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IMPACT OF THE MACS ON ELIMINATION OF APOPTOTIC SPERMATOZOA FROM RABBIT EJACULATES

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ABSTRACT

The objective of this study was to assess the effectiveness of the MACS technique used for the elimination of apoptotic rabbit spermatozoa from heterospermic pool (Experiment 1) as well as from the ejaculates of individual bucks (Experiment 2). The semen samples from control (untreated) and magnetically separated spermatozoa (in both E1 and E2) were evaluated by fluorescence analysis using Annexin-V-FLUOS Staining Kit (Roche Slovakia, Slovak Republic). Superparamagnetic microbeads conjugated with annexin V eliminated spermatozoa with externalized phosphatidylserine via MACS. MACS separation of spermatozoa yields two fractions: the annexin V-negative (AnV⁻) and the annexin V-positive (AnV⁺). The number of AnV⁺ sperm was significantly lower (P<0.001) in the AnV⁻ fractions than in the AnV⁺ fractions (in both E1 and E2). Our observations indicate that MACS technique could be an adequate method for the elimination of apoptotic spermatozoa with externalized phosphatidylserine from the rabbit ejaculates. However, further experiments are required in order to prove this suggestion.

Key words: rabbit; spermatozoa; MACS; annexin V

INTRODUCTION

When artificial insemination (AI) is applied in a rabbitry, it is estimated that one single buck may affect the fertility and prolificacy of about one hundred does (Seleem, 2005). Thus, the bucks employed in AI must have good genetic characteristics and provide a good semen yield both in terms of quality and quantity (Panella and Castellini, 1990; Battaglini, 1992; Castellini and Dal Bosco, 1998). There are many factors influencing the quality and quantity of rabbit semen such as breed (Amin *et al*., 1987), male (Castellini, 1996), age (Gogol *et al*., 2002), season (Bodnar *et al*., 2000), photoperiod (Theau-Clement *et al*., 1995), nutrition (Fodor *et al*., 2003), collection rhythms (Nizza *et al*., 2003) and transgenesis (Chrenek *et al*., 2007).

Henkel *et al.* (2004) and Seli *et al.* (2004) observed that the presence of apoptotic spermatozoa during in vitro fertilization (IVF) can be one of the reasons for obtaining suboptimal fertility results. The phenotypic expression of apoptosis has been in relation to the presence of abnormal spermatozoa in semen. The failure to eliminate these abnormal spermatozoa during the spermatogenesis can lead to their presence in semen (Sakkas *et al*., 1999; Barroso *et al*., 2000; Sakkas *et al*., 2002). Therefore, selection and elimination of apoptotic spermatozoa is one of the necessary requirements for achieving optimal assisted reproduction outcomes. For this purpose the MACS (magnetic-activated cell sorting) technique is used in human medicine (Said *et al*., 2006a).

Principle of the magnetic separation is difference in membrane characteristics of separated cells (Said *et al*., 2006b). The translocation of phospholipid phosphatidylserine (PS) is one of the earliest detectable features of cells undergoing the initial steps of apoptosis. PS has a high and selective affinity for annexin V (Van Heerde *et al.*, 1995). Superparamagnetic microbeads

conjugated with annexin V eliminated spermatozoa with externalized phosphatidylserine via MACS (Meng *et al.*, 1996). MACS separation of spermatozoa yields two fractions: the annexin V-negative (intact membranes, non-apoptotic) and the annexin V-positive (externalized PS, apoptotic) (Glander *et al.*, 2002). The selection of non-apoptotic spermatozoa may improve sperm quality complementary to other separation techniques and assure optimal conception rates in human and animal assisted reproduction (Said *et al.*, 2005; Vasicek *et al.*, 2010, 2011a).

The objective of this study was to assess the effectiveness of the MACS technique used for the elimination of apoptotic rabbit spermatozoa from heterospermic pool as well as from the ejaculates of individual bucks.

MATERIAL AND METHODS

Animals

Sexually mature (2 - 3 years old) and clinically health rabbit bucks (n = 11) of broiler New Zealand White (NZW) line reared in a partially air-conditioned hall of a local rabbit farm at APRC Nitra (Animal Production Research Centre, Lužianky, Slovak Republic) were used in the experiments. The animals were housed in individual cages, under a constant photoperiod of 14 h of light day. Temperature and humidity in the building were recorded continuously by means of a thermograph positioned at the same level as the cages (average relative humidity and temperature during the year was maintained at 60 ± 5 % and 17 ± 3°C). The rabbits were fed *ad libitum* with a commercial diet (KV; TEKRO Nitra Ltd., Slovak Republic) and water was provided *ad libitum* with nipple drinkers.

The treatment of the animals was approved by the Ministry of Agriculture and Rural Development of the Slovak Republic, no. SK P 28004 and Ro 1488/06-221/3a.

In this study the control (untreated) and magnetically separated spermatozoa from heterospermic pool (Experiment 1) as well as from the ejaculates of individual bucks (Experiment 2) were used for fluorescence analysis.

Semen collection and handling

Semen samples from 25 NZW bucks were collected using an artificial vagina. Each sample of fresh ejaculate was evaluated for the concentration and motility using Sperm Vision™ (Minitübe, Tiefenbach, Germany), a computer assisted sperm motion analyser (CASA). For magnetic separation, the best 11 bucks (Experiment 1) or the best four bucks (Experiment 2) were chosen basing on motility parameters. Ejaculates

from chosen bucks were collected using an artificial vagina once a week during each experiment. In the Experiment 1 (E1) the ejaculates from 11 bucks were mixed to make heterospermic pool and routinely diluted in a commercial insemination diluent (MiniTüb) at the ratio of 1:6, whereas in the Experiment 2 (E2) the ejaculates from four bucks were handled separately and diluted at the same ratio.

Before magnetic sperm separation, the sperm cells were washed out of seminal plasma to facilitate better annexin V binding to PS. For this purpose the diluted semen was carefully filtered through a Sartorius filter (2 ml per filter) with a pore size of 1.2 μm, so that seminal plasma with a diluent passed through a membrane, which was then discarded. The rabbit spermatozoa retained by filter membrane were carefully flushed out from the filter to the collection tube with 2 ml of a binding buffer (Annexin V Microbead Kit, Miltenyi Biotec, Germany). The filtered spermatozoa were diluted in a binding buffer at the ratio of 1:3.66 (E1) or 1:8 (E2). Filtered and diluted rabbit semen was divided into the experimental group, intended for the magnetic separation, and the control group (untreated semen).

MACS separation of rabbit spermatozoa

In the Experiment 1, the filtered rabbit spermatozoa were incubated with 200 μl of annexin V-conjugated nanoparticles (Annexin V Microbead Kit, Germany) for 15 min at room temperature according to the original protocol (Miltenyi Biotec). The MidiMACS Magnetic Cell Sorting system (Miltenyi Biotec, Germany) was used for MACS assay of rabbit spermatozoa at room temperature. The MACS LD column was placed into the magnetic field of a MACS Separator and prepared by washing with 1 ml of a binding buffer. The filtered rabbit spermatozoa (7 ml for LD column) incubated with annexin V-conjugated nanoparticles were applied onto the column. The annexin V-negative (AnV⁻) spermatozoa passed through the column into the collection tube. Then the column was rinsed with 2 ml of a binding buffer, removed from the separator and placed onto a suitable collection tube. For the recovery of an annexin V-positive (AnV⁺) fraction 1 ml of a binding buffer was pipetted onto the column and firmly flushed out using the plunger supplied with the column.

The filtered rabbit spermatozoa in the Experiment 2 were processed as described previously by Vasicek *et al.* (2011b).

Apoptosis assay *in situ* (annexin V/PI/DAPI)

For annexin V analysis semen samples obtained from control group (untreated), negative (AnV⁻) and positive (AnV⁺) fractions were in both experiments (E1 and E2) processed using Annexin-V-FLUOS Staining Kit (Roche Slovakia, Slovak Republic). Each sample

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(10^6 cells) was centrifuged at 670 g for 8 min, resuspended in 500 µl of binding buffer (provided with the Kit) and centrifuged again as previously. Semen suspension (30 µl) was mixed with 50 µl of AnV/PI staining solution and incubated for 25-30 min at room temperature. Annexin V – FLUOS (4 µl), PI (4 µl) and buffer (192 µl; Annexin-V-Fluos staining kit) were mixed together in order to prepare 200 µl of the AnV/PI staining solution. After incubation samples were washed in 500 µl of binding buffer and centrifuged. Then aliquots of the semen suspension (4 µl) were placed between microslide and coverslip into 4µl of the Vectashield anti-fade medium containing DAPI fluorescent dye (Vector Laboratories, Burlingame, CA, USA). At least 200 spermatozoa were checked for staining and counted under a Leica fluorescent microscope (Leica Microsystem, Germany) at magnification 400x using 488 nm, 535 nm or 420 nm wave-length filters, respectively. The spermatozoa with the annexin V-positive membrane exhibited green fluorescence, dead spermatozoa exhibited red fluorescence, whilst total spermatozoa count was identified by blue signal due to DAPI staining (Fig. 1).
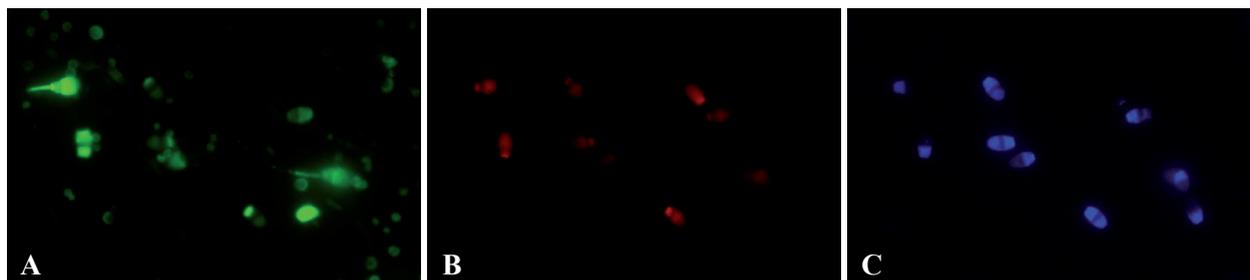
A – fluorescein - FITC (apoptotic spermatozoa), B – propidium iodide (dead spermatozoa), C – DAPI (total spermatozoa count)

Fig. 1: Fluorescent staining of the rabbit spermatozoa

Statistical analysis

Obtained results were evaluated statistically by one-way ANOVA (Holm-Sidak) using SigmaPlot software (Systat Software Inc., Germany) and expressed as the means ± SEM. P-values at $P<0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

In the Experiment 1, significantly higher ($P<0.001$) proportion of apoptotic spermatozoa was found in the AnV^+ fraction compared to the AnV^- fraction as well as to control samples. However, there were no significant differences in percentage of apoptotic cells between AnV^- spermatozoa and control samples as well as in proportion of dead cells among the all semen samples. Similarly, in the Experiment 2, we observed significantly higher ($P<0.001$) percentage of apoptotic as well as dead cells in AnV^+ fractions in comparison to AnV^- fractions and control samples, whereas there were no differences in percentage of apoptotic or dead cells between AnV^- spermatozoa and control samples (Table 1).

The use of the annexin V assay in livestock animals for the identification of different sperm subpopulations has already been documented (Chaveiro *et al.*, 2007; Peña *et al.*, 2003). Staining of cells with a combination of Annexin V and PI allows simultaneous distinguishing among live, apoptotic or necrotic sperm populations. This method has been used by two authors to investigate sperm apoptosis, but conflicting results have been obtained (Glander and Schaller, 1999; Oosterhuis *et al.*, 2000). In the first study the percentage of apoptotic sperm in the ejaculate positively correlated with motility, while in the second study a negative correlation was observed between apoptotic cells and sperm motility and concentration. This difference could be due to the different method used and/or to the different patient population studied, or more probably to the fact that in one study the analysis was carried out on a whole semen (Oosterhuis *et al.*, 2000) and in the other study it was carried out on sperm separated from seminal plasma by Percoll density gradient centrifugation (Glander and Schaller, 1999). In our experiments (E1 and E2) we noticed similar observation as in the second mentioned study (Glander and Schaller, 1999). Percentage of apoptotic sperm in AnV^+ fractions (Table 1) that were washed out from seminal plasma by filtration through Sartorius filter apparently negatively correlated with the total and progressive spermatozoa motility (data not published). Moreover, we compared PS externalization (annexin V assay) between the annexin V-negative and -positive fractions separated by MACS to assess the efficiency of MACS separation. The technique appears

Table 1: Proportion of apoptotic (AnV) or dead (PI) cells in MACS treated and control (untreated) rabbit spermatozoa

SEMEN SAMPLE		AnV/DAPI (%)	PI/DAPI (%)
Heterospermic pool	Control	4.69 ± 0.69a	2.74 ± 0.94
(Experiment 1)	AnV⁻	3.48 ± 0.78a	3.48 ± 1.00
	AnV⁺	16.83 ± 0.92b	3.00 ± 0.96
Individual buck	Control	5.97 ± 0.84a	7.12 ± 1.24a
(Experiment 2)	AnV⁻	6.60 ± 0.78a	5.22 ± 1.06a
	AnV⁺	59.53 ± 6.00b	34.95 ± 6.40b

Results are expressed as means ± SEM; a vs b were statistically significant at P<0.001

to be adequate because the number of PS-positive (annexin-positive) sperm was lower in the AnV⁻ fractions than in the AnV⁺ fractions (Table 1) similarly as reported by Said *et al.* (2006a) (3.4 ± 1.7 % vs. 54.9 ± 18.1 %, P<0.001), although there were no statistical differences in proportion of apoptotic and dead cells between AnV⁻ fractions and control samples. Minimal PS externalization was noted in the AnV⁻ fractions, whereas a considerable number of spermatozoa that stained negative for PS were found in the AnV⁺ fractions. The absence of PS externalization in some spermatozoa in the AnV⁺ fractions may be because beads have already blocked the PS binding sites. In addition, annexin V can also bind to other enzymes such as protein kinases, and phospholipids such as PE (phosphatidylethanolamine), despite high affinity for PS (Said *et al.*, 2006a).

We found some difference in the number of apoptotic spermatozoa within AnV⁺ fractions between Experiment 1 and Experiment 2 (Table 1). This could be due to several factors. Since experiments were carried out during different seasons, there may be a seasonal influence on male fertility parameters. Other factors may be different batch of the kit or the type of column used for magnetic separation in Experiment 1 and Experiment 2. The use of flow cytometry for the annexin V assay could be more objective. Moreover, the small size of microbeads, about 50 nm in diameter, is advantageous in flow cytometry because bound microbeads are unable to change the scatter properties of spermatozoa (Miltenyi *et al.*, 1990).

CONCLUSION

Our obtained results indicate that the MACS technique could be an adequate method for the elimination of apoptotic spermatozoa with externalized phosphatidylserine from the rabbit ejaculates. However,

because of some discrepancies further experiments are required in order to prove this suggestion.

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CHANGES IN MILK YIELD AND COMPOSITION AFTER LAMB WEANING AND START OF MACHINE MILKING IN DAIRY EWES

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ABSTRACT

Stress from weaning may cause problems with milk ejection resulting in remaining milk in alveolar part of the udder and thus changing the milk composition. It is important to know current health status of the udder immediately after lamb removal. The aim of this study was to determine changes in milk composition during first three milkings after lamb weaning and especially the frequency of distribution of ewes differed by somatic cell count (SCC). The study was performed on 36 lactating dairy ewes of two breeds - Tsigai and Improved Valachian within first three milkings after their lambs were weaned. Totally, 108 milk samples were collected for analysis. On the basis of SCC the animals were divided into three categories: low – SCC $< 0.5 \times 10^6$ cells.ml^{-1}, middle – $0.5 \times 10^5 <$ SCC $< 10^6$ cells.ml^{-1}, high – SCC $> 10^6$ cells.ml^{-1}. There were 64 percentages of ewes classified to low SCC category, 8 % - to middle and 28 % - to high SCC category at first milking after weaning. The average SCC was 5.39 ± 0.70 log$_{10}$.ml^{-1} at first milking, 5.66 ± 0.73 log$_{10}$.ml^{-1} at second, 5.68 ± 0.65 log$_{10}$.ml^{-1} at third and 5.26 ± 0.61 log$_{10}$.ml^{-1} at fourth milking. Significant negative correlation of SCC with lactose content ($r = -0.467$) and total milk yield ($r = - 0.196$) and order of milking and lactose ($r = - 0.319$) was found out. In conclusion, higher percentage of ewes with high SCC during first milking indicates health problems of the udder at the end of suckling period, and increasing SCC during next two milking could by caused by stress from weaning and starting of machine milking.

Key words: dairy ewes; weaning; somatic cells; stress

INTRODUCTION

Traditionally, dairy breed lambs are allowed to suckle after birth and the milking period for ewes begins after the lambs are weaned (Jaeggi *et al.*, 2008). In our traditional management lambs suckle 40-70 days after birth. When ewes are not exclusively machine milked immediately post-partum, the longer they remain in contact with their lambs during the suckling period, the more difficult it is for them to adapt to exclusive machine milking following weaning (Gargouri *et al.*, 1993; Labussière, 1988; 1978). Thus, the lamb removal from their mother and shift from suckling to machine milking could be considered as certain stress factors suppressing oxytocin release coming from the lost of lambs in combination with the new milk removal manner (Negrão

and Marnet, 2003). Not completely removed milk from the udder due to stress response causes reduction in milk yield and possible negative impact on immunity resulting in higher incidence of udder problems like mastitis (Paape *et al.*, 2002).

Somatic cells in milk are considered as an effective indicator of udder health (but also other factors are involved in their count in milk: parity, stage of lactation, season, herd, handling of ewes, diurnal variation (Gonzalo *et al.*, 1994; Gonzalo and San Primitivo, 1998), oestrus, vaccination, change in diet and change in the milking routine (Paape and Contreras, 1997; Barkema *et al.*, 1998). Many authors (Lafi, 2006; Bergonier and Berthelot, 2003; Fthenakis, 1994) reported that over 80 % of ewes which had somatic cell count (SCC) over 10^6 cells per ml, had positive reaction on California

mastitis test.

If machine milking not working and maintained correctly, is considered as adverse factor on the udder health, due to contamination of the teat skin, changes in teat condition, the penetration and spread of bacteria into the teat canal, and the inconsistent emptying of the udder (Hamann, 2000). The lambs' mouths and milkers' hands are the sources of milk contamination (Albenzio *et al.*, 2003). Suckling is still considered as positive factor in the prevention of mastitis as compared with machine milking (Krohn, 1999).

Hypothesis of the work was that lambs weaned from their mothers will change the milk composition as a possible negative effect of machine milking on udder health or/and stress from weaning due to the effect of milk removal disturbances. The aim of the experiment was to study the changes of milk composition during first three milkings after lamb weaning and mainly the frequency of distribution of ewes differed by SCC.

MATERIAL AND METHODS

The study was performed on experimental farm of the Animal Production Research Centre in Nitra, Slovakia. 36 lactating dairy ewes of two breeds - Tsigai (TS) and Improved Valachian (IV) were use in this investigation. Involved ewes were at their 3rd-9th lactations. The ewes were lambing from 2nd to 27th of February 2011 and were housed together with lambs and managed identically. The lambs were weaned from their mothers at 10th of April, and machine milking started twice daily on rotary parlour since next morning. Milking machine was set to provide 160 pulsations per minute at the ratio of 50:50 with a vacuum level of 38 kPa.

The experiment was performed during first three continuous milkings after lamb weaning and one control milking two weeks later (order of milking – OM). The first morning milking was performed approximately 12 hour after lamb weaning. Milk samples were collected individually from each ewe. Totally 108 samples from 36 ewes were collected during experimental milkings. Total milk yield (TMY) was recorded using electronic jars with 2-wire compact magnetostrictive level transmitters (NIVOTRACK, NIVELCO Ipari Elektronika Rt, Budapest, Hungary) connected to computer. Collected samples of SCC were analyzed on Somacount 150 (Bentley Instruments, Inc., Chaska, Minnesota). Milk components were analyzed by MilkoScan FT120 (Foss, Hillerød, Denmark).

According to Lafi (2006), Bergonier and Berthelot (2003), and Fthenakis (1994) animals on the basis of SCC were divided into the three categories (low – SCC $< 0.5 \times 10^6$ cells.ml^{-1}, middle – $0.5 \times 10^6 <$ SCC $< 10^6$ cells. ml^{-1}, high – SCC $> 10^6$ cells.ml^{-1}) to study the frequency of distribution of animals in selected category throughout experimental period. Also on the basis of above-mentioned SCC category at first milking only, three groups of animals were formed to study the frequency of animal distribution within group at next two milking and control milking.

Statistical evaluation of milk composition data from all four milkings was done by a One-Way ANOVA using Scheffe`s post-hoc test with order of milking (OM) as a factor. Before calculation the real data of SCC were transformed by log function. Relation between OM and TMY, milk composition at first three milkings was studied using regression analysis. Also, relation between SCC and TMY and milk composition was calculated with regression analysis – Linear model. Analyses were performed using IBM® SPSS® Statistics (version 20, IBM Corp.).
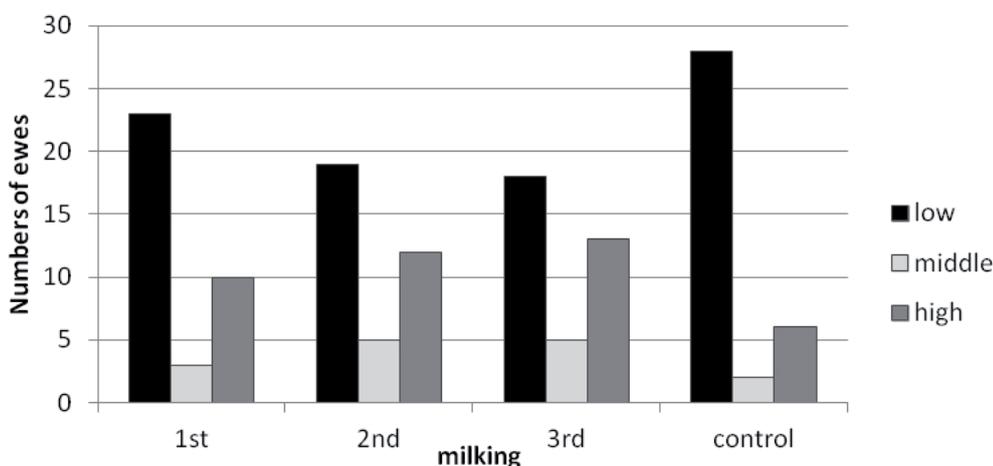
Fig. 1: **Numbers of ewes in SCC categories during three milkings after weaning**

RESULTS AND DISCUSSION

The effect of OM on all studied parameters is shown in Table 1. The most significant changes throughout three milkings were recorded for fat ($p <$ 0.001) and lactose ($p < 0.01$) content. Fat level rapidly increased from 3.07 % at first milking to 7.30 % at third one. After two weeks during control milking the fat level slightly decreased. Low fat content at first milking after lamb weaning may be caused by the lack of transfer of milk fat from the alveoli to the cistern due to possible stress from weaning of lambs (McKusick *et al.*, 2001, Antonič *et al.*, 2013) and/or from new milk removal (Tančin and Bruckmaier, 2001). The stress effect on ewe's response during first three milking is evident from TMY changes, when compared with control milking (Table 1). Explanation may be related to the stress effect causing the inhibition of oxytocin release during first milking as shown in ewes (McKusick *et al.*, 2001, Kulinová *et al.*, 2012) or cows (Tančin *et al.*, 2001). Inefficient removal of milk from alveoli significantly reduced total fat content in milk (Antonič *et al.*, 2013).

SCC during first milking after lamb removal can be considered as an indicator of health status of the udder during the period of suckling only. There were 64 % of ewes classified to low SCC category, 8 % to middle and 28 % to high SCC category at first milking after weaning (Fig. 1 – column 1st milking). The average SCC was 5.40 ± 0.69 $\log_{10}.ml^{-1}$. At second milking there was increase in SCC to 5.66 ± 0.73 $\log_{10}.ml^{-1}$, with next increase at third milking to 5.68 ± 0.65 $\log_{10}.ml^{-1}$ (Fig. 1). The trend of increase in SCC was also observed at the end of sucking (Margetín *et al.*, 1995; McKusick *et al.*, 2001) and milking periods (Margetín *et al.*, 1995). At control milking SCC decreased to 5.27 ± 0.61 $\log_{10}.ml^{-1}$ (Table 1). It may indicate the adaptation of ewes to machine milking – increase in number of ewes at low SCC category (Fig. 1).

From the health point of view the healthy udders regularly show a SCC value lower than 500 000 cells per ml (5.7 $\log_{10}.ml^{-1}$) without the effect of lactation period (Bergonier and Berthelot, 2003). The above mentioned authors pointed out that subclinical or chronically infected udder usually exceed one million cells per ml.

Table 1: The effect of order of milking on studied parameters

	Descriptive			ANOVA	
	Order of milking	Mean	Std. Dev.	F	*p-value*
TMY [l]	1st	0.65	0.34	2.259	0.084
	2nd	0.62	0.27		
	3rd	0.53	0.24		
	control	0.70	0.27		
Fat [%]	1st	3.07a	1.96	40.626	0.000
	2nd	5.43b	2.00		
	3rd	7.30c	1.92		
	control	6.84c	1.12		
Proteins [%]	1st	5.15	0.49	1.361	0.257
	2nd	5.19	0.43		
	3rd	5.30	0.57		
	control	5.35	0.44		
Lactose [%]	1st	5.27a	0.23	13.498	0.000
	2nd	5.25a	0.24		
	3rd	5.07b	0.25		
	control	4.97b	0.21		
SCC [$\log_{10}$]	1st	5.39	0.70	3.181	0.026
	2nd	5.66	0.74		
	3rd	5.68	0.66		
	control	5.27	0.61		

a,b,c – values within the same column with different letters are different at the level $p < 0.00$

Thus, our data demonstrate relatively high percentage of ewes with probably infected udder immediately after lamb weaning and during the period of shift to machine milking, but SCC was decreased during control milking. Our conclusion is coming from the results of Lafi (2006), who reported that from infected mammary gland only 9 % of samples had SCC less than 1×10^6 cells/ml, other ones had SCC over 10^6 cells/ml.
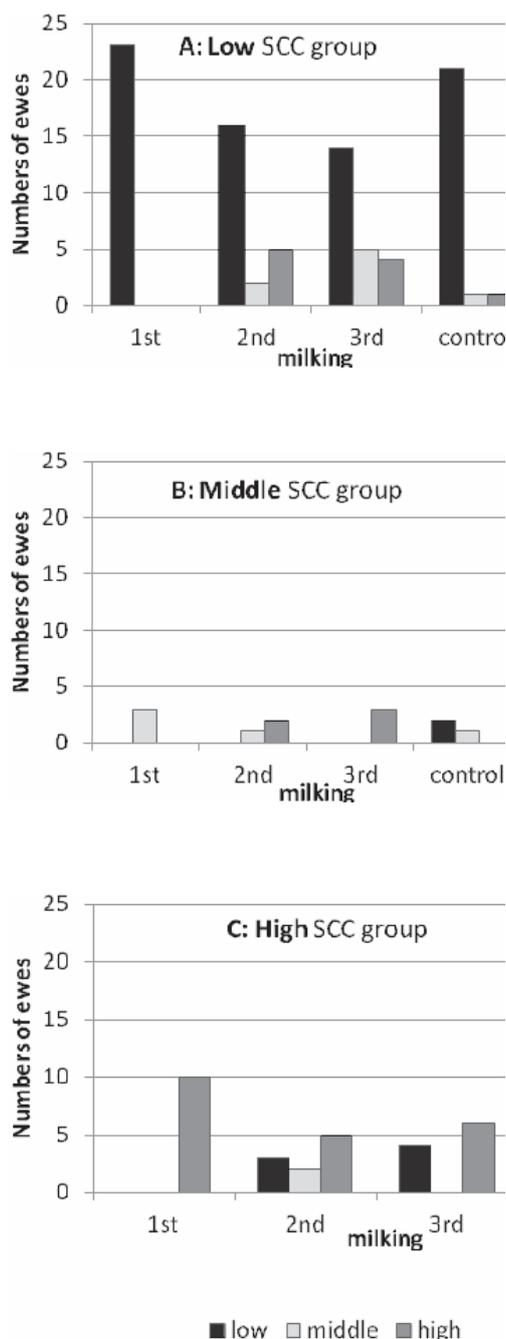
Fig. 2: Frequency of distribution of SCC categories within the groups differed by SCC during first milking

First milking could be considered as certain status of udder health during suckling period. Increase in SCC during next two milking could be related to stress response. As compared with short period of milking after weaning, the SCC status was improved two weeks later. Even the number of ewes in low category was higher during control milking than during first milking.

The changes in SCC within the each group formed at first milking (Fig. 1) are shown in Figures 2 (A-C). There were 23 animals in low SCC group at first milking (Fig. 2 A). During next two milkings the SCC was changed in the negative direction – the number of animals with low SCC was reduced. The fact that at control milking the number of ewes with low SCC increased again probably indicates stress effect from lamb weaning at the beginning. Three ewes were found out in the middle SCC group (Fig. 2 B) and 10 ewes were in the high SCC group (Fig. 2 C). Similarly, as it was in the low SCC category, there were also changes in SCC during second and third milking. It is not easy to explain the changes in SCC within each group because no bacterial evaluation of samples was done. The minimal changes in SCC within middle and high groups during first three milkings and at control milking could indicate possible problems with udder health caused by suckling. It was found out that almost 21 % of duct samples and 8 % of milk from udder cisterns are contaminated by bacteria during the period of suckling (Mavrogianni *et al.*, 2007), which could influence the further development of the udder after lamb weaning. The colonisation of teat duct could be eliminated by the immune system of ewes, but under the stress response the immunity of ewes (machine milking and lamb removal) is weaker, what could cause the health problem of the udder during second and third milking. As is shown on the 4th column in each Figure 2 A-D, the SCC within each group changed in the positive direction in control milking – the numbers of ewes with middle and high SCC were reduced.

Albenzio *et al.* (2003) have found that within 4 week lasting experiment there was higher SCC at machine milking of ewes when compared to suckled ones, as a consequence of higher bacterial positive samples at machine milking. On the other hand, in some cases lamb suckling could be responsible for increased SCC (Bergonier *et al.*, 1994; McKusick *et al.*, 2001). The risk is mainly related to "milk-robber" lambs, as they can spread microorganisms by suckling more ewes (Bergonier and Berthelot, 2003).

Although, regression analysis between OM and SCC ($r = 0.167$; $p = 0.085$) (Fig. 3 A) and ANOVA results ($p = 0.164$) did not significantly confirm increase in SCC during first three milkings, on the basis of analysis of regression lines between OM and lactose ($r = -0.319$; $p < 0.001$) (Fig. 3 B), SCC and lactose ($r = -0.467$; $p < 0.001$) (Fig. 3 C) we can indicate some problem
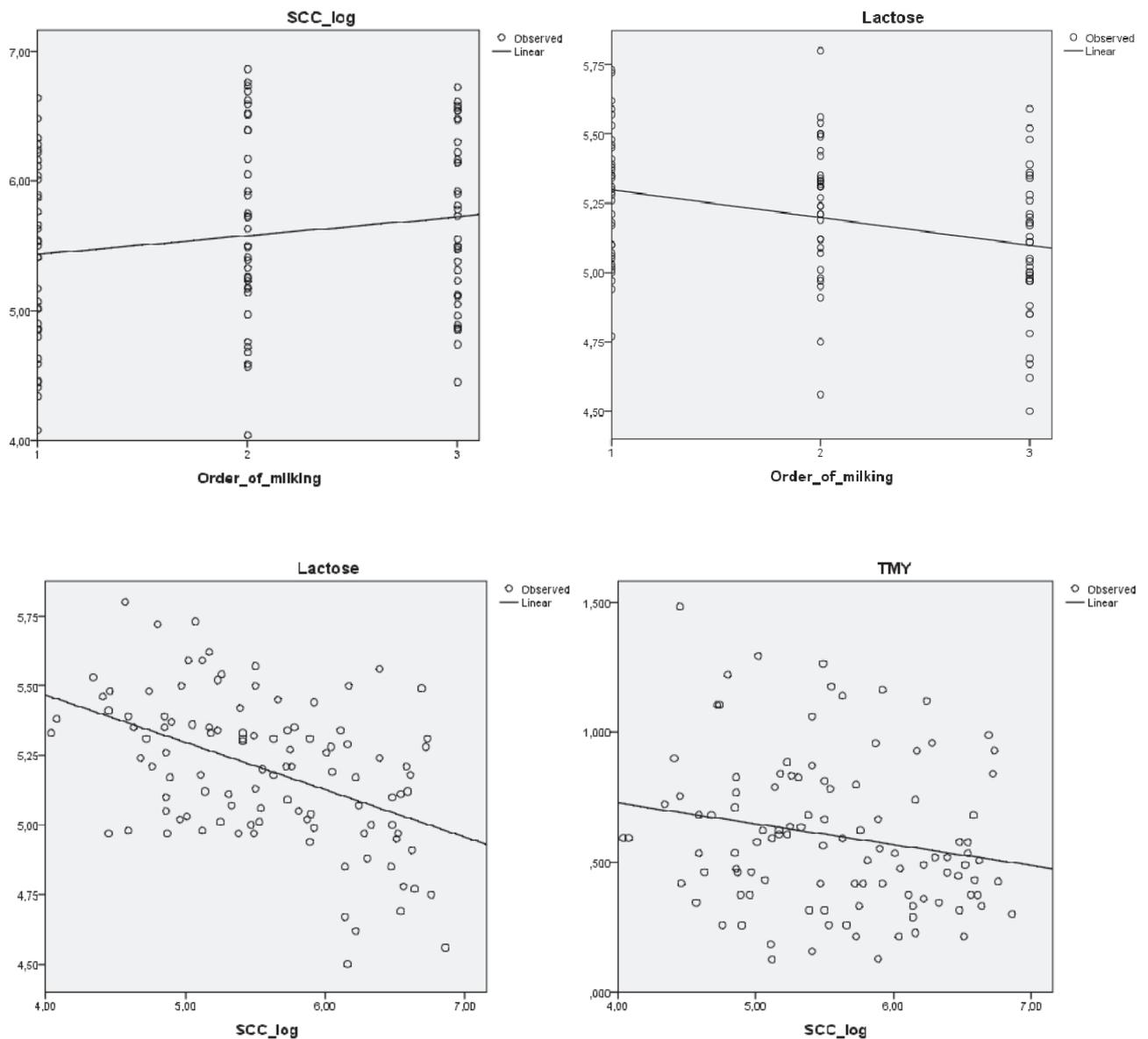
Fig. 3: Regression lines between OM and SCC (A) and Lactose content (B), and between SCC and Lactose content (C) and TMY (D)

with udder health. SCC had significant effect on TMY ($r = -0.196$; $p < 0.05$) (Fig. 2 D). Similar correlation between SCC and lactose and TMY was found by Margetín *et al.* (1994, 1996) during milking period of TS and IV. According to El-Tahawy and El-Far (2010) and Gonzalo *et al.* (2002), an increase in SCC caused a decrease in daily milk production and elevated risk of subclinical mastitis.

CONCLUSION

In conclusion, immediately after weaning there was relatively high percentage of ewes with high SCC indicating health problem of the udder during suckling period. Increase in SCC during next two milkings could by caused by stress from weaning and starting of machine milking. The SCC in milk during control milking supports this conclusion.

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MILKABILITY OF IMPROVED VALACHIAN, TSIGAI AND LACAUNE PUREBRED AND CROSSBRED EWES

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ABSTRACT

The objective of this study was to investigate the variation in milk yield and milk flow traits and to analyse the main factors influencing the milkability of ewes. Milk yield and milk flow traits were: milk yield to 10 s, milk yield to 30 s, milk yield to 60 s, machine milk yield, stripping yield, total milk yield, percentage milk yield to 30 s, percentage milk yield to 60 s, stripping percentage, machine milking time and average milk flow. Primiparous and multiparous Improved Valachian, Tsigai and Lacaune purebred and crossbred ewes were considered. Crossbred ewes were crosses of Improved Valachian or Tsigai ewes with Lacaune (genetic portion of Lacaune was 25, 50 and 75 %, respectively). A total of 359 to 370 ewes were measured depending on trait. Mixed model with fixed and random effects using the REML (restricted maximum likelihood) method was applied. All traits were significantly (P<0.01) influenced by genotype and year. Some traits were significantly (P<0.05 or P<0.01) influenced by parity, stage of lactation and interactions considered between genotype and parity and between genotype and stage of lactation. The repeatability varied from 0.23 to 0.43. Regardless of breed, mean values of machine milk yield, total milk yield and of stripping percentage were 318.3 ml, 436.6 ml and 27.7 %, respectively. Stripping percentage varied extensively, from 0 % to 95 %. The highest stripping percentage (37.8 %), the highest total milk yield (523.1 ml) and the second highest machine milk yield (330.3 ml) were found in Lacaune purebred ewes. The crossbred ewes were better than Improved Valachian and Tsigai purebred ewes in all examined traits, except for milk yield to 10 s, percentage milk yield to 30 s, percentage milk yield to 60 s, stripping percentage and machine milking time. Obtained results suggest that crossbreeding of local dairy breeds with Lacaune may be a good strategy for improvement of milkability of dairy sheep population in Slovakia.

Key words: dairy sheep; machine milking; milk yield; milk flow

INTRODUCTION

Milkability of ewes is a complex trait which can be described by milk yield (Rovai *et al.*, 1999), milk flow (Mayer *et al.*, 1989; Bruckmaier *et al*., 1997) and udder morphology (de la Fuente *et al*., 1996). A pattern of milk flow is influenced by milk storage in udder before milking and milk ejection (Labussiere, 1988; Bruckmaier *et al*., 1997). Udder milk consists of two fractions: cisternal and alveolar. The cisternal fraction is milk which has already been transferred from alveoli to the cistern during the interval between milkings and is immediately obtainable by the machine without milk ejection. The alveolar fraction (milk stored in the alveoli) is milk which can be removed from the udder only when milk ejection occurs during milking (Marnet and McKusick, 2001; Mačuhová *et al.*, 2008). Milk flow patterns depend on physiological response of ewes to machine stimulation, milk production and teat canal characteristics (Bruckmaier *et al*., 1997; Marnet *et al*., 1999; Tančin *et al*., 2011). Two (Labussiere, 1988) and three (Dzidic *et al.*, 2004) milk flow patterns were reported for ewes: one-peak, bimodal and plateau. Along with milking machine parameters (pulsation rate, milking vacuum etc.) and individual abilities of ewes, breed, parity and stage of lactation are the important factors of milkability as well. Ewes with

well shaped udder, vertically placed teats and having high milk emission flows eject their milk rapidly with only few manual interventions during machine milking (Marie-Etancelin, 2003). When ewes are adapted to show this aptitude, sheep farm profitability may increase.

Machine milking in Slovakia has been in place since 1960s. Early works on milkability were carried out in 1970s and 1980s (Masár, 1974, 1978; Mikuš 1974, 1985). Recent results on analyses of milk yield, milk flow and udder morphology were referred by Margetín *et al.* (2003, 2004, 2005) and Milerski *et al.* (2006). Recent results on analyses of milk flow curves were referred by Mačuhová *et al.* (2007, 2009, 2010), Kulinová *et al.* (2011) and Tančin *et al.* (2011). The complex work of such scope in terms of measured ewes and genotypes as presented here has not been done in Slovakia until now.

The objective of this study was to investigate the variation in milk yield and milk flow traits and to analyse the main factors influencing the milkability of ewes. A special emphasis was given on ewe genotype since milkability of Improved Valachian, Tsigai and Lacaune purebred and crossbred ewes was analysed.

MATERIAL AND METHODS

The study was performed in the experimental flock of the Animal Production Research Centre Nitra in Trenčianska Teplá between 2002 and 2008. Primiparous or multiparous Improved Valachian (IV), Tsigai (TS) and Lacaune (LC) purebred and crossbred ewes were considered. Crossbred ewes were crosses of IV or TS with LC (genetic portion of LC was 25, 50 and 75 %, respectively). Genotype acronyms for crossbred ewes were as follows: IVxLC 25 %, IVxLC 50 %, IVxLC 75 % and TSxLC 25 %, TSxLC 50 %, TSxLC 75 %. Ewes were milked twice a day. Milk yield and milk flow traits were measured during the morning milking, mostly in May and July. Machine milking was carried out in a 1x24 side by side milking parlour. Milking vacuum was 38 kPa, pulsation rate was 140 to 160 cycles/min at the ratio 1:1. Two to four measurements per lactation were taken. In ewes measured in two or more consecutive years, eight individual measurements were maximally taken.

Milk yield and milk flow were measured after the attachment of teat cups to ewe udder. A certified milkmeter (Farmtec, JSC Tabor, Czech Republic; accuracy ±10 ml) from routine milk performance testing was applied. Ewes were milked 60 s at least. The amount of milk extracted by the machine was recorded in 10 s intervals until milk flow ceased for 20 s. Machine stripping started afterwards and was recorded in 10 s intervals. Milk yield and milk flow traits were: milk yield to 10 s (MY10s), milk yield to 30 s (MY30s), milk yield to 60 s (MY60s), machine

milk yield (MMY), stripping yield (SY), total milk yield (TMY = MMY + SY), percentage milk yield to 30 s (MY30sP), percentage milk yield to 60 s (MY60sP), stripping percentage (SP), machine milking time (MMT) and average milk flow (AMF). MY10s, MY30s and MY60s are the amounts of milk extracted during the first 10, 30 and 60 s of machine milking, respectively. MMY is the amount of milk extracted by the machine before milk flow ceased for 20 s. SY is the amount of milk extracted during machine stripping performed after milk flow had ceased (not earlier than 60 s from the attachment of teat cups). MY30sP, MY60sP, SP and AMF were calculated as follows: (MY30s/TMY)x100, (MY60s/TMY)x100, (SY/TMY)x100 and MMY/MMT. A total of 359 to 370 ewes were measured depending on trait. Numbers of observations by trait, and genotype, parity, stage of lactation (according to traits) are reported in Tables 1 to 4b. The lower number of observations in traits MY10s and MMT was due to the fact that these indicators were assessed only in 2002-2005 and 2002-2007 respectively.

MEANS procedure (SAS, 2009) was used to calculate basic statistics for milk yield and milk flow traits. Mixed model with fixed and random effects (MIXED procedure; SAS, 2009) was applied to assess sources of variation for milk yield and milk flow traits (SY was excluded from the analysis; interactions were omitted in the model for MY10s, MMT and MMF). The model was as follows:

$$y_{ijklm} = \mu + G_i + P_j + S_k + Y_l + G_i * S_k + G_i * P_j + a_m + e_{ijklm}$$

where:

y_{ijklm}	is individual observation of trait i.e. MY10s, MY30s, MY60s, MMY, TMY, MY30sP, MY60sP, SP, MMT, AMF
μ	is intercept
G_i	is fixed effect of genotype with 9 levels; IV, TS, LC, IVxLC 25 %, IVxLC 50 %, IVxLC 75 %, TSxLC 25 %, TSxLC 50 %, TSxLC 75 %
P_j	is fixed effect of parity with 3 levels; first, second, third and further parity
S_k	is fixed effect of stage of lactation with 4 levels; from day 40 to 99, from day 100 to 129, from day 130 to 159 and from day 160 to 210
Y_l	is fixed effect of year of experiment with 4, 6 and 7 levels, respectively, depending on trait
$G_i * S_k$	is interaction between genotype and stage of lactation
$G_i * P_j$	is interaction between genotype and parity
a_m	is random effect of animal
e_{ijklm}	is random residual error

Fixed effects were estimated using the LSM (Least Squares Means) method. Statistical significance was tested by Fisher's F-test and differences between the estimated levels of fixed effects were tested by Scheffe's multiple range test. Ewe (σ^2_{ew}) and residual error variances (σ^2_e) were estimated using the REML (Restricted Maximum Likelihood) method. The estimated variances were used to calculate the repeatability within an individual ewe:

$$r^2 = \sigma^2_{ew} / (\sigma^2_{ew} + \sigma^2_e).$$

RESULTS

Basic statistics of milk yield and milk flow traits in Slovak sheep is summarised in Table 1. MMY was 317.5 ml and took 62.3 s (MMT) on average. SY was 118.8 ml and accounted for 27.7 % of TMY. The average value of TMY (435.9 ml) was low, taking into account the fact that LC purebred ewes were also measured. Ewes with milk yield to 10, 30 and 60 s (MY10s, MY30s and MY60s) as high as 400, 840 and 1200 ml were found. Ewes able of rapid udder emptying (MY30sP or MY60sP equal to 100 %) were also found. On the contrary, ewes with no milk ejection during the first 10, 30 or 60 s of machine milking also occurred.

Analysis of variance and estimates of repeatability for milk yield and milk flow traits are shown in Tables 2a and 2b. Effects of genotype and year showed highly significant ($P<0.01$) influence on all traits under study. The effect of parity influenced significantly ($P<0.05$) MMY and MY60s, and highly significantly ($P<0.01$) MY60sP and SP. The effect of stage of lactation was highly significant ($P<0.01$) or tended to be significant ($P<0.16$). Interactions between genotype and parity and between genotype and stage of lactation were included when MY30s, MY60s, MMY, TMY, MY30sP, MY60sP and SP were analysed. Effects of interactions (with exception of interaction between genotype and stage of lactation) was highly significant ($P<0.01$), significant ($P<0.05$) or tended to be significant ($P<0.15$) in all traits, except for MY30s and MY30sP. Repeatability equal to 0.34 or higher was found for all traits under study, except for MMT (0.23). The highest value of repeatability (0.43) was found for TMY.

Least-squares means and standard errors estimated for individual levels of effects of genotype, parity and stage of lactation are summarised in Tables 3a and 3b, and Tables 4a and 4b, respectively. Primiparous ewes had milk yield and milk flow traits higher (ML10s, ML30s, ML60s, ML60sP, AMF) or as high as multiparous ewes (MY, TMY, ML30sP, MMT). The exception was SP with the opposite trend. MY10s, MY30s, MY60s, MMY, TMY, MY30sP, MMT and AMF were decreasing with

Table 1: Basic statistics for milk yield and milk flow traits

Trait	n	$\bar{x}$	s	v	min.	max.
Milk yield to 10 s (MY10s), ml	796	116.4	67.1	57.6	0	400
Milk yield to 30 s (MY30s), ml	1218	220.4	102.8	46.6	0	840
Milk yield to 60 s (MY60s), ml	1159	307.2	154.1	50.2	0	1200
Machine milk yield (MMY), ml	1218	317.5	167.4	52.7	10	1200
Total milk yield (TMY), ml	1218	435.9	197.4	45.3	30	1339
Stripping yield (SY), ml	1218	118.8	91.8	77.3	0	775
Percentage milk yield to 30 s (MY30sP), %	1218	53.7	18.5	34.5	0	100
Percentage milk yield to 60 s (MY60sP), %	1159	69.4	17.0	24.5	0	100
Stripping percentage (SP), %	1218	27.7	15.6	56.3	0	95
Machine milking time (MMT), s	1088	62.3	16.4	26.3	15	160
Average milk flow (AMF), ml/s	1088	5.2	2.6	48.8	0	17.1

n: number of observations; $\bar{x}$: mean value; s: standard deviation; v: variation coefficient

Table 2a: Analysis of variance and estimates of repeatability for milk yield and milk flow traits

Source of variance	df	MY10s F-value	MY10s P	MY30s F-value	MY30s P	MY60s F-value	MY60s P	MMY F-value	MMY P	TMY F-value	TMY P
Genotype (G)	8	4.39	<0.0001	3.65	0.0003	10.29	<0.0001	14.10	<0.0001	30.92	<0.0001
Parity (P)	2	1.81	0.1642	0.59	0.5533	3.13	0.0441	3.49	0.0308	0.11	0.8991
Stage of lactation (S)	3	1.74	0.1586	29.99	<0.0001	56.28	<0.0001	89.16	<0.0001	125.35	<0.0001
Year	6 (3*)	13.78	<0.0001	32.13	<0.0001	24.51	<0.0001	23.30	<0.0001	32.48	<0.0001
GxS	24	-	-	1.04	0.4092	1.73	0.0169	2.46	0.0001	2.98	<0.0001
GxP	16	-	-	1.64	0.0530	1.48	0.1018	1.71	0.0406	1.54	0.0795
						Proportion of variance					
Repeatability		0.40		0.41		0.38		0.37		0.43	

df: degrees of freedom; *df for MY10s; for acronyms of traits see Table 1

Table 2b: Analysis of variance and estimates of repeatability for milk yield and milk flow traits

Source of variance	df	MY30sP F-value	MY30sP P	MY60sP F-value	MY60sP P	SP F-value	SP P	MMT F-value	MMT P	AMF F-value	AMF P
Genotype (G)	8	16.00	<0.0001	8.65	<0.0001	7.74	<0.0001	7.40	<0.0001	5.43	<0.0001
Parity (P)	2	1.07	0.3443	8.48	0.0002	7.35	0.0007	0.44	0.6439	6.42	0.0017
Stage of lactation (S)	3	7.18	<0.0001	1.79	0.1477	4.31	0.0050	31.33	<0.0001	78.84	<0.0001
Year	6 (5*)	9.85	<0.0001	4.88	<0.0001	6.41	<0.0001	30.38	<0.0001	26.97	<0.0001
GxS	24	1.23	0.2081	1.28	0.1645	1.51	0.0569	-	-	-	-
GxP	16	2.25	0.0034	1.89	0.0187	1.37	0.1480	-	-	-	-
						Proportion of variance					
Repeatability		0.42		0.37		0.35		0.23		0.34	

df: degrees of freedom; *df for MMT and AMF; for acronyms of traits see Table 1

an increasing number of days after parturition. SP showed the opposite trend as it increased with the increasing stage of lactation. MY60sP tended to be almost stable throughout the lactation.

The lowest MY10s (84.8 ± 6.9 ml) was found in LC purebred ewes, being 30 and 34 % lower than in IV and TS purebred ewes. TSxLC 25 % crossbred ewes had the highest MY10s, being 53 % higher than in LC purebred ewes. MY10s tended to decrease with an increasing portion of LC in crossbred ewes. TSxLC 25 % and IVxLC 25 % crossbred ewes had the highest MY30s (252.6 ± 37.0 ml and 237.7 ± 16.8 ml). TS and IV purebred ewes had the lowest MY30s, which was 31 % and 17 % lower than in TSxLC 25 % and IVxLC 25 % crossbred ewes. The same trend was revealed for MY60s; it was the highest in TSxLC 25 % and IVxLC 25 % crossbred ewes (362.6 ± 50.9 ml and 328.8 ± 22.8 ml). MY10s, MY30s, MY60s seem to have a potential to characterize the intensity of milk ejection in dairy ewes: the higher amount of milk is extracted during the first 60 (10, 30) s, the higher number of ewes can be milked per unit time. TMY in crossbred ewes was found lower than in purebred LC ewes, which had the highest TMY (523.1 ± 13.7 ml). Nevertheless, TMY in crossbred IV and TS ewes was higher than in purebred IV and TS ewes. MMY in IV and TS crossbred ewes (except for TSxLC 75 %) was higher than in LC purebred ewes. The lowest MMY (226.6 ± 13.2 ml and 200.9 ± 12.1 ml) and TMY (344.7 ± 14.3 ml and 273.3 ± 13.2 ml) were found in IV and TS purebred ewes. The highest MY30sP and MY60sP were found in IV and TS purebred ewes; the lowest MY30sP and MY60sP were found in LC purebred ewes. The higher MMT in LC purebred ewes than in IV and TS purebred ewes was found (by 6 and 8 s, respectively). MMT in crossbred ewes (except for IVxLC 75 % and TSxLC 75 %) was almost the same as in LC purebred ewes. The exceptions were TSxLC 25 % crossbred ewes with slightly lower MMT than TS purebred ewes. AMF calculated as the ratio MMY/MMT showed a similar trend, being of lower values in IV and TS purebred ewes than in IV and TS crossbred ewes and in LC purebred ewes. LC purebred ewes had higher SP than TS and IV purebred ewes (by 9 and 12 percentage points, respectively). SP in IV and TS crossbred ewes tended to differ from IV and TS purebred ewes to a lower extent (by 6 percentage points at maximum). As a general pattern, the differences in milk yield and milk flow traits between various genotypes within the same group of crossbred

Table 3a: Least squares means and standard errors of milk yield and milk flow traits by genotype

Source of variance		n *1	n *2	n *3	MY10s *1, ml LSM	SE	MY30s *2, ml LSM	SE	MY60s *3, ml LSM	SE	MMY *2, ml LSM	SE	TMY *2, ml LSM	SE
IV	(100)	148	218	200	128.9	7.1	196.8	9.3	271.8	12.8	226.6	13.2	344.7	14.3
IV xLC 25 %	(125)	42	68	67	124.0	13.6	237.7	16.8	328.8	22.8	339.0	24.0	428.5	25.9
IV xLC 50 %	(150)	43	93	91	128.5	13.0	225.2	13.6	325.4	18.4	348.2	19.3	468.7	20.9
IV xLC 75 %	(175)	68	82	82	111.6	10.6	219.9	14.2	344.7	19.1	367.9	20.1	498.4	21.8
TS	(200)	204	268	244	121.8	6.1	174.4	8.5	203.9	11.9	200.9	12.1	273.3	13.2
TSxLC 25 %	(225)	4	18	15	179.5	37.3	252.6	37.0	362.6	50.9	368.0	52.7	484.8	56.8
TSxLC 50 %	(250)	90	169	164	136.4	8.9	229.6	10.4	311.4	14.0	333.5	14.7	440.5	16.0
TSxLC 75 %	(275)	25	47	47	127.2	18.4	220.6	22.4	296.4	30.2	314.7	31.8	479.5	34.5
LC	(300)	172	255	249	84.8	6.9	211.4	8.9	312.7	12.1	330.3	12.6	523.1	13.7
Scheffé's multiple range tests					300:100,200,250+++; 300:125,150++		200:125,250+++; 200:150,175,300++; 100:125,250+; 200:225,275+		200:100,125,150,175, 250, 300+++; 100:175++; 200:225,275++; 100:125,150,250+		200:125,150,175,250, 275,300+++; 100:150,175,200,250, 300+++; 200:225++; 100:125++		300:250+++; 200:125,150,175,225, 250,275,300+++; 100:150,175,200,250, 275, 300+++; 125:100,300++; 100:125++; 175:125,250+;300:150+;	

+++ P<0.001; ++ P<0.01; + P<0.05; *1, *2, *3: number of observations (n) by trait; for acronyms of traits and genotypes see Table 1

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Table 3b: Least squares means and standard errors of milk yield and milk flow traits by genotype

Source of variance	n *1	n *2	n *3	MY30sP*1, % LSM	SE	MY60sP*2, % LSM	SE	SP*1, % LSM	SE	MMT*3, s LSM	SE	AMF*3, ml/s LSM	SE
IV (100)	218	200	196	58.5	1.7	74.7	1.6	24.8	1.5	56.3	1.4	4.77	0.22
IV xLC 25 % (125)	68	67	66	54.1	3.1	71.1	2.9	23.2	2.7	62.2	2.3	5.53	0.36
IV xLC 50 % (150)	93	91	79	50.6	2.5	69.2	2.3	27.5	2.1	62.3	2.0	5.50	0.33
IV xLC 75 % (175)	82	82	74	45.8	2.6	66.7	2.4	28.0	2.2	66.9	2.1	5.59	0.34
TS (200)	268	244	250	63.9	1.6	71.4	1.5	27.9	1.3	53.4	1.2	3.94	0.20
TSxLC 25 % (225)	18	15	12	52.6	6.7	73.7	6.5	25.3	5.9	52.6	5.2	5.48	0.83
TSxLC 50 % (250)	169	164	149	53.9	1.9	69.9	1.8	26.7	1.6	61.3	1.5	5.24	0.23
TSxLC 75 % (275)	47	47	37	47.8	4.1	63.1	3.8	34.1	3.5	66.0	3.1	4.86	0.51
LC (300)	255	249	225	41.8	1.6	58.7	1.5	37.8	1.4	61.4	1.3	5.30	0.21
Scheffe's multiple range tests				300:125,250+++; 200:150,175,250, 275,300+++; 100:175,300+++; 200:125; 100:150,300++; 100:200+,250,275+; 175:125,250+		300:100,125,150,200, 250++; 175:300+; 100:175,275++; 300:225+,275:125,200++; 100:150,250+		300:100,125,150,175, 200,250+++; 300:225+++; 275:100,125,250+; 200:100+		200:125,150,175,250, 275,300+++; 100:175++; 100:275,300++; 225:125,150,175,275, 300++; 175:250,300++; 100:125,150,200,250+		200:125,150,175,250, 300+++; 100:200++; 200:275++; 100:125,150,175,300+	

+++ P<0.001; ++ P<0.01; + P<0.05; *1, *2, *3: number of observations (n) by trait; for acronyms of traits and genotypes see Table 1

ewes (either IVxLC or TSxLC) tended not to be significant; the differences in milk yield and milk flow traits between purebred and crossbred ewes (either within IVxLC and IV or within TSxLC and TS) tended to be significant.

DISCUSSION

The mean values of TMY, MMY, MY60s and SP were consistent with the findings of Margetín *et al.* (2005), who examined ewes of the same genotypes. The values differed (except for SP) from the findings of Margetín *et al.* (2004) where only IV and TS purebred ewes and their crosses with LC were considered, and also from the findings of Margetín *et al.* (2003) where only TS purebred ewes were considered. SP was considerably lower (by 17 percentage points) than SP reported for TS purebred ewes and was slightly higher (by 7 percentage points) than SP reported for East Friesian crossbred ewes (McKusick *et al.*, 1996). With respect to ewes' adaptation to machine milking, SP is an important parameter affecting labour productivity and udder health and should be as low as possible.

Although studies on effects influencing milk yield and milk flow traits are reported in literature (e.g. Marie-Etancelin *et al.*, 2003), only some of them focus on the same effects. Thus, limited comparisons can be done. No significant effect of parity on milk yield and milk flow traits in Slovak dairy ewes was found by Mačuhová *et al.* (2008) and Tančin *et al.* (2011). According to Tančin *et al.* (2011), the effect of month of experiment shows significant influence on most of the traits. Dzidic *et al.* (2009) confirmed significance of effect of days in milk (60-, 90- and 120-days, respectively) in Istrian dairy crossbred ewes. Regarding the effect of genotype, Mačuhová *et al.* (2007, 2008, 2009) and Tančin *et al.* (2011) showed that this effect was significant in minority of studied traits. Nevertheless, the traits tended to differ between analysed purebred and crossbred ewes. Rovai *et al.* (1999) reported significant effects of breed, parity and stage of lactation on milk yield in Manchega and LC ewes. Almost the same repeatability for TMY, MMY and MMT was reported by Tančin *et al.* (2011), whereas these authors found higher repeatability for SP (by 10 %). Marie-Etancelin *et al.* (2003) reported higher repeatability for TMY and AMF in Sarda x Lacaune backcrossed ewes (0.65 and 0.50) and LC lines (0.53 and 0.50). Casu *et al.* (2008) and Fuente *et al.* (1997) also found higher repeatability in Sarda x Lacaune backcrosses and Churra ewes, respectively.

Although the rough approximation of daily milk yield requires TMY presented here to be

multiplied by 2, majority of studies devoted to milk emission characteristics reported breeds with higher milk production than Slovak dairy ewes. For instance, Peris *et al.* (1995) and Fernández *et al.* (1997) found MMY in Manchega ewes as high as 899 ± 38 ml and 992 ± 33 ml, respectively. According to Marie-Etanceline et al. (2003), TMY in LC lines and Sarda x Lacaune backcrossed ewes was 815 and 781 ml, respectively. The similar value of TMY (797.5 ± 262.6 ml) and MMY equal to 676.4 ± 244.4 ml in Sarda x Lacaune backcrossed ewes were reported by Casu et al. (2008). AMF in LC lines was 5.5 ml/s (i.e. it was found similar to AMF estimated for LC in this study), whereas AMF in Sarda x Lacaune backcrossed ewes was 8.23 ml/s (Marie-Etancelin *et al.*, 2003). Similar to the increasing trend of TMY with an increasing proportion of LC breed in IV crossbred ewes, Dzidic *et al.* (2004) reported TMY in Istrian 75 % x Awasi 25 %, Istrian 50 % x Awasi 50 % and Istrian 25 % x Awasi 25 % x East Friesian 50 % crossbred ewes as high as 0.52 ± 0.1 kg, 0.58 ± 0.1 kg and 0.75 ± 0.1 kg, respectively. The high values of TMY (1.14 ± 0.3 l) and MMY (0.92 ± 0.3 l) in East-Friesian crossbred ewes were reported by McKusick *et al.* (1996). Consequently, machine milking of East-Friesian crosses took 105.9 ± 38.6 s on average (i.e. AMF in Slovak ewes was found to be 40 % lower). In Boutsiko ewes (Sinapis *et al.*, 2006), MMY from morning milking investigated in independence on milking vacuum level which was between 338.5 ± 18 ml and 390.8 ± 21.4 ml i.e. similar to MMY found in IV and TS crossbred ewes and LC purebred ewes. MMT in Boutsiko ewes was by one third to one half lower. On the contrary, AMF was by one third to one half higher.

The comparisons between purebred and crossbred ewes presented here correspond with recent studies of Mačuhová *et al.* (2009) and Tančin *et al.* (2011) in most of the traits. Mačuhová *et al.* (2009) found the highest MY30s and MY60s in TSxLC 50 % crossbred ewes and the lowest MY30s and MY60s in TS and IV purebred ewes. According to them, the highest TMY and MMY was in IVxLC 50 % crossbred ewes and the lowest TMY and MMY was in TS purebred ewes. SP and MMT were the lowest in TSxLC 50 % crossbred ewes and the highest in LC purebred ewes and IVxLC 50 % crossbred ewes. Tančin *et al.* (2011) found the lowest TMY and MMY in IV purebred ewes and the highest TMY and MMY in IVxLC 50 % crossbred ewes. IV purebred ewes had the lowest SP, whereas LC purebred ewes and TSxLC 50 % crossbred ewes had the highest SP. The authors reported the lowest MMT in TS purebred ewes and the highest MMT in TSxLC 50 % crossbred ewes. MY30s was the lowest in IV purebred ewes; the highest MY30s was found in LC purebred ewes and TSxLC 50 % crossbred ewes.

The comparisons between IV and TS purebred ewes and their crosses with LC breed indicate that

Table 4a: Least squares means and standard errors of milk yield and milk flow traits by parity and stage of lactation

Source of variance		n *1	n *2	n *3	MY10s*1, ml LSM	MY10s*1, ml SE	MY30s*2, ml LSM	MY30s*2, ml SE	MY60s*3, ml LSM	MY60s*3, ml SE	MMY*2, ml LSM	MMY*2, ml SE	TMY*2, ml LSM	TMY*2, ml SE
Parity														
	(1)	434	289	425	133.1	6.0	225.1	5.8	329.2	9.8	340.8	9.7	442.0	10.4
	(2)	348	242	321	124.7	6.3	217.3	9.5	297.4	14.2	317.1	13.8	436.3	14.4
	(3+)	436	265	413	123.2	6.7	213.7	10.6	292.4	14.4	298.2	15.1	435.5	16.3
Scheffe's multiple range tests					ns		ns		1:2,3+		1:3+		ns	
Stage of lactation														
Day 40 to 99	(1)	261	173	251	131.5	6.7	262.69	9.2	415.5	13.6	448.1	13.4	593.0	14.0
Day 100 to 129	(2)	366	271	357	130.3	6.0	236.15	7.7	320.7	11.2	339.3	11.2	465.6	11.8
Day 130 to 159	(3)	335	217	316	127.3	6.3	188.90	7.5	238.6	10.5	243.9	10.9	352.2	11.5
Day 160 to 210	(4)	256	135	235	118.8	7.1	187.02	9.0	250.9	13.4	243.4	13.1	341.0	13.7
Scheffe's multiple range tests					1:4+; 2:4+		1:3,4+++; 2:3,4+++		1:2,3,4+++; 2:3,4+++; 1:2++		1:2,3,4+++; 2:3,4+++		1:2,3,4+++; 2:3,4+++	

+++ P<0.001; ++ P<0.01; + P<0.05; ns: not significant; *1, *2, *3: number of observation (n) by trait; for acronyms of traits see Table 1

Table 4b: Least squares means and standard errors of milk yield and milk flow traits by parity and stage of lactation

Source of variance		n *1	n *2	n *3	MY30sP*1, % LSM	MY30sP*1, % SE	MY60sP*3, % LSM	MY60sP*3, % SE	SP*1, % LSM	SP*1, % SE	MMT*2, s LSM	MMT*2, s SE	AMF*2, ml/s LSM	AMF*2, ml/s SE
Parity														
1	(1)	434	424	398	53.5	1.3	73.3	1.2	25.0	1.1	60.5	0.98	5.49	0.15
2	(2)	348	321	326	52.6	1.7	68.2	1.8	27.9	1.6	59.7	1.18	5.01	0.18
3+	(3)	436	413	364	50.3	1.9	64.8	1.8	32.2	1.7	60.6	1.21	4.91	0.19
Scheffe's multiple range tests					ns		1:3+++; 1:2++		1:3+++; 2:1,3+		ns		1:2,3++	
Stage of lactation														
Day 40 to 99	(1)	261	251	261	47.4	1.7	70.5	1.7	25.3	1.5	66.2	1.2	6.57	0.18
Day 100 to 129	(2)	366	357	346	52.0	1.4	69.0	1.4	27.6	1.3	61.8	1.1	5.41	0.16
Day 130 to 159	(3)	335	316	283	54.5	1.4	66.7	1.3	30.3	1.2	57.5	1.1	4.39	0.17
Day 160 to 210	(4)	256	235	198	54.7	1.6	68.8	1.7	30.2	1.5	55.5	1.3	4.26	0.19
Scheffe's multiple range tests					1:2,3,4+++; 2:3+		1:3+		1:3,4+; 2:3,4+		1:2,3,4+++; 2:3,4+++		1:2,3,4+++; 2:3,4+++	

+++ P<0.001; ++ P<0.01; + P<0.05; ns: not significant; *1, *2, *3: number of observation (n) by trait; for acronyms of traits see Table 1

crossbred ewes showed a good potential to benefit from desired traits of both local and LC breeds. As a partial disadvantage may be considered an increase in MMT and SP, nevertheless these seem to be balanced with better TMY, MMY, AMF, MY30s and MY60s. Knowledge gained from the analyses of milk yield and milk flow traits in various ewes' genotypes may be used as a basis for further improvement of local dairy ewes.

CONCLUSION

The experimental results suggest that crossbreeding of local dairy breeds with Lacaune breed may be a good strategy for improvement of milkability of Improved Valachian and Tsigai ewes. Selection based on such traits as machine milk yield or stripping percentage seem to be crucial for improving adaptation to machine milking and for increasing milk production. Both traits should be considered in a breeding programme since these traits require no additional costs when they are recorded routinely within milk performance testing.

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VARIABILITY IN BODY SHAPE CHARACTERS IN AN INDIGENOUS GUINEA FOWL (*NUMIDA MELEAGRIS* L.)

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ABSTRACT

Morphometric traits (body length, wing length, neck length, shank length, thigh length, keel length, chest circumference) and body weight obtained from 82 adult (both sexes) Nigerian indigenous guinea fowl, domesticated by rural farmers in three communities of Lafia local government area of Nasarawa State, were determined in the study. The study was aimed at obtaining the sources of shared variability among the body shape characteristics in adult guinea fowl and predicting live weight using both original and orthogonal traits. Sex effect on the traits was not significant (P>0.05). Correlations between traits were ranging from 0.07 to 0.98. Body conformation "shape" was controlled by both common and unique factors, communalities ranges between 0.371 to 0.996 for wing length and keel length, respectively. Common sources of variability in body dimensions of the bird were accounted for by factors representing general size and chest circumference. Original body dimensions were better predictors of body weight than the orthogonal traits derived from factor analysis.

Key words: guinea fowl; body dimensions; variability; factor analysis; communality

INTRODUCTION

There are two main guinea fowl subspecies found in Nigeria. The helmeted guinea fowl, *Numida meleagris galeata* Pallas, occur freely throughout the grassland areas spreading from the derived savanna near the forest zone in the south to the true savanna into northern guinea savanna vegetation zones. The second, the crested guinea fowl, *Guttera edouardi edouardi*, is restricted in distribution to the forest and derived savanna forest edges (Ayeni, 1979). The number of free ranging semi-wild guinea fowl kept in captivity in Nigeria alone is said to be about 45 million (Akinwumi *et al*., 1979) with more millions still in the wild. It is second to the domestic fowl in terms of number and supply of poultry protein in Nigeria. Thus a huge number exists for various studies and from which to select for improvement (Ayorinde, 1991; Smith 2000). They are described as a poor man's "pheasant" (Bond 1997). In north- central Nigeria, few farmers tend to domesticate the bird through collection of the egggs from the wild and hatching them at home, thereby growing them with the local chicken.

This practice is gaining wide acceptance among rural people. Some farmers keep the bird out of curiosity and as watch animal around home stead because they have an excellent eye sight, a hash cry and shrick at the slightest provocation.

Because of the ever increasing interest in consumption and domestication of this bird, deliberate efforts are required to promote the development of this species. This can be achieved through adoption of breeding programmes that are common to other livestock, there by evolving the know-how on the performance of the various traits and providing a blue print that will lead to improvement in performance and other economic traits in the bird.

Body measurement and its relationship to size and shape have been extensively studied in both large animals and poultry (Mendes *et al.*, 2005; Ogah *et al*., 2011; Shahin and Hassan 2000). Its use in predicting weight and other characteristics was also elucidated.

The objective of the study was to obtain the sources of shared variability among the body shape characters of adult guinea fowl and predict live weight

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using both original traits and orthogonal traits.

MATERIAL AND METHODS

The data used for the study were generated from 82 adult indigenous guinea fowl domesticated by rural farmers as described by Smith (2000), in three villages of Adogi, Ashige and Abu of Lafia local government area of Nasarawa State, Nigeria, located between latitude 08.30ºN and longitude 08.32ºE with annual rainfall ranging from 952 to1988 mm, and a mean monthly precipitation of 150 mm. Its minimum and maximum daily temperatures are in the range of 20-37ºC. Lafia has a mean relative humidity at noon varying between 14 and 74 %. It has two distinct seasons: the wet season covering late April to October and dry season covering November to early April.

The birds were managed under semi-intensive system, housing was provided, water was supplied *ad libitum*, and fed on brewer dried grain and whole corn seed and kitchen waste. They were also allowed to scavenge around at noon. The birds considered for measurement were adult birds of about a year and above.

Parameter measured (Body traits measurements)

Live body measurements included body weight (BW), body length (BL), wing length (WL), thigh length (TL), keel length (KL), neck length (NL), shank length (SL) and chest circumference (CC), as outlined by Gueye *et al.* (1998). Kitchen scale and graduated measurement tape were used to obtain the data. To ensure accuracy, each measurement was taken twice, the same person throughout took all measurements and weighing, thus eliminating error due to personal difference. The data from males and females are pooled since there was no significant difference between the sexes in the above mentioned traits, using multivariate Hotellings T^2 -test as described earlier (Ogah, 2012).

Statistical analysis

The data were subjected to a factor analysis procedure (SAS, 1999) after the descriptive statistics was initially obtained using same package. The main source of shared variation among the interdependence of body measurements (*p*) was expressed in terms of fewer mutually uncorrelated common factors F1, F2...., F*q* (where *q* < p), than the original measurements (Darton, 1980). The first factor contained the greatest portion of the original variation and in a morphometric application of factor analysis it was designated as a general size factor. Subsequent factors were mutually orthogonal to those preceding and to one another and contained less

variation. The model used is as follows:

$X = L F + U$, where

$X = a p ´ 1$ is a vector observational variables;

$L = a p ´ q$ a matrix of factor loading 'factor – variate correlations, the degree of correlation of the variable with factor' (the pattern matrix);

$F = a q ´ 1$ a vector of factors (non-observable) and

$U = a p ´ 1$ a vector of the specific 'unique' factor.

The total variance of a variable was equal to unity and can be written as the sum of common variance 'communalities' and unique variance 'uniqueness'. The communality represented the portion of the variable variance accounted for by all common factors and the uniqueness represented the portion of the variable variance not ascribable to its correlation with other variables. A build up stepwise multiple regression was used to predict body weight from the live measurements. Attaining the 5 % level of significance was the predetermined criterion for entering the independent variables. Their sequence of retention followed a descending order for the amount of variance explained. The programme terminated when the last independent variable entering the equation had an insignificant regression coefficient.

RESULTS AND DISCUSSION

The descriptive statistics of the morphometric traits of the indigenous guinea fowl is presented in Table 1. Most of the traits are similar to what was reported earlier by Ogah (2012). However, the body weight of the current work is higher. The reason for the differences in weight and other traits might be genetic and environmental, as variation in management could account for that. The result is similar to those of Saina *et al.* (2005), recorded from Zimbabwe, and higher than reported by Galor (1985) and Ayorinde (1991). The effect of sex using multivariate Hotellings T^2 test was not significant (P>0.05), which leads to pooling of the data for general analysis, thus agreeing to the submission of Ayorinde (1991).

Table 2 presents the correlation matrix between the morphometric traits. All traits were positively correlated with body weight, chest circumference had the highest phenotypic correlation (P<0.001) with body weight, while wing length had the least (0.17). Ogah *et al.* (2011) reported similar trend to male Muscovy duck. The positive and significant correlation of body weight and the other morphological traits (body length, keel length, chest circumference) suggests that the traits are under same gene action (pleitropism) and by implication selection for improvement of one result in improvement of the other trait as correlated response. Similar relationship between body weight and chest

Table 1: Descriptive statistics of morphometric traits of adult indigenous guinea fowl

Trait	mean ± se	min	max	cv
Body weight (kg)	1.42 ± 0.09	0.90	3.00	35.90
Body length (cm)	22.42 ± 0.17	20.00	24.00	4.46
Wing length (cm)	19.34 ± 0.21	15.00	22.00	6.62
Neck length (cm)	17.03 ± 0.10	16.00	17.60	3.58
Shank length (cm)	7.73 ± 0.08	7.10	8.10	5.90
Thigh length (cm)	11.87 ± 0.10	11.10	12.50	4.95
Keel length (cm)	2.80 ± 0.06	2.30	3.10	12.89
Chest circumference (cm)	35.37 ± 0.35	33.50	38.00	5.88

Table 2: Correlation matrix between morphometric traits of the indigenous guinea fowl

	BW	BL	WL	NL	SL	TL	KL	CC
BL	0.23							
WL	0.17	0.12						
NL	0.44**	0.16	- 0.09					
SL	0.67***	0.23	- 0.09	0.96***				
TL	0.31	0.12	- 0.12	0.98***	0.90			
KL	0.52**	0.18	- 0.07	0.96***	0.98	0.97		
CC	0.78***	0.24	0.23	- 0.11	0.18	- 0.27	- 0.12	

BW = body weight, BL = body length, WL = wing length, NL = neck length, SL = shank length,TL = thigh length, KL = keel length,
CC = chest circumference, ** = P<0.01, *** = P<0.001

Table 3: Explained variation associated with rotated factor analysis along with their common and unique factors

Trait	Common factors			
	FC1	FC2	communalities	unique factor
BL	0.214	0.618	0.428	0.577
WL	- 0.101	0.601	0.371	0.629
NL	0.998	- 0.042	0.997	0.003
SL	0.996	0.195	0.972	0.028
TL	0.980	- 0.166	0.987	0.013
KL	0.997	0.031	0.996	0.004
CC	- 0.072	0.806	0.655	0.345
% var	56.342	20.874		

BW = body weight, BL = body length, WL = wing length, NL = neck length, SL = shank length,TL= thigh length, KL = keel length,
CC = chest circumference, FC1 = first common factor, FC2 = second common factor

circumference was reported by Ogah *et al.* (2011) for Muscovy duck, Mendes *et al.* (2005) for America bronze turkey under different lightening programmes.

Table 3 outlines the communalities and unique factors for various variables. The result shows that 37 to 99 % of the variation in body measurement traits were brought about by the common factors, whereas 63 to 1 % of these variations were contributed by unique factors specific for each variable; keel length, neck length, shank length and thigh length had the highest common factors (0.993, 0.917, 0.972, and 0.987) with lowest variation contributed by the unique factors. While wing length had the least common factor (0.371) and the highest contribution of the unique factor.

The two common factors were obtained from varimax rotation, accounted for 77.22 % of the total variability of the original variables. The first factor (F1) general size was characterized by high positive loading (factor-variate correlaton) on all body dimensions other than wing length and chest circumference. Shank lenght, thigh length and neck length coefficients dominated the first factor and represent good estimator of general size (Shahin and Hassan, 2000). This first factor "general size" accounted for 56.34 % of the total variance, similar to those of Ricard and Rouvier (1968), obtained from principal component analysis of cockreal among body shape characters (Ogah *et al.*, 2009) 57 % for male muscovy duck.

The second factor which was mutually orthogonal to the first show pattern of variation independent of general size, it account for 20.87 % of the total variation and had high loading for chest circumference, body length and wing length. The most common variability here are general size and chest circumference similar

to that reported for New Zealand White rabbit (Shahin and Hassan, 2000).

Table 4 presents the results of stepwise multiple regression of body weight on both morphometric and orthogonal traits. Chest circumference alone accounted for 61 % of the variability in the body weight. These traits have been used as an indicator of animal size in number of studies (Shahin 1999; Ogah *et al.*, 2011). In addition of thigh length the R^2 increases to 86 %, this indicates that live weight can be predicted with fair degree of accuracy and reliability from chest circumference and thigh length. The result of the stepwise multiple regression of body weight could be outlined as following:

BW = -5.344 + 0.481TL + 0.27CC.

That of the orthogonal traits derived from factor analysis scores also show a progression from 41 % to 63 % R^2 . BW= 0.327 + 0.054FC1 + 0.054 FC2.

It shows that the regression coefficient in a stepwise multiple regression of body weight on the original traits was unstable and changed with the addition of variables into the equation. The instability could lead to probability to estimate the unique effect of individual variable in the regression equation and thus could lead to false inference.

Corresponding regression coefficient obtained from regression of the body weight on orthogonal traits obtained from factor analysis were stable with addition of factor scores in the equation (order of entry did not affect the result). The scenario was similar to what was reported by Shahin (1996) on analysis of muscles and bone weight variation of egyptian strain of Pekin duckling.

Table 4: Step-wise multiple regression of body weight on morphometric traits and their orthogonal variable from factor analysis scores

Step	indep. Var					
	Predictor					
A	Original body measurement	intercept	reg.coeff	se	R^2	VIF
1	Chest circumference	-5.344	0.191	0.026	0.61	01.000
2	Chest circumf.	-12.321	0.227	0.015	0.89	1.075
	Thigh length		0.481	0.052		1.075
B	Their orthogonal traits					
1	FC 2	1.421	0.327	0.067	0.41	1.000
2	FC 2	1.421	0.327	0.054	0.63	1.000
	FC 1		0.241	0.054		1.000

FC1 = first common factor, FC2 = second common factor, VIF = variance inflation factor

CONCLUSION

From the results it can be concluded that chest circumference and thigh length are good predictors of body weight in the bird, similarly the use of original interrelated traits was more appropraite than the orthogonal body shape characters derived from factor analysis for predicting body weight in guinea fowl.

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MASTITIS PATHOGENS IN MILK OF DAIRY COWS IN SLOVAKIA

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ABSTRACT

Mastitis, an inflammation of the mammary gland, is one of the most costly and complex diseases of the dairy cows. This study was done to evaluate the occurrence of mastitis pathogens in milk samples from cows with problematic udder health. Samples of milk for bacteriology were taken from dairy cows in an around Nitra region, Slovakia. For this purpose, the samples from udder quarters were cultured and bacteriologically evaluated. From 390 samples 73.85 % of positive samples were found. The predominant bacterial isolates were Coagulase negative staphylococci (17.95 %), followed by *Escherichia coli* (12.82 %), *Staphylococcus aureus* (9.74 %), *Bacillus* spp. (6.41 %), yeasts (5.64 %), *Streptococcus uberis* (4.1 %), *Staphylococcus epidermidis* (3.59 %), *Pseudomonas aerogenes* (3.33 %), others (bacteria and mould) (3.33 %), *Entrococcus* spp. (3.08 %), *Streptococcus agalactiae* (1.45 %), *Corynebacterium* spp. (1.28 %) and *Staphylococcus chromogenes* (1.03 %). In conclusion, high percentage of positive samples and relatively high occurrence of environmental microorganisms were identified in milk samples indicating the problem with the hygiene of the udder and environment in examined farms.

Key words: mastitis; milk bacteriology; dairy cows

INTRODUCTION

Mastitis can be considered as welfare, food safety and economic problem. Mastitis can cause chemical and bacteriological changes in milk and pathological changes in the mammary gland of the udder (Sharma, 2007). Somatic cell counts (SCCs) mean the number of cells in milk (in the case of mastitis there are mainly white blood cells as an immune response of mammary gland) (Sarikaya *et al.*, 2006) and can indicate intramammary infection (IMI) when elevated (Reksen *et al.*, 2008). SCC is used as a diagnostic tool to monitor subclinical mastitis in dairy herds worldwide (Schukken *et al.*, 2003).

In Slovakia, the problem of environmental mastitis has gradually increased since year 2000. The prevalent pathogens causing mastitis are *Streptococcus uberis*, Coagulase negative staphylococci (CNS), *Escherichia coli*, *Streptococcus dysgalactiae*, and the family of *Enterobacteriaceae* (Vasiľ, 2005). Milk products are influenced by milk quality related to consumer demands (Kubicová and Dobák, 2012).

The most important major pathogens involved in bovine mastitis worldwide are *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Escherichia coli* and *Klebsiella* spp. (Olde Riekerink *et al.*, 2008). The impact of CNS is increasing (Pyörälä and Taponen, 2009), probably because prevalence of major pathogens is decreasing (Sampimon *et al.*, 2009). *Strep. agalactiae* and *Staph. aureus* are considered to be contagious (Barkema *et al.*, 2009), but environmental *Staph. aureus* mastitis may also occur (Zadoks *et al.*, 2002). *E. coli* and *Klebsiella* spp. have mainly an environmental origin (Munoz *et al.*, 2007). Other pathogens have both routes of infection. *Strep. uberis* IMI (intramamary infection) originates mainly from the environment (Pullinger *et al.*, 2006), but can also behave contagious (Zadoks *et al.*, 2003). *Strep. dysgalactiae* behaves intermediate between contagious and environmental transmission (Baseggio *et al.*, 1997).

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For CNS, both environmental and contagious IMI occur (Taponen *et al.*, 2008).

Most of the intra-mammary infections arise during the process of milking or within 2 hours after it, i.e. to the time when the teat canal is fully closed. Tančin *et al.* (2006) described microbial contamination before and after preparation of the udder for milking. The aim of the study was to found out the microbiological contamination of raw milk by pathogens causing mastitis in milk of dairy cows.

MATERIAL AND METHODS

The study was conducted during the period from 2010-2012 in a surroundings Nitra region in Slovakia. A total of 390 milk samples were collected from dairy cows at some different small holder dairy farms, and pathogenic bacteria were examined. The samples were collected from farms with high bulk tank SCC and consequently from cows with possible problems with udder health.

Milk sample collection and laboratory analysis

After a quarter had been cleaned up by removing any possible dirt and washed with tap water, the teat end was dried and swabbed with cotton soaked in 70 % ethyl alcohol. Approximately 100 ml of milk was collected aseptically into sterile bottles, after discarding the first 3 milking streams. Milk samples from each quarter were transported to the Laboratory of Animal Production Research Center in an ice cooled box at 4 ºC and analysed immediately (max. 4 h after collection) either for identification of the clinical mastitis pathogen or to determine the reason for an increased somatic cell count (SCC). The milk samples were investigated for pathogenic mastitis according to a valid procedure of IDF (Bulletin, No.132, 1981).

Statistics: Statistical evaluation of the data was done using Excel program.

Table 1: Proportion of bacterial strains identified by complex examinations of milk from dairy cows within the period of 2010-2012 in Slovakia

Major mastitis pathogens	Year of examination						Proportion of pathogenic	
	2010		2011		2012			
	n_1	%	n_1	%	n_1	%	n_2	%
Contagious pathogens								
Staphylococcus aureus	14	16.47	21	10.82	3	2.70	38	9.74
Streprococcus agalactiae	1	1.18	5	2.58	0	0	6	1.54
Environmental pathogens								
Streptococcus uberis	4	4.71	7	3.61	5	4.50	16	4.10
Escherichia coli (E. coli)	5	5.88	23	11.86	22	19.82	50	12.82
Entrococcus spp.	0	0.00	6	3.09	6	5.41	12	3.08
Bacillus spp.	13	15.29	6	3.09	6	5.41	25	6.41
Minor mastitis pathogens								
Corynebacterium pyogenes	5	5.88	0	0.00	0	0	5	1.28
Coagulase-negative staphylococci	7	8.24	33	17.01	30	27.03	70	17.95
Pseudomonas aeruginosa	0	0.00	13	6.70	0	0.00	13	3.33
Staphylococcus epidermidis	4	4.71	6	3.09	4	3.60	14	3.59
Staphylococcus chromogenes	4	4.71	0	0.00	0	0.00	4	1.03
Yeasts	1	1.18	2	1.03	19	17.12	22	5.64
Others	3	3.53	8	4.12	2	1.80	13	3.33
Total of infected dairy cow quarters	61	71.76	130	67.01	97	87.39	288	73.85
Total of non-infected cow quarters	24	28.24	64	32.99	14	12.61	102	26.15
No. of dairy cow in the herd	85	100.0	194	100.00	111	100.0	390	100.0

n_1 = number of examined dairy cows, n_2 = total number of pathogens, % = the percentage of the number of examined dairy cows
Others = (different types of bacteria and mold)

RESULTS AND DISCUSSION

In Table 1, proportions of bacterial strains identified by complex examination in dairy cows milk are presented. Positive results (infected quarters) were found in 288 samples (73.8 % of the total number of samples) depending on the year of the study. The proportion of bacteriologically negative samples (non-infected quarters) was 26.2 % (102 samples) (and also the effect of year was observed, as shown in Table 1.

Of these 288 isolates, CNS was the most common prevalent in 70 isolates (17.95 %), followed by *E. coli* 50 (12.82 %), *Staph. aureus* 38 (9.74 %), *Bacillus* spp. 25 (6.41 %), yeast 22 (5.64 %), *Strep. uberis* 16 (4.1 %), *Staph. epidermidis* 14 (3.59 %), *Pseudomonas* spp. 13 (3.33 %), others (mixed bacterial and mould) 13 (3.33 %), *Entrococcus* spp. 12 (3.08 %), *Strep. agalactiae* 6 (1.54 %) and *Corynebacterium* spp. 5 (1.28 %) isolates (Table 1). Infections likely caused by *Strep. dsygalactiae* and *Arcanobacterium* spp. were not occurring.

The highest occurrence of intramammary infections in year 2010 was caused by *Staph. aureus* 16.47 %, followed by *Bacillus* spp. 15.29 %, CNS 8.24 %, *E. coli* 5.88 %, *Strep. uberis* 4.71 % and *Corynebacterium* spp. 5.88 % which hasn't occurred at the second and third years of study. While in 2011 the occurrence of CNS was 17.01 %, followed by *E. coli* 11.86 %, *Staph. aureus* 10.82 %, *Pseudomonas aeroginosa* 13.7 % which has only been detected in this year, and *Strep. uberis* 3.61 %. Whoever, in year 2012 only 14 dairy cows (12.16 %) was free from microorganism agents of mastitis. The most of the milk contamination was caused by CNS 27.03 %, *E. coli* 19.82 % and yeasts 17.03 %, while only 2.7 % by *Staph. aureus* , as is shown in Table 1.

Higher incidence of udder infections caused by pathogenic bacteria has been recorded by Ghazi and Niar (2006), and Fandrejewska (1993): 81.4 %, 66.8 % and 65.5 %, respectively. These results are similar to those in our study, where percentage of positive samples reached 73.85 %. Lower percentage of infected milk samples was published by Wilson *et al.* (1997) at the level of 48.5 %. The percentage of culture-negative samples in Netherland has been determined to be approximately 25 % (Barkema *et al.*, 1998), which corresponds to our observation (26.15 %).

In our study, the most frequent bacterial isolate has been found CNS 24.3 % (70 out of 288). We could also found out the increase in CNS occurrence during the study period. Coagulase-negative *Staphylococcus* spp. was isolated from 12.7 to 17.5 % by Makovec and Ruegg (2003). From the study performed on 20 conventional and 20 organic dairy farms, the prevalence of CNS IMI was 14 % on conventional farms and 17 % on organic farms (Pol and Ruegg, 2007). Last mentioned authors

also revealed CNS in 38 % and 30 % of milk samples on conventional and organic farms, respectively. In the study from Germany, 35 % of quarters with subclinical mastitis was caused by CNS (Tenhagen *et al.*, 2006). In the study carried out in the US and Canada, 15 % of new IMIs post-partum were due to CNS (Dingwell *et al.*, 2004). Among 77,051 routine mastitis samples submitted to laboratories in Finland during 2004-2006, CNS were the most frequently isolated bacteria in samples from clinical (18 %) and subclinical (24 %) mastitis cases (Koivula *et al.*, 2007).

Foltys and Kirchnerová (2005) found that the incidence of infections caused by *Staph. aureus* in 2001-2002 decreased from 29.30 to 10.30 %, respectively. Those results are similar to our findings. We found out only 2.7 % occurrence of *Staph. aureus* in 2012 indicating the improvement of the situation with contagious mastitis in dairy practice. There were also published reductions of *Staph. aureus* from 17.7 % in year 1997 to 9.7 % in year 2001 (Makovec and Ruegg, (2003).

E. coli and *Strep. agalactiae* were increased from 15.50 % to 28.20 % and 15.0 % to 20.40 % in 2003 2004, respectively (Foltys and Kirchnerová, 2005). The incidence of infections caused by *E. coli* is very difficult to eliminate in the environment where dairy cows are living. In our study incidence of *E. coli* mastitis was quite high and it superseded streptococcal mastitis. It could be due to poor hygiene conditions, as it infects the udder through teat canal (Sumathi *et al.*, 2008).

In our study incidence of mastitis due to yeast was found to be higher than *Strep. uberis* and *Strep. agalactiae.* Sporadic incidence of mastitis due to yeast has been reported by Ebrahimi and Nikookhah (2005). Stored antibiotics kept for repeated use may become contaminated with yeast and act as primary source of yeast and subsequent udder infection (Schalm, 1971). Tissue injury may also be helpful in establishing a mycotic mastitis. This obviously emphasizes the importance of strict aseptic measures in udder therapy with antibiotics.

CONCLUSIONS

Mastitis bacteriology, when used optimally as discussed, is an essential and cost effective tool in the ongoing control of mastitis and milk quality. Coagulase negative staphylococci (CNS) have been the most common bacteria identified in the whole survey. This means the impact of CNS is increasing, probably because prevalence of major pathogens is decreasing. Otherwise, the high frequency of CNS and *E. coli* occurrence indicated insufficient hygiene of housing and milking causing the risk of environmental mastitis.

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