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HAEMOGLOBIN POLYMORPHISM IN SELECTED FARM ANIMALS-A REVIEW

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Review paper

Abstract: Biochemical diversity or polymorphism is the occurrence of varieties attributed to biochemical differences which are under genetic control. It has created a leeway for the genetic improvement of farm animals. This is because it can be used as a useful tool for the characterization of livestock breeds and population. This way, the degree of similarity or differences within and between breeds can be ascertained and this differences or similarity are important raw materials for genetic improvement of animals. Data obtained on gene frequencies and genotypes through polymorphism study makes it not only possible to compare the gene stocks of animals, the possible effects of the genes on reproductive and performance traits, but also study genetic variability under different environmental conditions of selection. This study was carried out to review haemoglobin (Hb) polymorphism in selected farm animals with the view of finding out the type of polymorphism observed by starch gel electrophoresis due to variation in the amino acid sequence in the polypeptide chains of Hb. The review showed clearly that there is a gene-controlled diversity in the different farm animals considered. This could serve as a reference point for future studies earmarked for the improvement of the animals possibly via marker-assisted selection.

Key words: Biochemical polymorphism, haemoglobin, livestock, gene and genotype frequencies.

Introduction

Genetic differences exist in all farm animals which lead to variability in the reproductive and performance abilities of animals both within, and between breeds. Differentiating this variability could be a basis for selection and subsequent genetic improvement of farm animals. Biochemical polymorphism study is one way of delineating genetic variation in animals. *Osterhoff (1964)* defined polymorphism as the occurrence together of two or more varieties in the same population at the same

time in such proportions that the rarest of them cannot be maintained by mutation alone. Biochemical polymorphism on the other hand as defined by the same author, is the occurrence of varieties attributed to biochemical differences which are under genetic control. A population is said to show polymorphism when two or more distinctly inherited varieties coexist in the same individual *(Das and Deb, 2008).* This type of polymorphism is increasingly being used in the study of genetic variation within and between populations and to estimate genetic divergence *(Lee et al., 1995).* This is because the biochemical elements (blood proteins and enzymes) can be used widely as biomarkers of corresponding structural genes. These biomarkers are not affected or do not depend on environmental factors and this makes them suitable for genetic studies.

Recently, a large number of biochemical polymorphic characters of farm animals have been scrutinized. Characters such as transferrin, Hb, albumin, alkaline phosphatase etc have been reported to exhibit polymorphism *(Das and Deb, 1995).* This study has become imperative because of their importance in the improvement of farm animals, and the fact that some polymorphic alleles may be connected or linked with traits of economic importance due to pleiotropic effect, or general heterozygosity. For instance, marginally but not significant better body weight had been reported in Hb BB type animals *(Arora et al., 1971).* In sheep however, Hb AB and Hb BB had been observed to have an effect on sheep performance *(Dally et al., 1980; Barowicz and Pacek, 1984; Arora, 1984; Dratch et al., 1986).* Blood protein polymorphisms have been used to study evolutionary relationships in many animals *(Kalab et al., 1990; Emerson and Tate, 1993; Buchanan et al., 1994; Guney et al., 2003; Malan et al., 2003; Yang and Jiang, 2005; Al-Samarrae et al., 2010, Akinyemi and Salako, 2010; Yakubu and Aya, 2012; Agaviezor et al., 2013; Yakubu et al., 2014).* Although there are more advanced technologies for genetic studies of polymorphism in farm animals, the economic situations and inadequacy of infrastructural facilities in developing countries means that the importance of such studies using starch gel electrophoresis cannot be discountenanced. The objective of this write up therefore, is to undertake a review of relevant literature on Hb polymorphism in selected farm animals (cattle, sheep, goat and chicken).

Haemoglobin

Haemoglobin is a blood protein which contain four globin chains (two each of alpha and beta), and a prosthetic group called haem bound to each other *(de Souza and Bonilla-Rodriguez, 2007).* Human haemoglobin was the first protein analysed *(Osterhoff, 1964)* and the most studied blood protein *(Yakubu and Aya, 2012).* It is the respiratory carrier of oxygen and carbon dioxide to and from body tissues and cells. The haem portion of Hb is alike in all forms of Hb with genetic variation restricted to the structure of the globin portion only *(Chineke et al., 2007).*

Since there are structural differences at the globin portion of Hb, variants of it are likely to result and these variants may confer or limit animal's abilities/productivities, or confer certain advantages. *Ndamukong (1995)* reported on selective advantages in different geographical regions due to different Hb types. Such selective advantages include but not limited to resistance to helminthic infestation *(FAO, 1988; Ndamukong, 1995),* effect on meat quality *(Bezova et al., 2007),* productive traits *(Dally et al., 1980; Barowicz and Pacek, 1984; Arora, 1984; Dratch et al., 1986; Henkes et al., 2000; Boonprong et al., 2007)* and, hair and hair length *(Akinyemi and Salako, 2010). Di Stasio (1997)* opined that this could be due to better functional properties of the Hb molecule concern as a result of greater affinity for oxygen and higher Hb concentration and packed cell volume. *Thompson and Thompson (1980)* and *Peters et al. (2004)* reporting on the importance of Hb stated that, it has revealed more about the molecular basis of human, animal and medical genetics, vital for the part it plays in demonstrating the relationship between genetic information and protein structure, illustrated the mechanisms of new gene formation other than by point mutation as well as its importance in shedding more light on the causes of a variety of genetic disorders of the blood. The importance of Hb can therefore not be over-emphasized.

Haemoglobin polymorphism

Cattle. Haemoglobin polymorphism was first actually reported in the Algerian hill cattle *(Cabannes and Serain, 1957; Pal and Mummed, 2014).* Haemoglobin polymorphism in cattle seems to be breed influenced as some breeds have been shown to show a clear polymorphism with two alleles (AA, BB) and their possible phenotypes (AA, AB and BB), while others present only one allele type-AA *(Osterhoff, 1964).* Most studies have however reported on the occurrence of the two co-dominant alleles Hb A and Hb B *(Naik et al., 1968; Henkes et al., 2000; Pal and Mummed, 2014).* Apart from the co-dominant Hb A and Hb B types, other variants have also been reported such as Hb X, with a motility that is intermediate between those of Hb A and Hb B in Indian cattle *(Naik et al., 1965).* Another variant Hb C whose electrophoretic motility is similar to that of Hb X was also reported in the American Brahmin cattle by *Crockett et al. (1963).* Other variants reported include Hb Khillari with motility slower than that of Hb A in Indian cattle *(Naik and Sangvi, 1964),* and still another Hb variant which was slower than Hb A in Muturu cattle of West Africa *(Efremov and Braend, 1965). Majid et al. (1994)* observed a single type of Hb pattern; Hb AB in Tamaraw (*Bubalus mindorensis*) cattle in the Philippines. *Schwellnus and Guerin (1977)* also observed the presence of Hb C variant in Brahman and seven indigenous cattle breeds of Southern Africa. *Ahmed et al. (2010)* however, reported four Hb variants in their study with cows and buffaloes with allelic frequencies of 0.51, 0.33, 0.014 and 0.01 for Hb A, Hb B, Hb C and Hb D, respectively. *Pal and Mummed (2014)*

reported an allele frequency of 0.709 and 0.291 for genotypes Hb AA and Hb AB, and gene frequencies of 0.502, 0.411 and 0.084 respectively for Hb AA, Hb AB and Hb BB in Ogaden cattle in Ethiopia which is consistent with gene frequencies reported for Somalian Boran and Dawara cattles *(Di Stasio et al., 1980)* and Bunaji cattle of Nigeria *(Essien et al., 2011)*. High prevalence of Hb AA has also been observed in *Bos taurus* catlle *(Braend, 1972; Tejedor et al., 1986).* It has been suggested that Hb type may have been influenced by environmental factors *(Bangham and Blumberg, 1958; Balakrishnan and Nair, 1966; Sen et al., 1966; Ajuwape and Antia, 2000; Tibbo et al., 2005; Essien et al., 2011)*. The mechanism for such polymorphism has however not been made clear *(Al-Samarrae et al., 2010)* and needs to be further studied. Equally, no direct evidence exists of differences among the three Hb phenotypes (AA, AB and BB) for fitness has been found in cattle *(De Vito et al., 2002). Bangham (1963)* reported that Hb type did not significantly affect milk yield and butterfat percentage in dairy cattle.

Sheep. The existence of three major Hb types (AA, AB, and BB) caused by Hb A and Hb B genes and the existence of some rare Hb types have been reported in the sheep *(Evan et al., 1958a; Missohou et al., 1999; Miresa, 2003; Mohri et al., 2005; Al-Samarrae, 2006; McManus et al., 2009; Tsunoda et al., 2010; Al-Samarrae et al., 2010)*. In contrast to other animals, Hb A is the fastest moving Hb in the sheep based on electrophoretic motility *(Osterhoff, 1964)*. Different frequencies have been reported for the different Hb variants in the sheep. *Meyer (1963)* for instance, demonstrated the frequency of Hb A gene varying from 0.00 in the English Dartmoor breed, to 0.97 in the German Heid-Schnukle sheep. *Rao and Panandam (1998)* observed an allelic frequency of 0.16 (Hb A), 0.30 (Hb B), and 0.54 (Hb AB) with a gene frequency 0f 0.43 (Hb A) and 0.57 (Hb B) in Santa Ines hair type sheep population. *Al-Samarrae et al. (2010)* observed high frequencies; 0.98, 0.99 and 0.97 for Hb B in local Awassi, Arrabi and Karradi sheep breeds of Iraq while *Akinyemi and Salako (2010)* reported high frequency for Hb A in West African Dwarf sheep of Nigeria. Like in cattle, attempts have been made to link sheep Hb type with geographical location. *Huisman et al. (1958)* and *Evans et al. (1958a, b)* reported that sheep reared in higher altitude have a higher frequency of Hb A, and equally show a higher oxygen affinity. *Evans et al. (1958a)* observed that mountain and hill sheep breeds particularly of the Northern parts of Britain tend to have higher Hb A while those of the lowland tend to have Hb AB and Hb B, respectively. *Pieragostini et al. (2006)* observed that Hb A is found more frequently in sheep living above 40^{o} C latitude, while *Sun et al. (2007)* opined that sheep with Hb B were better able to withstand the stress associated with acute hypoxia compared to those with Hb A. *Akinyemi and Salako (2010)* reported a higher frequency of Hb A in West African Dwarf sheep of Nigeria as one moves towards the forest belt of the country. Similar trend was observed in the Yankasa sheep by *Tella et al. (2000)* as one move into the forest belt of Nigeria. In

their study of Balami, Uda and Yankasa sheep breeds in the Northern part of Nigeria however, *Akinyemi and Salako (2012)* were able to show that Hb B was more predominant with allele frequencies of 0.75, 0.90 and 0.81 recorded for the Balami, Uda and Yankasa sheep, respectively. The predominance of Hb B over Hb A in sheep has also been reported by other authors *(Clarke et al., 1989; Zanoti Cassati et al., 1990; Bunge et al., 1990; Boujenane et al., 2008; Shahrbabake et al., 2010; Mwacharo et al., 2002).* The difference in Hb allele of the sheep has been adduced to be due to selective advantages in the different geographical regions in which the animal finds itself. The selective advantage appears to be of adaptive significance in the regions in which the sheep finds itself, and possibly have an effect on its performance. For instance, *Tsunoda et al. (2006)* however reported that Hb A has a relatively high affinity for oxygen and is therefore very important for survival of the sheep in mountainous areas at latitude above 3000 m, while *Nihat et al. (2003)* in their study using Merino sheep crosses, showed that ewes with Hb types AB gave better lamb birth weight.

Goat. According to *Schmid (1962)*, there are two different Hb types in goat (Hb A and Hb B). Other authors who also reported on only two Hb types in the goat include: *Khanilkar et al. (1963), Garzon et al. (1976), Turker et al. (1983), Barbancho et al. (1984), Bhat (1986, 1987) and Tunon et al. (1987). Elmaci (2001)* also observed two Hb phenotypes controlled by the two co-dominant autosomal alleles Hb^A and Hb^B in hair goat breeds raised in Turkey with frequency of Hb^A being considerably higher than Hb^B allele. *Agaviezor et al. (2013)* however, reported the incidence of three Hb types (Hb AA, Hb AB and Hb BB) in the Red Sokoto goat, Hb AA and Hb AB in Sahelian goat, and only Hb AA in West African Dwarf goats sampled in the Niger Delta area of Nigeria. The gene frequencies for the alleles were: 0.80 and 0.20 (Red Sokoto goat), 0.77 and 0.23 (Sahelian goat), and 1.00 and 0.00 (West African Dwarf goat), respectively. In their own study of the West African Dwarf goat in the North Central region of Nigeria, *Yakubu et al. (2014)*, detected three co-dominant alleles, causing the presence of three genotypes (AA, AB and AC), with frequencies of 0.69, 0.30 and 0.01, respectively. They observed corresponding genotype frequencies of 0.37, 0.61 and 0.02, respectively. *Nafti et al. (2013)* reported frequencies of 0.779 and 0.757 (Hb AA), 0.206 and 0.226 (Hb AB), and 0.013 and 0.017 (Hb BB) for Arbi and Serti goats of Tunisia. In a pooled population of Indian Malabari goats, *Bindu and Raghavan (2010)* reported on the predominance of Hb A allele (0.927) over that of Hb B allele (0.012). *Johnson et al. (2002)* also discovered that Hb A was the only allele found in adult Batinah and Jebel Akhdar goats while studying three breeds of Omani goats. They equally observed that 34% of Dhofari goats were homozygous for Hb AB while 66% were heterozygous for Hb A and Hb B, respectively. The works of *Agaviezor et al. (2013) and Yakubu et al. (2014)* clearly reflected the predominance

of Hb A over Hb B in West African Dwarf Nigerian goat breeds. This could be an adaptive feature for survivability of the West African Dwarf goat.

Poultry. Haemoglobins are heterogeneous in the fowl *(Osterhoff, 1964). Rodnan and Ebaugh (1957)* were of the opinion that the synthesis of fowl Hb is controlled by at least three genes. *Dimri (1978)* reported that three types of Hb have been observed in poultry which are controlled by two autosomal alleles A1 and A2, respectively. *Mazumder and Mazumder (1989)* observed the frequency of normal Hb gene in White leghorn to be 0.96, 1.00 in broilers, 1.00 in local fowl, 1.00 in the guinea fowl, and 0.85 in quail with corresponding figures for the normal mutant alleles being 0.04, 0.00, 0.00, 0.00 and 0.15, respectively. *Al-Samarrae et al. (2010)* from their work with White leghorn and native Iraqi chickens, observed both A and B Hb alleles at a gene frequency of 0.65 and 0.35 (Leghorn) and, 0.54 and 0.46 for the native Iraqi chickens, respectively. *Yakubu and Aya (2012)* analysed three indigenous Nigerian chickens (normal feathered, naked neck and Fulani ecotype), respectively and observed the frequencies of the A and B genes to be 0.68 and 0.32 (normal feathered), 0.71 and 0.29 (naked neck), and 0.75 and 0.25 (Fulani ecotype), respectively. The corresponding genotype frequencies for AA, AB and BB alleles were 0.54 and 0.28 (normal feathered), 0.58, 0.27 and 0.15 (naked neck), and 0.62, 0.26 and 0.12 for the Fulani ecotype chicken, respectively. They equally reported that, while the gene and genotype frequencies of naked neck and Fulani ecotype chickens were in Hardy-Weinberg equilibrium, those of the normal feathered birds deviated from the theoretical proportions. The three Hb types reported by *Yakubu and Aya (2012)* are consistent with the earlier findings of *Ugur et al. (2006)* in Chuckars and Pheasant, and *Salako and Ige (2006)* in indigenous chickens of South-West Nigeria. *Okamoto et al. (2003)* however reported that in general, Asian native fowls were being fixed at the Hb B locus with Hb A detected at extremely low frequencies in some chickens. Haemoglobin polymorphisms have been linked to some performance or survivorship traits in the chicken. *Washburn et al. (1971)* inferred that chickens of the homozygous mutant Hb genotypes were approximately 20% less susceptible to Marek's disease. *Dimri (1978)* reported that Hb polymorphism affects growth rate and hatchability, with the highest hatchability recorded for AA (62.20%) followed by AB (48.20%) and BB (31.50%), respectively. *Al-Murrani et al. (1996)* however did not observed any significant relationship between Hb type and some measures of resistance to coccidiosis in both native Iraqi and White leghorn chickens.

Conclusion

It is clear that there is gene-controlled Hb polymorphism in the animals treated and this could be useful in marker-assisted selection of animals for

subsequent genetic improvement programmes. The possibility of tying the different genotypes to performance indices makes it all the more useful as animals could be selected based on their selective advantages for geographical region, resistance to pests and disease infestation and other economic factors of interest to farmers. Although simple starch gel electrophoresis could bring out the polymorphic differences existing in the Hb of farm animals, there is the need for more sophisticated screening of blood proteins using microsatellite markers and single nucleotide polymorphisms. This way more informed decisions could be made.

Polimorfizam hemoglobina u izabranim vrstama domaćih životinja

S.S.A. Egena, R.O. Alao

Rezime

Biohemijska različitost i polimorfizam je pojava varijeteta koja se pripisuje biohemijskim razlikama koje su pod genetskom kontrolom. Polimorfizam je stvorio slobodu za genetsko unapređenje domaćih životinja, zbog toga što se može koristiti kao alat za karakterizaciju rasa domaćih životinja i stanovništva. Na ovaj način, stepen sličnosti ili razlike unutar i između rasa može da se utvrdi, a ove razlike ili sličnosti su važne za genetsko unapređenje životinja. Dobijeni podaci o frekvencijama gena i genotipovima kroz studije polimorfizma čine mogućim ne samo poređenje gena životinja, mogućih uticaja gena na reproduktivne i performanse osobina, već i ispitivanje genetičke varijabilnosti pod različitim uslovima životne sredine selekcije. Ova studija je sprovedena sa ciljem da razmotri polimorfizam hemoglobina (Hb) u odabranim vrstama domaćih životinja sa ciljem pronalaženja tipa polimorfizma posmatrano preko elektroforeze na skrob gelu, usled varijacije u sekvenci amino kiselina u polipeptidnim lancima Hb. Pregledni rad jasno pokazuje da postoji diverzitet pod kontrolom gena u različitim vrstama domaćih životinja. To bi moglo da posluži kao referentna tačka za buduće studije namenjene unapređenju životinja možda korišćenjem selekcije pomoću markera.

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AMOUNTS OF ESSENTIAL AND NON-ESSENTIAL AMINO ACIDS AND THE RATIO IN LITHUANIA BRED CATTLE MEAT

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Abstract: The aim of the research was to determine the amounts of essential and non-essential amino acids and the ratio in various breed cattle meat. The content of amino acids and the ratio at the longest dorsal muscle (*musculus longissimus dorsi*) was determined analyzing Angus, Hereford, Charolais, Limousine purebreds, Lithuanian Black and White x Charolais (LTBWxCHA) crossbreed, Lithuanian Black and White x Limousine (LTBWxLI) crossbreed, Lithuanian Black and White x Simmental (LTBWxSI) crossbreed and Lithuanian Red x Limousine (LTRxLI) crossbreed. Analyzing the research results it was noticed that Lithuanian Black and White cattle breed meat contained the highest total amount of amino acids and Angus breed cattle meat contained the smallest amounts. The difference amounted to 33.87 g/kg or 4.1 percent ($p<0.01$). While comparing total amounts of amino acids at the purebred cattle breeds and the crossbreeds, it was noticed that the meat of purebred cattle contained higher amounts of amino acids, the difference ranging between 18.54 and 19.00 g/kg or 2.23-2.28 percent ($p<0.01$). Purebred cattle meat contains higher amounts of essential amino acids compared to crossbreed cattle meat. The meat of Aubrac and Angus breeds was determined to have the highest meat protein sufficiency rate. The lowest biological values were determined at Lithuanian Black and White x Charolais crossbreed meat. The highest amount of the amino acid leucine was observed in Aubrac breed cattle meat ($p<0.05$). The difference of the leucine amounts reached up to 0.45 g/kg ($p<0.01$) comparing to various crossbreed cattle meat.

Keywords: amino acids, essential, non-essential, breed, cattle, *longissimus dorsi.*

Introduction

Biological value of cattle meat is determined by essential or indispensable amino acids and lipid structure *(Serra et al., 2004; Raes et al., 2004)*.

The nutritional protein value depends upon its amino acid composition's ability to meet the organism's needs. *(Culioli et al., 2003)*. In order to determine the nutritional value of meat more exactly the tryptophan and oxiproline ratio is used, where tryptophan denotes the amount of complete proteins and oxiproline denotes the amount of incomplete proteins in the meat.

Meat quality is affected by many physical and (bio) chemical changes involved during the post-mortem conversion of muscle to meat *(Valin et al., 1992; Radovic et al., 2013)*. Significant differences in the balance of amino acids have been shown between species *(Sawyer, 1975)* and between muscles (*Lawrie, 1991; Purchas et al., 2009)*. Muscle enzymes are responsible for most of these changes and muscle aminopeptidases contribute to the generation of free amino acids in post *mortem* which improve the nutritional value *(Toldra et al., 1995)* and can affect flavour as taste enhancers or precursors of aroma compounds *(Toldra et al., 1997)*. Animal meats like beef have a score of approximately 0.9, compared with values of 0.5–0.7 for most plant foods. The amino acid glutamic acid/glutamine is present in meat in the highest amounts (16.5%), followed by arginine, alanine and aspartic acid *(Williams, 2007)*. The concentration for each amino acid is important for its contribution to taste *(Kato et al., 1989)*.

The aim of the research was to determine the composition of amino acids and their ratio at the longest dorsal muscle of different breeds and crossbreeds cattle grown in Lithuania under the same conditions. Controlled cattle rearing were carried at the company UAB "Šilutės Breeding Station" at identical feeding and storage conditions.

Material and methods

The study investigated the amounts of amino acids at the longest dorsal muscle (*Musculus longissimus dorsi*) of bulls reared at controlled rearing station at identical feeding and storage conditions, slaughtered at the age of 500 days concerning Angus, Hereford, Charolais and Limousine breeds, Lithuanian Black and White crossbred with Charolais (LTBW x CHA), Lithuanian Black and White crossbred with Limousine (LTBW x LI), Lithuanian Black and White crossbred with Simmental (LTBW x SI) and the Lithuanian Red crossbred with Limousine (LTR x LI).

The amounts of amino acids in bovine meat was determined while examinations of dry material applying following methods: technical regulation for assessment of the amounts of amino acids and olakvindox in the feed, Nr. 104-

3302 (EU directive 98/64/EB). The amount of amino acids in the meat was determined using automatic analyser for amino acids AAA 400.

"Microsoft Corporation Excell 2007" program was used for data analyses for calculating Arithmetic average (X), standard errors of arithmetic average (Sx), statistical reliability degree of groups (p).

Results and discussion

The Tables 1 and 2 present the amounts of essential or indispensable amino acids in the meat of different breed and crossbreed cattle.

Table 1. The amounts of essential amino acids in the meat of pure breed cattle, g/kg

	Cattle breed denomination					
Amino acids	**LTBW**	**AN**	**AU**	**HE**	**CHA**	**LI**
Threonine	36.76±1,02	33.43±0.25	35.97±2.47	35.67±2.22	35.82±0.07	35.74±0.34
Valine	42.29±2,08	46.09±1.98	41.87±2.01	41.43±0.98	41.65±0.93	41.54±1.01
Methionine	30.99±1,67	35.67±4.31	31.74±1.24	30.82±0.74	31.28±3.05	31.05±1.21
Isoleucine	43.24±0,94	42.06±1.73	42.14±2.60	41.70±2.23	41.92±0.66	41.81±0.67
Leucine	65.62±1,09	63.31±2.21	66.06±1.22	65.35±3.42	65.70±0.73	65.52±0.78
Phenylalanine	33.63±1,28	31.63±0.91	32.78±2.17	32.401.56	32.59±0.18	32.49±0.21
Lysine	66.00±2,42	71.83±1.67	68.27±1.99	69.61±4.33	68.74±1.27	68.48±1.78
Total:	318.53	322.53	318.83	316.98	317.70	316.63

Table 2. The amounts of essential amino acids in the meat of crossbreed cattle, g/kg

	Cattle crossbreed denomination		
Amino acids	**LTBWxCHA**	**LTBWxLI**	**LTBWxSI**
Threonine	35.78±0.45	35.76±0.67	35.77±1.54
Valine	41.60±1.28	41.57±2.02	41.58±0.56
Methionine	31.16±1.20	31.10±1.67	31.13±3.23
Isoleucine	41.86±0.87	41.84±0.95	41.85±0.84
Leucine	65.61±2.67	65.57±2.54	65.59±1.83
Phenylalanine	32.54±0.34	32.52±0.54	32.53±1.56
Lysine	68.61±1.32	64.54±2.87	68.57±1.84
Total:	317.16	312.90	317.02

The data in Table 1 and Table 2 proves, that the highest amount of essential amino acids is observed in AN cattle meat and the lowest amount is observed in LTBWxLI crossbred cattle meat. The difference amounted to 9.63 g/kg or 4.1 percent ($p<0.001$). Comparing the total amounts of essential amino acids in purebred cattle meat to the amounts in crossbreed cattle meat it is observed that purebred cattle meat contained higher amounts. AN breed cattle meat was noticed to have the highest amounts of following amino acids: valine, methionine and lysine ($p<0.01$). Similar results were obtained by other authors who have studied the ratio of amino acids in the bovine meat *(Lawrie, 1991; Purchas et al., 2009)*. The highest amount of the amino acid leucine was observed in AU breed cattle meat ($p<0.05$). The difference of the leucine amounts reached up to 0.45 g/kg ($p<0.01$) comparing to various crossbreed cattle meat. Similar results were obtained by other authors who have studied the amino acid amounts and the ratio in the bovine meat (*Williams, 2007*). The amounts of non-essential amino acids in the pure bred cattle meat are presented in the Table 3 and Table 4.

Table 3. The amounts of non-essential amino acids in the meat of purebred cattle, g/kg

	Cattle breed denomination					
Amino acids	**LTBW**	**AN**	**AU**	**HE**	**CHA**	**LI**
Aspartic acid	77.47±0.56	74.67±0.32	77.13±1.15	76.26±2.53	76.69±0.86	76.47±0.56
Serine	35,13±1.12	32.10±1.06	32.54±0.84	32.51±3.15	32.52±1.92	32.51±0.43
Glutamic acid	150.3±2.12	140.75±6.58	146.65±0.10	145.76±6.61	142.21±0.57	145.98±2.12
Proline	29.93±1.45	28.35±0.35	29.65±2.52	29.61±1.73	29.63±0.77	29.62±0.24
Glycine	39.42±2.10	40.64±1.14	37.39±0.11	37.10±2.11	37.24±4.19	37.17±0.43
Alanine	43.23±0.91	47.65±0.65	45.63±0.83	44.92±0.85	43.28±0.30	45.10±0.67
Tyrosine	27.61±1.19	25.62±0.64	26.92±2.02	26.15±2.17	26.53±0.07	26.34±1.69
Histidine	43.27±0.87	46.84±0.54	47.99±1.07	48.69±0.32	45.34±2.81	48.51±1.56
Arginine	50.15±3.01	50.53±0.06	56.43±3.00	54.18±3.23	51.31±1.12	56.24±2.56
Total:	495.51	488.15	500.33	495.18	496.75	479.94

Table 4. The amounts of non-essential amino acids in the meat of crossbreed cattle, g/kg

Amino acids	Cattle crossbreed denomination		
	LTBW x CHA	**LTBW x LI**	**LTBW x SI**
Aspartic acid	76.58±1.09	76.53±0.89	76.56±0.58
Serine	32.52±1.12	32.52±2.12	32.52±0.85
Glutamic acid	146.09±1.23	146.04±1.38	146.07±1.43
Proline	29.62±0.67	29.62±0.89	29.62±1.67
Glycine	37.21±0.56	37.19±0.65	37.20±0.79
Alanine	45.19±0.82	45.14±1.29	45.16±1.08
Tyrosine	26.44±0.78	26.39±1.23	26.41±1.59
Histidine	48.42±1.34	48.47±2.17	48.45±2.06
Arginines	56.27±1.02	56.26±1.93	56.27±1.02
Total:	498.34	498.16	498.26

The data in Table 3 and Table 4 illustrates the changes of amounts of non-essential amino acids in various breed cattle meat. The highest amount of non-essential amino acids was observed in CHA breed cattle meat, the lowest in the LI breed cattle meat. The difference amounted up to 16.81 g/kg ($p<0.01$). Concerning various crossbreed cattle meat, the amounts of non-essential amino acids were alike. The ratios of oxiproline and tryptophan amino acids are presented in the Table 5 and Table 6 below.

Table 5. The ratio of amino acids oxiproline and tryptophan in the meat of purebred cattle.

Amino acids	Cattle crossbreed denomination		
	LTBWxCHA	**LTBWxLI**	**LTBWxSI**
Oxiproline	69.43±0.92	69.60±0.12***	54.07±1.20
Tryptophan	295.70±0.32	330.91±0.28	266.11±1.52
Tryptophan / Oxiproline ratio	4.47	4,85	5.23

*p<0.05; **p<0.01; ***p<0.001.

Table 6. The ratio of amino acids oxiproline and tryptophan in the meat of various crossbreed cattle.

Amino acids	Cattle crossbreed denomination		
	LTBWxCHA	**LTBWxLI**	**LTBWxSI**
Oxiproline	69.43±0.92	69.60±0.12***	54.07±1.20
Tryptophan	295.70±0.32	330.91±0.28	266.11±1.52
Tryptophan / Oxiproline ratio	4.47	4,85	5.23

*p<0.05; **p<0.01; ***p<0.001.

The data in Table 5 and Table 6 presents the nutritional value of bovine meat. The data proves, that the highest meat protein sufficiency ratio is observed in Au and AN breed cattle meat. All the purebred cattle have a meat protein sufficiency ratio greater than 5, and the meat is of high biological value. Concerning crossbred cattle meat, the highest quality of meat was determined in LTBW x SI. The lowest biological value was observed in LTBW x CHA crossbred meat. Similar results were obtained by other authors who have studied the ratio of amino acids in the bovine meat *(Jukna et al., 2013).*

Conclusions

The highest amount of essential amino acids was observed in AN breed cattle meat, the lowest in LTBW x LI crossbreed meat. The difference amounted to 9.63 g/kg or 4.1 percent ($p<0.001$). The AN breed cattle meat was observed to have the highest amounts of following essential amino acids: valine, methionine and lysine ($p<0.05$).

The highest meat protein sufficiency ratio was observed in Au and AN breed cattle meat. All the purebred cattle have a meat protein sufficiency ratio greater than 5, and the meat is of high biological value. Concerning crossbred cattle meat, the highest quality of meat was determined in LTBW x SI. The lowest biological value was observed in LTBW x CHA crossbred meat.

The purebred cattle meat was observed to have a higher total amount of amino acids comparing to crossbred cattle meat. No significant differences were observed concerning the amounts of amino acids in meat of separate cattle breeds.

Having results concerning the composition of amino acids of different cattle breeds and the composition's changes following crossbreeding it is possible to obtain bovine meat with more appropriate ratio of amino acids.

Esencijalne i ne-esencijalne amino kiseline i njihov odnos u meso goveda gajenih u Litvaniji

V. Jukna, Č. Jukna, E. Meškinytė – Kaušilienė

Rezime

Cilj istraživanja bio je da se utvrde količine esencijalnih i ne-esencijalnih amino kiselina i njihov odnos u mesu različitih rasa goveda. Sadržaj aminokiselina i odnos u leđnom mišića (*musculus longissimus dorsi*) određen je analizom mesa goveda sledećih rasa: angus, hereford, šarole, limuzin, u čistoj rasi, melezu litvanske crno & bele rase x šarole (LTBVkCHA), melezu litvanske crno & bele rase x limuzina (LTBVkLI) melezu litvanske crno & bele rase x simentalske rase (LTBVkSI) i melezu goveda litvanske crvene rase x limuzina (LTRkLI). Analizirajući rezultate istraživanja uočeno je da je meso goveda litvanske crno & bele rase sadržavalo najviše ukupnih aminokiselina a meso goveda rase angus najmanje iznose. Razlika je iznosila 33,87 g/kg ili 4,1 % ($p<0,01$). U poređenju ukupne količinu aminokiselina u mesu čistokrvnih goveda i melez, primećeno je da meso čistokrvnih grla sadrži veće količine aminokiselina, razlika je bila u rasponu od 18.54 i 19.00 g/kg ili 2.23-2.28 % ($p<0,01$). Meso čistokrvnih goveda sadrži veće količine esencijalnih aminokiselina u poređenju sa mesom meleza. U mesu goveda rasa aubrac i angus rasa utvrđena je najveća najveća stopa dovoljnosti proteina u mesu. Najniže biološke vrednosti su određene u mesu meleza litvanske crno & bele rase x šarole. Najveći iznos amino kiseline leucin je registrovan u mesu goveda aubrac rase ($p<0,05$). Razlika u količini leucina dostigla je 0.45 g/kg ($P<0.01$) u odnosu na meso ostalih meleza.

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LIFETIME PRODUCTION OF HIGH-YIELDING DAIRY COWS

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Abstract: Lifetime milk production is a key success factor in fulfilling the production potential of high-yielding cows. Lifetime milk production traits are pronouncedly variable. The life expectancy and the length of productive life of dairy cows are repeatedly limiting factors for improving lifetime milk production. Lifetime milk production is greatly depended on age at first calving and the number of lactations during productive life. Previous researches have implied there are real chances for improving the lifetime milk production of high-yielding cows. The goal of this research was to investigate the significance of key systematic factors on the lifetime production of high-yielding Black-and-White cows. The animals included in the sample had different share of Holstein genes. The researchers determined systematic factors that caused some significant phenotypic variations of the investigated trait. The average lifetime milk production was 25,002.66±7,755.39 kg. When observed by cow genotypes, the mean values of the lifetime milk production varied from 27,061.37 kg (<58% HF) and 24,761.26 kg (58-73% HF) to 23,185.36 kg (>73% HF). The differences in lifetime milk production determined among the animals were due to a highly significant ($p \leq 0.01$) impact of the bulls – the sires of the cows and the year of culling; the impact of the class of HF genes was significant ($p \leq 0.05$), whereas the impact of the reason for culling was non-significant ($p > 0.05$).

Key words: Holstein-Friesian cows, production, factors of impact

Introduction

The key factor of milk production economic efficiency is the lifetime production of high-yielding cows. Higher lifetime milk production ensures better economic results per cow. Economic efficiency is mostly a result of achieved milk production and longevity (*Heins et al., 2012; Martens and Bange, 2013*). Lifetime

milk production depends on age at first calving, the length of productive life and milk yield in certain lactations (*De Vries, 2008*).

An important characteristic of lifetime production is a high variability. Many factors can affect the lifetime productivity of high-yielding cows, most important of which is a breed, breed selection, the environment, feeding and fertility *(Petrović et al., 2007; Terawaki and Ducrocq, 2009)*. The genetic potential of an animal, expressed in existing conditions on a farm, is particularly important (*Páchová et al., 2005*). Milk yield per lactation has greatly increased in the last couple of decades, whereas in the same period productive life has shortened (*Hare et al., 2006*).

Higher lifetime milk production means that fewer cows are needed for the same scope of milk production on farms. The productive life and life expectancy of high-yielding cows under normal conditions enable improved lifetime milk production. In herds with intensive milk production, there are some problems that can considerably decrease economic results. The problems are often manifested as health disorders, infertility problems, and a high percentage of culling, short productive life and short life expectancy of cows (*Dechow et al., 2008*).

The odds for culling increase greatly with every next lactation (*Fetrow et al., 2006*). More culling per year shortens the period of productivity, thus decreasing the lifetime milk production of cows. Longer productive life ensures more lactations with higher milk production *(Donaldson, 2006)*. Longer life, higher milk production and more calves are characteristics of cows with good body composition (*Norman et al., 2007*). The utilisation rate of a cow is important in terms of determining the proportion of non-productive period in the total lifetime of a cow. If cows have more lactations, breeding costs to the time they reach sexual maturity are to be apportioned to a larger amount of milk obtained during several lactations.

Previous researches have implied there are great possibilities for improving the lifetime production of high-yielding cows. It is necessary to know the importance of environmental impact on the results of the lifetime production of high-yielding cows in order to include it in the model. Considering the importance of each systematic factor, the authors of this research gave their unbiased assessment to evaluate the results as accurate as possible.

The goal of this research was to investigate the significance of key systematic factors on the lifetime production of high-yielding Black-and-White cows with different share of Holstein genes.

Material and Methods

The investigation and analysis of key systematic factors on achieving lifetime production was conducted on a herd of Black-and-White cows. The

animals included in the sample were European type of Black-and-White cattle. During the research the cows were at the final stage of intensive breeding using Holstein genes. The high-yielding cows that were the subject-matter of this research were kept under the same housing conditions, feeding, care and method of utilisation.

A mathematical-statistical analysis of the impact of certain systematic factors was conducted with the method of least squares (*Harvey, 1987*). The advantage of this method is a possibility for concurrent and simultaneous determination of multiple factors that have an impact on the investigated trait. The following table with investigated factors shows animal distribution (N = 331) according to the previously defined classes:

Table 1. Animal distribution according to defined classes

Class of HF genes	<58%	58-73%	>73%
n	83	125	123

Year of culling	1	2	3
n	88	140	103

Reason for culling	1	3	4
n	278	47	6

Applied statistical model:

$$Y_{ijklm} = \mu + O_i + HF_j + G_k + R_l + e_{ijklm}$$

Where:

Y_{ijklm} = result of **m** cow, daughter of **i** sire, belonging to **j** group according to share of HF genes, culled in **k** year, because of **l** reason

μ = general average
O_i = impact of **i** sire
HF_j = impact of **j** group of HF genes
G_k = impact of **k** year of culling
R_l = impact of **l** reason for culling
e_{ijklm} = random error

Results and Discussion

Achieved lifetime milk production can greatly affect economic results. The productive period is a period from the first calving to culling. Lifetime milk production is achieved within the productive period.

The analysis of mean values (lsm), mean value errors (Slsm) of least squares and the significance of the investigated factors for the lifetime production of high-yielding Black-and-White cows is shown in Table 2.

Table 2. Mean values (lsm) and mean value errors (Slsm) of least squares for the investigated factors for lifetime milk production (kg)

Factors	N	lsm	Slsm
Total			
μ	331	25,002.66	7,755.39
Class of HF genes (df_1=2, df_2=307, $f\text{-}_{exp}$=4.007*)			
<58%	83	27,061.37	7,796.64
58-73%	125	24,761.26	7,779.84
>73%	123	23,185.36	7,787.97
Sires - bulls (df_1=17, df_2=307, $f\text{-}_{exp}$=31.858**)			
23	42	33,299.09	1,490.42
28	20	24,636.17	2,014.13
33	3	40,446.99	4,506.45
35	29	15,645.69	1,813.27
36	45	20,519.79	1,557.39
38	11	7,400.24	2,576.62
270	22	39,538.36	1,929.98
283	5	48,946.03	3,550.72
293	8	1,304.13	2,909.00
337	7	5,789.60	3,036.78
762	21	35,476.32	1,975.43
795	6	49,058.76	3,283.66
816	33	32,587.44	1,719.46
879	7	10,904.66	3,016.05
927	31	21,711.56	1,643.02
1,040	19	9,763.58	2,021.80
1,304	15	24,039.53	2,236.11
5,368	7	32,710.09	3,028.12
Year of culling (df_1=2, df_2=307, $f\text{-}_{exp}$=49.147**)			
1	88	19,725.90	7,772.39
2	140	24,086.81	7,783.63
3	103	31,195.29	7,789.04
Reason for culling (df_1=2, df_2=307, $f\text{-}_{exp}$=2.740ns)			
1	278	26,514.41	7,696.92
3	47	28,026.35	7,759.58
4	6	20,467.24	8,279.71

N.S.- $p>0.05$ *-$p\leq0.05$ **-$p\leq0.01$

The average lifetime milk production was 25,002.66±7,755.39 kg. The lifetime milk yield by cow genotypes ranged from 27,061.37 kg (<58% HF), and 24,761.26 kg (58-73%HF), to 23,185.36 kg (>73%HF).

The average lifetime milk production of 145 Holstein cows was 36,000 kg milk with 4.23% fat and 3.38% proteins. For a long time, average productive life was 4.5 lactations, but actual productive life was lower, ranging up to 3.5 lactations. One of the reasons for less lactations was the introduction of a larger number of young, first-calving cows to increase the size of a herd (*Martens and Bange, 2013*).

In Slovenia, a comparison between lifetime milk production of Holstein and Simmental cows was carried out according to their origin (*Janžekovič et al., 2009*). The subject-matter of that study were cows of Holstein breed (461 domestic - Slovenian cows and 356 cows of foreign origin) and Simmental breed (261 domestic and 43 foreign). The study included only cows with 1-9 lactations. The lifetime milk production of the domestic Holstein cows was 28,857.00 kg, and of foreign cows was 27,912.00 kg. The lifetime milk production of the domestic Simmental cows was 17,169.00 kg, and of foreign cows 21,519.00 kg. Statistically, significant differences ($p \leq 0.05$) were determined between the cows of domestic and foreign origin.

Donaldson (2006) states that the lifetime milk production of Holstein breed cows increases with longer life and more lactations. The interval between calving should be 380 days. As for milk yield, it should be 7,000.00 litres in the first lactation, 8,000.00 litres in second and 9,000.00 litres in the following lactations. Based on these values, lifetime milk production per cow according to the number of its lactations is, as follows: 7,000.00 litres in first, 15,000.00 litres in second, 24,000.00 litres in third, 33,000.00 litres in fourth and 42,000.00 litres in the fifth lactation.

The lifetime milk production of Black-and-White cows from two regions in south-western Romania was 16,461.10 kg in one, and 14,173.29 kg in the other region. The difference between these two lifetime productions was significant ($p \leq 0.05$) (*Bognar et al., 2011*).

Moreover, the average lifetime milk production of cows culled in 2013 was 24,254.00 kg for Black-and-White, 22,356.00 kg for Brown breed cows, 18,856.00 kg for Simmental cows and 22,465.00 kg for Simmental crossbreds (*Kmetijski inštitut Slovenije, 2014*).

From the investigated systematic factors, the impact of the sires - bulls and the year of culling on the differences in lifetime milk production was highly significant ($p \leq 0.01$), the impact of the class of HF genes was significant ($p \leq 0.05$), the impact of the year of culling highly significant ($p \leq 0.01$), whereas the impact of the reason for culling was non-significant ($p > 0.05$).

Conclusions

The average lifetime milk production was 25,002.66±7,755.39 kg. When observed by cow genotypes, the mean values of lifetime milk production varied from 27,061.37 kg (<58% HF), 24,761.26 kg (58-73% HF) to 23,185.36 kg (>73% HF). The differences in lifetime milk production determined among the animals were due to a highly significant ($p \leq 0.01$) impact of the bulls – the sires of the cows and the year of culling; the impact of the class of HF genes was significant ($p \leq 0.05$), whereas the impact of the reason for culling was non-significant ($p > 0.05$).

The results on lifetime milk production were affected by duration of productive life and increased number of culled cows, which had direct consequences on breeding and economic results. Lifetime milk production, achieved in complex conditions on cattle farms, has a great economic importance. Lifetime milk production traits should enable more efficient and more productive use of the genetic potential and life expectancy of cows.

Životna proizvodnja visokomlečnih krava

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Rezime

Osobine životne proizvodnje visokomlečnih krava imaju veliki ekonomski značaj. Crno-bela goveda imaju značajan genetski potencijal za proizvodnju mleka. Visokoproizvodne krave crno-bele rase izložene su tokom produktivnog veka velikom broju složenih uticaja koji intenzivno deluju na nivo relizacije njihovog genetskog potencijala. Uslovi sredine često nisu u saglasnosti sa potrebama ove visokomlečne rase goveda. Povećanjem učešća gena holštajn-frizijske rase, tokom procesa oplemenjivanja evropskog tipa crno-belih goveda, došlo je do povećanja prinosa mleka. Dosadašnja istraživanja ukazuju na mogućnost za značajnije povećanje životne produktivnosti crno-belih krava. Poznavanje broja i nivoa uticaja faktora sredine, na životnu proizvodnju visokomlečnih krava, važno je zbog njihovog uključivanja u model. U skladu sa značajem pojedinih sistematskih faktora u okviru istraživanja je obavljena njihova objektivna procena. Cilj rada je bio da se primenom odgovarajuće metodologije ispita značajnost razlika, u ostvarenoj životnoj proizvodnji mleka kod visokoproizvodnih crno-belih krava, preko najvažnijih sistematskih uticaja. Prosečna životna proizvodnja mleka iznosila je 25002.66±7755.39 kg litara. Posmatrano po genotipovima krava prosečne vrednosti životne proizvodnje mleka iznosile su 27061.37 kg (< 58% HF),

24761.26 kg (58-73% HF) i 23185.36 kg (> 73% HF). Utvrđene razlike između grla u pogledu ostvarenog nivoa životne proizvodnje mleka nastale su kao posledica visoko značajnog uticaja ($P \leq 0.01$) bikova-očeva krava i godine izlučenja krava, klasa HF gena krava imala je značajan uticaj ($p \leq 0.05$), dok razlog izlučenja nije imao značajan uticaj ($P > 0.05$).

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ASSIGNMENT FOR GENES ENCODING THE TERMINAL COMPLEMENT COMPONENTS TO PORCINE CHROMOSOME

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Abstract: One of the major goals of the porcine genome projects is building a physical map. To assign the porcine genes encoding the complement components C6, C7, C8 and C9 to porcine chromosomes, we used a porcine 7000Rad Radiation Hybrid panel (IMpRH) containing 118 clones provided by INRA-University of Minnesota. It resulted in assignment of the porcine C6, C7 and C9 genes to chromosome 16q1.4, the porcine C8A and C8B genes to chromosome 6q3.1-q3.5 as well as the porcine C8G gene lonely to chromosome 1q2.13.

Key words: IMpRH mapping, C6, C7, C8A, C8B, C8G, C9, pig

Introduction

The assignment of genes using radiation hybrid (RH) panels is an efficient way to map genes and markers as well as to integrate the linkage and cytogenetic maps of a species (*Hawken et al., 1999; Yerle et al., 1998*). RH mapping enhances linkage map reliability because of unambiguous determination of marker order and provides a powerful tool for fine mapping (*Yerle et al., 1998*). Moreover, radiation hybrid allows gene assignments without the detection of genetic polymorphism as needed for linkage mapping. Genes encoding the complement factors of the terminal lytic sequence of the complement cascades are considered as candidate genes for immune traits and disease resistance. We aimed at mapping the porcine genes C6, C7, C8A, C8B, C8G, and C9 to porcine chromosomes by using IMpRH tool.

Materials and methods

Preparation of genomic DNA from tail or ear samples was performed by standard procedures involving Proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. Therefore, the samples were cut into small pieces of 2-3 mm, weighed about 0.1g, and then placed in 2 ml tube containing 700 µl of digestion buffer. To lyse the cells and digest proteins, 35 µl of Proteinase K solution (20 mg/ml) was added and the samples were then incubated at 55°C overnight with mixing. Addition of an equal volume of phenol-chloroform (1:1 v/v, 700 µl for each) was done thereafter. The two phases were mixed until they formed a homogenous emulsion and separated by centrifuging at 5000 rpm for 3 min at 4°C. The aqueous phase was collected in fresh tubes. Phenol-chloroform extraction was repeated and always followed by a chloroform extraction. One-tenths volume of sodium acetate (3 M, pH 6.0) and an equal volume of isopropanol (700 µl) were put in. The samples were shaken gently until precipitation of DNA. The DNA pellet was, then, washed three times with 1 ml ethanol (70%) and dried at room temperature. Finally, the DNA was resuspended in 1× TE buffer and stored at 4°C for further analysis.

In order to perform physical mapping of the candidate genes the INRA-University of Minnesota porcine 7000Rad Radiation Hybrid panel (IMpRH) containing 118 pig/ hamster DNA hybrid clones (*Hawken et al. 1999, Yerle et al. 1998)* was employed. Based on accession number sequence DQ333199, NM_214282, 5´flanking region of clone XX-1C1, DQ333201, DQ333202 and DQ333198, specific primer pairs, which allowed amplifying DNA fragments of 159 to 707 bp for mapping C6, C7, C8A, C8B, C8G and C9 were derived. Amplicon design, primer sequences and PCR conditions are summarized in table 1. Prior to mapping, PCR conditions were optimized so that specific amplification of porcine DNA but not DNA of the hamster parental lines or amplification of fragments unambiguosly distinguishable between the two species was achieved. The expected porcine DNA fragments were, then, sequenced to verify their identity.

PCR reaction was performed in a total 15 µl reaction mixture containing 25 ng of hybrid DNA, 100 µM of each dNTP, 0.1 µM of each primer, 1×supplied PCR buffer containing 1.5 mM $MgCl_2$ and 1 U Taq polymerase. PCR reactions were prepared for 118 DNA templates of the IMpRH panel (positive), a hamster DNA template (negative control), a blank-template without DNA (negative control) and a porcine genomic DNA (positive control). The PCR products were

amplified using standardized thermal profile as follows: 4 min of initial denaturation at 94°C, 40 cycles at 94°C for 30 sec, at the annealing temperature 58°C or 60°C (depending on the specific primer pairs shown in Table 1) for 30 sec, at 72°C for 1 min and a final extension at 72°C for 5 min. For mapping of the C7 gene a modified, touch-down thermal profile was employed: 94°C for 4 min, followed by 9 cycles at 94°C for 30 sec, annealing temperature from 60-51°C (-1°C per cycle) for 30 sec, 72°C for 1 min, followed by 40 cycles of 94°C for 30 sec with annealing temperature at 50°C for 30 sec, at 72°C for 1 min, ending with an extension step at 72°C for 5 min.

The entire PCR reactions were separated on 3% agarose gel stained with ethidium bromide and amplification products were independently scored as present (1), absent (0), or ambiguous (?). The results translated into vector format for submitting to IMpRH mapping tool. The PCR-screening of the IMpRH panel was performed twice. For ambiguous data, the PCR was repeated to minimize genotyping errors and the remaining discrepancies scored as ambiguous (Figure 1). Two-point linkage analysis was done using the IMpRH mapping tool available at the IMpRH Web Server (http://imprh.toulouse.inra.fr).

Linkage mapping of candidate genes was performed using CRIMAP version 2.4 *(Green et al. 1990).* Therefore, genotype information of the respective complement genes and of microsatellite markers previously obtained of the DUMI resource population was used (*Wimmers et al., 2002*).

Results and discussion

In this study, to determine the location and order of certain candidate genes, we used the IMpRH panel, which allows assigning unambiguously the six loci to porcine chromosomes from pig/hamster genomic DNA amplification products converted into vector sequences.

Two-point RH analyses were used for the identification of linkage groups using LOD score threshold of 5.0. With all information for markers, including vectors summarized in table 3, it was demonstrated that both porcine C8A and C8B loci were physically assigned to the same chromosome 6q3.1-q3.5 whereas the porcine C6, C7 and C9 genes were closely linked on the same q-arm of chromosome 16 (16q1.4). C8G was mapped to chromosomes 1q2.13. Moreover, the porcine C6, C7, C8A, C8B, C8G and C9 significantly linked to markers S0077, SW1069, SW322, SSC10D08 and SW403, respectively (Figure 2). All LOD scores are greater than 6. These were in a strong agreement with genetic linkage and chromosomal localization studies previously established that the porcine C8A and

C9 are located on chromosome 6q3.1-q3.3 *(Nakajima et al. 1998)* and 16q1.4 *(Thomsen et al. 1998)* using the porcine bacterial artificial chromosome (BAC) clone as a hybridization probe and fluorescence *in situ* hybridization, respectively. Genetic linkages using two-point analysis from CIRMAP 2.4 *(Green et al. 1990)* were confirmed between C7 and C6 with rec. fracs. = 0.02, LOD = 50.45, between C9 and C6 with rec. fracs = 0.06, LOD = 31.50, between C9 and C7 with rec. fracs = 0.06, LOD = 35.84, between C8B and C8A with rec. fracs = 0.05, LOD = 65.72.

Table 1. Oligonucleotide primers used for radiation hybrid mapping

Primer name (Length, bp)	Primer sequence (genome localization, exon)	Tm (^{o}C)
C6 (159)	up 5´-3´: ttccttttgcaaggatcaga (nt. 1437-1457, 10) down 3´-5´: tcaatcacagcaggattttcc (nt. 1575-1595, 10)	58
C7 (196)	up 5´-3´: agttatcagttgttggttgttca (nt. 739-761, 8) down 3´-5´: ctcctcctaaggacccagac (nt. 915-931, 8)	50 (*)
C8A (707)	up 5´-3´: tgcttctggaggtgttcattt (clone xx-1c1) down 3´-5´: cggttcaccttctcctgtatg (clone xx-1c1)	60
C8B (160)	up 5´-3´: gaaacaagagaagcagcatgg (nt. 1302-1322, 9) down 3´-5´: ttaattttgatgatgtctgggttg (nt. 1438-1461, 9)	60
C8G (295)	up 5´-3´: cctcttgacgctgctcct (nt. 75-92, 1) down 3´-5´: gagccacgtgcagtgaagt (nt. 268-286, 2)	58
C9 (511)	up 5´-3´: ggagcattgagacctttgga (nt. 283-302, 3) down 3´-5´: gccagctcagactcttccac (nt. 528-547, 4)	60

(*) A touch down PCR program was used to amplified a fragment of 196 bp under following thermal cycling conditions: 94^{o}C for 4 min, followed by 9 cycles at 94^{o}C for 30 sec, annealing temperature step-downs every 1 cycle of 1^{o}C (from 60-51^{o}C) for 30 sec, at 72^{o}C for 1 min, then followed by 40 cycles of 94°C for 30 sec, at 50°C for 30 sec, at 72°C for 1 min, and ending with an extension step at 72^{o}C for 5 min.

Table 2. Chromosomal assignments of the genes in difference species

	Mapping localization							
Gene	*B. taurus*	*D. rerio*	*G. gallus*	*H. sapiens*	*M. mulatta*	*M. musculus*	*R. norvegicus*	*P. troglodytes*
C6	20	21	-	5p13	6	15A1	2q16	5
C7	20	21	Z	5p13	-	15A1	2q16	5
C8A	3	-	-	1p32	1	4C6	5q34	1
C8B	3	-	8	1p32	1	4C6	5q34	1
C8G	-	24	-	9q34.3	-	2A3	3p13	-
C9	1	5	-	5p14-p12	6	15A1	2q16	-

Genetic mapping correspondingly revealed linkage of the porcine C6 and C7 to C9 and AGXT2 that have previously been assigned to the q-arm of chromosome 16 (*Ponsuksili et al. 2001, Thomsen et al. 1998, Wintero et al. 1998*) while on chromosome 6q3.1-q3.5, C8B links closed to the porcine C1q and C8A that have been reported by *Jorgensen et al. (1997)* and *Nakajima et al. (1998),* respectively. This is in agreement with the most recent human-porcine comparative map *(Meyers et al. 2005).*

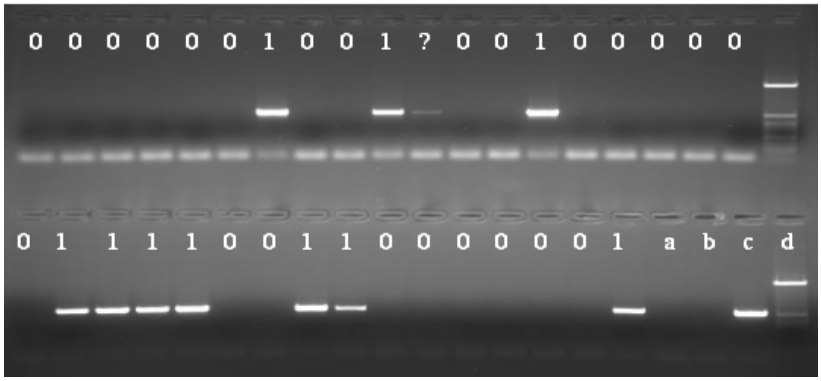

Figure 1. A representative pattern for PCR result of 118 DNA clones of the IMpRH. The symbols (0), (?), and (1) used for absent, ambigous and present results, respectively. The letters a, b, c, and d show a hamster sample (negative control), a blank sample without DNA (negative control), a pig DNA (positive control) and a marker to estimate length of DNA fragments.

The mapping results also fit the current pig-mouse and pig-rat comparative maps as accessible via the MGI webpage (http://www.informatics.jax.org/searches/homology_form.shtml). Further, the location of distinct C8 loci on chromosomes supports genetic evidence that C8 contains three separate genes encoding different proteins C8A, C8B and C8G.

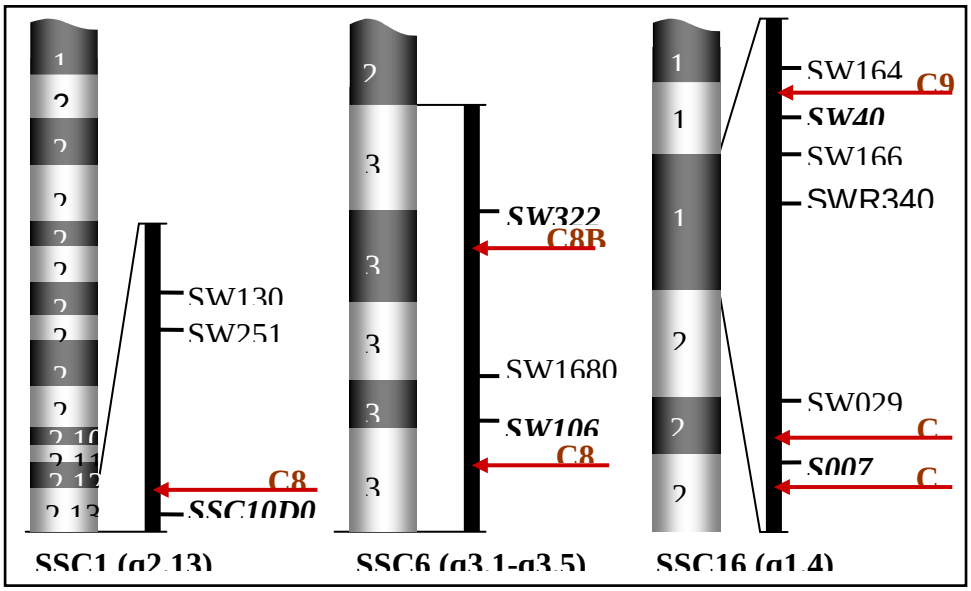

Figure 2. Position of the candidate genes on porcine chromosomes. Linkage is closed to markers in bold and italic

Table 3. Summary of RH mapping results of the candidate genes

Gene	Result in vector	LOD score, distance in cR, retention fraction (%), SSC
C6	00001000000100110000111100011100100100011001101000001101001001101101101101110000000011100000000100001000010111111111100	18.13, 21, 42, 16q1.4
C7	11011000000100010000011100010?0010010001100100101000000000000110010110110111000000001110000000100001000010111111111011	16.65, 23, 38, 16q1.4
C8A	00100001010110000000111000001000000000000000100010000000000000100001101000000001011010001100000001001011000000000010001	7.00, 56, 22, 6q3.1-q3.5
C8B	00100001010110000000111100000100000000100000100010000000000000100001100010000001011010001100000001001011000000000010001	8.18, 50, 24, 6q3.1-q3.5
C8G	00000000000010000110000000000000000000001000010000000100000011000000000000000000001100000010011000000000010100001001010	8.26, 44, 15, 1q2.13
C9	11011000000100010000000100010001100100010000001000000010000010001011011010100000001000000000011000100011111111000101	14.45, 27, 31, 16q1.4

Looking on the genetic maps of other species, C6 and C7 are mapped to the same chromosome while C8A and C8B components are always found on the same chromosome. Additionally, C9 complement component could be assigned to the same chromosome carrying C6 and C7 in all species addressed here except in *B. taurus* and *D. rerio*. Especially, C8G gene is always located in a different chromosome (Table 2).

In summary, the study revealed four new assignments and confirmed previous results.

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Zadatak za gene koji kodiraju terminalne komplementarne komponente hromozoma svinja

D. V. Anh Khoa, K. Wimmers

Rezime

Jedan od glavnih ciljeva projekata koji se bave genomom svinje je stvaranje fizičke mape. Da biste dodelili gene svinja koji kodiraju komplementarne komponente C6, C7, C8 i C9 hromozomu svinje, koristili smo 7000Rad Radiation Hybrid panel (IMpRH) koji sadrži 118 klonova koje je obezbedio INRA-Univerzitet Minesota. To je rezultiralo u dodeli C6, C7 i C9 geni svinje hromozomu 16q1.4, C8A i C8B gena hromozomu 6q3.1-q3.5, kao i gena C8G hromozomu 1q2.13.

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THE EFFECT OF PARENTAL GENOTYPE AND PARITY NUMBER ON PIGS LITTER SIZE

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Abstract: The aim of this study was to investigate the effect of parental genotype and parity number on the litter size properties of sows (number of live born, stillborn and weaned piglets). The investigation was conducted on a farm in Vojvodina. The analysis included 65535 litters that originated from five genotypes of dams (sows with unknown origin, n = 20980; Yorkshire, n = 3189; Landrace, n = 22426; $F1_{(YxL)}$, n = 14251; $F1_{(LxY)}$, n = 4689) and five genotypes of sires (Yorkshire, n = 21641; Landrace, n = 26623; Pietrain, n = 485; Duroc, n = 13463; Hampshire, n = 3323). Based on the obtained results it can be concluded that the genotypes of the dams had statistically significant ($p<0.01$) influence on the observed litters properties. Landrace sows achieved the highest average number of born alive piglets (10.12) with a statistically significant difference ($p<0.01$) compared with sows of other genotypes. The observed effect of sire genotype on litter size properties was statistically significant ($p<0.01$), where the terminal genotypes were superior when it comes to the number of live born and weaned piglets. Regression analysis of dependence between parity and litter size recorded positive regression coefficients: number of live born ($b = 0.007$), stillborn ($b = 0.09$) and weaned piglets ($b = 0.07$). Influence of parity on the observed traits of litter size was highly statistically significant ($p<0.01$).

Key words: genotype, sire, dam, liter size, piglets

Introduction

The aim of contemporary pig production is to improve the most important economic traits. The most important economic trait in swine production represents reproductive traits such as litter size, litter weight at birth and survival rates of piglets to weaning. The reason for this is the fact that the annual productivity of sows depends solely on litter size and number of parity during the year. Annual

productivity of sows and economic efficient of piglets production could be increased with increases of any of these parameters (*Vidović et al., 2012*). The objective of modern pig breeding is to exhaust the genetic potential in reproduction performance of sows regarding to litter size and number of weaned piglets per litter (*Stella et al., 2003*). Litter size is one of the most important reproductive traits which present a low heritable trait and the influence of environmental factors on its expression is significantly expressed, because it is necessary 8 to 10 generations of selection to increase litter size by one piglet (*Wähner and Brüssow, 2009*). Knowledge of parent genotype and genetic parameters of reproductive traits is an essential requirement in estimating the breeding value, selection, implementation and select the best method of breeding, because only in this way can the right to genetic improvement of the herd (*Irgang et al., 1994*). Litter size is a quantitative character of considerable complexity and improvement is likely to be slow since it is influenced to a large degree by environmental factors. The normal assumption is that the size of litter produced is primarily dependent on the female (*Roehe and Kennedy, 1993; Vidović, 2009*). The influence of the sire on litter size has been the subject of numerous studies (*Van der Lende et al., 1999; Klindt, 2003)*. Litter size at weaning is one of the most important traits in pig production. Direct selection for this trait is generally restricted in practice due to cross-fostering, which also makes it difficult to adequately estimate genetic parameters for litter size at weaning.

The aim of this study was to investigate the effect of parental genotype and parity number on the litter size properties of sows such as number of live born, stillborn and weaned piglets.

Materials and Methods

This paper analyses 65.535 pig litters, obtained by mating purebred Landrace and Yorkshire sows and F1 hybrid sows ($F1_{(Y\times L)}$ and $F1_{(L\times Y)}$) and sows with unknown origin, with Landrace and Yorkshire and terminal boars such as Pietrain, Duroc and Hampshire sire breeds. The animals originate from one commercial farm in Vojvodina, which has the entire cycle of production, where the collected data between January 2000 and December 2009 was analyzed. During data processing, all sows were distributed in the order of farrowing, and after that analysis of litter size traits (number of live births, stillbirths and weaned piglets) was performed.

To check the significance of the impact of parental genotypes and parity, general linear model (GLM) were used:

$$Y_{ijklm}=\mu+ S_i + D_j + P_k + E_{ijkl}$$

Where is:

Y_{ijkl} - observed traits

μ - mean of observed traits

S_i - sire effect

D_j - dam's effect
P_k – parity effect
E_{ijkl} - random error

For the traits adjusted mean (LSM - Least Square Means) were calculated and Fisher LSD post-hoc test for significance determination between the genotypes of sires and dams were performed. To test the dependence between parity and the observed reproductive traits, multiple linear regression were used with the statistical software XLSTAT 2013.

Results and Discussion

Tables 1 and 2, shows the average number of live births, stillbirths and weaned piglets according to dams and sires genotypes with the adjusted mean (LSM), standard error of the adjusted mean (SE_{Lsm}), and the effect of the parental genotype on the observed properties of litter sizes.

From the data in Table 1, it can be seen that the dams genotype had highly significant ($p<0.01$) influence on the observed properties. In the studied population, between the genotypes of pure sows breeds and hybrid sows statistically significant differences ($p<0.01$) in the number of live born and weaned piglets was observed, while the number of weaned piglets showed no significant differences ($p>0.05$). Greater variability was observed in litter sizes of pure sows breed (9.44; 10.12), while the hybrid sows have almost uniform litter sizes (9.79; 9.78 and 9.72). Almost the same number of weaned piglets in all observed genotypes can be explained by inadequate farm management in the growth process of suckling piglets, where maximum attention should be paid, because this phase is the most critical and most difficult phase of piglet production.

Table 1. Influence of dam genotype on litter size

Dam genotype	Number of litters	Live born piglets		Stillborn piglets		Weaned piglets	
		LSM	SE_{Lsm}	LSM	SE_{Lsm}	LSM	SE_{Lsm}
Hybrid	20980	9.79^{A}	0.02	1.29^{A}	0.01	8.30^{A}	0.02
Yorkshire	3189	9.44^{aB}	0.05	0.59^{aB}	0.02	8.40^{B}	0.06
Landrace	22426	10.12^{abC}	0.02	0.74^{ab}	0.01	8.34^{C}	0.02
$F_{1(YxL)}$	14251	9.78^{bc}	0.02	0.71^{abC}	0.01	8.45^{acD}	0.03
$F_{1(LxY)}$	4689	9.72^{bc}	0.04	0.54^{ac}	0.02	8.67^{abcd}	0.05
p value		*0.000*		*0.000*		*0.000*	
F value		*75.494*		*646.79*		*11.42*	

The same upper and lower case letters – statistically high significant differences $p<0.01$

The same lower and different upper case letters – no statistically significant differences p>0.05

The genotype of the sire had highly significant (p<0.01) effect on all observed characteristics what can be seen from the results given in Table 2. In addition, the sires genotypes was found to be statistically significant different (p<0.01) in the number of live born and stillborn piglets, where the largest number of live born piglets was found in Pietrain sires (10.55), while the lowest number was recorded in Hampshire sires line (9.65). Differences in the number of weaned piglets within the sires of all breed lines genotype found no significant (p>0.05) differences.

Table 2. Influence of sire genotype on litter size

Sire genotype	Number of litters	Live born piglets		Stillborn piglets		Weaned piglets	
		LSM	SE_{Lsm}	LSM	SE_{Lsm}	LSM	SE_{Lsm}
Yorkshire	21641	9.73^{A}	0.02	1.09^{A}	0.01	8.30^{A}	0.03
Landrace	26623	10.00^{aB}	0.01	0.86^{aB}	0.01	8.27^{B}	0.02
Pietrain	485	10.55^{abC}	0.12	0.84^{aC}	0.06	8.92^{ab}	0.17
Duroc	13463	9.95^{acD}	0.02	0.74^{abD}	0.01	8.62^{ab}	0.03
Hampshire	3323	9.65^{bcd}	0.04	0.43^{abcd}	0.02	8.58^{ab}	0.08
p value		*0.000*		*0.000*		*0.000*	
F value		*39.107*		*230.79*		*25.317*	

The same upper and lower case letters – statistically high significant differences p<0.01
The same lower and different upper case letters – no statistically significant differences p>0.05

The Table 3 shows the parity structure of the herd on the farm, on the basis of which can be seen very good parity structure of the population, where the number of litters till fifth farrowing is around 72%. The highest percentage of litters was in a first parity which was around 21.56%, and then in the second 15.81%, 13.39% in the third, fourth 11.41% and 9.57% in the fifth, respectively. According *Vidović and Šubara (2011)* the preferred structure of the sows herd parity is, when a number of zero-parity sows is around 20%, in the first 18%, in the second 15%, in the third 14%, in the fourth 12%, in the fifth10%, sixth 6%, and more than seven is around 7%. A similar parity structure was proposed by *Tretinjak et al. (2009), Chen et al. (2003).*

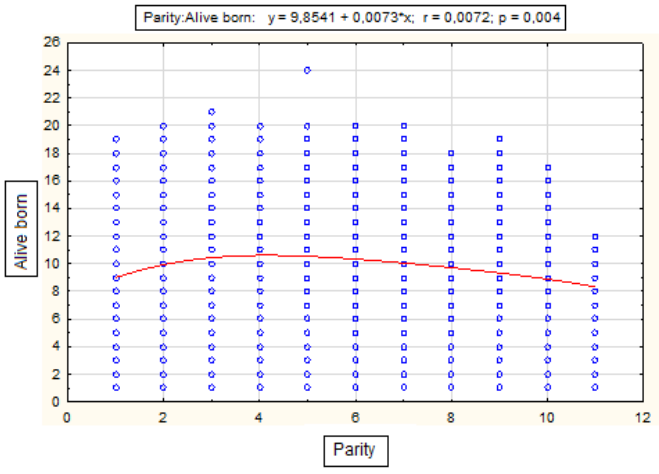
Farrowing parity as expected, had statistically significant (p<0.01) effect on the number of live births, stillbirths and weaned piglets. Once again the hypothesis that the first farrowing sows have smaller litters, and later with increasing the sows parity, the number of live born and weaned piglets was increased till the sixth farrowing, which after was gradually decreased, while the number of stillborn piglets was increased is confirmed. The similar results was

obtained by *Lukač (2013), Vidović et al. (2011a, 2011b), Tretinjak et al. (2009), Lucia et al. (2002)* and *Vincek (2005)* in their investigations.

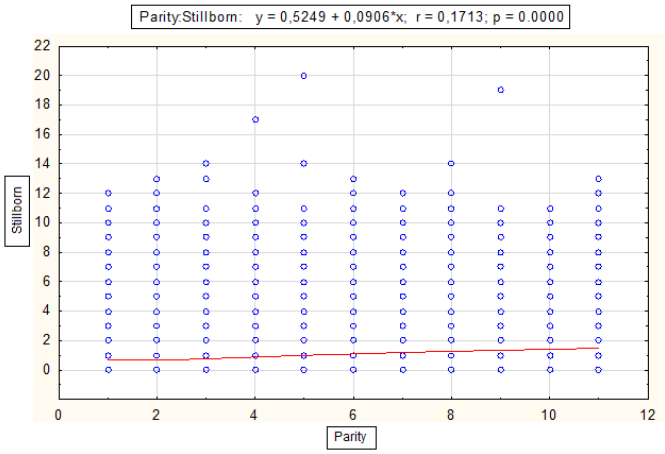
Table 3. Influence of parity on litter size

Parity	Number of litters		Live born piglets		Stillborn piglets		Weaned piglets	
	No	%	LSM	SE_{Lsm}	LSM	SE_{Lsm}	LSM	SE_{Lsm}
1	14132	21.56	8.96	0.02	0.67	0.02	7.00	0.03
2	10366	15.81	10.05	0.02	0.63	0.01	9.00	0.04
3	8779	13.39	10.52	0.03	0.74	0.01	9.02	0.04
4	7480	11.41	10.62	0.03	0.89	0.01	8.97	0.04
5	6277	9.57	10.47	0.03	1.02	0.02	8.90	0.05
6	5201	7.93	10.26	0.04	1.07	0.02	8.67	0.05
7	4219	6.43	9.98	0.04	1.19	0.02	8.45	0.06
8	3334	5.08	9.64	0.05	1.28	0.02	8.33	0.07
9	2578	3.93	9.29	0.05	1.32	0.03	8.22	0.07
10	1925	2.93	8.96	0.06	1.39	0.03	7.88	0.09
>10	1244	1.89	8.30	0.08	1.48	0.04	7.93	0.11
p value			*0.000*		*0.000*		*0.000*	
F value			*393.11*		*205.52*		*282.14*	

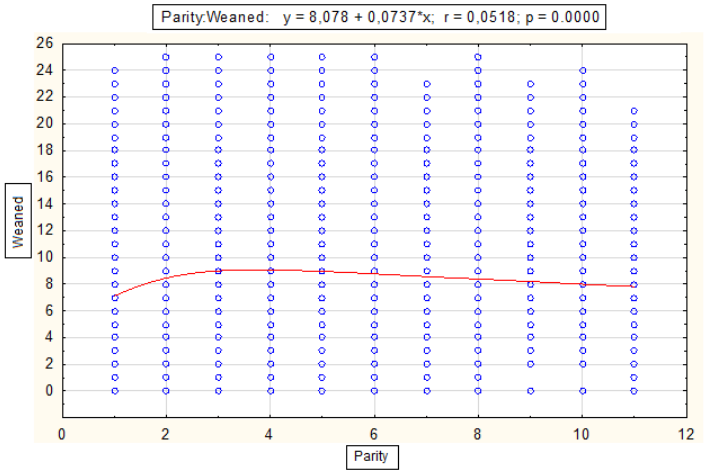
Regression analysis of dependence between parity and litter size recorded positive regression coefficients which can be seen from the results showed at Graphs 1, 2 and 3. With each unit increase in parity, there was an increase in the number of live born (b=0.007), stillborn (b=0.09) and weaned piglets (b=0.07). Observed properties of litter size was significant ($p<0.05$) and highly statistically significant ($p<0.01$).



Graph 1. Relation between parity and alive born piglets



Graph 2. Relation between parity and stillborn piglets



Graph 3. Relation between parity and weaned piglets

Conclusion

Based on the gained results, which have the aim to investigate the influence of parental genotype and parity number on the litter size properties of sows it can be concluded that the dams and sires genotype had highly significant influence on the observed properties of number of live born, stillborn and weaned piglets. The same tendency was observed in the influence of parity number on litter size with also high significance. In this regard, the maximum litter size, which is also an indicator of economic production, together with the number of farrowing

sow per year, can be achieved by proper selection of genotypes of future parents with properly define production technology and with the optimization of paragenetic factors.

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Uticaj genotipa roditelja i pariteta na veličinu legla svinja

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Rezime

Cilj ovog rada je bio da se ispita uticaj genotipa roditelja i pariteta prašenja na osobine veličine legla krmača (broj živorođene, mrtvorođene i zalučene prasadi). Podaci su sakupljeni sa jeden farme u Vojvodini. Analiza je obuhvatila 65535 legala koja vode poreklo od pet različizih genotipova majki (plotkinje sa nepoznatim poreklom, n= 20980; jorkšir, n= 3189; Landras, n= 22426; $F1_{(YxL)}$, n = 14251; $F1_{(LxY)}$, n = 4689) i pet genotipova očeva (jorkšir, n= 21641; landras, n= 26623; pietren, n= 485; durok, n= 13463; hempšir, n=3323). Na osnovu dobijenih rezultata može se zaključiti da su genotipovi majki imali statistički značajan uticaj ($p<0.01$) na posmatrane osobine veličine legla. Landras krmače su imale prosečno najveći broj živorođene prasadi (10,12) u poređenju sa krmačama druga četiri genotipa, gde je zabeležena statistički značajna razlika ($p<0.01$). Genotip oca je imao statistički značajan uticaj ($p<0.01$) na osobine veličine legla, gde su terminalnih genotipovi bili superiorniju u pogledu broja živorođene i zalučene prasadi. Regresiona analiza zavisnosti između pariteta i osobina veličine legla beleži pozitivne regresione koeficijente: broj živorođene prasadi (b= 0,007), broj mrtvorođene prasadi (b= 0,09) i broj zalučene prasadi (b= 0,07). Uticaj pariteta prašenja na posmatrane osobine veličine legla je bio statistički visoko značajan ($p<0.01$).

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EEFECTS OF DIFFERENT METHODS FOR MYOMETRIAL CONTRACTION STIMULATION PRIOR TO AI ON SOWS FERTILITY

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Original scientific paper

Abstract: These study compares farrowing rate and litter size in AI sows, treated with different methods for myometrial contraction stimulation, immediately around AI. The total of 249 sows, inseminated within 7 days after weaning, were divided into five groups. Group I: 10 IU oxytocine addition in semen dose immediately before AI (n=50), Group II: i/m vulvar injection of 5 IU oxytocin just prior to AI (n=50), Group III: cervix stimulation with AI catheter (n=49), Group IV: fence-line boar contact immediately around AI (n=50) and Group V: unstimulated AI (control) sows (n=50). Farrowing rate were significant higher ($P<0.05$) in all stimulated sows groups (92%, 88%, 90% and 84%), compared with unstimulated sows (78%). Subsequent litter sizes were not affected by treatment, ranged between 11.41 and 11.98 liveborn piglets. These results indicate that performed treatment for myometrial contractions stimulation can be useful method to improve sows fertility.

Key words: myometrium, contraction, stimulation, oxytocin, boar, AI, fertility, sow

Introduction

During mating or artificial insemination in sows, semen is deposited intra-cervically. From the site of deposition, sperm cells must be distributed over both horns and transported to the tubal end of the horns, i.e. utero-tubal junction, which serve as a sperm reservoir *(Hunter, 1981)*. The transport of sperm cells through the uterine horns is believed to be a passive process, in which intrinsic sperm cell motility plays no part *(Langendijk et al., 2005; Radović et al., 2006)*. This passive transport is probably driven by the flow of intrauterine fluid containing sperm cells,

due to gravitational force, movement of the sow and uterine contractions *(Scott and Glimpse, 2000)*. Inadequate stimulation of the sow during and after insemination result in reduced myometrial contractions *(Langendijk et al., 2002)* and a poorer sperm cell transport to the oviduct *(Langendijk et al., 2003; Stančić et al., 2006)*. It has been shown that uterine contractions is influenced by dramatically oxytocin concentration increases in the blood of sows, within 2 minutes of the onset of ejaculation by a mature boar *(Levis, 2000; Scott, 2000)*. This blood increasing of endogenous oxytocin is influenced by boar sexual stimuli (olfactory, visual, tactile and auditive) as well as direct stimulation of cervix by penis within the act of cupulation *(Langendijk et al., 2005)*.

An inadequate sperm transport within the uterus result in decreasing the sow fertility *(Langendijk et al., 2005)*. Namely, a sufficient number of spermatozoa in the oviductal sperm cell reservoir, ie. caudal isthmus in the 24-hour period preceding ovulation *(Hunter, 1981)*, is the ultimate factor for successful fertilization *(Soede et al., 1995)*. Any factors that reduce this reservoir may compromise fertility. In the AI, such reduction in the sperm cell reservoir may result of: (a) poor timing of semen deposition relative to time of ovulation *(Kemp and Soede, 1996; Stančić and Šahinović, 2001)*, (b) inadequate stimulation of the sow during and after insemination, resulting in reduced myometrial contractions *(Langendijk et al., 2002)* and a poorer sperm cell transport to the oviduct *(Langendijk et al., 2003; Stančić et al., 2006)*, and (c) excess semen reflux (backflow) during insemination *(Steverink et al., 1998)*. It has been shown that inadequate stimulation of sow during and immediately after insemination is the most common factor that affect the sows fertility rate *(Spronk et al., 1997)*. In farm practice, the reproductive performance of artificial inseminated sows is often lower than that achievable with natural breeding *(Spronk et al., 1997; Stančić, 2000)*. It is often result of inadequate myometrial stimulation, due to the small dose volume, high dilution rate of native ejaculate, as wel as an inadequate stimulation of the sow by boar presence and absence of mechanical stimulation of the cervix *(Langendijk et al., 2003; Beham and Watson, 2005; Kemp et al., 2005; Mezalira et al., 2005; Stančić et al., 2006)*. An adequat myometrial stimulation is most important in the intrauterine insemination technology with reduced dose volume and spermatozoa number per dose *(Roseboom et al., 2004; Mezalira et al., 2005; Stančić et al., 2010; Stančić et al., 2013)*.

The aim of the present study was to investigate whether farrowing rate and litter size can be enhanced by oxytocin addition in semen doses, sow injection by oxytocin, boar presence or by cervix stimulation, immediately around insemination, in the practical artificial insemination technology on pig farms in Serbia.

Materials and Methods

The study was conducted during September to November 2012 in an intensive pig farm, housing about 1,200 sows. Lactation length of herd was average 28 days. Average sows farrowing rate at the farm in 2011 were 76%, and average liveborn

piglets per litter were 10.68. Estrus detection of weaned sows involved full boar contact once daily starting on day 2 after weaning. Immediately after estrus detection, sows were inseminated with 4×10^9 sperm cells in 100 mL dose (BTS1-extender, Minitübe, Germany). Insemination was repeated 24 hours later if sows still exhibited estrous behavior, using disposable Safe Blue® AI catheters, lubricated and single wraped in protective sheaths, sterilized (Minitübe, Germany). Third estrus inseminations were not allowed. Age of semen at insemination was 4-6 hours to 1 day. AI doses were stored in thermo-box at +17°C up to insemination.

At the time of AI (4-5 days after weaning), experimental sows (2 to 5 parity) were assigned to five groups. Group I: AI doses supplemented with 10 IU oxyticin (10 IU/mL wather solution, Oxytokel®, Kelan N.V. - Belgium), immediately before insemination (n=50), Group II: sows injected with 5 $IUmL^{-1}$ oxytocin in the mucosa of the vulvar lips just prior insemination (n=50), Group III: stimulation of cervix by moving the top of the catheter within the cervix, about 1 minute before and 1 minute after sperm deposition (n=49), Group IV: fence-line boar contact with sow immediately before, within and about 5 minutes after insemination (n=50) or Group V: insemination without stimulation, control group (n=50).

Data recorded were farrowing rate after first postlactational insemination and subsequent litter size (liveborn, stillborn and total born piglets).

Obtained data were analyzed by using software package "Statistica 12". Data for litter size were testing by General linear model (GLM) and by LSD test. Farrowing rate was analyzed by test of proportion.

Results and Discussion

Our results demonstrated that farrowing rate were significant higher ($P<0.05$) after oxytocin addition in AI dose (92%), vulvar oxytocin injection (88%), cervix stimulation (90%) or boar presence (84%), compared with untreated (control) sows (78%).

Table 1. Farrowing rate and litter size in treated and control sows

Group	Stimulation method	Fertility parameters			
		Farrowing rate (%)	Litter size (average ± SD)		
			Liveborn	Stillborn	Total
I	10 IU oxytocin in AI dose (n=50)	92^{a} (46/50)	$11.41^{a}\pm2.37$	$0.93^{a}\pm1.29$	$12.34^{a}\pm2.69$
II	5 IU oxytocin injection (n=50)	88^{ab} (44/50)	$11.52^{a}\pm2.88$	$0.84^{a}\pm1.14$	$12.36^{a}\pm2.80$
III	Cervix stimulation (n=49)	90^{a} (44/49)	$11.98^{a}\pm2.96$	$0.66^{a}\pm0.80$	$12.64^{a}\pm3.17$
IV	Boar presence (n=50)	84^{b} (42/50)	$11.86^{a}\pm2.56$	$1.25^{a}\pm1.14$	$13.12^{a}\pm2.93$
V	Without stimulation, control (n=50)	78^{c} (39/50)	$11.79^{a}\pm2.75$	$0.77^{a}\pm0.77$	$12.56^{a}\pm2.78$

^{a,b,c}Values within a columns, with different superscripts differ ($P<0.05$). In parenthesis: No. farrowed/No. inseminated.

These value were not significant differ ($P>0.05$) between sows AI with oxytocin in dose, vulvar oxytocin injection or cervix stimulation. However,

farrowing rate were significantly lower ($P<0.05$) in sows stimulated by boar presence (84%) compared with oxytocin addition in AI dose (92%) or cervix stimulation (90%) and were not significantly lower ($P>0.05$) compared with sows injected with oxytocin (88%). Performed treatments has no significant effect ($P>0.05$) on litter size (Table 1).

The present results show that performed treatment with oxytocin, boar presence and cervix stimulation significantly increase farrowing rate, compared with untreated sows. The lower farrowing rate in sows stimulated by boar presence, compared with oxytocin treatment or with cervix stumualtion could have been caused due to absence of tactile boar stimuli (*Langendijk et al., 2005)*, because fence-line boar contact were performed in the present experiment. But, it is unclear which boar stimuli stimulate maximal uterine activity during estrus *(Gerritsen et al., 2005)*. Litter size was not affected by performed treatments, as it has been shown by other authors *(Gibson et al., 2004; Peláez et al., 2006; Stančić et al., 2006)*.

Establishment the optimal number of spermatozoa in the utero-tubal junction, caudal istmus and the site of fertilization (ampulo-isthmic junction of the oviduct) is the key factor for successful ovulated ova fertilization *(Hunter, 1981)*. Sperm cells have to be transported from the site of deposition to the utero-tubal junction within 15 minutes to 2 hours after deposition through the cervix. The rapid transuterine transport of spermatozoa to the utero-tubal junction and oviduct is extremely important for prevent spermatozoa to being phagocytized (killed) by leukocytes *(Levis, 2000)*. This passive transport is mainly driven by uterine contractions *(Scott, 2000; Umesiobi, 2010; Stančić and Dragin, 2011)*, influenced by dramatically oxytocin concentration increases in the blood of sows, within 2 minutes of the onset of ejaculation by a mature boar *(Levis, 2000)*. Elevation of plasmal endogenous oxytocin is induced by several boar sexual stimuli. These stimuli can be divided into sensory stimuli, i.e. tactile, olfactory, visual and auditory stimuli, on the one hand and seminal plasma-related stimuli (estrogen, oxytocin, prostaglandin $F_{2\alpha}$), on the other *(Langendijk et al., 2003; Langendijk et al., 2005)*. Additionally, the presence of a boar during estrus stimulate the estrus signs expression, particulary standing reflex *(Kemp et al., 2005; Stančić et al., 2008)*. Further more, the boar ejaculate contains high levels of estrogens *(Claus, 1990)*, which stimulates myometrial contractions *(Willenburg et al., 2004)* via an estrogen-induced local release of prostaglandin $F_{2\alpha}$ *(Claus, 1990; Willenburg et al., 2004)*. The synchronization of viable spermatozoa presence in oviduct and the time of ovulation is of extreme importance for successful fertilization. Whole boar semen or seminal plasma has been demonstrated to advance the time of ovulation *(Waberski et al., 2000)*. It is possible that semen-induced cytokines in the uterine lymph undergo counter-current transfer to the ipsilateral ovary and accelerate the final maturation of pre-ovulatory follicles *(O'Leary et al., 2004; Waberski et al., 2006)*.

If oxytocin was included in the semen, farrowing rate were higher (P =0.02) for weaned sows bred only once (84.9%) than for repeat sows (63.7%), but litter size was not affected *(Gibson et al., 2004)*. Authors conclude that inclusion of oxytocin in extended semen may benefit sow fertility when breeding management may otherwise result in a smaller sperm cell reservoir in the oviduct. Addition of 5 to 10 IUmL of oxytocin has no effect on boar sperm motility or morphology in the semen samples *in vitro* stored at +18^{o}C for 56 hours *(Çiftçi, 2005)*. The farrowing rate was 5.7 percent greater for sows inseminated with oxytocin-treated semen (83%) compared to sows injected with oxytocin (77.3%) immediately before insemination. The litter size was 11.50 piglets for sows inseminated with oxytocin-treated semen and 10.97 piglets for sows injected with oxytocin at the time of insemination *(Peña et al., 1998)*. Hormone (estrogens, oxytocin or prostaglandin $F_{2\alpha}$) addition to semen increased numbers of embryos 25 to 30 days after AI. Therefore, in situations of lowered fertility, hormone addition could be a strategy to limit infertility in swine *(Willenburg et al., 2003; Peláez, et al., 2006)*. According to results obtained by other authors, the conclusions from review paper of *Levis (2000)* are: (1) Adding 4 to 5 IU's of oxytocin to a dose of semen improves farrowing rate and litter size, (2) Use of oxytocin treated semen is more effective in multiparous sows than in gilts, (3) During the summer months, oxytocin-treated semen significantly increased farrowing rate and litter size and (4) In most studies, the use of oxytocin at the time of insemination was profitable.

Releasing the endogenous oxytocin and myometrial contraction can also be induced with cervix stimulation by moving catheter within the cervix, immediately before and after AI dose deposition (*Fülöp et al., 1992; Steverink et al., 1998; Grafenau et al., 2005; Stančić et al., 2006)*. It has been demonstrated *(Stančić et al., 2006)* that cervix stimulation by catheter immediately before and after insemination, significantly increase farrowing rate (83.3%), in comparison with unstimulated sows (71.1%).

According to mentioned facts, lower farrowing rate, after artificial insemination, my be the result of poorer uterine contraction caused by: (a) inadequate sow sexual stimulation, due to no full boar contact and act of coitus or (b) lower amount of semen oxytocin and estrogen in insemination dose, due to increase dilution rate of ejaculate.

Conclusion

Oxytocin addition to semen (10 IU per dose) immediately before AI, vulvar injection of 5 IU oxytocin just prior to AI, cervix stimulation with catheter or boar presence, significantly increase farrowing rate (92%, 88%, 90% and 84%) compared with untreated sows (78%). Subsequent litter sizes were not affected by treatment.

However, according to results of other authors, this method is controversial and the generalized recommendations for use should be made with caution, since the most profound effects occur in sub-fertile farms or groups of sows, seasonal infertility, and with sub-fertile boars. Nevertheless, in many cases, sows fertility rate are improved.

Uticaj različitih metoda za stimulaciju kontrakcije miometrijuma neposredno pre VO na fertilitet krmača

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Rezime

U istraživanju je izvršena komparacija vrednosti prašenja i veličine legla kod krmača tretiranih različitim metodama za stimulaciju kontrakcija miometriuma, neposredno pre VO. Ukupno 249 krmača, osemenjenih unutar 7 dana posle zalučenja, podeljeno je u 5 grupa. Grupa I: u inseminacionu dozu je dodato 10 i.j. oksitocina, neposredno pre VO (n=50), Grupa II: izvršena je i/m injekcija 5 i.j. oksitocina u vulvu krmača, neposredno pre VO (n=50), Grupa III: stimulacija cerviksa vrhom katetera, neposredno pre i nakon aplikacije inseminacione doze (n=49), Grupa IV: kontakt sa pono zrelim nerastom, neposredno pre, tokom i neposredno posle VO (n=50) i Grupa V: krmače su osemenjene bez ikakve stimulacije, kontrolna grupa (n=50). Vrednost prašenja je bila statistički značajno veća ($P<0,05$) kod svih stimulisanih krmača (92%, 88%, 90% i 84%), u poređenju sa krmačama koje nisu bile stimulisane (78%). Veličina legla se nije značajno razlikovala između stimulisanih i kontrolnih krmača. Ovi rezultati pokazuju da primenjeni tretmani za stimulaciju kontrakcija miometriuma mogu biti koristan metod povećanja fertiliteta krmača.

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THE INTERACTION BETWEEN THE SWINE INFECTIOUS DISEASES AGENTS AND LOW LEVELS OF MYCOTOXINS IN SWINE FEED

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Abstract: The aim of the paper was to evaluate the possible interaction between the presence of swine infectious diseases and low levels of mycotoxins in swine feed. The material for this research included the samples from three swine farms, where health disorders in different swine categories were detected. The applied research methods included: epidemiological and clinical evaluation, pathological examination, bacteriological and virological laboratory testing and microbiological feed testing, in order to examine the presence of fungi and mycotoxins by the method of thin layer chromatography. Beside this, the molecular diagnostic method, reverse transcripton-polymerase chain reaction (RT-PCR) and viral isolation was included. The obtained results support the existance of positive interaction between the mycotoxins and causative agents of bacterial and viral swine infective diseases.

Key words: infective diseases, swine mycotoxicoses

Introduction

Mycotoxins are secondary metabolites of fungi that can cause serious health problems in animals, and may result in severe economic losses (*Greinier et al., 2013*). At the global level, it is considered that 25% of the world crop production is contaminated by mycotoxins, which may be a risk factor affecting human and animal health (*Bouhet and Oswald, 2005; Weaver et al., 2013*). A recent study investigated the occurrence of mycotoxins in European feed samples and concluded that 82% of the samples were contaminated with mycotoxins, indicating that mycotoxins are omnipresent (*Goossens et al., 2012*). Climatic conditions and growing of cereals on large areas in Republic of Serbia are conducive to the growth of toxigenic species, such as *Fusarium* spp., *Aspergillus* spp. and *Penicillium* spp., resulting in frequent contamination of animal food by their secondary metabolites. In Republic of Serbia, the most often isolated species

in animal food are fungi of *Fusarium* species, as well as their mycotoxins *(Krnjaja et al., 2011).*

Consumption of feed contaminated with mycotoxins may result in immunosuppresion, which represent a predisposing factor livestock to infectious diseases (*Oswald et al., 2005; Prodanov-Radulović et al., 2011*). All farm animals can experience a negative impact form a dietary intake of mycotoxins but pigs are one of the species which are highly sensitive (*Goossens et al., 2012*). The influence of mycotoxin on immune system is of special interest in swine industry. The technology on swine farms demands frequent vaccinations, which may be a problem in the case of immunocompromised animal (*Prodanov et al., 2009*). The economic impact of mycotoxins includes increased mortality, increased veterinary care costs, reduced livestock production, disposal of contaminated foods and feeds (*Goossens et al., 2012*). However, the major problem associated with animal feed contaminated with mycotoxins is not acute disease episodes, but rather the ingestion of low level of toxins which may cause an array of metabolic, physiologic, and immunologic disturbances (*Greinier et al., 2013*). The aim of the paper was to evaluate the possible interaction between the presence of swine infectious diseases and low levels of mycotoxins in swine feed.

Material and Methods

The material for this research included the samples from three swine farms, where health disorders i.e. clinical and patomorphological signs resembling to the problem with bacterial and viral infectious diseases in different swine categories were detected. Depending on the specificity of each evaluated case and available material, the applied research methods included: epidemiological and clinical evaluation, gross pathological examination, standard laboratory testing for detection the precence of aerobic and anaerobic bacteria, virological testing and microbiological feed testing.

History of the pig units

The following details were ascertained by the interview and from farm records: number and category of pigs on the unit, production details (breeding, finishing unit; nucleus or commercial), disease status, current veterinary health plan, (vaccination programmes, routine medication), biosecurity protocols and feeding system used. The control of indoor pig environment was inspected with regards to basic zootechnical conditions for swine: ambient temperature, lighting, ventilation, stocking density, bedding and hygiene (*Kyriazakis and Whittemore, 2006*). The animals were observed and inspected for clinical signs of disease and abnormal behaviour. The clinical inspection was followed by the necropsy of dead pigs for pathomorphological and laboratory diagnosis.

Bacteriology testing

Isolation of bacteria from tissue samples deriving from dead pigs was performed by standard aerobic and microaerophilic cultivation. Microscopic examination determined whether the isolated bacteria were Gram positive or not and whether it is a coccoid or rod-like organisms. The determination was carried out by determining the biochemical characteristics of the isolated bacteria (*Quinn et al., 2011*).

Microbiological and mycotoxicological feed testing

The feed sampling were done according to The Official Gazette of SFRY, No 15/87. Microbiological examination of food animals for the presence of total molds and yeasts were performed by standard methods Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of yeasts and molds, Part 2: Colony count technique in products wit water activity less than or equal to 0.95. Determination of the isolated fungi was performed macroscopic examination of colonies in terms of their color and appearance and the microscopic examination of a certain shape of vesicles and conidia, or what the structure of conidia isolated molds (*Quinn et al., 2011*). The presence of mycotoxins in examined feed samples was determined by the method of thin layer chromatography (*Balcer et al., 1978*).

Virological testing

Isolation of Aujeszky's disease virus (ADV; pseudorabies virus PrV) was done by cultivation of tissue samples on cell culture line PK-15 (porcine kidney - ATCC CCL-33). Samples of brain, tonsil, and lung of died animals were homogenized by mortar and pestle and diluted in PBS 1:10 (1g of tissue and 9 mL of PBS) supplemented with antibiotics (200 IU/ml penicillin; 100 μg/ml gentamicin and 5 μg/ml amphotericin B) to prevent bacterial grow. The tissue homogenate is centrifuged on 2000 *g* for 10 min and 1 mL of supernatant was used for inoculation of 24 hours old PK-15 cell culture with 75% confluent cell layer in 25 cm^2 tissue culture flask. Before inoculation, the cell culture growing medium is decanted from the flask and 1 mL of tissue homogenate was added to the cell monolayer, gently shaking to distribute the inoculated material over the whole cell monolayer, and incubated for 1 hour on 37°C. After the incubation 10 mL cell growing medium (Eagle MEM, Sigma) with 10% fetal calf sera (EU grade, PAA, Austria) was added to the cells and cell monolayer was microscopically observed daily for the development of the characteristic herpes virus cytopathic effects (CPE - with rounded birefringent cells, followed by complete detachment of the cell sheet) in the next 7 days. In the absence of any obvious CPE, after the 7 days incubation period, one blind passage into the new 24 old cell monolayer was performed with 1 mL suspension of the first cell passage after 3 cycles of freezing-towing steps. If the visible CPE is observed, the virus presence was confirmed by neutralization with specific antiserum. Beside this, the molecular diagnostic

method, real time reverse transcriptase - polymerase chain reaction (RT-PCR) for for detection of *Mycoplasma hyopneumoniae* (*Strait et al., 2008*) and detection of ADV (*Ma et al., 2008*) were applied.

Serology testing

The serum neutralising test (SNT) was applied in order to estimate the specific antibody titer in farm pigs (sows), following standard procedure as described before (*O.I.E. Manual of Epizootic, 2004*). A total number of 15 blood samples were examined.

Results and Discussion

The first examined farm represent the modern commercial swine farm, located in Južnobački district in Vojvodina. In the time of examination, on the farm the following swine categories were included: 1400 sows, 7 boars, 120 growing gilts, 290 breeding gilts, 2060 suckling piglets, 5051 weaned piglets and 6050 fatteners. The farm represent the one-site production system (farrow-to finish) i. e. all production stages occuring at one site. Applying control of all production stages (farrowing, weaning, finishing), the correct stocking densities and housing requirements (ventilation, temperature, bedding and hygiene) (*Kyriazakis and Whittemore, 2006*) were detected. The farm have organised own veterinary services and swine health control programm include vaccination against *Classical Swine Fever (CSF), Porcine Parvovirus (PPV), Mycoplasma hyopneumoniae, Circovirus type 2 (PCV-2), Erysipelas* and sows vaccination against enteric bacterial infections (*Clostridium perfringes* and *Escherichia coli).* The last mentioned vaccination of dams is alpplied during gestation with the aim to prevent disease in piglets in the first days of life. In the case of disease outbreak (pneumonia, digestive problems), the sucklings, growers and fatteners are therapeutically treated (parenteral injection for clinically diseased animals and water/feed medication is given for in-contacts). Recently, the health disturbances in the youngest swine categories on the farm were registrated. Clinically, the diarrhoea in suckling piglets already in the first 3 days of life after farrowing were detected. After supervision of the farm records several facts were discovered: diarrhoea occurs in the piglets of normal birth body weight, the percent of mortality is higher in animals in good body condition and on the weaning there is 30% of small piglets. Therapeutic treatment of piglets by oral and parenteral antibiotics aplication did not improve health problems. By clinical examination the certain number of suckling piglets the clinical sign of vulvovaginitis (swelling and reddening of the vulva) were discovered. Carriyng health control in the weaned piglets the diarrhoea and signs of pneumonia (dyspnea, cough, purulent nasal discharge) were detected. The gross pathological examination of the dead suckling piglets revealed lesions dominantly

on the mucosal surface of the digestive tract *(Haemorrhagiae mucosae ventriculi, Enteritis catharralis acuta et haemorrhagica*). In dead weaners the prominent pathological changes in lungs were discovered (*Pleuropneumonia, Pneumonia fibrinosa in statu hepatisationis rubrae et griseae*). By bacteriological testing on tissue samples deriving from dead animals the following bacteria was detected: *Escherichia coli haemolytica, Arcanobacterium pyogenes, Pasteurella multocida, Haemophilus parasuis.*

Table 1. The clinical, gross pathology and bacteriology results overview for swine farm No. 1

Swine category	Clinical signs	Gross pathology	Bacteriology testing
Suckling piglets	Diarrhoea, vulvovaginitis	*Haemorrhagiae mucosae ventriculi, Enteritis catharralis, Enteritis haemorrhagica*	*E. coli haemolytica*
Weaned piglets	Diarrhoea, dyspnea, cough, purulent nasal discharge	*Pleuropneumonia, Pneumonia fibrinosa in statu hepatisationis rubrae et griseae*	*A. pyogenes, Pasteurella multocida, Haemophilus parasuis*
Pregnant sows	Not detected	Not observed	Not isolated

Table 2. The results of testing swine feed samples from farm No.1

Complete feed mixture for:	Results of swine feed testing			
	Microbiological testing	Levels of mycotoxines		
	Total fungi number *Fungi Species*	Investigated Mycotoxins	Level (mg/kg)	mpl (mg/kg)
Suckling piglets	**$163x10^3$/1g** *Fusarium sp, Penicillium sp., Aspergillus sp., Rhisopus sp.* mpn: $50x10^3$/1g	AF	**0.018**	0.01
		OCT-A	< 0.02#	0.1
		ZEA	< 0.05#	0.5
Weaned piglets	**88 $x10^3$/1 g** *Fusarium sp, Penicillium sp., Aspergillus sp., Rhisopus sp.* mpn: $50x10^3$/1g	AF	<0.005#	0.01
		OCT-A	**0.12**	0.1
		ZEA	< 0.05#	0.5
Pregnant sows	Not detected	AF	<0.01#	0.02
		OCT-A	< 0.02#	0.2
		ZEA	**0.75**	0.50

Legend: **mpl**- maximum permissible level and **mpn** - maximum permissible number according to Serbian national regulations (The Official Gazette of RS, No. 4/2010); # - limit of detection

Microbiological testing of complete feed mixture for piglets (starter) detected 3-fold increase in the number of fungi genera *Fusarium sp, Penicillium, Aspergillus, Rhisopus sp.* as compared to the level set by the regulation. Applying further laboratory testing, the presence of mycotoxins was detected: zearalenon (ZEA) in the feed for pregnant sows (0.75 mg/kg), total aflatoxin (AF) in the complete feed mixture for piglets (0,018 mg/kg) and ochratoxin (OCT) A in the grover (0.12 mg/kg).

Research investigating the influence of mycotoxins on the animal susceptibility to infectious diseases focuses mainly on exposure to single major mycotoxins. However, limited information is available on the interaction between low levels of mycotoxins and causative agents of swine infectious diseases (*Antonissen et al., 2014*). In our research we noticed the presence of various infections, which react poorly or do not react on the applied antimicrobial therapy (gastroenteritis, pneumonia). Also, the chronic disturbances and presence of infections of low intensity suggest on the potential presence of mycotoxins. As a consequence of immunosuppresive action of mycotoxins (*Kabak et al., 2006*), clinical and pathological lesions that correspond to the infective diseases of different ethiology occured on the examined farm. From the obtained results an example of potential immunosuppresive effect can be presented i.e. the occurence of enteroxemia in piglets, despite the fact that dams were vaccinated twice during gestation. The enterotoxemia is caused by pathogenic bacterial strains and occurs frequently as a cause of mortality in the examined production phase (*Prodanov et al., 2009*). It can be provoked with the feed quality i. e. the presence of mycotoxins. The gastrointestinal tract represents the first barrier against ingested food contaminants and natural toxins (*Bouhet and Oswald, 2007*). Stability of the intestinal flora appeared to be an important factor for animal health (*Oswald et al., 2005*). Thus an impaired balance of the intestinal microbiome, such as dysbiosis condition, could have many adverse effects on the health of the host. However, data on the influence of toxins on the intestinal microflora are still limited (*Greinier et al., 2013*). The biggest challenge with mycotoxicoses is the non-specific nature of symptoms in the affected animals (*Kabak et al., 2006*). Consequently, the health disorders due to mycotoxins in the feed are difficult to diagnose (*Prodanov et al, 2009*). Great potential in prevention of the diarrhoea syndrome of piglets and subsequent improvement in animal growth and feed conversion has been attributed to organic acids, probiotics or/and prebiotics. Although some studies do show little response, a number of studies have shown at least trends for improvements in growth performance, decrease in variation, mortality and morbidity, or decreased medicine costs when prebiotics are fed (*Živković et al., 2011*)

The second evaluated swine farm represent one-site production system i. e. on the farm there is only fatteners production, capacity 2000 fatteners. The pigs are delivered from one large farrow-to-finish, commercial swine farm at the body

weight 20-25 kg. The farm is located in Sremski district in Vojvodina. Applying control of fatteners production, the correct stocking densities and housing requirements (ventilation, temperature and hygiene) (*Kyriazakis and Whittemore, 2006*) were detected. The local veterinary service provide all necessary medication and vaccination against CSF. Anamnestically and clinically, the health problems included increased incidence of respiratory diseases. Analysing the existing data on the farm, the high incidence of morbidity in grovers and fatteners was noticed, which did not decreased after medical treatment. Therapeutic treatment of the diseased animals was intensive and multiple: the antibiotics were given through feed, water and parenterally. In the grover pigs the disease was clinically characterised with the signs of severe coughing, dyspnoea with open-mouth breathing, pyrexia. Prolonged non-productive coughing, worsened by exercise was the main clinical sign of the disease in affected fatteners. Appying gross pathological examination on the dead pigs, the prominant changes on the respiratory tract were detected: acute necrotizing and fibrinous pneumonia, areas of grey-pink consolidation in apical, cardiac and diaphragmatic lung lobes. By bacteriological testing on tissue samples from dead pigs the following bacteria were isolated: *Pasteurella multocida*, *Haemophilus parasuis*, *Streptococcus uberis*. Applying RT-PCR method on the lung tissue derived from dead fatteners, *Mycoplasma hyopneumoniae* was detected. By laboratory feed testing for grovers and fatteners the increase presence of total AF (0.04 mg/kg and 0.038 mg/kg) was discovered.

In the second evaluted case, the presence of AF in the feed for grovers and fatteners was detected. Consequently, on the farm an evident decrease in the swine immunity against infective diseases of the respiratory tract was noticed and no positive respond on the applied antibiotic therapy. Respiratory disease in pigs are often caused by the combined effects of multiple pathogens and predisposing factors (*Antonissen et al., 2014*). For AF, swine are one of the most sensitive species. When consumed, this mycotoxin can cause immune dysfunction or damage organs, even when consuming moderate concentrations of contaminating grains (*Weaver et al., 2013*).

The last examined farm represent the modern commercial swine farm, located in Južnobački district in Vojvodina. In the time of examination, on the farm the following swine categories were included: 650 sows, 10 boars, 155 pregnant gilts, 400 breeding gilts, 1400 suckling piglets, 2600 weaned piglets and 4000 fatteners. The farm represent the one-site production system (farrow-to-finish) i. e. all production stages occuring at one site. Applying control of all production stages (farrowing, weaning, finishing), the correct stocking densities and housing requirements (ventilation, temperature, bedding and hygiene) (*Kyriazakis and Whittemore, 2006*) were detected. The farm have organised own veterinary services and swine health control programm include: imunoprophylaxis i.e. the vaccination against *Classical Swine Fever (CSF), Porcine Parvovirus (PPV), Mycoplasma*

hyopneumoniae, Circovirus type 2 (PCV-2), Erysipelas and sows vaccination against *Clostridium perfringes* and *Escherichia coli.* In the case of health distrurbance, the animals are therapeutically treated (parenteral injections and in-water medication). However, recently the connection between the presence of mycotoxins in swine feed and an outbreak of viral infection of swine, *Morbus Aujeszky* (MA) was established. By microbiological testing in feed for lactating sows the precence of fungi (in 1g x 10^3 85,5 *Fusarium sp., Mucor*) and AF (0.09 mg/kg) were detected. Anamnestically, the health disorders in sows and in their litters were observed. By epidemiological investigation it was discovered that on the swine farm 4 months before in total 15 new sows had been introduced. Serologically, in most of the sows the presence of specific antibodies against MA by SNT was detected. However, despite the fact that these animals were serologically positive, the origin of that immunological status from the aspect of MA remained unknown: vaccination or infection. By clinical examination in sows the signs of inapetence, mild apathy, constipation and agalactiae were observed. In suckling piglets the sings of severe disturbance of the central nervous system (paddling, trembling, ataxia, paresis and paralysis) were clinically detected. In some cases the whole litter of piglets died within 48 hours. Clinically the fatteners also become anorectic, listless and apathic. The gross pathological changes that were detected in dead sucklings indicated the lesions characteristic for MA infection (*Necroses miliares hepatis et lienis, Tonsillitis diphtheroides necroticans).* Applying virological testing (VI on the susceptible cell culture PK-15) and RT-PCR from the tissues deriving from dead piglets the *Morbus Aujeszky* virus (ADV) was isolated.

Table 3. The clinical, gross pathology and bacteriology results for swine farm No. 3

Swine category	Clinical signs	Gross pathology	Virology testing
Suckling piglets	paddling, trembling, ataxia, paralysis	*Necroses miliares hepatis et lienis, Tonsillitis diphtheroides necroticans*	isolated ADV RT-PCR positive result
Lactating sows	inapetence, mild apathy, agalactiae	No dead sows	SNT positive antibody titer 1:16- 1:128

Suppressed immune functions by mycotoxins may decrease resistance to infectious diseases, reactivate chronic infections and reduce therapeutic efficacy (*Oswald et al., 2005*). In the last examined case, where the outbreak of MA on the farm were examined, AF in the feed can be connected with the possible reactivation of chronic (latent) infection in sows. Even when is present in low

doses, AF alters the immune response and this may predispose pigs to infectious diseases (*Prodanov-Radulović et al., 2011*).

Table 4. The results of testing swine samples farm No. 3

Complete feed mixture for:	Results of swine feed testing			
	Microbiological testing	Levels of mycotoxines		
	Total fungi number *Fungi Species*	Investigated Mycotoxins	Level (mg/kg)	mpl (mg/kg)
Suckling piglets	< $50x10^3$/1g *Fusarium sp, Penicillium sp., Aspergillus sp., Rhisopus sp.* mpn: $50x10^3$/1g	AF	< 0.005#	0.01
		OCT-A	< 0.02#	0.1
		ZEA	< 0.05#	0.5
Lactating sows	85.5 $x10^3$/1g *Fusarium sp, Mucor sp.* mpn: $200x10^3$/1g	AF	**0.09**	0.02
		OCT-A	< 0.02#	0.2
		ZEA	< 0.05#	0.50

Legend: **mpl**- maximum permissible level and **mpn** - maximum permissible number according to Serbian national regulations (The Official Gazette of RS, No.4/2010); # - limit of detection

One should remember that detected concentrations of mycotoxins in the feed are approximations, because sampling is never completely representative. Applying chemical analyses we can identify mycotoxin, but sometimes causative cereal that iniciated the problem is no longer available or representative sample (*Kabak, 2006*). From the other side, when we are discussing the mycotoxin problem, feed dilution may reduce exposure initially, but care must be taken that wet or contaminated grain can introduce new fungi, and conditions that eventually lead to the entire mixture being contaminated (*Oswald et al., 2005*).

Because of detrimental effects of mycotoxins, a number of strategies have been developed to decontaminate and detoxify mycotoxin-contaminated feed (*Krnjaja et al., 2011*). They may include inhibition of mycotoxin adsorption in the gastrointestinal tract. One of the most recent approaches to the prevention of mycotoxicoses is the addition of non-nutritionally adsorbents in the feed that bind mycotoxins in the gastrointestinal tract and reduce their bioavailability. The activated carbons, aluminosilicate, zeolites, bentonites and certain clays are well known (*Kabak et al., 2006*). Strategy which includes application of good agricultural practice and good storage practice with reduced effect of mycotoxins, with implementation of all regulations and measures, can provide production of safe food for humans and animals *(Krnjaja et al., 2011)*.

Conclusion

The achieved results support the existance of possible positive interaction between the mycotoxins and causative agents of bacterial and viral swine infective diseases. The continuos intake of small amounts of mycotoxins may leads to chronic intoxication which is clinically characterized by the loss of weight, insufficient weight gain and increased susceptibility for infectious diseases. The basic preventive measures in order to protect animals are usage of healthy feed and proper storage and managemant conditions for animals feed. Certainly, when mycotoxicosis occurs or is suspected, the first action should be to change the source of feed. Mycotoxicoses is generally a herd problem and not amenable to individual treatment. Practical preventive program should be part of every swine management program.

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Interakcija između uzročnika infektivnih bolesti svinja i niskih vrednosti mikotoksina u hrani za svinja

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Rezime

Cilj rada je bio ispitivanje mogućnosti interakcije između uzročnika infektivnih oboljenja svinja, bakterijske i virusne etiologije i niskih vrednosti mikotoksina u hrani za svinje. Materijal za ispitivanje je obuhvatao uzorke poreklom sa tri farme svinja, na kojima su registrovani zdravstveni problemi kod različitih kategorija svinja. Primenjene metode ispitivanja su obuhvatale epizootiološka i klinička ispitivanja, patomorfološki pregled, standardne laboratorijske bakteriološke i virusološke metode i mikrobiološko ispitivanje uzoraka hrane u cilju ustanovljavanja prisustva plesni i mikotoksina, metodom tankoslojne hromatografije. Pored toga, primenjena je i molekulatna metoda dijagnostike, reverzna transkripcija-lančana reakcija polimeraze (RT-PCR). Postignuti rezultati ispitivanja ukazuju na postojanje pozitivne interakcije između mikotoksina i uzročnika bakterijskih i virusnih infektivnih bolesti svinja.

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THE EFFECT OF GENOTYPE AND LACTATION ON YIELD AND PHYSICOCHEMICAL PROPERTIES OF EWE MILK

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Original scientific paper

Abstract: Two genotype of sheep have been utilized in the conduct of the experiment composed of 60 ewes from Pirot x Virtemberg as genotype 1 and 60 ewes of Improved Pirot as genotype 2. All the ewes were reared under identical conditions and without any differences in nutrition and management during the whole period under study. The collection of Milk sampling was done in morning and evening during periods (1, 2, 3) of lactation duration. The average lactation duration and average total milk of the two genotypes were very close and has a minimal difference of 0.467 day and 1.562 kg, in favor of genotype 2. The differences between genotypes were not significant (P>0,05). Regarding physical and chemical properties of milk for both genotypes, the difference were very minimal such as follows; viscosity Pa x s - 0.006, electrical conductivity Ω – 0.018, density kg/m^3 -0,001, freezing, t ^{0}C - 0.013, LD number – 0.028 total solids, % – 0.014, fat,% - 0,026, protein,% - 0.085, lactose,% - 0.038, ash,% - 0,021, acidity, ^{0}SH – 0.209. The results indicated that the properties of milk for both genotypes were very near to each other. It can be interpreted that the breeds utilized in the experiment were comparable due to similar characteristics perhaps. The effect of genotype was very significant only for the % protein of the milk. The lactation periods were highly significant in all physical and chemical properties of milk.

Key words: sheep milk, genotype, lactation, physical, chemical properties

Introduction

Milk plays a tremendous role in building a healthy society and can be used as vehicle for rural development, employment and slowing down the migration of the rural population *(Sarwar et. al., 2002)*. The milk is the secretion of the mammary glands and the only food of the young mammal during the first period of

its life. The substances in milk provide both energy and the building materials necessary for growth. It has a dietary properties that is important in human diet and children on the rise. Sheep milk contains higher levels of total solids and major nutrient than goat and cow milk *(Park et. al.,2007).* The goat and sheep milk is similar but sheep milk contain more fat, solids-non-fat, proteins, caseins, whey-proteins and total ash as compared with goat milk *(Jandal, 1996).*

Sheep milk is white but sometimes more or less yellowish depending on the milk fat contents, the size of fat globules and suspended protein, while colostrums has the normal yellow color. The taste of milk has a specific little sweet, depends mostly on the kind of food eaten by animals, and in milk may take the taste and smell of food. Colostrums milk is salty taste, depending on the food that is taken by the animal that can occur through bitter taste. Odor of milk is specific, and the milk belongs to the foods that easily take different scents due of milk fat.

The quantity of milk is characterized of the breed, feeding and the lactation period. Nutrition is a very important factor in milk production and duration of lactation period *(Petrovic et al.,2003, Ilic et al. (2007, 2010), Lalic, M. et al.,* (*1989).* Sheep milk, due to its chemical composition and physicochemical properties is an excellent raw material for the production of some types of dairy products *(Cais-Sokolińska et. al.,2008).*It has higher specific gravity, viscosity, refractive index, titratable acidity, and lower freezing point than average cow milk *(Haenleinand Wendorff, 2006).* Additionally the total solids, conductivity are important parameters in studying the physicochemical compositions and nutritional aspects of milk *(Imran et. al., 2008).* Furthermore, sheep milk composition and its production are influenced by large number of factors which most important are: breed, nutrition, health of the animals, environment and the large number and stage of lactation *(Kuchtik et.al.,2008).*

The aim of the study was to determine the influence of genotype and period of lactation on yield and the physico-chemical properties of milk.

Material and Methods

The experiment was conducted on private farm in Vrnjacka Banja. Two genotype of sheep have been utilized in the conduct of the experiment composing 60 ewes from Pirot x Virtemberg as genotype 1 and 60 ewes of Improved Pirot as genotype 2. All the ewes were reared under identical conditions and without any differences in nutrition and management during the whole period under study.

Testing of milk. The collection of milk sampling was done in morning and evening during periods (1, 2, 3) of lactation duration. The 1st and 2nd month of lactation will be period 1, the 3rd and 4th month will be period 2 and the 5th and 6th

month will be period 3. Milk is heated at a temperature of 50 º C, then for all analyzes cooled to 20 ° C and only for specific gravity (LD number) cooled to 15 ° C. Milk was kept in a refrigerator at 4 º C.

Physical properties of milk. Density of milk was determined Gerber lactodensimeter. As the temperature of milk ranged from 15 - 17 ° C, specific gravity (LD number) is calculated at 15 º C. In tube is poured 50 ml of milk and stir the mixture thoroughly with a diluted solution of $CaCl_2$ specific gravity of 1.135. The value of the refractive index of the serum obtained was read at Hilger refractometer at 20 º C.

Freezing points of milk were determined by methods cryoscope the Funke – Gerber. Viscosity was determined by measuring Hoppler viscometer at a temperature of 20 º C, so that the milk is maintained at 20 ° C. Electrical conductivity of milk was determined by conductivity meter, and for analysis were taken milk tempered at 20 ° C.

The chemical properties of milk. Milk fat content was made by Gerber method. The determination of dry matter (DM) was performed according to International Standard FIL / IDF 21-1962 by which 3 ml of milk dried at constant temperature to a constant weight.

The dried mass is the amount of dry matter (DM). The measurements were performed on an electronic scale.The dry matter without fat was obtained by calculation from the difference between total solids content and milk fat content.

Soxlet Henkel degrees (°SH): obtained by titrating 100 mL of milk with 0.25 NaOH, using phenolphthalein as the indicator. In tube is poured 50 ml of milk and stir the mixture thoroughly with a diluted solution of serum calcium chloride ($CaCl_{2)}$ specific gravity of 1.135. The content stayed 15 minutes in boiling water and then cooled. Thereafter, the refraction reading for certain percentage of milk sugar (lactose) was determined by refractometer method Ackermann.

Total proteins were determined by the Kjeldahl method, using the conversion factor 6.38 for total nitrogen in proteins. Determination of ash was carried out by incineration. Before burning vessel that is glowing and measured on an analytical balance. The container measured by pouring 5 ml of milk, and again the same extent. Thereafter, the vessel is brought into the incinerator at a temperature of 500 - 600C for a period of 3 hours. The percentage of ash is obtained from the difference of empty containers weight and pots with ashes. The resulting value is multiplied by 100 and divided by the weight of milk samples.

Statistical analysis. Results of the physical and chemical properties of milk by lactation periods have been analyzed using GLM methods, SPSS program version 20. Genotype and period of lactation were observed as fixed factors.

Results and Discussions

As presented in table 1, the average lactation duration and average total milk of the two genotypes were very close and has a minimal difference of 0.467 day and 1.562 kg, in favor of genotype 2. The differences between genotypes were not significant (P>0,05). The quantity of milk is characteristic of the breed, and less during the lactation period. The largest quantities are obtained at the beginning of the period of secretion *(Krajinović (1978)*and the least amount at the end of secretion. The quantity of milk produced affected diet *(Ilic et al. (2007), Lalic et al., (1989)*, the findings of these authors was comparable with ours. Nutrition is a very important factor in milk production and duration of lactation period.

Table 1. Average lactation duration and milk yield of sheep per genotype

Dependent Variable	Genotype	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Lactation, days	1.00	168.767	.42	167.940	169.593
	2.00	169.234	.42	168.408	170.060
Total milk, kg	1.00	69.189	.75	67.708	70.670
	2.00	70.751	.75	69.270	72.233

Table 2. The physical properties of milk during lactation period

Dependent Variable	Genotype	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Viscosity,Pa x s	1	3.340	.008	3.325	3.355
	2	3.334	.008	3.319	3.350
El.conduct., Ω	1	55.636	.016	55.605	55.667
	2	55.618	.016	55.586	55.649
Density,kg/m^3 (specfic gravity)	1	1.037	.001	1.036	1.039
	2	1.036	.001	1.034	1.037
Freezing, t ^{0}C	1	-.649	.007	-.664	-.635
	2	-.662	.007	-.677	-.648
LD number	1	36.557	.030	36.499	36.615
	2	36.585	.030	36.527	36.643

In tables 2 and 3, it can be noticed that the regarding physical and chemical properties of milk for both genotypes, the difference were very minimal such as follows; viscosity Pa x s - 0.006, electrical conductivity Ω – 0.018, density kg/m^3 - 0,001, freezing, t ^{0}C - 0.013, LD number – 0.028 total solids, % – 0.014, fat,% - 0,026, protein,% - 0.085, lactose,% - 0.038, ash,% - 0,021, acidity, ^{0}SH – 0.209. The results indicated that the properties of milk for both genotypes were very near to each other. It can be interpreted that the breeds utilized in the experiment were comparable due to similar characteristics perhaps.

The viscosity is changed during lactation, at least initially was 3 x 10^3 Pa x s, and the largest cluster at the end of the period of 3.6 x 10^3 Pa x s. The average

value of 3.3 x 10^3 Pa xs is nearly twice then that of cow's milk (*Djordjevic, 1982).* Based on this figures, the values we attained (Tables 2 and 4) in this study were under this ranges. The electrical conductivity is inversely proportional to the resistance of milk provides during power. Electrical conductivity depends largely on salt in milk and in most part of these salts influence of potassium and sodium chlorides. The electrical conductivity of milk, ranging from 55.586 to 55.667 Ω. Lowest results of electrical conductivity of milk was found by *Djordjevic, 1982).*

The result on the density of milk (specific gravity) was similar in the study of *Mahmood and Sumaira, (2010)* in sheep milk (1.032-1.037). The results in the study of *Yuksea et. al.,(2012)* relating to the lactodensimeter degree, protein content, fat and total solids on the next breeds were: Improved Sakiz breed- 30.4, Pure Sakiz -35.6, Kivircik breed- 35, whereas the protein content; fat and total solids on the said breeds ranges from: 4.3 - 8.7%; 4 - 9%; 15.5 - 24%, this means that our findings within the ranges of theirs. Other authors found the value for the milk fat content is about 7.40%, lactose 4.90 and ash 0.88% *(Memiši and the Bauman, 2002)* while *Stojanovic and Katic, (2004*) have described the average composition of sheep milk indicating the averagecomposition of sheep milk: 19.50% dry matter, 7.20% fat, fat-free dry matter 12.30, 5.70 protein, casein 4.90, lactalbumin and lactoglobulin 0.98%, lactose 4.30, ash 0.90% and the greatest amount of 80.50% is water. According to *Jovanovic (1996)* he commented that ewes receiving quality food could provide milk throughout the year (concentrate, green feed), and the feeding itself can influence milk fat of milk. This statement supported the result of this study.

Table 3. The chemical properties of milk during lactation period

Dependent Variable	Genotype	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Total Solids, %	1	19.027	.051	18.926	19.127
	2	19.041	.051	18.940	19.142
Fat, %	1	8.326	.029	7.956	8.936
	2	8.352	.029	8.005	8.806
Protein,%	1	6.405	.019	6.368	6.442
	2	6.320	.019	6.283	6.357
Lactose,%	1	4.366	.014	4.339	4.393
	2	4.404	.014	4.376	4.431
Ash,%	1	.962	.010	.942	.982
	2	.983	.010	.963	1.003
Non-fat, solids, %	1	10.701	0.49	10.605	10.797
	2	10.705	0.49	10.609	10.801
Acidity,^{o}SH	1	8.747	.076	8.598	8.896
	2	8.538	.076	8.389	8.687

^{0}SH- Soxhlet-Henkel degree

The interaction of genotype and period of lactation on the different properties of milk have shown in table 4 and 5. It can be observed that, almost all of the milk properties got the highest values in period 3 of lactation for both genotypes except for lactose that showed highest for both genotypes in period 1 of lactation while period 2 got second place in all properties for both genotypes. The differences for viscosity in genotype 1 were: 0.195 Pa x s (periods 1&2); 0.386 (periods 1&3); 0.191(periods 2&3) while for genotype 2 were: 0.202; 0.405; 0.203 Pa x s. Pertaining to electrical conductivity, the differences were: 0.35 Ω (for lactation periods 1&2); 0.807 (periods 1&3); 0.457(periods 2&3) for genotype 1 while for genotype 2 were: 0.354 Ω; 0.824; 0.47. The density for genotypes 1 and their differences in periods of laction were: 0.01 kg/m^3; 0.015; 0.005 while in genotype 2 were: 0.014; 0.018; 0.004. As for freezing point, the differences on periods 1&2; 1&3; and 2&3 for genotype 1 were: -.102^{0}C; -.187; -.085 and for genotype 2 were: -.066; -.15; and -.084^{0}C. When it comes to lactodensimeter degree (LD number) the differences for each genotype and periods were: 0.174 - 0.155 ; 0.474 - 0,461 ; 0.3 - 0.306. The differences in total solids for each genotype were: 4.23 - 3.64% (periods 1&2); 11.50 – 11.54% (periods 1&3) and 7.59- 8.2% (periods 2&3). The fat % differences for each genotype were: 4.21 – 4.21% (periods 1&2); 11.48- 11.49% (periods 1&3); 7.59 -7.60% (periods 2&3). Relating to the other properties the differences for each genotype were the next: for protein – 4.19 - 4.39% (periods 1&2); 11.48-11.63% (periods 1&3); 7.60-7.57% (periods 2&3); for lactose- 7.57 - 7,72% (periods 1&2); 11.54 - 11.50%(periods 1&3); 4.29 - 4.10% (periods 2&3); for ash - 4.42-4.33% (periods 1&2); 11.67-11.79% (periods 1&3); 7.59-7.79% (periods 2&3); for non-fat solids- 4.21-4.25% (periods 1&2); 11.49-11% (periods 1&3); 7.60-7.05% (periods 2&3). The differences in acidity,^{0}SH- (Soxhlet-Henkel degree) for each genotype for lactation periods 1&2 were - 0.315-0.226^{0}SH; 0.576-0.475^{0}SH ; 0.261-0.249^{0}SH.

Table 4. Interaction genotype x period of lactation on the physical properties of milk

Dependent variable	Genotype	Period of lactation	Mean	Std. Error	95% Confidence interval	
					Lower Bound	Upper Bound
Viscosity, Pa x s	1	1	3.146	.013	3.119	3.172
		2	3.341	.013	3.315	3.367
		3	3.532	.013	3.506	3.559
	2	1	3.132	.013	3.106	3.158
		2	3.334	.013	3.308	3.360
		3	3.537	.013	3.511	3.563
Electrical conductivity, Ω	1	1	55.250	.028	55.196	55.304
		2	55.600	.028	55.546	55.654
		3	56.057	.028	56.003	56.112
	2	1	55.225	.028	55.171	55.279
		2	55.579	.027	55.525	55.633
		3	56.049	.028	55.994	56.103
Density, kg/m^3 (specific gravity)	1	1	1.029	.001	1.026	1.031
		2	1.039	.001	1.037	1.042
		3	1.044	.001	1.042	1.047
	2	1	1.025	.001	1.022	1.041
		2	1.039	.001	1.036	1.042
		3	1.043	.001	1.041	1.046
Freezing, t ^0C	1	1	-.553	.013	-.578	-.528
		2	-.655	.013	-.680	-.630
		3	-.740	.013	-.765	-.715
	2	1	-.590	.013	-.615	-.565
		2	-.656	.013	-.681	-.631
		3	-.740	.013	-.766	-.715
LD number (Lactodensimeter Degree)	1	1	36.341	.051	36.240	36.441
		2	36.515	.051	36.414	36.616
		3	36.815	.051	36.714	36.916
	2	1	36.380	.051	36.279	36.481
		2	36.535	.051	36.435	36.635
		3	36.841	.052	36.740	36.943

Our results can be comparable with the result obtained by *Pavić et al, (2002)* for Travnik sheep in terms of total solids contained an average of 19.11% and lactose 4.55% while their results for 7.52% fat, 5.90% protein were lowered then our results but 11.45% non-fat solids they attained was higher with ours (Table 3). *Dario et. al., (1996)* who reported that a higher lactose content obtained at the beginning of lactation period (from milk of Leccese sheep) which was true with our study (Table 4). *Manfredini et al., (1993)* stated that protein content of sheep milk was significantly lower at the beginning than at the end of lactation (5.38 and 7.11%; 5.47 and 6.46%) agreed with the result we acquired in this study. According to the statement of *Storry et al., (1983)* that "high fat, protein and total solids concentration in the milk are associated with high yields in the resulting dairy products", supported the values we attained in the later mentioned. The

average % ash obtained in our study for each genotype were higher compared with the result of *Yilmaz et al., (2011)*, which was 0.91% for Red Karaman ewes. The result we gathered on the degree of acidity were lower compared with the result obtained by *Pavić et al., (2002)* which was 9.29 ^{0}SH but their result on freezing point was lower -0.566^{0}C then ours.

Table 5. Interaction genotype x period of lactation on the chemical properties of milk

Dependent variable	Genotype	Period of lactation	Mean	Std. Error	95% Confidence interval	
					Lower Bound	Upper Bound
Total Solids,%	1	1	17.983	.089	17.809	18.157
		2	18.777	.089	18.603	18.952
		3	20.320	.089	20.146	20.494
	2	1	18.030	.089	17.856	18.204
		2	18.711	.088	18.538	18.883
		3	20.382	.089	20.207	20.558
Fat, %	1	1	7.871	.029	7.565	8.421
		2	8.217	.030	7.857	8.862
		3	8.892	.030	8.497	9.512
	2	1	7.895	.027	7.565	8.322
		2	8.242	.029	7.901	8.693
		3	8.920	.030	8.550	9.405
Protein, %	1	1	6.055	.033	5.991	6.119
		2	6.320	.033	6.256	6.384
		3	6.840	.033	6.776	6.904
	2	1	5.967	.033	5.903	6.031
		2	6.241	.032	6.178	6.305
		3	6.752	.033	6.687	6.817
Lactose, %	1	1	4.663	.024	4.615	4.710
		2	4.310	.024	4.263	4.357
		3	4.125	.024	4.078	4.172
	2	1	4.705	.024	4.658	4.752
		2	4.342	.024	4.658	4.389
		3	4.164	.024	4.116	4.212
Ash, %	1	1	.908	.018	.873	.942
		2	.950	.018	.915	.985
		3	1.028	.018	.993	1.062
	2	1	.928	.018	.893	.963
		2	.970	.018	.936	1.005
		3	1.052	.018	1.016	1.087
Solids, non-fat, %	1	1	10.115	.085	9.949	10.281
		2	10.560	.085	10.394	10.726
		3	11.428	.085	10.394	11.594
	2	1	10.138	.085	10.394	10.304
		2	10.588	.084	10.423	10.753
		3	11.391	.085	11.223	11.558
Acidity, ^{o}SH	1	1	8.450	.132	8.191	8.709
		2	8.765	.132	8.506	9.023
		3	9.026	.132	8.767	9.284
	2	1	8.304	.132	8.046	8.563
		2	8.530	.130	8.274	8.787

Table 6. Tests of fixed effects and their interactions on quantity and quality of milk

Source	Dependent Variable	Sum of Squares	df	Mean Square	F	Sig.
Genotype	QTM	2.949	1	2.949	.184	.669
	Fat	.011	1	.011	.118	.732
	Viscosity	.002	1	.002	.226	.635
	Elec.Cond.	.030	1	.030	.660	.417
	Density	.000	1	.000	2.052	.153
	TotalMilk	219.685	1	219.685	2.152	.143
	Freezing	.015	1	.015	1.502	.221
	DM-solids	.018	1	.018	.038	.845
	Non-fat,solids	.002	1	.002	.004	.948
	Protein	.648	1	.648	10.187	.002
	lactose	.129	1	.129	3.710	.055
	Ash	.042	1	.042	2.240	.135
	Acidity	3.931	1	3.931	3.788	.052
	Lactoden.^{o}	.073	1	.073	.467	.495
Period	QTM	14232.409	2	7116.204	443.133	.000
	Fat	83.394	2	41.697	461.472	.000
	Viscosity	9.368	2	4.684	442.825	.000
	Elec.Cond.	39.992	2	19.996	438.930	.000
	Density	.019	2	.009	80.263	.000
	TotalMilk	5.112	2	2.556	.025	.975
	Freezing	1.698	2	.849	86.514	.000
	DM-solids	343.300	2	171.650	364.788	.000
	Fat	101.262	2	50.631	118.057	.000
	Protein	38.004	2	19.002	298.510	.000
	lactose	18.016	2	9.008	259.295	.000
	Ash	.912	2	.456	24.175	.000
	Acidity	16.495	2	8.247	7.947	.000
	Lactoden.^{0}	13.451	2	6.726	42.885	.000
Genotype * Period	QTM	.893	2	.446	.028	.973
	Fat	.010	2	.005	.054	.947
	Viscosity	.005	2	.002	.233	.792
	Elec.Con.	.004	2	.002	.048	.953
	Density	.000	2	.005	.664	.515
	TotalMilk	5.112	2	2.556	.025	.975
	Freezing	.026	2	.013	1.343	.262
	Solids	.300	2	.150	.318	.727
	Non-fat,solids	.077	2	.038	.090	.914
	Protein	.002	2	.001	.014	.986
	lactose	.002	2	.001	.024	.976
	Ash	.000	2	.000	.007	.993
	Acidity	.182	2	.091	.088	.916
	Lactoden.^{0}	.006	2	.003	.019	.981

As exposed in table 6, showed that there were no significant effect of genotype on the following properties such as; viscosity, electrical conductivity, density, freezing point, total solids, fat, ash, and non-fat solids (P>0.05) including lactose and the acidity^0 SH (with a significance of .055 and .052) but there was a highly significant effect of genotype on % protein (P<0.01). The lactation periods have highly significant (P<0.01) effect on all physical and chemical properties of milk. Meanwhile, interaction between genotype and lactation period have no significant effect for all tested properties (physical and chemical).

Conclusion

Based on the results obtained can be terminated that the two genotypes tested had a very closed lactation duration and total quantity of milk. Likewise, also having a very closed mean averages relating to the physical and chemical properties of their milk during periods of lactation. In this connection, the reason might be was that the genotype 2 had a 75% gene of Virtemberg. The effect of genotype was very significant only for the % protein of the milk. The lactation periods were highly significant in all physical and chemical properties of milk.

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Uticaj genotipa i perioda laktacije na količinu, fizičke i hemijske osobine ovčijeg mleka

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Rezime

Istraživanja su obavljena kod dve rase ovaca i to 60 grla Pirotska x Virtemberg kao genotip 1 i 60 ovaca pirotske oplemenjene populacije, kao genotip 2. Sve životinje su držane u istim proizvodnim uslovima na farmi u Vrnjačkoj Banji. Prosečne vrednosti trajanja laktacije i mleka dobijenog u periodu laktacije su bile vrlo ujednačene, tako da nije utvrđen uticaj genotipa na ova svojstva. Takođe, razlike između fizičko hemijskih osobina mleka, u većini slučajeva su bile nesignifikantne. Uticaj genotipa kao fiksnog faktora je bio vrlo signifikantan samo

kod sadržaja proteina u mleku ovaca (P<0.01). Međutim utvrđeno je da period laktacije ima vrlo signifikantan uticaj na sva posmatrana fizička i hemijska svojstva mleka.

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REARING BROILER CHICKENS UNDER MONOCHROMATIC BLUE LIGHT IMPROVE PERFORMANCE AND REDUCE FEAR AND STRESS DURING PRE-SLAUGHTER HANDLING AND TRANSPORTATION

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Abstract: The aim of this study was to evaluate the effect of monochromatic light on broiler performance, fear and stress response during pre-slaughter handling and transportation. Two thousand unsexed one-day old Cobb broiler chicks were used. At day 34, two hundreds broilers of similar live body weight were selected and divided into two equal groups (2 group x 5 replicates). Broilers were reared under white light (WL) from 0-34 day. From 35 to 49 day, the first group was reared under WL and the second group under blue light (BL). Final body weight (FBW), tonic immobility reactions (TI), respiratory rate (RR), heterophils to lymphocytes (H/L) ratio and interlukien-1β (IL-1β) were estimated at day 49 before and after transportation. After transportation, weight of internal organs (liver, spleen, heart and bursa of fabricius) as a percentage of FBW was calculated. Results showed that there was a significant ($P < 0.05$) increase in FBW and reduced weight loss due to transportation in broilers reared under BL. In broilers reared under BL: TI duration, RR, H/L ratio, IL-1β and weight of internal organs (except the heart) were significantly ($P < 0.05$) lower. The interaction effect of light and transportation on TI duration, RR, lymphocytes, H/L ratio and IL-1β were significant ($P < 0.05$). Therefore, it is suggested that BL may be a good tool for improving welfare and mitigating stress not only in pre-slaughter handling but also during transportation of broilers.

Key words: Broilers welfare, monochromatic BL, transportation, tonic immobility, H/L ratio

Introduction

In modern poultry husbandry, light has become an important factor that can be used to improve broiler welfare. Light color has been considered a powerful management tool that can be used for mitigating several stressors in broilers by modulating many physiological, immunological and behavioral pathways *(Xie et al, 2008 and 2011; Lewis and Morris, 1998).* It has also been found that broiler performance could be affected by the light spectra *(Rozenboim et al., 1999; Halevy et al., 1998)*. Broilers reared under blue or green light were significantly heavier than those reared under red or white light *(Rozenboim et al., 2004)*. Several studies indicate that blue light (BL) which characterized by short wavelength seems to stimulate broiler growth at the end of the production cycle (27-49 day) without significant effects on total feed consumption, food conversion ratio and/or mortality rate *(Halevy et al., 1998; Rozenboim et al., 1999; 2004; Cao et al., 2008; Ke et al., 2011)*. It also has an important role in reducing stress, decreasing fear, modulating the stress response and has been suggested to have a calming effect on broilers (*Prayitno et al., 1997; Glatz 2005; Mohamed 2011; Xie et al,. 2008).*

Broilers housed under intensive management systems are subjected to a lower degree of human contact, particularly at systems in which environmental conditions and provision of feed and water are automated. This may produce a fear of humans *(Zulkifli et al., 2002)*. It is well documented that procedures with high human contact such as catching and crating induce stress and fear reactions. At the end of the production cycle, broilers are harvested and transported to the slaughter plant for slaughtering. Several studies have been conducted to determine which stage of the pre-slaughter processes of transportation is the most stressful event for broilers. During harvesting and transportation, broilers are subjected to several stressors such as feed and water deprivation, physical contact with workers, social disruption, noise, overcrowding, vibration and thermal extremes *(Mench, 1992; Mitchell and Kettlewell, 1998; Delezie et al., 2007)*. It has also been found that improper handling and transportation of broilers may result in injuries, increase fear and decrease immune responses, resulting in high mortalities *(Savenije et al., 2002; Nijdam et al., 2004 and 2005; Vieira et al., 2010; Knowles and Broom, 1990)*. *Cashman et al. (1989)* reported that fear levels in birds were mainly determined by transportation and not just by catching and loading.

Many studies have been conducted to determine the effect of light on broiler performance, and many others on the effect of transportation. However, there has been a very few investigation on the influence of light color on transportation of broilers. The objectives of this study was to determine the effect of monochromatic light on broiler performance and to evaluate its role in reducing fear and stress in broiler during pre-slaughter handling and transportation.

Materials and methods

Ethical approval

Animal ethics committee, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, approved the protocol and conduct of the study.

Poultry housing and management

Two thousands unsexed one-day-old Cobb broiler chicks were used in this study. From day 0-34, all birds were reared in the same pen at a stocking density of 11 birds/m^2. The floor was concrete with wood shavings as a bedding material. Birds had ad libitum access to feed and water. Ration was formulated to meet the nutrient recommendations for broilers by the National Research Council *(NRC, 1994)*. At day 34, two hundreds birds of similar live body weight were selected and divided into two equal groups. From day 35-49, each group was reared in a separate pen divided into 5 equal replicates kept under the same conditions, except for the color of light.

Light treatment

All birds were reared under WL from zero to 34 day. From 35-49 day, two different types of light were used; the first group was reared under WL (400 to 700 nm) and the second one was reared under BL (480 nm). The light schedule was constant at 23L: 1D under 15 lx light intensity during the entire experiment. The duration of natural light was 10 h and 56 m, 10 h and 15 m and 10 h and 7m at day 0, 34 and 49 respectively then followed by artificial light either white or blue. The dark hour was set at 21.00: 22.00 h.

Handling and transporting conditions

Feed was withdrawn 6 h prior to birds harvesting. Water was available for ad libitum consumption until 1 h before the birds were manually caught and crated. Individual birds were picked up in an upright position with both hands and placed in the crates (100 x 50 x 25 cm) at a density of 10 birds/crate. Birds were handled as gently as possible in order to avoid physical damage or stress. The duration of transportation was 5 h with an average speed of 60 ± 5 km/h. The average environmental (external) temperature was 23 ± 1.2 °C and core (internal) temperature of 33 ± 2.7 °C.

Blood sampling and analysis

Blood samples (3mL/bird) were aspirated from the wing vein and transferred into vacuum tubes with or without ethylenediaminetetraacetic acid (EDTA). The whole blood (with EDTA) was used for heterophils (H) and lymphocytes (L) count and calculation of heterophils to lymphocytes (H/L) ratio. Serum samples were separated by centrifugation of blood at 3000 rpm for 10 m and were stored at −20°C until analysis.

Experimental treatments

1. Broiler performance

Chicks were individually weighed at day 0, 34, 49 before and after transport (using Sartorius balance produced by Sartorius– universal, made in Germany). Individual live body weights were totaled and divided by the number of chicks to obtain the average live body weight (LBW). The final body weight (FBW) was recorded at day 49 after handling and directly after transportation for 25 randomly selected birds from each light treatment.

2. Tonic immobility (TI) reactions

Immediately following the handling and directly after transportation and unloading of broilers, 25 randomly selected birds from each light treatment were tested individually for the duration of TI. The birds were carried to a separate room (no visual contact with other birds) and subjected to TI measurements. TI was induced as soon as the birds were carried to a separate room by gently restraining the bird for 15 s on its right side by the legs and wings. The researcher then remained observing the bird without unnecessary noise or movement. Direct eye contact between the observer and the bird was avoided as it may prolong TI duration *(Jones, 1986).* A stopwatch was used to record latencies until the bird righted itself. If the bird righted itself in less than 10 s, the process was repeated. If TI was not induced after three attempts, the duration of TI was considered as 0 s. The maximum duration of TI allowed was 600 s *(Campo and Carnicer, 1993).* The number of inductions required to perform TI were also recorded.

3. Respiratory rate (RR)

Respiratory rate was recorded for 25 randomly selected birds by counting the number of thoracic movement visually *(Kassim and Norziha, 1995)* before and after transportation for each light treatment.

4. Heterophils/ lymphocytes ratio (H/L ratio)

Heterophils, lymphocytes and H/L ratio in whole blood were measured using an automatic blood cell counter (exigo-Vet., BOULE MEDICAL AB Inc., Stockholm - Sweden.) after handling and immediately after transportation and unloading processes for 25 birds from each light treatment.

5. Serum IL-1β

The serum IL-1β of 25 birds from each light treatment was measured using a commercial broiler ELISA kit (BioSource International Inc., Beijing, China) before and after transportation *(Xie et a,. 2008).*

6. Weight of internal organs

Immediately after transportation and unloading processes, 10 birds of an average body weight from each light treatment were carefully euthanized via exsanguination from a neck cut that severed the carotid artery and jugular vein. This method is considered humane when performed by a trained person (Gracey 1986). The birds were eviscerated to harvest the liver, spleen, heart and bursa of Fabricius. The organs were gently soaked in 0.9% ice-cold saline to remove the remaining blood. Harvested organs were immediately weighted by digital balance (PW Balance, ADAM equipments Co., USA).

Statistical Analysis

Data were tested for distribution normality and homogeneity of variance. Data were reported as means ± SEM and analyzed by ANOVA with Minitab software version 16. Differences in parameters between groups were compared with Student-t test. The significance level was set at $P < 0.05$.

Results

Data analysis revealed that, there were no significant differences of all measured parameters between the five replicates within the experimental groups.

Broiler performance

Body weights (g) of chicks on day 0 were 40.72 ± 2.14 (minimum) and 47.42 ±1.09 (maximum). On day 34, mean body weight (g) of WL group was 1562.92 ± 53.24 and BL group was 1560.67 ± 35.73. The results for FBW are shown in table 1. Birds reared under BL had a higher FBW ($P < 0.05$) and lost significantly less body weight during transportation and unloading ($P > 0.05$) than those reared under WL.

Table 1. Effect of light treatment and transportation on final body weight, tonic immobility (TI) induction, TI duration, respiratory rate (RR), heterophils, lymphocytes, heterophil to lymphocyte (H/L) ratio and interlukien-1β (IL-1β) of broiler.

Variable	Before transportation		After transportation		P- value		
	WL	BL	WL	BL	light	Transport	Interaction
Final body weight (kg)	2.189 ± 0.018	2.372 ± 0.031	2.143 ± 0.017	2.336 ±0.025	0.0001	0.086	0.835
TI induction	1.480 ± 0.154	1.720 ±0.147	1.120 ±0.167	1.280 ±0.147	0.197	0.011	0.796
TI duration (s)	263.44 ± 1.42	224.60 ±1.26	462.24 ±2.36	340 ±4.09	0.0001	0.0001	0.0001
RR/ min	55.160 ±0.639	47.080 ±0.622	67.840 ±0.607	56.360 ±0.458	0.0001	0.0001	0.005
Heterophils (per 100 cell)	18.440 ±0.259	13.200 ±0.337	19.120 ±0.362	15.120 ±0.291	0.0001	0.0001	0.052
Lymphocytes (per 100 cell)	38.760 ±0.456	41.320 ±0.446	40.080 ±0.412	39.840 ±0.320	0.006	0.846	0.001
H/L ratio	0.477 ±0.009	0.320 ±0.008	0.478 ±0.011	0.380 ± 0.008	0.0001	0.001	0.001
IL-1β (pg/mL)	15.936 ±0.391	2.032 ± 0.082	36.340 ±0.477	8.575 ±0.280	0.0001	0.001	0.0001

The significance level was set at $P < 0.05$ within the same row.

Fear and stress indices

Analysis of TI duration (table 1) revealed significant differences between birds reared under BL and WL before and after transportation. The interaction effect of light and transportation (Fig. 1) was significant ($P < 0.05$). There was no significant ($P > 0.05$) effect of light on number of TI inductions, while transportation had a significant ($P < 0.05$) effect on TI induction number.

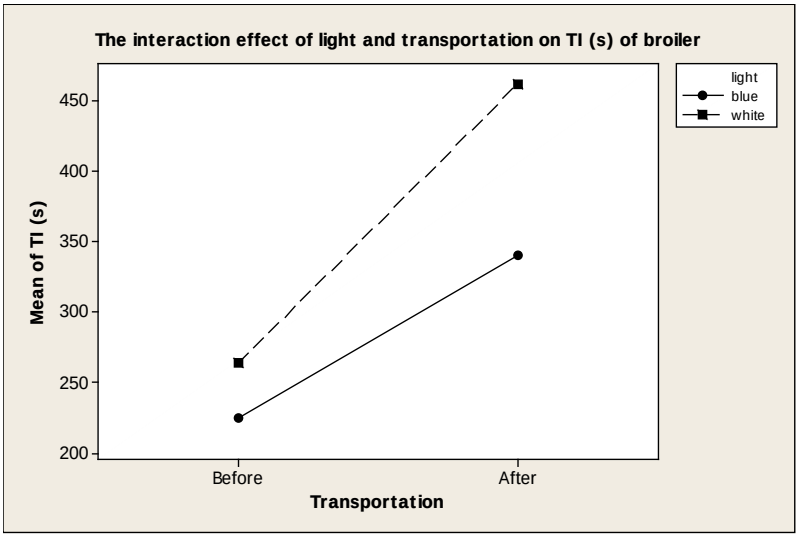

Figure 1.The interaction effect of light and transportation on TI duration(s) of broiler

Results in table one indicated that the RR/min was higher in birds reared under WL than BL before and after transportation. There were significant ($P < 0.05$) effects of light and transportation on RR/min, and the interaction between the two factors (Fig. 2).

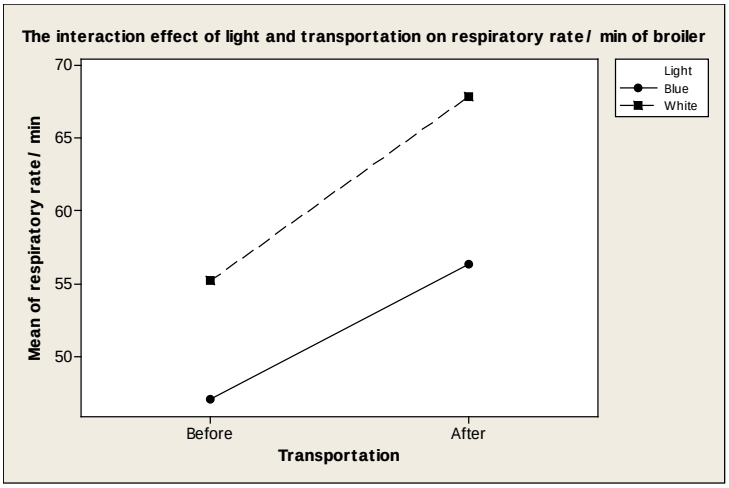

Figure 2.The interaction effect of light and transportation on respiratory rate (RR)/ min of broiler

Heterophils (table 1) was higher in broilers exposed to WL than those exposed to BL before and after transportation. The light colour and transportation had a significant ($P < 0.05$) effect on heterophils but the interaction effect was not significant.

There was a significant ($P < 0.05$) effect of light colour on lymphocytes (table 1). While the effect of transportation on lymphocytes was not significant and the interaction between light and transportation was significant (Fig.3).

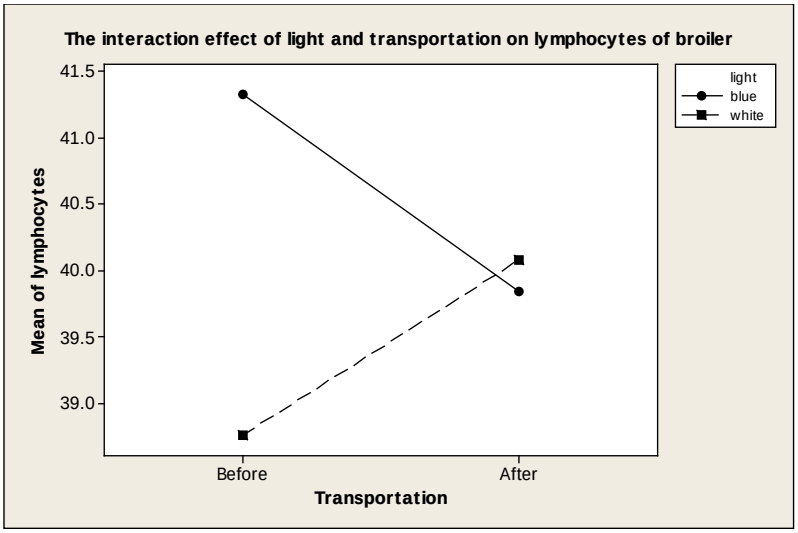

Figure 3.The interaction effect of light and transportation on lymphocytes of broiler

Broilers exposed to WL had significantly ($P < 0.05$) higher H/L ratios than those exposed to BL before and after transportation (table 1). The interaction effect of light and transportation was also significant (Fig. 4).

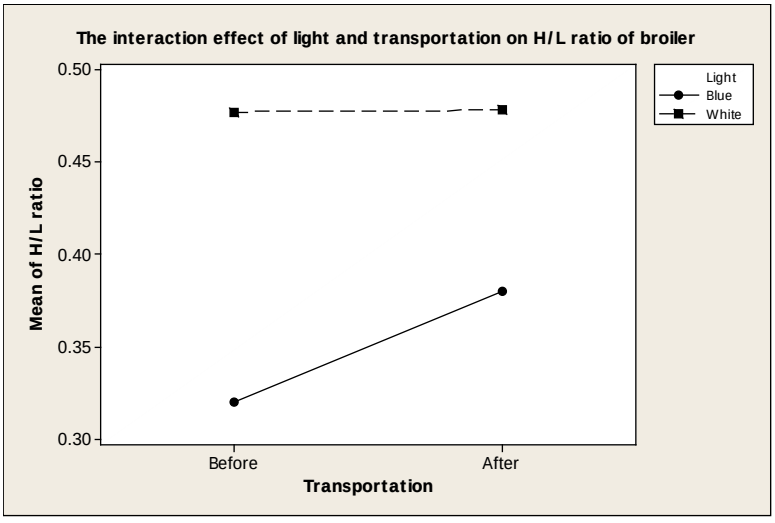

Figure 4.The interaction effect of light and transportation on H/L ratio of broiler

Serum samples of IL-1β in table one were significantly higher ($P < 0.05$) in broilers reared under WL than those reared under BL before and after

transportation. The interaction effect of light and transportation on IL-1β was significant (Fig. 5).

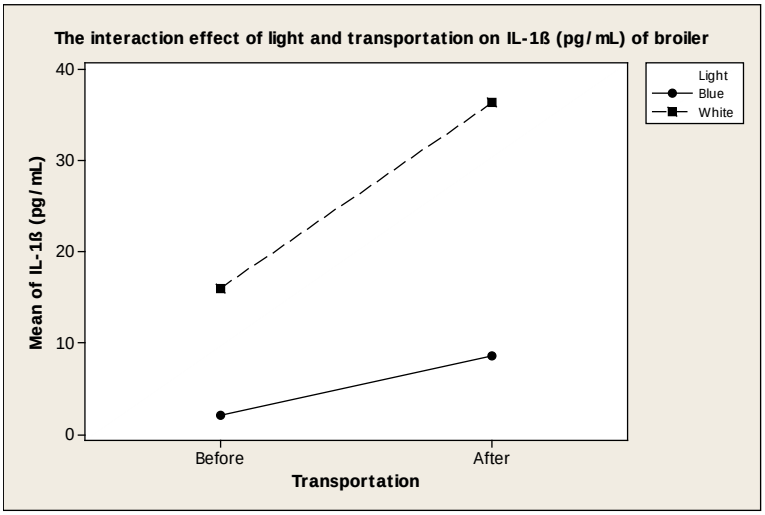

Figure 5.The interaction effect of light and transportation on interlukien-1β (IL-1β) of broiler

After the transportation and unloading processes, the weight of the liver, spleen and bursa of Fabricius in broilers reared under WL was significantly higher ($P < 0.05$) than BL (table 2). No significant difference between light treatments was detected in heart weight.

Table 2. Effect of light colour on weight of internal organs (to final body weight, %) of broiler chickens after transportation

Variable	WL	BL
Liver	2.9460 ± 0.0428^{a}	2.1700 ± 0.0610^{b}
Spleen	0.2008 ± 0.0216^{a}	0.1152 ± 0.0099^{b}
Heart	0.7118 ± 0.0660^{a}	0.5374 ± 0.0276^{a}
Bursa of Fabricius	0.09232 ± 0.00705^{a}	0.05376 ± 0.00235^{b}

Mean values within the same row with different superscript are significantly different ($P<0.05$).

Discussion

In this study, the effects of monochromatic light on growth performance and in mitigating stress during handling and transportation of broilers were

investigated. The welfare of broilers was assessed via performance, TI, RR, H/L ratio, IL-1β and weight of internal organs. The results showed that the spectrum of monochromatic BL positively affects the growth rate of broilers; rearing of broilers under BL from 35-49 d was found to increase the FBW. Our results are in agreement with the results from previous work by *Wabeck and Skoglund (1974); Prayitno (1994); Celen and Testik (1994); Rozenboim et al., (2004); and Cao et al., (2008).* This enhancement in growth may be due to the elevation of plasma androgens that increase protein synthesis and decrease destruction, consequently maintaining myofibrils and muscle growth *(Bates et al., 1987; Salomon et al., 1990; Crowley and Matt, 1996; Rozenboim et al., 1999; Cao et al., 2008).* Monochromatic BL may also improve the quality and antioxidation of muscles, which improves FBW of broilers at later stages of growth *(Ke et al., 2011).*

Body weight loss after transportation in birds reared under BL was lower than those reared under WL. This may be due to the birds being less active, calm and having a well-developed small intestine that indicates good feed absorption (*May et al. 1990; Prayitno et al., 1997; Buhr et al., 1998; Xie et al., 2011*).

Tonic immobility duration in broilers reared under BL was lower than broilers reared under WL. This may be due to the calming effect of BL, causing the birds to become less active and less nervous *(Prayitno et al. 1997).* BL provides adequate illumination for workers but not for broilers, and consequently reduces the movement and escape behavior while harvesting the broilers *(Gregory et al. 1993).*

The results showed that RR/min was increased immediately after handling, loading and during transportation and unloading of broilers reared under WL. However, RR was reduced in broilers reared under BL. This may be due to the exposure of broilers to high temperature, overcrowding and vibration during transportation *(Freeman 1984; Minka and Ayo 2007).* The previous factors may be mitigated in birds reared under BL through its calming effect.

Heterophils to lymphocytes ratio is used as an indicator of stress. Some previous studies *(Mahmoud and Yaseen 2005; Dozier et al., 2006; Mumma et al., 2006; Al -Aqil and Zulkifli, 2009; Kang et al., 2011)* reported that H/L ratio increased under the stressful conditions. In the present experiment, broilers reared under BL had lower H/L ratio than broilers reared under WL before and after transportation. This may be attributed to the calming effect of BL and its effect on modulation of stress response in broilers *(Prayitno et al., 1997; Xie et al., 2008)* and the birds become less aggressive and less active *(Glatz 2005; Mohamed 2011).*

Concentration of IL-1β in serum has been used to demonstrate the effect of monochromatic light on the stress response in broilers in many studies. The IL-1β can stimulate specific hypothalamic neurons to secrete corticotrophin releasing hormone, which stimulates the adrenal cortex to secrete corticosterone *(Berkenbosch et al., 1987).* Corticosterone is one of the most reliable indicators of stress in broilers, especially during handling and transportation *(Kannan et al.*

1997; Nijdam et al., 2005; Zhang et al., 2009). Therefore, IL-1β could be considered an indicator of the stress response in birds. In our study, rearing broilers under monochromatic BL has reduced the level of serum IL-1β both after handling and directly after transportation. Serum IL-1β levels were greater in the broilers reared under WL than BL. These results and results from other studies *(Xie et al., 2008)* indicate that BL may be helpful to prevent excessive IL-1β expression compared with WL. In addition, the results demonstrate that exposure of birds to BL (35-49 d) can reduce the adverse effects of stress during pre-slaughter handling and transportation.

There were significant differences in the weight of liver, spleen and bursa of Fabricius of broilers reared under WL and BL after transportation. These results indicated that BL reduced the weight of internal organs. This could be due to the reduction of stress and improvement of general health *(El-Saidy, 2011).*

Conclusion

Monochromatic BL improve broilers performance, welfare and reduce fear and stress during pre-slaughter handling and transportation. Our study recommends that the catching of broilers should be carried out under BL to calm the birds. Further studies are required to make additional development in this area of science.

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Uticaj uzgoja brojlera pod monohromatski plavim svetlom na poboljšanje performansi i smanjenje stresa tokom transporta i postupka pred klanje

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Rezime

Cilj ovog istraživanja je bio da se proceni uticaj monohromatske svetlosti na performanse brojlera, strah i stres kao odgovor na rukovanje pticama tokom transporta i pre klanja. Dve hiljade jednodnevnih Cobb brojlerski pilići, oba pola, su korišćena u istraživanju. U uzrastu od 34 dana, 200 brojlera slične telesne mase su izabrani i podeljeni u dve jednake grupe (2 grupe x 5 ponavljanja). Brojleri su gajeni u uslovima sa belom svetolosti (WL) od 0-34 dana. Od 35. do 49. dana, prva grupa je gajnae pod WL a druga grupa pod plavom svetlošću (BL). Završne telesne mase (FBV), tonične reakcije (TI), respiratorna stopa (RR), odnos heterofila i limfocita (H/L) i interlukien-1β (IL-1β) su procenjeni 49. dana, pre i posle transporta. Posle prevoza, težina unutrašnjih organa (jetre, slezine, srce i burza - *Bursa fabricii*) je izračunata kao procenat FBV. Rezultati su pokazali da postoji značajno ($p < 0,05$) povećanje FBV i smanjenje gubitka težine zbog transporta kod brojlera gajenih pod BL. Kod brojlera gajenih pod BL: trajanje TI, RR, H/L odnos, IL-1β i težine unutrašnjih organa (osim srca) bili su značajno ($P<0,05$) niži. Interakcija efekta svetlosti i transporta na trajanja TI, RR, limfocite, H/L odnos i IL-1β je bila značajna ($p<0,05$). Stoga se sugeriše da je BL možda dobar alat za poboljšanje dobrobiti i ublažavanje stresa, ne samo pred klanje, već i tokom transporta brojlera.

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CARCASS QUALITY OF CHICKENS OF DIFFERENT CONFORMATION

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Original scientific paper

Abstract: The aim of this study was to investigate the effect of conformation of chickens of different genotype on the yield of breast meat. As a typical example of the chickens of very poor conformation pure breed Naked neck chickens were taken, fattened 8 and 10 weeks (groups K_8 and K_{10}). As an example of good conformation, an imported hybrid of chickens was taken, known for its broiler qualities and as medium growing hybrid, Red Bro (R). The second experiment included commercial hybrids of fast growth (Ross, Cobb and Hubbard) reared according to all technological standards of intensive fattening until the age of 42 days. The results obtained were contrary to the conclusion obtained a few decades ago, at the beginning of the study the conformation of chicken, by *Scots and Darrow (1953),* according to which the selection of chickens of heavy type, despite the fact that, to some extent, it had improved meat yield of the breast, did not significantly improve slaughter traits of fattening chickens, confirming that better conformation and higher body weight had a positive impact on improving relative share of breast, i.e. white meat. The results regarding the slaughter traits of chicken genotypes of different conformation suggest that breeding - selection work to improve the conformation of broilers significantly improved slaughter yields and breast meat yield. In this sense, the conformation can be treated as an indicator of the slaughter value of carcasses, rather than an aesthetic category.

Key wards: genotype, conformation, share of meat

Introduction

In recent decades, in the selection of heavy-type hens, considerable attention has been given to body type or structure, or conformation of the body of chickens in broiler age, and in this regard has made remarkable progress. Even though, in broiler hybrids, in modern production certain minor differences in conformation may be established, virtually there are no significant differences in slaughter output ratios. Possible differences in these traits appear to be predominantly in genotypes of different body weight before slaughter *(Hopić 1999;*

Hopić et al., 2002; Vračar et al., 1996; Pavlovski et al., 2006; Pavlovski et al., 2007; Blagojević et al., 2009).

Back in 1951, *Asmundson and Lerner (1951)* have expressed the opinion that the selection to improve the conformation rather concerns vague aesthetic standards by which a consumer evaluates the appearance of dressed carcass than the actual amount of meat on the carcass. On the other hand, the results of some authors indicate no significant differences in the characteristics in slaughter traits of chicken of different conformation. If we compare the results of the research obtained in the last 20 years, it can be concluded that the genetic selection testing made a significant contribution to the increase in body weight of chickens and share of breast (white meat), and thus a better conformation. The results of our study in 2014 confirmed that genotype and body mass directly affect better conformation and a higher share of breast meat.

It seemed interesting to perform tests with genotypes of chickens of distinctly different conformations: Red Bro and indigenous breed Naked neck, on the one hand and commercial fast-growing hybrids, on the other hand, in terms of carcass quality.

Materials and Methods

The experimental research was conducted through two experiments. In the first experiment, as an example of good conformation, an imported hybrid of chickens was taken, known for its broiler qualities and as medium growing hybrid, Red Bro (R). In contrast, as the representative of the old, unimproved type of conformation, native breed Naked neck was taken, grown in pure blood without applying selection measures. Since the body weight of this population at the age of 8 weeks was significantly lower than that of the modern hybrids, in addition to these groups, a group of chickens was slaughtered at 10 weeks of age K_8 and K_{10}). Chickens of these groups were grown extensively in the same building, in the same conditions of keeping and feeding, except for the differences in the duration of fattening.

The second experiment included commercial fast growing hybrids (Ross, Cobb and Hubbard) which were reared according to all technological standards of intensive fattening until the age of 42 days.

Prior to slaughtering chickens spent 12 hours without food and water. After measuring of body weight, chickens were slaughtered in the experimental poultry slaughterhouse of the Institute for Animal Husbandry, Belgrade-Zemun. The following body dimensions (measures conformation) were measured: shank length, keel length, breast angle and thigh girth according to the method of *Pavlovski and Mašić (1983)*, on 15 and 12 chickens, respectively, per genotype and gender, and the obtained results are shown in absolute values. In recent years the presentation of conformation less precise but more comprehensive indexes are used which show

the relationship of live weight and linear measures or simply body weight (g) at 1 mm of corresponding body measure.

Slaughter yields (dressing percentage): traditionally dressed carcass, ready to cook, ready to grill were taken according to *Pravilnik o kvalitetu pernate živine (1981) (Rulebook on quality of poultry meat)*.

The software package STATISTICA, version 12 (Stat Soft inc.) was used for statistical analyses. The level of statistical significance of differences betwen groups was determined by Tukey-test.

Results and Discussion

Data on individual body measures that in some way reflect the body conformation of chickens are given in table 1.

Based on the data from the table, the following can be concluded:

- The difference in the average live weight of chickens between groups R and K_{10} was very strong and almost 500 g, so it does not seem justified to compare these groups in other traits;
- The average live weight of K_{10} chickens was less than the weight of chicks of group R, and in males by just over 500 g, and in females more than 300 g - in both cases the difference was statistically significant;
- Despite the significantly lower body weight, K_{10} chickens had greater shank length and the keel length compared to chickens of R group - in both case the difference was statistically significant;
- The breast angle was about 40 degrees lower in chicks of group K, and the difference compared to the R group was statistically significant;
- The differences in thigh girth of all the groups were statistically significant and approximately proportional to the difference in live weight;
- A relatively greater keel length and much smaller breast angle in chickens of group K_{10} indicated a significantly less favourable conformation of these chickens compared with chickens of group R.

Table 1. Conformation measures on carcass of chickens

Sex	Genotype	Body weight, g	Shank length, mm	Keel length, mm	Breast angle, degrees	Thigh girth, mm
Male	R	2060^{a}	71.83^{c}	92.83^{b}	113.50^{a}	133.50^{a}
	K_8	1163^{c}	84.93^{b}	93.67^{b}	71.60^{b}	114.46^{c}
	K_{10}	1588^{b}	10.29^{a}	107.65^{a}	73.18^{b}	125.53^{b}
Female	R	1513^{a}	65.50^{c}	94.67^{a}	119.50^{a}	118.17^{a}
	K_8	925^{c}	77.64^{b}	87.07^{b}	70.71^{b}	105.21^{c}
	K_{10}	1179^{b}	86.85^{a}	96.77^{a}	71.46^{b}	112.92^{b}

a-c average values in each column without common marks are significantly different on the level of 5%

Table 2 shows the index values of conformation measures measured on typical 15 chickens for each provenience and gender. Due to the uneven live weight of investigated chicken proveniences, as an relative comparable indicator of the conformation the ratio between the live weight and certain linear measures (index g / mm) was taken. Most suitable index for all the studied measures were recorded in chickens of both sexes of genotype Red Bro (R).

Table 2. Index value conformation measures, g/mm

Sex	Genotype	BW/SL	BW/KL	BW/TG
Male	R	24.24	18.74	13.09
	K_8	13.69	12.41	10.16
	K_{10}	15.83	14.75	12.65
Female	R	23.11	16.04	12.83
	K_8	11.91	10.62	8.79
	K_{10}	13.57	12.18	10.44

BW – body weight KL – keel length
SL – shank length TG – thigh girth

Table 3. Dressing percentage in % of body mass

Sex	Genotype	Yield "Traditionally dressed carcass" %	Yield "Ready to cook" %	Yield "Ready to grill" %
Petlići	R	84.43	67.81	80.32[a]
	K8	85.29	66.16	77.57[b]
	K10	85.91	67.04	77.99[b]
Kokice	R	84.57	68.68[a]	81.21[a]
	K8	82.99	64.46[b]	77.69[b]
	K10	85.14	67.22[a]	78.95[b]

a-b average values in each column without common marks are significantly different on the level of 5%

Table 3 shows the values obtained for slaughter yields of tested chickens per genotype and gender. The most favourable yield "traditionally dressed carcass" had male and female chickens of genotype K10 (85.91% and 85.14%), which were not statistically significant. The resulting yields were significantly better than the yield in the trials with the same pure breed (*Pavlovski et al., 2009*), which can be correlated with a higher body mass live chickens the measured before slaughter.

Significantly best dressing percentage "ready to cook" and "ready to grill" recorded by male and female chickens of genotype Red Bro.

Table 3 shows the values obtained for slaughter yields of tested chickens per genotype and gender. The most favourable yield "traditionally dressed carcass" was established for male and female chickens of genotype K_{10} (85.91% and 85.14%), which were not statistically significant. The resulting yields were significantly better than the yield in the trials with the same pure breed *(Pavlovski et al., 2009)*, which can be correlated with a higher pre-slaughter body weight of live chickens. Statistically significantly best dressing percentages/yields "ready to cook" and "ready to grill" were recorded in male and female chickens of genotype Red Bro.

The hypothesis that chickens of poor conformation expressed through breast angle, achieve also lower share of the breast, or the share of breast meat, is confirmed by our study and data presented in Table 4. Statistically significantly lower share of breast meat in live pre-slaughter body weight of chickens (14.7%) showed the chickens of Hubbard genotype, which had the lowest body weights and the worst conformation expressed by the breast angle.

Table 4. Effect of live mass and breast angle on share of breast meat

Genotype	n	Body mass (g)		Breast angle, degrees		Share of breast (g)		Share of breast meat (%)	
		x	Sd	x	Sd	x	Sd	x	Sd
Ross 308	12	2402.5^{a}	272.3	129.5^{x}	1.4	439.3^{x}	50.9	18.3^{x}	1.1
Cobb 500	12	2406.7^{a}	225.8	129.7^{x}	0.6	458.0^{x}	81.3	18.9^{x}	2.1
Hubbard	12	2180.0^{b}	208.5	125.0^{y}	5.6	321.7^{y}	42.8	14.7^{y}	1.3
Significance									
Genotype		p<0.05		p<0.01		p<0.01		p<0.01	

a-b average values in each column without common marks are significantly different on the level of 5%

x-y average values in each column without common marks are significantly different on the level of 1%

Bearing all this in mind, and contrary to the conclusion from a few decades ago, at the beginning of the study of the conformation of chickens, made by *Scots and Darrow (1953)*, according to which the selection of chickens of heavy type, despite the fact that, to some extent, it improved meat yield of chicken breast, it did not improve significantly slaughter traits of fattening chickens. Contrary to this claim, the present study confirms that better conformation and higher body weight have a positive impact on improving the relative shares of the breast or white meat.

Conclusion

The results of the study of slaughter traits of chicken genotype of different conformation suggest that breeding - selection work to improve the conformation of broilers significantly improved slaughter yields and meat yield of chicken breast. In this sense, the conformation can be treated as an indicator of the slaughter value of carcasses, rather than an aesthetic category.

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Kvalitet trupa pilića različite konformacije

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Rezime

Cilj rada je bio da se ispita uticaj konformacije pilića različitog genotipa na prinos mesa grudi. Kao tipični predstavnici pilića izuzetno loše konformacije uzeti su pilići čiste rase nacked neck, koji su tovljeni 8 i 10 nedelja (grupe K_8 i K_{10}). Kao primer povoljne konformacije uzeta je jedna uvozna provenijenca hibridnih pilića, poznatih po svojim brojlerskim kvalitetima i kao provenijenca srednjeg porasta, Red Bro (R). Drugim ogledom su obuhvaćeni komercijalni hibridi brzog porasta (Ross, Cobb i Hubbard) koji su gajeni po svim tehnološkim standardima intenzivnog tova do uzrasta 42 dana.

Dobijeni rezultati suprotno od zaključka koji su pre nekoliko decenija, na početku ispitivanja konformacije pilića, dali *Scots and Darrow (1953)*, a prema kojima selekcija kokoši teškog tipa, i pored toga što je u izvesnoj meri popravila mesnatost grudi, ipak nije bitno poboljšala klanične osobine pilića u tovu, potvrđuju da bolja konformacija i veća telesna masa pozitivno utiču na poboljšanje realivnog udela grudi, odnosno belog mesa.

Rezultati ispitivanja klaničnih osobina pilića genotipa različite konformacije ukazuju na to da odgajivačko – selekcijski rad na poboljšanju konformacije brojlera je bitno poboljšao klanične randmane i mesnatost grudi. U tom smislu, konformacija se pre može tretirati kao indikator klanične vrednosti trupova, nego kao estetska kategorija.

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FUNGAL CONTAMINATION AND NATURAL OCCURRENCE OF OCHRATOXIN A (OTA) IN POULTRY FEED

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Original scientific paper

Abstract: Total fungal count, the presence of potentially toxigenic fungi and natural occurrence of ochratoxin A (OTA) were studied in 30 poultry feed samples (14 samples of feed for chickens and 16 samples of feed for laying hens), which were collected from different farms in Serbia at the beginning of year 2014. The total number of fungi was determined by the method of dilution and OTA was detected using the imunoadsorption enzymatic assay (ELISA).
In most of the samples of chickens feed (50%) the total number of fungi was 1 - 3 x 10^2 CFU g^{-1}, and in feed for laying hens the highest number of samples (37.50%) had the total fungal count from 1.4 to 4.8 x 10^4 CFU g^{-1}. The species of genera *Aspergillus* and *Penicillium* were identified as producers of OTA in 21.43% and 42.86% of chickens feed samples and in 68.75% and 25% of samples of feed for laying hens. The presence of OTA was detected in 100% of samples of feed for chickens and laying hens, with average concentrations of 34.40 μg kg^{-1} (feed for chickens) and 43.89 μg kg^{-1} (feed for laying hens).
The total fungal count and content of OTA were not above the maximum allowed quantities, even though the presence of *Aspergillus* and *Penicillium* species was found in a large number of samples (up to 68.75%). These results indicate that the tested samples of poultry feed were mycologically and mycotoxicologically correct.

Key words: poultry feed, total fungal count, ochratoxin A (OTA)

Introduction

The fungi are ubiquitous and produce mycotoxins that can occur in all agricultural products in appropriate conditions in the field and in storage. In livestock production, one of the main tasks is to provide healthy food for animals. Feed spoilage by fungi can be a problem for feed security *(Bryden, 2012)*. Poultry

feed is frequently contaminated with mycotoxins. Ochratoxin A (OTA) is a mycotoxin, a secondary metabolite produced by the fungi *Aspergillus* and *Penicillium* species. After aflatoxin, OTA is the second most important mycotoxin in terms of economic losses and is considered the most toxic mycotoxin for birds *(Indresh and Umakanth, 2013).* Ochratoxin often causes lower performances in poultry production, and the level of losses depends on the dose and duration of feeding poultry with contaminated food. The largest amount of this toxin accumulates in the kidneys and liver *(Resanović et al., 2009).* Food contaminated with OTA results in lower egg production, reduced performance and body weight, as well as reduced feed conversion ratio in poultry *(Hassan et al., 2012).*

OTA was detected around the world as a natural contaminant of various grains such as barley, wheat, oats, rye, and maize. OTA is a potent nephrotoxin, immune suppressant, teratogen and carcinogen *(Joo et al., 2013).* Through the food chain, OTA may be involved in the pathogenesis of various forms of human nephropathies, including kidney cancer *(Denli and Perez, 2010).* High concentrations of OTA in food and urine and blood samples in humans are found in rural areas of Bulgaria, Romania, Bosnia and Herzegovina, Croatia, Serbia and Tunisia, where endemic nephropathy (chronic kidney disease) is widespread, where a nephropathy of unknown aetiology occurred. Also, it is considered a possible cause of cancer of the urinary tract in humans and animals *(Klarić, 2013; Kocić-Tanackov and Dimić, 2013)*.

Because of insufficient data on fungal and ochratoxin contamination of poultry feed in Serbia, the aim of the study was to examine the total fungal count, presence of potentially toxigenic fungi, and the level of food contamination with OTA in prepared mixtures of feed for poultry and thereby establish the mycological and mycotoxicological quality of the tested feed samples.

Materials and Methods

At the beginning of 2014 from different poultry farms in Serbia a total of 30 samples of poultry feed (14 chickens and 16 laying hens feed samples) were collected according to *European Commission (2006).* By using a moisture analyzer (Ohaus MB35, USA) the moisture content of tested samples was determined.

According to the pour-plate method, the total fungal count was determined. In the Erlenmeyer bottle the sample (20 g) was homogenized with 180 ml of normal saline (NaCl, 8.5 g/l) in the course of a few minutes on the orbital shaker (GFL 3015, Germany). From homogenate the serial dilutions to 10^{-4} concentration were made and 1 ml of dilutions to 10^{-2}, 10^{-3} and 10^{-4} each, and applied with micro pipette (1000 µl) on Sabouraud maltose agar in a Ø90 mm Petri plates. In incubator (Memmert, Germany) the Petri plates were kept at 25ºC for 5-7 days. Total fungal count was presented as colony-forming units (CFU) per gram of sample. Based on

morphological characteristics the fungal genera were identified according to fungal key of *Watanabe (1994)*.

The presence of ochratoxin A was detected by ELISA assay according to the instructions Tecna S.r.l. (Italy) ELISA kits on an ELISA reader (Biotek EL x 800TM, USA). The limit of detection was 1 μg kg^{-1} for OTA.

Correlation between individual values obtained for total fungal count, concentration of OTA and grain moisture content was determined using Pearson's correlation coefficient.

Results

By analyzing of investigated it was established that the number of fungi ranged from 0 to 14 x 10^4 CFU g^{-1}. Most of chickens feed samples (50%) had a total fungal count from 1 to 3 x 10^2 CFU g^{-1}, whereas 37.50% of laying hens feed samples had a 1.4 to 4.8 x 10^4 CFU g^{-1}. No fungi were detected in 14.29% of chickens feed samples and 6.25% of laying hens feed samples (Table 1). Statistically insignificant negative correlation (r = - 0.31) was determined between the total fungal count and the moisture content in chickens feed samples and positive correlation (r = 0.46) between the total fungal count and the moisture content in samples of feed for laying hens. The moisture content of the chickens feed samples ranged from 8.81 to 12.67% with an average of 11.71%, and of laying hens feed samples ranged from 5.80 to 11.77% with an average of 10.14%.

Table 1. Level of fungal contamination of chickens and laying hens feed samples

Fungal counts (CFU g^{-1*})	Chickens feed	Laying hens feed
	Frequency (%)	
5.4 – 14 x 10^4	0	12.50
1.4 – 4.8 x 10^4	14.29	37.50
1 – 9 x 10^3	21.42	12.50
1 – 3 x 10^2	50	31.25
0	14.29	6.25

*Colony forming units per g of sample

Mycological survey of investigated chickens feed samples identified four fungal genera, *Aspergillus*, *Fusarium*, *Penicillium* and *Rhizopus*, and in addition to above mentioned genera also *Alternaria* and *Mucor* were identified in feed samples for laying hens. In most chickens feed samples the species from genera *Fusarium* (50% positive samples) and *Penicillium* (42.86%) were isolated, followed by species from genera *Aspergillus* (21.43% positive samples) and *Rhizopus* (7.14% positive samples). In most laying hens feed samples the species from genera *Aspergillus* (68.75% positive samples) and *Fusarium* (43.75% positive samples)

were isolated, followed by species from genera *Alternaria*, *Penicillium* and *Rhizopu*s (each with 25% positive samples) and *Mucor* (18.75% positive samples) (Table 2).

Table 2. Fungal genera in investigated chickens and laying hens feed samples

Fungal genera	Chickens feed	Laying hens feed
	Frequency (%)	
Alternaria	0	25
Aspergillus	21.43	68.75
Fusarium	50	43.75
Mucor	0	18.75
Penicillium	42.86	25
Rhizopus	7.14	25

Mycotoxicological analysis showed the presence of 100% OTA positive samples, with mean concentration of 34.40 μg kg^{-1} (chickens feed samples) and 43.89 μg kg^{-1} (laying hens feed samples) (Table 3). In chickens feed samples between the concentrations of OTA, and the moisture content and the concentration of OTA, and the total fungal count, statistically insignificant positive correlations r = 0.21 and statistically insignificant negative r = - 0.34, respectively, were established. In laying hens feed samples between the concentrations of OTA, and the moisture content and the concentration of OTA, and the total fungal count, statistically insignificant positive correlations r = 0.07 and r = 0.09, respectively, were established.

Table 3. Concentration ochratoxin A (OTA) in investigated chickens and laying hens feed samples

Item	Ochratoxin A (OTA)	
	Chickens feed	Laying hens feed
Sample size[a]	16/16	14/14
Incidence %	100	100
Range (μg kg^{-1})	19.04 – 51.30	28.34 – 65.30
Mean[b] (μg kg^{-1})	34.40	43.89

[a] Number of positive samples/Number of total samples
[b] Mean concentration in positive samples

Discussion

The assesment of total fungal count in animal feed is important criteria in the determination of hygienic quality and a necessary tool for assessing the potential risks and dangers of the increased presence of mycotoxins. According to the Regulation on quality of animal feed *(Službeni glasnik Republike Srbije, 2010)*, mixtures and forage raw materials do not correspond to the hygienic quality if they

contain more than 200,000 spores in 1 g of mixture for older animals or 50,000 spores in feed for young animals. In Serbia the maximum allowed level of OTA is 1000 μg kg^{-1} in chickens feed and 250 μg kg^{-1} in laying hens feed *(Službeni glasnik Republike Srbije, 2010).*

In the present studies, most frequently isolated fungal species were from genera *Aspergillus*, *Fusarium* and *Penicillium*. The values for total fungal count and content of OTA in investigated chickens and laying hens feed samples have not exceeded maximum allowed limit confirmed by the Regulation. In Serbia, there is scant data on the presence of OTA and only in some components of the feed, such as for example wheat. Thus, in the mycotoxicological analysis of wheat originating from different localities in Serbia, *Škrinjar et al. (2005)* have detected the presence of OTA in 5 of 20 samples originating from Niš and Leskovac with concentration from traces to 40 μg kg^{-1} and in 70% of samples originating from Kikinda, with a concentration of 8 to 48 μg kg^{-1}. Also, by studying different types of flour, *Škrinjar et al. (2005)* have found a total fungal count of 10 (graham flour) to 8.5 x 10^3 (rye flour), with the most frequently isolated species from the genera *Aspergillus*, *Eurotium* and *Penicillium*. During the five-year period (2007-2012) *Radulović et al. (2013)* have analyzed 104 samples of poultry (broilers and laying hens) for the presence of OTA and found values for ochratoxin in feed mixtures for broilers which ranged from 2 to 650 μg kg^{-1}, and the values in feed mixtures for laying hens from 4 to 100 μg kg^{-1}. *Milićević et al. (2011)* have found low concentrations of OTA in chicken tissue samples and found that chicken nephropathy observed in Serbia has multitoxic etiology with possible synergistic effect between natural toxins and microorganisms, usually present in low concentrations.

In countries with similar geographical and climatic conditions, there is also little data on contamination of poultry feed with fungi and OTA mycotoxin. Thus, in Poland, *Cegielska-Radziejewska et al. (2013)* have found that the level of fungal contamination in 45 samples of feed for broiler chickens was on average 7 x 10^2 CFU g^{-1}, and the most commonly isolated fungal genera were *Aspergillus* and *Rhizopus*. In most of the studied samples of poultry feed originating from south-western Poland, mycoflora contamination has not exceed the allowed level of 2 x 10^5 CFU g^{-1} and in the majority of cases it was in the range of 10^2-10^4 CFU g^{-1} *(Kubizna et al., 2011).* During the two-year study (2006-2007) of the share of mycotoxins that have a synergistic effect in the etiology of nephropathy from 50 feed samples from various pig/chick farms in Bulgaria, *Stoev et al. (2010)* have determined the presence of OTA in concentrations of 188.8±27.3 μg kg^{-1} (2006) and 376.4±63.9 μg kg^{-1} (2007) and range of fungal contamination (*Aspergillus ochraceus* and *Penicillium verrucosum*) from 4 x 10^3 to 6 x 10^5 CFU g^{-1}. In Croatia, in the analysis of 34 samples of poultry feed, *Pleadin et al. (2012)* have detected low concentrations of OTA with an average of 1.42 μg kg^{-1}.

Conclusion

Given the importance of the OTA as a nephrotoxic and carcinogenic agent that causes many kidney diseases, the best way to control the formation of this mycotoxin is to prevent the growth of fungi on wheat as the main component of poultry feed and on other susceptible commodities. In the fields crops should be protected with appropriate fungicides, and moisture content at harvest should be reduced to a safe level. Regulated aeration of the storage facilities also prevents the increase in fungal contamination. The obtained results revealed the presence of potentially toxigenic fungi and OTA, but the levels of these contaminants did not exceed allowed limits. Since the *Aspergillus* and *Penicillium* species were isolated with relative high frequency (up to 68.75% positive samples) and OTA was present in 100% of investigated samples, it is necessary to emphasize the need for continuous monitoring of the mycologically and mycotoxicologically safe animal feed as an important measure to prevent conditions for increased production of mycotoxins.

Acknowledgment

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Kontaminacija gljivama i prirodna pojava ohratoksina A (OTA) u hrani za živinu

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Rezime

Ukupan broj gljiva, prisustvo potencijalno toksigenih rodova gljiva i prirodna pojava ohratoksina A (OTA) proučavani su u 30 uzoraka hrane za živinu (14 uzoraka hrane za piliće i 16 uzoraka hrane za nosilje), koji su sakupljeni iz različitih farmi u Srbiji početkom 2014. godine. Ukupan broj gljiva određen je primenom metode razređenja a OTA je detektovan primenom imunoadsorpcione enzimske metode (ELISA).

U najvećem broju uzoraka hrane za piliće (50%) ukupan broj gljiva je bio od 1 - 3 x 10^2 CFU g^{-1}, a u hrani za nosilje najveći broj uzoraka (37,50%) imao je ukupan broj gljiva od 1,4 do 4,8 x 10^4 CFU g^{-1}. Kao producenti OTA

identifikovane su vrste iz rodova *Aspergillus* and *Penicillium* u 21,43% and 42,86% uzoraka hrane za piliće i u 68,75% and 25% uzoraka hrane za nosilje. Prisustvo OTA je detektovano u 100% uzoraka hrane za piliće i nosilje, sa prosečnim koncentracijama od 34,40 μg kg^{-1} (hrana za piliće) i 43,89 μg kg^{-1} (hrana za nosilje).

Vrednosti za ukupan broj gljiva i sadržaj OTA nisu bile iznad maksimalno dozvoljenih količina, iako je ustanovljeno prisustvo *Aspergillus* i *Penicillium* vrsta u velikom broju uzoraka (do 68,75%). Ovi rezultati ukazuju da su ispitivani uzorci hrane za živinu mikološki i mikotoksikološki ispravni.

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COMPARATIVE HISTOLOGY OF TESTES OF BROWN (*SALMO TRUTTA M. FARIO)* AND CALIFORNIA *(ONCORHYNCHUS MYKISS)* TROUT DURING THE SPAWNING PERIOD

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Original scientific paper

Abstract: The testes of fish are paired organs, of a variable shape in different species of fish. Their structure in the salmonid species is lobular. With the histological assays, we established that the lobes were separated by the connective tissue septa, which, given the intensity of spermatogenesis in the studied groups of fish (*Salmo trutta m. Fario; Oncorhynchus mykiss*) sporadically disappear, in fact, they break. In the space between the lobes there are also cross-sections of blood vessels with visible erythrocytes. During the spermatogenesis, in the interstitium there are clearly observable interstitial endocrine (Leydig) cells that excrete steroid hormones. The intensity of the spermatogenesis in the studied fish varies, which is concluded on the basis of the presence of the spermatogenesis cells. In nature, the reproductive cycle in fish is mostly based on an annual cycle, and that is why different stages of reproduction take place at a different temperature and during a different photoperiod. Hence, regardless of the same time period, the spawning time in November, different types of breeding, and finally salmon farming, point to the very important factors that influence reproduction - diet and microclimatic conditions.

Key words: Brown trout, California trout, histology of testes, spawning

Introduction

Bosnia and Herzegovina is a country with high potentials for fish production, considering that it has natural resources of unpolluted water, which is the main pre-condition for the development of fishing industry. Natural water in Bosnia and Herzegovina consists of 20,000 km of rivers and streams, 1,400 ha of coastal line and 4,000 ha of lakes. Fish producers often note that fish is the only food of animal origin that has made it to the tables in the EU countries. According to the data from

the Bosnia and Herzegovina Agency for Statistics for 2012, Bosnia and Herzegovina produced 3,584.2 t of fish, by 11.5 % less than in 2011. According to the agency's data, carp accounts for 16% of the total amount of consumer fish, trout for 78.6% and other fish for 5.4 %. There are many EU regulations and frequently their changes, amendments or replacements are in line with the new scientific findings, including this area of animal production. According to the EC 2013 Progress Report on Bosnia and Herzegovina - European Commission (Brussels, October 16, 2013, SWD (2013) 700 FINAL), "Regarding fisheries, the Republika Srpska and the Federation of Bosnia and Herzegovina adopted legislations on freshwater fisheries that are partially aligned with the *acquis.* Bosnia and Herzegovina needs to step up efforts to implement the *acquis* for this area in order to facilitate an increase in exports of fish and fishery products to EU." All of the above stated facts point to Bosnia and Herzegovina's great opportunities to increase production of consumer fish, fish progeny and ikra, as the main or additional source of income for population that would be sufficient not only for domestic but for the demanding international market as well. Given that many experts from different scientific aspects invest great efforts to realize these ideas, keeping in mind that this branch of production is very demanding and complicated, both from the aspects of production and placement - uncertainty of placement, a special attention is directed at increasing the reproductive capacity of fish cultured in our fish ponds. With histological assays of the testes of Brown *(Salmo trutta m. Fario*) and California trout (*Oncorhynchus mykiss*) taken from different sites during the spawning time, we attempted to study the intensity of spermatogenesis in these species.

Material and methods

The material required for the histological assays was sampled from different sites of the rivers Vrbas and Neretva in November. The total of 20 males was taken, 10 from different locations and of different breeding. Brown trout was taken from the free watercourse of the river Vrbas, while California trout was cultured in cages on the river Neretva.

Upon fishing, testes were carefully removed from the surrounding tissue and placed in powdered solution of 10% formaldehyde until the moment of their embedding in the paraffin blocks. The testes were placed in 70% alcohol for two days, then in 96% alcohol for one day, and in the end in 100% alcohol for one day. Later, they were transferred into a mixture of 100% alcohol and toluol for two hours and at the end in toluol for four hours. Such prepared testes were placed in paraffin I for five hours and paraffin II for twelve hours, completing the embedding procedure in the paraffin blocks. The processing of the testes from fixation to paraffin embedding was performed on a rotational tissue processor (MICROM

model STP 120). After the embedding, the testes were cut using digital microtome (LEICA RM 2145), several serial cuts from 0.5 to 1.5 micrometer thick. The pieces were placed on glass slides and then they were deparaffinized by being taken through a set of alcohol ranging from lower to higher concentrations. After that, they were stained with hematoxylin-eosine, covered by glass cover and glued with Canada balsam. The histological assays were done with a light microscope under magnification of 100, 200 and 400 times. The results of the histological assays were descriptively displayed in the form of microphotographs.

Results and discussion

It should be emphasized that in fish with external fertilization, spermatozoids are excreted together with semen into their immediate surroundings during the spawning period, where they are momentarily activated by flagella motions and they engage in different physical interactions: osmotic pressure appears on the membrane of a spermatozoid; the surface-to volume-ratio of the fish spermatozoids is much larger than in the majority of other species, as well as the physical connection between the flagella and the surfaces they react with (such is egg shell), (*Cosson et al., 2008*). The same authors note the effect of the bioenergetics aspects, due to which spermatozoids swim fast, reaching a high frequency of flagellum (from 70-100 Hz), implying a large consumption of ATP. It is a known fact that the fish reproductive system, especially that of the salmonid species, is significantly affected by climate factors, especially by water temperature, saturation with oxygen, pH of water, altitude, breeding method, diet and so on. Photoperiod and temperature have different impact on different species of fish, and they can even have different impact on an individual within one species of fish, which depends on the reproductive cycle of the individual itself – there is no universal rule. Of course, all of it is regulated primarily by neuroendocrine system, i.e. the pituitary gland, its hormones that directly act on gonads. The quality and morphology of semen and sperm, i.e. anatomic and functional parameters of the reproductive system in male fish, as well as the content and the quantitative and qualitative composition of sperm are influenced by ambient conditions, water temperature (*Krol et al., 2006*). Early studies of cyclic gonads in male fish also bring into the connection the factors mentioned, as well as the connection of reproductive physiology and activity of the pituitary (*Khannai et al., 1966*). The pituitary gland in fish is built of two basic parts, adenohypophysis and neurohypophysis. Adenohypophysis is divided on three areas marked as proadenohypophysis, mesoadenohypophysis and metaadenohypophysis (*Kozaric, 2001*). All three parts of the pituitary gland are built of different types of cells excreting different hormones with different activity. The cells of mesoadenohypophysis are particularly interesting for the salmonid species. They are divided in three groups,

and the highest in number are those that excrete growth hormone, which, among other things, act on the reproductive organs of fish, and gonadotroph cells, which are basophile cells, and their number varies depending on the sexual cycle of fish (*Bone et al., 1995*).

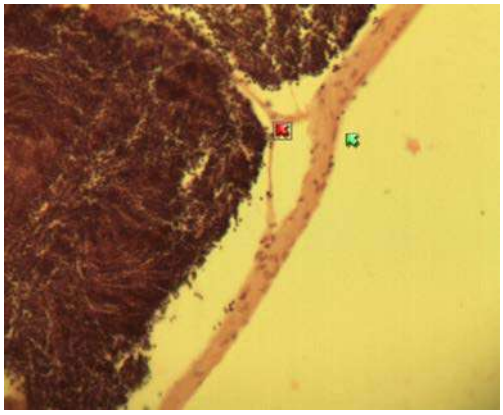

Figure 1. Connective-tissue lining with visible septa (200x, hematoxylin-eosine), Neretva

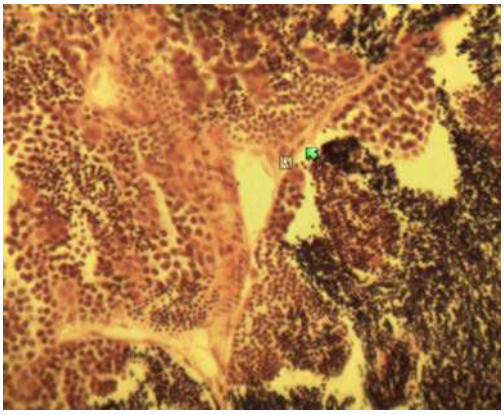

Figure 2. Arrows indicate Myoepithelial and Leydig cells in the interstitum (200x, hematoxylin-eosine), Vrbas

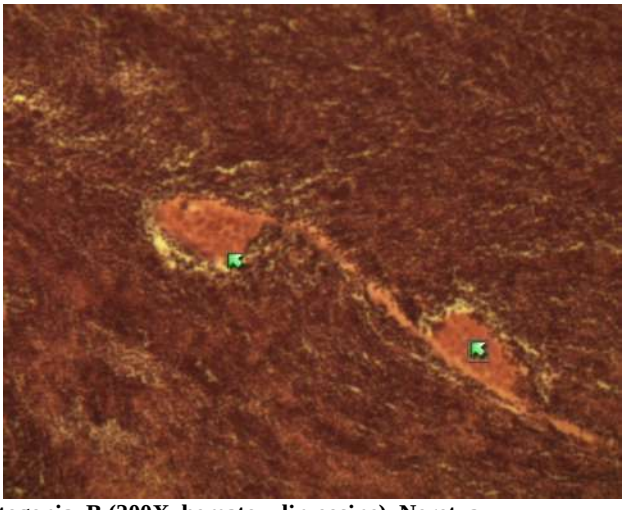

Figure 3. Spermatogonia B (200X, hematoxylin-eosine), Neretva

The process of spermatogenesis is generally a seasonal phenomenon in fish that begins several months before the start of the spawning period, depending on the fish species. Spermatogenesis is the process that occurs throughout the year, but in male fish it is active once a year.Spermatogonia A and B make the first phase of

spermatogenesis, and they contain a diploid number of the chromosome, 2nxy. Mitosis of each spermatogonium B creates two spermatocytes of the first order, 2nxy. A further stage of meiosis I forms spermatocytes of the II order with a haploid number of chromosomes, either nx or ny. Spermatocytes of the II order are short lived; they are divided through meiosis II, forming the spermatids. They are not being divided but rather transformed into mature sexual cells - spermatozoids (*Vergilio et al., 2012*), and it should also be noted that the endocrine system of vertebrates is involved as the main controlling system of spermatogenesis. Males reach maturity usually before females, which is usually around age two in the salmonid species, while females become sexually mature at age three. The spawning period for the salmonid species in our climate conditions (moderate-continental climate) is from November until March. The histological assays of testes in Brown trout (*Salmo trutta m. Fario*) and California trout (*Oncorhynchus mykiss*) that were sampled at different locations in Bosnia and Herzegovina, established discrepancies in the micro-structure and the presence of cells of spermatogenesis during the same time period, but under different microclimatic conditions and diet. The testes of Brown trout, fished from Vrbas, in Central Bosnia, show more intensive picture of spermatogenesis. The water temperature at the time of fishing was 6 °C, while oxygen saturation was 11 ppma. On the surface of testes, there is a very thin connective tissue lining, the starting place of the connective tissue septa, which are broken and disappear in an abundant, active part of testes – parenchyma (Figure 7). Along with the visible connective tissue septa, which imply lobularity of testes, there are also elongated, spindle-like myoepithelial cells and clearly observable polymorphic Leydig cells with spherical nucleus, which are the endocrine cells that excrete steroid hormones (Figure 2). In the parenchyma of testes, there are numerous spermatogonia A, large, round cells with clearly visible, large, light colored basophilic nucleus, and spermatogonia B, which are somewhat smaller and with darker colored nucleus (Figure 4). Division of spermatogonia creates primary spermatocytes that following the meiotic division create the secondary ones. On the histological preparations, spermatocytes of the first order are somewhat bigger than the spermatocytes of the second order, and with somewhat larger, dark colored nuclei. Both types of spermatocytes are grouped in irregular groups within the parenchyma of testes (Figure 4). In the parenchyma of Brown trout, there are Sertoli cells, predominantly located beside spermatogonia A and B, of irregular, pear-like shape, with somewhat darker colored nucleus in relation to spermatogonia. Sertoli cells have a nutrient role, and they also excrete steroid hormones in fish. They show cyclical changes that are connected with spermatogenesis, and they enable differentiation of spermatogonia into sperm (*Katarzyna Dzewulska et al., 2002).* In semen channels, there are also Sertoli cells, supportive, nutrient cells, light colored, inserted among the cells of spermatogenesis. During the spermatogenesis, they are subjected to cytomorphological changes, vacuolization, degeneration, i.e. disorganization of the

ultra-structure, even the necrotic material of Sertoli cells (*Tavchiovska-Vasileva et al., 2012*). The secondary spermatocytes are divided by another meiotic division and they create spermatids that are stained intensively basophilic and have extremely scarce cytoplasm. Each spermatid will develop into spermatozoa (Figure 6).

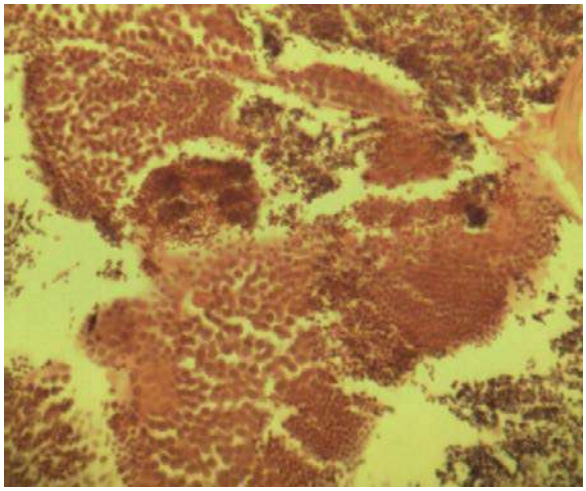

Figure 4. Cells of spermatogenesis; Spermatid, Sertoli cells, spermatozoa and spermatocytes of the I and II order, Spermatogonia A and B (200x, hematoxylin-eosine), Vrbas

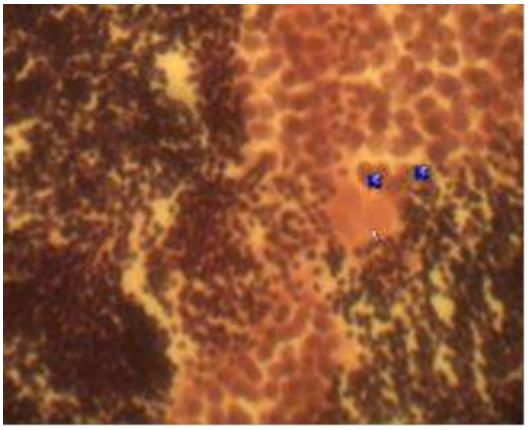

Figure 5. Spermatogonia A and B, Sertoli cells (400x, hematoxylin-eosine), Neretva

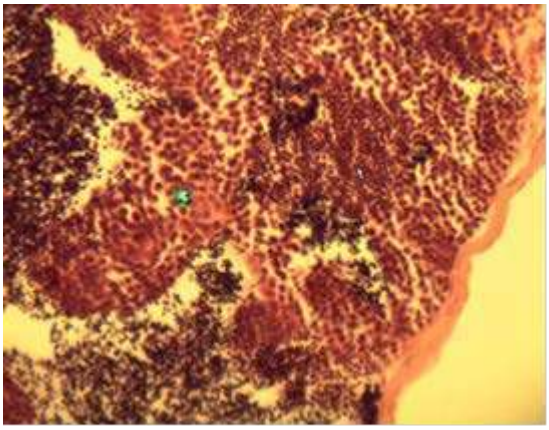

Figure 6. Spermatozoa, spermatid (200x, hematoxylin-eosine), Vrbas

The histological characteristics of California trout fished from the river Neretva in November, previously cultured in the cage system and fed with pelleted, commercial food, show discrepancies in the microstructure in relation to the previously described species. The temperature of the river Neretva at the time of fishing was 8.5 °C, and oxygen saturation was 10 ppm. The surface of testes shows

clearly differentiated connective-tissue lining, where there are noticeable smooth muscle and spindle cells and a few intersections of capillaries, the starting place of the connective tissue septum that separates testes into lobes, therefore, the lobularity is more clearly expressed than in Brown trout (Figure 1). Within the lobes, there is a large number of present spermatozoa, the cells of smaller dimension and with darkly colored nuclei, and on the lobes, where the connective tissue septum is not in continuity, there are sporadically observable Spermatogonia A, the largest spermatogenesis cells, with clearly visible nucleus and cytoplasm (Figure 5), and among which one can notice the nuclei of the nutrient oval Sertoli cells. Spermatogonia B (Figure 3) are smaller cells with darkly colored nuclei and more scarce cytoplasm. In adult, sexually more mature salmonid species, spermatogenesis occurs seasonally, which is closely associated with climate conditions - microclimatic conditions *(Genten et al., 2009*). The continuing changes take place at the cellular level, starting with germinative cells located in the parenchyma of testes, representing the largest cells of spermatogenesis, which are going through the mitotic division, the first division, and then the second division, followed by the first meiotic division of the cells that produce the secondary spermatocytes with a haploid number of chromosomes. These then go through the second meiotic division and they produce spermatids with extremely basophilic nucleus and scarce cytoplasm. Each spermatid will then give a spermatozoon (*Ando et al., 2000*).

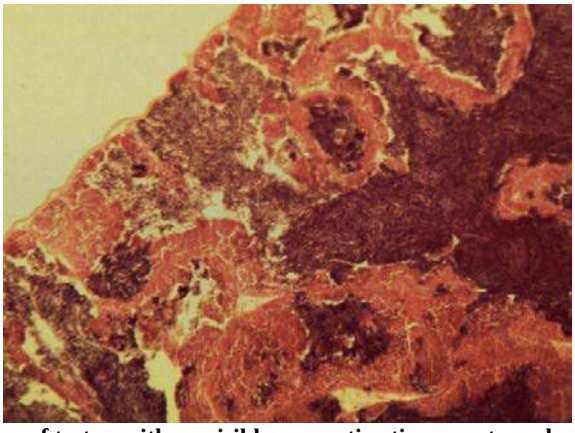

Figure 7. Parenchyma of testes, with no visible connective tissue septa and very thin connective tissue capsules (200x, hematoxylin-eosine) Vrbas

Conclusions

- The testes of Brown trout and California trout are lobular structures; reproduction cycle is based on an annual cycle and

different reproduction stages – spermatogenesis is conditioned by different exogenous and endogenous factors.

- The intensity of spermatogenesis in these species during the spawning time point to the diversities of the microstructure and spermatogenesis cells under variable microclimatic conditions and diet.
- The presence of Sertoli cells within the lobes and their morphs confirm their supportive and nutrient functions to the cells of spermatogenesis
- Leydig cells, their presence point to their neuroendocrine function and participation in the regulation of reproductive cycle, from the aspect of endogenous factors.
- The intensity of spermatogenesis, based on the presence of the spermatogenesis cells varies, i.e. spermatogenesis in Brown trout in relation to California trout is intensified, clear and advanced spermatogenesis and presence of all spermatogenesis cells in Brown trout.

Uporedna histologija testesa potočne (*salmo trutta m. fario)* i kalifornijske *(oncorhynchus mykiss)* pastrmke u periodu mresta

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Rezime

Testesi-semenici riba su parni organi, različitih oblika kod različitih vrsta riba. Kod pastrmskih vrsta su režnjevite građe. Histološkim istraživanjima smo utvrdili da su režnjići odeljeni vezivno-tkivnim pregradama, koje se obzirom na intenzitet spermatogeneze u istraživanim skupinama riba (*Salmo trutta m. Fario; Oncorhynchus mykiss*) mestimično gube, zapravo pucaju. U prostorima između režnjića se mogu zapaziti i preseci krvnih sudova sa vidljivim eritrocitima. Tokom spermatogeneze u intersticijumu su jasno uočljive endokrine, intersticijske (Leydigove) ćelije koje luče steroidne hormone. Intenzitet spermatogeneze u istraživanim grupama riba je različit, što se zaključuje na osnovu prisustva ćelija spermatogeneze. U prirodi, reproduktivni ciklus riba je uglavnom baziran na godišnjem ciklusu i zato se različite faze reprodukcije odigravaju na različitoj temperaturi i fotoperiodu. Dakle, bez obzira na isti vremenski period, vreme mresta u mesecu novembru, ali različite načine uzgoja, te na koncu i salmonikulture,

upućuju na veoma bitne činioce koji utiču na reprodukciju - ishrana i mikroklimatski uslovi.

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IMPACT OF COPPER CYANIDE ON THE KEY METABOLIC ENZYMES OF FRESHWATER FISH *CATLA CATLA* (HAMILTON)

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Abstract: Short term toxicity experiments were conducted to study the effect of metal cyanide complex (copper cyanide) on the key metabolic enzymes viz., lactate dehydrogenase (LDH), succinate dehydrogenase (SDH), glucose-6 phosphate dehydrogenase (G6PDH), aspartate amino transferase (AST) alanine amino transferase (ALT), acid phosphatase (AcP) and alkaline phosphatase (ALP) activity in *Catla catla* juveniles. A total of 60 fingerlings were (2±0.5 cm; 1.5±0.2 g) exposed to two sublethal concentrations (0.253 and 0.152 mg/L) for a period of 15 days. Copper cyanide had significant (P> 0.05) effect on the key metabolic enzymes, the highest activities were observed in the group exposed to 0.253 mg/L. Results suggest that metal cyanide complex significantly altered enzyme activities of fish in both the sublethal concentrations.

Key words: Copper cyanide, enzymes, metabolism, subacute, *Catla catla*

Introduction

Pollution of the aquatic environment is a matter of concern. Around 1500 chemical substances are been listed as pollutants of freshwater ecosystem. Indiscriminate use of such chemicals lead to the contamination of our natural water resources such as lakes, reservoirs, rivers, ponds, paddy-fields, streams, and other low-lying areas all across the globe *(Dellinger et al., 2011).* These chemicals disturb the whole ecosystem, mainly the aquatic ones, leading to needless mortality of aquatic fauna, in particularly fish as revealed by several workers *(Hosetti et al., 2010; Osman et al., 2010; Suneetha, 2012).*

Among the different sources which cause environmental deterioration cyanide is the most important one *(Bhattacharya et al., 2009).* The use of cyanide in mining causes an unreasonable risk to the health of people, wildlife, and

fish *(Eisler and Wiemeyer, 2004)*. Apart from mining, cyanide is also used in photographic processes, production of synthetic rubber, chemical synthesis, manufacture of plastic, pesticides, dehairing of hides, laboratory processes, manufacture of dyes and pigments. These industries release an estimated amount of more than 14 million kg/yr of cyanide *(Dube and Hosetti, 2011)*. Cyanide and its metals complexes are one of the most potentially harmful chemicals due to their adverse effects on non-target organisms, primarily due to the formation of complexes with metal ions that are present as enzyme cofactors. Most notably this occurs with Fe^{3+} ion in cytochrome, thereby inhibiting respiration and hence, oxidative phosphorylation *(Shwetha et al., 2012)*.

Algae and other macrophytes have the ability to tolerate higher environmental concentrations of cyanide, and show no adverse effects until 160mg/L or more when compared to fish and invertebrates *(Heath, 1991)*. Freshwater fish are the most cyanide-sensitive group of aquatic organisms tested, with high mortality documented at free cyanide concentrations >20 microg/L and adverse effects on swimming and reproduction at >5 microg/L *(Eisler and Wiemeyer, 2004)*. Studies carried out on freshwater fish species like *Cyprinus carpio (David et al., 2010)*, *Oreochromis mossambicus (Prashanth, 2012)*, *Cirrhinus mrigala (Shwetha et al., 2012)* found that when exposed to toxic concentrations of cyanide, their tissues get damaged, show abnormal behaviour such as hyper-active and restless swimming, and movements such as burst swimming, jerking, partial jerking and increase in darting.

The present paper is a contribution to the assessment of the toxicity effects of copper cyanide on the Indian major carp *C. catla*. Since biochemical assessment is a useful tool for measuring environmental quality, the present work is aimed to study the effect of copper cyanide on key metabolic enzymes of fish.

Materials and methods

For the present study, freshwater fish *C. catla* (2±0.5 cm; 1.5±0.2 g) were collected from State fisheries Department, Bhadra Reservoir Project, Shimoga and experiment was conducted in the laboratory at Department of Applied Zoology, Kuvempu University, Shimoga, Karnataka India. Fishes were acclimated to the laboratory condition in glass aquarium (20 L) for subacute studies *(APHA, 2005)*. Average water quality parameters during the present investigation were, temperature 27 ± 1°C, pH 7.2 ± 0.2, dissolved oxygen 6.3 ± 0.4 mg/l, hardness 23.2 ± 3.4 mg/l as $CaCO_3$, phosphate 0.39 ± 0.002 µg/L, salinity 0.01ppm, specific gravity 1.001 and conductivity less than 10 µS/cm. Fishes were fed with commercial fish feed pellets (Nova Aquatic Pvt Ltd, not less than 3% of body weight) and water was renewed on every day to maintain water quality.

The toxicant used in the present study was copper cyanide (97% purity), obtained from Loba chemicals Pvt. Ltd, Mumbai, Maharashtra, India. Total of 60 fingerlings of *C. catla* were divided into three groups (20 each). First two groups were exposed to two sublethal concentrations (0.253 and 0.152 mg/L) of copper cyanide for 15 days and third group was maintained as control. These concentrations were selected on the basis of $1/3^{rd}$ and $1/5^{th}$ of 96h LC_{50} *(Hosetti and Dube, 2010)*. At the end of exposure period, fishes were sacrificed and tissues such as liver, gill and muscle were dissected and used for estimating the enzymatic activity. Results obtained were tested by one-way Analysis of Variance (ANOVA). ANOVA effects and treatments differences were considered significant when $p<0.05$.

Five percent of tissue homogenates were prepared in 0.25M ice-cold sucrose solution using a glass homogenizer and centrifuged. Supernatant is used for the estimation of enzymes viz, LDH, SDH, G6PDH, ALT and AST. LDH activity in different tissues was assayed following method of *Govindappa and Swami (1965)* and SDH by the method of *Nachlas et al. (1960)*. The formazon extracted was measured spectrophotometrically at 495 nm and the activity of enzyme was represented in µmol formazon/mg of tissue. For the estimation of G6PDH activity method described by *Bergmeyer and Bernt (1965)* was followed and the activity expressed as µM Pi liberated/mg protein/h, using phosphate standards *(Fiske and Subbarow, 1925)*. ALT and AST activity were estimated using method of *Reitman and Frankel (1957)*. Where as for the estimation of AcP and ALP method of *Kind and King, (1954)* modified by *Agorey (2010)* was followed.

Results

Exposure of fish to both sublethal concentrations of copper cyanide resulted significant changes in the key enzymatic activity of the fish over a period of 15 days. The activity of LDH, G6PDH, ALT and AST exhibited increasing trend in all the tissues under cyanide treatment, where as the activity of SDH, AcP, ALP shown declining trend.

Table 1. Effect of sublethal concentrations of copper cyanide on LDH, SDH (μmol formazon/mg protein/h) and G6PDH activity (μmol of Pi formed/mg protein/h) in different tissues of *C. catla*

	Tissue	Control	Sublethal 1/3rd (0.253 mg/L)	Sublethal 1/5th (0.152 mg/L)
LDH	Liver	1.38 ± 0.03	1.97 ± 0.04	1.91 ± 0.04
	% Change		42.30	38.62
	Muscle	1.15 ± 0.20	1.60 ± 0.10	1.42 ± 0.16
	% Change		39.62	23.59
	Gills	1.59 ± 0.21	2.51 ± 0.19	2.10 ± 0.13
	% Change		57.28	31.67
SDH	Liver	0.74 ± 0.02	0.25 ± 0.02	0.33 ± 0.02
	% Change		-66.46	-55.79
	Muscle	0.75 ± 0.03	0.46 ± 0.01	0.50 ± 0.02
	% Change		-38.81	-33.40
	Gills	1.20 ± 0.02	0.80 ± 0.02	0.86 ± 0.02
	% Change		-33.14	-28.52
G6PDH	Liver	6.08 ± 1.11	8.32 ± 1.06	7.62 ± 0.73
	% Change		36.68	25.25
	Muscle	1.90 ± 0.37	2.84 ± 0.32	2.19 ± 0.21
	% Change		49.58	15.64
	Gills	1.28 ± 0.23	1.70 ± 0.22	1.68 ± 0.30
	% Change		32.43	30.84

Data are means ± SD (n = 5) for an organ in a row followed by the same letter are significantly different ($p < 0.05$) from each other.

Maximum increase in LDH activity was observed in gills (57.28%) at 1/3rd sublethal concentration and liver (38.62%) at 1/5th sublethal concentration. Similarly G6PDH activity was found maximum in liver (49.58%) at 1/3rd sublethal concentration and in gills (30.84%) at 1/5th sublethal concentration. In contrast, SDH activity was declined in both the concentration, showing maximum inhibition in liver (66.46% and 55.79%), at both 1/3rd and 1/5th sublethal concentration (Table 1). Fish exhibited higher ALT and AST activities in both sublethal concentrations. The maximum increase in ALT activity observed in gills (49.45% and 42.27%) at 1/3rd and 1/5th of sublethal concentrations. Similarly the activity AST also exhibited maximum increase in the gills (35.19% and 28.43%) at 1/3rd and 1/5th of sublethal concentrations (Table 2).

Table 2. Effect of sublethal concentrations of copper cyanide on ALT (µmol of pyruvate formed/mg protein/h) in different tissues of *C. catla*

	Tissue	Control	Sublethal 1/3rd (0.253 mg/L)	Sublethal 1/5th (0.152 mg/L)
ALT	Liver	6.41 ± 0.22	9.41 ± 0.12	8.94 ± 0.14
	% Change		46.77	39.49
	Muscle	3.38 ± 0.15^{g}	4.81 ± 0.15	4.57 ± 0.20
	% Change		42.39	35.27
	Gills	4.85 ± 0.19	7.25 ± 0.21	6.90 ± 0.15
	% Change		49.45	42.27
AST	Liver	10.50 ± 0.11	13.60 ± 0.11	13.14 ± 0.07
	% Change		29.54	25.15
	Muscle	7.61 ± 0.12	9.95 ± 0.11	9.28 ± 0.09
	% Change		30.65	21.88
	Gills	12.65 ± 0.21	17.11 ± 0.20	16.25 ± 0.14
	% Change		35.19	28.43

Data are means ± SD (n = 5) for an organ in a row followed by the same letter are significantly different ($p < 0.05$) from each other.

Table 3 shows the activity of acid and alkaline phosphatase activity in the fish exposed to both the sublethal concentration. Maximum inhibition in the AcP was observed in the liver (45.32%) at 1/3rd and muscle (39.86%) at 1/5th sublethal concentration, where as ALP activity exhibited maximum decline in liver (41.76 and 38.74%) at both the concentration.

Table 3. Effect of sublethal concentrations of copper cyanide on AcP and ALP (µmol of p-nitro phenol formed/mg protein/h) in different tissues of *C. catla*

	Tissue	Control	Sublethal 1/3rd (0.253 mg/L)	Sublethal 1/5th (0.152 mg/L)
AcP	Liver	4.10 ± 0.11	2.24 ± 0.21	2.76 ± 0.16
	% Change		-45.32	-32.75
	Muscle	2.63 ± 0.01	1.51 ± 0.06	1.58 ± 0.06
	% Change		-42.62	-39.86
	Gills	1.17 ± 0.03	0.71 ± 0.12	0.79 ± 0.12
	% Change		-38.83	-32.26
ALP	Liver	6.96 ± 0.21	4.05 ± 0.21	4.26 ± 0.16
	% Change		-41.76	-38.74
	Muscle	4.17 ± 0.08	3.04 ± 0.07	2.92 ± 0.02
	% Change		-27.06	-30.00
	Gills	2.69 ± 0.17	1.63 ± 0.06	1.81 ± 0.06
	% Change		-39.47	-32.74

Data are means ± SD (n = 5) for an organ in a row followed by the same letter are significantly different ($p < 0.05$) from each other.

Discussion

Cyanide is one of the most toxic chemical to fish, as fish are one thousand times more sensitive to cyanide than human *(Hosetti and Dube, 2010)*. This active sensitivity of fish to cyanide therefore makes fish an excellent biomarker for the presence of cyanide in water *(Adeyemo, 2005)*. Pollution of the aquatic ecosystem stresses the animals and disturbs their metabolism by altering the enzyme activity, damage and dysfunction the tissues and hindering growth all that associated with biochemical changes (*Osman et al., 2010).* Analysis of biochemical parameters could help to identify the target organs of toxicity as well as the general health status of animals. It may also provide an early warning signal in stressed organism *(David et al., 2010; Prashanth, 2012).*

LDH is a tetrameric enzyme recognized as a potential marker for assessing the toxicity of a chemical *(Suneetha, 2012)*. In the present study gill tissue exhibited maximum decrease in LDH activity compared to muscle and liver. This may be due to the direct effect of the cyanide on disruption of the gill epithelium, resulting to the inhibition of cytochrome oxidase activity *(Bhattacharya et al., 2009)*. This situation might favor anaerobic respiration due to the mild stress of hypoxia in *C. catla*; thus, aerobic processes might be operating at a very slow rate. Similar observations were made by *David et al. (2010)* in the fish *C. carpio*, exposed to sodium cyanide. Further, conversion of pyruvate to lactate at the expense of NAD contributed to increase in LDH activity. Resultantly, to fulfill the energy demands, there may be increase in the operation of glycolysis under cyanide stress *(Rees et al., 2009).*

SDH is an important active regulatory enzyme of the Kreb's cycle of mitochondria. Depletion of SDH can be noticed from the present study in all tissues treated with sublethal doses of cyanide when compared to controls. Suppression of SDH activity in subacute conditions indicates derailment of metabolic cycle. This may also be due to the out come of mitochondrial disruption leading to a decrease in activities of oxidative enzymes *(Prashanth, 2012).* The induced decrease of SDH activity can be attributed to the ability of cyanide to inhibit mitochondrial enzymatic activities *(Shwetha et al., 2012).* Consequently, the decline in SDH activity shows a shifting of aerobic respiration to anaerobic respiration. The results of the present study are also in conformity with those of the earlier observations *(David et al., 2010*).

G6PDH enzyme is extra mitochondrial in location and highly specific for NADP as an electron acceptor *(Barroso et al., 2001)* and is the first enzyme in the pentose phosphate pathway. The effects of different chemical substances on the activity of G6PDH enzyme have been investigated in many *in vitro* and *in vivo* studies, performed with various organisms *(Murat et al., 2009).* In the present study cyanide significantly stimulated G6PDH activity in the fish indicating mobilization of glucose through pathways other than glycolysis-Krebs cycle. High

G6PDH activity indicative of high rates of PPP or HMP (Hexose monophosphate) shunt under stress condition as reported by *Surendranath et al. (1991)* and substantiates the present work.

Transaminases are important enzymes known to play a key role in mobilizing L-amino acids for gluconeogenesis and function as links between carbohydrate and protein metabolism under altered physiological, pathological conditions *(Prashnath, 2012; Shwetha et al., 2012).* Increase in the levels of these enzymes in liver, muscle and gills of fishes can be considered as a response to the stress induced by cyanide to generate ketoacid-like ketoglutarate and oxaloacetate for contributing to gluconeogenesis and/or energy production necessary to meet the excess energy demand under the toxic manifestation *(Okafor et al., 2008).* The increase in the ALT and AST activities in our study supports earlier findings and serves as indicator of tissue damage *(Okolie and Osagie, 2000).* Similar findings were also observed by *Naveed et al. (2010)* in *C. punctatus. Agrahari et al., (2007)* observed an increase in the ALT and AST of the catfish exposed to pesticide and opined that this increase in the activity of these enzymes is an indicator of cellular damage.

Acid phosphatase is a lysosomal enzyme that hydrolyses the phosphorous esters in acidic medium. This enzyme is hydrolytic in nature and acts as one of the acid hydrolyses in the autolysis process of the cell after its death. Alkaline phosphatase splits various phosphorous esters at alkaline pH, its activity is related to the cellular damage. Since, cyanide has anti-phosphatase activity; inhibition of phosphatase activity may be due to reduced protein synthesis, as such phosphatase plays an important role in protein synthesis *(Okolie and Osagie, 2000). Ogundele et al. (2010)* illustrated inhibition in ALP and AcP activity in the adult Wistar rats, administered with the cassava. *Inyang et al. (2011)* reported inhibition of AlP and AcP activity in the fish *C. gariepinus* resulting from the Diazinon exposure.

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Uticaj bakar-cijanida na ključne metaboličke enzime slatkovodnog Indijskog šarana (Hamilton)

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Rezime

Eksperimenti kratkoročne toksičnosti su sprovedeni sa ciljem da se prouči efekat metal cijanid kompleksa (bakar-cijanid) na ključne metaboličke enzime, aktivnost laktat dehidrogenaze (LDH), sukcinat dehidrogenaze (SDH), glukoza-6 fosfat dehidrogenaza (G6PDH), aspartat amino transferaze (AST) alanin amino transferaze (ALT), kisele fosfataze (ACP) i alkalne fosfataze (ALP) u mladim primercima Indijskog šarana. Ukupno 60 mladih šarana su (2 ± 0,5 cm, 1,5 ± 0,2 g) bili izloženi subletalnim koncentracijama (0,253 i 0,152 mg/l) u periodu od 15 dana. Bakar cijanid je imao signifikantan (p> 0,05) uticaj na ključne metaboličke enzime, najviše aktivnosti su zabeležene u grupi izloženoj koncentraciji od 0,253 mg/l. Rezultati ukazuju na to da metal cijanid kompleks značajno menja aktivnost enzima ribe u obe subletalne koncentracije.

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PERMANENCE OF SOWN SWARD SITUATED ALONG THE SLOPES OF THE CENTRAL BALKAN MOUNTAIN

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Abstract: The state of mixed swards of red fescue, Kentucky bluegrass and bird's foot trefoil was studied. The experiment was situated along the slopes of the Central Balkan Mountain, during the period of the 1st to the 13th year of their creation. At a high degree of soil gleying, the low part of the slope, the dry matter yields were within the limits of 2.8 t/ha (1997, south-eastern exposure) up to 10.66 t/ha (1999, north-eastern exposure). At a low degree of soil gleying, high part of the slope, the dry matter yields were within the limits of 2.34 t/ha (1994, western exposure) up to 14.34 t/ha (1995, east exposure). The most prominent in productive terms for the period of the study are the variants at the east and south-eastern exposure, slightly gleyed soil. The participation of the sown species in the total forage yield is variable quantity. They reach (at their most) up to 96% in 1998, north exposure, slightly gleyed soils and up to 97% in 2000, north-east exposure, highly gleyed soils. Their share was small in 2004 (44%) and in 2006 (42%) on a western slope, highly eroded soils.

Key words: red fescue, Kentucky bluegrass, bird's foot trefoil, Balkan Mountain, slopes

Introduction

In the previous study (*Mitev et al., 1992*) a number of positive results have been determined in the growing of mixed sward of red fescue, Kentucky bluegrass and bird's foot trefoil of local origin, which are a subject of patent work. We share the view of some authors that the combination of certain species could ensure priority of the mixed swards through better use of the resources of the environment (*Sanderson et al., 2001*). The combination of components in the swards, as well as the determining of their number should be a result from their behaviour in the conditions of the habitat. When the number of components was increased from 2 to 3, the aggressiveness in the system raised fourfold (*Mitev and Petrov, 1999*).

The aim of the study was to estimate the permanence of a mixed sward consisting of red fescue, Kentucky bluegrass and bird's foot trefoil with a local origin during the thirteen years period, along the slopes of the Central Balkan Mountain.

Materials and Methods

The conditions of creation and conducting of the experiment were described in the methodical part of a previous publication (*Mitev and Belperchinov, 2000*). The main feature in it is the spreading of swards of red fescue, Kentucky bluegrass and bird's foot trefoil along the foothill slopes at a different disposure against the four cardinal points under correspondent soil differences, insolation etc. The variants are given under the enclosed tables. Data were processed by statistical software Stratgraphics Plus v.2.1, with analysis of variance (ANOVA) and multiple comparison of mean values through the smallest statistically proven differences (LSD 0.05).

The dry matter yield and botanical composition of swards were studied.

In relation to soil, the region belongs to the foothill subzone of Northern Bulgaria forest-steppe zone, about which is typical the significant presence of pseudopodzolic soils. Pseudopodzolic horizon (A_1; A_2) has a thickness of 30 – 40 cm and it is saturated abundantly by gleyic spots and iron-manganese concretions. Illuvial horizon (B_1; B_2; B_3) has a thickness of 80 – 100 cm and it is abundantly saturated at the top with light grey and rusty gleyic spots. Brown and brown-yellowish tones are predominant in the lower parts (*Palaveev and Totev, 1983*).

The soils of high degree of gleying (A) were characterized by $pH_{(KCl)}$ 3.9–4.0; exchangeable cations in meqv/100 g soil, Al 1.3–1.6, Mn 0.6–1.3, Ca and Mg 3.6–4.5. The soils with low degree of gleying (B) were characterised by pH (KCl) 4.7; exchangeable cations in meqv/100 g soil, Al 0.6–1, Mn 0.3–0.8, Ca and Mg 9.1–11.1.

The experiment was established in 1994 in a randomized complete block design with four replicates, and a plot size of 4 m^2. After the autumn ploughing in depth of 18-20 cm and the following pre-sowing procedures, 800 germinating seeds were sown at 1m^2 of local populations of red fescue, Kentucky bluegrass and bird's foot trefoil in equal proportions. Each of the above mentioned species participate with 1/3 of its sowing rate for creation of independent swards. Sward was fertilized with N (80 kg/ha) annually and with P (80 kg/ha) every second year, starting from 1995. The dry matter yields were studied in the following way: green mass samples were dried at 105^oC till a permanent weight was reached. The dry matter yield was recalculated on the base of the determined percentage proportion. The botanical composition of swards was determined in the following way: four plant samples were taken from random places in the diagonals of the plots from each variant, for each replication of the experiment, till the weight of the pattern of

two kilograms was reached. The botanical composition of swards was determined in this sample. The swards were cut at the phase of beginning of flowering of legume. Variants A_1 and B_1 were accepted as a control, which was conventionally chosen (*Iglovikov et al., 1971*). The average amount of precipitation for a period of 35 years (1965-2000) was 737.3 mm. It was closer to that in 1994, 1995, 1998. In 1996, 1997, 1999, 2001, 2003 the precipitation was less in comparison with the average for the region. In 2002, 2004, 2006 it was greater. Particularly significant were the differences in 2000, when the precipitation was almost the half in comparison with the average for the region. In 2005 it was almost twice as high. The average annual temperature (1965-1994) for the region was 9.7°C, and for the vegetation period March-October 13.6°C. It was lower in 1996, 1997, 2000, and higher in the rest years.

Results and Discussion

At a high degree of soil gleying, low part of the slope, the dry matter yields were within the limits from 2.8 t/ha (1997, south-eastern exposure) to 10.66 t/ha (1999, north-eastern exposure) (Table 1).

Table 1 Dry matter yield in t/ha of mixed sward from red fescue, Kentucky bluegrass and bird's foot trefoil

Harvest year	Low part of the slope, high degree of soil gleying			High part of the slope, low degree of soil gleying				
	Variant							
	A_1 (k)	A_2	A_3	B_1 (k)	B_2	B_3	B_4	B_5
1994	-	-	2.88	7.98b	9.08a	2.34d	3.79d	4.57c
1995	9.25A	6.38B	4.49B	14.34a	11.90b	7.88c	7.34c	10.24b
1996	4.46B	4.22B	9.07A	5.74b	6.69b	4.54c	5.35b	7.60a
1997	3.21C	2.80C	7.21A	9.30b	12.64a	7.31c	6.12c	7.00c
1998	4.22B	4.82B	7.21A	6.77a	7.58a	7.92a	5.39b	7.33a
1999	6.36B	6.92B	10.66A	8.88b	12.58a	9.00b	8.12b	7.68b
2000	3.42A	3.24A	4.82A	4.08a	4.62a	3.54b	2.97b	4.73a
2001	3.10B	3.41B	7.07A	7.14a	7.59a	3.58b	4.64b	7.36a
2002	6.42B	7.21B	10.10A	8.40b	10.41a	6.77c	7.08c	8.53b
2003	3.41A	3.11A	4.49A	5.46a	5.32a	4.57a	3.15b	4.92a
2004	6.53A	6.99A	-	10.85a	9.09b	7.11c	7.07c	7.14c
2005	6.25A	6.90A	-	6.78b	7.22a	6.37b	5.88c	8.00a
2006	6.31A	6.05A	-	9.68b	11.22a	5.58c	6.12c	-
Average for the period	5.24B	5.17B	6.80A	8.10a	8.18a	5.88c	5.61c	7.91b

The same letters - are not significantly different at the 0.05 probability levels; A1 East exposure (Control of A); A2 South-east exposure; A3 North-east exposure; B1 East exposure (Control of B);

B2 South-east exposure.; B3 West exposure.; B4 West exposure (highly eroded soils); B5 North exposure.

At a low degree of soil gleying, high part of the slope, the dry matter yields were, in the range, from 2.34 t/ha (1994, west exposure) to 14.34 t/ha (1995 eastern exposure). The most prominent in productive terms for the period of the study were the variants at south-east exposure, slightly gleyed soils.

On a slope at south-eastern exposure with heavily gleyed soils the highest productivity was recorded in 2002 (9^{th} vegetation). During the last three year period (2004-2006), the productivity of that variant was higher than that in the previous periods. In a previous publication was presented the thesis that each 'structural unit' (….species, population, variety, ...) probably has a specific energy configuration where a proper energy balance is achieved, which allows the formation of a specific “projection in Time” (including permanence in ontogenetic and phylogenetic aspect), which in a peculiar way is included in the cycle of nature. In this situation, the use of the environmental factors is strictly individual, and a part of them remains conditionally not mastered for ever. The access to them from the part of the plant material, the sustainability of the development of the swards, and so on differ (*Mitev, 2004; Mitev and Naydenova 2012*). In this case it is not difficult to assume that the components in a sward interact on "time level", creating peculiar "energy-informational systems" with their corresponding durability (*Mitev and Naydenova, unpublished*).

The botanical composition of the swards manifests the sustainability of their development (Table 2).

The participation of the sown species in the total forage yield is a variable. They reached up to 96% in 1998, north exposure, slightly gleyed soils and up to 97% in 2000, north-eastern exposure, highly gleyed soils. Their share in the initial and final period of the study usually is smaller: 29%, north exposure, low degree of soil gleying, 1994; 36% west exposure, highly eroded soils, 1994; 42%, west exposure, highly eroded soils, 2006. The red fescue is a predominant species in the swards. In previous publications has been mentioned that the participation of red fescue reached up to 84% in 2004, north exposure, slightly gleyed soils (*Mitev et al., 2006*). It had 89% from the total yields at north-eastern exposure, highly gleyed soils, in 1998 (*Belperchinov and Mitev, 2004*).

The presence of weeds was higher in low level of soil gleying, at a high part of the slope. It should be noticed that the species, included here, are not Setaria viridis (L.) P.B., Setaria glauca P.B., Agropyrum repens (Scop.) etc. We found the presence Galium aparine (L.), Galium tricorne (With), the broadleaf plantain (*Plantago major L.*), narrowleaf plantain (*Plantago lanceolata L.*) etc., which are characteristic for the neighbouring natural swards. These species are herbs that have a positive influence over the animal organism, respectively over the human one.

Table 2 Botanical Composition of swards in %, I cutting

Year		Variant							
		A_1 (k)	A_2	A_3	B_1 (k)	B_2	B_3	B_4	B_5
1994	sown species	0	0	41	46	38	67	36	29
	weeds	0	0	59	54	62	33	64	71
1995	sown species	73	69	77	93	86	89	86	58
	weeds	27	31	23	7	14	11	14	42
1996	sown species	95	89	95	57	83	91	67	95
	weeds	5	11	5	43	17	9	33	5
1997	sown species	89	86	90	94	91	92	88	89
	weeds	11	14	10	6	9	8	12	11
1998	sown species	92	90	94	93	88	93	86	96
	weeds	8	10	6	7	12	7	14	4
1999	sown species	94	94	88	94	60	67	75	92
	weeds	6	6	13	6	40	33	25	8
2000	sown species	96	96	97	88	81	75	84	94
	weeds	4	4	3	12	19	25	16	6
2001	sown species	91	87	86	89	88	80	68	95
	weeds	9	13	14	11	12	20	32	5
2002	sown species	88	92	84	43	84	90	69	92
	weeds	12	8	16	57	16	10	31	8
2003	sown species	89	58	61	94	89	85	86	82
	weeds	11	42	39	6	11	15	14	18
2004	sown species	73	72	-	94	83	64	44	88
	weeds	27	28	-	6	17	36	56	12
2005	sown species	72	87	-	78	81	84	91	51
	weeds	28	13	-	22	19	16	9	44
2006	sown species	88	64	-	71	86	81	42	-
	weeds	12	36	-	29	14	19	58	-

A1 East exposure with a high degree of soil gleying (Control of A); A2 South-east exposure with a high deg. of soil gley.; A3 North-east exposure with a high deg. of soil gley.; B1 East exposure with a low deg. of soil gley. (Control of B); B2 South-east exposure with a low deg. of soil gley.; B3 West exposure with a low deg. of soil gley.; B4 West exposure with a low deg. of soil gley. (highly eroded soils); B5 North exposure with a low deg. of soil gley.

The mentioned species, as well as the other self-sown meadow grasses and legumes, with a local origin such as white and hybrid clover, etc, display the level of balance in the system, as well as the change in the balance. The specificity of the interrelation among productivity and the category of the so called weeds impose a careful analysis according to the type of swards, as well as continuation of the experiment in the future.

Conclusion

The opportunity for prolonged use of artificial meadow wards has been found in the slopes of the Central Balkan Mountain.

At a high degree of soil gleying, low part of the slope, the dry matter yields were within the limits from 2.8 t/ha (1997, south-eastern exposure) to 10.66 t/ha (1999, north-eastern exposure).

At a low degree of soil gleying, high part of the slope, the dry matter yields were within the limits from 2.34 t/ha (1994, west exposure) to 14.34 t/ha (1995 eastern exposure). The most prominent in productive terms for the period of the study were the variants at east and south-east exposure, slightly gleyed soils.

The participation of the sown species in the total forage yield is a variable quantity. They reached up to 96% in 1998, north exposure, slightly gleyed soils and up to 97% in 2000, north-eastern exposure, highly gleyed soils. The grass components, and especially red fescue, are predominant in the swards. The presence of self-sown other legumes of local origin was determined, such as white clover, hybrid clover, etc.

Weed infestation, in view of the durability of swards (1st - 13th year since their establishment) has been slight.

Postojanost sejanih travnjaka koji se nalaze na padinama duž padina centralnih Balkanskih planina

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Rezime

Stanje mešovitih travnjaka crvenog vijuka, livadarke i žutog zvezdana je analizirano u ovoj studiji. Eksperiment je smešten duž padina Centralnog Balkanskog planinskog venca , u periodu od 1. do 13. godine njihovog postojanja.

Na izrazito glejnim zemljištima, na nižim delovima padina, prinosi suve materije bili su u granicama 2,8 t/ha (1997, jugoistočni izloženosti) do 10,66 t/ha (1999, severno-istočni izloženosti).

Na slabo glejnim zemljištima, na višim delovima padina, prinosi suve materije bili su u granicama 2,34 t/ha (1994, zapadne ekspozicije) do 14,34 t/ha (1995, istočne ekspozicije). Najistaknutiji u proizvodnim uslovima za period istraživanja su varijante na istoku i jugo-istoku, na blago glejnom zemljištu.

Učešće posejanih vrsta u ukupnom prinosu travnjaka je promenljivo. Dostiže 96% u 1998, na severnim ekspozicijama i blago glejnim zemljištima i do 97% u 2000.godini na severno-istočnim ekspozicijama i visoko glejnim zemljištima. Njihov udeo je bio mali u 2004. (44%) i 2006. godini (42%) na zapadnoj padini, gde su veoma erodirana zemljišta.

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INFLUENCE OF INTER-ROW SPACING AND CULTIVAR ON THE PRODUCTIVITY OF SOYBEAN

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Original scientific paper

Abstract: Influence of inter-row spacing on a productivity of soybean yield was studied on the experimental field on low carbonate chernozem soil. The greatest grain weight per plant (13.22 g) was achieved at the smallest row spacing. It decreased at higher row spacing, except for cultivar Balkan, where value of these parameters was the highest (13.09 g). The highest grain yield (4,868 kg ha^{-1}) was determined at the 20 cm inter-row width. It decreased equally at bigger row spacing for 7.0-12.9%. The highest grain yield was achieved with Balkan cultivar (4,773 kg ha^{-1}), and the lowest with Dragana cultivar (4,284 kg ha^{-1}).

Keywords: soybean, inter-row spacing, cultivars, yield components

Introduction

Soybean (*Glycine Max.* [L.] Merr.) is importance of comes first and foremost from the chemical composition of its grain, which is about 40% protein and 20% oil (*Popovic et al. 2012, Popovic et al.,2013).* Soybean meal, as a by-product in processing, is indispensable protein component for animal feed. In addition to traditional sources of animal proteins, soybean is used in developed world for controlled nutrition of certain population groups *(Miladinovic et al., 2008).* Grain seed are in the usage for humans and domestic animals diet (*Nikolic et al.,* 2013). Protein content accounts for about 40% of dry soybeans while carbohydrates and oils account for about 35% and 20%, respectively. Because soybeans have high protein content, they are a major ingredient in livestock feed. A smaller percentage is processed for human consumption and made into products including soy milk, soy flour, soy protein, tofu and many retail food products. Soybeans are also used in many non-food (industrial) products. Recently, soybean oil has caused considerable attention due to its increased use for biodiesel production. High quality soybeans are grown, harvested and purchased by the seed industry to be used as seed for the next year's crop *Anđelovic et al.,* (2011).

Sown area under soybean in the world is constantly increasing. About 90% of the area is concentrated in the U.S., Brazil, Argentina, China and India. In Serbia, the area under soybean was over 160.000 ha in recent years *(Miladinovic et al., 2008, Popovic et al., 2013).*

High and stable yields of soybean can be achieved only when they are based on cultivation of varieties of high yielding capacity and the implementation of intensive cropping. A wide range of local high-yielding cultivars that were selected in our climatic conditions, i.e. adapted to our climate, is available to soybean producers in our country *(Popovic, 2010).*

The proper arrangement of plants in appropriate plant density is one of the requirements to achieve high and stable yields during intensive production of soybean. It is well known that the ideal vegetation space is a square shape. However, in practice it is difficult to achieve a square shape if soybean is sown at inter row-spacing of 50 cm and intra row-spacing of 3-5 cm. Changing the shape of growing space and row spacing leads to change in microclimate growing conditions (light, relative humidity, aeration), where soybean is very sensitive, especially in the flowering stage. Therefore, a form of vegetative area or sowing modes was study object in almost all areas of growing soybeans. When sowing with greater spacing is performed, large portion of the sunlight falls between the rows and remains unused, especially in the initial part of soybean growing season *(Glamočlija,2004, Kolarić, 2010).*

The aim of this study was to examine the effect of inter row spacing at the same density on productivity of soybean yield. This would give quite a contribution to a better understanding of the impact of row spacing, and in this regard, specific recommendations related to modern production technology of soybean.

Materials and Methods

Research of the effect of inter-row spacing and cultivar on the productivity of soybean was conducted at the experimental field of Maize Research Institute in Zemun Polje on low carbonate chernozem soil in 2003 and 2004. Field micro-experiments were carried out as a two-factorial, using split-plot method in four replications.

This research covered two factors: 1. Inter-row spacing (A): 20 cm distance between rows, 45 cm distance between rows, 70 cm distance between rows and 2. Cultivar (B): Bosa (0 maturity group), Maize Research Institute, Zemun Polje, Balkan (I maturity group), Institute of Field and Vegetable Crops, Novi Sad; Dragana (II maturity group), Selsem.

Crop density within cultivars was the same for all variants, which was 500,000 plants per hectare for cultivar Bosa, 450,000 plants per hectare for cultivar

Balkan, and 400,000 plants per hectare for cultivar Dragana. Different densities were taken for each cultivar because of previous research that found that they exert maximum genetic potential in these conditions. The size of experimental plots was 5.4 m (6.0 x 0.9 m) for a combination of sowing at 45 cm between rows, 6.0 m (6.0 x 1.0 m) for a combination of sowing at 20 cm between rows and 8.4 m (6.0 x 1.4 m) for sowing at 70 cm spacing between rows.

Standard agricultural practices for soybean production were applied in the experiments, with the exception of the studied factors. In both research years, preceding crop to soybean was corn. Deep plowing was performed to a depth of 25 cm in fall, immediately after maize harvest and on this occasion 100 kg ha^{-1} of UREA (46% N) was applied. Seedbed soil preparation was performed in spring. Sowing was performed on April 23 in the first year of study and May 5 in the second year of study. Just before sowing, seeds were inoculated by microbiological chemical preparation, NS-Nitragin. Hand weeding and hoeing was performed two times during the growing season. Harvesting was performed by hand on September 10 and September 17, in the first and second year of the study. After harvest, samples from each plot and all replications consisting of ten plants were taken for laboratory analysis of following important characteristics of fertility: the number of pods per plant, grain weight per plant and 1,000-grain weight. Grain yield was reduced to 13% moisture content, determined for each plot and converted to yield kg per hectare.

The obtained experimental data were analyzed by analytical and descriptive statistics using the statistical package STATISTICA for Windows 1*0*. Significance of differences between the calculated mean values of the studied characteristics (year and genotype) was tested by the two-way analysis of variance. All significant values obtained in the LSD test were calculated for significance levels of 0.05% and 0.01.

Meteorological conditions. An analysis of thermal conditions concluded that the temperature in 2003 was higher 1.9°C compared to 2004 and 1.6°C compared to long-term average (Tab. 1).

In 2004, the temperature was close to multi-year average. It should be noted that an average monthly temperature in May and June 2003 was higher compared to 2004 and multi-year average for about 4°C and 4.5°C. Very high average monthly temperature in August 2003, which was a 3°C higher than in 2004 and multi-year average. That significantly influenced on the yield, since the soybean crop was in the stage of grain filling. In September 2003, high temperature in the first ten-day period accelerated seed ripening and soybean harvest (Tab. 1).

Table 1. Average air temperature (°C) and sum of rainfall (mm), 2003-2004, Zemun Polje

Month	Temperature		Rainfall		Temperature	Rainfall
	2003	2004	2003	2004	Average	
4.	11.5	12.9	14.6	27.2	11.5	49.1
5.	20.9	16.6	36.4	53.6	17.1	62.4
6.	24.6	20.4	19.0	125	19.9	79.9
7.	22.6	22.9	105.4	66.4	21.8	61.5
8.	24.7	21.7	26.4	39.4	21.6	51.5
9.	19.2	16.2	41.2	35.8	17.2	44.7
Total/Average	20.6	18.5	243.0	347.4	18.2	349.1

Amount and distribution of rainfall per year varied so that water regime in a year with less rainfall (2003) significantly affected the production of soybean (Tab. 1). In 2004, rainfall during growing season was at multi-year average and higher by about 105 mm, compared to 2003. In the first year, when weather was unfavorable for growing soybean, there was less rainfall in April, May and especially in June (only 19 mm). In relation to a long-term average, rainfall deficit, combined with high temperatures especially in May and June, has caused a drastic reduction in grain yield of soybean. Higher amount of rainfall was recorded in July (105.4 mm). Far better distribution and quantity of rainfall were recorded in 2004, a year with more favorable weather. Higher amount of precipitation, as well as its favorable distribution especially in the critical stages of water, combined with favorable temperatures, had favorable impact on the growth and development of soybean. It has certainly influenced better yield and quality of soybean genotypes. Our study is consistent with *Popovic et al. (2013)* research, where authors stated that there was a significant effect of temperature and rainfall on soybean yield.

Soil conditions. Parental material, calcareous forest (soil organic matter) is very well connected with the mineral part, so it is a well-formed organic-mineral complex.

According to the pH factor, it is evident that this is a soil of neutral to slightly alkaline reaction. $CaCO_3$ content at a depth of 20 cm was 1.6%, while at a depth of 40 cm was 2.2% and indicates that the soil was slightly calcareous. The humus content is variable and gradually decreases with depth. Its percentage at a depth of 20 cm is 2.87%, and at depths of 20 to 40 cm was 2.72%, which indicates high coverage with soil humus and substantial share of nitrogen therein. In addition, soil supply with phosphorus and potassium is higher. Chernozem, with its favorable chemical and physical properties, is an ideal pursued by every user of the land because it provides high yields of major agricultural land.

Results and Discussion

Influence of row spacing and cultivar on grain weight per plant

Grain weight per plant, in two-year study, was 12.26 g for factors included in this study, averagely. The highest grain weight per plant in the two-year average was achieved on a square sowing (20 cm between rows) and was 13.22 g, which is higher by 8.2% compared to the standard sowing (45 cm between rows) and 13.5% in relation to the largest sowing (70 cm between rows). On average for row spacing, the highest grain weight per plant in two-year study was achieved by cultivar Balkan (13.09 g), which is higher by 7.4% compared to cultivar Dragana, and 14.3% compared cultivar Bosa (Table 2).

Individually, in all three cultivars, the highest grain weight per plant was achieved at the smallest spacing (20 cm between rows). Similarly to the number of pods per plant, grain weight uniformly decreased with increase of row spacing, except for Balkan cultivar. This important parameter of soybean productivity ranged from 8.05 g with cultivar Bosa in wide sowing (70 cm between rows) in 2003 to 18.6 g with Balkan genotype in weather favorable 2004 at smallest sowing (20 cm between rows), (Table 2).

In the first year of study, grain weight per plant was 8.92 g, on average for the factors included in studies. Averagely for genotypes, the highest grain weight per plant was recorded at smallest row spacing (20 cm) and was 9.68 g. With increase of row spacing to 45 and 70 cm, grain weight decreased from 0.96 to 1.31 g per plant. There was no statistically significant difference in grain weight per plant between standard (45 cm) and largest row spacing (70 cm) (Table 2).

Table 2. Grain weight per plant (g) of estimated soybean cultivars in different row spacing

Year	Row spacing (A)	Cultivar (B)			Average	Index (%)
		Bosa	Balkan	Dragana		
	20	8.96	9.98	10.1	9.68	100.0
	45	8.20	8.87	9.08	8.72	90.1
2003	70	8.05	8.30	8.77	8.37	86.5
	Average	8.40	9.05	9.33	8.92	100.0
	Index (%)	100.0	107.7	111.1	-	-
	20	15.28	18.60	16.38	16.75	100.0
	45	14.72	16.77	15.13	15.54	92.8
2004	70	13.47	16.01	13.97	14.48	86.4
	Average	14.49	17.13	15.16	15.59	174.8
	Index	100.0	118.2	104.6	-	-
	20	12.12	14.29	13.24	13.22	100.0
Average	45	11.46	12.82	12.11	12.13	91.8
	70	10.76	12.16	11.37	11.43	86.5
Total average		11.45	13.09	12.24	12.26	-
Index (%)		100.0	114.3	106.9	-	-

LSD	year 2003				year 2004			
	A	B	BxA	AxB	A	B	BxA	AxB
0.05	0.37	0.41	0.72	0.69	0.85	0.95	1.65	1.59
0.01	0.56	0.57	0.98	0.97	1.29	1.30	2.26	2.24

Averagely for row spacing, the highest grain weight per plant was found at the cultivar Dragana (9.33 g). It was higher by 3.4% in comparison to the cultivar Balkan, and 11.1% with respect to cultivar Bosa. There was no statistically significant difference in grain weight per plant between genotypes Balkan and Dragana. The interaction of row spacing x genotype (AxB), at a significance level of 99%, is present in all variants of row spacing between cultivars Bosa and Dragana, while the variant of square sowing (20 cm spacing) between genotypes Bosa and Balkan is statistically significant. Statistically significant interaction BxA is determined in all cultivars between square (20 cm) and the largest sowing (70 cm), while at cultivar Bosa statistically significant difference in the grain weight per plant was found between 20 and 45 cm of row spacing (Table 2).

Table in the second year of study, on average, significantly higher values of grain weight per plant were obtained as a result of better branching plants, higher number of pods per plant due to favorable conditions of humidity and temperature. Similar to the previous year, the highest grain weight per plant, on average for the cultivars included in the study, was obtained at smallest sowing (15.59 g). The difference in grain weight per plant, on average, in the smallest sowing compared to 2003 amounted to 6.67 g or 74.8%. With increase of row spacing, grain weight had almost uniform trend of decreasing from 7.2 to 13.6%.

Statistically significant or very significant difference is present in grain weight per plant between the studied row spacing. On average for the genotypes included in the study, the highest grain weight per plant, similar to the number of pods per plant, was determined at the cultivar Balkan (17.13 g) in 2004. It was statistically significantly higher than in cultivars Bosa and Dragana. AxB interaction has not been established in any of sowing variants between cultivars Bosa and Dragana. The interaction of cultivar x row spacing is statistically significant in all studied cultivars between smallest (20 cm) and largest sowing (70 cm distance between rows), but in cultivar Balkan it is statistically justified between smallest (20 cm) and standard spacing (45 cm between rows) (Table 2).

By summarizing the data on grain weight per plant, it is noted that in both years of study it decreased with increase of row spacing. These data are consistent with results of *Nenadić (2003)* and *Kolarić (2010).*

Influence of row spacing and cultivar on soybean grain yield

Soybean grain yield was 4,545 kg/ha in a two-year study, on the average for the factors included in the research (Table 3). On the average for the cultivars, the highest yield of soybean in this two-year study was achieved with a square sowing (20 cm of row distance) and was 4,868 kg/ha. With increase of row spacing to 45 cm and 70 cm, the yield almost uniformly decreased by 7.0% and 12.9%, (Table 3).

The grain yield in two-year study, on the average for cultivars and row spacing, ranged from 3,997 kg/ha in a cultivar Dragana at wide-spacing (70 cm

between rows) to 5.151 kg/ha in cultivar Balkan at smallest spacing (20 cm between rows) (Table 3).

Table 3. Grain yield (kg/ha) of estimated soybean cultivars in different row spacing

Year	Row spacing (A)	Cultivar (B)			Average	Index (%)
		Bosa	Balkan	Dragana		
	20	3,584	3,792	3,636	3,671	100.0
	45	3,280	3,459	3,359	3,366	91.7
2003	70	3,220	3,237	3,245	3,234	88.1
	Average	3,361	3,496	3,413	3,424	100.0
	Index (%)	100.0	104.0	101.5	-	-
	20	6,112	6,510	5,569	6,064	100.0
	45	5,888	6,037	5,144	5,690	93.8
2004	70	5,388	5,604	4,749	5,247	86.5
	Average	5,796	6,050	5,154	5,595	163.4
	Index (%)	100.0	104.4	88.9	-	-
	20	4,848	5,151	4,603	4,868	100.0
Average	45	4,584	4,748	4,252	4,528	93.0
	70	4,304	4,421	3,997	4,241	87.1
Total average		4,579	4,773	4,284	4,545	-
Indeks (%)		100.0	104.2	93.6	-	-

LSD	year 2003				year 2004			
	A	B	BxA	AxB	A	B	BxA	AxB
0.05	61.53	56.92	98.59	101.06	165.74	123.62	214.11	240.21
0.01	93.21	93.21	135.05	143.33	251.08	169.34	293.3	344.23

On average for studied row spacing, the highest yield of soybean in a two-year period was achieved at cultivar Balkan (4,773 kg/ha). The yield was increased to 194 kg/ha in comparison to cultivar Bosa and 489 kg/ha relative to Dragana cultivar (Table 3).

The seed yield in less favorable year 2003, on average for the tested factors, was 3.424 kg/ha. The highest yield of soybean was achieved at lowest spacing (20 cm between rows) and amounted to 3,671 kg/ha. It was higher by 8.3% compared to standard sowing (45 cm between rows) and 11.9% compared to wide sowing (70 cm between rows). These differences in grain yield are evaluated as a statically significant.

Observing the interaction AxB in this year, its presence at the variant of smallest sowing (20 cm) and between all three genotypes was noted, as well as the standard sowing (45 cm) between cultivars Bosa and Balkan (Table 3).

Analyzing the cultivars individually, the highest grain yield, on average for tested spacing, is recorded at cultivar Balkan (3,496 kg/ha) and was higher by 4.0% or 135 kg/ha compared to cultivar Bosa, and only 25% or 83 kg/ha in comparison

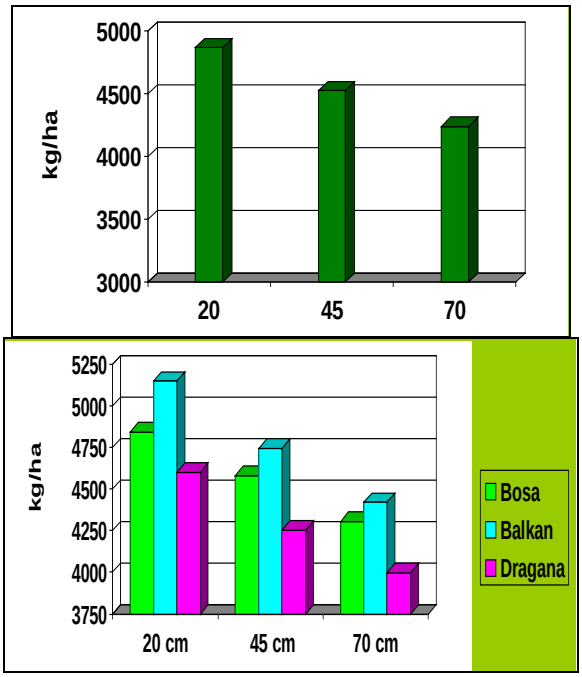
to cultivar Dragana. However, in this case, a statistically very significant difference in grain yield was noted.

The interaction of cultivar x row spacing is present on the following variants: at the cultivar Bosa between small (20 cm) and standard spacing (45 cm) at a significance level of 99%; at the cultivar Balkan, between all varieties of sowing at a very high significance; at cultivar Dragana between variants of standard (45 cm) and wide sowing (70 cm) is highly significant (95%), and among others is statistically very highly significant (Table 3).

Year 2004 was much more favorable for soybean cultivation, both in temperature and in amount and distribution of rainfall during vegetation period. Soybean yield, on the average of tested parameters, was 5,595 kg/ha and was higher by as much as 63.4% compared to less favorable year 2003. The highest grain yield, similar to the year 2003, on average of varieties included in study, was achieved at the lowest row spacing (20 cm between rows) and amounted to 6,064 kg/ha. The difference compared to yield in standard sowing (45 cm spacing) is 6.2% and compared to wide sowing (70 cm between rows) 13.5%. Statistically significant differences in grain yield were also present at the level of very high significance (99%). The interaction of row spacing x cultivar was not present in variants of standard (45 cm) and wide sowing (70 cm) between cultivars Bosa and Balkan (Table 3).

In addition, on the average for row spacing, similar to the year 2003, the highest yield of soybean gave cultivar Balkan (I maturity group) and amounted to 6,050 kg/ha. It was higher by 4.4% compared to cultivar Bosa and 15.5% compared to cultivar Dragana. The difference in grain yield between studied cultivars in this year's survey is statistically significant.

Interaction BxA, at a significance level of 95%, was found at early-maturing cultivar Bosa between square (20 cm) and standard spacing (45 cm), and other types of interaction cultivar x row spacing are statistically very highly significant (99%). The results of our study show that grain yield nearly equally decreased in both years with increasing distance between the rows.



a. b.

Figure 1. Average yield of soybean in different row spacing (a) and average yield of estimated soybean cultivars in different row spacing (cm) (b)

Our results are recorded by Bowers et al. (2000), Bullock et al. (1998), Holshouser and Whittaker (2002), Heatherly et al. (2002), Nenadic et al. (2003), as well as under irrigation conditions, noting that medium and smaller distances of sowing soybean are more favorable for achieving high yields.

Planting soybean row spacing of 25 cm was achieved on average for both years a higher yield of 11.74% in relation to the sowing row spacing of 70 cm of row (Dozet and Crnobarac, 2007).

Conclusion

Based on our two-year research of influence of row spacing and cultivar on grain weight and yield of soybean, following conclusions may be suggested:

- Planting at different spacing, as well as selected soybean cultivars, had a significant impact on productivity parameters of soybean.
- Grain weight per plant was 12.26 g in two-year's average. The highest grain weight per plant was at the smallest spacing (20 cm), i.e. 13.22 g. With increase of distance to 45 cm and 70 cm, grain weight per plant decreased by 8.2% and 13.5%. Cultivar Balkan had, averagely, the largest

grain weight per plant, which was higher by 7.4% compared to cultivar Dragana and 14.3% compared to cultivar Bosa.

- The average grain yield of soybean was 4,545 kg/ha. The yield was significantly higher in the second year of study (5,595 kg/ha) by 63.4% as compared to the first year of research. The average yield of a thick crop was increased by 7.5% as compared to standard sowing (45 cm), and by 14.8% as compared to wide sowing (70 cm). The highest average yield of soybean was achieved by cultivar Balkan (4,773 kg/ha). The yield was increased by 4.2% in comparison to cultivar Bosa and 11.4% in relation to cultivar Dragana. The highest yield of soybean in both years was achieved by cultivar Balkan (3,496 kg/ha and 6,050 kg/ha).
- It can be concluded from our study that in terms of arid and semiarid climate, which encompasses the majority of the country, significantly higher productivity can be achieved with a smaller spacing, which in our studies is 20 cm.

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Uticaj međurednog rastojanja i sorte na produktivnost soje

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Rezime

Istraživanja uticaja međurednog rastojanja i sorte na produktivnost soje obavljena su na oglednom polju Instituta za kukuruz u Zemun Polju.

Najveća masa zrna po biljci (13,22 g) zabeležena je pri najmanjem međurednom rastojanju. Ravnomerno se smanjivala sa povećanjem međurednog rastojanja, izuzev sorte Balkan. Kod ove sorte ujedno je zabeležena i najveća vrednost ovog parametra, 13,09 g.

Uskorednom setvom ostvaren je i najveći prinos zrna soje (4.868 kg/ha). Sa povećanjem rastojanja između redova skoro ravnomerno se smanjivao za 7,0 do 12,9%. Najveći prinos dala je sorta Balkan (4.773 kg/ha), a najmanji sorta Dragana (4.284 kg/ha).

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GENETIC VARIABILITY OF RED CLOVER SEEDLINGS IN RELATION TO SALT STRESS

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Abstract: Red clover is highly salt-sensitive plant, especially during germination and early seedling growth stages. The aim of this investigation was to estimate the effects of different saline conditions (0, 50, 100, 150, and 200mM NaCl) on germination and early seedling growth in four red clover varieties (Kolubara, K-32, K-17 and K-39). Germination test was conducted in the laboratory conditions using sterile plastic vessels on filter paper moistened with 10ml of the appropriate salt test solutions. It was observed that the germination energy (GE), germination (G), percentage of dead or infected seeds (DIS), normal seedlings (NS), root length (RL), shoot length (ShL), fresh weight (FW) and dry weight of seedling (DW) and seedling vigor index (SVI) were significantly decreased with increasing concentrations of NaCl in the growing medium. The tested varieties of red clover showed different NaCl tolerance at the seedling stage. Generally, studied red clover varieties are very sensitive to salt, especially K-32 which has the lowest values for GE, G, NS and SVI and highest for DIS. Variety K-17 proved to be a variety that the best tolerates conditions of salt stress because the values for GE, G, NS, RL and SVI were highest. Testing of varieties of red clover in the early seedling growth at different concentrations of NaCl in the growing medium could be helpful in the identification and selection of varieties for cultivation on saline soils.

Key words: variety, germination, early seedling growth, red clover, salinity

Introduction

Red clover is the second most important perennial forage legume in Republic of Serbia. Red clover is planted on area of about 120.000 ha in Republic of Serbia with average production on 4 t ha^{-1}, especially in mountainous regions

(Mandić et al., 2011). The soil salinity is one of the important abiotic factors affecting crop production. Estimates are that about 7% of the world's total land area is affected by salt *(Munns et al., 2002)*. There is 5.112.000 ha of total agricultural land in Serbia, of which are 233,000 ha of saline and alkaline soils *(Ličina et al., 2011)*. Legume species are significant genotypic differences with respect to salt tolerance *(Asgharipour and Rafiei, 2011)*. Legumes are generally more sensitive to salinity, especially red clover *(Asci, 2011)*. *Shereen and Ansari (2001)* reported that the salt salinity might affect legume growth and development independently. *Asci (2011)* reported that germination is an important stage in the life cycle of crop plants, particularly in saline soils as it determines the degree of crop establishment. *Kara and Kara (2010)* concluded that salinity has toxic effect on germinating seeds, and excessive salt hinders seed from water uptake during germination. *Machado et al. (2004)* pointed out that the main negative effect of salinity on seed water uptake difficult. Also, *Khan et al. (2001)* reported that salinity is a major environmental stress factor that affects seed germination. Salinity resistance of germination seeds of forage rape, berseem clover, alfalfa, and red clover has been shown to be a heritable trait which could be used as an efficient criterion for the selection of salt-resistant populations *(Ashraf et al., 1987)*. *Rogers et al. (1995)* found that germination significantly decreased with increasing NaCI concentrations between 60 to 200 mol m^{-3} NaCl in three populations of white clover. Many researchers reported that the highest concentration of NaCl strongly affected germination and growth of seedlings in several species of leguminous fodder crops: Egyptian, red and Persian clovers *(Gravandi, 2013)*, Persian clover *(Ates and Tekeli, 2007)*, strawberry clover *(Can et al., 2013)*, white clover and Egyptian clover *(Saberi et al., 2013)*, alfalfa *(Zhanwu et al., 2011)*, *Medicago ruthenica (Guan et al., 2009)*, yellow sweet clover *(Ghaderi-Far et al., 2010)* and sainfoin *(Majidi et al., 2010)*.

The aim of this paper was to estimate the effects of various NaCl concentrations (0, 50, 100, 150, and 200mM NaCl) on germination and early seedling growth in four Serbian red clover varieties (Kolubara, K-32, K-17 and K-39).

Materials and methods

The experiment was carried in March 2011 in the laboratory conditions at the Institute for Animal Husbandry in Belgrade. The research included the seeds of four varieties of red clover: Kolubara, K-32, K-17 and K-39. Seeds were taken from the second growth in 2010. The seeds were stored in paper bags in laboratory room. Seeds were sterilized in 6% sodium hypochlorite solution during 5 min and washed 3 times in sterile distilled water. Before sowing the seeds scarified in a ceramic mortar with fine quartz sand. Germination tests were carried out at 20 ± 1°C, in darkness in sterile plastic vessels (15 cm wide, 21 cm long and 4 cm high) on filter paper moistened with 10ml of the appropriate salt test solutions (0, 50,

100, 150, and 200mM of NaCl), using four replicates of 100 seeds. The experimental design was arranged in a Randomized Complete Block Design (RCBD).

According to *ISTA (1999)* seeds germinate when root elongation of about 2 mm. Germination energy (GE) and germination (G) were evaluated after 4 and 7 days after sowing, respectively *(ISTA, 2008)*. Percentage of dead or infected seeds (DIS), percentage of hard seed (HS), normal (NS) and abnormal seedlings (AS), root length (RL), shot length (ShL), fresh weight (FW) and dry weight (DW) of seedling were evaluated after 14 days. Values for DW were obtained after drying NS at 80 °C for 24 hours. Seedling vigor index (SVI) was calculated as per formula *(ISTA, 1999)*: Vigor Index = (Root length + Shoot length) x Germination percentage.

Data were subjected to an ANOVA using Statistica version 10, a Randomized Complete Block Design and Duncan's Multiple Range Test was used to compare differences among treatment means ($P < 0.05$).

Results and discussion

Seed traits. Results of ANOVA indicated that variety had significant effect on GE, G, DIS, and HS (Table 1). The salt had highly significant effect on GE, G and DIS. The interaction of salinity and varieties had significant effect on GE, G and DIS.

GE and G of studied red clover varieties were affected by salt treatment. Varieties Kolubara and K-17 have higher GE (53.6% and 49.2%) and G (63.6% and 62.6%) than varieties K-32 (24.4% and 34.3%, respectively) and K-39 (34.3% and 46.6%, respectively). GE and G were decreased with increasing salinity. Maximal GE (86.1%) and G (88.6%) were at 0 mM NaCl, and minimal at 200 mM NaCl (5% and 17.8%, respectively). This can be explained due to compression of membranes at high osmotic potential under salt stress conditions. Varieties Kolubara and K-17 have tolerance to low salt stress (50mM NaCl). Generally, seeds of red clover were as sensitive to high levels of NaCl concentrations. *Asci (2011)* and *Atis et al. (2011)* also concluded that increasing the salinity decreases the G of red clover. Salinity affects germination of seeds either by creating osmotic potential which prevent water uptake, or by toxic effects of Na^+ and Cl^- on embryo viability *(Lianes, 2005)*. *Ates and Tekeli (2007)* have found that G of different *Trifolium resipinatum* sp. was 5% in dose of 150 mM salt. In our study red clover germinated in a dose of 200 mM salt. Higher salt doses were not used since *Nichols et al. (2009)* have concluded that the G in pasture legumes did not germination on higher NaCl levels.

NaCl concentrations and varieties significantly effect on DIS. The highest DIS values were determined in K-32 (60.7%) and lowest in K-39 (24.4%). Minimal DIS was recorded at control (3.6%) and maximal at 200mM NaCl (72.2%). *Mandić et al. (2011)* found that variety and pH did not significantly affect on DIS in two Serbian red clover varieties (Kolubara and K-17). Also, in our

studies there were not differences for DIS between these Serbian red clover varieties, indicating of similar their behavior in the environment conditions.

HS were affected by variety. The number of seed who not germinate within 10 days after placement on germination is the number of HS. The HS ranged from 0.2% (Kolubara) to 29% (K-39). Results indicate that the hard seed characteristic is under genetic control. This result is consistent with the researches *Mandić et al. (2011)*. The NaCl concentrations did not affect the HS. *Elçi (2005)* concluded that the genetic and environmental factors during plant growth determine the maximum proportion of hard seeds in *Fabaceae* family.

Table 1. The effects of variety and different NaCl concentration level on seed properties of red clover

Parameters	Variety (A)	NaCl concentration effects mM NaCl (B)					Means
		0	50	100	150	200	
Germination energy (GE), %	Kolubara	94.8	84.0	60.0	22.0	7.0	53.6[a]
	K-32	87.0	21.0	10.0	3.0	1.0	24.4[c]
	K-17	94.0	81.0	44.0	20.0	7.0	49.2[a]
	K-39	68.5	54.0	32.0	12.0	5.0	34.3[b]
	Means	86.1[a]	60.0[b]	36.5[c]	14.2[d]	5.0[e]	40.4
Germination, %	Kolubara	98.0	91.0	72.0	40.0	17.0	63.6[a]
	K-32	88.5	30.0	21.0	21.0	11.0	34.3[c]
	K-17	98.0	91.0	62.0	34.0	28.0	62.6[a]
	K-39	70.0	67.0	53.0	28.0	15.0	46.6[b]
	Means	88.6[a]	69.8[b]	52.0[c]	30.8[d]	17.8[e]	51.8
Dead or infected seeds (DIS), %	Kolubara	2.0	9.0	28.0	60.0	82.0	36.2[b]
	K-32	6.5	65.0	74.0	74.0	84.0	60.7[a]
	K-17	2.0	7.0	35.0	63.0	69.0	35.2[b]
	K-39	4.0	3.0	18.0	43.0	54.0	24.4[c]
	Means	3.6[a]	21.0[b]	38.8[c]	60.0[d]	72.2[e]	39.1
Hard seed (HS), %	Kolubara	0	0	0	0	1.0	0.2[c]
	K-32	5.0	5.0	5.0	5.0	5.0	5.0[b]
	K-17	0	2.0	3.0	3.0	3.0	2.2[c]
	K-39	26.0	30.0	29.0	29.0	31.0	29.0[a]
	Means	7.8[a]	9.2[a]	9.2[a]	9.2[a]	10.0[a]	9.1
F test	GE	G	DIS	HS			
A	**	**	**	**			
B	**	**	**	ns			
AB	**	**	**	ns			

Legend: [(1)] Means followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test at the 5% level ($p \leq 0.05$), ** - significant at 1% level of probability, * - significant at 5% level of probability and ns - not significant

Table 2. The effects of variety and different NaCl concentration level on seedling traits of red clover

Parameters	Variety (A)	NaCl concentration effects mM NaCl (B)					Means
		0	50	100	150	200	
Normal seedling (NS), %	Kolubara	88.0	79.0	58.0	27.0	10.0	52.4[a]
	K-32	74.5	15.0	9.0	9.0	1.0	21.7[c]
	K-17	88.0	83.0	53.0	25.0	9.0	51.6[a]
	K-39	62.0	58.0	43.0	23.0	5.0	38.2[b]
	Means	78.1[a]	58.8[b]	40.8[c]	21.0[d]	6.2[e]	41.0
Abnormal seedling (AS), %	Kolubara	10.0	12.0	14.0	13.0	7.0	11.2
	K-32	14.0	15.0	12.0	12.0	10.0	12.6
	K-17	10.0	8.0	9.0	9.0	19.0	11.0
	K-39	8.0	9.0	10.0	5.0	10.0	8.4
	Means	10.5	11.0	11.2	9.8	11.5	10.8
Root length (RL), cm	Kolubara	2.0	2.1	2.0	0.9	0.4	1.5[b]
	K-32	2.4	2.0	1.7	1.5	1.0	1.7[ab]
	K-17	2.6	2.5	2.2	1.5	0.93	1.9[a]
	K-39	2.7	2.6	2.1	1.2	0.6	1.8[a]
	Means	2.4[a]	2.3[ab]	2.0[b]	1.3[c]	0.7[d]	1.7
Shoot length (ShL), cm	Kolubara	7.4	6.2	5.2	2.9	2.1	4.8
	K-32	7.2	6.9	5.7	4.0	2.4	5.2
	K-17	7.9	7.4	5.5	3.5	2.4	5.4
	K-39	8.1	7.0	5.1	3.2	1.6	5.0
	Means	7,6[a]	6,9[b]	5,4[c]	3,4[d]	2,1[e]	5.1
Fresh weight (FW), g	Kolubara	17.03	15.85	6.84	5.07	0.95	9.15
	K-32	17.48	17.10	10.70	6.56	0.26	10.42
	K-17	14.65	14.54	11.95	5.54	2.32	9.80
	K-39	15.91	13.95	9.71	5.27	2.04	9.38
	Means	16.27[a]	15.36[a]	9.80[b]	5.61[c]	1.39[d]	9.69
Dry weight (DW), g	Kolubara	1.57	1.60	1.06	1.07	0.29	1.12
	K-32	2.02	1.79	1.62	1.05	0.70	1.44
	K-17	1.67	1.59	1.31	1.08	0.99	1.33
	K-39	1.60	1.40	1.38	1.28	0.48	1.22
	Means	1.71[a]	1.59[a]	1.34[ab]	1.12[b]	0.61[c]	1.28
Seedling vigor index (SVI)	Kolubara	916.5	753.3	510.6	140.4	43.6	472.9[b]
	K-32	853.3	260.5	157.8	118.2	38.9	285.8[d]
	K-17	1026.0	899.4	481.4	161.1	84.9	530.6[a]
	K-39	758.6	641.8	379.6	124.5	35.2	388.0[c]
	Means	888.6[a]	638.7[b]	382.3[c]	136.1[d]	50.7[e]	419.3
F test	NS	AS	RL	ShL	FW	DW	SVI
A	**	ns	*	ns	ns	ns	**
B	**	ns	**	**	**	**	**
AB	**	ns	ns	ns	ns	ns	**

Legend: [(1)] Means followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test at the 5% level ($p \leq 0.05$), ** - significant at 1% level of probability, * - significant at 5% level of probability and ns - not significant

Seedling traits. Results of ANOVA indicated that variety had significant effect on NS, RL and SVI (Table 2). The salt had highly significant effect on NS, RL, ShL, FW, DW and SVI. The interaction of salinity and varieties had significant effect on NS and SVI.

Varieties Kolubara and K-17 have statistically significant higher NS (52.4% and 51.6%) than K-32 (21.7%) and K-39 (38.2%). The NS was significantly decreased when seeds were subjected to higher salinity levels. Result shows that at 0 mM NaCl the NS is 78.1%, at 50 mM NaCl is 58.8%, at 100 mM NaCl is 40.8%, at 150 mM NaCl is 21.0% and at 200 mM NaCl is 6.2%. *Atis et al. (2011)* concluded that 240 mM salinity level had the most negative influence on NS of red clover all studied seeds lots.

According to *ISTA (2009)* the AS are those which do not show capacity for continued development into normal plants when grown in good quality soil, under favorable condition of heat, light ad water supply. Variety and NaCl concentrations did not significantly affect on AS. The AS was higher when seeds were germinated at higher levels of salinity. However, the difference is not significant. AS are incapable of normal growth and are therefore incapable of developing into healthy seedlings in the field. Previous research indicates that the initial phase of seed deterioration is seed degradation in which there is a reduction in ATP synthesis, respiration and biosynthesis rates, resulting in reduced emergence and development of AS *(Dornbos, 1995)*.

Varieties K-17 and K-39 have significantly higher RL (1.9 cm and 1.8, respectively) than Kolubara (1.5 cm). Salt concentrations significantly decreased RL. The highest RL (2.4 cm) was obtained at 0 mM NaCl and lowest (0.7 cm) at 200 mM NaCl.

Varieties of red clover did not have significant differences in ShL. Salt concentrations significantly decreased ShL. The highest ShL was obtained at 0 mM NaCl (7.6 cm) and lowest at 200 mM NaCl (2.1 cm). *Asci (2011)* reported that populations of red clover have not significant effect on RL and ShL, and that RL and ShL decreased with increasing levels of salinity and the lowest value for both traits was obtained at 180 mM NaCl. Reduced seedling growth under salt stress conditions are also reported *Ates and Tekeli (2007)* on persian clover and *Zhanwu et al. (2011)* on alfalfa. High salinity levels led to a decrease in these parameters due to retardation in water and essential mineral nutrients absorption from soil by plant.

The varieties did not have significant differences in FW and DW. The NaCl concentrations had significant effect on the FW and DW. Minimal FW (1.39 mg) and DW (0.61 mg) were at 200 mM NaCl and maximal FW (16.27 mg) and DW (1.71 mg) at 0 mM NaCl. The reduction in DW could be due to the high concentration of Na^+ and Cl^-.

Varieties and NaCl concentrations have significant effect on SVI. K-17 had significantly higher SVI (530.6) than Kolubara (472.9), K-32 (285.8) and K-39

(388.0). Maximal SVI (888.6) was at 0 mM NaCl and minimal SVI (50.7) at 200 mM NaCl. This indicates that increased NaCl concentration caused a harmful effect in the seed. Seed vigor is the ability of a seed to germinate and grow rapidly to establish a normal seedling. Good seed vigor means rapid and uniform emergence and development of normal seedlings under a wide range of field conditions. *Ferguson (1995)* concluded that seed vigor is concept describing the interaction of several characteristics (the rate and uniformity of germination and growth, tolerance of environmental stresses after sowing, and retention of performance capacity after storage). *Vieira and Carvalho (1994)* reported that the vigor comprises a set of characteristics that determine seed vigor and is influenced by environmental conditions and handling during the stages of pre-and post-harvest. In addition to the above, vigor index determines the longevity of seed, without adverse consequences *(ISTA, 2009)*.

Conclusion

The properties GE, G, DIS, NS, RL and SVI of red clover were significantly affected by variety and NaCl concentration. Also, the variety had significant effect on HS and the NaCl concentration on ShL, FW and DW. Results indicate genetic variability existing among Serbian red clover varieties for salinity tolerance. Generally, studied commercial varieties of red clover are sensitive to salt stress conditions during seed germination and early seedling growth although variety K-17 had best salt tolerance performance. Results indicate on ability growth of seedling of red clover of different NaCl concentration. Also, results indicate that testing of genotypes of red clover in the early seedling growth at different NaCl levels would be helpful in the identification and selection of genotypes for cultivation on saline soils.

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Genetička varijabilnost klijanaca crvene deteline u odnosu na soni stres

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Rezime

Crvena detelina je vrlo osetljiva biljka na veću koncentarciju soli, posebno tokom klijanja i rane faze porasta klijanaca. Cilj ovog istraživanja bio je da se proceni uticaj različite zaslanjenosti (0, 50, 100, 150 i 200mM NaCl) na klijanje i rani porast klijanaca četiri sorte crvene deteline (Kolubara, K-32, K-17 и K-39). Test klijavosti sproveden je u laboratorijskim uslovima u sterilnim plastičnim posudama na filter papiru natopljenom sa 10 ml odgovarajuće koncentracije soli. Uočeno je da energija klijanja (EK), klijavost (K), neklijala i bolesna semena (NB), normalni klijanci (NK), dužina korena (DK), dužina hipokotila (DH), sveža (SvMK) i suva masa klijanaca (SuMK) i vigor indeks klijanaca (VIK) se značajno smanjuju sa povećanjem koncentracije NaCl u podlogama za naklijavanje. Ispitivane sorte imale su različitu toleranciju na soni stres u fazi klijanaca. Generalno, proučavane sorte su veoma osetljive na veću koncentraciju soli, posebno K-32 koja je imala najniže vrednosti za EK, K, NK i VIK, kao i najveći broj NB. Sorta K-17 se pokazala kao sorta koja najbolje toleriše soni stres jer su vrednosti za EK, K, NK, DK i VIK bile najviše. Testiranje sorti crvene deteline u ranom porastu klijanaca na podlogama za naklijavanje sa različitom koncentracijom NaCl može pomoći u indentifikaciji i izboru sorti za gajenje na zaslanjenim zemljištima.

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FORAGE QUALITY AND ENERGY CONTENT OF PERENNIAL LEGUME-GRASS MIXTURES AT THREE LEVEL OF N FERTILIZATION

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Abstract: The aim of this study was to investigate fodder quality and nutritive value of different grass-legumes mixtures influenced by various level of N fertilization. Studied factors had an impact only on the content of crude protein (CP), crude protein yield (CPY) and nitrate content in the forage. The level of N fertilizer showed a highly significant and positive impact on the CP and nitrate content. Treatment with 210 kg N ha^{-1} is characterized by the highest content of CP and nitrate of 189.7 g kg^{-1} DM and 2524 ppm, respectively, and the highest protein yield of 1.95 t ha^{-1}. The value of nitrate in the forage does not exceed the limit that is considered hazardous to the health of animals. Energy value of forage obtained from the grasslands of ME ≈7.75 and NEL≈4.32 MJ kg^{-1} DM is lower than values obtained in other studies.

Key words: lucern,grass, mixture, quality, energy content

Introduction

Lucerne is one of the most commonly used legumes for animal feeding in Serbia because of its high yielding, good nutritional quality, resistance to drought, uniform yield during the growing season and ability to fix nitrogen. Total area on which it is cultivated is 176 178 ha, of which 128 495 ha in Central Serbia, and 47 683 ha in Vojvodina. Total dry matter production, in four or five swaths per year, ranges evenn over 18 tonnes per hectare. The resulting hay, depending on the time of cutting and way of preserving, is of excellent quality with high crude protein content (18-22%), excellent digestibility, favorable amino acid composition with a high content of minerals, especially calcium and phosphorus, carotene and vitamins. Grass-legume mixtures are rarely used in production, mainly as a mixture of field peas or vetch with grains (*Avena sativa* L.). The reason for this is absence

of knowledge of the benefits of growing mixtures as compared to pure crop. Mixtures offer several adventages over pure crops. These adventage include the possibility of good fermentation and preparation of high quality silage (*Dinić et al., 1996*), the successful grazing without the risk of bloat occurrence, prolonged stand longevity, the control of erosion, weed control (*Casler and Walgenbach, 1990*) and reduced use of N fertilizer which is useful for solving the problem of N surplus (*Danso et al., 1991*). N fixation of legume species can be enhanced with competition from nonlegume species (*Pirhofer-Walzl et al., 2012*). Lucern usually grown with grasses similar morpho physiological traits like cocksfoot, tall fescue, perennial ryegrass, meadow fescue. In the research of *Albayrak et al. (2011),* mixture of lucerne and sainfoin with grasses gave fodder of high quality, that were not significantly different from pure legume crops in regard to the content of ADF, NDF and TDN. The crude protein content in the mixture of lucerne and grasses was not significantly different (15.42 to 16.50%) from the content of CP in pure crop of sainfoin (16.30 to 17.45%). Quality of feed produced from grassland is primarily influenced by the components, species and the cultivares, the proportion of their participation in the mixture and the level of nitrogen fertilization (*Samuil et al., 2012*).

The aim of this study was to investigate fodder quality and nutritive value of different grass-legumes mixtures influenced by various level of N fertilization.

Matherials and methods

The experiment was conducted in 2006 and 2007 and located on the experimental field of Institute for Animal Husbandry (44° 49′ 10″ N, 20° 18′ 45″ E) with an average annual rainfall of 693 mm. The soil type was low carbonate chernozem, with pH 7.18, humus 3.25% and organic mater 6.44%. The experiment was arranged in split plot design, in four repetitions, with a plot size of 2 m x 5 m. The main experimental factor was the type of fodder mixture and the second different level of nitrogen fertilization. Seeds of the lucerne *Medicago sativa* L. (cv. K-28) was sown individually and in the mixture with perennial grass species *Dactylis glomerata* L. (cv. K-40), *Festuca arundinacea* Schreb. (cv. K-20) and with perennial legume species *Onobrychis sativa* L. (cv. Krajina).

Table 1. Studied fodder mixture sructure

Species	Mixture structure %			
	L	L+C	L+C+F	L+C+F+S
Medicago sativa L.	100	50	33.3	25
Dactylis glomerata L.	-	50	33.3	25
Festuca arundinacea Schreb.	-	-	33.3	25
Onobrychis sativa L.	-	-	-	25

L-lucern; C-cocksfoot; F-tall fescue, S-sainfoin.

The plots were sown in the spring, 2005, with sowing rate of 20 kg ha^{-1} lucern, 30 kg ha^{-1} cocksfoot, 25 kg ha^{-1} tall fescue and 140 kg ha^{-1} sainfoin.

There were four levels of nitrogen fertilization: control with no nitrogen, 70 kgN ha^{-1}, 140 kgN ha^{-1} and 210 kgN ha^{-1}. Nitrogen fertilizers were applied twice, half the amount at the beginning of vegetation and half after first cut.

Cutting in the experiment was done in phase of one third of blooming of lucerne flowers. Samples of 1 kg for chemical analysis were taken after cutting, and were dried at a temperature of 105°C.

To determine the chemical composition of the fodder the following methods of analysis were used: the Kjeldahl method to determined crude protein content, the Wende method for crude fiber analysis and nitrate with colorimetric method. Metabolizable energy (ME) and net energy for lactation (NEL) were calculated by formula acording Obračević (1990):

GE(MJ kg^{-1}) = (0.02414 x CP) + (0.03657 x Cf) + (0.02092 x CF) + (0.01699 x NFE)

ME(MJ kg^{-1}) = (0.01715 x dCP) + (0.03766 x dCf) + (0.0138 x dCF) + (0.01464 x dNFE)

NEL(MJ kg^{-1}) = ME x kl

kl= 0.6 x (1+ 0.004 x (q-57)) x 0.9752

q(%)= (ME / GE) x 100

CP - crude protein (g kg^{-1});

Cf - crude fat (g kg^{-1});

CF – crude fibre (g kg^{-1});

NFE – nitrogen free extracts (g kg^{-1});

d – digestible

q - metabolizability coefficient

The experimental data were processed by the method of analysis of variance (two factor experimental design), applying the programme ANOVA and means were compared using Fisher's protected least significant difference (LSD) test. A linear regression was also carried out between the content of CP, nitrate and level of N fertilization. Results of the variance analysis are presented in tables as averages over two years of research by individual factors, and regression analysis are presented graphically.

Results and discussion

The crude protein content of the fodder significantly increased, as the aplication of the N fertilizer increased. The highest CP was recorded in treatment with the highest N rate (210 kg ha^{-1}) of 189.7 g kg^{-1} and the lowest in control treatment of 170.2 g kg^{-1}. *Komarek et al. (2007)* and *Tomić et al. (2012)* have

proved in their research that the crude protein content in forage of grass - legume mixtures progressively increases with the addition of N fertilizers. Although the type of the fodder mixture did not have a significant influence on the content of crude protein, the degradation of the contents of the CP according to the share of legumes in the mixture is observed, which is in agreement with the research of *Mika et al. (2004).* Protein yield changed significantly compared to the tested factors and their interaction. Pure lucerne crop had significantly higher protein yield as compared to lucerne mixtures. The two-component mixture of lucerne and cocksfoot stands out in regard to the yield of protein of 1.62 t ha^{-1}. Contrary to the present study, in the research of *Lättemäe and Tamm (1997),* CPY did not show statistically significant differences between individual species and mixtures thereof. The highest yield of CP, has been realized by mixture of white clover and grasses of 1.54 t ha, which is less compared to the average yield of CP of examined grasslands. The mixture of white clover and grass with 1.54 t ha of CP stands out. CPY increased progressively with the addition of N fertilizer. Treatment with 210 kg N ha^{-1} gave a significantly higher CP yield than the control and fertilization with lower quantities of N.

Table 2. Content of crude protein (g kg^{-1}), yield of crude protein (t ha^{-1}) depending on the type of fodder mixture and N fertilization

	CP (g kg^{-1})				Mean	CPY (t ha^{-1})				Mean
	0	70	140	210		0	70	140	210	
L	175.2	183.4	191.5	194.8	186.2	1.62	1.98	2.00	2.30	1.97^{a}
L+C	170.1	173.7	174.1	191.4	177.3	1.48	1.51	1.54	1.94	1.62^{b}
L+C+F	163.4	169.3	175.2	184.7	173.2	1.35	1.42	1.55	1.92	1.57^{bc}
L+C+F+S	172.2	175.4	183.2	187.9	179.7	1.32	1.59	1.65	1.62	1.55^{c}
Mean	170.2^{b}	175.4^{b}	181.0^{ab}	189.7^{a}		1.44^{c}	1.63^{b}	1.69^{b}	1.95^{a}	
Level of significance										
Mixture	ns					**				
Level of N	**					**				
Mixture x Level of N	ns					**				

L-lucern; C-cocksfoot; F-tall fescue, S-sainfoin; CP- crude protein; CPY- crude protein yield; ns- non significant, *- significant at $p \leq 0.05$; **-significant at $p \leq 0.01$.

The results of crude fiber (CF) are presented in Table 3. The examined factors had no significant impact on the content of CF. In spite of this, CF content had a slight tendency of increase with the increase in the share of grasses in the mixture. The minimum CF content was recorded for lucerne monoculture of 290.0 g kg^{-1}, and the highest in cocksfoot and lucerne mixture of 300.1 g kg^{-1}. Fertilization also had a significant impact, but there are some differences between the control and treatment with the least amount of nitrogen and the other two treatments. Large amounts of N fertilization decreased the content of CF in monoculture and mixture. Also *Tomić et al. (2012)* concluded that type of fodder crop and level of N fertilization didn′t have significant influence on the CF content.

Nitrogen from the soil is taken up by plant in the form of nitrate. Plants convert nitrate (NO_3) to nitrite (NO_2) which converted to ammonia and then to amino acids and proteins. However, in certain conditions like prolonged cool temperatures, shade, disease and high levels of soil nitrogen, the roots will accumulate nitrate faster than the plant can convert the nitrate to protein. That nitrate can cause noninfectious disease called nitrite poisoning. According to *ARC (1980),* if the values of nitrate in the forage exceed 3000 ppm, the forage is considered potentially dangerous and should be avoided in certain categories of animals. The nitrate content in the observed mixtures increased proportionately relative to the quantity of added N fertilizer (Table 3). The control treatment had the lowest nitrate content of 956 ppm, the highest content of 2524 ppm treatment with 210 kgN ha^{-1}. Although fertilization increases the content of nitrate up to 164%, the value of nitrates does not exceed values that are regarded as hazardous to animal nutrition.

Table 3. Content of crude fibre (g kg^{-1}) and nitrates (ppm) depending on the type of fodder mixture and N fertilization

	CF (g kg^{-1})				Mean	Nitrate (ppm)				Mean
	0	70	140	210		0	70	140	210	
L	283.7	282.1	276.7	282.8	290.0	869	1180	1640	2150	1460
L+C	286.8	298.7	294.0	297.6	300.1	999	1289	1636	2694	1655
L+C+F	289.5	288.3	287.8	287.1	299.0	988	1400	1618	2590	1649
L+C+F+S	297.8	290.6	288.9	285.0	299.6	969	1287	1847	2663	1691
Mean	289.4	289.9	286.9	288.1		956^{c}	1289^{bc}	1685^{b}	2524^{a}	
Level of significance										
Mixture	ns					ns				
Level of N	ns					**				
Mixture x Level of N	ns					ns				

L-lucern; C-cocksfoot; F-tall fescue, S-sainfoin; CF- crude fibre; ns- non significant, *- significant at $p \leq 0.05$; **-significant at $p \leq 0.01$.

The content of metabolizable energy (ME) was fairly uniform according to individual factors of observation. It ranged from 7.73 to 7.80 MJ kg^{-1}, depending on the type of the mixture and from 7.65 to 7.86 MJ kg^{-1}, depending on the level of fertilization. Net energy for lactation (NEL) also showed no significant variation with respect to the investigated factors. Values ranged from 4.22 MJ kg^{-1} in lucerne without fertilization to 4.43 MJ kg^{-1} in lucern and cocksfoot mixture, fertilized with 210 kgN ha^{-1}. Energy value of the tested mixture was lower than the value obtained by other researchers, for instance *Lättemäe and Tamm (1997),* reporting the ME content of grass-legume mixtures on average 10.6 MJ kg^{-1} DM, and *Mika et al. (2004)* reporting the content of NEL of 5.85 - 6.10 MJ kg^{-1} DM. According to *NRC (2001),* the ME requirements of pregnant heifers (450 kg mature weight) are 87.4 - 109.2 MJ day^{-1}, and cows in early lactation, which give 30 liters of milk with 4%

milk fat, NEL 125 MJ day $^{-1}$. From the results we can conclude that pregnant heifers must consume 11-14 kg day^{-1} of DM (12-16.7 kg hay at 15% moisture) to meet the daily requirements for ME and cows in early lactation 29 kg day $^{-1}$ of DM (34 kg hay at 15% moisture) to meet the NEL needs.

Table 4. The content of metabolizable energy (MJ kg^{-1}) and net energy for lactation (MJ kg^{-1}) depending on the type of fodder mixture and N fertilization

	ME (MJ kg^{-1})				Mean	NEL (MJ kg^{-1})				Mean
	0	70	140	210		0	70	140	210	
L	7.58	7.76	7.76	7.82	7.73	4.22	4.33	4.33	4.36	4.31
L+C	7.60	7.67	7.73	7.94	7.73	4.24	4.27	4.31	4.43	4.31
L+C+F	7.64	7.71	7.73	7.87	7.74	4.26	4.30	4.31	4.39	4.31
L+C+F+S	7.77	7.78	7.85	7.81	7.80	4.33	4.34	4.38	4.36	4.35
Mean	7.65	7.73	7.77	7.86		4.26	4.31	4.33	4.38	
Level of significance										
Mixture				ns				ns		
Level of N				ns				ns		
Mixture x Level of N				ns				ns		

L-lucern; C-cocksfoot; F-tall fescue, S-sainfoin; ME- metabolizable energy; NEL- net energy for lactation; ns- non significant, *- significant at p≤0.05; **-significant at p≤0.01.

The results of regression analysis are shown in Figure 1 and Figure 2. The level of N fertilization is in a positive linear relationship with both quality parameters, the content of CP and NO_3 in the forage.

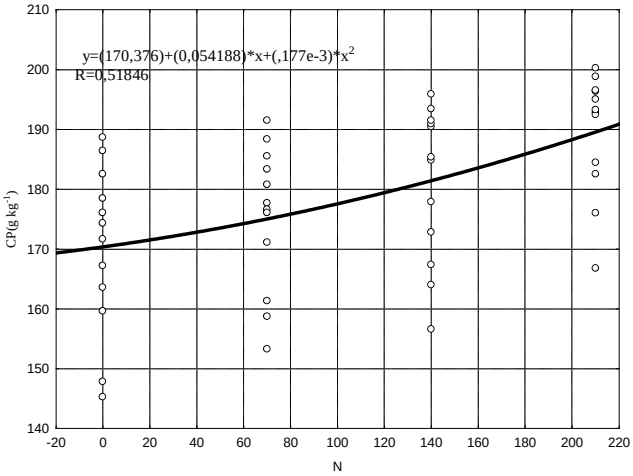

Figure 1. Regression analysis of crude protein content (g kg^{-1} DM) relative to N fertilization

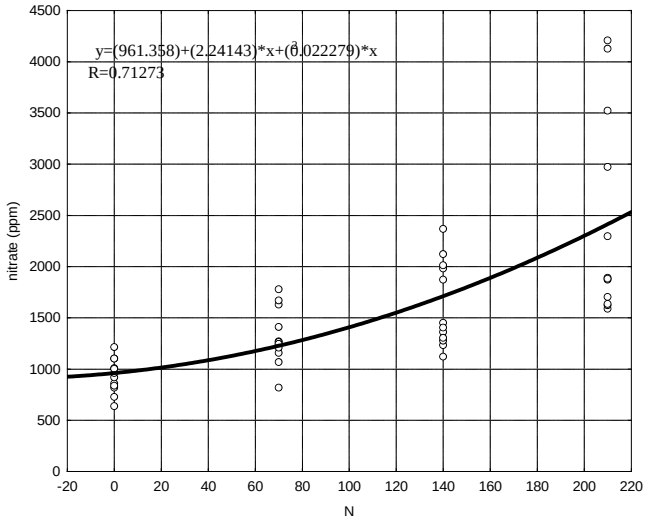

Figure 2. Regression analysis of nitrate content (ppm) relative to N fertilization

The changes in the content of crude protein with a coefficient of determination of i^2 = 26.88% are explained by changes in the utilization of N mineral fertilizer. A medium positive dependence of 0.51846 (Figure 1) is observed between the studied parameters. The changes in the content of nitrate with i^2 = 50.80% are explained by changes in the utilization of N mineral fertilizers, and a medium positive dependence of 0.71273 (Figure 2) is determined between the investigated parameters.

Conclusion

Examining the impact of the structure of forage mixture and the level of N fertilization on the quality and energy value of feed, we can conclude that the studied factors had impact only on the CP content, protein yield and nitrate content in the forage. The level of N fertilizer showed a highly significant and positive impact on changes of the CP and nitrate content. Nitrate levels in the forage did not exceed the critical limit considered harmful to the health of certain categories of animals.

Reducing the share of lucerne in the mixture and increasing levels of N fertilizer increased the yield of protein.

Energy value of the tested mixtures was relatively low, so in order to meet the energy requirements of animals it is necessary, in addition to lucerne hay and its mixtures, to include concentrated feed and silage of better energy performance in the animal diet.

Acknowledgement

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Kvalitet i energetski sadžaj krme višegodišnjih travno-leguminoznih smeša u tri nivoa N djubrenja

Z. Bijelić, Z. Tomić, D. Ružic-Muslić, V. Krnjaja, V. Mandić, S. Vučković, D. Nikšić

Rezime

Cilj ovog istraživanja je bio da se ispita kvalitet stočne hrane i hranljiva vrednost različitih travno-leguminoznih smeša pod uticajem različitih nivoa N đubrenja. Ispitivani faktori imali su utisaja samo na sadržaj sirovih proteina (CP), prinos proteina (CPY) i sadržaj nitrata u krmi. Nivo N đubriva ispoljio je visoko značajan i pozitivan uticaj na promunu sadržaja CP i nitrata. Tretman sa 210 kgN ha^{-1} karakteriše se najvećim sadržajem CP i nitrata od 189,7 g kg^{-1} DM odnosno 2524 ppm kao i najvećim prinosom proteina od 1,95 t ha^{-1}. Vrednost nitrata u krmi ne prelazi limit koji se smatra opasnim za zdravstveno stanje zivotinja. Energetska vrednost krme dobijene sa travnjaka je niži u odnosu na druga istraživanja i iznosi za ME ≈7,75 i NEL≈4,32 MJ kg^{-1} DM.

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