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THE EFFECT OF HERD SIZE ON DAIRY COWS' WELFARE QUALITY – PROVISION OF GOOD FEEDING AND HOUSING

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Original scientific paper

Abstract: In the last decades, there has been a trend present in the world to increase the size of dairy herds while increasing the yield of milk per head. In addition to environmental and economic benefits, this trend carries certain risks for the welfare of cows because in conditions of increased agglomeration of cattle the possibility of spreading of pathogens is also increased, there are less opportunities for adequate control and cows are exposed to greater selection and production stress. Research of the relationship between herd size and welfare quality parameters is still not sufficient to make relevant conclusions. Starting from that, the aim of this study, conducted in Serbia, is to examine the influence of herd size on parameters related to providing good feeding and housing conditions as important segments of the overall welfare of dairy cows. The assessment of given welfare parameters was done by *Welfare Quality® Assessment Protocol for Cattle (2009)* on 16 dairy farms of different herd sizes (large, medium and small) and housing management. The results indicate that there are significant variations in welfare indicators in each of the observed groups, which is why the size of the herd cannot be taken as a parameter that explicitly determines the quality of welfare. However, individual observation and comparison of welfare parameters between groups indicate that small herds in our production conditions could be identified as the greatest risks to the welfare of cows. In small herds, the highest share of cows of poor (4.62%) and fattened condition (8.76%) was found, as well as the lowest freedom of movement because cows on small farms are mostly reared in a tied system. Average values of indicators: lying down time (6.24s), frequency of collisions with equipment (13.25%) and high dirt contamination of cows (65.6-89.8%) further emphasize the issue of providing comfort in small herds.

Key words: dairy cows, welfare, herd size, feeding, housing, comfort

Introduction

The relationship between herd size and welfare quality has not been extensively investigated in the past. Based on a review of the available literature, it is evident that this topic was considered mainly from the perspective of the impact of herd size on the incidence of various disorders and diseases of dairy cows (*USDA, 1996; Wells et al., 1999; Waage et al., 1998*). Although some studies indicate significant variation in welfare parameters under the influence of herd size (*Gieseke et al., 2018; Beggs et al., 2019*), there is currently no reliable scientifically based confirmation of this relationship. However, it is widely believed that the quality of welfare of cows in large herds is generally worse compared to smaller herds. There are several reasons for this. In conditions of higher concentration of animals, the possibility of spreading infectious agents increases, and thus the frequency of the disease, while the identification of health and other problems is difficult. In herds of large size, production usually takes place with higher intensity, which implies greater pressure on the physiological functions of farmed animals and their welfare (*Rauw et al., 1998; Royal et al., 2000*). Intensive production is often accompanied by infrastructural solutions that support higher economy of production (fewer beds and feeding places, higher population density, etc.) but adversely affect the welfare of cows (*Leonard et al., 1996; Tucker et al., 2005; Popescu et al., 2007).*

The mentioned influences should be taken seriously, especially since in the developed countries of the world, there has been a significant increase in the size of farms in the past decades. In the past 30 years, the average herd size in New Zealand and Australia has almost tripled *(Dairi New Zealand, 2014; Dairi Australia, 2015*), while in the United States the size of dairy herds has increased sixfold (*MacDonald et al., 2007*). In many countries, the increase in the herd was accompanied by changes in the way of housing of animals, because the development and application of the lying boxes resulted in a reduced stay of cows on pasture and free ranges. Consequently, only 20% of lactating cows and 34% of dry cows accessed pasture in 2013 (*USDA, 2014).*

A similar development of herd size has been observed on the European continent. In the EU-10 Member States (Belgium, Denmark, Germany, Ireland, Greece, France, Italy, Luxembourg, the Netherlands and the United Kingdom), herd size has been increased from 17 to 54 dairy cows per farm (*Eurostat, 2015*). It is indicative, however, that in these EU countries, milk production is relatively stable (100 million tons) despite a significant reduction in number of farms of about 80% and a reduction in dairy cattle populations by about 30% (*Eurostat, 2015*). This indicates a significant improvement in milk yield per cow as well as increased stress to which cows are exposed in production, which could affect the

welfare of dairy cows, especially due to the increased frequency of productive diseases (*Coignard et al., 2014*).

At the same time, public awareness of farm animal welfare issues is growing in the EU (*European Commission, 2016*), and many consumers are concerned about the industrialization of livestock production. Observed from the consumer point of view, natural housing conditions are the main precondition for animal welfare (*Spooner et al., 2014*), while the industrial farms induce serious animal health and welfare problems (*Vanhonacker and Verbeke, 2014*).

Starting from the need to contribute to a more thorough understanding of the relationship between the quality of welfare and the size of dairy herds, as well as the main challenges arising from it, this study examined the impact of herd size on parameters related to providing good nutrition and housing, as important segments of overall welfare of dairy cows on farms in Serbia.

Material and Methods

The farms

The study was conducted on 16 selected conventional dairy farms of different herd sizes and housing management (free-stall housing-FSH; tie-stall housing-TSH). Therefore, farms were classified by the number of cows into three herd size groups: large (>301 cows), medium (101–300 cows), and small (30 - 100 cows). FSH was implemented in 60% of large, 75% of medium, and 15% of small size herds. Presences of the races were 80% and 20% for Domestic Simmental and Holstein Friesian cattle, respectively. Due to discretion and simpler presentation, the analyzed farms were assigned codes (1 - 16).

Welfare assessment

Welfare assessment of cows was done according to the *Welfare Quality® Assessment Protocol for Cattle -WQP (2009)*. This is a standardized indicator system for on-farm animal welfare assessment. It focuses mainly on animal-based measures, which directly reflect the actual welfare state of the animals. Three trained assessors (experienced in cows' welfare assessment) evaluated the cows on each farm. To avoid seasonal effects on the animal welfare assessment, each farm was visited twice a year, in the winter and summer season, and the average value of each welfare measure was calculated.

Processing of data collected on the farms was carried out using the *Welfare Quality® Scoring System Software Program (2012)*. More than 30 animal welfare indicators covering aspects of feeding, housing, health, and behavior are measured and aggregated to 12 welfare criteria and 4 welfare principles (Good feeding; Good

housing; Good health and appropriate behavior). Finally, farms are assigned to 1 of 4 overall welfare categories, representing an "excellent" (81-100 points), "enhanced" (56-80 points), or "acceptable" (21-55 points) animal welfare state. In cases where minimum requirements could not be achieved, the farms are rated as "not classified" (under 20 points). Since this research focuses on the effect of herd size on the provision of good feeding and housing, only the parameters included in the assessment of these principles (principles of "Good feeding" and "Good housing") are shown in Table 1. A detailed description of the assessment of each measure can be found in the WQP.

Table 1. Criteria and measures used in the assessment of "Good feeding" and "Good housing" principles ***(Welfare Quality® Assessment Protocol, 2009)***

Principles	Criteria	Measures
Good feeding	Absence of prolonged hunger	Body condition score
	Absence of prolonged thirst	Water provision; cleanliness of water points; water flow; functioning of water points
Good housing	Comfort around resting	Time needed to lay down; animals colliding with housing equipment during lying down; animals lying partly or completely outside the lying area; cleanliness of udders, flank/upper legs, lower legs
	Ease of movement	Presence of tethering; access to outdoor loafing area or pasture

Statistical analysis

All statistical analyses were performed using Statistica v.10 commercial software (*StatSoft, Inc., USA, 2010*). Descriptive statistical parameters were determined (mean, standard deviation, minimal and maximal values) for the assessed measures, and for the scores of the criteria and principles. The statistical significance of the herd size effect on the welfare in the studied farms was determined by the t-test or the Mann-Whitney test, depending on the normality of data distribution, established with the Kolmogorov-Smirnov test. P values less than 0.05 were considered as significant.

Results and Discussion

Welfare measures and categorizations of studied farms

Table 2 provides an overview of welfare parameters on the studied farms (1-16). For a more complete presentation, the categories of welfare quality for each of the farms are given, determined on the basis of the overall assessment, i.e. values for all four principles. The results show that one half of the surveyed farms are classified in the category of acceptable and the other half in the category of appropriate quality of welfare. None of the studied farms' quality of welfare was evaluated as unacceptable or excellent, based on which it can be argued that the observed farms provided cows with conditions that meet more than the basic needs of animals in terms of nutrition, health, comfort and behavior.

In relation to herd size, category of enhanced welfare quality was established in all farms of medium herd size, about 60% of small herd size and 20% of large herd size. Lower, acceptable welfare quality was determined predominantly in large herds (80%) whereas about 40% of small herds were assessed by this category.

The principle of "Good feeding" was evaluated as twice as good as the principle of "Good housing" and generally indicates that the welfare of dairy cows on the surveyed farms is not endangered by prolonged starvation and thirst. However, within this principle, great variability has been determined, so there are evident deficiencies on some farms (score ≤ 20) that have a threatening effect on the nutritional status of farmed animals.

Conditions of housing were assessed on average as acceptable, with a rather low score for the "Comfort around resting" criterion, which indicates a more pronounced problem of providing appropriate rearing conditions (space, hygiene and collision). In contrast, freedom of movement was assessed more favourably, but with pronounced variability on the surveyed farms.

Table 2. Welfare measures and categorizations of farms

Farm code (1 - 16)	1	2	3	5	6	4	7	8	9	10	11	12	13	14	15	16	Average score	Standard deviation
Herd size (L; M; S)	L	L	L	L	L	M	M	M	M	S	S	S	S	S	S	S		
Housing (FSH; TSH)	FSH	TSH	TSH	FSH	FSH	FSH	FSH	TSH	FSH	TSH	TSH	TSH	FSH	TSH	TSH	TSH		
Principle Good feeding	94.40	89.10	80.30	13.95	94.80	67.40	95.85	49.10	61.60	100.00	59.40	86.00	69.65	95.65	100.00	58.30	75.97	25.31
Absence of prolonged hunger	92.30	85.05	73.00	94.50	92.85	55.35	94.30	80.35	73.15	100.00	44.40	80.80	58.45	94.05	100.00	42.90	78.84	19.51
Absence of prolonged thirst	100.00	100.00	100.00	3.00	100.00	100.00	100.00	51.50	60.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	88.41	29.58
Principle Good housing	47.30	13.15	13.80	56.50	53.90	62.25	56.35	19.00	53.90	27.90	30.35	30.35	62.90	13.60	15.50	28.65	36.59	19.37
Comfort around resting	16.40	14.70	14.20	30.90	26.70	40.10	30.75	26.70	26.70	26.85	30.75	30.75	41.10	13.55	16.40	25.75	25.77	12.50
Ease of movement	100.00	15.00	15.00	100.00	100.00	100.00	100.00	15.00	100.00	34.00	34.00	34.00	100.00	15.00	15.00	34.00	56.94	39.25
Welfare category of farm	Acceptable	Acceptable	Acceptable	Acceptable	Enhanced	Enhanced	Enhanced	Enhanced	Enhanced	Enhanced	Acceptable	Acceptable	Enhanced	Acceptable	Enhanced	Acceptable		

The effect of herd size on provision of “Good feeding”

Although small farms were, in summary, rated for the principle of "good nutrition" with the best score, the results presented in Table 3 show that the criterion "absence of hunger" was rated as the worst, while the criterion "absence of thirst" was given the maximum number of points. On large and medium-sized farms, this relationship is exactly the opposite, based on which it can be assumed that when calculating the score of the main principle based on the score of the

corresponding criteria, there is some compensation. However, this outcome is actually the result of the application of a specific mathematical operation (Choquet integral) in computational processing, which gives one criterion greater importance (weight) than another. In this particular case, "absence of thirst" is more important for welfare than "absence of hunger", which is reflected in the score for the main principle "good feeding". However, due to the high variability within the groups, statistical analysis showed no significant impact ($p \geq 0.05$) of herd size on the provision of good feeding, i.e. analyzed principle and criteria.

Table 3. Effect of herd size on provision of good feeding

Herd size	Large (>301 cows)				Medium (101-300 cows)				Small (30 - 100 cows)				F	LSD test		
Welfare criterion/ measures	$\bar{x}$	SD	Min	Max	$\bar{x}$	SD	Min	Max	$\bar{x}$	SD	Min	Max		L/M	L/S	M/S
Principle Good feeding, points	74.51	32.71	13.30	100.00	68.49	27.03	12.20	100.00	81.29	18.07	56.40	100.00	ns	ns	ns	ns
Criterion Absence of prolonged hunger, points	87.54	10.75	70.90	100.00	75.79	15.55	52.40	100.00	74.37	24.74	40.30	100.00	ns	ns	ns	ns
Very lean, %	1.68	1.51	0.00	4.17	3.65	2.71	0.00	8.20	4.62	4.88	0.00	12.50	ns	ns	ns	ns
Regular body condition, %[N]	97.00	2.26	94.00	100.00	96.26	2.63	91.80	100.00	86.35	6.73	75.87	97.26	**	ns	**	**
Very fat, %[N]	1.32	1.68	0.00	4.17	0.09	0.25	0.00	0.72	8.76	5.21	1.22	18.00	**	ns	**	**
Criterion Absence of prolonged thirst, points	80.60	40.90	3.00	100.00	77.88	35.24	3.00	100.00	100.00	0.00	100.00	100.00	ns	ns	ns	ns

[N]=not included in software analysis, *P* = significance of differences between the means calculated by t-test, ns = not significant ($P > 0.05$),
* = significant statistical differences at $P < 0.05$, ** = significant statistical differences at $P < 0.01$

The criterion "absence of prolonged hunger" was assessed on the basis of the cows' body condition (BCS), which represents the nutritional history of the animal, rather than the current nutritional status. Including BCS within welfare assessment has the aim to identify the proportion of animals that are under nutrition or over nutrition. Both of these conditions can lead to serious health problems and thus can be regarded as a potential welfare risk. BCS is determined regard to breed (dairy or dual purpose) and four body region condition (cavity around tail head;

vertebrae; tail head, hipbones, spine and ribs) according to descriptive scale (o-regular; 1-very lean or 2-very fat). On herd level, the calculated percentage of very lean cows served as indicator for food provision on farm. The highest share of cows with regular body condition was determined on large and medium farms (Table 3). On the other hand, cows were nutritionally provided the worst on small farms, where the highest share of cows of poor body condition (4.62%) and fattened cows (8.76%) was found. Similar results were obtained in a study by *Adams et al. (2017)* where the highest share of lean cows (BCS $\leq$2.5) was also found on small farms (9.1%) compared to medium (3.0%) and large herds (2.0%). The association of lower percentages of lean cows with increasing herd size was also found in studies by *de Vries et al. (2016)* and *Gieseke et al. (2018).*

The high share of cows of undesirable body condition (lean and fat) on small farms may be due to inadequate diets as a result of insufficient education and poor financial capabilities of farmers, while on medium and especially large farms, due to more demanding production conditions, balancing the diet is responsibility of permanently employed experts or consultants. However, it should be noted that the determined share of cows of poor body condition on farms of different herd sizes, from 1.68% to 4.62%, does not endanger the quality of animal welfare as it corresponds to the range 0-11% stated by *Webster (2005)* for farms of best welfare quality. Contrary to that, the share of fattened cows of 8.76% in small herds corresponds to the range established by *Webster (2005)* on farms of lower welfare categories and may pose a risk to the welfare of cows in terms of disposition to dystocia and fatty liver degeneration *(Reid et al., 1986).*

Animal health, welfare and productivity are significantly affected by adequate water intake (*Beede, 2012*). Limited access and/or poor water quality inevitably lead to reduced production performance and endanger animal health. It is therefore very important to provide cows with unrestricted access to drinking water of appropriate quality (*Häbich and Kamphues, 2009*). Water provision (availability of at least two power supplies per head), cleanliness and functionality of drinkers, as well as water flow are indicators on the basis of which the criterion "absence of prolonged thirst" was assessed. The average value of this criterion indicates that in our conditions cows were not exposed to prolonged thirst (Table 2), but unexpectedly, the best water provision for cows was determined on farms with small herd size. The average score for this criterion was 88.41 points with 6.25% of farms with a value of less than 10 points. In the EU, the average value is 64.6 points with a significant share of farms (20%) on which the value is less than 10 points (*Welfare Quality Network, 2012*). The range of minimum and maximum values of the criteria is the same in Serbia and the EU (3 - 100 points). This indicates a significant variation in the assessment of this criterion between farms, which in our conditions is mainly due to insufficient number of drinkers per head, while other indicators (functionality and cleanliness of drinkers) are satisfactory on all surveyed farms.

The effect of herd size on provision of "Good housing"

Based on the results of the research presented in Table 4, it is evident that the size of the herd had no significant influence ($p \geq 0.05$) on the principle of "Good housing". However, the best score of this principle was achieved on farms of medium herd size (47.88 points) and the worst (29.89 points) on small farms, up to 100 heads, with significant differences at the level of $p \leq 0.05$

Housing comfort was rated very low in herds of all sizes and worst in large herds. Some indicators within this criterion were significantly influenced by herd size. Thus, the longest duration of cows' lying down of 6.65 seconds on average was observed in large herds and the shortest 5.78 seconds in medium-sized herds ($p \leq 0.05$). The laying down duration determined in the present study (Table 4), according to *Forkman and Keeling (2009)*, on large-capacity farms is a serious and on small and medium farms a moderate problem from the aspect of dairy cow welfare. Differences in the values of the mentioned indicator can be explained by different housing conditions *(Pleisch et al., 2010; Ostojić Andrić et al., 2011).* Namely, studies have shown that a deep mat, more often used in medium-sized herds, provides better comfort to animals, which can result in reduced lying down time (*Wechler et al., 2000*). Also, inadequate dimensions of accommodation, typical of small farms with tied animals, reduce comfort and may increase collisions with equipment (*Veissier et al., 2004*). Finally, some painful conditions such as laminitis and mastitis, which are more common in intensive production conditions, can cause prolonged lying down, as found in study of *Popescu et al. (2013).* Considering that the longest duration of lying down was determined on large and small farms, which at the same time had a higher share of collisions with equipment and lying out of lying area, it can be concluded that their interaction resulted in a worse score for comfort and adequate principle.

Cleanliness of cows is defined as the degree of dirt on the lower hind legs, hind quarters and the udder considered splashing (e.g. faeces, mud) and plaques (three-dimensional layers of dirt). Firstly it is estimated on individual level (scale: 0-no dirt/minor splashing or 2-separate or continuous plaques of dirt) and then on herd level by calculating percentage of animals with clean (score 0) and dirty body parts (score 2). Most of the recent studies including ours (Table 4) showed that alarm thresholds set by WQP for the dirtiness of lower hind legs (50%) and dirtiness of flank and udder (20%) were widely exceeded *(Heath et al., 2014; Zuliani et al., 2017).* The results of *Gieseke et al. (2018)* confirmed the significant effect of herd size on the proportion of cows with dirty lower legs ($p \leq 0.05$), unexpectedly with the lowest dirtiness in large herds.

Table 4. Effect of herd size on provision of "Good housing"

Herd size	Large (>301 cows)				Medium (101-300 cows)				Small (30 - 100 cows)				F	LSD test		
Welfare measures	$\bar{x}$	SD	Min	Max	$\bar{x}$	SD	Min	Max	$\bar{x}$	SD	Min	Max		L/M	L/S	M/S
Principle Good housing, points	36.93	20.70	7.30	59.10	47.88	18.83	19.00	65.40	29.89	16.75	11.00	65.40	ns	ns	ns	*
Criterion Comfort around resting, points	20.58	9.77	2.70	35.10	31.06	10.01	16.40	45.10	26.45	14.64	8.60	45.10	ns	ns	ns	ns
Time needed to lie down, s	6.65	0.73	5.33	7.58	5.78	0.65	4.50	6.70	6.24	0.62	5.40	7.10	*	**	ns	ns
Colliding with equipment during lying down, %	12.39	14.11	0.00	37.00	1.03	1.90	0.00	4.35	13.25	10.71	0.00	28.60	*	*	*	ns
Lying outside the lying area, %	39.05	29.49	0.00	83.78	17.29	16.39	0.00	41.90	45.74	40.72	0.00	100.00	ns	ns	ns	ns
Cows with dirty lower legs, %	72.57	28.07	14.81	95.80	90.65	10.68	72.60	100.00	89.83	14.88	61.30	100.00	ns	ns	*	ns
Cows with dirty udder, %	49.17	29.00	9.26	91.70	63.95	13.14	42.70	87.75	65.64	23.73	20.00	100.00	ns	ns	ns	ns
Cows with dirty flank and upper legs, %	58.33	26.07	3.70	92.10	82.50	8.05	68.30	95.92	74.27	16.53	39.70	100.00	*	**	*	ns
Criterion Ease of movement, points	66.00	43.89	15.00	100.00	78.75	39.35	15.00	100.00	38.00	27.64	15.00	100.00	*	ns	ns	*
No. of days with access to outdoor loafing area, per year	72.00	92.95	0.00	180.00	128.75	159.84	0.00	365.00	121.07	115.91	0.00	300.00	ns	ns	ns	ns
No. of days with access to outdoor loafing area, daily	7.20	10.12	0.00	24.00	12.00	12.83	0.00	24.00	8.57	8.72	0.00	24.00	ns	ns	ns	ns
No. of days with access to pasture, per year	0.00	0.00	0.00	0.00	15.00	27.77	0.00	60.00	30.00	76.26	0.00	210.00	ns	ns	ns	ns
No. of hours with access to pasture, daily	0.00	0.00	0.00	0.00	6.00	11.11	0.00	24.00	1.71	4.36	0.00	12.00	ns	ns	ns	ns

P = significance of differences between the means calculated by t-test, ns = not significant (P> 0.05), * = significant statistical differences at P< 0.05, ** = significant statistical differences at P< 0.01

In our research (Table 4), the cleanliness of cows as an indicator of housing comfort was the biggest problem in medium-sized herds where the highest share of cows with dirty legs and flank was observed, as opposed to large herds where cow hygiene was scored as the best. In the interpretation of obtained results, significant influence could also be attributed to the housing system. Namely, in our study, most (3 out of 4) medium-sized herds were reared in free housing conditions for which studies *(Regula et al., 2004; Ostojić Andrić et al., 2011)* have found to have a worse effect on cow hygiene compared to the tied system. Nevertheless, it is certain that the type of lying area *(Cook et al., 2016; Cramer et al., 2009*) as well as the regularity of cleaning the facility (*Gieseke et al., 2018*), i.e. the organization of farm business management, play an important role in ensuring hygiene.

In small herds, where the tied system was mostly used, the highest number of cows lying partially out of lying area (45.74%) and the highest frequency of collisions when lying down (13.25%) were found in contrast to the herd size of 100 to 300 head where these phenomena were least represented. The fact is that in our conditions, small farms are located within family farms, and their construction is often unplanned and does not follow the appropriate technical - technological standards and norms.

Freedom of movement, a welfare criterion assessed on the basis of the applied housing system and the time spent in the free range and on the pasture, was significantly influenced by the size of the farm ($p \leq 0.05$). This criterion of welfare was scored the best on medium-sized farms and worst on small-capacity farms with significant differences at the level of $p \leq 0.05$. The results of the research presented in Table 3 show that freedom of movement was most endangered on large-capacity farms where cows were allowed an average of 21.6 days in the free range per year, without access to pasture, while on medium-capacity farms the average annual stay of cows in the free ranges was 64 days, with 3.75 days on pasture.

Conclusion

This study showed no statistically significant effect of herd size on the provision of good feeding and housing as significant preconditions for ensuring the overall welfare of farmed animals. Given that large variations in welfare indicators were found in each of the observed groups, herd size could not be used, on its own, as a valid indicator of animal welfare. On the contrary, housing and management conditions appear to have a greater impact on welfare than the number of dairy cows per farm. Based on that, overcoming the identified risks in herds of different

sizes can be achieved by investing in continuous education of breeders, optimization and innovation of technical characteristics of facilities and technological processes in a way to adequately adapt to the needs of livestock and production conditions.

Uticaj veličine stada na kvalitet dobrobiti mlečnih krava - obezbeđenost adekvatne ishrane i uslova držanja

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Rezime

U svetu je poslednjih decenija prisutan trend povećanja veličine mlečnih stada uz istovremeno povećanje prinosa mleka po grlu. Pored ekoloških i ekonomskih benefita, ova tendencija nosi i određene rizike po dobrobit krava sa obzirom da se u uslovima povećane aglomeracije grla povećava mogućnost širenja patogena, manje su mogućnosti adekvatnog nadzora dok su istovremeno krave izložene većem selekcijskom i proizvodnom pritisku. Istraživanja odnosa veličine stada i parametara kvaliteta dobrobiti još uvek nisu zastupljena u dovoljnom obimu kako bi se izveli relevantni zaključci. Polazeći od toga, cilj ove studije izvedene u Srbiji, bio je da se ispita uticaj veličine stada na parametre koji se odnose na obezbeđivanje dobrih uslova ishrane i držanja kao važnih segmenata celokupne dobrobiti mlečnih krava. Ocena datih parametara dobrobiti obavljena je prema *Welfare Quality® Assessment Protocol for Cattle (2009)*, na 16 mlečnih farmi različitih veličina stada (velike, srednje, male) i načina držanja. Rezultati ukazuju da postoje značajne varijacije indikatora dobrobiti u svakoj od posmatranih grupa, zbog čega se veličina stada ne može uzimati kao parametar koji eksplicitno determiniše kvalitet dobrobiti. Ipak, pojedinačno sagledavanje i upoređivanje parametara dobrobiti između grupa ukazuje da bi se stada male veličine u našim uslovima proizvodnje mogla označiti kao nosioci najvećih rizika po dobrobit krava. U malim stadima utvrđen je najveći udeo krava slabe (4,62%) i utovljene kondicije (8,76%), kao i najmanja sloboda kretanja jer se krave na malim farmama uglavnom gaje u vezanom sistemu. Prosečne vrednosti indikatora: vreme leganja (6,24s), učestalost kolizija sa opremom (13,25%) i visoka zaprljanost krava (65,6-89,8%) dodatno naglašavaju problematiku obezbeđenja komfora u malim stadima.

Ključne reči: mlečne krave, dobrobit, veličina stada, ishrana, uslovi držanja, komfor

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FUTURE PERSPECTIVES IN BREEDING THE INDIGENOUS LOCAL STARA ZAGORA SHEEP AND IMPROVING THE PHENOTYPIC AND GENETIC PARAMETERS OF THE BREED

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Abstract: One of the most phenotypically attractive breeds in Bulgaria is the local Stara Zagora sheep. It is a local - indigenous breed specialized in dairy direction. In recent years, this valuable genetic resource is on the verge of being lost as the breed is threatened with extinction. The aim of the study is to monitor the genetic and phenotypic parameters of the main reproductive and productive traits in local Stara Zagora sheep.The research includes a total of 9495 ewes of the local Stara Zagora breed reared in 15 farms, produced during the period from 2011 to 2020 including. The studied traits were: fertility - biological of the first, second and third lambing, milk yield for a 120-day period of first, second and third standard lactation and live weight of different age categories. The statistical model that we used was based on the model of animal /Animal model/, using the software product PEST and VCE (Groeneveld), SYSTST 13 and SPSS for Descripive statistics. The average phenotypic values of the traits: are respectively - fertility of 1^{st} – 113%, 2^{nd} – 125% and 3^{rd} – 129% lambing; live weight of weaning - 29.79 kg, at 18 months - 63.87 kg, and at 2.5 years 72.92 kg; milk yield of the 1^{st} – 98.37 l; 2^{nd} – 104.60 l and 3^{rd} – 108.80 l. lactation. Heritability (h^2) in the main selection traits milk yield of the first, second and third lactation is characterized by moderate values - 0.191; 0.225 and 0.184, respectively, and we report from low to moderate values of h^2 on the fertility in all three studied groups - fertility in the 1^{st} - 0.183; 2^{nd} - 0.149 and 3^{rd} lambing 0.137. Milk yield is in high positive correlation and with a high statistical significance at different stages of lactation, between the 1^{st} and 2^{nd} - 0.849, between the 2^{nd} and 3^{rd} - 0.628 and between the 1^{st} and 3^{rd} - 0.447.

Key words: milk yield, fertility, heritability, correlation, genetic

Introduction

Worldwide, there are many indigenous breeds of farm animals and in particular endangered small ruminants. All these initial forms are the "genetic basis" of modern high-yielding widespread sheep breeds. They represent a valuable genetic resource, not only for the countries where their origin is, but also globally *(Belabdi et al., 2019)*. Such is the local Stara Zagora sheep breed. The need to preserve these breeds of sheep is the motivation for this study. The monitoring will be useful for further breeding efforts with the breed.

The sheep reared and bred in Bulgaria before 1939 are mostly local and indigenous and bear their name from the region of their distribution. Such is one of the most attractive in phenotypic aspect breeds, namely the local Stara Zagora sheep. It is considered to be an intermediate form derived from the cross between Tsigai and Tsakel, but closer to Tsigai phenotypic and genetically. The most typical representatives of the breed are in the Stara Zagora region, where the origin of its name comes from. The area of distribution is not only in the low part of Stara Zagora, but also in Sliven, Haskovo and Yambol regions, as well as in the Thracian lowlands of southern Bulgaria. There are separate herds in other parts of the country. For many years it has been one of the most valuable in our country dairy breeds *(Kalaydzhiev et al., 2012)*.

Figure 1. Local Stara Zagora sheep area of distribution, "Livestock breeds in the Republic of Bulgaria" 2011

Figure 2. Ram from local Stara Zagora sheep breed, from the archives of "ABSSBB" National Sheep Breeding Fair - Arbanasi 2021

The local Stara Zagora sheep in the recent past was one of the most common local milk breeds in Bulgaria, this fact provoked us to carry out this study, giving rise to the idea to study the basic phenotypic and genotypic indicators of some reproductive and productive traits of the sheep in the population. Phenotypically the breed is characterized by large and elongated body shapes, the typical features of dairy sheep. The head is relatively elongated, narrow and tender, not covered with wool. The breed is characterized by a curved profile of the nasal

line. The ears are long, wide and drooping, often wavy at the end. Basically, sheep are hornless, although 50-60 years ago it was considered normal to have horns as well. The legs are long, strong and thin. The tail is long and reaches below the hocks of the animals. The color of the fleece is white and the wool is uniform. In the descriptions of the first authors who studied the breed, animals with a black fleece color were also found. Their phenotypic traits are belonging to realm of cultivation - different economic years. Especially recently, the most preferred are the so-called "sandlot type" - with a pronounced long body, very long ears and tail, curved sometimes even very deformed profile of the head.

Milk production is of strategic importance for Bulgaria. The most numerous part of the sheep population in our country is dairy, that fact determines their headed place in the structure of the national gene pool *(Stancheva et al., 2013)*.

The local Stara Zagora sheep is one of the breeds included in the schemes for complex reproductive crossbreeding and creation of the Bulgarian Dairy Synthetic Population (BDSP) *(Stancheva et al., 2014)*.

One of the most important features of the existing local forms of domestic animals in the past is their adaptation to a given ecological zone or microdistrict of the country *(Bowles, 2015; Belabdi et al., 2019)*. Created and bred for centuries in these areas, individual animal populations have gradually adapted to its characteristics - climate, terrain, soil and food and often bore the name of the region.

Stara Zagora sheep are much more sensitive, especially to the changing conditions of feeding and care *(Djorbineva, 1984)*. These qualities do not make them attractive in today's market economy, despite their relatively good productivity. Many breeders have significantly reduced the number of ewes, and many have even given up, due to a number of difficulties, both financial and objective - related to the characteristics of both the market and the breed. This has led to a large reduction in the population.

Despite the developed program for the establishment of dairy sheep breeding in the country in the 80s the number of ewes of this breed did not increase significantly. After 1989 the number of controlled sheep decreased dramatically, as most of the animals were slaughtered, and others were distributed to private owners *(Djorbineva et al., 1995)*. Thus, in this period, extremely valuable genetic material modeled and maintained over the decades is irretrievably lost.

The core of the Stara Zagora sheep breeding population has been reduced in recent years. There are very few farmers raising more than 50-60 sheep of the breed. Small farms with flocks of 20-30 purebred sheep predominate, which are extremely insufficient to conduct adequate selection *(Djorbineva et al., 2011)*. Due to market situation, more breeders are turning to nurture of imported highly productive breeds in search of bigger profit.

Preservation of the typical phenotype of the population is also a serious problem, due to the fact that in recent years some farmers raising Stara Zagora sheep, cross them with introduced breeds for milk, and some with meat-breeds. Preservation of the breed in its authentic appearance requires purebred breeding *(Kalaydzhiev et al., 2012)*.

Effective part of the population which is controled and work out in the direction of preserving the authentic appearance of the breed and at the same time its selection improvement varies over the years. In the last ten years the number of purebred Stara Zagora sheep included in the selection program of ABSSBB NGOs varies from 600 to 1150 ewes.

The aim of the study is to monitor the genetic and phenotypic parameters of the main reproductive and productive traits in local Stara Zagora sheep.

Materials and Methods

The study included private farms from Bulgaria - Stara Zagora, Haskovo and Sofia districts, flocks are owned by members of the NGO "Association for Breeding of Stara Zagora sheep breed in Bulgaria" (ABSSBB), based in Stara Zagora.

The study covers a total of 9495 ewes of the local Stara Zagora breed reared in 15 farms, produced during the period from 2011 to 2020 including - 10 year period. The sheep are being raised by a traditional technology typical for the low lands of our country - semi-intensively, not only on pasture but additionally fed.

All fifteen farms participating in the study are members of the NGO “Association for Breeding the Stara Zagora Sheep Breed in Bulgaria”. The studied traits were: fertility - biological of the first, second and third lambing, milk yield for a 120-day period of first, second and third standard lactation and live weight of different age categories.

The control of milk yield was performed during the milking period, and over the years four controls were performed. The milk yield data refer only to the milk obtained from the ewes after complete weaning of the lambs. The quantity of milk is presented in volume units (ml). The individual milk yield of each ewe for the control day was calculated by multiplying the amount of milk obtained by the morning individual control by a herd ratio representing the ratio: morning + evening milk / morning milk. The milk yield for a 120-day milking period was calculated as the sum of the milk yields from the individual control periods of each sheep. Live weight was measured individually at weaning, at 18 months and 2.5 years.

The examined traits are controlled and registered according to a standard method and instructions, provided in the Instruction for control of the productive qualities and grading of the sheep in Bulgarian legistration *(MAFF 2003-2013)*.

The necessary primary information for the study was obtained from the herd books and primary documentation kept in the "Association for breeding local Stara Zagora sheep breed in Bulgaria".

The phenotypic and genotypic parameters of the productive and reproductive indicators of the local Stara Zagora sheep were studied taking into account the influence of genetic and non-genetic factors on the studied traits.

The analysis of genetic and environmental varianses is based on the hypothesis that genetic variation is influenced by the effects: herd-year-season, year of birth, parity, litter size, permanent environmental effect and other effects reported in the error.

The general statistical working model is based on the model of animal /Animal model/:

Yijklm = HYM **i** +Lam **j** + SL **k** + Lact **l** + LW **m** + eijкlmno

where:

Yijklm - observation of the respective trait;
HYM **i** - fixed effect of **i** th herd-year season;
Lam **j** - fixed effect of the size of **j** th lambing;
SL **k** - fixed effect of **k** th consecutive lambing;
Lact **l** - effect of the **l** th consecutive lactation;
LW **m** - effect of the **m** th live weight level of the animal – on weening, 18 months and 2,5 years of age;
Eijklmno - random effect of unobserved factors;

Used software products to perform the statistical analysis of phenotypical values of the productive and reproductive traits in local Stara Zagora sheep, mean phenotypic values of ferttility traits in 1st, 2nd and 3rd lambing and mean phenotypic values of traits live weight at weaning, at 18 months and 2.5 years, mean phenotypic values of the trait milk yield for 120 days at the 1st, 2nd and 3rd lactation were SYSTST 13 and SPSS.

Software products PEST was used to perform statistical analysis for calculation heritability and VCE (Groeneveld) for calculation the genetic correlations between the main productive and reproductive traits of local Stara Zagora sheep.

The method applied by us is similar to these used by *Kalydzhiev (2021)*.

Results and Discussion

Over time and under the influence of market changes, objective changes occur in the exterior of the animals in order to meet the specific needs of the market and to ensure the normal profitability of producers. In this way, the genome of the population is directly modeled and phenotypic changes occur. It is necessary to be traced in purebred breeding, which is carried out in the particular breed, whether it would bring qualitative and quantitative changes in the main traits, through selection based on phenotype. This would be possible only with a higher genetic diversity of the examined traits in the local Stara Zagora sheep. The studied population is one of the smallest in the country. The number of animals under control from 2010 to the present varies from 600 to 1,150 ewes.

The main statistical parameters of the traits: biological fertility; live weight and milk yield in different stages of development and producing of the animal, are presented on Table 1.

In recent years, the direction of selection in the population is aimed at increasing fertility, due to the fact that the realized lambs for slaughter and breeding represent over 45% of revenues from ewes, this determines the trait as a priority (*Slavova et al., 2017*). The average values of this trait obtained by us for the studied period are the following: fertility of 1st – 113%, 2nd – 125% and 3rd – 129% lambing.

Table 1. Phenotypic values of the main statistical parameters of the productive and reproductive traits in local Stara Zagora sheep

Trait	Main statistical parameters	STZ n= 9495	Trait	Main statistical parameters	STZ n= 9495	Trait	Main statistical parameters	STZ n= 9495
Ferility at 1st lambing	Min. value	1	Live weight at weaning	Min. value	27	Milk yield at 1st lactation	Min. value	78
	Max. value	2		Max. value	32		Max. value	116.5
	Mean value	1.13		Mean value	29.79		Mean value	98.37
	SD	0.427		SD	0.686		SD	3.346
	CV	34		CV	3		CV	4
Ferility at 2nd lambing	Min. value	1	Live weight at 18 months	Min. value	60	Milk yield at 2nd lactation	Min. value	83
	Max. value	3		Max. value	65		Max. value	124.7
	Mean value	1.25		Mean value	63.87		Mean value	104.60
	SD	0.519		SD	1.005		SD	3.780
	CV	34		CV	2		CV	4
Ferility at 3rd lambing	Min. value	1	Live weight at 2.5 yars	Min. value	68	Milk yield at 3rd lactation	Min. value	85
	Max. value	3		Max. value	76		Max. value	129.6
	Mean value	1.29		Mean value	72.92		Mean value	108.80
	SD	0.498		SD	1.264		SD	4.425
	CV	35		CV	2		CV	3

STZ – local Stara Zagora sheep; SD - Standard Deviation; CV - Coefficient of variation

Compared to highly productive milk sheep breeds, the results are relatively low *Kalydzhiev (2021)*, but for local - indigenous breed are significant. According to *Djorbineva et al. (2011)* the average fertility of the Strao Zagora sheep in 1980. was 112% in 1989. - 108% and in 1999. - 96%. The reported standard deviation for the trait is low, but the coefficient of variation is high, with values of 34 for the 1st and 2nd lambing and 35 for the 3rd.

As mentioned above, increasing the profitability of a sheep breeding is also associated with increasing the number of animals realized for slaughter, which automatically makes live weight one of the important signs. The local Stara Zagora sheep is one of the largest sheep breeds in our country, the values for the trait exceed the average for sheep in Bulgaria. The obtained results prove it, as the average live weight of weaning is 29.79 kg, at 18 months - 63.87 kg, and at 2.5 years is 72.92 kg. Over the last 15 years, an increase of 8% has been reported for this sign.

The main selection trait for the Stara Zagora breed is milk yield. It provides about 50% of the income in the farms rearing the breed. The reported milk yield in our study is moderate, with a standard deviation in the range of 3 to 5, and the coefficient of variation with values from 3 to 4, which determines a not so variable sign with more constant values. The average values for milk yield per lactations are: 1st – 98.37 l; 2nd – 104.60 l and 3rd – 108.80 l. According to *Djorbineva et al. (2011)* the average milk yield of the Stara Zogora sheep in 1980 was 74.6 l, in 1989 - 89.0 l and in 1999. - 76.7 l. Conducting purposeful breeding activity on the trait of the last 40 years has led to its significant increase. For the studied period there are reported low minimum and relatively high maximum values of the trait in individual farms, this is a fact resulting from the diversification in the level of selection activity in individual farms. Some farmers still have difficulties in achieving better milk production, as breeding conditions are more primitive and the genetic potential of animals is lower.

Table 2 presents heritability of the selection traits: live weight; fertility and milk yield during different stages of development and production in the local Stara Zagora sheep breed.

Table 2. Heritability (h2) of the main selection traits of local Stara Zagora sheep

Trait	h^2	Trait	h^2	Trait	h^2
Fertility at 1st lambing	0.183 ± 0.021	Live weight at weaning	0.078 ± 0.021	Milk yield at 1st lactation	0.191 ± 0.067
Fertility at 2nd lambing	0.149 ± 0.025	Live weight at 18 months	0.022 ± 0.004	Milk yield at 2nd lactation	0.225 ± 0.044
Fertility at 3rd lambing	0.137 ± 0.074	Live weight at 2.5 years	0.033 ± 0.004	Milk yield at 3rd lactation	0.184 ± 0.039

Biological fertility in the 1st, 2nd and 3rd lambing are the main reproductive traits included in our study. We report moderate values of h^2 at: fertility in the 1st - 0.183; 2nd - 0.149 and 3rd lambing 0.137 respectively. The values of heritability established by us are a reliable basis for conducting a purposeful breeding activity in the direction of increasing fertility. They also correspond to those established by *(Kalydzhiev, 2014)*.

Coefficient of heritability of the traits characterizing the intensity of growth: live weight at weaning, live weight at 18 months and live weight at 2.5 years obtained by us are identify with low values in the three groups - Table 2.

The lowest values of the indicator were reported at a live weight of 18 months - 0.022 h^2. The heritability values were also low in the other two periods of development of the animals included in the study: live weight at 2.5 years - 0.33 and live weight at weaning - 0.078, the last was slightly higher than the other two periods.

Milk yield is the productive trait wit main importance for the local Stara Zagora sheep breed. The results obtained by us show moderately high levels of hertabils in the milk yield of the 1st, 2nd and 3rd lactation - 0.191; 0.225 and 0.184 respectively. Published by *Kalaydzhiev et al. (2012)*, correspond to those received from us. The genetic diversity established by us for the trait of milk yield in the local Stara Zagora sheep is a favorable fact that would contribute to positive results in conducting targeted selection on the trait.

Table 3 presents results describing the genetic correlations between traits: live weight; biological fertility and milk yield during different stages of development and production in the local Stara Zagora sheep.

The correlations regarding the fertility trait are high and positive. It is highest between the 2nd and 3rd lambing 0.814 and has a degree of statistical significance, and the lowest between the 1st and 3rd lambing 0.471. Conducting selection for higher fertility of the 1st lambing would lead to a high positive result in the following, ie. can rely on selection carried out at an earlier stage of animal development, which will indirectly lead to a positive change in this trait in late stages of development.

Correlations within respect to the same trait in terms of age, ie. the recurrence of the trait in different phases of the development of the organism is evincible of the degree of its age variability. Earlier assessment of the potential of the animals is of great importance for selection and allows optimization of the control on productive traits and earlier indirect selection.

Correlation between biological fertility and live weight at different development stages of individuals characterizes with positive values. Between the fertility of the 3rd lambing and live weight of 2.5 years of age we established a medium negative correlation (- 0.428). Due to the obtained results we can conclude that in future breeding activity on the traits biological fertility and live weight

should be conducted purposeful selection according to the selection limits for the indigenous breed.

Table 3. Genetic correlations between the main productive and reproductive traits in local Stara Zagora sheep

Trait	Fertility at 1st lambing	Fertility at 2nd lambing	Fertility at 3rd lambing	Live weight at weaning	Live weight at 18months	Live weight at 2.5 years	Milk yield at 1st lactation	Milk yield at 2nd lactation	Milk yield at 3rd lactation
Fertility at 1st lambing		0.631*	0.471	0.173*	0.188**	0.122	0.454**	0.268	-0.113
Fertility at 2nd lambing			0.814*	0.134	0.076*	0.351**	0.084	-0.128**	-0.187
Fertility at 3rd lambing				0.205	0.312**	-0.428	-0.725	0.144	-0.639**
Live weight at weaning					0.193*	-0.055	-0.041	-0.336	-0.077
Live weight at 18 months						-0.449	-0.791***	-0.294	-0.194
Live weight at 2.5 years							-0.213	-0.172**	0.318
Milk yield at 1st lactation								0.849***	0.447**
Milk yield at 2nd lactation									0.628**
Milk yield at 3rd lactation									

statistical significance *: $p<0.05$ **: $p<0.01$ ***: $p<0.001$

The dependence between the signs determining live weight at different stages of development of animals are from low to moderate negative, with the exclusion of the relationship between live weight at weaning and live weight at 18 months 0.193. This is exponential that it is suitably to carry out live weight selection independently and at least up to 18 months of age.

The trait milk production is in a high positive correlation and with a high degree of statistical significance at different stages of lactation. Between the 1st and 3rd lactation we found the lowest value 0.447, between the 2nd and 3rd 0.628 and between the 1st and 2nd lactation we report the highest value 0.849. The conducted

results gives us a reason to believe that targeted selection for the trait of milk yield of the 1st lactation would lead to a positive effect on the levels of the trait in the later stages of production. This aftermaths are also an strong argument for proposition optimization of milk control in different stages (up to lactation II), given their complexity.

It is important to note that the trait milk production is negatively correlated, in dissimilar extent, with most of the sights included in the study. An exclusion is the fertility of the 1st lambing, as the dependence between it and the milk production of the 1st and 2nd lactation is 0.454 and 0.268, respectively, but the correlation of the trait with the third lactation is negative (-0.113). We also establish a positive correlation between the milk yield of the 3rd lactation and the live weight at 2.5 year 0.318. This sign is straight related to both fertility and milk yield of ewes. According to our founding's, animals with a higher level of milk yield of third - lactation have a higher live weight at 2.5 years. Low positive dependence is observed between the signs of milk yield of the 1st lactation and fertility of the 2nd lambing 0.084, as well as in the milk yield of the 2nd lactation and fertility of the 3rd lambing 0.144. It should be mentioned that the results show very high negative correlations with a high degree of statistical significance in milk yield of 3rd lactation and fertility at 3rd lambing (-0.639), as well as in milk yield at 1st lactation and live weight of 18 months of age (-0.791).

The established results and the made analyzes are the reason to consider that for the future selection activity with the local Stara Zagora breed it is necessary to continue purebred breeding in order to keep the breed in its current phenotype, and it would be appropriate in view of the results independent selection on some of the main productive and reproductive traits - live weight, milk yield and fertility, according to the accepted selection limits for the breed. Recommendations that can be made are: the live weight of female animals be monitored until at least 18 months of age; fertility on the 1st and 2nd lambing; and milk yield on the 1st and 2nd lactation.

Monitoring the population and tracking its development during the years in which the selection was conducted by ABSSBB, leads to a clearer idea of what is happening inside it. The analyzes will lead to a more adequate direction and goal that must be pursued by breeders of the breed. Above all, it should be aimed at preserving the existing population of the local Stara Zagora sheep breed. This determines the task of preserving the genetic variability present in the breed in all morphological, qualitative and quantitative traits.

Conclusions

The average phenotypic values of the traits: of the local Stara Zagora sheep, respectively - fertility of 1st – 113%, 2nd – 125% and 3rd – 129% lambing; live weight of weaning 29.79 kg, 18 months - 63.87 kg, and 2.5 years 72.92 kg; milk yield of the 1st – 98.37 l; 2nd – 104.60 l and 3rd – 108.80 l lactation, as the established coefficient of variation and the standard deviations are moderate and prove that the direction of breeding during the development of the breed over time is aimed at establishing the above-mentioned traits as the main selection ones.

Heritability (h^2) in the selection traits milk yield at first, second and third lactation is characterized by moderate values - 0.191; 0.225 and 0.184, respectively, and we report from low to middle range values of h^2 for the three studied groups - fertility in the first - 0.183; second - 0.149 and third lambing 0.137. Moderate levels of genetic diversity in the studied traits of the local Stara Zagora sheep are reason to believe that breeding activities on phenotype would lead to positive results in increasing milk production and fertility.

The low rate of h^2 for the signs characterizing the growth intensity show vicinity and low variability in the studied part of the population. Live weight at weaning - 0.078, live weight at 18 months - 0.022 and live weight at 2.5 years - 0.33. The possibility of effective selection based on phenotype on the trait minimized by low levels of genetic diversity.

The positive correlations between the two traits fertility and growth intensity in all their phases of development are a reliable basis for leading a mass selection (by phenotype) and indirect selection on them.

Milk yield is in high positive and with a high degree of statistical significance at different stages of lactation, between the 1^{st} and 3^{rd} - 0.447, 2^{nd} and 3^{rd} - 0.628 and between the 1^{st} and 2^{nd} - 0.849. The established results gives us a reason to believe that targeted selection for improving milk yield of the 1^{st} lactation would lead to a positive effect on the levels of the trait in the later stages of production.

Positive correlations were also found between fertility of the 1st lambing and milk yield of the 1^{st} and 2^{nd} lactation - 0.454 and 0.268 respectively. Which is also a basis for conducting indirect selection on the traits.

Perspektive uzgajanja autohtone lokalne starozagorske ovce i poboljšanje fenotipskih i genetskih parametara rase

Georgi Ivanov Kalaydzhiev

Rezime

Jedna od fenotipski najatraktivnijih rasa u Bugarskoj je lokalna starozagorska ovca. To je lokalna autohtona rasa specijalizovana za proizvodnju mleka. Poslednjih godina, ovaj vredni genetski resurs je na ivici ugroženosti jer rasi preti izumiranje. Cilj rada je praćenje genetskih i fenotipskih parametara glavnih reproduktivnih i produktivnih osobina lokalnih starozagorskih ovaca. Istraživanjem je obuhvaćeno ukupno 9495 ovaca domaće starozagorske rase gajenih na 15 farmi, proizvedenih u periodu od 2011. do 2020. godine. Ispitivane osobine su: plodnost - biološka prvog, drugog i trećeg jagnjenja, mlečnost za 120-dnevni period prve, druge i treće standardne laktacije i masa živih grla različitih starosnih kategorija. Statistički model koji smo koristili baziran je na Animal modelu, koristeći softverski proizvod PEST i VCE (Groeneveld), SYSTST 13 i SPSS za deskriptivnu statistiku. Prosečne fenotipske vrednosti osobina su: - plodnost 1. jagnjenje – 113%, 2. jagnjenje – 125% i 3. jagnjenje – 129%; masa grla na odbijanju - 29,79 kg, sa 18 meseci - 63,87 kg, a sa 2,5 godine 72,92 kg; mlečnost 1. laktacija – 98,37 l; 2. Laktacija – 104,60 l i 3. Laktacija – 108,80 l. laktacija. Heritabilnost (h^2) u glavnim selekcijskim osobinama - mlečnost u prvoj, drugoj i trećoj laktaciji, karakterišu umerene vrednosti - 0,191; 0,225 i 0,184, sa niskim do umerenim vrednostima h^2 za plodnost u sve tri ispitivane grupe - plodnost u 1. Jagnjenju - 0,183; 2. Jagnjenju - 0,149 i 3. Jagnjenju - 0,137. Mlečnost je u visokoj pozitivnoj korelaciji i sa visokom statističkom značajnošću u različitim fazama laktacije, između 1. i 2. - 0,849, između 2. i 3. - 0,628 i između 1. i 3. - 0,447.

Ključne reči: prinos mleka, plodnost, heritabilitet, korelacija, genetika

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ANALYSIS OF VITALITY AND BIOCHEMICAL PARAMETERS IN FREEZE-THAWED SEMINAL PLASMA OF RAMS

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Abstract: The current study aimed to examine the percentage changes of viability sperm and the activity of the enzymes LDH, ALP, GOT / AST, GPT / ALT in the sperm plasma of Lacaune rams, before and after cryopreservation. For this purpose, five rams were examined, and two ejaculates were obtained from each ram. Ejaculates are collected by the method of artificial vagina, during the insemination campaign. All ejaculates were diluted with a 6AG extender and frozen by the Cassou's sequin method. Sperm viability was determined by eosin and nigrosine smears, and enzyme activity was examined spectrophotometrically. As a result, the percentage of vital sperm after cryopreservation decreased by 15% ($P \leq 0.001$). The freezing and thawing process also reduced the activity of the enzymes LDH, ALP, GOT / AST and GPT / ALP. In conclusion, the observed enzymes, in relation to sperm vitality, could be used as indicators to optimize the protocols for cryopreservation of ram's sperm.

Key words: ram, sperm, cryopreservation, biochemical parameters, vitality

Introduction

Mammalian sperm plasma is a composite mixture of epididymal secretions and accessory gonads (*La Falci et al., 2002*). It creates the mandatory environment for the normal functioning of sperm, and the present amount and type of enzymes and metabolites strongly affects sperm quality and freezing stability (*Asadpour, 2012; Juyena and Stelletta, 2012; Mráčková et al., 2015; Atroschenko et al., 2019*). Some of the enzymes are found in sperm, and they play an important role in fertilization, metabolic processes and the conversion of chemical energy

into mechanical, which ensures the movement of sperm (*Baychev et al., 2007*). Some authors have also established the role of various enzymes in semen. For example, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are crucial for processes of metabolism that provide energy for sperm survival, motility and fertility (*Sirat et al., 1996; Duan and Goldberg, 2003*). According to some studies in horses, γ-glutamyl transferase (GGT) correlates with sperm motility (*Pesch et al., 2006; Dogan et al., 2009*). Also, plasma aminopeptidases of sperm, such as alanyl aminopeptidase (APN), are involved in many physiological processes. Alkaline phosphatase (ALP) can be used to differentiate azoospermia (*El-Bishbishy et al., 2013*).

Some studies show that if a certain enzyme is present in total semen (that is, in both sperm and plasma), there is often a large difference in its activity (*Andreeva, 2020*). Most often, intracellular enzymes (found in sperm) enter the sperm plasma after an effusion resulting from disruption of the cell membrane during sudden cooling, after deep freezing or ultrasonic disintegration by a sonifier (*Murdoch and White, 1967; Andreeva et al., 2022*). Therefore, evaluations of these enzymes are endorsed as indicators of quality of the sperm (*Sirat et al., 1996; Tvrda et al., 2013; Tejaswi et al., 2016*). Vitality assessment is also one of the major part of analysis of sperm. This is an extremely important method of distinguishing the dead from the living sperm (*Björndahl et al., 2004*). Currently, there are numerous available techniques for staining and evaluating quality of sperm in different animal species (*Suttiyotin and Thwaites, 1992; Łącka et al., 2016; Gerzilov and Andreeva, 2021*).

The aim of our research is to study the percentage changes of viable sperm and the enzymatic activity of LDH, ALP, GOT / AST, GPT / ALT in the sperm plasma of Lacaune rams, before freezing and after thawing.

Material and Methods

Animals and sperm production

The experiment included five clinically healthy rams, aged 3-4 years of the Lacaune breed, during the insemination campaign. Two sperm samples were obtained from each ram, given that the interval between ejaculations was 1-5 minutes. All ejaculates were diluted 1:12 with a 6AG sperm extender, prepared by us and containing sodium citrate, lactose, sucrose, egg yolk and glycerin. Semen from rams were collected using the method of artificial vagina, by an experienced operator. All of the obtained ejaculates underwent a preliminary macroscopic evaluation and those that did not meet the criteria were discarded. After that, vitality and enzyme activity were examined.

Analysis

Sperm viability was determined with a solution containing the dyes eosin and nigrosine. To 1 mL of the staining solution 30 µl of diluted semen was added using a micropipette. The mixture was incubated for 10-15 minutes at 37 ° C. The total of 10 µl of the prepared mix was smeared on glass while allowing to dry at room temperature. Microscopic analysis was performed on a Boeco BM-180 binocular microscope, 100X magnification, oil immersion. 100 spermatozoa were counted from each smear. The smears were made before freezing and after thawing the ejaculate.

Sperm freezing was carried out in semen straws according to the method of *Cassou (1964)*. All samples were thawed after one week. Thawing process was carried out in water bath at 37°C for 30 seconds.

Biochemical analysis

In an Eppendorf tube with a capacity of 1 ml, the ejaculates (diluted 1:12) were centrifuged at 3500 rpm for 15 minutes. The sperm plasma obtained from each Eppendorf was gently aspirated into sterile tubes with a micropipette and the enzymatic activity was determined. The activity of enzymes was determined spectrophotometrically with a set of reagents from Via Campania - Italy, according to the manufacturer's protocol. The enzymes LDH, GOT / AST and GPT / ALT were determined at a wavelength of 340 nm, and ALP of 405 nm. The activity was determined before freezing and after thawing the ejaculate. The data obtained are presented in U / L.

Statistical analysis

Data sets were analyzed using SPSS 23 to compare characteristics of the sperm using a Paired T-test. Differences between groups were assessed for significance by Student's t-criteria, and results were considered statistically significant at $P < 0.05$.

Results and Discussion

Freezing-thawing processes can cause irreversible damage to ram semen. Some authors reported that about 40-60% of sperm retain their motility after cryopreservation, but only about 20-30% retain biological function (*Medeiros et al., 2002*). In our study, the viability of sperm decreased by nearly 15% after cryopreservation (Fig. 1). Our results are in line with the 12% obtained for the same breed by *Andreeva and Stefanov (2020a)*. *Salmon and Maxwell (1995)* also reported reduction in sperm viability after freezing-thawing.

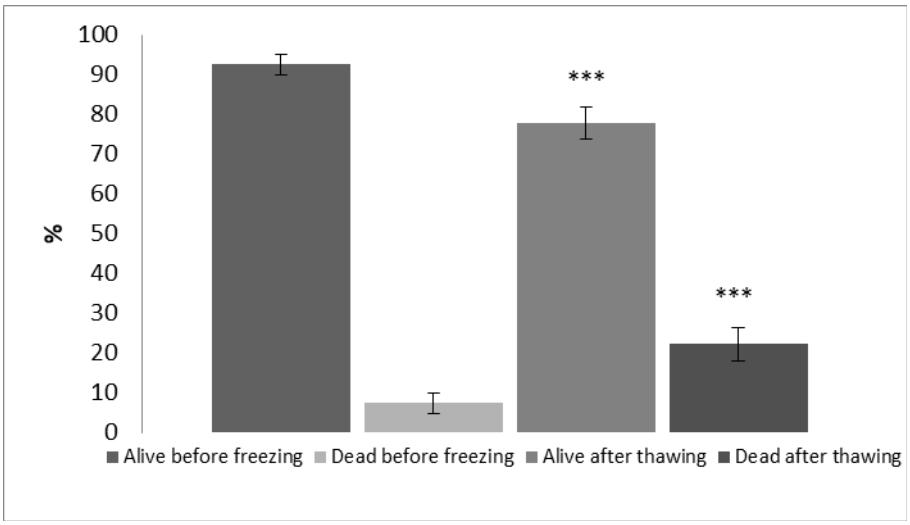

Figure 1. Sperm viability before freezing and after thawing (Mean ± SD, Significant differences *** P ≤0.001)

An important role in capacitance and fertilization has enzyme LDH (*Duan and Goldberg, 2003*). The activity we observed before freezing was lower than reported by some authors (*Zakrzewska et al.,2002; Tejaswi et al., 2016*), but significantly higher than that found by others (*Asadpour, 2012; Andreeva and Stefanov, 2020b*). After thawing of the ejaculates, the activity of the LDH enzyme in the sperm plasma decreased (P≤0.001) (Table 1). *Tejaswi et al. (2016)* obtained results different from ours. They reported an increase in enzyme activity after 24-hour refrigeration, explaining the result with damage to the membrane of the sperm and subsequent leaking of enzymes into the extracellular fluid due to cold shock. Our results are in line with those of *Fatihah et al. (2015)* who also observed decreased enzymatic activity after cryopreservation. According to *Brooks (2001)*, one of the main reasons for low fertility when using frozen-thawed ram sperm is the loss of LDH activity in most sperm after cryopreservation. An intracellular enzyme that regulates protein phosphorylation through the cAMP-dependent protein kinase pathway, which is required for sperm motility, is ALP. *Ciereszko et al. (1992)* reported a high correlation between ALP enzyme activity and sperm quality when sperm were under the stress. In our studies, its activity after cryopreservation decreased (Table 1). The leakage of ALP in the sperm plasma from the sperm can be used as a indicator to optimize the cooling and freezing steps during thawing for cryopreservation of ram semen (*Upreti et al. 1996*).

Table 1. Enzyme activity before freezing and after thawing

Parameters	Before freezing	After thawing
LDH, U/L	253.00 ± 73.97	178.88 ± 20.25***
ALP, U/L	731.13 ± 128.24	703.13 ± 164.16
GOT/AST, U/L	5.16 ± 2.03	2.25 ± 1.04**
GPT/ALT, U/L	46.13 ± 17.9	3.13 ± 1.55***

Note: Results are presented as Mean ± SD, Significant differences ** $P \leq 0.01$; *** $P \leq 0.001$

Transaminases (ALT-AST) are also intracellular enzymes used as a marker in sperm membrane damage. Leakage of these enzymes into sperm plasma is a sign of damage to sperm membranes (*Tuli and Singh, 1982; Katila, 2001; Alamaary et al., 2020*). In our studies, the activity of both enzymes after thawing decreased, and cryopreservation showed a significant depletion in ALT ($P \leq 0.001$) (Table 1). Most likely the process of cryopreservation led to inhibition of the enzyme and from there to the reporting of lower results. The values obtained for the extracellular activity of the enzyme are close to those reported by *Tejaswi et al (2016)*, who studied the activity of the enzyme in fresh samples and samples refrigerated at 4° C. *Rastegarnia et al. (2010)* obtained a different correlation than ours in studies conducted with buffalos. They report an increase in the enzyme activity of AST and ALT after cryopreservation. In our studies, the activity of enzymes in sperm plasma decreased, which is an indicator of good cryopreservation of samples and preservation of sperm fertility.

Conclusion

Cryopreservation reduces sperm viability, along with the activity of the enzymes LDH, ALP, GOT / AST and GPT / ALT. The studied enzymes can be used as indicators to optimize the protocols for cryopreservation of ram's sperm.

Analiza vitalnosti i biohemijski parametri seminalne plazme ovnova prilikom zamrzavanja i odmrzavanja

Bogdan Cekić, Hristiyana Kanzova, Georgi Petrov, Nevena Maksimović, Ivan Ćosić, Aleksandar Milovanović

Rezime

Istraživanje je imalo za cilj ispitivanje promena u procentu vijabilnih spermatozoida i promena u aktivnosti enzima LDH, ALP, GOT / AST, GPT / ALT u seminalnoj plazmi ovnova rase Lakon, pre i nakon krioprezervacije. Za tu svrhu, ispitivana je sperma pet ovnova, sa dva ejakulata po ovnu. Ejakulati su dobijeni metodom veštačke vagine, u toku sezone parenja. Svi ejakulati su razređeni razređivačem 6AG i zamrznuti po metodi Cassou sequin. Vijabilnost spermatozoida je određena na osnovu razmaza sa eozinom i nigrozinom, a aktivnost enzima je određena spektrofotometrijski. Rezultati istraživanja pokazuju da je udeo vitalnih spermatozoida nakon krioprezervacije smanjen za 15% ($P \leq 0.001$). Procesi zamrzavanja i odmrzavanja takođe smanjuju i aktivnost enzima LDH, ALP, GOT/ AST i GPT / ALP. Ispitivani enzimi, združeno sa vitalnošću spermatozoida, mogu da se koriste kao markeri za optimizaciju stope hlađenja i koraka u procesima zamrzavanja i odmrzavanja sperme ovnova.

Ključne reči: ovan, sperma, krioprezervacija, biohemijski parametri, vitalnost

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SEQUENCE ANALYSIS OF EXON 1 AND INTRON 1 OF GROWTH HORMONE GENE IN SIX CHICKEN GENOTYPES RAISED IN TROPICAL ENVIRONMENT

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Abstract: Chicken growth hormone (*cGH*) is a polypeptide hormone secreted by the pituitary gland which is responsible for several functions such as tissue growth and reproduction in chickens. This study was conducted to characterize six chicken genotypes using exon 1 and intron 1 regions of *cGH* gene sequences. One hundred and thirty-four (134) chickens comprising Normal feather (19), Naked neck (21), Frizzle feather (8), Arbor Acre (24), FUNAAB Alpha-1 (dihybrid) (31), and FUNAAB Alpha-2 (trihybrid) (31) were used for the study. Blood samples were collected from the birds into EDTA bottles for DNA extraction. The exon 1 and intron 1 regions of *cGH* were amplified using published primers. The product of the polymerase chain reaction was subjected to Sanger sequencing. DnaSP5 software was used to determine the diversity indices and MEGA6 software was used to determine the phylogenetic relationships among the six chicken genotypes and other chicken sequences. Fifteen (15) SNPs were identified in intron 1 and none in exon 1 of the *cGH* gene in all the six genotypes, and nine (9) of the SNPs occurred as transitions while others were transversions. The allele frequency ranged from 0.30 to 0.95 while the highest heterozygosity (0.66) was observed in mutation 410A>C in Naked neck genotype and lowest heterozygosity observed in Arbor Acre at SNP 330C>T. Polymorphic Information Content (PIC) was at the maximum in SNP 410A>C in Naked neck genotype with a value of 0.92. The exon 1 phylogeny tree revealed two clades where all the genotypes diverged. Intron 1 revealed two clades where Frizzle feather clustered with FUNAAB Alpha-1, Naked neck and FUNAAB Alpha-2 clustered together at one of the sub-clades in the second clade. Network analysis revealed Normal

feather chicken as the major ancestor of all the genotypes. The study concluded that intron 1 of *cGH* is polymorphic in all the six chicken genotypes investigated, and this can be used as candidate gene for selection in growth-related traits.

Key words: Diversity, FUNAAB Alpha**,** Growth hormone gene, Nigerian indigenous chicken, SNPs

Introduction

Poultry production is an important aspect in agriculture that contributes to the enhancement of the living standard of the people where income is provided, socio-cultural, and religious needs of farmers are met (*Akinola and Essien, 2011*). Chicken, out of all the poultry birds, is mostly consumed in the tropics because of its quality meat, egg, and short generation interval with reasonable feed efficiency (*Oluyemi and Roberts, 2007*). These attributes make it more preferred compared to other animal protein sources (*Peters, 2000*). Attention has been drawn to indigenous chickens in the tropics due to their adaptability and the quality of meat they produced (*Kaya and Yildiz, 2008*). Previously, it had been reported that Nigerian indigenous chickens exhibited slow growth rate and lower egg production compared to the exotic strains. *Oladeji (2016)* revealed that these inadequacies aided quest for better poultry chicken in Nigeria. However, the challenge being faced by breeders and geneticists in importing commercial chickens is enormous and this can be reduced by making use of the existing indigenous chickens to reduce the cost of importing exotic ones *(Chineke, 1998).* The indigenous birds are more adapted to the tropical environment with high disease resistance than their exotic counterpart (*Nwosu, 1987).* This led to the development of improved indigenous chicken, FUNAAB Alpha (Dual-purpose and broiler). The breed was developed for over six generations of selection and inbreeding, growth and productive performance were improved upon through crossbreeding with exotic lines *(Adebambo et al., 2018)*.

Growth rate in chicken is an economic trait and a set of multiple genes control growth performance in broiler production (*Vasilatos et al., 1997).* Growth hormone (*GH*) gene is one of the important genes responsible for various physiological functions in animals especially in growth and reproduction of chicken (*Enayati and Rahimimianji, 2009*). *Tanaka et al. (1992)* reported a nucleotide length of 4,101 base pairs with five exons and four introns. Polymorphisms in chicken growth hormone gene (*cGH*) have been studied generally using sequencing and restriction fragment length polymorphism (RFLP) and this had earlier been reported by different authors. One of these studies earlier reported is the polymorphisms in the intron 1 of chicken growth hormone gene which was positively associated with egg production in chickens according to

Kuhnlein et al. (1997). SNPs identified in intron 1 and exon 1 regions of *cGH* had been reported to be significantly associated with chicken body weight (*Ghelghachi et al., 2013; Kaur et al., 2008*) while some of the alleles identified in the introns had been linked to egg production traits and Marek's disease resistance in chickens (*Ip et al., 2001; Yan et al., 2003*). This present study aimed to determine the genetic diversity in the exon 1 and intron 1 of growth hormone gene of, Nigerian indigenous Normal feather, Naked neck, Frizzle feather, FUNAAB Alpha-1, and FUNAAB Alpha-2 chicken genotypes in comparison with the exotic Arbor Acre commercial strain.

Materials and Methods

Experimental birds and their sources

A total of 134 birds were sourced from FUNAAB Alpha project farm, Federal University of Agriculture, Abeokuta. The chickens consist of the following: 19 Normal feather, 8 Frizzle feather, 21 Naked neck, 24 Arbor Acre, 31 each of FUNAAB Alpha-1 and FUNAAB Alpha-2.

Blood sample collection

Blood samples were collected from 134 birds at their 8th week of age at the Biotechnology Laboratory of the Department of Animal Breeding and Genetics, Federal University of Agriculture, Abeokuta. This was done by collecting about 0.5ml of blood from brachial vein using a new needle and syringe per bird and stored in an ethylene diamine tetra acetic acid (EDTA) bottle to prevent coagulation.

DNA Extraction and Quantification

DNA was extracted from the whole blood samples using Qiagen DNA extraction kit following the manufacturer's procedure. The DNA was quantified using NanoDrop Spectrophotometer in order to determine the purity and the concentration of the DNA.

Amplification and sequencing of exon 1 and intron 1 of six chicken genotypes

Polymerase Chain Reaction (PCR) was carried out using designed primers Fwd 5'-ATCCCCAGGCAAACATCCTC-3' and Rev 5'-GACTATACAGAAAGAACCCAC-3' primers to amplify 776 bp region covering the exon 1 and intron 1 of the *cGH* gene. During the amplification, 5µL of genomic

DNA was added to a reaction mixture containing 17.8 µl of nuclease-free water, 2.5 µl of 10x PCR buffer, 1.5 mM of 25 mM MgCl2, 1mM of 5 mM dNTP, 1 µM of 10 µM forward and 1 µM of 10 µM reverse primer, and 0.2 U of 10 U/µl surf Hot Taq.

The PCR condition involved an initial denaturation step at 96°C for 15 minutes, 35 cycles of final denaturation at 95°C for 30 seconds, annealing at 60°C for 1 second and extension at 72°C for 90 seconds and a final extension at 72°C for 5 minutes carried out in a thermocycler (Agilent Surecycler 8800). To visualize the PCR product, gels were stained using ethidium bromide, microwaved and ran through a distinct well and a voltage of 100 volts after being loaded with a DNA ladder of 100 base pairs. The amplified fragment was visualised by an ultraviolet (UV) trans-illuminator and photographed. Sequencing of PCR product was carried out using Sanger-based capillary automatic sequencers (ABI prism 3130 system).

Trimming, Cleaning and Multiple Sequence Alignment

The nucleotide sequences were viewed with Bioedit software and trimmed using MEGA 6 software to remove noise in the sequences. The sequence obtained for exon 1 and intron 1 were aligned with *cGH* reference sequence NM_204359 and the alignment was carried out using Clustal W software (*Thompson et al., 1994*) integrated in MEGA 6 software.

Identification and Analysis of SNPs

Codon Code Aligner (Codon Code Corporation, Dedham, MA, USA) and MEGA 6 software were used to identify the SNPs in *cGH* gene in the six genotypes. The allele frequencies, heterozygosity and polymorphic information content were calculated manually.

Genetic Diversity

The genetic diversity indices were determined using DnaSP version 5 software (*Librado and Rozas, 2009).*

Evolutionary Analysis

The nucleotide frequency present in exon 1 and intron 1 of c*GH* gene in six chicken genotypes was determined using MEGA 6 software. MEGA6 software was also used to determine the phylogenetic relationship among exon 1 and intron 1 of *GH* gene in the six chicken genotypes used for this study and other downloaded sequences from the Genbank (Table 1). The phylogenetic tree was inferred using

unweighted pair group method and the reliability of the inferred tree was evaluated using bootstrap analysis of 1000 replications.

Table 1. Retrieved chicken growth hormone (*cGH*) gene sequences from NCBI with their accession numbers

Common name	Accession number	Base pair	Region	Origin
Dokki-4	MG906785	467	Exon	Egypt
Elmandra	MG906787	467	Exon	Egypt
El-salam	MG906782	467	Exon	Egypt
Inchas	MG906789	467	Exon	Egypt
Yellow wai chow	EF472953	776	Intron	China
Indian Cornish-3	JN403373	776	Intron	India
NG	JN403372	776	Intron	India
Rhode Island Red	EF452679	776	Intron	USA

Results and Discussion

PCR amplification of the *cGH* gene surveyed a region of 776 bp in six chicken breeds. Fifteen SNPs were identified at the intron 1 region of *cGH* gene (Table 2) while no SNP was found at the exon 1 region. The first SNPs identified frequently occurred in all the genotypes and SNPs 673T>C and 696G>A occurred frequently in Frizzle feather chickens while SNPs 279G>A was found specifically in Naked neck chicken genotype only. SNPs 330C>T was only peculiar to Arbor Acre and one of the FUNAAB Alpha chicken genotypes (TRH) and SNPs 410C>A was found only in Arbor Acre chicken genotype. The identified SNPs represent the occurrence of genetic variations in the intron 1 of growth hormone gene of the six chicken genotypes. This SNPs identified was higher than the one earlier reported by *Nie et al. (2005). Lyons et al. (2005)* reported more transition mutation more than transversion in their research and this is in line with the result in this research but in contrast with the report of *Nie et al. (2005)* where transversion was more than transition. According to *Ip et al. (2001*), high substitution rate of transition than transversion showed that selection had occurred severally among the chicken genotypes and this generally does not in any way favour transversion mutation.

Table 2. Single nucleotide polymorphisms identified in intron 1 of *GH* gene of Nigerian and improved indigenous chicken genotypes

Polymorphism	Type of mutation
267T>C	Transition
279G>A	Transition
282C>A	Transversion
310A>G	Transition
330C>T	Transition
410A>C	Transversion
410C>A	Transversion
453A>C	Transversion
453C>A	Transversion
454A>C	Transversion
492C>T	Transition
576C>T	Transition
576T>C	Transition
673T>C	Transition
696G>A	Transition

The high heterozygosity observed in some of the loci at the intron 1 in this study in Table 3 could be as a result of large heterozygous alleles present (*Chatterjee et al., 2010*). According to *Guo and Elson (1999),* polymorphic information content measures the informativeness of a genetic marker. *Hildebrand et al. (1992)* reported that a PIC greater than 0.7 is highly informative while bi-allelic markers PIC of 0.375 is at the maximum value and this can be observed in Naked neck with PIC 0.92 in Table 3. The Tajima's D estimated for all the chicken genotypes were not significant. Normal feather and Naked neck had negative values of -0.033 and -0.459 respectively. The negative and non-significant Tajima's D observed at the intron 1 in Normal feather in the first two indigenous chickens showed that the genotype has undergone positive selection with no demographic changes happening to them at the studied loci. This negative Tajima's D compares the average number of pairwise nucleotide differences with the total number of segregating sites (*Alonso and Armour, 2001; Durvasula, 2015*). This negative Tajima's D can be as a result of rare alleles produced by population expansion and purifying selection at the first intron (*Hahn et al., 2002*). The positive Tajima's D observed in other genotypes indicated that each population was undergoing balancing selection at each particular locus where alleles are at the intermediate frequencies (*Larsson et al., 2013; Durvasula, 2015*).

Table 3. Allele Frequencies, Heterozygosity and Polymorphic Information Content (PIC) of SNPs identified in intron 1 of *GH* gene of the six chicken genotypes

Genotype	SNP	MAF	H	PIC
Normal Feather	267T>C	0.89	0.19	0.17
	410A>C	0.78	0.34	0.28
	492T>C	0.89	0.19	0.17
	576T>C	0.78	0.34	0.28
Naked Neck	267T>C	0.88	0.21	0.19
	279G>A	0.88	0.21	0.19
	282C>A	0.82	0.29	0.29
	310A>G	0.88	0.21	0.19
	410A>C	0.71	0.66	0.92
	453A>C	0.82	0.29	0.25
	576T>C	0.88	0.21	0.19
Frizzle Feather	267T>C	0.50	0.50	0.38
	282C>A	0.62	0.47	0.36
	310A>G	0.87	0.23	0.20
	410A>C	0.62	0.47	0.36
	454A>C	0.50	0.50	0.38
	492C>T	0.62	0.47	0.36
	576T>C	0.75	0.38	0.37
	673T>C	0.87	0.23	0.20
	696G>A	0.87	0.23	0.20
Arbor Acre	267T>C	0.67	0.12	0.62
	282C>A	0.95	0.12	0.10
	310A>G	0.56	0.49	0.37
	330C>T	0.95	0.10	0.10
	410A>C	0.78	0.34	0.28
	453C>A	0.56	0.47	0.37
	454A>C	0.89	0.19	0.17
	492C>T	0.83	0.28	0.24
	576C>T	0.72	0.40	0.32
FUNAAB Alpha-1	267T>C	0.89	0.19	0.17
	310A>G	0.67	0.12	0.62
	410A>C	0.39	0.48	0.37
	453C>A	0.39	0.48	0.37
	454C>A	0.94	0.11	0.10
	492C>T	0.50	0.54	0.38
	576C>T	0.78	0.34	0.28

	267T>C	0.81	0.30	0.26
	282C>A	0.92	0.15	0.14
	310A>G	0.73	0.39	0.31
FUNAAB Alpha-2	410A>C	0.31	0.43	0.34
	453C>A	0.69	0.43	0.34
	454A>C	0.31	0.43	0.43
	492C>T	0.38	0.47	0.36
	576C>T	0.30	0.42	0.33

MAF= Major Allele Frequency; H= Heterozygosity; PIC= Polymorphic Information Content; SNP= Single Nucleotide Polymorphism

The diversity of chicken growth hormone gene at the intron 1 in all the genotypes studied is presented in Table 4. The number of polymorphic site is similar in three of the genotypes (Frizzle feather, Arbor Acre, and FUNAAB Alpha-2) studied while FUNAAB Alpha-1 with (7) polymorphic sites was closer to Naked neck genotype. The nucleotide diversity was highest in Frizzle feather and lowest in Normal feather (0.002) and this was lower than the one earlier reported by *Nie et al. (2005).* This was in line with the report of *Ilori et al. (2016)* where they reported that differences in effective population size of indigenous chicken could be responsible for a higher nucleotide diversity and this was recorded in Frizzle feather, an indigenous chicken. *Frankham et al. (2002)* had earlier reported that effective population size is one of the factors that affect SNP frequency and nucleotide diversity. The average number of nucleotide difference lowest value was observed in Normal feather (1.150) while the highest value was observed in Frizzle feather (3.929). According to *Abebe et al. (2015),* mating individuals that are genetically identical can result to a low heterozygosity and this was observed in Arbor Acre having the lowest heterozygosity in all the genotypes used. The genotypes used in this study were sourced and raised on the same farm and this may likely have contributed to the low heterozygosity observed.

The phylogenic tree at the exon 1 (Figure 1) showed that the six chicken genotypes can be separated into two clades with first clade, Dokki-4 chicken distinct itself on the evolutionary scale while other chickens diverged progressively. The second clade consists of the other chickens with Frizzle feather and Naked neck forming a sub-clade. This suggests that there was no difference within the genotypes and that they all shared a common ancestor.

Table 4. Diversity indices of intron 1 *GH* gene in Nigerian indigenous and improved indigenous chickens

Diversity indices	n	Eta	pi	k	Tajima's D
Normal feather	18	4	0.001	1.150	-0.033
Naked Neck	17	8	0.002	2.059	-0.459
Frizzle feather	8	9	0.006	3.929	0.646
Arbor Acre	18	9	0.004	3.033	0.563
FUNAAB Alpha-1	18	7	0.003	2.693	1.097
FUNAAB Alpha-2	26	9	0.005	3.382	1.394

n= Number of sample; Eta= total number of mutation; pi= nucleotide diversity; k= Average number of nucleotide change.

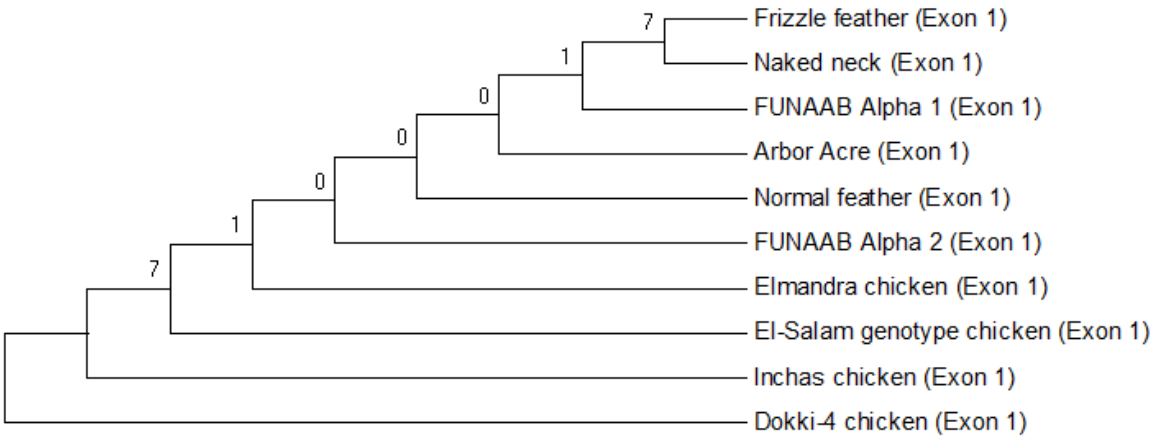

Figure 1. Phylogenetic relationship based on exon 1 of *GH* gene of the six chicken genotypes and other chicken genotypes.

At the intron sequence, commercial strain Rhode Island Red and Normal feather indigenous chicken are closely related since they form a sub-cluster with one another and they clustered with Indian Cornish-3 chicken and Yellow Wai Chow and also sub-cluster with Frizzle feather and FUNAAB Alpha-1 (Figure 2). This may be because the indigenous chicken was improved with an Indian chicken

to develop FUNAAB Alpha chickens and the it also suggested that all Indian chicken breeds are genetically similar at the inron1 of the growth hormone gene. The FUNAAB Alpha-2 closely related with Naked neck indigenous chicken and clustered with sub-cluster of NG and Arbor Acre chickens. This could be because of the development that has taken place in all the genotypes. Close relationship between intron 1 of normal feather and Rhode Island Red implied high comparability and evolution from a most common ancestor.

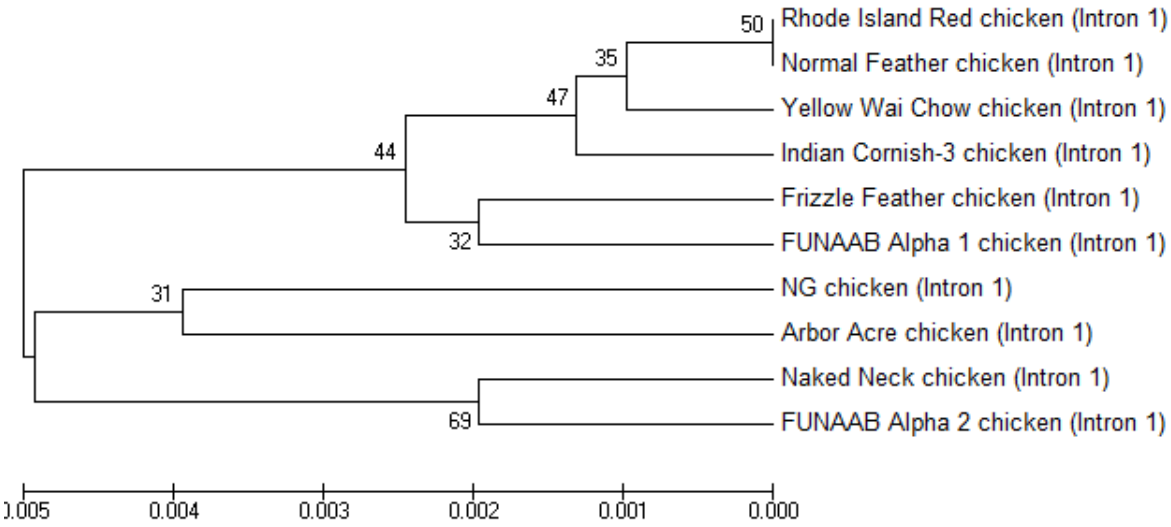

Figure 2. Phylogenetic relationship based on intron 1 of *GH* gene of the six chicken genotypes and other chicken genotypes.

Conclusion

Analysis of exon 1 and intron 1 of growth hormone gene in Normal feather, Naked neck, Frizzle feather, Arbor Acre, FUNAAB Alpha-1 and FUNAAB Alpha-2 chickens showed the existence of polymorphisms at the intron 1 region in all the chicken genotypes other than the exon 1. The Frizzle feather chicken had the highest number of polymorphism with higher nucleotide and haplotype diversity. Number of polymorphic site, haplotype diversity and nucleotide diversity showed the level of allelic variation in the GH gene of chicken which result in the genetic diversity of the GH gene in all the chicken genotype used. The phylogenetic tree showed that small genetic differentiation exists at the exon 1 among the chicken genotype studied while the intron 1 *GH* gene of Arbor Acre was closely related with NG strain from Indian while all the genotypes were

related with the same NG strain except Normal feather. This can also be seen in intron 1 phylogenetic tree where Normal feather sub-clustered with a commercial strain (Rhode Island Red) and clustered with an Indian strain (Yellow wai chow chickens). Associating the alleles with the genotypes of Normal feather, Naked neck, Frizzle feather, FUNAAB Alpha-1, and FUNAAB Alpha-2 is very necessary in order to know if they actually associate with production traits. This will help to channel the selection in any breeding program in order to improve their growth.

Analiza sekvence egzona 1 i introna 1 gena hormona rasta kod šest genotipova pilića uzgajanih u tropskom okruženju

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Rezime

Hormon rasta (*cGH*) je polipeptidni hormon koji luči hipofiza i koji je odgovoran za nekoliko funkcija kao što su rast i reprodukcija tkiva kod pilića. Ovo istraživanje je sprovedeno kako bi se okarakterisalo šest genotipova pilića korišćenjem egzon 1 i intron 1 regiona sekvenci gena *cGH*. Grupa od sto trideset i četiri (134) pileta, koja se sastojala od pilića normalnog perja (19), golovratih pilića (21), pilića kovrdžavog perja (8), Arbor Acre (24), FUNAAB Alpha-1 (dihibrid) (31) i FUNAAB Alpha- 2 (trihibrid) (31), je korišćena za ispitivanje. Uzorci krvi su sakupljeni od ptica u EDTA boce za ekstrakciju DNK. Regioni egzona 1 i introna 1 *cGH* su amplifikovani korišćenjem objavljenih prajmera. Proizvod lančane reakcije polimeraze je podvrgnut Sangerovom sekvenciranju. Softver DnaSP5 je korišćen za određivanje indeksa diverziteta, a softver MEGA6 je korišćen za određivanje filogenetskih odnosa između šest genotipova pilića i drugih sekvenci pilića. Petnaest (15) SNP-ova je identifikovano u intronu 1 i nijedan u egzonu 1 gena *cGH* u svih šest genotipova, a devet (9) SNP-ova se dogodilo kao tranzicije, dok su ostali bili transverzije. Frekvencija alela se kretala od 0,30 do 0,95 dok je najveća heterozigotnost (0,66) primećena kod mutacije 410A>C u genotipu golovratih pilića, a najniža heterozigotnost uočena u Arbor Acre na SNP 330C>T. Polimorfni informacioni sadržaj (PIC) bio je na maksimum u SNP 410A>C u genotipu golovratih pilića sa vrednošću od 0,92. Stablo filogenije egzona 1 otkrilo je dve klade u kojima su se svi genotipovi razišli. Intron 1 je otkrio dve klade gde su pilići kovrdžavog perja grupisani sa FUNAAB Alpha-1, golovratim i FUNAAB Alpha-2 grupisanim u jednoj od podklasa u drugoj kladi. Mrežna analiza je otkrila da su pilići sa normalnim perjem glavni predak svih genotipova. Studija je zaključila da

je intron 1 *cGH* polimorfan u svih šest ispitivanih genotipova pilića i da se može koristiti kao gen kandidat za selekciju u osobinama vezanim za rast.

Ključne reči: diverzitet, FUNAAB Alpha, gen hormona rasta, nigerijski autohtoni pilići, SNP

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Conflict of interest

The authors declare no conflict.

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COMMERCIAL POULTRY FEED IN SERBIA – CALCIUM AND PHOSPHORUS CONTENT SURVEY

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Abstract: Calcium and phosphorus represent very important nutrients when it comes to poultry diet formulations. In this paper, we will briefly discuss the relevance and nutritional requirements of these minerals in poultry feedstuffs as well as the average amounts in poultry feed commercially sold in the Serbian market. A total of 1,058 samples of standard complete feed mixtures for broilers and laying hens were collected from the Serbian market, produced by the four major Serbian manufacturers (I-IV) of animal feed over a period of five years (2017-2021). The samples were classified into five groups: broiler starter feed (n = 198) - SF, grower feed (n = 239) - GF, and finisher feed (n = 204) – FF; layers feed 1 (n = 204) – LF1, and layers feed 2 (n = 213) – LF2. This research suggests that the mineral composition of poultry feed is highly variable among manufacturers, but also among the batches of the same manufacturers. All manufacturers for the analyte in focus had values for certain batches that were outside the limits set by the Rulebook. In general, the results of our research indicate that the average content of total phosphorus in feed for broilers and laying hens in Serbia was mostly close to the minimum-to-mid value of the defined (and declared) range of permitted concentrations by the Rulebook, while the calcium content was predominantly close to the maximum-to-middle value. Based on the results of this study, it is recommended that feed manufacturers more frequently conduct an external analysis of samples of feed components and poultry feed products for the composition of these nutrients. Quality control of animal feed could be advised for poultry farms as well in order to make sure that the feed is actually within the parameters given by the manufacturers' declaration.

Key words: broilers, laying hens, feed quality, calcium content, phosphorus content

Introduction

Skeletal abnormalities are of economic importance to the poultry broiler industry as they result in increased mortality, culling, downgrading of carcasses, and trimming of deformed legs at processing. On the other hand, in layers, this is experienced through smaller or misshapen eggs, thinner shells, and changes in color, resulting in downgrades or rejections of the eggs by the industry. Some of these conditions and malformations can be prevented by reducing the growth rate and helping animals through diet modification (*Tablante et al., 2003; Jones et al., 2018*). Taking into account nutritional factors, the attention has to be turned to the two of the major macroelements involved - the amount and ratios intake of calcium and phosphorus which are necessary to ensure bone strength, especially during early life, as well as normal egg production. These elements are the main components of bone mineral structure, where they are present in the form of hydroxyapatite (*Schaible, 1941*; *Scott, 1971; Lukić, 2008*). In poultry and other animals, the physiological roles of these two macro-minerals are intricately linked (*Rath et al., 2000*).

Calcium metabolism is highly efficient and well regulated in poultry as it represents the most abundant mineral in the body and 99% of it can be found in the skeleton. Thus, the predominant role of Ca provides structural strength and support in bones (*Wilkinson, 2011*). According to *Rath et al. (2000)*, in the case of broiler chickens, deformities of the skeletal system will highly depend on the strength of leg bones (femur, tibia, fibula, and pad skeleton), considering their weight supporting and carrying functions. Bone strength is determined by various factors, such as bird growth rate, age, sex, genotype, and endocrinal metabolism, as well as anthropogenic factors that include feeding systems, quality of feedstuffs (presence of toxins in feed), and bird handling, e.g. mechanical damages caused during catch and slaughter. Severe calcium deficiency in broiler chickens causes bone injuries and poor growth, and it can lead to the development of conditions such as tibial dyschondroplasia and rickets, as well as increased mortality of the young population of poultry and reduced body weight in mature animals. Calcium also take a significant part in many of the biochemical pathways in the body such as muscle and nerve conduction, blood coagulation, eggshell calcification, and control of hormone secretions such as vitamin D3 and parathyroid hormone (*Wilkinson, 2011; Matuszewski et al., 2020; Han et al., 2022*).

Under certain physiological conditions, the need for calcium is greatly increased. For example, laying hens have a much higher calcium demand during reproduction than other vertebrates, as this mineral is necessary for maintaining the integrity of the skeleton as well as the eggshell formation. This can be seen from the mere fact that the laying hen must excrete about 2 - 2.5 g of calcium almost every day during the laying period, and 60 – 75% of the eggshell calcium can be provided by the feed (*Fleming, 2007; Lukić, 2008; Wilkinson, 2011*). Calcium

deficit in laying hens, as the short term occurrence (e.g. overnight during the process of eggshell formation) or a long term one (during a part of the production cycle), increases the transport of calcium from the bone depots in order to maintain normal eggs production, which can negatively reflect on the quality of bones, depending on the level and duration of deficit (*Lukić et al., 2011*). Research conducted by *Lukić et al, 2009* showed that the use of diets with 2.5 – 3.0 % calcium in the nutrition in young layers, regardless of the form of calcium incorporated in feed, can have a negative impact on, not only the productivity of layers, but also on and eggshell quality, with an increased incidence of defected eggs. On the other hand, excessive dietary calcium can lead to a decrease in body weight gain (BWG) and feed intake of broilers (*Han et al., 2022)*. An issue that is recognized industry-wide is that diets high in calcium concentrations used to achieve optimal bone density also result in a significantly wetter litter thus, there is an additional cost of excess calcium to be considered, particularly in winter or in humid environments (*Bedford et al., 2017*). Excess dietary calcium interferes with the bioavailability of other minerals such as phosphorus, magnesium, manganese, and zinc (*Selle et al., 2009*). In addition, high dietary calcium concentrations may reduce the energy value of the diet through the chelation of lipids (*Li et al., 2016*).

The second most prevalent mineral critical for the normal functioning of the poultry body is phosphorus. Almost 80% of it can be found in bones where is involved, along with calcium, in the bone mineralization process and normal growth performance. As an essential component of normal physiological functions, phosphorus is very important as it is used in energy pathways (e.g, phosphorus-containing compound is adenosine 5'-triphosphate (ATP)) and during the synthesis of phospholipids which are the building blocks of cell membranes. It also represents a major component of the phosphate buffer system which helps maintain osmotic and acid-base balance. Phosphorus cannot be synthesized in avian organisms, so it must be obtained from different dietary sources (*Wilkinson, 2011; Li et al., 2016; NRC, 1994*). The deficit in levels of phosphorus in poultry leads to similar outcomes as calcium deficiency which includes loss of skeletal integrity, changes in poultry appetite, subnormal growth, and weight loss in older animals (*Wilkinson, 2011).* On the other hand, according to *Li et al. (2016),* studies on the effects of dietary phosphorus levels on animal growth and bone development showed that high phosphorus intake can negatively impact calcium metabolism and bone properties, whereas low phosphorus diets will limit the growth of the animals. Research in poultry has concentrated on the amount of phosphorus used in feed to optimize its utilization because of its high cost and potential as an environmental pollutant (*Li et al., 2017).*

In order to meet calcium and phosphorus requirements, the diet is usually enriched with limestone, inorganic phosphorus supplements such as dicalcium phosphate, and, where allowed, meat and bone meal (*Han et al., 2022).* Since only a few of the known plants are rich in calcium, with extracted rapeseed meals being

one of them, calcium in its inorganic form has to be added as a supplement in poultry feed. It has been reported that approximately 20–30% of calcium found in plants occurs in the form of oxalates which birds cannot absorb. However, quite considerable amounts of phosphorus (around 50 – 90%) are present in plant-sourced feed ingredients. These forms of phosphorus, if present in plants, can be digested by poultry; however, such digestible forms usually account for only 30 – 40 % of the total phosphorus. The remaining phosphorus is present as phytate-bound in a complex that is only partially, and variably, available to the avian family (*NRC, 1994; Selle et al., 2009; Matuszewski et al., 2020*). Only about 10 percent of the phytate phosphorus in corn and wheat is digested by poultry (*NRC, 1994*). This led to the necessity of the incorporation of inorganic phosphates (mono and di-calcium phosphates) and external enzyme phytase in poultry diets. And since these mineral sources are rather expensive, coming from non-renewable sources, and partly responsible for the increased phosphorus load in the environment, they are less and less acceptable (*Elwinger et al.*, *2016*). Limestone is low-cost and most frequently used very good source of calcium consisting of ~97% calcium carbonate (CaCO3). It has a smooth structure and occurs in two forms: fine-grained and coarse-grained (most often used in laying hen feeds).

The nutritional requirements of the poultry animals are essential for good performance, and the poultry industry relies on the supply of commercially available ready-to-use feed. There are many commercial producers of poultry feed in Serbia and raw materials for the production of this feed are of different origins and quality, as well as final products. Also, the regulations in Serbia provide the ranges of required quantities for calcium and total phosphorus for different categories of poultry feed, which are very wide, but generally in line with very precise recommendations given by poultry hybrid breeder companies. All this in practice implies that different feed producers in Serbia may have different concentrations in the same poultry feed category.

Therefore, the present study aimed to investigate the concentrations of calcium and phosphorus in poultry feed available on the Serbian market. The established calcium and phosphorus levels in analyzed poultry feeds were, then, also compared to allowed ranges described in the Rulebook on the quality of animal feed (*Official Gazette R.S. No. 4/10, 113/12, 27/14, 25/15, 39/16, and 54/17*), by categories.

Material and Methods

Sample collection

A total of 1,058 samples of different poultry feeds were collected from the Serbian market from four different major Serbian manufacturers of animal feed over five years (from the beginning of January 2017 to the end of December 2021).

The samples were classified into five groups: Broiler Starter Feed, standard commercial feed mixture intended for hybrid broiler chickens aged 0-21 days (n = 198) – SF; Grower Feed intended for hybrid broiler chickens aged 22-35 days (n = 239) - GF, Finisher feed for broilers aged 36-42 days (n = 204) – FF; Layers feed 1, standard commercial feed mixture for hybrid laying hens aged 20-50 weeks (n = 204) – LF1; Layers feed 2 for hybrid laying hens over 50 weeks of age (n = 213) – LF2. After collection, feeds were labeled in accordance with laboratory practice and stored in polyethylene bags until analysis.

Sample preparation and measurement of calcium and phosphorus concentrations

Prior to analysis, all samples were homogenized and ground in a laboratory mill (ϕ 1 mm). Determination of calcium levels was performed using Atomic Absorption Spectrophotometer Varian AA-175 according to the official method SRPS ISO 27085:2008. Phosphorus concentration was determined using the spectrophotometry method, on spectrophotometer SPECOL 1300 (Analytik Jena, Germany) in accordance with official method SRPS ISO 6491:2002. Total calcium and phosphorus levels were expressed as % of the calcium/phosphorus of the feed sample. Each sample was carried out in triplicate for both, calcium and phosphorus levels.

Statistical analysis

The results of our research were statistically processed (Statsoft Inc. Statistics for Windows, Version 5.0) and presented in tables as the arithmetic mean ($\overline{X}$), the standard deviation (SD), coefficient of variation (CV), and the variation interval (minimum-maximum).

Results and Discussion

Table 1 shows the results of total calcium (%) content in samples collected from four major manufacturers of animal feed available on the Serbian market.

Table 1. Calcium content (%) in poultry feed by categories

Manufacturer	I (n = 267)	II (n = 222)	III (n = 272)	IV (n = 257)	Rulebook on the quality of animal feed*
Feed sample	$\bar{x}$ ± SD (minimum; maximum) (number of samples) (CV, %)				
SF	1.07 ± 0.12 (0.88; 1.40) (n = 65) (CV=11.59)	0.93 ± 0.11 (0.68; 1.08) (n = 48) (CV=11.90)	0.88 ± 0.08 (0.73; 1.01) (n = 52) (CV=8.54)	0.96 ± 0.09 (0.82; 1.10) (n = 33) (CV=9.34)	0.9-1.10
GF	1.05 ± 0.12 (0.78; 1.34) (n = 65) (CV=11.68)	0.85 ± 0.15 (0.48; 1.14) (n = 57) (CV=17.98)	0.86 ± 0.13 (0.65; 1.15) (n = 57) (CV=6.09)	0.96 ± 0.06 (0.84; 1.06) (n = 60) (CV=6.71)	0.80-1.00
FF	0.99 ± 0.23 (0.79; 1.27) (n = 60) (CV=23.08)	0.80 ± 0.16 (0.43; 0.99) (n = 42) (CV=19.60)	0.84 ± 0.07 (0.64; 0.90) (n = 53) (CV=8.18)	1.02 ± 0.05 (0.96; 1.18) (n = 49) (CV=4.98)	0.70-0.90
LF1	3.61 ± 0.27 (3.22; 4.00) (n = 58) (CV=7.60)	3.33 ± 0.19 (3.00; 3.66) (n = 38) (CV=5.71)	3.27 ± 0.16 (2.98; 3.48) (n = 56) (CV=4.86)	3.54 ± 0.23 (3.22; 3.83) (n = 52) (CV=6.38)	3.20-4.00
LF2	3.60 ± 0.25 (3.40; 3.87) (n = 59) (CV=6.97)	3.38 ± 0.18 (3.17; 3.65) (n = 37) (CV=5.19)	3.52 ± 0.21 (3.11; 3.76) (n = 54) (CV=6.09)	3.57 ± 0.24 (3.04; 3.84) (n = 63) (CV=6.71)	

*Rulebook on the quality of animal feed (*Official Gazette R.S." No.27/14, 25/15, 39/16, and 54/17*);
I, II, III, IV – manufacturers in the Serbian market;
n – number of samples; SD – standard deviation; $\bar{x}$ - arithmetic mean; CV - coefficient of variation
SF – Starter feed; GF – Grower feed; FF – Finisher feed; LF1 – Layer feed 1; LF2 – Layer feed 2

While observing, we can see that the contents of calcium varied not only between the manufacturers but also within the different batches of the same manufacturers over five years.

The arithmetic mean was somewhat higher for the groups SF, GF, and FF from the manufacturer I as well as the FF group from manufacturer IV than the limits given by the Rulebook on the quality of animal feed, while this value is slightly lower for the SF group from the manufacturer III. However, in the Rulebook on the quality of animal feed, there is section 105. that is referring to feed also meeting the quality requirements if the chemical quality analysis reveals deviations within the limits set by the Rulebook if they meet allowed tolerances for mixtures: if the calcium content is lower than 1%, the tolerance equals 0.15 % of the absolute value of the result (e.g. result of the arithmetic mean for the group SF from manufacturer III is 0.88 ± 0.15, which now when calculated, can be considered acceptable); if the calcium content is in range of 1 – 6 %, the tolerance equals to 15 % of the results relative value (e.g. result of the arithmetic mean for

the group GF manufacturer I is 1.13 ± 0.17, which when calculated may be considered within the acceptable ranges). This tolerance gives a rather wide range of results that would be acceptable. When this principle is applied to all of the arithmetic means from every feed group from each manufacturer it can be concluded that all of the mixtures were within the ranges described by the Rulebook. Special attention has to be turned to several batches from each manufacturer that, even when tolerance by the Rulebook applied, were not acceptable in quality, as their calcium content was higher than allowed for this poultry feed mixtures.

Taking into consideration maximum levels of total calcium content prescribed by the Rulebook, the research showed that all four of the manufacturers had values for certain batches that were outside the limits, with the exception of manufacturer III for feed LF1. The same manufacturer had calcium results for the FF feed (100% of them) above the maximum of the defined ranges. With the ranges calculated to adhere to allowed tolerances for each poultry feed, as described by the Rulebook, the results for total calcium for mixtures SF, GF, and FF for the manufacturer I had several batches results above permitted (7.69%, 15.38%, and 36.84% respectively, of all samples). Also, the portion of samples that were above the specified values, with applied tolerance by the Rulebook, for manufacturer II for the same three poultry feed categories were 8.33%, 7.02%, and 9.52%, respectively. For manufacturer III for the feed FF, 22.45% of samples were above the ranges with applied tolerances, and in manufacturer IV for feed SF that share was 5.77%. These batches are considered to be unacceptable in quality, by the Rulebook.

The research results of the analyzed feeds for broilers and laying hens in Serbia indicate that the average calcium content was predominantly close to the maximum-to-middle value of the defined (and declared) range of permitted concentrations according to the Rulebook. With that in mind, the research showed relatively high variability of calcium concentration in poultry feed, especially in broiler feed, both between different manufacturers and among different batches of the same product from the same manufacturer.

Table 2. Total phosphorus content (%) in poultry feed by categories

Manufacturer	I (n = 267)	II (n = 222)	III (n = 272)	IV (n = 257)	Rulebook on the quality of animal feed*
Feed sample	$\bar{x}$ ± SD (minimum; maximum) (number of samples) (CV, %)				
SF	0.77 ± 0.11 (0.56; 1.0) (n = 65) (CV=13.92)	0.60 ± 0.04 (0.52; 0.68) (n = 48) (CV=6.18)	0.74 ± 0.10 (0.56; 0.86) (n = 52) (CV=13.99)	0.64 ± 0.07 (0.54; 0.76) (n = 33) (CV=11.66)	0.65-0.85
GF	0.74 ± 0.09 (0.58; 0.95) (n = 65) (CV=11.68)	0.55 ± 0.06 (0.42; 0.64) (n = 57) (CV=11.14)	0.66 ± 0.06 (0.57; 0.77) (n = 57) (CV=8.76)	0.60 ± 0.04 (0.52; 0.66) (n = 60) (CV=5.81)	0.60-0.80
FF	0.65 ± 0.08 (0.47; 0.82) (n = 60) (CV=11.66)	0.47 ± 0.06 (0.36; 0.54) (n = 42) (CV=11.85)	0.62 ± 0.07 (0.53; 0.74) (n = 53) (CV=10.89)	0.63 ± 0.03 (0.57; 0.70) (n = 49) (CV=5.47)	0.50-0.70
LF1	0.73 ± 0.08 (0.52; 0.85) (n = 58) (CV=10.41)	0.56 ± 0.04 (0.51; 0.62) (n = 38) (CV=6.35)	0.69 ± 0.06 (0.62; 0.82) (n = 56) (CV=8.83)	0.61 ± 0.07 (0.52; 0.77) (n = 52) (CV=11.40)	0.65-0.85
LF2	0.74 ± 0.06 (0.57; 0.83) (n = 59) (CV=7.70)	0.53 ± 0.03 (0.46; 0.58) (n = 37) (CV=6.28)	0.59 ± 0.05 (0.52; 0.68) (n = 54) (CV=8.43)	0.62 ± 0.06 (0.54; 0.84) (n = 63) (CV=10.42)	0.60-0.80

*Rulebook on the quality of animal feed (*Official Gazette R.S." No.27/14, 25/15, 39/16, and 54/17*);
I, II, III, IV – manufacturers in the Serbian market;
n – number of samples; SD – standard deviation; $\bar{x}$ - arithmetic mean; CV - coefficient of variation
SF – Starter feed; GF – Grower feed; FF – Finisher feed; LF1 – Layer feed 1; LF2 – Layer feed 2

Table 2 shows the results of total phosphorus (%) content in samples of the same feed category. The same tolerance rules are applied to the total phosphorus content, as the one applied to the calcium content. The average value for the contents of this element differed, as manufacturer II showed the lowest results for all of the poultry feed mixtures, lower than prescribed (and declared). When taking into consideration the tolerance of 0.15 % from the absolute value of the results given as calculated arithmetic mean, these values could be regarded as acceptable, as there is a chance of them being in the ranges given by the Rulebook. However, it has to be mentioned that some of the results of the certain batches for the categories GF and FF sampled by manufacturer II, in regards to total phosphorus content were lower even with the tolerance level by the Rulebook applied.

Based on the ranges for the content of total phosphorus, set by the Rulebook, all manufacturers for the analyte in focus had values for certain batches that were outside the limits, with the exception of manufacturer III for the feed FF who had all of the samples within the ranges. Manufacturer II had results for all of

the mixtures from the group LF1 and LF2 below the minimum given by the Rulebook. But when we take into account the tolerance allowed by the Rulebook, the total phosphorus content was acceptable for almost all manufacturers with the exception of the manufacturer I, who had results of total phosphorus for the mixtures SF, GF, and FF (6.15%, 15.38%, and 18.33% respectively, of all samples) that were lower even with the allowed tolerances for mixtures applied. Also, manufacturer II for the GF mixture had 10.53% of the total samples that were lower than the ranges calculated to adhere to the Rulebook by applying the tolerance. Hence, these batches could be considered unacceptable.

In general, the results of our research indicate that the average content of total phosphorus in feed for broilers and laying hens in Serbia is close to the minimum-to-mid value of the defined (and declared) range of permitted concentrations by the Rulebook. The reason can be found in the fact that phosphorus sources are among the most expensive components for the production of poultry feed, as well as the established practice of adding phytase enzymes to commercial poultry feed (*Li et al., 2017*).

The research results also suggest that Serbian commercial food producers are relatively more precise in formulating total phosphorus than calcium in poultry feed (the average coefficient of variation in all producers was 9.03% for total phosphorus and 13.36% for calcium in broiler feed, and 6.72 % for calcium in food for laying hens). The source of these variations may be different, but it is usually associated with the variability in the quality of raw materials for production and the efficiency of the quality control system. For this reason, manufacturers are recommended to work on more efficient and frequent quality control, both raw materials that go into food mixtures and finished products.

Conclusion

This research suggests that the composition of two main minerals in poultry feed is highly variable among manufacturers. The variation was noticeable even between the batches of the same manufacturers, and results found in several batches were unacceptable and mislabeled. Based on the results of this study it may be advisable to conduct an extern analysis of poultry feed samples more frequently for the composition of these nutrients as well as the components that go into these mixtures prior to production. Quality control of animal feed could be advised for poultry producers as well in order to make sure that the feed is actually within the parameters given by the manufacturers' declaration.

Komercijalna hrana za živinu u Srbiji – analiza sadržaja kalcijuma i fosfora

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Rezime

Kalcijum i fosfor predstavljaju važne mikronutrijente u hrani za živinu. U ovom radu ćemo ukratko govoriti o značaju i nutritivnim potrebama ovih minerala u ishrani živine, kao i o prosečnim količinama ovih nutrijenata u hrani živine koja se može komercijalno naći na tržištu Srbije. Sa tržišta Srbije prikupljeno je ukupno 1.058 uzoraka od četiri velika srpska proizvođača stočne hrane u periodu od pet godina - od januara 2017. do decembra 2021. Uzorci su klasifikovani u četiri grupe: Potpune smeše za tov pilića I (n = 198) - SF, Potpune smeše za tov pilića II (n = 239) - GF, Potpune smeše za tov pilića III (n = 204) - FF, Potpune smeše za nosilje jaja za konzum I (n = 204) – LF1, i Potpune smeše za nosilje jaja za konzum II (n = 213) – LF2. Ovo istraživanje ukazuje na to da je mineralni sastav hrane za živinu veoma različit među proizvođačima, ali i među šaržama istog proizvođača. Nekoliko šarži proizvođača I (kod grupa SF, GF, FF) i IV (kod FF grupe), čak i kada se primene pravila za dozvoljena odstupanja za smeše iz Pravilnika o kvalitetu hrane za životinje, nisu bile prihvatljive po kvalitetu, jer je njihov sadržaj kalcijuma bio veći od dozvoljenog za analiziranu smešu hraniva. U pogledu sadržaja ukupnog fosfora, rezultati pojedinih šarži za kategorije GF i FF proizvođača II bili su niži i po primeni računice za dozvoljena odstupanja za smeše prema Pravilniku, pa se kao takve, smatraju neprihvatljivim. Na osnovu rezultata ove studije može se preporučiti da se češće vrše eksterne analize uzoraka hrane za živinu na sastav ovih nutrijenata, kao i komponenta koje ulaze u ove smeše. Kontrola kvaliteta stočne hrane može se savetovati i uzgajivačima živine kako bi se uverili da je hrana koju daju životinjama zaista u okviru parametara datih u deklaraciji proizvođača.

Ključne reči: brojleri, nosilje, kvalitet hraniva, sadržaj kalcijuma, sadržaj ukupnog fosfora

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CHANGES IN MEAT QUALITY OF *MUSCULUS LONGISSIMUS THORACIS ET LUMBORUM* AFTER 1 AND 4 MONTHS OF FROZEN STORAGE AT -18 ˚C, OBTAINED FROM LAMBS

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Abstract: The present study aimed to evaluate the effect of freezing and frozen storage duration (1 and 4 months) on the chemical composition and technological properties of lamb meat. Meat samples from Musculus Longissimus thoracis et lumborum were collected from Bulgarian Dairy Synthetic Population and their crosses with Ile-de-France and Mouton Charollais breed, with a slaughtering weight of 22-23 kg. Samples were frozen in a freezer at −18 °C. Frozen meat samples were thawed in a refrigerator at 4 °C 1 month and 4 months after freezing and analysed for determination of chemical composition and technological properties. It was established that lamb meat from the control group kept frozen for 1 month had statistically significantly higher water content compared to crosses of Bulgarian Dairy Synthetic Population with the Ile-de-France breed. Furthermore, dairy lambs were outlined with substantially higher meat fat content compared to crosses of Bulgarian Dairy Synthetic Population with the with Mouton Charollais. The chemical analysis of meat kept frozen for 4 months demonstrated significantly lower water content and higher dry matter and protein content in Ile-de-France crosses with Bulgarian Dairy Synthetic Population than in the control group. The meat protein percentage was higher while fat content – was lower in crossed Bulgarian Dairy Synthetic Population with Mouton Charollais compared to respective values in controls. Meat pH values of Mouton Charollais crosses differed significantly between the 1st and 4th month of frozen storage ($P \leq 0.01$). It was found out that the tenderness of meat frozen for 1 and 4 months was statistically significantly elevated in crosses of Bulgarian Dairy Synthetic Population with Ile-de-France and Mouton Charollais breeds compared to meat from control lambs.

Key words: sheep farming, meat quality, freezing

Introduction

It is common knowledge that meat provides essential nutrients e.g. protein, lipids, vitamins and minerals (*Akhtar et al., 2013; Akram et al., 2019; Devi et al., 2019*). According to these authors, the demands of today's busy and stressful life drive some consumers to buy meat for future consumption and store it frozen.
The primary cause for meat spoilage are microorganisms that replicate onto its surface and cause spoilage.To prevent this, conditions should be created so that microorganisms are either destroyed or could not replicate (*Kim et al., 2015; Ishevskiy and Davydov, 2017*). A technique for the creation of such conditions is freezing of products (*Lisitsyn et al., 2019*). Meat freezing is a practice for increasing its shelf-life (*Kiani and Sun, 2011; Akhtar et al., 2013; Akram et al., 2019; Tan et al., 2021*).

Literature data demonstrates that freezing of meat from various animals at different temperatures and for different periods causes both deterioration of some along with improvement of other technological and chemical properties of meat are available. It is generally agreed that meat tenderness was enhanced following freezing and thawing, in other words, the meat becomes more tender (*Shanks et al., 2002; Akhtar et al., 2013; Lagerstedt et al., 2008*). It is considered that the mechanism involved in meat tenderness increase is a combination of muscle fibres' degradation, meat aging and loss of structural integrity consequently to ice crystals formation (*Vieira et al., 2009*).

According to some researchers, the water content of cooled and frozen meat is different (*Khadeeja and Husseiny, 2017*). Others affirm that meat pH values do not change during freezing and thawing (*Akhtar et al., 2013; Daszkiewicz et al., 2017*). The cooking loss percentage of four bovine muscles increases both after meat freezing and with frozen storage period prolongation (*Cho et al., 2017*).

The present study aimed to evaluate the effect of freezing and frozen storage duration (1 and 4 months) on the chemical composition and technological properties of lamb meat.

Materials and Methods

The experiments were performed in the sheep farm of the Agricultural Institute, Stara Zagora, Bulgaria. A total of 27 lambs were studied: 18 F_1 crosses of Bulgarian Dairy Synthetic Population (BDSP) ewes and rams from meat breeds Mouton Charollais (MC) and Ile-de-France (IF), as well as 9 BDSP lambs. The animals were distributed into 3 groups – 9 BDSP lambs (first group, control), 9 F_1 BDSP×IF crosses (second group, experimental) and 9 F_1 BDSP×MC crosses (third group, experimental), according to the principle of initial body weight, sex

and type of lambing. All lambs were reared in group boxes supplied with feeders and drinkers. They were fed ad libitum (+ 5 to 10% residue) a ratio compliant with their age and met all requirements from nutrients and biologically active substances. The ration included concentrate and alfalfa hay. All animals had free access to tap water.

For slaughter analysis, three male twin lambs from each group were slaughtered in a licensed slaughterhouse in the Stara Zagora region at 22-23 kg body weight. The animals were transported to the slaughterhouse early in the morning with the licensed vehicle. From slaughter carcasses, samples from *Musculus Longissimus thoracis et lumborum* were collected for analysis of technological properties and chemical meat composition at the level of 11^{th}-12^{th} thoracic vertebrae. Meat samples were frozen in a freezer at −18 °C for 1 and 4 months.

Frozen meat samples were thawed in a refrigerator at 4 °C on post freezing months 1 and 4 and analysed for determination of chemical composition and technological properties. Meat pH was measured on Testo 205 pH meter. The water holding capacity of meat (WHC) was evaluated by the classical pressing method of *Grau and Hamm (1953)*, described by *Zahariev and Pinkas (1979)* with modifications of *Petrov (1982).* The water absorption capacity of meat (WAC) was determined by the method proposed by *Kyosev and Danchev (1979).* Cooking loss was determined by roasting a meat sample at 150° C for 20 min in a convection oven. Cooking loss percentage was calculated as the difference in sample weight prior to and after roasting. Meat tenderness was determined with DSD VEB Feinmess penetrometer (Dresden, Germany) and reported in penetrant degrees - °P. The water content of meat was determined by drying in a dryer at 105° C as per *BSS 15437:1982*. The protein content of meat was determined as per *BSS 9374:1982.* To this end, a Kjeldahl automatic distiller UDK 149 VELP Scientifica, Italy was used. Fat content was determined by Soxhlet extraction as per *BSS 8549:1992*. Mineral content was determined by the method described in *ISO 936:1998* based on ashing a meat sample in a muffle furnace.

The results were statistically processed with descriptive statistics and paired samples t-test.

Results and Discussion

Table 1 presents the results from the chemical analysis of lamb *Musculus Longissimus thoracis et lumborum* samples, stored frozen at −18 °C for 1 and 4 months. Meat water content after 1-month frozen storage varied from 75.03% to 76.08%. The meat of BDSP lambs was outlined with the highest water content – 76.08%. The differences between groups 1 and 2 were statistically significant

(P≤0.05). *Ablikim et al. (2016)* reported a water percentage of 76.16% in *Musculus longissimus dorsi* meat of Bashbay and Xinjiang Merino, 7-9 months of age, kept frozen for 1 month. This value is comparable to ours. The highest dry matter content of meat frozen for 1 month was obtained for IF crosses – 24.97%, while the lambs from the control group had the lowest meat dry matter percentage – 23.92%. The table showed that meat dry matter of group 2 exceeded substantially that of controls (P≤0.05). As for meat fat content after 1 month of frozen storage (Table 1), BDSP×MC crosses exhibited the lowest value – 2.63%, while the control group with the highest meat fat content - 3.32%. There was a statistically significant difference with respect to this trait between dairy lambs and BDSP×MC crosses (P≤0.05). The meat of group 3 had by 20.78% less fat compared to the control group. No significant differences between the groups in meat protein and mineral contents were observed.

Table 1. Chemical composition of lamb *Musculus Longissimus thoracis et lumborum*, stored at –18 °C for 1 and 4 months

Traits	Groups of animals						
	Group 1 BDSP (a)		Group 2 BDSP x IF (b)		Group 3 BDSP x MC (c)		Significance
Frozen storage at –18 °C for 1 month	n	$\bar{x}$±SD	n	$\bar{x}$±SD	n	$\bar{x}$±SD	
Water, %	6	76.08±0.60	6	75.03±0.21	6	75.96±0.25	a:b*
Dry matter, %	6	23.92±0.42	6	24.97±0.25	6	24.04±0.26	a:b*
Protein, %	6	19.50±0.70	6	20.77±0.26	6	20.21±0.12	NS
Fat, %	6	3.32±0.25	6	3.02±0.12	6	2.63±0.07	a:c*
Minerals, %	6	1.10±0.11	6	1.18±0.07	6	1.20±0.04	NS
Frozen storage at –18 °C for 4 months	n	$\bar{x}$±SD	n	$\bar{x}$±SD	n	$\bar{x}$±SD	Significance
Water, %	6	75.92±0.22	6	75.06±0.12	6	75.84±0.10	a:b*
Dry matter, %	6	24.08±0.12	6	24.94±0.10	6	24.16±0.22	a:b***
Protein, %	6	19.75±0.10	6	20.84±0.22	6	20.47±0.12	a:b*; a:c*
Fat, %	6	3.18±0.10	6	2.89±0.10	6	2.51±0.10	a:c*
Minerals, %	6	1.15±0.10	6	1.21±0.10	6	1.18±0.10	NS
Within-group statistical significance between months 1 and 4	1st month	4th month	1st month	4th month	1st month		4th month
Groups of lambs	Group 1 BDSP		Group 2 BDSP x IF		Group 3 BDSP x MC		
Water, %	NS		NS		NS		NS
Dry matter, %	NS		NS		NS		NS
Protein, %	NS		NS		NS		NS
Fat, %	NS		NS		NS		**
Minerals, %	NS		NS		NS		NS

BDSP - Bulgarian Dairy Synthetic Population, IF - Ile de France, MC - Mouton Charollais, * - P≤0.05, ** - P≤0.01, *** - P≤0.001, NS - not significant

According to Table 1, meat samples frozen for 4 months showed the highest water content in the control group - 75.92%. Dry matter of *Musculus Longissimus thoracis et lumborum* after 4 months of frozen storage was the highest in IF crosses – 24.94%, followed by MC crosses with 24.16% and finally, controls (24.08%). There were very significant differences between meat dry matter of lambs from control group and BDSP×IF crosses ($P \leq 0.001$) – in the latter, it was by 3.57% higher. The BDSP×IF crosses had also the highest meat protein percentage - 20.84%, followed by BDSP×MC crosses and BDSP lambs -20.47% and 19.75%, respectively. The meat of crosses with MC and IF had statistically significantly higher protein content compared to controls ($P \leq 0.05$). Protein content of frozen lamb similar to ours – 18.60% was reported by other authors (*Khadeeja and Husseiny, 2017*). As fat content was concerned, the highest values were found out in BDSP lambs – 3.18%. This trait differed considerably between controls and BDSP×MC crosses: the latter had by 21.07% less fat.

The chemical composition of meat after 1 and 4 months of frozen storage did not show any statistically significant within-group differences in groups 1 and 2. On the contrary, BDSP×MC crosses showed substantially difference meat fat content between the two periods ($P \leq 0.01$).

Table 2 presents the chemical composition of cooled meat from the same crosses from a previous study of ours (*Ivanov, 2019*). Data demonstrate that meat water content of the three groups varied with a tendency for reduction from cold storage to 1-month and 4-month frozen storage. Water percentage in cooled meat in control lambs according to *Ivanov (2019)* was 76.69%, after 1 month frozen storage it was 76.08% and after 4 months: 75.92%. Cooled BDSP×IF meat had a water content of 75.37% (*Ivanov, 2019),* whereas the values after 1 and 4 months of frozen storage were 75.03% and 75.06% respectively. For BDSP×MC crosses, cooled meat contained 76.59% water (*Ivanov, 2019*), and frozen meat: 75.96% after 1 month and 75.84% after 4 months. Our results were in line with those of *Khadeeja and Husseiny (2017)* who reported higher water content in cooled meat compared to frozen meat.

On the contrary, meat dry matter content of lamb meat tended to increase in association with cold to 1-month or 4-month frozen storage. Data of *Ivanov (2019)* demonstrated that cooled meat of BDSP lambs had a dry matter content of 23.31%, which increased to 23.92% after 1 month in the freezer and to 24.08% after 4 months of frozen storage. The same tendency was observed for dry matter content of BDSP crosses with either Ile-de-France or Mouton Charollais. The reduced water content in frozen vs fresh meat is associated with water extraction during freezing and release of more liquid during meat thawing. Dry matter increase is explained with reduction of water during the freezing process. Meat is composed of water and dry matter. The increase in water leads to decrease in dry matter and vice versa, as both are negatively correlated. Meat dry matter is represented by protein, fat and minerals. Thus, increased dry matter corresponds to increased proportions of protein, fat and minerals, as shown in Tables 1 and 2.

Table 2. Chemical composition of *Musculus Longissimus thoracis et lumborum* from light carcasses at post mortem hour 24 (*Ivanov, 2019*)

Traits	Groups of animals						
	Group 1 BDSP (a)		Group 2 BDSP x IF (b)		Group 3 BDSP x MC (c)		Significance
	n	$\bar{x}\pm S_{\bar{x}}$	n	$\bar{x}\pm S_{\bar{x}}$	n	$\bar{x}\pm S_{\bar{x}}$	
Water, %	3	76.69±0.70	3	75.37±0.20	3	76.59±2.19	NS
Dry matter, %	3	23.31±0.70	3	24.63±0.20	3	23.41±2.19	NS
Protein, %	3	2.70±1.29	3	3.08±1.49	3	2.53±1.20	NS
Fat, %	3	19.37±0.55	3	20.48±1.39	3	19.69±2.37	NS
Minerals, %	3	1.23±0.15	3	1.07±0.07	3	1.19±0.34	NS
Fat, % DM,	3	11.53±5.20	3	12.48±6.00	3	10.80±5.10	NS
Protein, % DM	3	83.18±4.43	3	83.17±5.96	3	84.10±5.76	NS

DM - dry matter, NS - Not Significant, n - number of samples, BDSP - Bulgarian Dairy Synthetic Population, IF - Ile de France, MC - Mouton Charollais

Table 3 presents the results about the technological properties of lamb meat, kept frozen for 1 and 4 months. It demonstrated that meat pH after 1 month of frozen storage varied within a narrow range for the three groups. The lowest meat pH was found out in BDSP – 5.30, and the highest values were found in BDSP×Mouton Charollais crosses - 5.41. The differences between controls and Mouton Charollais crosses were statistically significant ($P\leq0.05$). Meat pH of 7-9-month-old Bashbay's and Xinjiang Merino's *Musculus longissimus dorsi* after 1-month frozen storage was reported to be 5.83 *(Ablikim et al., 2016)* which is slightly higher than our values. As WHC results were concerned, values were comparable and insignificant. The same was true for WHC in distilled water which were 3.76%, 3.00% and 3.47% in group 1, 2 and 3 respectively. Inconsistent differences were found out for WHC in saline. The lowest values were found out in BDSP×Mouton Charollais crosses – 11.43%, while the highest meat WHC was exhibited by lambs from control group - 16.54%. The tenderness of meat stored at –18 °C for 1 month, the highest values corresponding to the most tender meat were those of BDSP×Mouton Charollais crosses – 313.28 °P, and the lowest meat tenderness (e.g. the toughest or hardest meat) was that of BDSP lambs - 236.11°P. This parameter differed statistically significantly between controls and crosses with Ile-de-France ($P\leq0.05$) as well as between controls and crosses with Mouton Charollais ($P\leq0.001$). The most significant meat cooking losses occurred in lambs from the control group – 39.07%, and the lowest – in crosses with Ile-de-France – 35.36%. Average cooking loss in the meat of BDSP×Mouton Charollais lambs was 37.96%. *Ablikim et al. (2016)* reported cooking loss percentage of 34.95% in the meat from two Chinese sheep breeds, stored frozen for one month at –18 °C.

Others *(Choi et al., 2018)* gave proofs for cooking losses of 41.59% in lamb meat kept frozen at – 18 °C for one month.

Table 3. Technological properties of lamb *Musculus Longissimus thoracis et lumborum*, stored at – 18 °C for 1 and 4 months

Traits	Groups of animals						Significance
	Group 1 BDSP (a)		Group 2 BDSP x IF (b)		Group 3 BDSP x MC (c)		
Frozen storage at –18 °C for 1 month	n	$\bar{x}$±SD	n	$\bar{x}$±SD	n	$\bar{x}$±SD	
pH	9	5.30±0.05	9	5.35±0.14	9	5.41±0.09	a:c *
WHC, %	9	29.24±4.71	9	27.95±2.76	9	29.42±3.39	NS
WHC – distilled water	9	3.76±2.33	9	3.00±2.16	9	3.47±2.66	NS
WHC – saline	9	16.54±5.88	9	12.99±3.09	9	11.43±3.17	NS
Tenderness,°P	18	236.11±51.68	18	280.56±49.12	18	313.28±28.21	a:b*; a:c***
Cooking losses, %	9	39.07±5.23	9	35.36±3.98	9	37.96±4.64	NS
Frozen storage at –18 °C for 4 months	n	$\bar{x}$±SD	n	$\bar{x}$±SD	n	$\bar{x}$±SD	Significance
pH	9	5.40±0.11	9	5.40±0.09	9	5.27±0.08	a:c***
WHC, %	9	26.19±3.39	9	26.08±1.56	9	29.62±1.83	NS
WHC – distilled water	9	7.65±4.53	9	3.67±2.97	9	7.04±4.53	NS
WHC – saline	9	17.87±4.72	9	11.87±5.89	9	18.84±5.03	NS
Tenderness,°P	18	233.83±39.39	18	292.28±35.83	18	316.39±28.69	a:b***; a:c***
Cooking losses, %	9	37.59±1.65	9	34.14±3.28	9	36.44±4.51	a:b*
Within-group statistical significance between months 1 and 4	1st month	4th month	1st month	4th month	1st month		4th month
Groups of animals	Group 1 BDSP x BDSP		Group 2 BDSP x IF		Group 3 BDSP x MC		
pH	*		NS		**		
WHC, %	NS		NS		NS		
WHC – distilled water	*		NS		NS		
WHC – saline	NS		NS		*		
Tenderness,°P	NS		NS		NS		
Cooking losses, %	NS		NS		*		

BDSP - Bulgarian Dairy Synthetic Population; IF - Ile de France; MC - Mutton Charollais;
n - number of samples, °P - Penetrant degrees, * - P ≤ 0.05, ** - P ≤ 0.01, *** - P ≤ 0.001, NS - not significant

The lowest pH of meat stored for 4 months (Table 3) was observed in BDSP×Mouton Charollais crosses – 5.27, whereas pH values of lambs from the other two groups were equal (5.40). The differences between control meat pH and that of BDSP×Mouton Charollais crosses were significant (P≤0.001).

WHC values showed the same tendency for samples frozen for 1 month. Again, the lowest WHC of meat was found out in Ile-de-France crosses – 26.08%, the highest values - 29.62% were those of BDSP×Mouton Charollais crosses.

There were no significant differences with respect to WHC is water and WHC in saline among the three groups. Similarly, meat tenderness after 1 months of frozen storage, showed the highest values in BDSP×Mouton Charollais crosses, followed by lambs from group 2 and BDSP lambs - 316.39 °P, 292.28 °P and 233.83 °P, respectively.

Similarly to the first analysis of cooking losses (meat frozen for 1 month), the same tendency was outlined. The lowest cooking losses in the meat kept frozen for 4 months was found out in BDSP×Ile-de-France crosses, followed by BDSP×Mouton Charollais crosses and control lambs – 34.14%, 36.44% and 37.59 respectively.

Technological properties of meat frozen for 1 and 4 months demonstrated statistically significant differences with respect to meat pH ($P \leq 0.05$) and WHC in saline ($P \leq 0.05$). In group 2, there were no relevant within-group differences. In group 3 (BDSP×Mouton Charollais crosses), substantial differences were observed for meat pH ($P \leq 0.01$), WHC in saline ($P \leq 0.05$) and cooking losses ($P \leq 0.05$).

Table 4 presents the results for technological properties of cooled lamb Musculus Longissimus Lumborum from another study of ours *(Ivanov et al., 2017).* The comparison of data from Tables 3 and 4 indicated that meat pH in BDSP lambs did not change considerably after freezing for 1 and 4 months compared to cooled samples. The cooled meat pH was 5.40, after 1-month frozen storage: 5.30, and after 4-month frozen storage: 5.40. No statistically significant differences were found between meat pH of crosses with Ile-de-France after either cold storage or frozen storage for 1 and 4 months: 5.37, 5.35 and 5.40. Higher differences were observed in meat pH of BDSP×Mouton Charollais lambs: pH 5.38 in cooled meat, 5.41 in meat frozen for 1 month and 5.27 for meat frozen for 4 months.

Data from Table 3 and 4 regarding WHC showed increased percentages from cooled to frozen meat in the three groups of lambs. The reported value in BDSP lambs for cooled meat was 19.19% (*Ivanov et al., 2017*). Meat WHC of the same group after 1-month frozen storage was 29.24%, and after 4-month frozen storage: 26.19%. In the first groups of lambs, WHC was deteriorated after freezing for one month by 52.37%, and after freezing for 4 months: by 36.48%. A similar trend was outlined for BDSP×Ile-de-France crosses – worse WHC by 17.78% after one month and by 9.90% after 4 months. In BDSP×Mouton Charollais crosses, the water holding ability of meat became worse after 1 month of frozen storage – by 26.00%, whereas after the 4th month: by 26.85%. This worsening of meat WHC was due to tissue damage caused by the formation and movement of ice crystals and its reduced ability to hold water *(Choi et al., 2018).*

Table 4. Technological properties of lamb *Musculus Longissimus Lumborum* after 24 hours of cold storage at 0-4 °C (*Ivanov et al., 2017*)

Characteristics	Group						Significance
	Group 1 BDSP (a)		Group 2 BDSP x IF (b)		Group 3 BDSP x MC (c)		
	n		n		n		
pH values on the 24th hour post mortem	9	5.40±0.07	9	5.37±0.09	9	5.38±0.12	a:b**
WHC, %	9	19.19±3.59	9	23.73±1.07	9	23.35±3.60	a:c*
WAC/distilled water, %	9	7.91±3.69	9	7.60±3.54	9	7.17±6.73	a:c*
WAC/saline solution, %	9	14.33±6.43	9	15.77±2.57	9	13.46±3.65	NS
Meat tenderness values, °P	15	236.47±61.69	15	330.80±52.69	15	240.00±70.57	a:b***
Cooking losses, %	9	41.75±2.92	9	43.96±2.32	9	45.02±2.27	a:c***

BDSP - Bulgarian Dairy Synthetic Population; IF - Ile de France; MC - Mutton Charollais;
N - number of samples; °P - Penetrant degrees; * - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$, NS - not significant

As meat tenderness was concerned, BDSP lambs showed no consistent differences between cooled *(Ivanov et al., 2017)* samples and those kept frozen for 1 and 4 months – 236.47 °P, 236.11 °P and 233.83 °P respectively. The meat tenderness in IF crosses varied between cooled samples - 330.80 °P, those stored frozen for a month - 280.56 °P and 4 months - 292.28 °P. Tenderness values decreased by 15.19% and by 11.64% in meat frozen for 1 month and 4 months respectively compared to cooled meat in the same group. Tenderness of meat in BDSP×Mouton Charollais crosses was also variable. The tenderness of cooled meat was 240.00 °P, that kept frozen for 1 month – 313.28 °P, and after 4 months of frozen storage – 316.39 °P, It was noted that the meat of MC crosses that have been frozen for 1 month was more tender by 30.53% than cooled meat whereas the meat frozen for 4 months: by 31.83%. Data from other researchers evidence that the tenderness of lamb meat increased after freezing, e.g. shear force values were higher for cooled meat compared to frozen/thawed meat (*Wiklund et al., 2009; Qi et al., 2012*). The trend is the same for frozen and then thawed horse meat samples (*Seong et al., 2017*) and veal meat from *Longissimus dorsi* (*Shanks et al., 2002*) – the shear strength of the chilled sample is higher than that of the thawed sample.

Cooking losses decreased from cooled to frozen meat (for 1 and 4 months) in all three groups of lambs. Cooking loss percentages of BDSP meat were 41.75% (cooled meat), 39.07% (1-month frozen storage) and 37.59% (4-month frozen storage). In the dairy lamb group, cooking losses of cooled meat were by 6.42% and 9.96% higher than those of meat frozen for 1 and 4 months respectively. For the BDSP×Ile-de-France crosses, cooking losses were 43.96% for cooled meat,

35.36% for meat kept frozen for a month and 34.14% for meat strode frozen for 4 months. Cooled meat of BDSP×Mouton Charollais crosses demonstrated by 19.07% higher cooking loss than meat stored frozen for 1 month and by 21.86% compared to meat frozen for 4 months. Cooking loss values for these crosses was 45.02% (cooled meat), 37.96% and 36.44% (meat stored frozen for 1 and 4 months respectively). Thus, compared to frozen meat stored for 1 and 4 months, respective cooking loss percentages of cooled meat were by 15.68% and 19.06% higher.

Conclusion

The results from the present study allowed concluding:

The meat samples from control lambs had statistically significantly higher water content than the meat of Ile-de-France crosses after 1 month of frozen storage.

The meat of lambs from the Bulgarian Dairy Synthetic Population, kept frozen for 1 month, demonstrated statistically significantly higher fat content compared to BDSP×Mouton Charollais crosses.

In the meat stored frozen for 4 months, water content was substantially reduced along with significantly higher dry matter and protein percentages in BDSP×Ile-de-France cross compared to lambs from the control group. Furthermore, protein content in the meat of BDSP×Mouton Charollais crosses was significantly higher, yet fat content: was significantly lower compared to those in dairy lambs.

Meat water content showed as insignificant decrease from cold to frozen storage. Meat water is encountered in three forms – bound, free and immobilized. Free and immobilized water is important during meat storage. Free water is easily removed from the meat and lost, being held by weak forces. Entrapped water is extracted by drying or pressing and is easily transformed into ice during freezing. The decrease in water content in meat from cold to frozen storage is due namely to its extraction during freezing and release after thawing. On the other hand, the insignificant increase in dry matter is due to the negative water-dry matter relationship, so as water decreases, dry matter (protein, fat and minerals) in meat becomes greater.

Meat tenderness after 1 and 4 months of frozen storage was statistically significantly higher in crosses with Ile-de-France and Mouton Charollais breeds compared to that of meat of control lambs.

Cooking losses of meat stored frozen for 4 months were statistically significantly lower in BDSP×Mouton Charollais crosses compared to those in controls.

Promene u kvalitetu *Musculus Longissimus thoracis et lumborum* jagnjadi nakon jednog i četiri meseca skladištenja na -18 °C

Nikolay T. Ivanov, Stayka S. Laleva, Georgi I. Kalaydzhiev, Daniela N. Miteva

Rezime

Cilj ovog istraživanja je bio da se proceni uticaj trajanja zamrzavanja i zamrznutog skladištenja (1 i 4 meseca) na hemijski sastav i tehnološka svojstva jagnjećeg mesa. Uzorci mesa *Musculus Longissimus thoracis et lumborum* prikupljeni su od grla bugarske sintetičke mlečne populacije i njihovih meleza sa rasama il de frans i šarole, klanične težine 22-23 kg. Uzorci su zamrznuti u zamrzivaču na -18°C. Uzorci smrznutog mesa su odmrznuti u frižideru na 4°C jedan, odnosno četiri meseca nakon zamrzavanja i podvrgnuti analizi u cilju određivanja hemijskog sastava i tehnoloških svojstava. Utvrđeno je da je jagnjeće meso iz kontrolne grupe koje je držano zamrznuto jedan mesec imalo statistički značajno veći sadržaj vode u poređenju sa melezima bugarske mlečne sintetičke populacije sa rasom il de frans. Štaviše, jagnjad mlečne populacije je označena sa znatno većim sadržajem masti u mesu u poređenju sa melezima bugarske sintetičke mlečne populacije sa šaroleom. Hemijska analiza mesa koje je držano zamrznuto 4 meseca pokazala je značajno manji sadržaj vode i veći sadržaj suve materije i proteina kod meleza rase il de frans sa populacijom bugarskih mlečnih grla nego u kontrolnoj grupi. Procenat proteina u mesu je bio veći, dok je sadržaj masti – bio niži kod meleza bugarske sintetičke mlečne populacije sa rasom šarole u poređenju sa odgovarajućim vrednostima u kontrolama. Vrednosti pH mesa meleza sa šaroleom značajno su se razlikovale između 1 i 4 meseca zamrznutog skladištenja ($P \leq 0,01$). Utvrđeno je da je mekoća mesa zamrznutog 1 i 4 meseca statistički značajno povećana kod meleza bugarske mlečne sintetičke populacije sa rasama il de frans i šarole u poređenju sa mesom kontrolne jagnjadi.

Ključne reči: ovčarstvo, kvalitet mesa, zamrzavanje

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Correction of the paper "Effect of year, lambing season, sex and birth type on early performance in MIS lambs" by Bogdan Cekić, Dragana Ružić-Muslić, Nevena Maksimović, Violeta Caro-Petrović, Krstina Zeljić Stojiljković, Ivan Ćosić, Radmila Beskorovajni, published in Biotechnology in Animal Husbandry vol. 37 (4), pages 255-262, 2022 (DOI: 10.2298 / BAH2104255C):

In Tables 1 and 2 instead of BW90, kg correct is BW60, kg.
In Table 1 the values of statistical parameters for ADG2, g and ADG3, g are changed:

Table 1. Average values and variability of studied traits

Traits	n	x̄	SD	Variance	Min	Max	CV (%)
BW0, kg	1573	4.41	0.99	0.99	1.80	7.80	22.55
BW30, kg	1414	14.11	2.80	7.86	4.10	23.50	19.87
BW60, kg	1383	24.05	3.86	14.93	12.00	36.50	16.07
ADG1, g	1412	319.87	74.97	5619.89	6.67	653.33	23.44
ADG2, g	1382	**329.84**	**70.90**	**5026.81**	**33.33**	**706.67**	**21.45**
ADG3, g	1382	**324.99**	**55.48**	**3078.03**	**133.33**	**520.00**	**17.07**

*BW0= birth weight; BW30= body weight at 30 days; BW60= body weight at 60 days; ADG1= average daily gain from birth to 30 days; ADG2= average daily gain from 30 days to 60 days; ADG3= average daily gain from birth to 60 days

Editorial

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

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[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

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

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