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BIOTECHNOLOGY IN ANIMAL HUSBANDRY

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Dragana Ružić-Muslić,Milan P. Petrović, Bogdan Cekić, Ivan Ćosić, Ivan Pavlović, Nevena Maksimović, Violeta Caro Petrović
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MORPHOMETRIC CHARACTERIZATION OF PIROT PRAMENKA

Dragana Ružić-Muslić[1], Milan P. Petrović[1], Bogdan Cekić[1], Ivan Ćosić[1], Ivan Pavlović[2], Nevena Maksimović[1], Violeta Caro Petrović[1]

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Abstract: The strategy of conservation of endangered sheep populations implies morphological and genetic characterization, as basic preconditions for their conservation. The aim of this study was to determine the morphometric characteristics, their correlations and the index of physical development of Pirot pramenka, which has the status of the most endangered population in Serbia. The measuring was performed on 30 sheep, aged 3 years, reared in the area of Stara Planina. The descriptive statistical procedure was performed using the statistical package Statistica (version 8). The average height at the withers was 56.31 cm, body length 62.93 cm, chest width 18.37 cm, chest depth 25.96 cm, chest circumference 77.59 cm, shin circumference 6.70 cm. The strongest and significantly positive correlation ($P < 0.05$) was found between chest depth and height at withers (0.65), body length (0.58) and body weight (0.56). Pirotska pramenka has slightly higher indices of format, chest and massiveness, in relation to breeds Vitoroga Žuja and Travnik Pramenka. These morphometric traits suggest that, compared to previous research, the body frame of Pirot pramenka sheep did not change significantly, which means that there were no crosses with other breeds due to geographical isolation and enthusiasm of breeders to preserve the indigenous Pirot pramenka which was the starting point for following authentic brands: Pirot lamb, cheese and carpet. Hence the biological and moral imperative: to preserve this highly endangered population.

Key words: Pirot pramenka, morphometric properties, correlations, indices

Introduction

Strains (ecotypes) of the indigenous breed of sheep - Pramenka were formed during a long process of evolution in certain biological areas, limited by geographical entities, in different feeding and housing conditions, which resulted

in their specific morphological and production performance. However, the expansion of high yielding breeds in the race for profit, on the one hand, as well as the depopulation of the rural environment, on the other hand, caused some strains of Pramenka to become endangered in their biological survival. Pirotska pramenka is one of the most endangered sheep populations in Serbia. According to data of the Domestic Animal Diversity Information System (DAD-IS), in 2021, 207 females and 25 males are reared in Serbia. The effective size of the population is 89 heads, which classifies it as highly endangered and is at risk of complete extinction. At the same time, it is the source of extraordinary national brands: Pirot lamb, Pirot carpet and Pirot cheese, which imposes its preservation as a biological, economic and moral imperative. The first step in the concept of sustainable use of genetic resources is their identification, description, development and monitoring, and subsequently their conservation. The variability and differentiation of different strains of pramenka in the Balkans has been the subject of numerous studies: *Mioč et al. (1998; 2003)* performed tests on Lika and Dubrovnik Pramenka, while *Antunović et al. (2013)* and *Novoselec et al. (2020)* determined the morphometric properties of Travnik pramenka. *Važić et al. (2016; 2017a; 2017b)* compared the exterior measures of the Privor, Dub and Kupreška pramenka, while *Ćinkulov et al. (2003)* conducted research on Tsigaia and *Pihler et al. (2019)* examined the phenotypic variability of Vitorog Žuja breed.

Morphometric measurements of sheep are important because they are a reflection of breed standards (*Verma et al., 2016*). They provide us with valuable information about the morphological structure and ability to develop an animal. Linear body measurements are an indicator of an animal's growth during life (*Attah et al., 2004*) and are helpful in predicting body weight and carcass characteristics (*Thiruvenkadan, 2005*). Determining different body measurements of animals is of great importance in assessing the quantitative parameters of meat, and helps in the development of appropriate selection methods *(Kumar et al., 2017)* and proper implementation of breeding and selection work. Body weight of farm animals is useful information in determining daily feeding need, growth assessment, medication administration, and its changes are a possible indicator of certain animal health problems on the farm or herd (*Paresd et al., 2014*). There are no current researches in the available literature that refer to the morphometric characterization and indices of physical development of the Pirot Pramenka.

Taking into account the above, the aim of this paper is to present the results of external measurements and body development indices of Pirot Pramenka, which can be used as a basis for morphometric characterization, which is a necessary prerequisite for the preservation of this highly endangered sheep.

Material and Methods

Pirot pramenka originated in eastern Serbia, in the vicinity of Pirot, after which it was named. It is also reared in the municipalities of Dimitrovgrad, Bela Palanka and Babušnica. It is a small, lively, mobile and late-maturing sheep, with combined production traits, meat-milk-wool. It belongs to the long-tailed Pramenka. The sheep have white, fine wool, and there are also heads with black and thick wool. The fleece is semi-open, with funnel-shaped strands. The sheep are hornless, and the rams are horned. Milk yield is 75-80 kg in lactation, which lasts about 180 days. Pirot Pramenka is mature and ready for breeding at the age of 16-18 months. Fertility is 100-115%. The characteristics of this sheep, like most Pramenka, are pronounced resistance, adaptability and modesty. The research was conducted on 30 sheep of Pirot Pramenka, aged about 3 years, reared in the area of Stara Planina, in the winter. The diet consisted of meadow hay (ad libitum) and 0.3 kg of corn, per head/day. Taking body measurements of sheep was done with the help of Litin's stick and ribbon, and determining body weight, with the help of livestock weight scales. The measurement was performed by one person, with the help of an assistant. The influence of the evaluator was excluded in this study. Each sheep was measured on a flat surface, on the left side of the animal. The following body measurements were taken: height at withers, body length, chest width, chest depth, tail length, chest circumference, pelvic width, shin circumference and body weight.

HW: The height at withers represents the vertical distance from the base, behind the front hoof, to the highest point at the withers (the area between the 2nd and 5th dorsal vertebrae).

DT: The body length represents the distance from the anterior edge of the shoulder-blade joint to the posterior point of the sciatic hump.

CHW: Chest width represents the distance at the narrowest point behind the shoulder blades.

CHD: The chest depth represents the vertical distance from the lower edge of the sternum to the highest point at the withers.

CHC: Chest circumference is the body circumference, measured at the chest just behind the shoulder blades and measured with a ribbon

PW: The width of the pelvis represents the distance between the outer edges of the tuber ischii.

SHC: The shin circumference is measured with a ribbon at the thinnest point on the shin of the front leg.

The body weight of the animal is measured using livestock weight scales.

Indices are the absolute values of a measurement in relation to another body measurement, expressed as a percentage. These indices are used to determine the proportions of the animal body and to more precisely compare the development of individuals (*Ćinkulov et al., 2003*). According to *Ćinkulov et al. (2003*), the

following are calculated: format index, chest index, chest depth index, body compactness index, massiveness index, pelvis and chest index, leg length index and forehead width index.

$$\text{Format index} = \frac{\text{Body length}}{\text{Visina Height at withers grebena}} \times 100$$

$$\text{Chest index} = \frac{\text{Chest width}}{\text{Chest depth}} \times 100$$

$$\text{Chest depth index} = \frac{\text{Chest width}}{\text{Height at withers}} \times 100$$

$$\text{Body compactness index} = \frac{\text{Chect circumference}}{\text{Body length}} \times 100$$

$$\text{Massiveness index} = \frac{\text{Chest circumference}}{\text{Height at withers}} \times 100$$

$$\text{Pelvis and chest index} = \frac{\text{pelvis width}}{\text{Chest width}} \times 100$$

$$\text{Leg length index} = \frac{\text{Height at withers} - \text{Chest depth}}{\text{height at withers}} \times 100$$

$$\text{Forehead width index} = \frac{\text{Forehead width}}{\text{Forehead length}} \times 100$$

Descriptive statistical processing of data related to external measurements and indices in the population of Pirot Pramenka was performed using the statistical package Statistica (version 8).

Results and Discussion

Table 1. Morphometric properties of Pirot Pramenka

Indicator	Mean	SD	SEM	CV,%
Height at withers, cm	56.31	3.35	0.59	5.95
Body length, cm	62.93	3.26	0.57	5.18
Body width, cm	18.37	2.32	0.41	12.65
Chest depth, cm	25.96	1.59	0.28	6.14
Chest circumference, cm	77.59	6.39	1.13	8.24
Pelvis width, cm	11.08	1.70	0.30	15.41
Shin circumference, cm	6.70	0.56	0.10	8.44
Head length, cm	25.87	1.71	0.30	6.62
Head width, cm	14.50	1.23	0.21	8.53
Ear length, cm	11.20	1.57	0.27	14.06
Horn length, cm	9.77	3.48	1.74	35.63
Tail length, cm	33.75	6.02	1.06	17.85
Body weight, kg	33.42	4.69	0.82	14.04

Mean = arithmetic mean; SD = standard deviation; SEM = mean standard error; CV = coefficient of variability

Table 1 shows that the highest value of the coefficient of variation was established in the following traits: horn length (35.63%) and tail length (17.85%). Pirot pramenka sheep in this study had lower body weight, lower height at withers, lower values for body length and chest depth, and higher values for chest width and tail length, compared to the desirable body measures listed in the Main Breeding Program for Indigenous Sheep Breeds 2020-2024. According to body development, Pirot Pramenka belongs to the group of less developed sheep, with a smaller body format. If we compare it with Liplje Bardoka (*Mitić, 1987*), Dubrovnik Pramenka (*Mioč et al., 2003*) Dublje, Privor and Kupreš Pramenka strains (*Važić et al., 2017*), Lika (*Mioč et al., 1998*) and Travnik Pramenka strains *(Novoselec et al., 2020*) and Vitorog Žuja *(Pihler et al., 2019*), we can conclude that in the present study, in terms of height at withers (56.31 cm), Pirot Pramenka had similar values with Pag and Rab sheep and slightly higher vaues than Krk sheep. The value obtained for body length (62.93 cm) was higher compared to the Krk strain and lower than other Pramenka strains. In terms of chest depth (25.96 cm) and shin circumference (6.7cm), Pirot pramenka had lower values than the mentioned Pramenka strains, while it had higher value for chest width (18.37 cm), compared to the Lika Pramenka (16.64 cm) and the Pag sheep (17.11 cm) and similar to the Vitoroga Žuja (18.89 cm). If we compare the above exterior measures of Pirot Pramenka with older data (*Mitić, 1987*) for the same population, we can conclude that, in regard to its exterior, this Pramenka remained almost the same (height at withers 55 cm), which means that it was least exposed to other

breeds of sheep and changes in production technology. Differences in terms of morphometric properties compared to other populations of Pramenka are a consequence of different nutritional and production statuses of the animals (Table 2).

Table 2. Morphometric measures of individual populations of Pramenka

Population	Indicator, cm						Source
	HW	BL	CHW	CHD	CHC	SHC	
Dubrovnik sheep	60.12	65.05	19.81	30.32	86.45	7.54	Mioč et al.,2003
Dublje Pramenka	73.37	74.66	22.72	34.50	98.72	9.31	Važić et al., 2017
Privor Pramenka	70.28	73.04	20.83	32.49	88.89	8.45	Važić et al., 2017
Kupreš Pramenka	69.71	72.84	21.12	31.98	90.75	7.91	Važić et al., 2017
Lika Pramenka	60.75	67.35	16.64	29.28	83.83	7.48	Mioč et al., 1998
Travnik Pramenka	69.63	74.78	20.15	31.40	93.61	7.31	Novoselec et al., 2020
Vitoroga žuja	64.31	69.56	18.89	37.97	85.25	7.99	Pihler et al., 2019
Pag sheep	56.14	64.27	17.11	28.98	83.26	7.04	Pavić i sar., 2005
Krk sheep	54.96	61.78	16.26	28.29	77.18	6.99	Mioč i sar., 2004
Rab sheep	56.83	64.60	16.60	28.29	82.28	7.51	Mioč i sar., 2006

HW = height at withers; BL = body length; CHW = chest width; CHD chest depth; CHC = chest circumference; SHC = shin circumference

Table 3 shows the body development indices of Pirot Pramenka sheep. The highest coefficient of variation (15.75%) was recorded in the chest and pelvis index and the lowest (4.25) in the leg length index.

Table 3. Indices of body development of Pirot Pramenka

Indicator	Mean	SD	SEM	CV,%
Format index	111.95	5.80	1.02	5.18
Chest index	70.87	8.79	1.55	12.40
Chest depth index	46.16	2.28	0.40	4.95
Body compactness index	123.51	10.83	1.91	8.77
Massiveness index	136.16	12.88	2.27	9.32
Pelvis and chest index	169.35	26.62	4.78	15.72
Leg length index	53.83	2.28	0.40	4.25
Forehead width index	56.19	5.38	0.95	9.59

Mean = arithmetic mean; SD = standard deviation; SEM = mean standard error; CV = coefficient of variability

The determined indices of body development follow their body measures. These indices are used to determine the proportions of animal bodies as well as for a more precise comparison of individuals (*Ćinkulov et al., 2003*).

Table 4. Indices of body development of Vitoroga Žuja and Travnik Pramenka

Indicator	Vitoroga žuja (Pihler et al, 2019)	Travnik Pramenka (Novoselec et al.,2020)
Format index	108.49	107.39
Chest index	62.75	64.12
Body compactness index	122.67	125.25
Massiveness index	132.86	134.70
Leg length index	52.89	54.82

Pirot pramenka had slightly higher indices of format, chest and massiveness in relation to Vitoroga Žuja and Travnik Pramenka, which is in accordance with the research of *Ćinkulov et al (2008*) as well as *Ćurković et al. (2016),* who have determined minimal genetic differentiation between seven strains of Pramenka, which is explained by similar agroecological rearing conditions as well as population mixing throughout the long history of population migrations.

Table 5. Correlations between individual body measures of Pirot Pramenka

	HW	BL	CHW	CHD	CHC	PW	SHC
HW	1.00						
BL	0.58*	1.00					
CHW	0.25	0.43*	1.00				
CHD	0.65*	0.52*	0.31	1.00			
CHC	0.18	0.20	0.21	0.45*	1.00		
PW	0.25	0.19	0.37*	0.29	0.26	1.00	
SHC	0.40*	0.27	-0.10	0.34	0.47*	0.02	1.00
BW	0.56*	0.46*	0.30	0.68*	0.47*	0.44*	0.50*

* ($P<0.05$)

The correlation coefficients between individual phenotypic traits of Pirot pramenka are shown in Table 5. A positive correlation between phenotypic traits and body weight of sheep was determined. The strongest and significantly positive correlation ($P<0.05$) was found between chest depth and height at withers (0.65), followed by body length (0.58) and body weight (0.56). High correlation coefficients between individual morphometric traits of Pirot Pramenka sheep indicate that these variables and their combinations can be used in breeding selection procedures.

These morphometric traits suggest that, compared to previous research, the body frame of Pirot Pramenka sheep has not changed significantly, which is useful information and the first step in the program of conservation of this genetic resource from extinction. The next step within the global strategy of preservation is determination of the genetic structure of this population, which will be the subject of our further research.

Conclusion

Pirot Pramenka has the status of a highly endangered population and is at risk of complete extinction. Its preservation is a biological and moral imperative since it is source of important national brands: Pirot lamb, Pirot cheese and Pirot carpet. The first step in this strategy is morphometric characterization of the population. Compared to previous studies, the body frame of this Pramenka did not change significantly, which means that there were no crosses with other breeds due to geographical isolation and the desire of breeders to preserve the autochthonous Pirot Pramenka. By analyzing the correlations between individual body measures and the weight of Pirot Pramenka sheep, a positive correlation was determined. The strongest and significantly positive correlation ($P<0.05$) was found between chest depth and height at withers (0.65), body length (0.58) and body weight (0.56). Pirot pramenka had slightly higher indices of format, chest and mass in relation to Vitorog žuja and Travnik Pramenka. Since the morphometric characteristics of the animals are a reflection of the breed standard, their determination is a necessary precondition for the preservation of this highly endangered sheep population.

Morfometrijska karakterizacija pirotske pramenke

Dragana Ružić-Muslić,Milan P. Petrović, Bogdan Cekić, Ivan Ćosić, Ivan Pavlović, Nevena Maksimović, Violeta Caro Petrović

Rezime

Strategija očuvanja ugroženih populacija ovaca podrazumeva morfološku i genetsku karakterizaciju, kao osnovne preduslove za njihovu konzervaciju. Cilj ovog rada je utvrđivanje morfometrijskih osobina,njihovih korelacija i indeksa telesne razvijenosti pirotske pramenke, koja ima status najugroženije populacije u Srbiji.Merenje je sprovedeno na 30 ovaca, uzrasta 3 godine, gajenih na području Stare planine.Deskriptivna statistička procedura je urađena korišćenjem statističkog paketa STATISTICA (version 8). Prosečna visina grebena ovaca je iznosila 56.31cm, dužina trupa 62.93cm, širina grudi 18.37cm, dubina grudi 25.96cm,obim grudi 77.59cm, obim cevanice 6.70cm. Najjača i značajno pozitivna korelacija je ($P<0.05$) je ustanovljena između dubine grudi i visine grebena (0.65), dužine trupa (0.58) i telesne mase (0.56). Pirotska pramenka je imala neznatno veće indekse formata, grudi i masivnosti, u odnosu na vitorogu žuju i travničku pramenku. Navedene morfometrijske osobine upucuju na zakljucak da se, u odnosu na ranija istrazivanja,telesni okvir ovaca pirotske pramenke nije značajnije menjao što znači da nije bilo ukrštanja sa drugim rasama iz razloga geografske izolovanosti i entuzijazma odgajivača da sačuvaju autohtonu pirotsku pramenku koja je iznedrila autentične brendove:pirotsko jagnje, pirotski kačkavalj i pirotski ćilim.Otuda je biološki i moralni imperativ:očuvati ovu visoko ugroženu populaciju.

Ključne reči: pirotska pramenka, morfometrijske osobine, korelacije, indeksi

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EGG QUALITY OF BANAT NAKED NECK HENS DURING STORAGE

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Abstract: In less intensive production systems, native poultry breeds can be used in order to diversify the products and achieve self-sustainability of these breeds through production. Given the missing data on the sustainability of quality of eggs obtained from indigenous, native hens, during storage, the aim of the study was to determine the most important parameters of egg quality of indigenous breed of Banat Naked Neck hens during a four-week period in different storage conditions. The design of the experiment was two-factorial with 4 levels of storage time factors (fresh eggs - 0, 1, 2, 3 and 4 weeks of storage) and 2 levels of temperature storage condition factors (room temperature and refrigerator). The room temperature was on average 21.3°C and the refrigerator temperature 8°C. Quality analysis was performed on a total of 200 eggs, and it included following parameters: egg weight, egg weight loss, weight and proportion of structural components: shell, yolk and albumen, albumen height, yolk colour, Haugh Units and albumen pH. The storage time had a significant effect on all properties of egg quality, except for the yolk colour, which was under the impact of the interaction of storage time and temperature. Storage temperature influenced egg weight loss (<0.001), shell weight (<0.05), albumen height (<0.0001), Haugh Units (<0.0001) and albumen pH (<0.0001). By storing in the refrigerator, changes in internal quality were significantly slowed down. After 28 days of storage in the refrigerator, the values of albumen and Haugh Units were higher than the same parameters of eggs stored for only 7 days at room temperature.

Key words: egg quality, Banat Naked Neck, storage time, storage temperature

Introduction

Egg is a nutritionally high quality product, with low energy values and high digestibility, which makes it a desirable food product. The specific structure of the egg, thanks to the shell and membranes, has a protective role in terms of biosafety and quality, which in certain conditions of farm management and storage can be maintained for different periods of time.

It is known that the table egg has the best internal quality at the time of oviposition. Subsequently, depending on the handling on the farm, the storage duration and conditions, the quality of the eggs decreases at different rates. In addition to the storage time, factors recognized as important for the sustainability of egg quality are temperature and relative humidity (*Jin et al., 2011; Chung and Lee, 2014; Kopacz and Drazbo, 2018).* According to *Feddern et al. (2017), Kopacz and Drazbo (2018)*, storing eggs at room temperature leads to a decrease in quality, which is measured by unusability after 4 weeks. Egg quality parameters that change significantly under the influence of storage conditions and time are the height of the thick albumen, the egg weight, Haugh Units, the pH values of the albumen and egg yolk.

The effect of storage time in some studies is related to the effect of age and genotype of hens, breeding systems and nutrition on egg quality *(Jin et al., 2011; Jones et al., 2014; Feddern et al., 2017; Perić et al., 2018 ; Vlčkova et al., 2019; Santos et al., 2019).* In most studies, egg quality during storage is tested on eggs from hybrid hens, which are the basis of commercial egg production.

With the development of various forms of alternative egg production in which, in addition to different hybrids and pure breeds, native hens can be used, the question of sustainability of egg quality of these hens during storage is opened. Earlier research by *Škrbić et al. (2020)* points out the possibility of using Banat Naked Neck hens in alternative egg production systems, in order to diversify the products and achieve self-sustainability of this breed, whose population according to FAO (2020) is increasing, but still very vulnerable. In addition, the results of the research by *Škrbić et al. (2011)* show that the initial quality, as well as changes in egg quality traits during the laying period, differ significantly between the indigenous breed of the Banat Naked Neck from traditional, extensive production and hybrid laying hens in the cage breeding system.

Considering the missing data on the sustainability of the egg quality of autochthonous, native hens during storage, the aim of the research is to determine the most important parameters of egg quality of the autochthonous breed of Banat Naked Neck hens during a four – week period in different storage conditions.

Material and Methods

The experimental part of the research involving storage time influence, temperature conditions and their interaction on egg quality was conducted in a two-factor experiment, with 4 levels of storage time factors (fresh eggs - 0, 1, 2, 3 and 4 weeks of storage) and 2 levels of temperature storage condition factors (room temperature and refrigerator temperature). The room temperature was on average 21.3°C and 8°C in the refrigerator. Eggs of native Banat Naked Neck hens in the second production cycle were analyzed. The Banat Naked Neck hens were reared in a floor system divided into boxes, with a space of 3 hens per m^2. The diet was a complete mixture with 16.25% CP, 11.7 MJ/kg ME, 4.1% Ca, 0.33% P. The duration of daylight was 15 hours.

A total of 200 eggs were analysed and collected in two weeks. Eggs were collected in the morning and immediately the initial quality was examined on a sample of 20 eggs. From the remaining eggs, groups of 20 eggs were formed and stored for one, two, three and four weeks at room temperature, and in the refrigerator. Egg quality analysis was performed based on following parameters: egg weight, egg shell weight (without drying), albumen height (tripod micrometer), yolk weight, yolk colour (Roche Color Fan) and albumen pH (pH meter Consort C830). Haugh units (HU) were calculated based on egg weight and albumen height (*Haugh, 1937*). The weight of the albumen was calculated based on the difference between the weight of the egg, the weight of the shell and the weight of the yolk. For each structural part of the egg, the share in the weight of the egg was calculated. By measuring the weight on a sample of 20 eggs stored at room temperature, and in the refrigerator, the weight loss of the egg was determined at weekly intervals.

The test results were statistically analysed by two-way ANOVA analysis of variance in the Statistica software package (StatSoft Inc., 2012). Significance of differences was assessed at the probability level $p \leq 0.05$ by LSD post hoc test.

Results and Discussion

The effects of storage temperature and time on egg weight, egg weight loss and shell weight are shown in Table 1. The results show that longer storage time at room temperature affects ($p<0.05$) the reduction in egg weight. A significant difference in the weight of stored eggs in relation to fresh eggs was determined after 14 days of storage. The difference was maintained at the same level of significance in relation to the fresh eggs until the end of the examined storage period (28 days). The weight of eggs stored in the refrigerator was not affected ($p> 0.05$) by storage time.

A more accurate indicator of the effect of storage time and temperature on egg weight is the loss of egg weight during storage. Egg weight loss was significantly influenced by storage temperature ($p<0.001$), storage time ($p<0.001$), as well as the interaction of temperature and storage time ($p<0.001$). With a longer storage time, the weight loss of the egg was higher at both storage temperatures. Storage temperature determined the level of egg weight loss during storage (graph. 1). After 4 weeks of storage at room temperature, eggs lost 5.68% of their weight, or an average of 3.45 g, and eggs stored in the refrigerator lost 2.66% of their weight, or an average of 1.59 g.

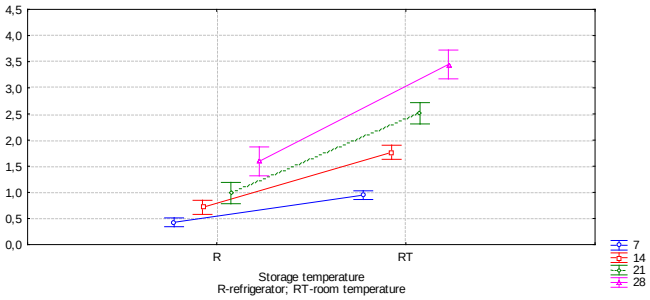
Similar to the egg weight, the egg shell weight stored at room temperature was also influenced ($p<0.001$) by the time of storage. Significant differences were found after 14 and 28 days of storage at room temperature compared to fresh eggs. The absence of difference after 21 days of storage is probably a consequence of the more difficult separation of albumen from the shell during the measurement of the shell, which was performed without drying. Observed as a percentage, as a share in the egg weight, the weight of the shell did not show the significance of differences in relation to the time of storage. The weight and proportion of the shell in eggs stored in the refrigerator were not affected ($p> 0.05$) by storage time. *Chung and Lee (2014)* find no significant changes in shell weight under the influence of storage temperature and time, while *Kopacz and Drazbo (2018)* find a significantly higher share of shell in eggs stored at room temperature compared to fresh eggs without significant changes in other egg shell quality properties.

The egg begins the aging process from the moment it is laid. The basis of this process is the evaporation of water through the pores on the shell, which results in a reduction in the weight of the egg. The results of our study show that by storing eggs in the refrigerator, it is possible to maintain the weight of the eggs without a significant difference during 4 weeks of storage. Significantly lower egg weight compared to fresh eggs was recorded after 14 days of storage at room temperature (21°C). *Jin et al. (2011)* report a significant reduction in egg weight after 10 days of storage at 29°C but not at 5°C or 21°C. A better indicator of the changes in the egg that occur during storage, regardless of temperature, is the loss of egg weight. According to *Grashorn (2016),* higher egg weight loss indicates faster changes in the internal quality of eggs. Storage at lower temperatures reduces egg weight loss *(Chung and Lee, 2014; Feddern et al., 2017; Kopacz and Drazbo, 2018),* which is confirmed by our results. According to *Lewko and Gornowicz (2011),* water loss is faster in smaller eggs due to the unfavourable surface-to-egg ratio, which explains the higher weight loss of native hen eggs in our study compared to the results of other studies conducted on hybrid hen eggs (*Jin et al., 2011; Perić et al., 2017; Đukić Stojčić and Perić, 2018*).

Table 1. Effects of storage temperature and time on egg weight and egg shell weight

Storage temperature	Storage time, day	Egg		Egg shell	
		Weight, g	Loss, %	Weight, g	%
Room temperature	0	61.79^{ab}	-	8.01^{a}	12.98
	7	62.24^{a}	1.56^{d}	7.67^{a}	12.31
	14	58.16^{c}	2.91^{c}	6.88^{b}	11.83
	21	58.74^{c}	4.15^{b}	7.40^{ab}	12.60
	28	57.26^{c}	5.68^{a}	6.91^{b}	12.06
	SEM	0.57	0.22	0.12	0.15
	p	0.012	< 0.001	0.004	0.105
Refrigeration	0	62.06	-	8.20	13.20
	7	62.80	0.72^{d}	7.93	12.65
	14	60.67	1.20^{c}	7.39	12.19
	21	60.16	1.65^{b}	7.43	12.38
	28	59.10	2.66^{a}	7.54	12.77
	SEM	0.53	0.11	0.11	0.16
	p	0.181	< 0.001	0.1	0.336
Source of variation					
Storage temperature	p-value	0.08	<0.001	0.037	0.192
Storage time		0.001	<0.001	<0.001	0.041
Time x temperature		0.877	<0.001	0.740	0.743

$^{a-c}$ Different letters indicate significant differences among the means in each column with the same storage temperature



Graph. 1. Egg weight loss(g) during the storage period in different temperature conditions

Changes in albumen weight, egg yolk weight and egg yolk colour under the influence of storage time and temperature are shown in Table 2. Storage temperature did not have a significant effect on the weight and proportion of albumen and yolk in the egg. Changes in these structural parts of the egg occurred

under the influence of storage time ($p<0.05$). In eggs stored at room temperature, the percentage of albumen was at the limit of significance ($p = 0.054$) of the storage time effect. The percentage of albumen in eggs stored in the refrigerator decreased significantly ($p<0.05$) only after 4 weeks of storage compared to fresh eggs.

In both storage temperatures, an increase in the percentage of yolks was recorded with a longer period of egg storage. In eggs at room temperature, a significantly higher percentage of egg yolk was found after 14 days of storage, and in eggs stored in the refrigerator, after 28 days.

The yolk colour was not significantly influenced by temperature and storage time, but the significance of the interaction effect of the examined factors was established. Colour changes in egg yolks *Santos et al. (2019)* explain as the degradation of carotenoids by oxidative processes that occur as a consequence of water diffusion from albumen into yolks under conditions of longer storage periods and higher storage temperatures.

Table 2. Effects of storage temperature and time on the albumen weight, yolk weight and yolk colour

Storage temperature	Storage time, day	Albumen		Yolk		Yolk color, Roche
		Weight, g	%	Weight, g	%	
Room temperature	0	34.15^{a}	55.09	19.63	32.94^{c}	13.33
	7	33.94^{ab}	54.37	20.63	33.32^{bc}	13.07
	14	30.89^{c}	53.03	20.39	35.13^{ab}	13.07
	21	31.22^{bc}	53.03	20.12	34.36^{ab}	12.93
	28	29.96^{c}	52.23	20.39	35.71^{a}	13.67
	SEM	0.47	0.34	0.17	0.36	0.09
	p	0.009	0.054	0.429	0.006	0.082
Refrigeration	0	34.10	54.67^{a}	19.76^{c}	32.13^{b}	13.20
	7	33.90	53.89^{a}	20.97^{ab}	33.45^{b}	13.80
	14	33.45	55.03^{a}	19.84^{bc}	32.77^{b}	13.13
	21	32.17	53.43^{ab}	20.56^{abc}	34.20^{ab}	13.20
	28	30.37	51.22^{b}	21.19^{a}	36.01^{a}	13.07
	SEM	0.48	0.42	0.19	0.38	0.10
	p	0.078	0.031	0.05	0.01	0.117
Source of variation						
Storage temperature	p-value	0.236	0.846	0.366	0.436	0.61
Storage time		0.001	0.003	0.03	<0.001	0.306
Time x temperature		0.69	0.391	0.543	0.385	0.026

$^{a-c}$ Different letters indicate significant differences among the means in each column with the same storage temperature

During storage of eggs, their structural components, albumen and yolks, change their percentage (Table 2). The basis of the process of egg freshness loss is the diffusion of water and gases that occurs between the egg and the environment but also inside the egg (*Lewko and Gornowicz, 2011). Chung and Lee (2014)* find a significant decrease in albumen weight under the influence of egg storage and storage time. Egg yolk weight increased with longer storage time. Similar results are reported by *Jin et al. (2011). Khan et al. (2013)* report a significant increase in yolk weight during storage, while a significant effect of storage time on albumen weight is absent, in accordance with our results for eggs stored at room temperature. In another study, *Khan et al. (2014)* find that prolonged storage time leads to a decrease in albumen weight and egg yolk weight. The absence of changes in the weight of albumen and yolks during storage of eggs at temperatures of 4°C and 20°C is determined by *Akyiurek and Okur (2009).* A slower albumen weight decrease is reported by *Feddern et al. (2017)* for eggs stored in the refrigerator. In our study, we found that storing at a lower temperature slowed down the change in the percentage of yolks. The inconsistency of the stated research results is probably a consequence of the inconsistency of the examined factors in different studies, as well as the initial quality of the eggs that were stored.

Table 3. Effects of storage temperature and time on albumen quality, Haugh Units and albumen pH

Storage temperature	Storage time, day	Albumen hight, 0.1 mm	Haugh Units	Albumen pH
Room temperature	0	61.60[a]	75.93[a]	8.46[c]
	7	45.80[b]	61.87[b]	9.27[b]
	14	40.80[b]	59.07[b]	9.36[a]
	21	33.07[c]	48.93[c]	9.34[a]
	28	24.60[d]	38.00[d]	9.31[ab]
	SEM	1.79	1.82	0.04
	p	<0.001	<0.001	<0.001
Refrigeration	0	61.33[a]	75.47	8.48[c]
	7	56.93[a]	71.93	8.85[b]
	14	55.67[ab]	71.53	9.00[a]
	21	54.13[ab]	69.67	9.06[a]
	28	47.20[b]	64.47	9.07[a]
	SEM	1.45	1.24	0.03
	p	0.035	0.073	<0.001
Source of variation				
Storage temperature	p-value	<0.0001	<0.0001	<0.0001
Storage time		<0.0001	<0.0001	<0.0001
Time x temperature		<0.0001	<0.0001	<0.0001

[a-d] Different letters indicate significant differences among the means in each column with the same storage temperature

The impact of storage temperature and time on albumen height, Haugh Units and albumen pH is shown in Table 3. The presented results confirm a highly significant ($p<0.0001$) influence of storage temperature, storage time and their interaction on internal egg quality. Height of albumen stored at room temperature was significantly reduced after only 7 days of storage and after that the trend continued to a value of only 24.6 (0.1mm). In concordance with these results are the values of Haugh Units, which decreased from the initial 75.93 to 38. Storage of eggs in the refrigerator contributed to a moderate (slower) decrease in albumen height during storage. In relation to the initial value of fresh eggs, a significant difference was found after 28 days of storage. In regard to the Haugh Units, there were no significant differences in refrigerated eggs during the four-week storage period. The pH value of the albumen increased significantly with the storage time of the eggs, both at room temperature and in the refrigerator.

The reduction in albumen height during storage is an expected consequence of the egg aging process during which CO_2 is released from the albumen. Thinning of the thick albumen leads to a decrease in the number of Haugh Units. Under refrigerated storage conditions, these processes are less intense than at room temperature. Preservation of internal quality, based on Haugh Units, was better with eggs from the refrigerator thanks to a more moderate reduction in albumen height during storage. Similar results on the impact of cooling and room temperature on Haugh Units are presented by *Feddern et al. (2017).* Since Haugh Units are considered a biased quality parameter *(Silversides and Scott, 2001)* according to *Jin et al. (2011),* the pH of the albumen objectively shows the freshness of the egg. A rapid increase in pH was recorded after only two days of storage, regardless of temperature conditions, however, the largest increase occurred during the first 5 days of storage. In our study, the increase in albumen alkalinity was intense during the first 14 days at both storage temperatures, subsequently the processes were more moderate and the pH maintained at values that did not differ statistically from the 14th to the 28th day of storage. Similar tendencies are reported by *Feddern et al. (2017)* but only for refrigerated eggs. Lower storage temperatures contributed to a slower rate of increase of albumen pH value.

Conclusion

The eggs of the native Banat Naked Neck hen, in the second year of production, were of satisfactory initial quality, which significantly decreased under the influence of storage time. Storage temperature affected egg weight loss (<0.001), shell weight (<0.05), albumen height (<0.0001), Haugh Units (<0.0001) and albumen pH (<0.0001). When stored in the refrigerator, changes in internal quality were significantly slowed down. After 28 days of storage in the refrigerator,

the values of albumen height and Haugh Units were higher than the same parameters in eggs stored for only 7 days at room temperature. The obtained results indicate the speed of changes in the internal quality of Banat Naked Neck eggs at room temperature, which indicate the necessity of storing these eggs in the refrigerator, regardless of the duration of storage.

Kvalitet jaja Banatske gološijanke tokom skladištenja

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Rezime

U manje intenzivnim sistemima proizvodnje nativne rase živine se mogu koristiti u cilju diverzifikacije proizvoda i samoodrživosti ovih rasa kroz proizvodnju. S obzirom na nedostajuće podatke o održivosti kvaliteta jaja autohtonih, nativnih kokoši tokom skladištenja, cilj istraživanja je bio da se tokom četvoronedeljnog perioda u različitim temperaturnim uslovima skladištenja utvrde najvažniji parametri kvaliteta jaja autohtone rase kokoši banatska Naked Neck. Dizajn ogleda je bio dvofaktorijalni sa 4 nivoa faktora dužina skladištenja (sveža jaja - 0, 1, 2, 3 i 4 nedelje skladištenja) i 2 nivoa faktora temperaturni uslovi skladištenja (sobna temperatura i frižider). Sobna temperatura je bila prosečno 21.3°C i u frižideru 8°C. Analiza kvaliteta je izvršena na ukupno 200 jaja, obuhvatajući parametre: težina jajeta, gubitak težine jajeta, težina i udeo strukturnih komponenti: ljuska, žumance i belance, visina belanca, boja žumanca, Haugh Units i pH belanca. Dužina skladištenja je imala signifikantan efekat na sve osobine kvaliteta jaja, osim na boju žumanca koja je bila pod interakcijskim uticajem vremena i temperature skladištenja. Temperatura skladištenja je uticala na gubitak mase jajeta, težinu ljuske, visinu belanca, Haugh Units i pH belanca. Skladištenjem u frižideru, promene u unutrašnjem kvalitetu se značajno usporavaju. Nakon 28 dana skladištenja u frižideru vrednosti visine belanca, Haugh Units i pH belanca su bile veće, odnosno bolje u odnosu na iste parametre jaja skladištenih samo 7 dana na sobnoj temperaturi.

Ključne reči: kvalitet jaja, Banatska gološijanka, dužina skladištenja, temperatura skladištenja

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INFLUENCE OF FLOOR EGG SHELL CLEANLINESS AND CLEANING TREATMENT ON HATCHABILITY AND CHICK QUALITY

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Original scientific paper

Abstract: A total of 3,600 floor eggs from a 59-week-old Cobb 500 parent flock were collected to examine the effects of shell cleanliness and cleaning treatment on incubation results. The eggs were divided into two equal groups according to the cleanliness of the shell: eggs with a visually clean shell (clean eggs) and eggs with a dirty shell (dirty eggs). Depending on the cleaning treatment, clean and dirty eggs were divided into three equal groups: eggs that were not cleaned at all (intact), eggs that were cleaned with metal wire (scraped eggs) and eggs that were washed (washed eggs). Cleaning treatment significantly affected egg weight loss ($p = 0.057$). The hatchability of set eggs was under significant influence of egg cleanliness ($p = 0.018$), while the hatchability of fertile eggs was under significant influence of egg cleanliness ($p = 0.003$) and cleaning treatment ($p = 0.029$). Significant influence of shell cleanliness ($p = 0.000$) and cleaning treatment ($p = 0.000$) on egg contamination was also observed. Early, middle and total embryonic mortality were not significantly influenced by shell cleanliness and cleaning treatment, in contrast to late mortality which was under significant influence of egg cleanliness ($p = 0.028$). The number of first grade chicks per incubator tray was significantly influenced by egg cleanliness ($p = 0.018$). Chick weight and length were not significantly affected by shell cleanliness and cleaning treatment. The study showed that washed eggs had a higher weight loss compared to intact and scraped eggs. Dirty eggs had a lower hatchability, a higher percentage of contamination and late mortality as well as a lower number of first grade chicks per incubation tray, compared to clean eggs. Cleaning treatments did not have a significantly positive effect on the incubation results of either clean or dirty eggs. Washing treatment had a particularly negative effect on dirty eggs as they had reduced hatchability and increased contamination. The absence of a positive effect of scraping and washing treatment on the incubation results makes justification of these cleaning treatments for floor eggs doubtful.

Key words: hatching eggs, floor eggs, egg cleaning, hatchability, chick quality

Introduction

A broiler hatching egg should not only meet certain criteria in terms of weight, shape, shell quality, cleanliness and freshness, but should also be laid in a nest *(King'ori, 2011)*. However, it is known that a certain number of eggs are laid outside the nest, most often on the part of the floor covered with bedding or on grid, which is why they are known as floor eggs. According to field data, the percentage of floor eggs on broiler parents' farms varies from 0.1 to almost 18%, which can be related to a number of factors such as genotype, bird health, flock management, facility design, ambient conditions, and the number of nests, their location, type and attractiveness *(Hulzebosch, 2006)*. In addition to requiring additional collection work, the percentage of contamination is higher in floor eggs than nest eggs from the same flock, which results in lower hatchability and making them a source of contamination for other eggs *(Van den Brand et al., 2016; Ahamed et al., 2019)*. Namely, during the cooling of the eggs after oviposition, the contents of the egg are shrinking, so the resulting negative pressure creates the suction effect that promotes the entry of microorganisms through the shell pores *(Berrang et al., 1999)*. Floor eggs are cooled in an environment rich in microorganisms, which is why they have higher level of initial contamination than nest eggs *(Deeming et al., 2002)*. Also, floor eggs have a higher percentage of cracked shell, which is known from the production of table eggs *(De Reu et al., 2009)*, which can facilitate bacterial penetration *(Berrang et al., 1999)* and adversely affect the hatchability and chick quality *(Khabisi et al., 2012)*.

The use of floor eggs in incubation for higher production of chicks or due to insufficient available nest eggs is not a rare practice in commercial hatcheries *(Fasenko et al., 2000; Van den Brand et al., 2016)*, which is why there is interest in examining the possibility of increasing of their hatchability. The possibility of cleaning dirty nest and floor eggs in order to increase the incubation results has been considered in previous studies. *Buhr et al. (1994)* did not improve hatchability of clean and dirty nest eggs by sanitizing them, nor did *Yoho et al. (2008)* who wiped dirty nest eggs with a damp cloth or scraped them. *Van den Brand et al. (2016)* found a higher percentage of contamination and lower hatchability in washed and unwashed floor eggs compared to clean nest eggs, in contrast to *Fasenko et al. (2000)* who reported that washed floor eggs were suitable for incubation, as they did not differ in hatchability from washed nest eggs.

Therefore, the aim of this study was to examine the influence of floor egg cleanliness at the time of collection (clean and dirty eggs) and egg cleaning treatment (intact, scraped and washed) on the hatchability and chick quality.

Materials and Methods

The experiment used eggs produced from a 59-week-old Cobb 500 broiler parent flock, reared in accordance with the recommendations of the producer of a given hybrid in a typical closed facility equipped with system for mechanical egg collection.

A total of 3,600 eggs were collected, of which 1,800 were floor eggs with a visually clean shell (clean eggs, without any visible dirt or materials on the shell), and 1,800 were eggs with a visually soiled shell (dirty eggs, with visible dirt or materials on the shell). The design of the experiment was set up to determine whether shell cleaning treatments had a positive effect on incubation parameters in dirty eggs. The same cleaning treatments were applied to clean eggs (as a control), to determine whether cleaning procedures as such had an impact on the incubation parameters. Clean and dirty eggs are further divided into three equal subgroups (n = 600 eggs) depending on the cleaning treatment: intact - eggs that are not cleaned at all, washed - eggs that are washed in water, then wiped with a damp cloth and left to dry before storage, scraped - eggs that were cleaned by scraping with a dry metal wire. All eggs in the study were stored for five days in the same ambient conditions. Each group consisted of four incubator trays of 150 eggs each, representing four replicates. Preheating and incubation of eggs was performed identically and simultaneously in all groups, following the standard procedure of the commercial hatchery where the research was conducted. Egg weight was determined before setting and when transferring eggs from the setter to the hatcher (18th day of incubation) to calculate egg weight loss during incubation *(Ahamed et al., 2019)*. After removing the hatchery crates, the number of first and second grade chicks was determined, as well as the number of dead chicks. Chicks that were dry, clean, with a closed and clean navel, acceptable body size and without deformities or lesions were classified as the first grade, and all other chicks as the second grade chicks *(Reijrink et al., 2010)*. All unhatched eggs were examined to determine the number of contaminated eggs, unfertile eggs, as well as the number of eggs with dead embryos, classified according to the day of death as early (0-9 days), medium (10-17 days) or late mortality (18 -21 days) *(Reijrink et al., 2010)*. The length of the chick (cm) was determined using a ruler, as the distance from the tip of the middle toe to the tip of the beak, as described by *Reijrink et al. (2010)*, while a technical scale (Kern EMB 200-2) was used to measure the chick weight. The length and weight of the chicks were individually measured in a sample of ten randomly selected chicks per replicate in each group. Hatchability percentage was calculated for set and fertile eggs, and embryonic mortality parameters as a percentage of fertile eggs *(Reijrink et al., 2010)*.

The statistical analysis was performed using Statistica 13 (Tibco Software Inc, 2017). The incubation tray was considered the experimental unit. The two-way ANOVA was used to determine the effects of treatments. The means were sepa-

rated using the Tukey *post hoc* test and values were considered statistically different if $p<0.05$ i $p<0.01$.

Results and Discussion

The results of the examination of the influence of shell cleanliness and cleaning treatment of floor eggs on the incubation results are shown in Table 1.

Egg weight loss was not significantly influenced by the shell cleanliness ($p = 0.376$), but it was significantly influenced by the cleaning treatment ($p = 0.057$). On average, higher values of weight loss were determined in the washed group compared to scraped and intact eggs. The cuticle is a surface shell layer that participates in the regulation of gas exchange through the shell *(Samiullah and Roberts, 2014). Peebles et al. (1998)* reported that eggs with the removed cuticle had a higher weight loss compared to intact eggs, which in this study may be related to a washing treatment that probably removed the cuticle to a greater extent and increased the number of open pores, ultimately causing higher weight loss.

Table 1. Effect of egg shell cleanliness and cleaning treatment on incubation results (mean ± stan. dev.)

Parmeters	Clean eggs			Dirty eggs			p values for main effects		
	Int	Scr	Wash	Int	Scr	Wash	C	T	C x T
Egg weight before setting, g	72.52 ± 0.58	73.26 ± 0.77	73.32 ± 0.64	72.98 ± 0.65	72.98 ± 0.46	72.87 ± 0.46	0.719	0.382	0.279
Egg weight at transfer, g	62.73 ± 0.75	63.76 ± 0.76	63.25 ± 1.10	63.61 ± 0.75	63.50 ± 0.74	60.99 ± 3.11	0.379	0.141	0.128
Egg weight loss, %	13.51 ± 0.45^{b}	12.96 ± 0.28^{b}	13.74 ± 0.83^{ab}	12.84 ± 0.28^{b}	13.00 ± 0.52^{b}	16.31 ± 4.11^{a}	0.376	0.057	0.176
Hatchability of set eggs, %	66.8 ± 3.2^{a}	75.2 ± 3.6^{a}	71.8 ± 9.4^{a}	71.8 ± 4.8^{a}	64.3 ± 13.0^{a}	52.3 ± 9.1^{b}	0.018	0.127	0.021
Hatchability of fertile eggs, %	80.5 ± 2.7^{ab}	86.2 ± 4.2^{a}	81.4 ± 7.4^{ab}	81.7 ± 6.1^{ab}	73.9 ± 11.8^{b}	59.7 ± 10.1^{c}	0.003	0.029	0.030
Contaminated eggs, %	2.0 ± 0.5^{bc}	1.2 ± 1.1^{c}	2.8 ± 1.7^{bc}	1.3 ± 1.2^{bc}	6.3 ± 3.5^{b}	19.0 ± 7,2^{a}	0.000	0.000	0.000
Early mortality, %	10.5 ± 1.9	8.1 ± 3.6	11.6 ± 5.8	6.9 ± 2.3	9.1 ± 5.6	14.1 ± 4.2	0.978	0.094	0.334
Middle mortality, %	0.8 ± 0.6	0.2 ± 0.4	0.6 ± 0.7	0.4 ± 0.8	0.4 ±0.5	1.2 ±0.5	0.574	0.172	0.245
Late mortality, %	6.2 ± 3.4	3.7 ± 1.7	3.7 ± 2.1	7.5 ± 2.6	8.7 ± 3.9	7.8 ± 3.4	0.028	0.704	0.665
Total mortality, %	16.6 ± 2.5	11.2 ± 5.2	15.5 ± 5.9	14.1 ± 3.8	16.5 ± 9.2	21.8 ± 5.3	0.210	0.254	0.256
First grade chicks / incubator tray, n	103.0 ± 4.9^{a}	112.8 ± 5.4^{a}	107.8 ± 14.1^{a}	108.0 ± 7.1^{a}	96.5 ± 19.5^{a}	78.5 ± 13.6^{b}	0.018	0.124	0.021

Int – intact eggs; Scr – scraped eggs; Wash – washed eggs
C – Egg cleanliness, T – Cleaning treatment
^{abc} – Values in the same row with different letters are statistically different

The hatchability of set eggs differed statistically significantly in relation to the egg shell cleanliness ($p = 0.018$), but not in relation to the cleaning treatment ($p = 0.127$), while the hatchability of fertile eggs was influenced by both egg shell cleanliness ($p = 0.003$) and cleaning treatment ($p = 0.029$). Significantly higher hatchability of set and fertile eggs was obtained with clean eggs compared to dirty eggs. Also, intact and scraped eggs had a higher hatchability compared to washed eggs. The hatchability of intact dirty eggs was significantly higher than washed and scraped dirty eggs, while no significant differences in hatchability were found between intact, scraped and washed clean eggs. These results are consistent with the results of *Buhr et al. (1994)* who reported that sanitized and unsanitized dirty nest eggs have significantly lower hatchability compared to sanitized and unsanitized clean eggs, which indicates the absence of a positive sanitation effect in both clean and dirty eggs. Also, *Yoho et al. (2008)* did not improve the hatchability of dirty nest eggs by wiping them with a damp cloth or sanded them with an abrasive pad, as they did not differ significantly from uncleaned dirty nest eggs. On the other hand, *Fasenko et al. (2000)* found a significantly higher hatchability of fertile eggs in washed floor eggs compared to unwashed floor eggs, while washed nest eggs did not differ from these groups.

Egg contamination in this study was influenced by shell cleanliness ($p = 0.000$) as well as cleaning treatment ($p = 0.000$). Higher values were found for dirty compared to clean eggs, as well as for washed compared to intact and scraped eggs. A drastic increase in contamination was observed in washed dirty eggs compared to other groups in the study. *Fasenko et al. (2000)* determined a significantly higher percentage of contamination in washed floor eggs compared to washed nest eggs, while unwashed floor eggs did not differ from these two groups.

No significant effect of shell cleanliness and cleaning treatment was found for early, middle and total embryonic mortality. Late mortality was significantly influenced by egg shell cleanliness ($p = 0.028$), so that dirty eggs had a significantly higher value compared to clean eggs. *Deeming et al. (2002)* found that dirty floor eggs have a higher degree of contamination compared to nest eggs, which is why they also had a higher percentage of late mortality. *Buhr et al. (1994)* also reported higher late embryonic mortality after incubation of dirty nest eggs compared to clean nest eggs. *Fasenko et al. (2000)* observed significantly higher mortality between 8 and 14 days of incubation in unwashed floor eggs compared to washed floor eggs, while washed nest eggs did not differ from these groups. The cuticle is a physical barrier to the penetration of microorganisms into the egg because it closes the pore openings and contains certain antimicrobial substances *(Samiullah and Roberts, 2014)*, so any damage to this layer, such as scraping or washing, can impair its defense function *(Berrang et al., 1999)*.

The number of first grade chicks per incubator tray was significantly influenced only by egg shell cleanliness ($p = 0.018$), but not by cleaning treatment ($p = 0.254$). A higher number of first grade chicks were obtained from clean

compared to dirty eggs. The value of this parameter was particularly low in dirty scraped and washed eggs compared to other eggs in the study. The absence of a significant difference in the percentage of first grade chicks between washed and unwashed floor and clean nest eggs was reported by *Van den Brand et al. (2016)*, as well as *Fasenko et al. (2000)* in a comparison of washed nest, washed and unwashed floor eggs.

The average weight and length of chicks obtained in this study are shown in Table 2.

Table 2. Effect of egg shell cleanliness and cleaning treatment on chick quality (mean ± stan. dev.)

Parameters	Clean eggs			Dirty eggs			p values for main effects		
	Int	Scr	Wash	Int	Scr	Wash	C	T	C x T
Chick weight, g	48.42 ± 0.78	48.54 ± 1.97	47.88 ± 0.85	49.42 ± 1.67	48.40 ± 2.70	48.91 ± 1.19	0.369	0.792	0.729
Chick length, cm	19.91 ± 0.22	19.77 ± 0.25	19.57 ± 0.04	19.71 ± 0.10	19.52 ± 0.24	19.84 ± 0.50	0.579	0.473	0.133

Int – Intact eggs; Scr – scraped eggs; Wash – washed eggs
C – Egg cleanliness, T – Cleaning treatment

Chick weight did not differ significantly depending on egg shell cleanliness ($p = 0.369$) and egg cleaning treatment ($p = 0.792$). Similarly, the absence of a significant influence of shell cleanliness ($p = 0.579$) and cleaning treatment ($p = 0.473$) was determined for chick length. *Buhr et al. (1994)* also found no significant difference in chick weight between sanitized and unsanitized clean and dirty nest eggs. *Van den Brand et al. (2016)* found a higher chick weight in the group of clean nest eggs and mixed groups (floor and clean nest eggs) compared to the groups of washed and unwashed floor eggs.

Conclusion

Based on the obtained results, it can be concluded that clean eggs had significantly higher hatchability and the number of first grade chicks per incubator tray compared to dirty eggs. Scraping and washing as cleaning treatments did not have a positive effect on incubation results in both clean and dirty eggs. Also, washing of dirty eggs significantly reduced the hatchability and number of first grade chicks and increased the percentage of egg contamination. Chick weight and length were not influenced by the cleanliness of the shell and the cleaning treatment.

Efekat čistoće ljuske i tretmana čišćenja na inkubacione rezultate podnih jaja

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Rezime

U cilju ispitivanja uticaja čistoće ljuske i tretmana čišćenja na rezultate inkubacije sakupljeno je ukupno 3.600 podnih jaja od 59-nedelja starog roditeljskog jata Cobb 500. Jaja su bila podijeljena u dvije jednake grupe po čistoći ljuske: jaja sa vizuelno čistom ljuskom (čista jaja) i jaja sa prljavom ljuskom (prljava jaja). Čista i prljava jaja su zavisno od tretmana čišćenja bila podiljena u tri jednake grupe: jaja koja uopšte nisu čišćena (intaktna), jaja koja su očišćena pomoću metalne žice (ostrugana jaja) i jaja koja su oprana (oprana jaja). Tretman čišćenja je značajno uticao na gubitak mase jaja ($p = 0.057$). Leženost uloženih jaja značajno je bila uslovljena čistoćom jaja ($p = 0.018$), dok je leženost oplođenih jaja bila značajno uslovljena čistoćom jaja ($p = 0.003$) i tretmanom čišćenja ($p = 0.029$). Zapažen je značajan uticaj čistoće ljuske ($p = 0.000$) i tretmana čišćenja ($p = 0.000$) na kontaminaciju jaja. Rani, srednji i ukupan embrionalni mortalitet nisu bili značajno uslovljeni čistoćom ljuske i tretmanom čišćenja, za razliku od kasnog mortaliteta koji je bio značajno uslovljen čistoćom jaja ($p = 0.028$). Broj pilića prve klase po ljesi bio je značajno uslovljen čistoćom jaja ($p = 0.018$). Masa i dužina pileta nisu bili pod značajnim uticajem čistoće ljuske i tretmana čišćenja. Istraživanje je pokazalo da su oprana jaja imala veći gubitak mase u odnosu na intaktna i ostrugana jaja. Prljava jaja su imala nižu leženost, veći procenat kontaminacije i kasnog mortaliteta kao i manji broj pilića prve klase po ljesi u odnosu na čista jaja. Tretmani čišćenja nisu imali značajno pozitivan uticaj na inkubacione rezultate ni čistih ni prljavnih jaja. Pranje je imalo posebno negativan uticaj na prljava jaja zbog snižene leženosti i povišene kontaminacije. Izostanak pozitivnog efekta struganja i pranja na inkubacione rezultate dovodi u pitanje opravdanost ovih tretmana čišćenja kod podnih jaja.

Ključne reči: jaja za nasad, podna jaja, čišćenje jaja, leženost, kvalitet pilića

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WELFARE PARAMETERS AND KEEL BONE DAMAGE IN LAYING HENS REARED IN DIFFERENT PRODUCTION SYSTEMS

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Abstract: The aim of this study was to determine the effect of housing system and the age of hens on welfare parameters and the prevalence of keel bone damage in laying hens. In this study two housing systems were evaluated: aviary system and enriched cages. From each system and age, we used 50 randomly selected hens from different cages and tiers. The results showed significant differences between systems in the type of keel bone damage. There was no significant difference in keel deviation between systems, but the higher prevalence of keel fractures was found in aviary system. In addition, significant effect of the age of hens was found on the occurrence of keel fractures. Footpad dermatitis had statistically higher occurrence in enriched cages at 62 weeks of age. Hens from aviary system had significantly better plumage score compared to hens from enriched cages but only at 42 weeks of age.

Key words: keel bone damage, housing system, welfare, laying hens, aviary, enriched cages

Introduction

In 2012 EU countries banned conventional cages for the welfare reasons, when Directive 1999-74-EC came to force. In Serbia this directive will came to force in 2024. This will lead to transitioning to new housing systems in poultry production which are significantly better when it comes to animal welfare. However, new systems generated new problems, such as keel bone damage (KBD), which also endangers hen's welfare. There are two types of KBD - keel fractures (KF) and keel deviation (KD) and both can be painful to the bird and reduce productivity *(Harlander-Matauschek et al., 2015)*. Fractures are characterized by sharp bends, shearing and fragmentation of the keel bone. Keel fractures can lead to pain and stress response in hens *(Riber et al., 2018; Wei et al., 2019)*. Deviations

are characterized by an abnormally sharped structure that deviates from a theoretically perfect 2-dimensional straight plane *(Casey-Trott et al., 2015)*. Prevalence of KBD ranges from 5% to as high as 97% depending on housing system and age of hens *(Rodenburg et al., 2008; Wilkins et al., 2011; Petrik et al., 2015; Riber and Hinrichsen, 2016; Regmi et al., 2016)*.

Besides keel bone condition there are many other traits that indicate welfare of laying hens. *Nicol et al. (2009)* described plumage score as one of the major welfare indicators because feather pecking is one of the most important causes of feather damage, and presents major welfare problem *(Habig and Distil, 2013)*. Also, foot pad dermatitis and skin lesions are important because they are forms of contact dermatitis, affecting skin in contact with irritating materials *(Green et al., 1985; Martins et al., 2016; Thofner et al., 2019)*.

The aim of this study was to determine the effect of housing system and the age of hens on welfare parameters and the prevalence of keel bone damage in laying hens.

Material and Methods

In this research two types of housing systems were examined: enriched cages with full equipment and aviary system. Fifty hens were randomly selected from each housing system at the middle and at the end of production cycles (43 and 63 weeks of age, respectively). From enriched cages hens were taken from different cages and levels, and from aviary system hens were taken from the floor and different tiers.

Assessment of keel bone damage was performed by palpation. The prevalence of KBD was assessed using the technique of palpation according to the method described by *Scholz et al. (2008)*. Palpation was done by running fingers alongside and over the keel bone. It was determined whether the damage was present or not.

Other welfare parameters included in this research were: plumage condition, skin lesions, comb pecking wounds, footpad dermatitis and claw length. Plumage condition was assessed by using the method described by *Tauson et al. (2005)*, scale ranging from 0 (highly damaged plumage) to 4 (very good plumage). Footpad dermatitis, skin lesion and comb pecking wounds were assessed on the scale from 0 to 2 depending on severity, according to Welfare quality assessment protocol for poultry (2009) (0 presenting no visible damages, and 2 presenting severe damage). Claws were assessed by its length, and were described either as normal or long.

Statistical analysis was performed using Statistica 13.5. Analysis of variance ANOVA was used to compare the mean values between evaluated parameters among housing systems and age of hens. Post hoc analysis was

performed using Mann Whitney test. Results of statistical analysis were considered significant when the $p \leq 0.05$.

Results and Discussion

The results of this research showed high occurrence of KBD in both housing systems. The higher prevalence of KBD was detected in aviary system compared to enriched cages. Besides that, the main difference in these two housing systems is in the type of KBD. In aviary system main cause of KBD were keel fractures (KF), and in enriched cages the main type of KBD were keel deviations (KD) (table 1). Regarding the keel deviation (KD), it's occurrence was relatively uniform between housing systems, without statistically significant differences. These results are expected since the KD is caused mainly by the pressure of the keel bone on metal perches which are present in both housing systems. Also, there was no effect of age of hens on the occurrence of KD, since the keel bone ends its ossification until 40 weeks of age (*Toscano et al., 2020*) and keel deviations are not likely to develop after that time.

Table 1. Prevalence of keel bone damage in laying hens in different housing systems and in different phase of production cycles

Type of KBD	43 weeks of age		62 weeks of age	
	Aviary system	Enriched cages	Aviary system	Enriched cages
Keel deviation (KD), %	27.3	29.2	23.3	30
Keel fracture (KF), %	16.4[a]	4.2[b]	30[c]	3.3[b]
KF+KD (KBD), %	43.7[ab]	33.4[ab]	53.3[a]	33.3[b]

[a,b,c] Values with different superscript within the same row are statistically significant

Đukić Stojčić et al. (2017) investigated the influence of the housing system on the occurrence of KBD, and found that as many as 39% of hens in enriched cages had KBD. High frequency of KBD in this housing system can be attributed to metal perches. The assumption that perches have a key role in developing KBD was confirmed by other authors too *(Rodenburg et al., 2008; Wilkins et al., 2011; Đukić Stojčić et al., 2017)*.

Statistical analysis showed significantly higher occurrence of keel fractures in aviary system compared to enriched cages (table 1). Some authors explain this by the increased risk of accidents and falls in more extensive housing systems *(Vits et al., 2005; Sandilands et al., 2009; Wilkins et al., 2011; Lay et al., 2011).* Also, in our research, keel fractures were highly related to the age of laying hens, but only in aviary system. This can be explained by the fact that in aviary system birds are more active, and have more chances of injury which only get worse with the age of

hens. Similar results were reported by *Eusemann et al. (2018b, 2020). Habig and Distil (2013)* and *Sherwin et al. (2010)* found that the incidence of fractures in laying hens at the end of the production cycle in enriched cages ranged from 30 to 53.3%.

Table 2. Prevalence of welfare parameters in different housing systems and in different phase of production cycles

Welfare parameters	43 weeks of age		62 weeks of age	
	Aviary system	Enriched cages	Aviary system	Enriched cages
Footpad dermatitis (FPD), %	9.1[a]	6.6[a]	10[a]	33.3[b]
Plumage	3.89[a]	3.62[b]	3.60[b]	3.58[b]
Skin lesions	-	-	-	-
Comb pecking wounds	-	-	-	-
Long claws, %	-	-	-	6.6

[a,b]Values with different superscript within the same row are statistically significant

Higher occurrence of FPD was established in hens housed in enriched cages compared to aviary system (table 2). It is interesting that there was no significant difference in young age, but the difference was significant at the age of 62 weeks. One of the explanations for the higher occurrence of FPD in enriched cages is the existence of metal wire from which the cage is made. Other authors reported that up to 39% of bird had foot pad dermatitis in non-cage systems *(Abrahamsson and Tauson, 1995; Gunnarsson et al., 1995; Wang et al., 1998; Röngen et al., 2008).*

Generally, the plumage condition of the birds in both housing systems was satisfactory. The best plumage score had hens housed in aviary system at 43 weeks of age. Statistically significant differences were found between hens housed in aviary system and enriched cages at 43 weeks of age, but not at the 62 weeks of age. *Staaveren et al (2021)* found that the housing system affected plumage, and that plumage from cage systems had a poorer assessment of plumage compared to hens from non-cage housing systems, which is in accordance with the results obtained in this study. Perches and other equipment can lead to problems in plumage condition in laying hens *(Sepeur et al., 2015).* Significant deterioration of plumage was found with increasing age of hens, which is in accordance with the results of other authors *(Rönchen et al., 2007; Habig and Distl, 2013, Schreiter et al., 2020).*

Only 6.6% of laying hens at the end of the production cycle had long claws in the enriched cages, while in the aviary system there were no hens with long claws. Also, no long claws were found in younger hens reared in enriched cages. No statistically significant differences were found between the housing system as well as the age of the laying hens, regarding claw length. Further, no hens with skin

lesions and comb pecking wounds were found in either housing systems at both ages.

Conclusion

Significant differences in occurrence and the type of KBD were found between the housing systems. Generally, higher occurrence of KBD was found in aviary system compared to enriched cages. Regarding the type of the KBD higher prevalence of keel fractures was found in aviary system, while in case of KD there were no significant differences between systems. In addition, significant effect of the age of hens was found only on the occurrence of keel fractures.

Regarding the welfare parameters, higher incidence of footpad dermatitis was detected in enriched cages compared to aviary system, but only at 62 weeks of age. Also, housing system had significant effect on plumage condition which was better in aviary system at 42 weeks of age.

Based on all of the above, it can be concluded that housing systems and age have a significant impact on the occurrence and the type of keel bone damage and some welfare parameters (FPD, plumage score and claw length). Further research is needed to determine specific risk factors of KF and KD in order to develop strategies for reducing the incidence of this multifactorial welfare issue.

Parametri dobrobiti i deformacija grudne kosti kokoši nosilja u različitim sistemima držanja

Sava Spiridonović, Mirjana Đukić Stojčić, Lidija Perić, Marko Pajić, Slobodan Knežević

Rezime

Cilj ovog rada bio je da se utvrdi efekat sistema držanja i starost na parametre dobrobiti i učestalost deformacija grudne kosti kod kokoši nosilja. U ovom istraživanju su ocenjena dva sistema držanja: avijarni sistem i obogaćeni kavezi. Iz svakog sistema i proizvodnih ciklusa koristili smo 50 slučajno odabranih kokoši iz različitih kaveza i nivoa. Rezultati su pokazali značajne razlike između sistema u tipu oštećenja grudne kosti. Nije bilo značajne razlike u devijacijama grudne kosti između sistema, ali je veća učestalost fraktura grudne kosti utvrđena u avijarnom sistemu. Pored toga, utvrđen je značajan uticaj starosti kokoši na pojavu fraktura grudne kosti. Dermatitis tabanskih jastučića imao je statistički veću pojavu u obogaćenim kavezima u starosti od 62 nedelje. Kokoši iz avijarnog sistema imale su znatno bolju ocenu operjalosti u poređenju sa kokošima iz obogaćenih kaveza,

ali samo u starosti od 42 nedelje.

Ključne reči: deformacija grudne kosti, sistem držanja, dobrobit, kokoši nosilje, avijarni sistem, obogaćeni kavezi

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HAEMATOLOGICAL AND BIOCHEMICAL BLOOD PROFILE OF AFRICAN CATFISH (*CLARIAS GARIEPINUS*) CULTURED IN PONDS OF DIFFERENT WATER DEPTH AND FED SINKING VERSUS FLOATING DIET

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Original scientific paper

Abstract: This study contributes data on haematological and biochemical parameters of African catfish, *Clarias gariepinus*. It employed a 3 × 2 factorial design with three ponds of different water depth (0.5, 1 and 1.5 m) and two types of feed (floating and sinking). Twelve earthen ponds (1 m x 2 m) were stocked with 16 fingerlings catfish each (mean weight ~100g) and their blood parameters were monitored over 12 weeks. Differences in hematological parameters related to water depth were mostly significant, and better results were recorded in fish reared in shallower water ponds. Feed type showed improved hematological parameters with using of sinking diet. Most biochemical parameters showed significant differences in pond waters depth and feed type with better results coincided with rearing fish in shallower water depth and with sinking feed. Conclusively, culturing Catfish in shallow ponds (0.5 m) and use of sinking feed improve physiological response and health condition.

Key words: pond water depth, floating and sinking fish feed, African catfish (*Clarias gariepinus)*, physiological response

Introduction

Intensive aquaculture production of African catfish (*Clarias gariepinus*) has increased in the last few decades. Although intensive production delivers the maximum benefits from cultured area but it subject the fish to many stressors as high stocking density, handling, transportation, bad water quality, sorting and

grading (*Thanikachalam et al., 2010*). In order to overcome these stressors and improve fish health and welfare, several attempts have been done. For example, improve husbandry system used for fish rearing, use optimum nutrient level, improve water quality and the using of immunostimulants and growth promoters (*Gabriel et al., 2019*).

Haematological and biochemical studies of cultured fish are important in order to monitor the health of fish during cultivation. Such studies are particularly useful in assessing a fish's physiological and physiopathological status since morphological and biometric parameters alone do not always give a complete picture (*Adakole, 2012; Tavares-Dias and Moraes, 2007*). However, in order for data on haematological and biochemical parameters to be meaningful, there have to be reliable reference ranges for comparison. For a number of fish species, there is still a scarcity of studies establishing normal blood values and reference ranges. Normal values exist for only a handful of hematological parameters and the established values tend to have a wide range due to a lack of standardization between methods (*Fazio et al., 2019*). This study contributes important data on the normal blood parameters of African catfish (*Clarias gariepinus*) for which little data exists. The data from this study are part of a study on the effect of water depth and sinking versus floating feed on the growth of catfish (*Abdel-Hay et al., 2019*).

Materials and Methods

Ethical approval

Ethical approval for this study was obtained from the Committee of Aquatic Animal Care and Use in Research, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Egypt.

Experimental design

A 3 × 2 factorial treatment design was used to evaluate the effect of three pond water depth (0.5, 1 and 1.5 m) and two types of feed (floating and sinking) on the growth performance of African catfish. There were two replicates per treatment and the study was carried out for 12 consecutive weeks. These water levels were chosen to reflect the traditional water depth used throughout Africa of approximately 1 m (*Hecht et al., 1996*).

Animal husbandry system

The experiment was conducted in 12 equal-sized earthen ponds (1 m x 2 m) with different water depth (0.5, 1 and 1.5 m). The animals were exposed to the following treatments; one receiving floating feed and the other sinking feed at three water depths (0.5, 1 and 1.5 m), with two replicates each. Water exchange was carried out every two days at a rate of 5 – 10%. Each pond was randomly stocked

with 16 animals at an average weight of 100.15 ± 3.480 g. The animals were left to acclimatize for one week prior to the start of the experiment. All animals were fed a quantity of approximately 3% of body mass each morning for 12 weeks and were weighed every three weeks at which point feeding rate was adjusted accordingly. Ethical approval for this study was obtained from the Committee of Aquatic Animal Care and Use in Research, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Egypt (approval number: IAACUC-KSU-2-2018).

Experimental feeds

A commercial floating and sinking feed (Table 1) were purchased from a local feed factory (Al-Ekhwa® feed factory, Kafrelsheikh, Egypt). The content of both feeds was identical, and they differed only in form (floating versus sinking).

Table 1. Composition and chemical analysis of the experimental diets on dry matter basis

	Ingredients composition (%)	
Fish meal (72%CP)	10	**10**
Soybean meal (45%CP)	40	**40**
Yellow corn	24	**24**
Wheat bran	10	**10**
Rice bran	10	**10**
Corn oil	3	**3**
Di calcium phosphate	1	**1**
Vitamin and mineral mixture	2	**2**
Total	100	**100**
	Chemical analysis (%)	
Dry matter (DM %)	93.00	**92.15**
Crude protein (CP %)	30.85	**30.84**
Ether extract (EE %)	7.94	**7.91**
Crude fiber (CF %)	4.95	**5.01**
Ash %	8.66	**8.29**
Nitrogen free extract (NFE %)	48.00	**47.85**
	Calculated energy value	
Gross energy (kcal/kg)	4496.36	**4492.48**
Digestible energy (kcal/kg)	3372.27	**3394.36**
Metabolizable energy (Kcal/kg)	**365.63**	**362.09**

Haematological and biochemical parameters

Blood sampling and serum separation

Blood samples were taken from the caudal vein of 16 animals in each treatment (eight fish per replicate) using a sterile syringe. Each sample taken was

split in two: the first part was transferred into a sterile 2 ml test tube with added KEDTA for future analysis in a haematological assay and the second part was stored in a 2 ml Eppendorf tube which would be later used for serum separation. Blood was left to coagulate at 4°C for 60 minutes. After that, tubes were centrifuged at 3000 rpm for 10 minutes in order to separate serum which was then transferred into clean Eppendorf tubes and stored at −40°C until the time of analysis.

Haematological parameters

The following blood parameters were measured: red blood cell count (RBC), haemoglobin concentration (HB), packed cell volume (haematocrit), mean corpuscular volume (MCV), mean corpuscular haemoglobin count (MCH), mean corpuscular haemoglobin concentration (MCHC) and white blood cell count (WBC) using an automatic blood cell counter (Exigo-Vet., Boule Medical AB Inc., Stockholm, Sweden). For estimation of the differential leucocytes count, two thin smears were prepared from each blood sample on clean microscope slides and were left to air-dry before being stained with a modified Wright's stain and covered. A total of 100 cells were counted under a ×100 oil immersion lens and the percentage of heterophils, lymphocytes, and monocyte was estimated following the method outlined by *Anderson and Siwicki (1995*).

Biochemical parameters

Serum total protein (TP) was determined calorimetrically using commercial kits (TP0100, Sigma-Aldrich, USA). Serum albumin was measured using the bromocresol green binding method (*Doumas et al., 1971*). Serum globulin was calculated by subtracting albumin values from total protein. Albumin/globulin (A/G) ratio was calculated by dividing albumin values by globulin values. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine were assessed following the method outlined in *Palti et al. (1999).*

Statistical analysis

Data were tested for distribution normality, linearity and homogeneity of variance. Log-transformation of the raw data was used for some measured parameters because of the large range across the data. Data were analysed and visualized in GraphPad Prism 6 and all results were reported as means with SEM. A two-way ANOVA was used for comparison of the main effects of pond water level and feed type. The interaction of the two factors was tested using Tukey's multiple comparison test as a *post hoc* test where appropriate. The level of significance was set at $p \leq 0.05$.

Results and Discussion

Haematological parameters

Differences in blood cell formation and function are considered as good indicators of nutritional status, stress response, health condition and welfare of fish (*Buentello et al., 2007*). Most blood parameters of the animals in this study differed significantly between the different treatments except for red blood cell count (RBCs), mean corpuscular volume, and mean corpuscular haemoglobin and concentration (see Table 2). The highest levels were recorded in the shallowest water for the following parameters: haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC), and lymphocytes whilst the lowest values were recorded for the following parameters: white blood cells (WBC) and heterophils. Differences in blood parameters between animals fed different feed types were not significant except for WBCs, heterophil percentage, lymphocytes and monocytes. Higher values of haematological parameters were recorded in the animals fed sinking pellets for Hb, PCV, MCV, MCH, MCHC, heterophils, and monocytes while lower values were recorded for RBC, WBC, and lymphocytes. The interaction between pond water levels and feed type was not significant for most blood parameters except WBC, lymphocytes, heterophils, and monocytes.

The results from this study are similar to those reported by *Owolabi (2011), Al-Dohail et al. (2009)* and *Acharya and Mohanty (2014).* All haematological parameters in the present study are within the normal reference range of catfishes (*Lim et al., 2000; Owolabi, 2011*). This indicates that both the pond water levels and feed types used in this study meet the basic needs of the African catfish. An erythrogram (RBC, Hb, PCV, MCV, MCH, MCHC) is commonly used to detect conditions such as anaemia and other basic health issues in animals (*Owolabi, 2011*) while, the leukogram (WBC, lymphocytes, heterophils, and monocytes) is used to get a picture of the status of an animal's immune system (*Fagbenro et al., 2000*). The increase in the values of the erythrogram in the animals reared in the shallowest ponds in this study (0.5 m) may be related to an increase in fish activity (*Acharya and Mohanty, 2014*) and improved health condition (*Buentello et al., 2007*). The results from this section of the study would imply that African catfish attain optimal physiological status in 0.5m deep ponds; however, larger sample sizes and more detailed studies would be needed to explore this further.

Table 2. Effect of pond water level and feed type on the hematological parameters of African catfish, *Clarias gariepinus*

	Hb (g/l)	RBC (10^6/L)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (g/l)	WBC (10^3/L)	Heterophils (%)	Lymphocytes (%)	Monocytes (%)
Water level										
0.5 m	11.93^{a}	3.533	36.63^{a}	105.7	35.53	33.52	34.68^{b}	3.333^{b}	90.83^{a}	5.917^{b}
1.0 m	10.88^{b}	3.650	33.63^{b}	93.59	30.55	32.32	41.03^{a}	4.625^{ab}	86.42^{b}	8.958^{a}
1.5 m	10.38^{b}	3.258	31.58^{b}	97.41	31.98	32.86	35.13^{b}	5.417^{a}	89.42^{b}	5.167^{b}
Feed type										
Floating	10.92	3.489	33.56	97.62	31.98	32.56	37.17	3.556	90.78	5.778
Sinking	11.02	3.472	34.34	100.2	33.40	33.24	36.73	5.361	87.00	7.583
SEM	0.384	0.203	1.159	6.460	2.456	0.512	1.236	0.543	1.516	0.429
P-value										
Water level	0.002*	0.169	0.001*	0.190	0.142	0.091	0.001*	0.004*	0.027*	0.001*
Feed type	0.194	0.921	0.419	0.637	0.487	0.120	0.669	0.001*	0.007*	0.001*
Interaction	0.386	0.396	0.780	0.377	0.497	0.113	0.006*	0.024*	0.048*	0.001*

Means within a column and effect that lack common superscripts differ significantly (Tukey's multiple comparison test, $p \leq 0.05$). Asterisks indicate significant differences between groups (two-way ANOVA* $p \leq 0.05$). SEM= standard error of the mean.
Hb=hemoglobin; RBCs=red blood cells; PCV=packed cell volume; MCV=mean corpuscular volume; MCH=mean corpuscular hemoglobin; MCHC=mean corpuscular hemoglobin concentration; WBCs= white blood cells

Biochemical parameters

All serum biochemical parameters in the animals in this study differed significantly between the different water level treatments except for total protein (TP) and creatinine (see Table 3). The highest value of serum biochemical parameters were recorded in the fish kept in shallowest ponds for the following parameters: TP and albumin while, globulin, ALT, AST, cholesterol, triglycerides and glucose recorded the highest values in deeper ponds. Differences in serum biochemical parameters between animals fed floating and sinking pellets were significant for all parameters except for TP, albumin, cholesterol and creatinine. Higher serum biochemical parameters were recorded in the animals fed floating pellets for albumin, albumin-to-globulin (A/G) ratio, ALT, AST, triglycerides and creatinine while the lower values were recorded in the following parameters: TP, globulin, cholesterol and glucose. The interaction effect between pond water level and feed type was significant for all biochemical parameters except for TP, globulin and creatinine.

These results are similar to those reported by *Al-Dohail et al. (2009), Owolabi (2011)* and *Acharya and Mohanty (2014).* All biochemical parameters in this study are within the normal reference range of catfish (*Owolabi, 2011*). This indicates that both the pond water levels and feed types used in this study meet the

basic needs of the African catfish. The fish reared in the shallowest ponds and fed sinking pellets had the highest TP and globulin levels. These biochemical parameters are all known to play an important role in fish nutrition, health and immunity (*Swain, 2007*). In contrast, the other biochemical parameters in fish reared in the shallowest ponds and fed sinking pellets were higher than the other groups but still within the normal range.

Table 3. Effect of pond water level and feed type on the serum biochemical parameters of African catfish, *Clarias gariepinus*

	TP (g/dl)	Albumin (g/l)	Globulin (g/dl)	A/G ratio	ALT (U/L)	AST (U/L)	Cholesterol mmol/l)	Triglycerides (mmol/l)	Glucose (mmol/l)	Creatinine (mmol/l)
Water level										
0.5 m	5.360	2.169^{a}	3.191^{a}	0.695^{ab}	61.90^{b}	446.6^{c}	405.6^{b}	69.80^{b}	36.40^{b}	0.377
1.0 m	5.080	2.073^{ab}	3.007^{b}	0.705^{a}	66.90^{b}	585.9^{b}	440.2^{b}	59.60^{c}	45.00^{b}	0.441
1.5 m	5.240	1.980^{b}	3.260^{a}	0.637^{b}	74.50^{a}	661.0^{a}	575.0^{a}	88.60^{a}	58.20^{a}	0.425
Feed type										
Floating	5.260	2.100	3.067	0.706	82.13	819.6	462.0	79.73	31.87	0.441
Sinking	5.460	2.048	3.239	0.653	53.40	309.4	485.2	65.60	61.20	0.388
SEM	0.133	0.047	0.051	0.024	2.624	27.97	24.60	4.043	3.555	0.037
P-value										
Water level	0.128	0.002*	0.001*	0.018*	0.003*	0.001*	0.001*	0.001*	0.001*	0.225
Feed type	0.279	0.189	0.001*	0.012*	0.001*	0.001*	0.259	0.003*	0.001*	0.097
Interaction	0.298	0.006*	0.277	0.004*	0.001*	0.025*	0.012*	0.039*	0.001*	0.311

Means within a column and effect that lack common superscripts differ significantly (Tukey's multiple comparison test, $P \leq 0.05$). Asterisks indicate significant differences between groups (two-way ANOVA*$p \leq 0.05$). SEM= standard error of the mean.
TP= total protein; A/G ratio= albumin-to-globulin ratio; GPT= glutamic pyruvic transaminase; GOT= glutamic oxaloacetic transaminase.

Conclusion

Rearing African Catfish in shallow ponds water depth (0.5 m) and the use of sinking feed significantly improve its physiological response, health condition and welfare.

Hematološki i biohemijski profil krvi afričkog soma (*Clearas gariepinus*) uzgajanog u ribnjacima različite dubine vode i hranjenog tonućim odnosno plutajućim obrokom

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Rezime

Ova studija daje podatke o hematološkim i biohemijskim parametrima krvi afričkog soma, *Clarias gariepinus*. Korišćen je faktorski dizajn 3 × 2 sa tri ribnjaka različite dubine vode (0,5, 1 i 1,5 m) i dve vrste hrane (plutajuća i tonuća). Dvanaest zemljanih ribnjaka (1 m x 2 m) bilo je opskrbljeno sa po 16 somova (prosečna težina ~ 100 g) i praćeni su njihovi parametri krvi tokom 12 nedelja. Razlike u hematološkim parametrima u vezi sa dubinom vode bile su uglavnom značajne, a bolji rezultati zabeleženi su kod riba uzgajanih u plićim vodenim ribnjacima. Tip hrane je pokazao poboljšane hematološke parametre uz upotrebu tonućeg obroka. Većina biohemijskih parametara pokazala je značajne razlike sa stanovišta i dubini vode u ribnjaku i tipu hrane, sa boljim rezultatima koji su se podudarali sa uzgojem ribe u manjoj dubini vode i sa tonućom hranom. Zaključno, uzgoj soma u plitkim ribnjacima (0,5 m) i upotreba tonuće hrane poboljšavaju fiziološki odgovor i zdravstveno stanje.

Ključne reči: dubina vode u ribnjaku, plutajuća i i hrana koja tone, afrički som (*Clarias gariepinus*), fiziološki odgovor

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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DISTRIBUTION OF *EUDIPLOZOON NIPPONICUM* (MONOGENEA, DIPLOZOIDAE) IN FARMED COMMON CARP (*CYPRINUS CARPIO*, L. 1758) FROM AQUACULTURE FACILITIES IN MACEDONIA

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Abstract: A total of 958 specimens of common carp from the most significant and larger cyprinid aquaculture facilities in Macedonia, including fish farms and reservoirs, were examined for parasitological investigations. *Eudiplozoon nipponicum* was found on gills in 121 specimens of common carp in spring, summer and autumn, with a prevalence of 10.67 %, and a mean intensity of 2.55. Our findings of *E. nipponicum* in common carp from aquaculture are first recorded in Macedonia.

Key words: diplozoon, parasites, common carp, aquaculture

Introduction

Diplozoids (Diplozoidae, Monogenea) are fish ectoparasites with a direct life cycle without intermediate hosts. Their free-swimming larva is called oncomiracidium. It hatches from eggs, invades a fish host and metamorphoses into a post-oncomiracidial larval stage, which is called dipopra. During their adult life, two dipoprae fuse and live as a pair in cross-copulation (*Pečínková et al., 2007*).

A typical and unique representative of the genus *Eudiplozoon* which belongs to the family Diplozoidae is *Eudiplozoon nipponicum*. According to *Matejusova et al. (2004)*, about 18 parasite species belonging to 2 genera, *Diplozoon* and *Paradiplozoon*, have been identified in Europe. *Denis et al. (1983)* considered that *Eudiplozoon nipponicum* has been introduced through the importation of carp from Asia to Europe.

Valigurová et al. (2011) state that the parasite has a complex digestive tract that is well equipped for hematophageal nutrition. According to *Milne and Avenant-Oldewage (2012)*, *Eudiplozoon nipponicum* infestation occurs as soon as the potential host swims through a group of oncomiracidiums. After attachment on

the host gills, the oncomiracidium develops into a dipopra with two pairs of clamps and can only reach maturity after merging with another dipopra, and the complete set of four pairs of clamps develops during the maturation phase.

Hermaphroditic adult forms are constantly fused, forming a characteristic "X" shape. They divide the reproductive and digestive systems (*Kamegai, 1976*). Such a reproductive strategy, in which two independent heterogeneous individuals unite in a hermaphroditic organism, without the need to look for a mating partner, demonstrates the high specialization of diploids to their parasitic life. Haptoral attachment flaps are permanent structures that are present at all stages of the diploid life cycle, from free-floating to parasitic adult stages (*Khotenovsky, 1977*).

The hatched larva of the oncomiracidium settles on the gills of the host fish, followed by transformation into a dipopra larva. The ventral growth is formed after three days and the mating of the two dipopra occurs after four days at T of 25°C (*Hirose et al., 1987*). The parasite attaches with the help of four pairs of clamps to the posterior part of the body and feeds on the blood of the host.

This study aimed to determine the distribution of *Eudiplozoon nipponicum*, prevalence, mean intensity, as well as, seasonal dynamic in farmed common carp (*Cyprinus carpio*, L. 1758) from the aquaculture facilities in Macedonia.

Material and Methods

This parasitological study was carried out by seasons, in three years. A total of 958 specimens of common carp from the most significant and larger cyprinid aquaculture facilities in Macedonia, including fish farms and reservoirs, were examined for parasitological investigations (Fig. 1). Only fresh fishes were subjected to routine identification, dissection and observation methods. Cleaned parasites were separated and put in certain fixatives, prepared for determination with determined techniques of staining and clearing (*Vasiljkov, 1983; Gussev, 1983; Stojanovski, 1997, 2003*). The gill filaments of fish were inspected by stereomicroscope. All parasites found in each fish were identified and enumerated. Preparation, fixing, staining and mounting of parasites for morphological determination was made by common methods used in parasitology. Classical epidemiological variables (prevalence and mean intensity) were calculated according to *Bush et al. (1997)*. The parasite specimens were identified according to reference keys of *Bauer (1985)* and *Gussev (1983)*.

During the examinations at Laboratory for fish diseases in Hydrobiological Institute in Ohrid (Macedonia), stereomicroscopes „Zeiss”- Stemi DV4 and „MBS 10”, as well as, light microscope „Reichart” were used.

Data of the number of fish examined, fish infected, prevalence and mean intensity (total and by seasons) are given in Tables 1, 2 and 3.

Fig.1. Sampling points –aquaculture facilities
(Blazhekovikj - Dimovska Dijana & Stojanovski Stojmir)

Legend:
1 – Fish pond Zhabeni
2 – Fish pond Bukri
3 – Fish pond Dolneni
4 – Fish pond Zhelezara
5 – Tikvesh reservoir
6 – Mladost reservoir
7 – Globochica reservoir
8 – Gradche reservoir

Results and Discussion

A total of 958 fish specimens of common carp from the most significant and larger cyprinid aquaculture facilities in Macedonia were comprised by a parasitological investigation (Fig. 1). In this study, *Eudiplozoon nipponicum* was found on gills in 121 specimens of common carp in spring, summer and autumn.

Our findings of *Eudiplozoon nipponicum* in common carp from aquaculture are first recorded in Macedonia.

Table 1. Determination of *Eudiplozoon nipponicum* by aquaculture facility and season

Season / Aquaculture facility	Spring	Summer	Autumn
Zhabeni	√	√	
Bukri		√	√
Dolneni			√
Zhelezara			√
Tikvesh Reservoir	√		
Mladost Reservoir	√		√
Globochica Reservoir	√		
Gradche Reservoir			√

Table 2. Total prevalence and mean intensity with *Eudiplozoon nipponicum* in common carp(*Cyprinus carpio,* L. 1758) from aquaculture facility

Fish species	Number of examined fish	Number of infected fish	Mean intensity	Prevalence (%)
Common carp (*Cyprinus carpio*, L. 1758)	958	121	2.55	10.67

Table 3. Prevalence (P) and mean intensity (I) with *Eudiplozoon nipponicum* in common carp (*Cyprinus carpio*, L. 1758) from aquaculture facility, by season

Parasite species	Spring		Summer		Autumn		Winter	
	I	P (%)	I	P (%)	I	P (%)	I	P (%)
Eudiplozoon nipponicum	1.89	4.76	2.39	6.53	3.70	11.73	/	/

The prevalence with *Eudiplozoon nipponicum* in common carp was 10.67 %, while the mean intensity was 2.55.

The prevalence with *Eudiplozoon nipponicum* in common carp by seasons was as following: spring – 4.76 %, summer – 6.53 %, autumn – 11.73 %; while the mean intensity was: spring – 1.89, summer – 2.39 and autumn – 3.70.

Eudiplozoon nipponicum has the following dimensions: body length 4.4 - 5.4 mm; length of the anterior part 3.0 - 3.5 mm; length of posterior part 2.0 - 2.2 mm. There are two suckers on the anterior part of the body with a diameter of 0.09 - 0.11 mm. Characteristic of *Eudiplozoon nipponicum* are the two circular glandular formations in front of the suckers. The posterior part of the body consists of three parts. One part of the body has 12 - 14 large wrinkles, which are equal to each other on both sides. The middle part forms an enlargement, on which there are large lateral oblique wrinkles. The other part is provided with four adhesive clamps

with the following dimensions: I - 0.08 × 0.12 mm; II - 0.07 × 0.15 mm; III - 0.09 × 0.14 mm; IV - 0.08 × 0.14 mm. The middle hooks are 0.024 mm long.

The covering of free-floating oncomiracidiums occurs in two types: ciliary and non-ciliary, with numerous non-ciliary sensory structures. The attachment apparatus begins to form during the oncomyracidium stage. The cover groove becomes visible later and plays a role in attaching the parasites. According to the reproductive strategy of *Eudiplozoon nipponicum*, a very important role is played by the two morphological structures of the dipopra (ventral suction cup and dorsal papilla). The posterior part of the body in adult parasites is highly modified for attachment. Haptor, grooves, and lobular dilatations are highly developed. The anterior part of the body is flexible and able to connect with the host gill tissue through the mouth and associated oral structures.

Fig. 2. *Eudiplozoon nipponicum* in common carp (whole parasite) (Blazhekovikj - Dimovska Dijana & Stojanovski Stojmir)

Fig. 3. *Eudiplozoon nipponicum* in common carp (whole parasite) (Blazhekovikj - Dimovska Dijana & Stojanovski Stojmir)

Fig. 4. *Eudiplozoon nipponicum* in common carp (whole parasite) (Blazhekovikj - Dimovska Dijana & Stojanovski Stojmir)

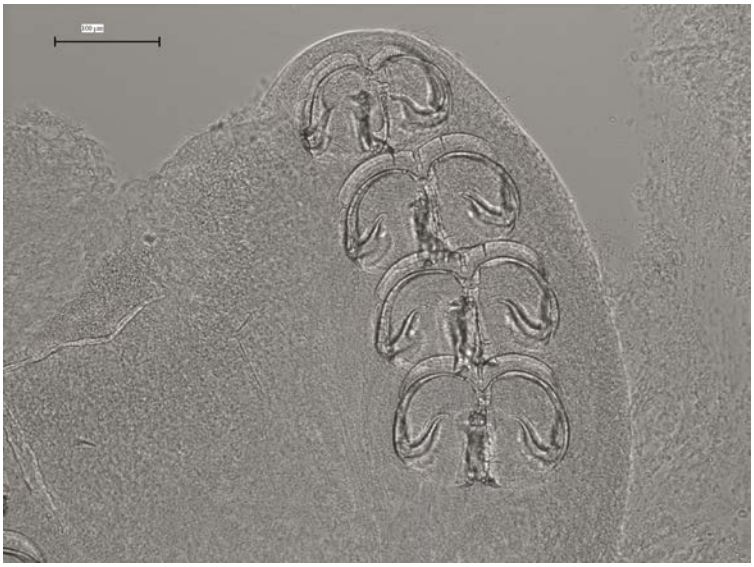

Fig. 5. *Eudiplozoon nipponicum* in common carp (4 clamps) (Blazhekovikj - Dimovska Dijana & Stojanovski Stojmir)

Fig. 6. *Eudiplozoon nipponicum* in common carp (clamps) (Blazhekovikj - Dimovska Dijana & Stojanovski Stojmir)

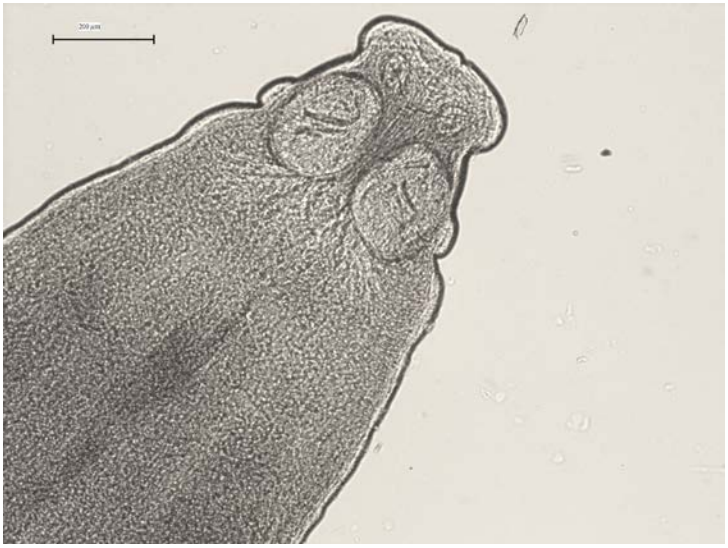

Fig. 7. ***Eudiplozoon nipponicum*** **in common carp (glandular formations on the anterior part) (Blazhekovikj - Dimovska Dijana & Stojanovski Stojmir)**

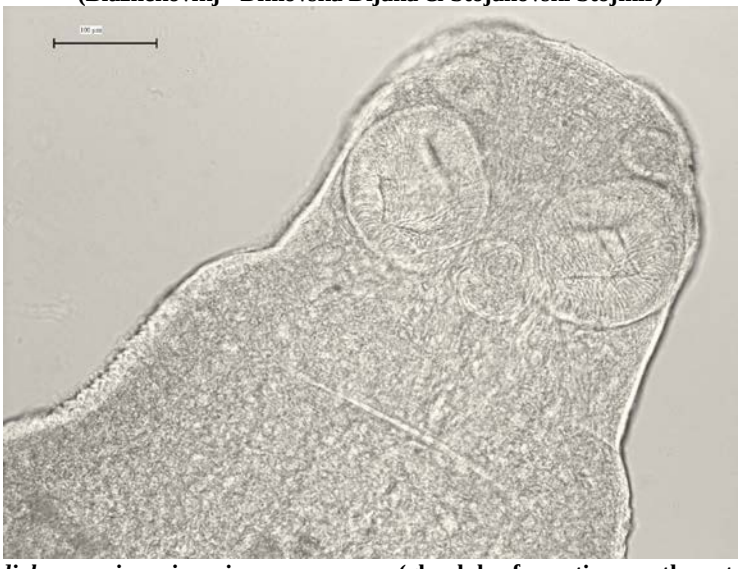

Fig. 8. ***Eudiplozoon nipponicum*** **in common carp (glandular formations on the anterior part) (Blazhekovikj - Dimovska Dijana & Stojanovski Stojmir)**

According to the data from previous parasitological researches in Macedonia, *Eudiplozoon nipponicum* in common carp was first determined by *Stojanovski (2003)* in Lake Prespa, with a prevalence of 3.58% and mean intensity of 3.25, as well as, in Lake Dojran, with a prevalence of 1.76% and mean intensity of 1.31. Also, tha data for the presence of *Eudiplozoon nipponicum* established

Hristovski et al. (2003) during the parasitological examinations in Lake Dojran Lake and *Hristovski et al. (2006, 2012)* during parasitological examinations in Lake Prespa, with a prevalence of 44.4%.

According to world literary reviews, the presence of *Eudiplozon niponicum* in common carp has been established by *Nedeva (1991)* in waters in Bulgaria; *Szekely and Molnar (1996 - 1997)* in Lake Balaton, Hungary and *Rohlenová et al. (2011)* and *Ondračková et al. (2012)* in fishponds in the Czech Republic.

According to *Kawatsu (1978)*, infested fish develop hypochromic microcytic anemia characterized by an increase in immature erythrocytes, which can lead to death, especially in carp juveniles. *Shindo (1997)* states that there is hyperplasia around the place where the parasite is attached to the gill epithelium, as we noticed in our research.

Conclusion

A total of 958 specimens of common carp from the most significant and larger cyprinid aquaculture facilities in Macedonia, including fish farms and reservoirs, were examined for parasitological investigations. *Eudiplozoon nipponicum* was found on gills in 121 specimens of common carp in spring, summer and autumn, with a prevalence of 10.67 %, and a mean intensity of 2.55.

In our study, hyperplasia of the gill epithelium and pale gill around the place where the parasite is attached to the gill epithelium was noticed, but not more serious damage to fish health or higher percentage of mortality.

Our findings of *Eudiplozoon nipponicum* in common carp from aquaculture are first recorded in Macedonia.

Due to the high number of parasites found and the pathological changes they cause, continuous follow-up of the condition with the presence of *Eudiplozoon nipponicum* in fish from aquaculture facilities is required.

Rasprostranjenost *Eudiplozoon nipponicum* (monogenea, diplozoidae) u gajenom običnom šaranu (*Cyprinus carpio*, l. 1758) iz objekata za akvakulturu u Makedoniji

Dijana Blažekovikj - Dimovska, Stojmir Stojanovski

Rezime

U okviru parazitološkog istraživanja, ispitano je ukupno 958 primeraka običnog šarana iz najznačajnijih i većih objekata akvakulture u Makedoniji, uključujući

ribnjake i rezervoare. *Eudiplozoon nipponicum* pronađen je na škrgama u 121 primerku običnog šarana u proleće, leto i jesen, sa prevalencijom od 10,67%, i srednjim intenzitetom od 2,55. Naši nalazi *E. nipponicum* kod običnog šarana iz akvakulture prvi put su zabeleženi u Makedoniji.

Ključne reči: diplozoon, paraziti, šaran, akvakultura

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FUNGAL CONTAMINATION OF PIG FARM FEEDS

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Abstract: The aim of this study was to establish the total fungal (mould) count in 79 commercial pig farm feed samples (42 samples for piglets, 29 samples for fattening pigs and 8 samples for sows) collected from the Serbian feed producers during the three-year period (2017–2019), as well as to evaluate the percentage of contamination samples by fungi, especially species from *Aspergillus*, *Fusarium* and *Penicillium* genera. Using standard mycological methods, the total count and identification of fungi were determined. Total fungal count was ranging from 1×10^2 to 1.41×10^5 cfu g^{-1} in the feed samples for piglets. Furthermore, in the feed samples for fattening pigs it ranged from 1×10^2 to 2.54×10^5 cfu g^{-1}, and from 1×10^2 to 1.93×10^5 cfu g^{-1} in the feed samples for sows. It has established the impermissible limit of total fungal count in 3.45% and 4.76% of a feed for fattening pigs and feed for piglets, respectively. Statistical analysis of the total number of fungi did not establish significant differences between the examined feed groups. *Fusarium* species were present in the most feed samples for fattening pigs (65.52%), followed by the feed for sows (62.50%) and piglets (47.62%). *Aspergillus* species were determined in 59.52, 58.62 and 37.50% feed samples for piglets, fattening pigs and sows, respectively. *Penicillium* species contaminated the lowest percentage of feed samples for fattening pigs (27.59%) and the highest percentage of feed samples for sows (37.50%). In a small number of samples *Alternaria*, *Mucor,* and *Rhizopus* species were identified. Based on these results, it can be concluded that the investigation of fungal contamination is an important indicator of a hygienic condition of feed intended for the nutrition of farm pigs. In addition, the percentage of fungal contamination of the examined samples indicates a potentially high risk to animal health. Due to that, a regular mycological evaluation is necessary to assess nutritional quality as one of the fundamental criteria for feed safety.

Key words: pig farm feed, total fungal count, fungal species

Introduction

Cereal grains, as the main component of the feed mixture, are an excellent substrate for development of fungi (moulds). Fungal species from genera *Aspergillus*, *Fusarium* and *Penicillium* and their secondary metabolites (mycotoxins) are inevitable contaminants in cereals and feeds. These fungi produce mycotoxins causing adverse effects in animal health and production (*Harčárová et al., 2018; Chiotta et al., 2020*).

High moisture content and water activity influence negatively on microbial stability of grains and in association with high temperature contribute favorably to the fungal growth and mycotoxin synthesis (*Bakutis et al., 2006; Giorni et al., 2012*). It was reported that temperatures ranging from 25 to 35°C were optimal for *Aspergillus* species growth and toxin production. Unlike *Aspergillus* species, *Fusarium* species have ability to grow at wide temperature values, ranging from 4–30°C, but production of toxins does not occur until temperature reaches bellow 15°C (*Manstretta and Rossi, 2016; Mannaa and Kim, 2017*). *Fusarium* species growth was also influenced by moisture content which must be in the ranges of 5–25%, while water activity must be from 0.87–0.99 (*Gebremeskel et al., 2019; Camardo Leggieri, 2017*).

Striving to provide human and animal healthy diet, the implementation of feed safety strategy is a priority. Intensive livestock production has high demands for animal feed hygiene. Total fungal count in animal feed is one of the fundamental parameters of feed quality. The quality of feed and raw cereal grains has an impact on animal health and growth (*Biagi, 2009; Dänicke et al., 2007*). According to the Serbian *Regulation on the quality of animal feed*, the limit values for total fungal count in 1g of feed mixtures was determined and it ranges from 50 000 and 200 000 in 1g of the feed mixture for young animals and adults, respectively.

Since there is not much data in the literature on fungal contamination of pig feed, the main aim of this research was to evaluate the total fungal count and the percentage contamination of pig feed samples by fungi, especially with potentially toxigenic fungi from *Aspergillus*, *Fusarium* and *Penicillium* genera. Given that fungal contamination is common in animal feed, these investigations should provide valuable information of the risk assessment for these contaminants in feed produced for farm pigs.

Materials and Methods

A total of 79 commercial pig feed samples (42 samples for piglets, 29 samples for fattening pigs and 8 samples for sows) collected from different Serbian feed producers during three years (2017–2019) were examined by mycological

tests. The sample size was approximately 1 kg. The moisture content of examined samples was determined using a moisture analyzer (OHAUS MB35, Parsippany, NJ, USA).

Total fungal count was determined using the dilution method according to ISO 21527-2 (2008). To prepare an initial suspension (primary dilution), 20 g of sample was added to 180 mL 0.1% of peptone water broth (PWB) and homogenized for 10 minutes on an orbital shaker (GFL 3015, Germany). A series of dilutions (10^{-2}, 10^{-3} and 10^{-4}) was prepared by transffering 1 mL of primary dilution (10^{-1}) into 9 mL of PWB. From prepared dilutions, 1 mL of suspension was transferred by sterile pipette and spread over the surface of Dichloran Glycerol agar (DG18) in Petri plates (Ø90) which were then kept in an incubator (Memmert, Germany) at 25ºC for 5 days. Total fungal count was expressed as colony-forming units per gram of sample (cfu g^{-1}).

Based on macroscopic and microscopic observations, the determination of fungal genera was done according to the descriptions of *Watanabe (2002*). The results were shown as the percentage of samples contaminated with fungal species per examined feed group.

Statistical data were analysed with the non-parametric test, independent-samples Kruskal-Wallis Test, using the SPSS software (IBM, Statistic 20). The correlation among individual values for total fungal count and the moisture content was determined using the Pearson correlation coefficient.

Results and Discussions

The total fungal count and fungal contamination of pig farm samples were studied. These parameters are good indicators of feed quality and essential for feed safety management. Constant evaluations of hygienic feed quality can reduce detrimental health effects in animals.

According to Serbian *Regulation on the quality of animal feed*, the total fungal count that has been recorded were above the permissible limit for older (2×10^5 cfu g^{-1}; 5.3 log10cfu g^{-1}) and younger categories of pigs (5×10^4 cfu g^{-1}; 4.7 log10cfu g^{-1}) in 3.45% and none of the feed samples for fattening pigs and sows, respectively and in 4.76% of the feed samples for piglets (Table 1).

Total fungal count was ranging from 1×10^2 (2 log10cfu g^{-1}) to 1.41×10^5 cfu g^{-1} (5.15 log10cfu g^{-1}) in the feed samples for piglets, from 1×10^2 (2 log10cfu g^{-1}) to 2.54×10^5 cfu g^{-1} (5.40 log10cfu g^{-1}) in the feed samples for fattening pigs and from 1×10^2 (2 log10cfu g^{-1}) to 1.93×10^5 cfu g^{-1} (5.29 log10cfu g^{-1}) in the feed samples for sows. The mean moisture content was 10.60, 13.38 and 9.79% in feed samples for piglets, fattening pigs and sows, respectively. Regarding total fungal count, there was no statistically significant difference between examined groups of pig farm feed samples (Table 2).

Table 1. Percentage of contaminated pig feed samples with total fungal count above the regulation limits

Total fungal count		Percentage (%) / Number of samples		
cfu g^{-1}	log_{10}cfu g^{-1}	Feed for piglets	Feed for fattening pigs	Feed for sows
$> 2 \times 10^5$	> 5.3	0/42	3.45/29	0/8
$> 5 \times 10^4$	> 4.7	4.76/42	24.14/29	25/8

*Colony forming units per g of sample

Table 2. Median, minimum and maximum total fungal count and mean moisture content in examined pig feed samples

Item	Total fungal count (cfu g^{-1} / log_{10}cfu g^{-1})			Mean moisture content (%)
	Median	Minimum	Maximum	
Feed for piglets [a]	6×10^2/ 2.78	1×10^2 / 2	1.41×10^5 / 5.15	10.60
Feed for fattening pigs [b]	2.10×10^2 / 3.32	1×10^2 / 2	2.54×10^5 / 5.40	13.38
Feed for sows	2.80×10^2/ 3.45	1×10^2 / 2	1.93×10^5 / 5.29	9.79
Level of significance	ns	-	-	-

[a] Animals from 15 to 25 kg; [b] Animals from 30 to 90 kg; ns - not significant at $P \geq 0.05$

In this study, considering mean moisture contents, the highest moisture content was in the samples of feed for fattening pigs (13.38%), followed by feed for piglets (10.60%) and sows (9.79%). Likewise, the maximum value of total fungal count (2.54×10^5 cfu g^{-1}; 5.40 log_{10}cfu g^{-1}) was in samples of feed for fattening pigs, feed group with the highest mean moisture content (Table 2).

The recommendation of maximum limited moisture content in cereal grains (a main component of feed) as good storage practice is 14.5% (*Fleurat-Lessard, 2015*). However, in stored grains, fungal development at lower moisture ($a_w \leq 0.70$) was found (*Magan et al., 2003*). In addition to the moisture content as a key factor for fungal growth on pre- and post-harvest grains, environmental relative humidity and temperature during storage are also important factors (*Ezekiel et al., 2020*). *Kukier and Kwiatek (2011)* have also reported that total fungal count in cereal grains was strongly dependent on weather conditions during the growing season.

Analogous to our results, *Milićević et al. (2010)* have established total fungal count ranging from 1×10^5 to 40×10^5 cfu g^{-1} in 18 pig feed samples collected from different provinces in Serbia, of which 39% samples exceeded the limit according to the Serbian legislation. They have also reported that with the storage period of pig feed extending, the moisture content is increasing. Similarly, by analysing 756 pig and poultry feed samples from Serbia over a ten-year period (1995–2004), *Marković et al. (2005)* have detected fungal count above the permissible limit in 35.1% feed samples for young animals and in 7.54% of feed samples for adults. These authors have pointed out that the high number of

contaminated feed samples was a consequence of inadequate environmental conditions (temperature and humidity levels) during storage. Furthermore, in the mycological analysis of pig feed samples from northwest of Croatia, *Pleadin et al. (2012)* have determined the total number of fungi ranged from 1×10^3 to 1×10^5 cfu g^{-1}. Then, *Almeida et al. (2009)* have established the mean number of fungi of 6.6×10^2 cfu g^{-1} (range from 2.7×10^1 cfu g^{-1} to 2.7×10^3 cfu g^{-1}) in pig feed samples from Portugal. In Argentina, *Pereyra et al. (2011)* analysed mycobiota contamination in 90 samples of raw materials (milled maize, whole soybean, wheat bran and soybean pellets) and finished feed for fattening pigs (suckling pig, piglet, weaner, growing and boar). It has been observed that high temperatures affected a greater amount of total fungal count in milled maize and all finished feed samples.

In this study, the examined pig farm feed samples were contaminated by fungal species that mainly belong to *Fusarium*, *Aspergillus* and *Penicillium* genera. *Fusarium* species contaminated the most feed samples for fattening pigs (65.52%) and the least samples for piglets (47.62%). *Aspergillus* species were identified in the most feed samples for piglets (59.52%), followed by feed samples for fattening pigs (58.62%) and sows (37.50%). The least percentage of examined feed samples was contaminated with *Penicillium* species ranging from 27.59% of feed for fattening pigs to 37.50% of feed for sows (Table 3). In addition to potentially toxigenic fungi from *Fusarium*, *Aspergillus* and *Penicillium* genera, *Alternaria*, *Mucor* and *Rhizopus* species were also identified but in a small percentage of examined samples (data not presented).

Table 3. The percentage of contaminated pig feed samples with *Aspergillus*, *Fusarium* and *Penicillium* species

Fungal species	Percentage of contaminated samples (%) / Number of samples		
	Feed for piglets	Feed for fattening pigs	Feed for sows
Aspergillus spp.	59.52/42	58.62/29	37.50/8
Fusarium spp.	47.62/42	65.52/29	62.50/8
Penicillium spp.	28.57/42	27.59/29	37.50/8

Contrary to our results, *Milićević et al. (2010)* registered the most number of pig feed samples contaminated with *Penicillium* species (94.4%). *Fusarium* species were present in 55.5% and *Aspergillus* species in 22% of pig feed samples. Other fungi from *Alternaria* and *Mucor* genera were represented in a smaller amount, while *Paecilomyces* spp. were registered in 44.4% of pig feed samples (*Milićević et al., 2010*). Similar to that, *Marković et al. (2005)* and *Pleadin et al. (2012)* have also reported that *Penicillium* species were the most common in analysed feed samples. Furthermore, according to the reports of *Almeida et al. (2009)* in Portugal and *Pereyra et al. (2011)* in Argentina, *Fusarium*, *Aspergillus* and *Penicillium* were also the prevalent fungal genera in commercial pig feeds,

while other fungi belonged to *Phoma, Rhizopus* and *Paecilomyces* genera (*Almeida et al., 2009).*

Moderate positive correlations were found between the total fungal count and moisture content, in the feed samples for piglets (r = 0.50) and fattening pigs (r = 0.40). A fairly strong negative correlation was observed between the total fungal count in feed for sows and moisture content (r = -0.79) (data not presented). Fungal growth is affected by many abiotic factors, such as moisture, temperature, nutrient availability, oxygen and pH. Of these, temperature and especially moisture are considered as the most significant factors for fungal proliferation. It was expected that the total fungal count increases with higher moisture content. However, some fungal species require a certain limit of moisture content for optimal growth *(Christensen and Kaufmann, 1965).* In addition, a weak positive correlation between feed microbial count and moisture content was found by *Vlachou et al. (2004).*

Conclusion

Based on obtained results in this study, the total fungal count was above the permissible limit in 3.45% and 4.76% feed for fattening pig and piglet samples, respectively. Potentially toxigenic fungi from *Fusarium, Aspergillus* and *Penicillium* genera were identified in the most samples. The most of feed samples for fattening pig (65.52%) were contaminated by *Fusarium* species, while *Aspergillus* and *Penicillium* species contaminated the most samples of piglets (59.52%) and sows (37.50%) respectively. A small number of examined pig farm feed samples were contaminated with *Alternaria, Mucor* and *Rhizopus* species.

These results indicate a potential risk in animal health, but also potential reduction in animal production. Hence, continuous mycological analysis of animal feed and risk assessment of fungal contaminants must be basic measures in feed safety strategy. Since cereals are the main components of animal feed, field and storage monitoring of grains/seeds is also a basic measure to reduce fungal contamination in the food/feed chain. Measures such as moisture content reduction, maintenance of low temperature, ventilation and fumigation should be used in the storage of cereal grains. During storage, additionally, adsorbents may reduce the incidence of fungal contaminants.

Kontaminacija hrane za farmske svinje gljivama

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Rezime

Cilj ovog istraživanja bio je da se utvrdi ukupan broj gljiva (plesni) u 79 komercijalnih uzoraka hrane za farmske svinje: za prasad (42 uzoraka), tovne svinje (29 uzoraka) i krmače (8 uzoraka), sakupljenih od različitih proizvođača stočne hrane u Srbiji tokom trogodišnjeg perioda (2017–2019), kao i da se oceni procenat kontaminiranosti uzoraka gljivama, posebno vrstama iz rodova *Aspergillus*, *Fusarium* and *Penicillium*. Primenom standardnih mikoloških metoda određen je ukupan broj i identifikacija gljiva.
Ukupan broj gljiva bio je u rangu od 1×10^2 do 1.41×10^5 cfu g^{-1} u uzorcima hrane za prasad, od 1×10^2 do 2.54×10^5 cfu g^{-1} u uzorcima hrane za tovne svinje i od 1×10^2 do 1.93×10^5 cfu g^{-1} u uzorcima hrane za krmače. Ustanovljen je nedozvoljen ukupan broj gljiva u 3,45% uzoraka hrane za tovne svinje i u 4,76% uzoraka hrane za prasad. Statističkom analizom ukupnog broja gljiva nisu utvrđene značajne razlike između ispitivanih grupa hrane.
Fusarium vrste bile su prisutne u najvećem broju uzoraka hrane za tovne svinje (65,52%), zatim u uzorcima hrane za krmače (62,50%) i prasad (47,62%). *Aspergillus* vrste bile su prisutne u 59,52% uzoraka hrane za prasad, 58,62% uzoraka hrane za tovne svinje i 37,50% uzoraka hrane za krmače. *Penicillium* vrste su kontaminirale najmanji broj uzoraka hrane za tovne svinje (27,59%) a najveći broj uzoraka hrane za krmače (37,50%). U malom broju uzoraka identifikovane su *Alternaria*, *Mucor*, and *Rhizopus* vrste.
Na osnovu dobijenih rezultata može se zaključiti da je ukupan broj gljiva odličan pokazatelj higijenske ispravnosti hrane za farmske svinje. Pored toga, procenat kontaminiranih uzoraka gljivama ukazuje na potencijalno visok rizik za zdravlje životinja. Stoga, redovna mikološka analiza je neophodna za ocenu nutritivnog kvaliteta kao jednog od osnovnih kriterijuma za bezbednost hrane za životinje.

Ključne reči: hrana za farmske svinje, ukupan broj gljiva, vrste gljiva

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TOXIC ELEMENTS IN SERBIAN SUNFLOWER HONEY ORIGINATING FROM VARIOUS REGIONS

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Abstract: The concentrations of As, Cd, Hg, Ni and Pb were determined in fifteen sunflower honey samples collected from 9 locations in the Republic of Serbia during 2019. The elements were analysed using inductively coupled plasma mass spectrometry (ICP-MS). Mean levels of elements (mg kg^{-1}) in all sunflower honey samples were as follows: 0.004 for As, 0.003 for Cd, 0.077 for Ni and 0.051 for Pb. The mercury content in all honey samples was below the detection limit of the applied method (< 0.001 mg Hg kg^{-1} honey). The lead level in all the examined honey samples was below maximum permissible value (0.10 mg kg^{-1}). The highest values of elements (mg kg^{-1}) were: 0.096 for Pb (in Kanjiža), 0.025 for As (Kikinda sample), 0.008 for Cd (Senta) and 0.125 for Ni in the honey originating from Svrljig. None of the 5 toxic elements analysed exceeded the maximum permissible level.

Key words: sunflower honey, toxic elements

Introduction

Honey is a foodstuff with nutritional, sensorial and potentially therapeutic properties (*Przybyłowski and Wilczyńska, 2001; Bilandžić et al., 2011; Sergalio et al., 2019*). These properties are related to the chemical composition of honey. As food, honey must be free from contaminants. Honey contains about 200 substances, mainly sugars such as glucose, fructose and sucrose. It also contains, but in much smaller quantities, proteins, organic acids, vitamins, minerals, pigments, phenolic compounds, volatile compounds, and solid particles derived from honey harvesting (*Bogdanov et al., 2008; Da Silva et al., 2016*). Mineral concentrations in honey depend on the botanical origin, climate conditions, but also significantly on geographical origin and type of soil where plant grows (*Bilić-Šobot, 2020; Živkov Baloš et al., 2018; Lazarević et al., 2017; Uršulin-Trstenjak et al., 2015).* Mineral content of honey contributes to the colour of honey. Darker honey types are richer

in minerals. Black locust and sunflower honey are characterized by low concentrations of ash and minerals, compared to meadow, chestnut and honeydew honey (*Lasić et al., 2018; Dhahir and Hemed, 2015; Uršulin-Trstenjak et al., 2015*).

Honey can be a useful indicator of environmental pollution *(Đogo Mračević et al., 2020; Sergalio et al., 2019; Lazarević et al., 2017; Moniruzzaman et al., 2014; Bilandžić et al., 2011).* Honeybees may be exposed to toxic elements pollution (arsenic, mercury, lead, cadmium, nickel) in an area of around 7 km^2 surrounding the hives (*Đogo Mračević et al., 2020*). During foraging, bees are exposed to pollutants. Their hairy bodies can gather various particles from the atmosphere or they may be exposed to contaminated pollen or water *(Porrini et al., 2003; Lambert et al., 2012; Costa et al., 2019).* Contamination of honey by toxic elements may be a result of industrial development, urbanization and transport (*Hamad et al., 2020; Tutun et al., 2019; Lambert et al., 2012; Bilandžić et al., 2011).* In addition to the listed sources, contamination of honey may be caused by incorrect procedures applied during harvesting, fumigation, extraction and processing, storage and conservation phases (*Bartha et al., 2020*).

Sunflower honey is traditional honey with exceptional healing properties and nutritional value (*Sari and Ayyildiz, 2012*). Sunflower is cultivated in the southern regions, with abundant sunshine and where the climate is favourable for growing this plant. It is important to point out that the literature clearly shows that sunflower can accumulate high concentrations of toxic elements (As, Pb, Cu, Cd, Ni, Cr, Co), mainly in shoots and roots (*Dhiman et al., 2017; Stoikou et al., 2017; Angelova et al., 2016; Garcia et al., 2006*). Since growing sunflower plants has considerable potential to accumulate toxic elements contaminants, they are considered “hyperaccumulators” of toxic elements (*Dhiman et al., 2017*).

In Serbia, honey production is well-developed thanks to the suitable climate and geographic location, so sunflower honey is one of the most commonly produced kind of honey (*Živkov Baloš et al., 2021*). There is very little data on toxic elements in sunflower honey available in the literature, so the purpose of this study was to determine concentrations of trace (toxic) elements (As, Cd, Hg, Ni and Pb) in sunflower honey in order to obtain information about honey safety.

Material and Methods

Samples: A total of 15 samples of sunflower honey harvested in 2019 were collected from beekeepers in various regions of the Republic of Serbia. The sampling included locations in the following municipalities: Kanjiža (4 samples), Kikinda (2 samples), Čelarevo (4 samples), Sremska Mitrovica (1 sample), Senta (1 sample), Žabalj (1 sample), Osečina (1 sample) and Svrljig (1 sample). All the collected samples were in their original packaging (jars) and were transferred to the

laboratory of Scientific Veterinary Institute "Novi Sad" for examination. Manufacturers used field observations for botanical origin determination. Honey analyses were carried out immediately after sampling. The honey analysis procedures were performed in two replicates.

Sample preparation: The samples (1 g) were prepared applying the microwave (Ethos, Labstation Microwave, Milestone), digestion method (14) with the use of the mixture H_2O_2/HNO_3 (1:4, v/v). After this process, the samples were transferred to 50 mL volumetric flasks and diluted with deionized water.

Determination of elements: The contents of Pb (NoG-M, IT 0.1 s/P), Cd (NoG-M, IT 1 s/P), As (He-M, IT 1 s/P), Ni (He-M, IT 1s/P) and Hg (NoG-M, IT 1 s/P) were analyzed by inductively coupled plasma mass spectometry (ICP-MS) on ICP-MS 7700 mass spectrometer (Agilent Technologies). Solutions used for calibration were prepared from commercial stock standard solutions with 1000 mg/l of each element (Accustandard). To calculate the recovery percentage, 6 samples has been spiked with known amounts of Cd, As, Hg, Ni and Pb analytical standards. Obtained results are presented in Table1.

Table 1. Isotopes, limit of detection (LOD) and recovery rates for monitored elements

Element	Isotope	LOD (mg kg^{-1})	Recovery (%)
Cd	^{111}Cd	0.001	96.1
As	^{75}As	0.001	100.4
Hg	^{201}Hg	0.001	83.7
Pb	^{208}Pb	0.001	88.1
Ni	^{60}Ni	0.001	92.6

Data analysis: All the calculations and statistical analyses were performed using the PAST software package, version 2.12, Oslo, Norway. The data were grouped according to the samples of sunflower honey and presented as mean, standard deviation, minimum, and maximum values.

Results and Discussion

Table 2 shows the values of toxic elements detected in sunflower honey samples. The obtained results were compared with the literature data presenting the highest and lowest mean values of investigated elements.

In the present study, lead concentrations ranged from 0.002 to 0.096 mg kg^{-1} with a mean value of 0.051 mg kg^{-1} in fifteen sunflower honey samples from all the examined locations. The highest Pb level was 0.096 mg kg^{-1} found in the sample from Kanjiža, and the lowest was 0.002 mg kg^{-1} in the sample from Čelarevo. The average lead level found in our investigation was similar to the levels found in honey samples from Croatia (65.2 µg kg^{-1}; reported by *Bilandžić et al., (2011)* and 0.02-0.11 mg kg^{-1}; reported by *Uršulin-Trstenjak et al., (2015)),*

Romania (51.674 µg kg^{-1}; *Oroian et al., 2016*) and Poland (0.048 mg kg^{-1}; *Przybyłowski and Wilczyńska, 2001*). However, the Pb concentrations were lower than those found in sunflower and other types of honey from Turkey (with maximum content of 0.48 mg g^{-1}*; Citak et al., 2012*), (0.80 mg kg^{-1}; *Tutun et al., 2019*), Iran (507.58 µg kg^{-1}; *Aghamirlou et al., 2015*), Iraq (0.108 – 0.820 mg kg^{-1}; *Dhahir and Hemed, 2015*), Malaysia (0.36 mg kg^{-1}; *Moniruzzaman et al., 2014*), and from polluted areas in Egypt (*Hamad et al., 2020*) and Romania *(Bartha et al., 2020).* In the current study, the average lead level was higher than those reported in other studies carried out in our country and Croatia, Malaysia, France, and Saudi Arabia (*Đogo Mračević et al., 2020; Lasić et al., 2018; Bilandžić et al., 2017; Chua et al., 2012; Lambert et al., 2012; Aljedani, 2017*).

Table 2. Toxic elements content in sunflower honey from different location in Serbia

Location	Pb	As	Hg	Cd	Ni
	mg kg^{-1}				
Kanjiža	0.033	0.005	<0.001	0.002	0.053
Kanjiža	0.028	0.004	<0.001	0.002	0.058
Kanjiža	0.096	0.005	<0.001	0.001	0.045
Kanjža	0.008	0.003	<0.001	0.002	0.048
Sr. Mitrovica	0.034	0.004	<0.001	0.002	0.099
Kikinda	0.063	0.025	<0.001	0.001	0.057
Kikinda	0.016	0.004	<0.001	0.003	0.078
Senta	0.089	0.005	<0.001	0.008	0.095
Osečina	0.040	0.005	<0.001	0.005	0.071
Čelarevo	0.046	0.004	<0.001	0.005	0.069
Čelarevo	0.043	0.005	<0.001	0.004	0.094
Čelarevo	0.002	0.003	<0.001	0.002	0.053
Čelarevo	0.081	0.005	<0.001	0.005	0.123
Žabalj	0.085	0.004	<0.001	0.004	0.093
Svrljig	0.094	0.004	<0.001	0.003	0.125
Mean value	0.051	0.006	< LOD	0.003	0.077
Standard deviation	0.032	0.005		0.002	0.026
Minimum	0.002	0.003		0.001	0.045
Maximum	0.096	0.025		0.008	0.125

LOD- limit of detection

Lead is a natural component of the biogeosphere. It enters the environment from metal smelters, coal-fired power plants, from sewage sludge, waste oil, or is a result of solid waste combustion. However, the dominant anthropogenic emission of Pb in the environment is the result of the use of organo-lead compounds - additives in the oil industry. The lead used in automobile fuel was forbidden a few years ago. However, air and water contamination is still high (Lambert et al., 2012). Lead is one of most widespread contaminants in the environment, and its

content is examined in all environmental studies (*Bilandžić et al., 2011*). Lead is the only metal whose maximum content in honey is limited by regulations. The maximum permissible value of lead is prescribed by national regulation on maximum concentrations of certain contaminants in food (Official Gazette, 81/2019). This regulation is harmonized with the European regulation (*Commission Regulation, 1005/2015*). Maximum permissible value is set at 0.10 mg of Pb/kg for honey. The lead content in all the examined honey samples was below 0.10 mg kg^{-1}. However, lead content in some samples (Kanjiža, Senta, Svrljig) was very close to the maximum permissible value (0.096, 0.089 and 0.094 mg kg^{-1}). Higher concentrations of Pb in sunflower honey samples from these three locations may be the result of the location of hives in the areas, as they were near roads, industrial or building sites.

Arsenic levels ranged from 0.003 to 0.025 mg kg^{-1} and mean content in all honey samples was 0.006 mg kg^{-1}. The highest As level was 0.025 mg kg^{-1} in the sample from Kikinda, while the lowest was 0.003 mg kg^{-1} in the samples from Kanjiža and Čelarevo. In comparison with the levels found in the literature, mean As level was higher than the values found in Malaysia (< LOD; Chua et al., 2012) and Iran (< LOD; *Aghamirlou et al., 2015*). Similar results for As in honey were reported in Croatia (1.97 μg kg^{-1} reported by *Bilandžić et al., (2011*) and 0.62 – 6.95 μg kg^{-1} reported by *Bilandžić et al., (2017))* and Romania (3.49 μg kg^{-1}; *Oroian et al., 2016*). Arsenic (As) is a common contaminant, found both naturally and as a result of human activity. Industrially produced arsenic mostly originates from agricultural products such as insecticides, herbicides, fungicides, algicides, wood preservatives, and growth stimulators for plants and animals. The use of pesticides containing arsenic and other chemical products in agriculture results in arsenic accumulation in soil and plants. Consequently, arsenic is usually found as a trace element in both food and feed (*Roy and Saha, 2002; Mandal, 2017*). Natural distribution of As is associated with igneous and sedimentary rocks. High As concentrations in groundwater and drinking water are registered throughout Pannonian Basin (*Kristoforović-Ilić et al., 2009; Kostić et al., 2016; Senila et al., 2017).* Most of As-contaminated areas in Vojvodina are in the region of alluvial formation along the banks of the rivers Danube and Tisa, and confluent rivers Zlatica, Begej, Tamiš and Nera (*Kristoforović-Ilić et al., 2009*). Taking into account these facts, it can be assumed that the high concentration of As in the sample from the area of Kikinda (0.025 mg kg^{-1}) is related to naturally contaminated groundwaters.

The presence of any form of mercury is considered undesirable and dangerous in the natural environment (*Dobrowolska and Melosik, 2002*). The source of mercury soil contamination are mineral fertilizers, fungicides and disinfectants in agriculture, as well as the use of waste sludge to fertilize arable land. Emitters of mercury in the atmosphere are metal smelters, burning fossil fuels and burning waste material. There is almost no literature data on mercury

content in honey. In the present study, the content of Hg was below the limit of detection by ICP MS method (< 0.001 mg kg^{-1}). *Bilandžić et al. (2011*) reported that mean Hg content in honey from Croatia was 2.72 µg kg^{-1} honey, while *Oroian et al. (2016)* found 0.73 µg Hg/kg in honey samples from Romania.

The concentration of cadmium in the environment increases significantly due to the industrial production of plastics, dry batteries, paints and other products that contain this element, and also through phosphate fertilizers that contain significant amounts of Cd (*Satarug et al., 2003*). The mean cadmium content in the tested sunflower samples was 0.003 mg kg^{-1} (0.001 to 0.008 mg kg^{-1}). These results were similar to those from Croatia (with mean Cd amounting 0.005 mg kg^{-1} reported by *Lasić et al. (2018*) and ranging from 0.003 to 0.011 mg kg^{-1} reported by *Uršulin-Trstenjak et al., (2015))* and Romania (1.19 µg kg^{-1}; *Oroian et al., 2016*). Mean Cd level in sunflower samples was higher than the levels found in Malaysia (< LOD; *Chua et al., 2012*) and Saudi Arabia (< LOD; *Aljedani, 2017*). Cadmium concentrations in this study were lower than those found in honey samples from Egypt (0.01 - 0.03 mg kg^{-1}; *Hamad et al., 2020*), Iran (27.62 µg kg^{-1}; *Aghamirlou et al., 2015),* Iraq (0.210 – 0.894 mg kg^{-1}; *Dhahir and Hemed, 2015*), and Malaysia (0.35 mg kg^{-1}; *Moniruzzaman et al., 2014*). *Bartha et al. (2020)* have found very high concentrations of Cd (0.05 – 3.81 mg kg^{-1}) in polyfloral honey from polluted areas in Romania.

The mean nickel content in the tested sunflower honey samples was 0.077 mg kg^{-1} and the range of concentration was from 0.045 to 0.125 mg kg^{-1}. The highest Ni level was 0.125 mg kg^{-1} in the sample from Svrljig, while the lowest amounted 0.045 mg kg^{-1} in the sample from Kanjiža. Similar to cadmium, Ni concentration in our study was higher than the levels found in Malaysia (< LOD; Chua et al., 2012), Turkey (mean 0.05 mg kg^{-1}) and Saudi Arabia (< LOD; *Aljedani, 2017*). The authors from Croatia (*Uršulin-Trstenjak et al., 2015*), Iraq (*Dhahir and Hemed, 2015*), Egypt (*Hamad et al., 2020*), Iran (*Aghamirlou et al., 2015*) and Romania (*Oroian et al., 2016*) have found higher Ni concentrations in honey samples (ranging between 0.09 and 1.86 mg kg^{-1}; ranging between 0.117 and 0.440 mg kg^{-1}; ranging between 0.24 and 1.29 mg kg^{-1}; with mean value of 651.78 µg kg^{-1}; mean 122 µg kg^{-1}, respectively), in comparison to the results of our study. Similar results for Ni in honey were reported from Turkey (< LOD - 9.86 µg kg^{-1}; *Citak et al., 2012*). Nickel is an essential and toxic element for humans, animals, plants and microorganisms. In the nature, this element is found in various forms, and is widely used in metallurgy, chemical and food industries, especially as a catalyst and pigment. Nickel has been studied more as a toxic element. High concentrations of Ni can cause allergies, cancer and non-malignant diseases of the respiratory tract. It has a toxic effect on the immune system. Also, nickel can interfere with DNA repair and lead to the production of free radicals, which causes an increase in the degree of lipid peroxidation and protein degradation (*Bangyuan et al., 2013).*

The data obtained in this research were also compared with the data from our previous research. In this study, a total of 40 samples of multi and polyfloral honey were collected from various localities in Serbia. The concentrations of lead and cadmium were in the range between 0.009 and 3.26 mg Pb kg^{-1}, and < LOD to 0.235 mg Cd kg^{-1} (*Mihaljev et al., 2001*). Since the research did not include certain locations, it can be concluded that the concentrations of lead and cadmium examined in this study are generally lower.

Conclusion

In this study, the mean concentrations of elements were measured in 15 sunflower honey samples from various locations in Serbia and they were decreasing in following order: Pb > Ni > As > Cd > Hg. The lead content in all the examined sunflower honey samples was below maximum permissible value. Regarding the fact that sunflower accumulates large amounts of metals in its tissues, the concentrations of toxic elements obtained in sunflower honey in our study are in line with the literature data on the concentrations of these elements in the honey originating from different botanical and geographical areas. In comparison to the data on sunflower honey from other countries, the concentrations of the examined elements in sunflower honey from Serbia are generally lower.

The obtained results are useful for improving the quality of honey production chain. Beekeepers should choose the location of their hives with caution. The procedures applied during the production and processing of honey should be in accordance with hygiene standards. It is very important to monitor the levels of elements in terms of their toxicity and because they can enter through root system or leaf surface of plants and thus access nectar.

Toksični elementi u suncokretovom medu sa različitih lokacija u Republici Srbiji

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Rezime

Cilj ovog istraživanja bio je dobijanje podataka o sadržaju toksičnih elemenata - As, Cd, Hg, Ni i Pb - u petnaest uzoraka suncokretovog meda koji su prikupljeni sa 9 lokacija u Republici Srbiji tokom 2019. godine. Koncemtracije ispitivanih

elemenata su su dobijene primenom induktivno kuplovane plazme sa masenom detekcijom (ICP-MS). Srednje vrednosti koncentracija elemenata (mg kg^{-1}) u svim uzorcima suncokretovog meda bile su: 0,004 za As, 0,003 za Cd, 0,077 za Ni i 0,051 za Pb. Sadržaj žive u svim uzorcima meda bio je ispod granice detekcije primenjene metode (<0,001 mg Hg kg^{-1} meda). Sadržaj olova u svim ispitivanim uzorcima meda bio je ispod maksimalno dozvoljene vrednosti (0,10 mg kg^{-1}). Najviši nivoi elemenata bili su (mg kg^{-1}): za Pb 0,096 (Kanjiža), za As 0,025 (Kikinda), za Cd 0,008 (Senta) i za Ni 0,125 (Svrljig). Nijedan od analiziranih toksičnih elemenata nije premašio maksimalno dozvoljeni nivo.

Ključne reči: suncokretov med, toksični elementi

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Authors must follow the instructions for authors strictly, failing which the manuscripts would be rejected without review.

The manuscript should be prepared in Microsoft Word for Windows, maximum 8 pages of typed text using, Paper size: Custom size, Width 17 cm, Height 24 cm; format (Portrait), normal spacing (Single Space). Margins: Top 2.0 cm, 2.0 cm Left, Bottom 2.0 cm, 2.0 cm Right, no pagination.

Use font Times New Roman, size 11 (except where it is stated otherwise), single space, justify

Title of the paper should be Times New Roman, font size 14, bold, capital letters, justify

Authors – Times New Roman, font size 12, bold, specify the full names of all authors on the paper. Use 1,2, … numbers in suffix to refer to addresses of authors, only in the case of different affiliations (institution)

Affiliations of authors – Times New Roman, font size 9, normal, under affiliations of authors should be mentioned e-mail of corresponding author and after that category of paper.

Example 1

POTENTIALS OF SERBIAN LIVESTOCK PRODUCTION – OUTLOOK AND FUTURE

Milan M. Petrović[1], Stevica Aleksić[1], Milan P. Petrović[1], Milica Petrović[2], Vlada Pantelić[1], Željko Novaković[1], Dragana Ružić-Muslić[1]

[1]Institute for Animal Husbandry, Belgrade – Zemun, 11080 Zemun, Serbia
[2]University of Belgrade, Faculty of Agriculture, Nemanjina 6, 11080 Zemun, Serbia
Corresponding author: Milan M.Petrović, **e-mail address**
Review paper

Example 2

EFFECTS OF REARING SYSTEM AND BODY WEIGHT OF REDBRO BROILERS ON THE FREQUENCY AND SEVERITY OF FOOTPAD DERMATITIS

Zdenka Škrbić, Zlatica Pavlovski, Miloš Lukić, Veselin Petričević

Institute for Animal Husbandry, Autoput 16, 11080 Belgrade, Serbia
Corresponding author: Zdenka Škrbić, **e-mail address**
Original scientific paper

Original scientific paper should contain following paragraphs with single spacing (title of paragraphs should be in Times New Roman 14 **bold**, except for **Abstract** and **Key words** where font size is 11 **bold**):

Abstract: up to 250 words, Times New Roman, font size 11, justify. Abstract should contain a brief overview of the methods and the most important results of the work without giving reference. Abstract submitted in English language.

Key words: not more than 6. The selection carried out by relying on widely accepted international source such as a list of keywords Web of Science.

Introduction – present the review of previous research and objective of the paper.

Materials and Methods – state methods applied in the paper; experimental research design. Use SI system of measurement units.

Results and Discussion – present investigation results separately from discussion or together in one paragraph. Presentation of the results should be precise and without repetitions, and include the evaluation of significant differences and other parameters.

Text and titles of tables, figures and graphs, Times New Roman, font size 9, **bold**, in the following form:

Table 1. Least square means for the reproductive traits of cows

Tables and figures should be numbered and with adequate title and legend, width and height not exceeding 12 cm and 17 cm, respectively. Tables should be prepared according to instruction for forming of tables in Office Word. Each column in table must have heading and, when necessary, abbreviations should be explained in the legend/footnote.

Conclusion – containing the most important issues of the paper

After Conclusion the title of the paper in Serbian in Times New Roman 14 **bold**, is stated, followed by authors in Times New Roman 11 *italic*, example:

Potencijali srpske stočarske proizvodnje – izgledi i budućnost

Milan M. Petrović, Stevica Aleksić, Milan P.Petrović, Milica Petrović, Vlada Pantelić, Željko Novaković, Dragana Ružić-Muslić

Summary – in Serbian language, 250 max. words (non-Serbian authors should provide Summary in English language that will be translated to Serbian by Editor's office)

Key words: not more than 6 (in Serbian language)

Acknowledgment – for example:

Research was financed by the Ministry of Science and Technological Development, Republic of Serbia, project TR 6885.

References – should be in alphabetical order. Names of the authors must be given in capital letters followed by the year of publication in brackets, titles in the language of the original. Use only the full name of the journal.

In scientific journals:

PETROVIĆ M. M., SRETENOVIĆ LJ., BOGDANOVIĆ V., PERIŠIĆ P., ALEKSIĆ S., PANTELIĆ V., PETROVIĆ D. M., NOVAKOVIĆ Ž. (2009): Quantitative analysis of genetic improvement of milk production phenotypes in Simmental cows. Biotechnology in Animal Husbandry, 25,1-2, 45-51.

ŠKRBIĆ Z., PAVLOVSKI Z., LUKIĆ M. (2007): Uticaj dužine tova u različitim sistemima gajenja na klanične osobine brojlerskih pilića genotipa Redbro. Biotechnology in Animal Husbandry 23, 3-4, 67-74.

WEBB E., O'NEILL H. (2008): The animal fat paradox and meat quality. Meat Science, 80, 28-36.

PhD Thesis:

RUŽIĆ-MUSLIĆ D. (2006): Uticaj različitih izvora proteina u obroku na proizvodne rezultate jagnjadi u tovu. Doktorska disertacija. Univerzitet u Beogradu, Poljoprivredni fakultet.

CAETANO A.R. (1999): Comparative mapping of the horse (*Equss caballus*) genome by synteny assignment of type-I genes with a horse-mouse somatic cell hybrid panel. Ph.D. Dissertation, University of California, Davis.

In Scientific Books:

PETROVIĆ P.M (2000): Genetika i oplemenjivanje ovaca. Naučna knjiga, Beograd, pp365.

FITZGERALD M. (1994): Neurobiology of Fetal and Neonatal Pain. In: Textbook of Pain. 3rd edition. Eds Wall P. and Melzack R. Churchchill Livingstone, London, UK, 153-163.

At Scientific Meetings:

ŠKRBIĆ Z., LUKIĆ M., BOGOSAVLJEVIĆ-BOŠKOVIĆ S., RAKONJAC S., PETRIČEVIĆ V., DOSKOVIĆ V., STANOJKOVIĆ A. (2015): Importance of farm management in reducing broilers skin lesions. Proceedings of the 4th International Congress "New Perspectives and Challenges of Sustainable Livestock Production", October 7 – 9, Belgrade, 145-158.

Citations in the text are presented in italic form, examples: …results of *Petrović (2009)*; *Petrović et al. (2009)*; *Webb and O'Neill (2008)….*; *(Škrbić et al., 2015); (Ružić-Muslić, 2006); (Webb and O'Neill, 2008)*

Editor's office

13th International Symposium
"Modern Trends in Livestock Production"
6th – 8th October 2021, Belgrade, Serbia

Organizer

INSTITUTE FOR ANIMAL HUSBANDRY, BELGRADE-ZEMUN

e-mail: biotechnology.izs@gmail.com
website: www.istocar.bg.ac.rs

SECOND ANNOUNCEMENT

On behalf of the International Scientific and Organizing Committee, it is our pleasure to invite you to participate at the **13th International Symposium on Modern Trends in Livestock production**, which will be held **from 6th to 8th October 2021, in Belgrade**.

We invite you to take part with an oral or poster presentation. You also have the opportunity to present your institution or company at the Symposium.

At the Symposium, the experts from Serbia and abroad will present the results of their research in order to enable a better transfer of scientific achievements in all fields of animal husbandry and science and making them available to the scientists, researchers and practitioners in livestock production, as well as students, in the private sector and to the general public.

The aim of the scientific meeting is to establish better cooperation between researchers in the field of animal science from different institutions, and experts from the industry, trade and other related fields, as well as producers from Serbia, Western Balkans, EU and other parts of the world in the field of science, education and good livestock production practice.

MAIN TOPICS OF THE SYMPOSIUM

1. Breeding, Selection, Genetics, and Reproduction of Farm Animals
2. Nutrition of Farm Animals
3. Animal Welfare and Health Care
4. Organic Livestock Production
5. Technology and Quality of Animal Products
6. Protection of the Environment and Bidoversity in Animal Production
7. Livestock Production and Food Security in a Context of Climate Change
8. Livestock Feed and Ecology

OFFICIAL LANGUAGE

The official language of the Symposium is English.

REGISTRATION AND PAYMENTS

Registration and submission of abstracts and full papers to the e-mail address: biotechnology.izs@gmail.com The authors shall submit full papers prepared acording to the **Instruction for Authors** for scientific journal "Biotechnology in Animal Husbandry" (www.istocar.bg.ac.rs). All submitted papers will be peer reviewed. Accepted papers will be published in the Proceedings.

REGISTRATION FEE	**Before 30th June 2021 (Early registration)**	**After 30th June 2021 (Late registration)**
Registration Fee, covers publishing of paper, Symposium material, participation in all sessions of the Symposium, coffee/tea break	**80** €	**100** €
Registration Fee, covers publishing of paper, Symposium material, participation in all sessions of the Symposium, coffee/tea break, tourist program and gala dinner	**120** €	**150** €

The first author of the Invited paper does not pay Registration Fee

IMPORTANT DATES

Deadline for full paper submission **May, 31st 2021**

Request for Proforma invoice for Registration fee to the e-mail address: biotechnology.izs@gmail.com

Symposium participants from Serbia can make the payment (in RSD value on the day of payment according to the exchange rate), on the following account:

Institut za stočarstvo, Beograd-Zemun
11080 Zemun, Autoput 16
Tekući račun br. 205-65958-94
Komercijalna banka

INSTRUCTION FOR EUR PAYMENTS
AIK BANKA AD BEOGRAD

Please pay as per instruction given below:

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59: Beneficiary customer:	**RS35105050120000062319** **INSTITUT ZA STOČARSTVO ZEMUN** **Autoput Beograd-Zagreb 16** **Zemun** **REPUBLIKA SRBIJA**

ACCOMMODATION AND SYMPOSIUM LOCATION

The Symposium will be held in Hotel Tulip Inn Putnik Belgrade, Palmira Toljatija street 9, 11070, Belgrade, Serbia
(https://tulip-inn-putnik-belgrade.goldentulip.com/en-us/)

Single room at special rate of 55 € daily per room
Double room at special rate of 65 € daily per room
City tax is not included and is approximately 1.3 € per person daily.

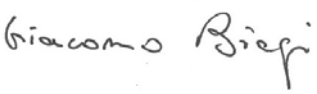
Accommodation at SPECIAL RATES is possible for reservations before **August, 31st 2021.**

Hotel reservation telephone: + 381112259999
Hotel reservation e-mail: info@tulipinnputnikbelgrade.com

On behalf of
Organizing Committee

Dr Čedomir Radović,
Senior Research Associate
Serbia

On behalf of
International Scientific Committee



Prof. Dr. Giacomo Biaggi
Italy