proteins	0.4290	0.3792	0.3025	0.3349	0.2170	0.1532
P2 Total	0.8891	0.9244	0.8645	0.8519	0.8512	0.8681
P3 Microsatellites	0.9966	0.9988	0.9969	0.9958	0.9972	0.9971
P3 Proteins	0.7938	0.7884	0.7649	0.7515	0.6785	0.5943
P3 Total	0.9993	0.9998	0.9993	0.9990	0.9991	0.9988

P1 = the probability of exclusion of an alleged parent if the genotypes of a mother, its offspring and a putative sire are known

P2 = the probability of exclusion if the genotypes of only an offspring and one parent are known

P3 = the probability of exclusion of both parents if the genotypes of two parents and one offspring are known (a substituted offspring)

Our average heterozygosity in *IDVGA9* 0.215 was comparable with that of Ferretti *et al.* (1994): 0.278.

In general, the probability of two individuals' genotypic identity, each belonging to a different breed should reflect the degree of relationship between the populations. But one must realize the existence of genetic drift in small populations, it concerns the low probability between Czech Red and German Red cattle, Czech Red and Czech Pied, Czech Red and Polish Red (Table 4). The high probability between Black and White cattle and German Black and White cattle reflects their relationship, the Black and White cattle of Czech origin has arisen from absorptive crossing of Czech Pied cattle and Black and White bulls both of American and European origin. The high probability between Czech Red and Black and White, and between Polish Red and German Red is also in accordance with the phylogeny.

The exclusion probabilities are given in Table 5. Taking into account all loci, an exclusion probability >0.98 was obtained if the genotypes of a mother, its offspring and a putative sire are known (P1). Due to the different number of loci the figures for microsatellites and protein markers are not directly comparable, the average exclusion probability in microsatellites in all breeds under study exceeded the probability in proteins (in Czech Red cattle by 0.2163 and 0.1735, in Czech Pied by 0.2591 and 0.1698, in German Black and White by 0.2233 and 0.1576, in Black and White by 0.2121 and 0.1541, in Polish Red by 0.2234 and 0.1303, in German Red by 0.2230 and 0.1126, respectively). Thus, the conclusion of Peelman *et al.* (1998) about the suitability of microsatellites for identifying the individuals in parentage control or forensic medicine has been confirmed. Nevertheless, they mention the fact that the high mutation rates of microsatellites can complicate parentage testing, and the alleged parent should never be excluded on the basis of incompatibility for one marker only. As expected, the probability of exclusion P2 was lower if the genotype of one parent was unknown (Table 5). Heyen *et al.* (1997) gave the probability of exclusion involving 22 microsatellites >0.99999 if the genotypes of both parents and an offspring are known, and >0.9986 if the genotype of the confirmed parent is unknown. They also draw attention to a possible mistake when the alleged parent is excluded by a single marker.

Except for German Red cattle, the total exclusion probability of both parents P3 if the genotype of both parents and offspring is known exceeded 0.999. Jamieson *et al.* (1997) gave maximal theoretical probabilities of parentage exclusion at loci with different numbers of alleles, naturally, the practical probabilities are lower.

In conclusion, the high degree of diversity in Czech Pied cattle, and the possible risk of random drift and inbreeding in small populations was stated. The suitability of microsatellites for population studies and parentage testing was confirmed.

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Comparison of several growth models for cattle

Srovnání několika růstových modelů pro skot

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ABSTRACT: Five growth models (logistic, Gompertz, Richards, Morgan-Mercer-Flodin and Weibull) and their suitability for modelling of body growth in cattle are studied in this paper. As these models are nonlinear regression models and their properties can be studied only in the combination model/data, growth data on breeding bulls of Czech Pied cattle were used for the purposes of the present analysis. Residual variance was used as a criterion of the quality of fit (Table 1); this criterion was supplemented by statistical properties of the model parameter estimators (Tables 2 and 3), degree of nonlinearity of individual models for the considered data (Tables 4 and 5) and visual inspection of the growth curve (Figures 1–5). Considering these criterions, the better of the three-parameter models was Gompertz model in parametrization 2, among the four-parameter models Richards model proved to be unsuitable for the data due to high values of residual variance and high degree of nonlinearity. The remaining two four-parameter models (Morgan-Mercer-Flodin and Weibull) had low residual variances but the curves lacked elasticity and both models overestimated the position of the asymptote which is important for breeders (final weight of an animal).

Keywords: growth models; nonlinear regression; cattle growth; breeding bulls

ABSTRAKT: V článku je studována vhodnost pěti růstových modelů (logistický, Gompertzův, Richardsův, Morgan-Mercer-Flodinův a Weibullův) pro modelování růstu skotu. Protože se jedná o nelineární regresní modely a vlastnosti takových modelů je možné studovat pouze v kombinaci model/data, jsou pro účely této analýzy použita data o růstu plemenných býků českého strakatého skotu. Základním kritériem pro posouzení vhodnosti modelu je reziduální rozptyl (Tab. 1), toto kritérium je doplněno sledováním statistických vlastností odhadů parametrů jednotlivých modelů (Tab. 2 a 3), stupně nelinearity modelů pro uvažovaná data (Tab. 4 a 5) a vizuálního posouzení průběhu růstové křivky (Obr. 1–5). Na základě těchto kritérií se jako lepší z tříparametrických modelů jevil Gompertzův model v parametrizaci 2, ze čtyřparametrických modelů se jako nevhodný ukázal Richardsův model kvůli vysokému reziduálnímu rozptylu a vysokému stupni nelinearity modelu. Další dva čtyřparametrické modely (Morgan-Mercer-Flodinův a Weibullův) mají podstatně menší reziduální rozptyly, ale jsou málo pružné a výrazně nadhodnocují polohu asymptoty modelu, která je z chovatelského hlediska důležitá (hmotnost zvířete v dospělosti).

Klíčová slova: růstové modely; nelineární regrese; růst skotu, plemenní býci

INTRODUCTION

Growth modelling has been permanently in the focus of attention of animal breeders, biologists and geneticists. In cattle, Gompertz and Richards models are among the most frequently used models for modelling body growth. These two growth models were supplemented by three others and their statistical properties were studied in more detail. Due to the fact that growth models are nonlinear regression models, their properties are determined only in combination with a particular data set. For the purposes of this study, data on individual growth of breeding bulls of the Czech Pied cattle were used.

For modelling the body growth of cattle usually nonlinear regression models with three or four parameters are considered. Among the three-parameter models, Gompertz model is frequently used. It is a model with a fixed inflexion point (weight at the inflexion point is approximately one third of the final, asymptotic, weight). The inflexion point of the model corresponds to the age with maximum growth rate. Thus the maximum growth rate is determined by the animal's final weight in Gompertz model. The logistic model in its classical form is used rarely because the weight at the inflexion point is one half of the asymptotic weight while the growth rate in cattle has its maximum earlier (Kníže, 1987). The growth models with four parameters are more flexible in general because the position of the inflexion point is not fixed like in the case of three-parameter models. Among the models with four parameters, Richards model is frequently used (Fitzhugh, 1976; Beltran *et al.*, 1992; Pulkrábek *et al.*, 1980; Nešetřilová and Pulkrábek, 1995). Nevertheless, the estimation of its parameters might cause difficulties as the least squares estimates are often unstable and the number of necessary iterations is usually high or sometimes the convergence of the estimates is not reached at all.

METHODS

Properties of five growth models (1) to (5) for modelling the individual growth curves of live weight in breeding bulls of the Czech Pied cattle were studied:

logistic model

$$y = \frac{\alpha}{1 + \exp(\beta - \gamma t)} \qquad (1)$$

Gompertz model

$$y = \alpha \exp(-\exp(\beta - \gamma t)) \qquad (2)$$

Richards model

$$y = \alpha(1 + \exp(\beta - \gamma t))^{-1/\delta} \qquad (3)$$

Morgan- Mercer-Flodin model

$$y = \frac{\beta\gamma + \alpha t^{\delta}}{\gamma + t^{\delta}} \qquad (4)$$

Weibull type model

$$y = \alpha - \beta \exp(-\gamma t^{\delta}) \qquad (5)$$

In these models y is live weight of an animal at age t. Symbols α, β, γ and δ denote parameters of the models. Parameter α represents the asymptote in all models, parameter β relates to the intercept of growth curve and y-axis, parameter γ relates to growth rate and parameter δ in four-parameter models influences the position of inflexion point. More details about presented models can be found in Kníže (1987), Fitzhugh (1976), Ratkowsky (1983).

Models (1) to (5) are nonlinear regression models. Their properties can be studied only in combination with the data for which the model is to be used. The properties of the models are documented in this paper on individual data sets of 8 bulls selected from a group of 101 breeding bulls of the Czech Pied cattle (the animals with the maximum number of weighings in the whole group were selected, the live weight in the selected group was recorded approx. till the age of 4 years).

The least squares estimates of the model parameters were computed by SPSS 8.01 using modified Levenberg-Marquardt iteration method. The estimation of vector $\boldsymbol{\vartheta}$ of the model parameters

where: $\boldsymbol{\vartheta} = (\alpha, \beta, \gamma)$ or
$\boldsymbol{\vartheta} = (\alpha, \beta, \gamma, \delta)$

is based on finding the minimum of the function:

$$S(\boldsymbol{\vartheta}) = \sum_i (y_i - f(t_i, \boldsymbol{\vartheta}))^2$$

The residual sum of squares $S = S(\hat{\boldsymbol{\vartheta}})$ for the estimate $\hat{\boldsymbol{\vartheta}}$ is often used as a criterion of the goodness of fit of a model to the empirical data. Nevertheless, due to an unequal number of observations for individual animals and also partly due to the unequal number of parameters of models (1) to (5) the residual variance s^2 was preferred as a criterion of the goodness of fit,

$$s^2 = \frac{S}{n - p}$$

where: n = denotes the number of observations
p = number of model parameters

Another important aspect of a nonlinear regression model is the degree of its nonlinearity which determines the statistical properties of its parameter estimators obtained by the least squares method. Nonlinear regression models differ in their properties from linear regression models: when the assumption of independent and identically distributed normal random errors is made, least squares estimators of linear model parameters are generally unbiased, normally distributed and have minimum variance while in the case of nonlinear models the least-squares estimators have these properties only asymptotically when the size of a sample becomes very large (Ratkowsky, 1983). Thus, the estimators are generally biased and their properties are not known (in the case of a finite sample). The extent to which a nonlinear model differs from a linear one (bias, degree of nonnormality, increase of estimators' variability) can vary greatly for different nonlinear models; thus it is not possible to give a general recommendation as to how large the sample size must be so that the properties of a model are close to its asymptotic behaviour. Moreover, it is always necessary to analyse properties of such a model together with particular data because even the properties of the same model can differ for different data sets (due to the different curvature of the solution locus at different points). Simulation studies have proved that the magnitude of an estimator bias and increase of the estimator variability are related to the degree of "nonlinearity" of its distribution. Thus it is generally recommended to prefer close-to-linear nonlinear models (Ratkowsky, 1983). Besides the above-mentioned advantages (close-to-asymptotic behaviour), predicted response values y will have only a small bias and also computational complexity and problem of the initial estimates of the parameter vector will decrease.

The degree of "nonlinearity" for a particular model/data combination can be measured theoretically. One such measure, based on the geometric notion of surface curvature, was proposed by Bates and Watts (1980). These authors showed that the nonlinearity of a model/data combination could be split into two parts. The first part is so called intrinsic nonlinearity, the second one is called parameter-effects nonlinearity (for a mathematically precise definition of these notions see Zvára, 1989 or Ratkowsky, 1983). Intrinsic nonlinearity has an impact on the extent of bias of y predictions while high parameter-effects nonlinearity may negatively influence the convergence to the least-square estimates of model parameters. Parameter-effects nonlinearity may sometimes be decreased by suitable reparametrization of a model while intrinsic nonlinearity does not depend on parametrization (Zvára, 1989). It is generally recommended to use models with low both intrinsic and parameter-effects nonlinearity. For models with acceptably low intrinsic nonlinearity Ratkowsky recommends to seek models with low parameter-effect nonlinearity using reparametrization of a model.

RESULTS AND DISCUSSION

The main criterion that was used for comparing the fit of the considered growth models to the data on Czech Pied bulls was residual variance. The values of residual

Table 1. Residual sum of squares (S), residual variance (s^2) and number of observations (n) for individual growth curves in models (1) to (5)

Model		Logistic		Gompertz		Richards		Morgan-Mercer-Flodin		Weibull	
Bull	n	S	s^2	S	s^2	S	s^2	S	s^2	S	s^2
1	28	50 669	2 026.8	26 432	1 057.3	26 433	1 101.4	10 800	450.0	11 739	489.1
2	26	44 087	1 916.8	22 108	961.2	22 109	1 005.0	8 338	379.0	8 489	385.9
3	32	35 748	1 232.7	19 945	687.8	19 946	712.4	11 126	397.4	11 533	411.9
4	32	36 357	1 253.7	15 241	525.6	15 242	544.4	6 934	247.7	6 711	239.7
5	32	38 262	1 319.4	17 806	614.0	17 807	636.0	5 495	196.2	5 829	208.2
6	26	34 249	1 489.1	18 340	797.4	18 341	833.7	7 206	327.5	7 236	328.9
7	25	46 800	2 127.3	31 268	1 421.3	31 268	1 489.0	8 067	384.1	8 098	385.6
8	27	47 389	1 974.5	25 648	1 068.7	25 649	1 115.2	12 484	542.8	14 689	638.7

Table 2. Parameter estimates and their asymptotic standard errors of three parameters growth models

	Parameter α		Parameter β		Parameter γ	
Bull	estimate	ASE*	estimate	ASE*	estimate	ASE*
Logistic model						
1	867.78	29.57	1.956	0.115	0.0042	0.0004
2	972.28	27.29	1.953	0.105	0.0043	0.0003
3	864.71	25.42	1.925	0.087	0.0042	0.0003
4	1059.88	23.39	1.999	0.072	0.0041	0.0002
5	935.76	22.83	1.888	0.082	0.0041	0.0003
6	958.55	27.90	1.916	0.091	0.0037	0.0003
7	903.73	50.57	1.614	0.098	0.0032	0.0004
8	866.11	23.76	1.895	0.127	0.0047	0.0004
Gompertz model						
1	923.98	30.67	0.926	0.047	0.0027	0.0002
2	1023.31	27.11	0.931	0.043	0.0028	0.0002
3	956.73	28.34	0.913	0.040	0.0027	0.0002
4	1142.03	23.35	0.937	0.026	0.0025	0.0001
5	999.00	23.06	0.889	0.032	0.0026	0.0001
6	1016.96	29.81	0.910	0.039	0.0024	0.0002
7	966.45	59.90	0.736	0.045	0.0021	0.0003
8	902.26	22.94	0.927	0.056	0.0031	0.0002

*asymptotic standard error

Table 3. Parameter estimates and their asymptotic standard errors of four parameters growth models

	Parameter α		Parameter β		Parameter γ		Parameter δ	
Bull	estimate	ASE*	estimate	ASE*	estimate	ASE*	estimate	ASE*
Richards model								
1	923.97	40.27	–8.58	1 742.03	0.0027	0.00041	0.00007	0.12953
2	1 023.30	27.72	–8.62	949.82	0.0027	0.00023	0.00007	0.06773
3	926.72	30.73	–8.54	1 155.10	0.0027	0.00022	0.00008	0.09032
4	1 142.02	37.66	–8.58	735.20	0.0025	0.00020	0.00007	0.05433
5	999.00	24.13	–8.69	849.29	0.0026	0.00018	0.00007	0.05848
6	1 016.95	35.75	–8.70	963.17	0.0024	0.00022	0.00007	0.06442
7	966.45	61.33	–9.37	1 394.42	0.0021	0.00027	0.00004	0.05693
8	902.26	24.00	–8.65	1 222.31	0.0031	0.00026	0.00007	0.08444
M-M-F model								
1	1 551.00	261.69	19.76	22.49	2 835.89	2 002.42	1.14	0.14
2	1 688.60	262.00	1.21	30.47	2 052.92	1 498.93	1.11	0.15
3	1 495.30	230.32	29.83	22.49	3 875.67	2 819.48	1.20	0.14
4	1 782.19	155.36	49.32	13.85	6 088.98	2 679.78	1.263	0.09
5	1 672.59	172.62	26.41	16.37	2 582.20	1 146.00	1.13	0.09
6	1 855.20	383.93	–8.40	36.36	1 760.58	1 351.72	1.04	0.16
7	438.10^7	449.10^{13}	–40.79	42.06	340.10^6	348.10^{12}	0.59	0.12
8	1 184.65	97.11	22.88	25.83	5 587.51	4 649.17	1.35	0.15
Weibull model								
1	1 236.49	194.20	1 231.51	212.39	0.0010	0.00058	0.99	0.12
2	1 287.62	145.50	1 301.62	169.81	0.0012	0.00070	0.98	0.11
3	1 138.45	135.47	1 118.39	152.40	0.0007	0.00039	1.08	0.11
4	1 330.45	75.56	1 287.70	84.59	0.0004	0.00013	1.16	0.06
5	1 267.18	101.21	1 252.36	113.78	0.0010	0.00035	1.01	0.07
6	1 358.14	205.09	1 378.93	233.97	0.0013	0.00078	0.95	0.12
7	5 2995.34	2 440 088.00	53 034.78	2 440 158.00	0.0002	0.01082	0.60	0.24
8	1 005.94	66.70	1 004.07	88.23	0.0008	0.00056	1.09	0.11

*asymptotic standard error

variance s^2 for growth models (1) to (5) are for the sample data presented in Table 1. When evaluating these results the precision of the model parameter estimates (characterised by asymptotic standard error) was also taken into account, namely estimates of parameter α, which is usually interpreted as the final weight of an animal. Parameter estimates and their asymptotic standard errors are presented in Table 2 (models with three parameters) and Table 3 (models with four parameters). Visual inspection of the model growth curve also provided additional information (Figures 1–5).

As argued above, the measures of nonlinearity of a model/data combination represent useful information which could complement the residual variance criterion for selecting the best nonlinear model. These measures for the considered growth data are presented in Tables 4 and 5. Nonlinearity is usually classified as high if the maximum of the corresponding measure exceeds

$$(F_{0.95}\,(p,\, n-p))^{-1/2}$$

where: n = the size of data sample

p = the number of model parameters (Zvára, 1989, p. 230).

Several parametrizations of models (1) to (5) were considered, the results for models (1), (3) to (5) and two different parametrizations of Gompertz model (2) are presented.

Parametrization 1 $\quad y = \alpha \exp(-\exp(\beta - \gamma t))$

Parametrization 2 $\quad y = \alpha \exp(-\beta \exp(-\gamma t))$

As expected, the comparison of three-parameter models (1) and (2) leads to a conclusion that Gompertz model is more suitable for the considered data than the logistic one. Residual variances of Gompertz model are approx. one half in most cases when compared to those of the logistic model.

Both models overestimate the live weight of an animal in the first period of life and underestimate it slightly in

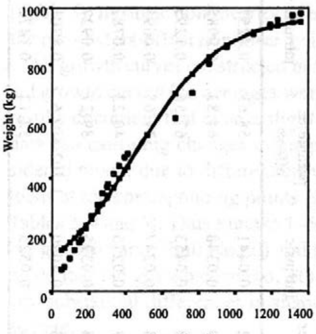

Figure 1. Logistic model

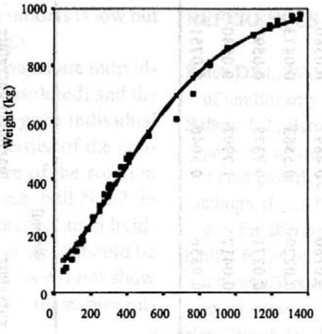

Figure 2. Gompertz model

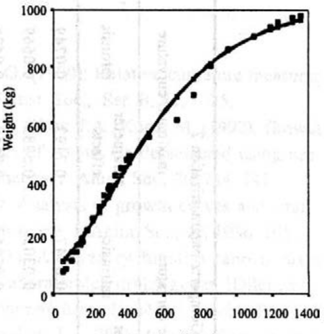

Figure 3. Richards model

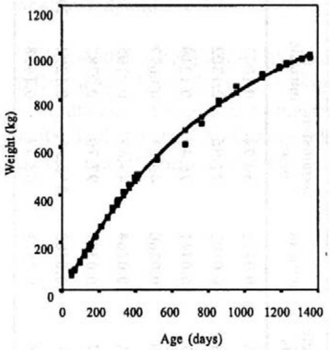

Figure 4. Morgan-Mercer-Flodin model

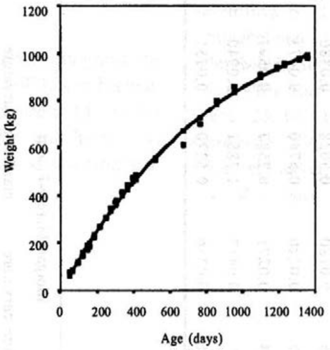

Figure 5. Weibull type model

the last period, the differences are more distinct for the logistic model. The intrinsic nonlinearity (Table 4) is low for both models, this implies good estimates of live weight and residual sum of squares (low bias of estimates). Parameter-effects nonlinearity for both models is on the edge of acceptability, parametrization 2 of Gompertz model is slightly better than parametrization 1.

Analysis of the four-parameter models shows that Richards model performs very poorly for the bull data, its residual variances are comparable with those of Gompertz model in spite of the fact that the model has more parameters. Richards model has highly correlated ($r > 0.98$) estimates of parameters β and δ (as reported also by others, see e.g. Fitzhugh, 1976). Very high are also asymptotic standard errors of these parameter estimates, which indicate a high degree of nonlinearity in these parameters (Table 3). This is confirmed by high values of nonlinearity characteristics (Table 5). As the intrinsic nonlinearity of the model is very high, it has no sense to seek better parametrization of the model.

The other two four-parameter models (Morgan-Mercer-Flodin and Weibull models, see (4) and (5)) have substantially lower residual variances than Richards model and both three-parameter models and are mutually comparable in this respect. Nevertheless, both models substantially overestimate the value of parameter α, which represents the final weight (asymptote of the model) and which is very important for breeders. Moreover, both growth curves seem to lack elasticity (e.g. they do not model well the first convex part of growth, Figures 4 and 5). The low elasticity might be caused by high correlations between parameters ($r > 0.9$) in both cases.[1] High asymptotic variability of estimates of some model parameters indicates high nonlinearity in those parameters (β and γ in Morgan-Mercer-Flodin model and γ in Weibull model). This is confirmed by nonlinearity characteristics

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[1] It is often assumed that the nonlinear behaviour of a model is related to (or is manifested by) high correlations between model parameters. This assumption is wrong. High values of correlation coefficients in asymptotic correlation matrix do not imply a high degree of nonlinearity of the model. Neither is the reverse statement true: correlations between parameters in close-to-linear models are not necessarily low (Ratkowsky, 1983).

Table 4. Degree of nonlinearity of three parameters growth models

Model		Logistic				Gompertz-parametrization 1				Gompertz-parametrization 2			
		average curvature		maximum curvature		average curvature		mamaximum curvature		average curvature		maximum curvature	
Bull	$(F_{0.95}\ (p.n-p))^{-1/2}$	parameter effects	intrinsic	parameter effects	intrinsic	parameter effects	intrinsic	parameter effects	intrinsic	parameter effects	intrinsic	parameter effects	intrinsic
1	0.5783	0.2913	0.0604	0.6408	0.1334	0.3346	0.0347	0.7408	0.0749	0.3284	0.0347	0.7192	0.0749
2	0.5751	0.2579	0.0543	0.5682	0.1207	0.2802	0.0305	0.6207	0.0669	0.2761	0.0305	0.5983	0.0669
3	0.4892	0.2685	0.0364	0.5922	0.0785	0.3488	0.0234	0.7747	0.0495	0.3388	0.0234	0.7559	0.0495
4	0.4892	0.2022	0.0342	0.4457	0.0744	0.2265	0.0180	0.5028	0.0380	0.2210	0.0180	0.4897	0.0380
5	0.4892	0.2278	0.0393	0.5020	0.0861	0.2587	0.0220	0.5740	0.0473	0.2523	0.0220	0.5583	0.0473
6	0.5751	0.2834	0.0447	0.6267	0.0987	0.3414	0.0271	0.7587	0.0597	0.3332	0.0271	0.7375	0.0597
7	0.5390	0.5977	0.0604	1.3283	0.1351	0.8015	0.0417	1.7851	0.0940	0.7917	0.0417	1.7792	0.0940
8	0.5764	0.2267	0.0589	0.4951	0.1296	0.2441	0.0356	0.5370	0.0751	0.2458	0.0356	0.5046	0.0751

Table 5. Degree of nonlinearity of four parameters growth models

Model		Richards				Morgan-Mercer-Flodin				Weibull			
		average curvature		maximum curvature		average curvature		maximum curvature		average curvature		maximum curvature	
Bull	$(F_{0.95}\ (p.n-p))^{-1/2}$	parameter effects	intrinsic	parameter effects	intrinsic	parameter effects	intrinsic	parameter effects	intrinsic	parameter effects	intrinsic	parameter effects	intrinsic
1	0.5783	1.2×10^9	9 941	3.4×10^9	28 119	20.95	0.0411	59.94	0.1067	21.02	0.0441	58.63	0.1257
2	0.5751	1.0×10^9	6 708	2.9×10^9	18 194	27.34	0.0501	77.96	0.1302	23.09	0.0348	64.83	0.0985
3	0.4892	1.5×10^9	10 253	4.2×10^9	29 000	26.77	0.0441	76.43	0.1219	24.10	0.0453	67.56	0.1295
4	0.4892	0.9×10^9	6 566	2.5×10^9	18 573	14.10	0.0260	40.23	0.0692	11.28	0.0247	31.65	0.0695
5	0.4892	0.9×10^9	6 673	2.7×10^9	18 876	15.34	0.0264	43.87	0.0705	14.37	0.0275	40.23	0.0782
6	0.5751	1.3×10^9	8 517	3.7×10^9	24 090	34.17	0.0307	97.70	0.0783	31.84	0.0325	89.27	0.0927
7	0.5390	4.1×10^9	17 881	1.2×10^9	50 575	124.23	0.0500	358.95	0.1294	389 932.93	0.3090	1 102 892.6	0.8701
8	0.5764	0.9×10^9	7 174	2.6×10^9	20 293	24.15	0.0521	68.47	0.1428	18.50	0.0525	52.12	0.1472

(Table 5). Intrinsic nonlinearity of both models is low but the parameters-effect nonlinearity is high.

All growth curves constructed in this paper are individual growth curves (no averages were considered) and the results document that even a slight change in individual data can cause big changes in the properties of the considered model due to different curvature of the solution locus at the corresponding points (see e.g. bull No. 7. in Tables 3, 4 and 5). Thus Figures 1–5 represent an individual growth curve (bull No. 2.) and therefore it should be noted that the growth curves of other bulls did not show any substantial differences in shape from those presented here.

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Effect of enzyme lysis on the digestibility of brewer's yeasts included in the diets of chicken broilers

Vliv enzymatické lyze na stravitelnost pivovarských kvasnic zařazených do krmných dávek pro kuřecí brojlery

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ABSTRACT: Digestibilities both *in vitro* and *in vivo* of intact and enzyme treated brewercs yeasts were studied. The enzyme disruption of yeast cell walls was carried out with lytic enzyme complex produced by *Streptomyces roseolus* 274. It was established that with 20U enzyme complex/g yeasts at pH 5.0, 45°C for 3 h increase digestibility *in vitro to* 87.7%, which was 1.8–2.0 time higher than that of whole yeast cells. Investigations of digestibility *in vivo* were carried out with three groups of chicken broilers: the first group, chickens fed with basic diet; the second group were chickens fed with a diet in which 20% of soybean protein in basic diet was replaced with 5% intact yeasts; the third group in which 20% of soybean protein was replaced with 5% enzyme treated yeasts. The highest weight gain and best feed consumption were recorded with the third group of chickens, confirming that 5% enzyme treated brewercs yeasts successfully replaced 20% of soybean protein in the basic diet.

Keywords: enzyme lysis; brewer's yeasts; chicken broilers; digestibility *in vitro* and in *vivo*; performance

ABSTRAKT: V pokusech *in vitro* a *in vivo* jsme sledovali stravitelnost pivovarských kvasnic v původním stavu a upravených pomocí enzymatické lyze. K enzymatickému štěpení buněčných stěn kvasnic jsme použili lytický enzymatický komplex, který produkuje bakterie *Streptomyces roseolus* 274. Zjistili jsme, že s použitím 20 U enzymatického komplexu na 1 g kvasnic při pH 5,0 a teplotě 45 °C po dobu tří hodin bylo dosaženo 87,7% stravitelnosti *in vitro*, což bylo o 1,8 až 2,0 % více než při použití kvasnic v původním stavu. Šetření stravitelnosti *in vivo* jsme prováděli ve třech skupinách kuřecích brojlerů: první skupina kuřat dostávala základní směs; druhá skupina kuřat dostávala směs, kde jsme 20 % sójových bílkovin ze základní směsi nahradili 5 % kvasnic v původním stavu; ve třetí skupině bylo 20 % sójových bílkovin nahrazeno 5 % enzymaticky štěpených bílkovin. Nejvyšší hmotnostní přírůstek a nejnižší spotřeba krmiv byla dosažena ve třetí skupině kuřat, kde 5% obsah enzymaticky štěpených pivovarských kvasnic úspěšně nahradil 20 % sójových bílkovin v základní směsi.

Klíčová slova: enzymatická lyze; pivovarské kvasnice; kuřecí brojleři; stravitelnost *in vitro* a *in vivo*; užitkovost

INTRODUCTION

Yeasts are known to have a relatively high nutritive value and can be used as a source of protein in animal fodder. However, the effective utilisation of the yeast components and particularly the protein has not been possible because of the rigid yeast cell walls, which make the yeasts to be relatively indigestible and nonassimilable. The specific ability of yeast lytic enzymes to break down the bands in the structural components of the wall resulting in increased permeability and improved access for digestive enzymes towards intracellular content. Kobayashi *et al.* (1982) reported that preliminary destruction of yeast cell walls increases their digestibility.

The high protein content of yeasts allows a part of plant protein to be replaced with microbial protein in the diets of different animals. The supplementation of diets with yeasts could be in amounts 1.5–10% without negative effect on feed conversion, weight gain and health status. Consequently, investigations have been carried out with fodder yeasts grown on different substrates-plant hydrolysates, molasses, sulfite liquor, cheese whey (Savage and Mirosh, 1990a, b; Surdjiiska *et al.*, 1983).

The incorporation of yeasts mannan-oligosaccharides (Bio-Mos) into the diets of chicken broilers (Hutton and Mehrle, 1993; Kumprecht and Zobač, 1997, 1999), turkeys (Cragoe, 1994; Olsen, 1996; Savage *et al*., 1996) layer breeders (Savage and Mirosh, 1990a, b) leads to improvements in performance such as the average daily gain, feed consumption and immune status (Spring, 1996).

We did not find any information about application of brewercs yeast as a supplement into the animal diets. The present article reports the results of the effect of lytic enzyme system from *Streptomyces roseolus* 274 on the digestibility *in vitro* of waste brewer's yeasts. Data about the biological effect of their inclusion into diets of chicken broilers are also reported.

MATERIAL AND METHODS

Yeast biomass

Waste brewer's yeasts were taken from the plant "Kamenitza" – Plovdiv, Bulgaria.

Enzyme

Yeast lytic enzyme complex was produced by *Streptomyces roseolus* 274 (Pavlova, 1980). The procedure for its production and preparation was described previously (Pavlova *et al*., 1994). The activity of the crude enzyme complex was expressed as β-1,3 glucanase activity using laminarin as substrate. The reducing sugars were determined according to the method of Somogyi (1952). One unit (U) of the β-1,3 glucanase activity was defined as the amount of the enzyme able to liberate 1 μmol of reducing sugars (calculated on the basis of glucose) of this substrate at 45°C for 30 min in 0.2M acetate buffer pH 5.0. An enzyme concentration 75 U/cm was used in the experiments.

Cell lysis

Yeast biomass was washed twice in water, suspended in water (5 g/100 ml) and then was treated with various lytic enzyme concentrations (10.0–30.0 U/g yeast) at pH values 5.0 and 7.0, temperature 45°C for 1, 3 and 6 h.

Table 1. Chemical composition of yeasts

Index (%)	Intact brewer's yeasts	Enzyme treated brewer's yeasts
Moisture	4.94	5.30
Protein	44.35	44.98
Lipids	1.31	2.30
Fiber	1.54	–
Non-nitrogen substances	46.35	42.43
Ash	6.45	10.29

In vitro estimation

Determination of the digestibility on enzyme treated yeasts was performed according to the procedure described by Hotyanovich *et al.* (1972).

In vivo experiments

Brewer's yeasts were suspended in water (5 g/ 100 ml), treated with lytic enzyme system (20 U/g yeast), at pH 5.0 incubated at 45°C for 3 h and then were dried. After determination of the chemical composition of intact and enzyme treated yeasts (Table 1), they were included into the basic diet of chicken broilers (Table 2). Experiments were carried out with 90 chickens divided into three groups: group I – chickens were fed with the basic diet; group II – chickens were fed with feed in which 20% of soybean protein was replaced with 5% intact brewer's yeast; group

Table 2. Composition of the basic diet

Component	%
Yellow corn	59.332
Soybean meal	36.400
Calcium phosphate	2.000
Calcium carbonate	1.00
Salts	0.340
Methionine	0.060
Vitamin premix*	0.600
Microelement mixture**	0.200
One kilogram of the diets contained:	
Metabolizable energy (MJ/kg)	13.0
Crude protein (g)	211.2
Crude filaments (g)	36.7
Lysin (g)	12.1
Methionine + Cystine (g)	7.9
Calcium (g)	9.5
Phosphorus (g)	7.5

*One kilogram of the vitamin premix contained: vitamin A 600 000 I.U., vitamin D_3 120 00 I.U., vitamin E 26 I.U., vitamin B1 100 mg, vitamin B2 600 mg, vitamin B6 200 mg, panthotenic acid 900 mg, nicotinic acid 1 400 mg, choline chloride 300 000 mg, folic acid 10 mg

**One kilogram of the microelement mixture contained: manganese 12 000 mg, iron 20 000 mg, copper 200 mg, iodine 100 mg, cobalt 10 mg and Zinc 8 000 mg

Note: The content of crude protein in the combined diets for the three groups is group I – 20.59%, group II – 20.60%, group III – 20.55%

III – chickens were fed with a diet in which 20% of soybean protein was replaced with 5% enzyme treated brewer's yeasts.

During investigations, the live weight and feed consumption in kg per 1 kg weight gain on 14th and 28th day were determined.

Protein balance was determined by calculation of protein retention as follows:

$$\text{protein retention (\%)} = \left(1 - \frac{\text{protein in diet} - \text{retained protein}}{\text{protein in diet}}\right).100$$

The chemical composition of chicken carcass was determined using conventional methods.

RESULTS AND DISCUSSION

The digestibility *in vitro* on both intact yeasts and yeasts treated with increased concentrations of lytic enzyme complex at pH 5.0 (optimum for β-glucanase activity) under different lysis duration is presented in Table 3. During the first hour of enzyme treatment with 20 U/g yeasts the cell walls began to disrupt which resulted in higher digestibility *in vitro* by 32.2% in comparison with that of the intact yeasts. The digestibility of yeasts was increased by 38.9% after 3 h enzyme treatment while lysis duration to 6 h had only marginal effect.

The protease included in the lytic enzyme complex also took part in the degradation of yeast cell walls. The enzyme treatment of the yeast cells was carried out at optimal pH 7.0 (Table 3). By this treatment at pH 7.0 the digestibility *in vitro* on yeast cells was increased by 29.0%, but it was lower in comparison with that of the cells treated at pH 5.0. Therefore, pH 5.0 was more suitable for the enzyme treatment of the waste brewer's yeasts. At optimal pH 5.0 value for β-glucanase, a higher degree of disruption of the structural glucan was achieved, improving access of pepsin to cellular proteins. This result confirmed the opinion of other authors for the first – rate role of β-1,3-glucanase in disruption of yeast cell walls (Andrews and Asenjo, 1987; Bielecki and Calas, 1991a, b). The destruction of brewer's yeasts with lytic enzyme system from *Streptomycen roseolus* 274 was a suitable biochemical method for the increasing of the digestibility *in vitro*.

The positive results for digestibility *in vitro* of the enzyme treated brewer's yeasts were a precondition to be carried out investigations *in vivo* with chicken broilers. Both intact and enzyme treated yeast cells were added into the basic corn-soybean diet in conformity with the protein content. The biological effect depending on the

Table 3. Digestibility *in vitro* (%) of brewer's yeasts after enzyme lysis at pH 5.0 and 7.0

Concentration of enzyme complex (U/g) yeasts	pH 5.0			pH 7.0		
	duration of enzyme lysis (h)					
	1	3	6	1	3	6
Control	47.80	47.80	48.90	52.20	53.10	53.10
10.0	70.30	73.00	77.10	59.40	66.60	68.20
15.0	73.10	84.20	87.20	73.60	79.60	80.30
20.0	80.00	86.70	87.70	74.90	81.20	82.60
25.0	79.50	81.20	83.30	73.40	81.00	80.60
30.0	79.30	78.40	81.00	72.10	80.40	79.30

Table 4. Changes in the live weight of the chickens

Group	Number	Live weight of the chickens			
		on 14th day		on 28th day	
		g	%	g	%
I	30	239	100.00^{a}	616	100.00^{a}
II	30	219	91.63^{b}	570	92.53^{b}
III	30	240	100.42^{c}	619	100.49^{c}

a, b, c = $P < 0.05$

Legend: group I – control, group II – intact yeasts, group III – enzyme treated yeasts kind of the protein source, included in the diets, was expressed by live weight gain of the chickens and feed consumption for definite period of time. The changes in the live weights of the chickens fed with the basic diet and the diets in which a part of the plant protein was replaced with protein of intact or enzyme treated yeast are shown in Table 4. There were no remarkable differences in the changes in the live weights of the chickens fed with the basic diet (group I) and those received enzyme treated yeasts (group III) on 14th and 28th day. Chickens, fed with diet containing intact yeast cells had about 8.5% and 7.6% lower live weight in comparison with the control group and the third group on 14th and 28th day respectively. The differences were statistically significant at $P < 0.05$.

The established increased digestibility *in vitro* of enzyme treated yeasts was confirmed by the tests *in vivo*. The higher live weights of the chickens was in conjunction with more complete utilisation of the nutritive components of enzyme treated yeasts. The difference in live weight between the third group and the control group on 14th day was established at $P < 0.05$. This tendency was maintained on 28th day. The chickens receiving intact yeasts (group II) consumed more feed per kg live weight than those fed with enzyme treated yeast cells (Table 5). The reason for lower weight gain was incomplete utilisation of the nutritive components of the whole yeast cells.

The chemical composition of chicken carcass (average of six carcases) showed that there were no differences in mean of the indexes between the control and tested groups, which indicated that the biotransformation of the feed diets was similar (Table 6).

The data for protein balance (Table 7) had the same tendency as the changes in live weight. The chicken fed with the diet of intact yeast cells had lower protein retention in comparison with the control group and chicken fed with the diet in which enzyme treated yeasts were added. In both cases, the existence of differences was established at $P < 0.05$.

In conclusion the lytic enzyme system from *Streptomyces roseolus* 274 had positive effect on the digestibility *in vitro* of brewer's yeasts which was increased by 1.8–2.0

Table 5. Feed consumption in kg per kg of weight gain in relation to the protein source

Group	Number	Feed consumption in kg/kg weight gain			
		between 1st and 14th day		between 14th and 28th day	
		kg	%	kg	%
I	30	1.57	100.00	2.14	100.00
II	30	1.52	96.82	2.22	103.64
III	30	1.44	91.72	2.14	99.95

$P < 0.05$

The legend see Table 4

Table 6. Chemical composition of the carcass

Group	Moisture (%)	Protein (%)	Lipids (%)	Ash (%)
I	68.32 ± 0.84	18.50 ± 0.57	10.02 ± 1.13	3.21 ± 0.21
II	68.72 ± 1.88	18.41 ± 0.98	8.92 ± 1.82	2.95 ± 0.22
III	68.23 ± 0.81	18.63 ± 0.73	8.73 ± 0.86	2.99 ± 0.28

$P < 0.05$

The legend see Table 4

Table 7. Protein balance

Group	Absorbed protein (g)	Retained protein (g)	Protein retention (%)
I	254.63 ± 11.74	106.90 ± 3.28^a	41.98 ± 1.29
II	242.99 ± 17.23	97.97 ± 5.22^b	40.31 ± 2.15
III	255.10 ± 11.23	108.20 ± 4.22^c	42.43 ± 1.65

a, b, c = $P < 0.05$

The legend see Table 4

times in comparison with the digestibility *in vitro* of the intact cells. The investigations *in vivo* carried out with chicken broilers showed that 5% enzyme treated yeasts could successfully replace 20% of the soybean protein in the basic corn-soybean diet.

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