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CONTENTS

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BIODIVERSITY AND PREDICTED METABOLIC FUNCTIONS OF THE RUMEN MICROBIOTA DEPENDING ON FEEDING HABITS AT DIFFERENT STAGES OF THE PHYSIOLOGICAL CYCLE OF DAIRY COWS

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Abstract

Under intensified cattle breeding, combined stress factors, in particular, extremely high milk productivity, inconsistency of neuro-humoral and hormonal regulation of feed intake and milk production, negative energy balance, feeds excessive in starch negatively impact the rumen microbiota and, consequently, a cow's physiology. This paper for the first time shows the phases of dairy cow lactation cycle as an important factor that determines the relative abundance of non-attributable bacteria from the candidate families vadinBE97 and WCHB1-41 which functions are practically not studied. The most pronounced changes in the metabolic potential of the microbiota, namely the inhibition of various metabolic pathways in the rumen chyme, e.g., energy (tricarboxylic acid cycle), protein, carbohydrate, lipid, including volatile fatty acid (VFA) synthesis, occurred in cows during stable and declining milk production phases as compared to dry, fresh and milked cows. The aim of this work is to study the composition and metabolic potential of the rumen microbiome in dairy cows during different physiological phases. The experiment (the JSC Agrofirma Dmitrova Gora, Tver Province, the summer 2020) was performed on 15 black-and-white Holsteinized dairy cows (*Bos taurus*) of the second and third lactations. The cows were assigned to five groups (5 cows each), including the dry cows (on average 30 days before calving, group I), the cows of 20 milking days (group II), of 90 milking days (group III), at day 208 of lactation (group IV), and in late lactation phase when the milk production is declining (day 310, group V). Dairy cows' diets were calculated using AMTS.Cattle.Professional software in accordance with the accepted requirements. Total DNA was extracted from rumen chyme samples (a Genomic DNA Purification Kit, Fermentas, Inc., Lithuania). The NGS procedure (a MiSeq platform, Illumina, Inc., USA) was performed using primers to the 16S rRNA V3-V4 region and reagents for NGS library preparation (Nextera® XT IndexKit, Illumina Inc., USA), PCR product purification (Agencourt AMPure XP, Beckman Coulter Inc., USA), and sequencing (MiSeq® ReagentKit v2, 500 cycle, Illumina Inc., USA). Bioinformatic analysis was performed with Qiime2 ver. 2020.8 software. Noise sequences were filtered by the Deblur method. The de novo phylogeny was constructed using the MAFFT software package. To analyze the taxonomy, the reference database Silva 138 (https://www.arb-silva.de/documentation/release-138/) was used. Reconstruction and prediction of the functional content of the metagenome was performed using PICRUSt2 software package v.2.3.0 with MetaCyc database for metabolic pathways and enzymes. Total RNA was isolated from the chyme samples (Aurum Total RNA kit, Bio-Rad, United States) followed by cDNA synthesis (iScript

RT Supermix kit, BioRad, USA). The relative expression of the bacterial L-lactate dehydrogenase gene *Ldh-L* and the *Ldb 0813* gene associated with D-lactate dehydrogenase synthesis was assessed using quantitative PCR (SsoAdvanced Universal SYBR Green Supermix kit, Bio-Rad, USA). The16S metagenomic sequencing revealed a decrease ($p \leq 0.05$) in the rumen bacteria α-diversity in group IV and group V. We have found twelve superphila and phyla of microorganisms. The superphylum *Bacteroidota* and the phylum *Firmicutes* we refer to the dominant rumen bacteria (up to 59.94±1.86 and 46.82±14.40 % of the population, respectively). The superphylum *Actinobacteriota* bacteria not found in lactating cows appeared only in dry cows. The bacteria of the superphylum *Armatimonadota* disappeared from the rumen of fresh cows and during stable lactation phase, and of the phylum *Chloroflexi* — during early and stable lactation phases. The cows differed significantly in eight bacterial families, the *Muribaculaceae*, *Prevotellaceae*, *Erysipelatoclostridiaceae*, *Oscillospiraceae*, *Ruminococcaceae*, *Saccharimonadaceae*, and candidate families WCHB1-41 and vadinBE97. The rumen genera *Asteroleplasma*, *Sharpea*, *Moryella*, *Oribacterium*, *Shuttleworthia* appeared after calving and persisted in the next phases of lactation. These bacteria were absent in dry cows. The predicted functional capability of 17 metabolic pathways of the microbiome varied ($p \leq 0.01$) in cows of different groups. The most pronounced changes, namely the suppression of various metabolic pathways in the rumen chyme, occurred in groups IV and V compared to group I, group II, and group III ($p \leq 0.01$). An increase in the expression of the *Ldh-L* ($p \leq 0.01$) and *Ldb 0813* ($p \leq 0.05$) genes associated with the synthesis of lactate dehydrogenases was characteristic of fresh cows compared to dry cows. There was a significant increase in the expression of the rumen bacteria genes *Ldh-L* (10.6-fold, $p \leq 0.001$) and *Ldb 0813* (2.8-fold, $p \leq 0.05$) when lactation declined as compared to group IV.

Keywords: rumen microbiome, ruminants, dairy cows, diet, starch, cellular tissue, NGS-sequencing, PICRUSt2, MetaCyc, metabolic pathway

Today's dairy farming strategy needs to maximize the utilization of nutrients in the feeds whilst minimizing the risks of rumen ecosystem dysbiosis, digestive and metabolic disorders. Microbial fermentation covers 70% of dairy cows' energy needs. This points to the critical role of rumen microbiota in animals' metabolism and to the need to investigate which microorganisms are present there and how they function [1].

Ruminants have one unique metabolic feature: due to the evolutionary symbiosis with the rumen microbiota, they are able to digest plant fiber that contains cellulose, hemicellulose, and xylans [2-4]. Microorganisms present in the digestive system, albeit of different phylogeny, are inextricably linked; their symbiotic relations and metabolic networks play a central role in the rumen functioning, especially in fiber digestion [5]. Thus, Akin et al. [6] and Janssen et al. [7] have shown the interdomain interdependence in the case of bacteria and archaea. Bacteria degrade lignocellulosic material and produce hydrogen [6] that methanogenic archaea need [7]. Similarly, most of the lactate produced by one category of microorganisms is further metabolized by the bacteria that need this substance [8]. This results in the production of volatile fatty acids (VFAs) that are absorbed through the stratified squamous epithelium of the rumen. On the one hand, VFAs directly replenish the energy substrates, mainly for gluconeogenesis [9], and thus make an important contribution to the formation of the animal's metabolic pool [10]. On the other hand, lactate-to-VFA conversion promotes the buffering of the rumen's contents, which is an important acidosis prevention mechanism [11].

Under intensified cattle breeding, combined stress factors, in particular, extremely high milk productivity, inconsistency of neuro-humoral and hormonal regulation of feed intake and milk production, negative energy balance, feeds excessive in starch negatively impact the rumen microbiota and, consequently, a cow's physiology. As it is known, dry cows' diet should be designed to lower the risks of postpartum complications, which dictates the use of high-quality feed rich in fiber and moderate in concentrates. This stimulates chewing activity and rumen motility, raises pH, restores rumen microbiota, and thus helps recover the animal's general metabolic activity [12-14]. Some researchers [4, 15, 16] note the extreme diversity of rumen microbiota that in dry cows mainly consists of cellulo-

lytic bacteria.

According to Henderson et al. [17], unlike in monogastric animals, a ruminant's diet contributes much more to the formation of the rumen microbiome than the host's genotype and individual physiology. Physiological status and milk productivity are most responsive in newly calved cows [18]. Such cows have rapidly rising glucose demand; thus, energy output for milk production exceeds energy intake from feed, which results in a negative energy balance [14]. Low glucose and insulin concentrations in blood plasma trigger a physiological mechanism designed to overcome energy deficiency: the body actively mobilizes triglycerides from adipose tissue in order to cover the rising energy demands [19]. All this induces an imbalance of glucogenic and lipogenic compounds in plasma, which has dire consequences for metabolism [20].

Starch- and monosaccharide-based feeds, which are staple foods for lactating cows, are known to negatively affect the physiology of recently calved cows [21, 22]. Yet, starch and monosaccharides are the key sources of glycogenic precursors (e.g., propionate) and fermentable energy for the rumen microorganisms. Nevertheless, excessive production of short-chain fatty acids lowers rumen pH [23], as increased VFA production increases the population of acid-resistant bacteria of the phylum *Bacteroidetes* and culls the population of *Firmicutes* [24]. *Bacteroidetes* actively synthesize lactate as an intermediate of starch fermentation; coupled with the microbiota's inability to maintain an appropriate acid-base balance in the rumen, this process results in intraluminal accumulation of protons, with pH further falling below the physiological range [14, 25, 26]. The condition is commonly known as rumen acidosis. Acidosis multiplies the risks of fatty liver and ketosis, metritis, and abomasum displacement. Such disorders have far-reaching consequences for cattle health and productivity, resulting in lameness [27, 28], worsening reproduction, lower milk productivity, and shorter productive lifespan [29, 30]. Zebeli *et al.* argue [14] that the incidence and severity of metabolic disorders depend on feed intake and on the starch-richness of the diet. A gradual increase in concentrates by 0.25 kg of dry matter (DM) per day after calving enables better rumen microbiota adaptation than a 1 kg increase in the daily intake of concentrate (DM) [18]. This combination of a fast transition to a high-energy diet, postpartum and lactation stress, and negative energy balance is associated with a high risk of metabolic disorders in newly calved cows [31, 32].

During an increase in yield, stabilization and decline of lactation, cows are still at high risk of rumen microbiome disturbances and the resulting metabolic disorders [14]. Some researchers [26, 33, 34] have observed that high concentrations of soluble starch in cow rumen during early lactation were associated with a rising population of *Lactobacillus* sp. and of the amylolytic microorganism *Streptococcus bovis*, which produce lactate [34]. This induced lowering pH and suppression of cellulolytic and VFA-producing bacteria; it also disrupted the VFA synthesis processes.

Studying the rumen microbiome is one of the fundamental approaches to developing effective measures to prevent metabolic disorders at dairy farms. Of interest is a comprehensive analysis of change in the composition and metabolic potential of the rumen microbiome in various physiological periods in dairy cows. Both Russian researchers using classical methods of microbiology [35-37] and their international colleagues [13, 14, 23] have studied in detail the effects that physiological periods and diets have on the rumen microbiome taxonomy. However, the specific biological and metabolic functions of the rumen microbiome as exhibited in different physiological periods in dairy cows are yet to be fully discovered. Besides, microbiomes in animals raised in accordance with the Russian dairy practices have been little studied by means of molecular biology.

This paper for the first time shows the phases of the dairy cow lactation

cycle ~~as~~ an important factor that determines the relative abundance of non-attributable bacteria from the candidate families vadinBE97 and WCHB1-41 whose functions have been understudied thus far. The most pronounced changes in the metabolic potential of the microbiota, namely the inhibition of various metabolic pathways in the rumen chyme, e.g., energy (tricarboxylic acid cycle), protein, carbohydrate, lipid, including volatile fatty acid (VFA) synthesis, occurred in cows during stable and declining milk production phases as compared to dry, freshly calved, and milked cows.

The aim of this work is to study the composition and metabolic potential of the rumen microbiome in dairy cows during different physiological phases.

Materials and methods. The experiment was performed on 15 black-and-white Holsteinized dairy cows (*Bos taurus*) of the second and third lactations at JSC Agrofirma Dmitrova Gora, Tver Region, summer 2020. The choice of a commercial farm as a pilot site was based on the experiments described in earlier studies of microbiome in dairy cows [38, 39].

The animals were kept under the same conditions in tie-stall barns. They were split into five groups, 3 cows each: Group 1 of dry cows (30 days before calving on average), Group 2 of newly calved cows (20 days of milking on average), Group 3 of cows in early lactation (90 days in milk in average), Group 4 of cows in mid-lactation (208th day in milk), and Group 5 of cows in late lactation (310th day in milk). Dry cows were selected on the basis of the expected calving day. The number of animals per group was adjusted following the guidelines found in earlier studies [40, 41]. Group 1 had an average live weight of 703 kg. The daily average yield was 27.0 l/head, the fat content of milk was 3.7%, and the weight was 637 kg in Group 2. The daily average yield was 38.5 l/head, the fat content of milk was 3.7%, and the weight was 650 kg in Group 2. Group 4 and 5 animals had an average live weight of 667 and 681 kg, respectively.

Chyme (30-50 g per cow) were sampled manually from the upper ventral sac of the rumen using a sterile probe under as aseptic conditions as possible.

Total DNA was isolated from the samples using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) per the kit manual [3]. Testing was based on DNA sedimentation from the substrate using solutions for cell wall lysis, DNA sedimentation, 1.2 M sodium chloride and chloroform.

The bacterial community of the rumen was detected by NGS sequencing using MiSeq (Illumina, Inc., USA) with primers for the V3-V4 region of 16S rRNA (forward primer 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′, reverse primer: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGATCTAATCC-3′).

Sequencing was performed using Nextera® XT IndexKit for library preparation (Illumina, Inc., USA), Agencourt AMPure XP kit for PCR product purification (Beckman Coulter, Inc., USA) and MiSeq® ReagentKit v2 (500 cycles) for sequencing (Illumina, Inc., USA). The obtained sequences had a maximum length of 2×250 bps.

Bioinformatics data analysis was performed by Qiime2 ver. 2020.8 (https://docs.qiime2.org/2020.8/). Paired reads were aligned after the initial import of sequences into the Qiime2 format. Then the sequences were filtered by quality using the default settings. Noise sequences were filtered by the Deblur method using the maximum trimming length of 250 bps (https://msystems.asm.org/content/msys/2/2/e00191-16.full.pdf). To construct the de novo phylogeny, the MAFFT software was used with subsequent masked sequence alignment. For taxonomy analysis, we used the Silva 138 reference database (https://www.arb-silva.de/documentation/release-138/).

The resulting table of operational taxonomic units (OTUs) was used to

compute α-diversity indices in Qiime2 plugins and to plot curves of the OTU number as a function of the number of reads. No additional transformations were applied for the statistical analysis of diversity indices.

Reconstruction and prediction of the functional content of the metagenome were performed using the PICRUSt2 software package v.2.3.0 (https://picrust.github.io/picrust/). The software was used as recommended; all default settings were kept. Metabolic pathways and enzymes were analyzed using the MetaCyc database (https://metacyc.org/). The predicted MetaCyc metabolic pathway profiles were tested for the abundance of amplicon sequence variants [42].

Total RNA was isolated from chyme samples using the Aurum Total RNA kit (Bio-Rad, United States) per the manufacturer's manual. cDNA was synthesized on the RNA matrix using the iScript RT Supermix kit (Bio-Rad, USA) [43]. Quantitative PCR was used to analyze the relative expression of the *Ldh-L* and *Ldb 0813* genes of lactic acid fermentation-capable bacteria. Amplification with the primers of the *Ldb 0813*, the gene associated with the synthesis of D-lactate dehydrogenase (F: 5′-CTGGGATCCGTTGAGGGAGATGCTTAAG-3′, R: 5′-TCCGAAGCTTTTAGTTGACCCGGTTGAC-3′) and L-lactate dehydrogenase (gene *Ldh-L*) (F: 5′-CATCAAAAAGTTGTGTTAGTCGGCG-3′, R: 5′-TCAGCTAAACCGTCGTTAAGCACTT-3′) was run on a DT Lite-4 unit (a detection amplifier, NPO DNA-Technology LLC, Russia). Amplification conditions: 1 min at 95 °C (1 cycle); 15 s at 95 °C, 1 min at 50 °C (45 cycles). The reaction mixture for amplification (SsoAdvanced Universal SYBR Green Supermix kit, Bio-Rad, USA) was prepared in accordance with the manufacturer's protocol.

Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method [44] using the 16S-ribosomal subunit gene of prokaryotes (primer pair: F — 5′-AGGCC-TTCGGGTTGTAAAGT-3′, R — 5′-CGGGGATTCACATCTCACT-3′) for reference.

The Chao1, Shannon (H), and Simpson (D) biodiversity indices for the rumen microbiome were calculated as described in [45].

For mathematical and statistical processing of the results, a single-factor analysis of variance (ANOVA) was applied (Microsoft Excel XP/2003, R-Studio Version 1.1.453, https://rstudio.com). To correct Type I error, Tukey's HSD test was applied (https://www.rdocumentation.org/pack-ages/stats/versions/3.6.1/topics/TukeyHS). The results are shown as means (*M*) and standard errors of the mean (±SEM), with $p \leq 0.05$ as a threshold for the significance level verified by Student's *t*-test.

Results. Cows' diets for each group were designed in AMTS.Cattle.Professional (https://agmodelsystems.com) in accordance with the common standards [46-48], see Table. Premix recipes were adjusted for each group specifically. The premixes were designed to fully cover micronutrient needs for required productivity.

NGS sequencing revealed composition and structure of the bacterial community of bovine rumens. The Shannon and Chao1 indices were found not to differ between Groups 2 (newly calved) and 1 (dry cows), $p \geq 0.05$, see Fig. 1. This is an interesting finding, as both metrics estimate species diversity [49]. Newly calved cows were expected to show a more pronounced change in the diversity, whether an upward or downward change, from their dry counterparts, as they had been exposed to several negative factors on top of drastic change in the diet structure, calving stress, and the onset of lactation. Thus, Bach et al. [50] observed a decline in the H index of the digestive microbial community in freshly calved cows as compared to dry cows. In this study, the H index rose slightly ($p \leq 0.05$) in early lactation as compared to newly calved cows. A sharp decline ($p \leq 0.05$) in the H and Chao1 indices occurred as compared to the preceding physiological periods during mid-lactation in Group 4 and late lactation in Group 5. Digestive

microbiota is generally known to become less diverse in cases of dysbiosis, particularly in cases of treatment with antimicrobials [51] or gastroenteritis [52]. Nevertheless, the diversity of the microbial communities in the rumen is a fairly stable indicator, as in this study, Shannon and Chao1 indices did not decline in newly calved cows nor in early lactation. The Simpson index calculated for each tested group did not have significantly different values ($p \geq 0.05$), see Fig. 1, C.

Nutritional factors of diets for black-and-white Holsteinized dairy cows (*Bos taurus*) in different physiological periods

Parameter	Group				
	I	II	III	IV	V
Dry matter (DM) content, %	39.02	45.10	42.31	43.50	42.50
DM consumption, kg	11.82	18.94	25.00	25.60	27.60
Metabolic energy provided by the diet, % of the normal value	106.86	103.82	104.87	105.52	100.20
Metabolic protein provided by the diet, % of the normal value	103.47	97.08	104.57	105.33	105.43
Crude protein (CP), %	14.86	16.44	17.48	17.50	17.60
Net energy of lactation, MJ/kg	6.29	6.47	6.86	6.60	6.30
Neutral detergent fiber, % of DM	41.63	25.75	28.28	29.28	30.20
Acidic detergent fiber, % of DM	26.64	16.25	17.75	18.50	18.80
Rumen-degradable protein, % of CP	56.0	57.0	52.0	55.0	56.0
Rumen-nondegradable protein, % of CP	44.0	43.0	48.0	45.0	44.0
Starch, % of DM	16.20	23.54	26.64	26.45	24.50
Ca, % of DM	0.49	0.82	0.74	0.71	0.68
P, % of DM	0.51	0.39	0.40	0.40	0.41
Mg, % of DM	0.47	0.39	0.37	0.39	0.42

N o t e. For description of the groups, see the Material and methods section.

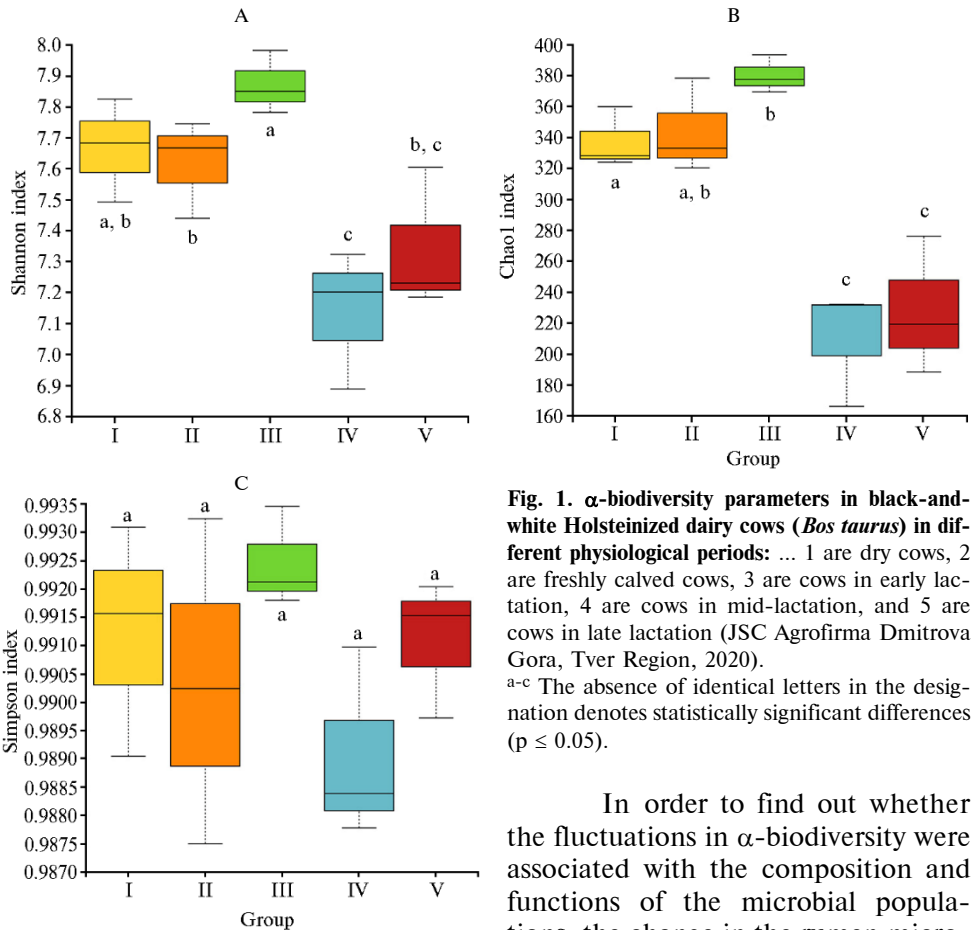

Fig. 1. α-biodiversity parameters in black-and-white Holsteinized dairy cows (*Bos taurus*) in different physiological periods: ... 1 are dry cows, 2 are freshly calved cows, 3 are cows in early lactation, 4 are cows in mid-lactation, and 5 are cows in late lactation (JSC Agrofirma Dmitrova Gora, Tver Region, 2020).

[a-c] The absence of identical letters in the designation denotes statistically significant differences ($p \leq 0.05$).

In order to find out whether the fluctuations in α-biodiversity were associated with the composition and functions of the microbial populations, the change in the rumen micro-

biota taxonomy was estimated using NGS sequencing data.

Twelve superphyla and phyla of microorganisms were found in the rumen microbiota, see Fig. 2, where the superphylum *Bacteroidota* and the phylum *Firmicutes* could be considered the dominant bacteria in the rumen, as they represented up to 59.94±1.86 and 46.82±14.40% of the microbiota, respectively. These two taxonomic groups can be considered to be the core of the bacterial microbiome, as they have a significant presence in nearly all *Bos taurus* [12, 53, 54]. The dominant bacteria found in this study were likely responsible for most of the substance transformation in the rumen, in particular, that of cellulose, hemicellulose, starch, organic acids, and protein, as these substances are diet components and intermediate energy substrates [1].

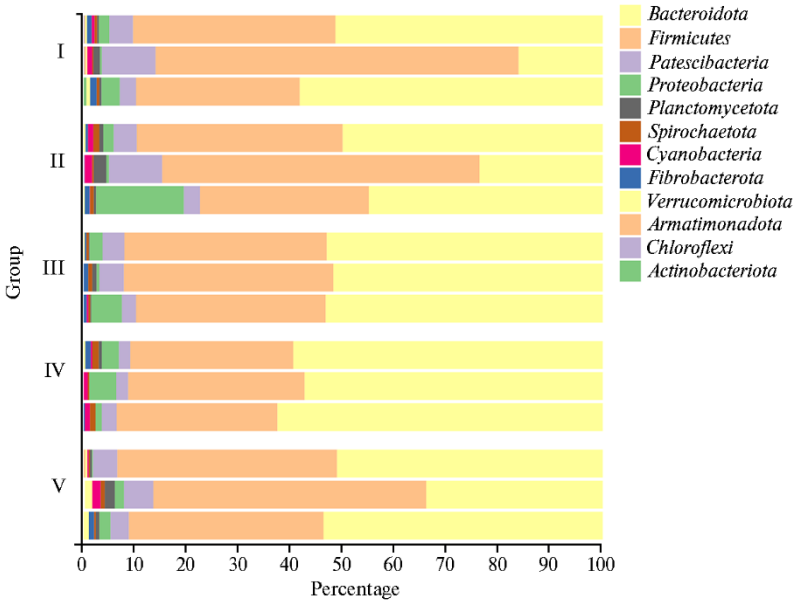

Fig. 2. Rumen microbiome taxonomy in black-and-white Holsteinized dairy cows (*Bos taurus*) in different physiological periods: I — dry cows, II — fresh cows, III — lactating cows, IV — stable lactation, V — late lactation period (NGS sequencing; JSC Agrofirma Dmitrova Gora, Tver Province, 2020).

The decline in the α-diversity of the rumen microbiome was found to be related to the reduction in the relative population of some taxa, which was registered at the phylum level. Thus, bacteria of the superphylum *Actinobacteriota* were eliminated completely from the rumen in lactating cows but were found in dry cows; *Armatimonadota* were not found in newly calved cows or cows in mid-lactation; the phylum *Chloroflexi* was not found in cows in early or mid-lactation. The disappearance of these microorganisms might have contributed negatively to metabolism in lactating cows. Thus, *Actinobacteriota* are common symbionts in eukaryotes [55]; their cellulolytic enzymes enable more efficient fiber breakdown [56].

The superphylum *Verrucomicrobiota* had a lesser presence in cows in early or mid-lactation compared to dry cows ($p \leq 0.05$). Despite the ubiquity of these microorganisms, pure cultures only contain a few isolates [57], making their ecological significance ambiguous still. *Verrucomicrobia* are known to have a significant presence in termite intestines where they metabolized plant polysaccharides into acetate [57]. Some members of the superphylum *Verrucomicrobia* have recently been discovered to be able to oxidize methane (an ability that had earlier been observed in proteobacteria only) in an acidic environment (pH 0.8-2.0) [58]. Thus, a decrease of *Verrucomicrobiota* in this experiment, which was observed in early and mid-lactation, could be associated with the suppression of fiber

digestion and methanogenesis in cases of high-concentrate feeding.

Of special interest is that the superphylum *Patescibacteria* had a lesser presence in mid-lactation than in dry cows ($p \leq 0.05$). The fact is that *Patescibacteria* have significantly reduced genomes compared to most other microorganisms; they use simple intermediates of the host's digestive system in order to obtain energy, e.g., glucose and pyruvic acid; they have lost the ability to digest complex polysaccharides [59]. Loss of the *Patescibacteria* population in mid-lactation could be due to the disruption in the synthesis of these nutrient substrates in the rumen.

Fig. 3. Rumen microbiome taxonomy (family level) in black-and-white Holsteinized dairy cows (*Bos taurus*) in different physiological periods: I — dry cows, II — fresh cows, III — lactating cows, IV — stable lactation, V — late lactation period (NGS sequencing; JSC Agrofirma Dmitrova Gora, Tver Province, 2020).

Detailed analysis of the microbiota composition (Fig. 3) showed some of the families to have a relatively even presence in the animals of different groups. However, eight families showed significant differences as well: *Muribaculaceae*, *Prevotellaceae*, *Erysipelatoclostridiaceae*, *Oscillospiraceae*, *Ruminococcaceae*, *Saccharimonadaceae*, and candidate families WCHB1-41, vadinBE97, see Fig. 3.

The family *Prevotellaceae* of the superphylum *Bacteroidota*, mainly of the genus *Prevotella*, has a greater presence in Group 4 than in Group 1 ($p \leq 0.05$). Such greater presence of *Prevotellaceae* in cows fed with starch- and monosaccharide-rich diets is only logical. These microorganisms are known [60, 61] to use starch to synthesize VFAs; however, excess production of short-chain fatty acids is associated with a decrease in ruminal pH and can lead to lactate acidosis accompanied by dysbiosis [62]. Besides, cows in Group 4 had smaller populations of the families *Oscillospiraceae* (the phylum *Firmicutes*) and *Saccharimonadaceae* (the superphylum *Patescibacteria*) than those in Group 1 ($p \leq 0.05$). This could be a sign

of dysbiosis due to high-concentrate feeding as *Oscillospiraceae* have a substantial set of glycoside hydrolases. The latter have a variety of functions and are involved in the degradation of cellulose and hemicellulose in feeds; thus, they are fundamental to the biological apparatus behind the destruction of glycosidic bonds [63].

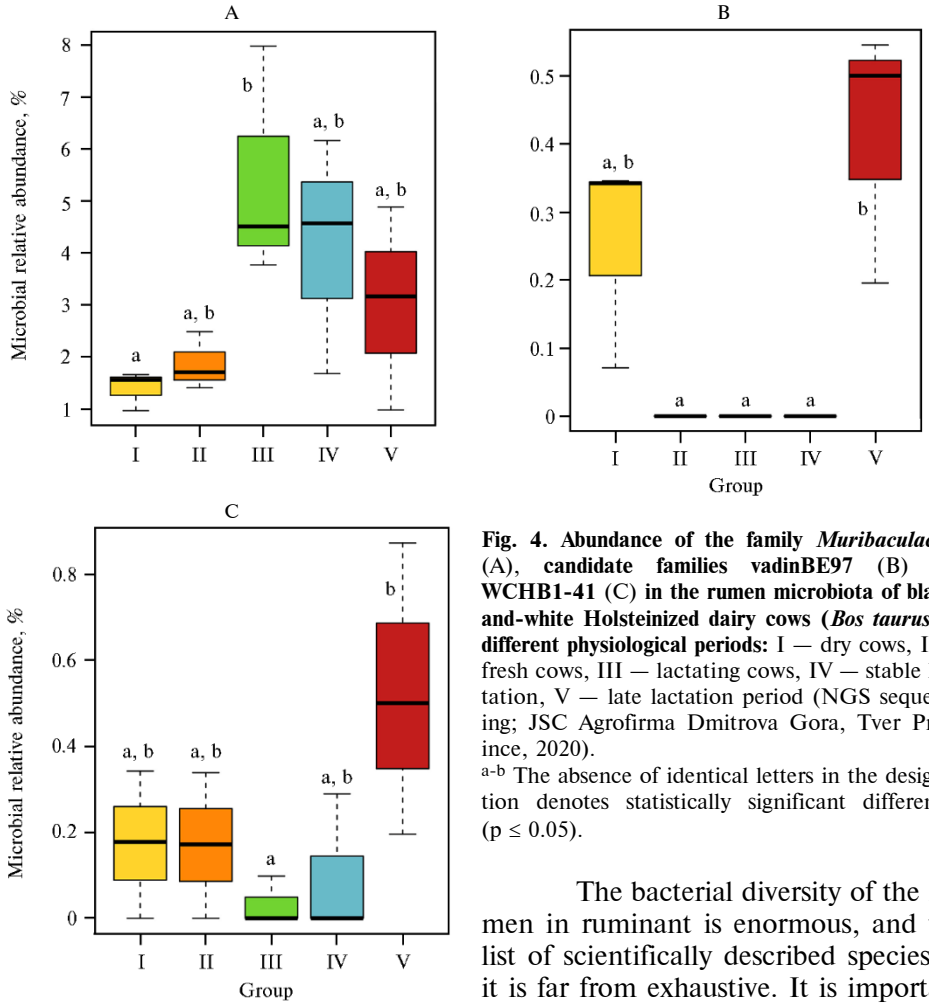

Fig. 4. Abundance of the family *Muribaculaceae* (A), **candidate families vadinBE97** (B) **and WCHB1-41** (C) **in the rumen microbiota of black-and-white Holsteinized dairy cows (*Bos taurus*) in different physiological periods:** I — dry cows, II — fresh cows, III — lactating cows, IV — stable lactation, V — late lactation period (NGS sequencing; JSC Agrofirma Dmitrova Gora, Tver Province, 2020).
[a-b] The absence of identical letters in the designation denotes statistically significant differences ($p \leq 0.05$).

The bacterial diversity of the rumen in ruminant is enormous, and the list of scientifically described species in it is far from exhaustive. It is important to focus on the microorganisms that are yet uncultured in order to properly analyze such important processes as nutrient metabolism cycles and modulation in the body's functions associated with the host's health. In this regard, it was interesting to identify the family *Muribaculaceae* of the superphylum *Bacteroidota* in the rumen microbiome, see Fig. 4. Until 2019 [64], this taxon was classified as an uncultured family S24-7 despite being ubiquitous in the intestinal microbiota in many vertebrates. Taxonomic ambiguity made agent-host interaction analysis difficult.

In this experiment, *Muribaculaceae* had a greater presence ($p \leq 0.05$) in lactating than in dry cows, see Fig. 4. Earlier, Ormerod et al. [65] demonstrated the presence of a substantial and versatile set of carbohydrate breakdown-associated enzymes in the analyzed genomes of S24-7 species. The ability to degrade carbohydrates might explain the presence of these microorganisms in cow rumen during high-concentrate feeding. The fact that *Muribaculaceae* have mechanisms to protect themselves from some organic acids [64] might explain their colonization behavior.

The facts that the non-attributable bacteria from the candidate family vadinBE97 disappeared from the rumen microbiota in lactating cows in Groups 2, 3, and 4, and that the presence of the candidate family WCHB1-41 was reduced in Groups 3 and 4 (see Fig. 4) confirms that feeding is an important factor of the relative abundance of these microorganisms in the rumen. This finding is of fundamental interest, as the functions of the bacteria of these families in the superphylum *Verrucomicrobiota* remain virtually unstudied due to the impossibility of laboratory culturing.

Attention-worthy is the fact that microbiota of the genera *Asteroleplasma*, *Sharpea* (fam. *Erysipelatoclos-tridiaceae*), and *Moryella*, *Oribacterium*, *Shuttleworthia* (fam. *Lachnospiraceae*), which were not found in dry cows' microbiota, appeared in the rumen shortly after calving and persisted through the subsequent lactation periods. These genera could be referred to as marker taxa that appeared in response to calving-related stressors, negative energy balance, and dietary changes.

Interestingly, *Asteroleplasma anaerobium*, being the only *Asteroleplasma* in the microbiome of the tested cows, has lactate dehydrogenases that are activated by fructose-1,6-biphosphate [66]. Lactate dehydrogenases are key enzymes of lactic acid fermentation that produces lactate [67, 68]. Lactate is also one of the key metabolic end products of *Moryella* [69], *Oribacterium* [70], and *Shuttleworthia* [71]. The fact that these taxa appeared in the microbiome during lactation could be due to metabolic disorders that are often observed in high-concentrate feeding [34], as lactate induces a lower pH and causes cellulolytic and VFA-synthesizing microorganisms to die [34].

Let us focus on *Sharpea azabuensis*, the only *Sharpea* species found in newly calved cows' microbiome as well as during early and mid-lactation. In the rumen, this bacterium is able to synthesize intermediate trans-11-isomers of linoleic and linolenic acid [72]. Concentrate-based diets of ruminants mainly contain C18 unsaturated fatty acids (α-linolenic, linoleic, oleic, etc.) [73]. A diet rich in starch and low in fiber is known to cause the rumen metabolism to shift towards producing trans-10 isomers of fatty acids via trans-11 intermediates [73]. The presence of trans-10 intermediates in the rumen is often associated with a decrease in milk fat [74].

Nevertheless, there were no typical changes [34, 75] indicative of possible metabolic disorders in the cows' microbiome. In particular, there was no increase in the population of *Lactobacillaceae* that in most cases [34] induce a decrease in pH and trigger metabolic disorders on top of a high-concentrate diet. *Fusobacterium necrophorum*, a highly virulent ruminant pathogen capable of producing hemagglutinin, endotoxin, and leukotoxin [76], often gaining a competitive advantage from high-concentrate feeding [76], was totally absent from the microbiome.

To find out whether the physiological period-related compositional changes in the rumen microbiome were associated with functional changes, the research team reconstructed and predicted the functional content of the metagenomic community of the rumen using PICRUSt2 and the Kyoto Encyclopedia of Genes and Genomes (KEGG: Kyoto Encyclopedia of Genes and Genomes) (https://www.genome.jp/kegg/). This reconstruction made it possible to annotate 282 various metabolic pathways. For 17, the predicted functional potential changed ($p \leq 0.01$) in cows of different groups, see Fig. 5. The most pronounced ($p \leq 0.01$) changes in the metabolic potential of the microbiota, namely the inhibition of various metabolic pathways in the rumen chyme, e.g., energy (tricarboxylic acid cycle), protein, carbohydrate, lipid, including volatile fatty acid (VFA) synthesis, occurred in cows of Groups 4 and 5 as compared to Groups 1, 2, and 3, see Fig. 5.

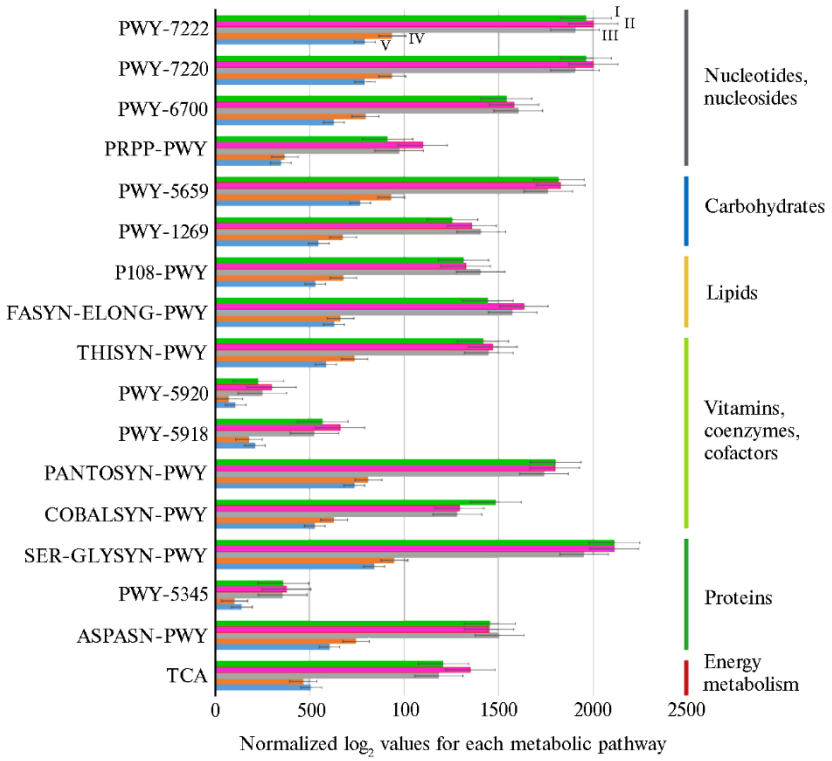

Fig. 5. Data ($p \leq 0.01$) of the functional annotation of metabolic pathways of metagenomics community of the rumen microbiota of black-and-white Holsteinized dairy cows (*Bos taurus*) in different physiological periods: I — dry cows, II — fresh cows, III — lactating cows, IV — stable lactation, V — late lactation period (NGS sequencing; JSC Agrofirma Dmitrova Gora, Tver Province, 2020). TCA stands for the Krebs cycle, ASPASN-PWY is for the biosynthesis of L-aspartate and L-asparagine, PWY-5345 is for the biosynthesis of L-methionine via sulfhydrylation, SER-GLYSYN-PWY is for the biosynthesis of L-serine and glycine, COBALSYN-PWY is for the biosynthesis of adenosylcobalamin from cobinamide I, PANTOSYN-PWY is for the biosynthesis of pantothenate and coenzyme A I, PWY-5918 is for the biosynthesis of heme from glutamate, PWY-5920 is for the biosynthesis of heme from glycine, THISYN-PWY is for the biosynthesis of thiamine diphosphate I, FASYN-ELONG-PWY is for the chain lengthening of saturated fatty acids, P108-PWY is for pyruvate fermentation into propanoate I, PWY-1269 is for the biosynthesis of CMP-3-desoxy-D-mannooctulosonate I, PWY-5659 is for the biosynthesis of GDP-mannose, PRPP-PWY is for the biosynthesis of histidine, purine, and pyrimidine, PWY-6700 is for the biosynthesis of queuosine , PWY-7220 is for adenosine deoxyribonucleotide biosynthesis, PWY-7222 is for guanosine deoxyribonucleotide synthesis.

Groups 4 and 5 had lower ($p \leq 0.01$) Krebs cycle-related predicted metabolic capacities of the microbiome than Groups 1, 2, and 3, which could disrupt energy metabolism in the rumen as the Krebs cycle is the central regulator in macroorganisms and microorganisms alike. It involves a complex multistep sequence of reactions that supply energy and plastic substrates, reduced and phosphorylated cofactors for the major biosynthetic pathways [77] that largely determine the subsequent intensity and direction of the major metabolic flows [78].

Consequently, it seems natural that the microbiome had a lower capacity to synthesize pantothenate and coenzyme A (CoA) in Groups 4 and 5 than in Groups 1, 2, and 3 ($p \leq 0.01$). Acetyl coenzyme A (acetyl-CoA) is a key link in the regulation of the activity of pyruvate and alpha-ketoglutarate dehydrogenases in the Krebs cycle [79]. CoA biosynthesis precursor 3-methyl-2-oxobutanoate functions as an intermediate in the biosynthesis of L-valine. First, this compound converts into (R)-pantoate, then into (R)-4′-phosphopantothenate. Further L-cysteine and (R)-4′-phosphopantothenate produces (R)-4′-phosphopantetheinyl-

L-cysteine, which is then decarboxylated into 4′-phosphopantetheine. The final reaction is catalyzed by dephospho-CoA kinase (EC 2.7.1.24) that converts 4′-phosphopantetheine into CoA [80, 81].

Groups 4 and 5 had a lower energy balance potential in the rumen than Groups 1, 2, or 3 ($p \leq 0.01$), possibly due to the disrupted synthesis of microbial amino acids (L-aspartate, L-asparagine, L-methionine, L-serine, and glycine), see Fig. 5. Indeed, energy and nitrogen are the key determinants of how much microbial protein is synthesized in the rumen [82]. Microbial protein is the primary source of amino acids for ruminants [83]. The aforementioned changes in the potential could be due to feeding. Microbial protein synthesis rates had been shown [84] to drop in cows on a high-concentrate diet.

Cows in Groups 4 and 5 could have health issues due to less genes being involved in pyruvate-propanoate conversion, as propanoate is an important gluconeogenesis precursor in ruminants and can only be produced by the rumen microbiota [9]. Phase I: succinyl-CoA is converted into (R)-methylmalonyl-CoA that breaks down and transfers a C1 unit onto pyruvate. This reaction is catalyzed by methylmalonyl-CoA-carboxyl transferase and produces propanoyl-CoA and oxaloacetate. Newly produced propanoyl-CoA donates coenzyme A for another cycle phase where it is transferred onto the succinate. The subsequent succinate CoA transferase-catalyzed reaction produces the final product: propanoate [85]. High-concentrate feeding-induced lower pH in the rumen inhibits the reproduction of acidification-sensitive VFA producers, in particular propanoate producers [86]. Thus might have lowered the rates of propanoate production in Groups 4 and 5, resulting in inhibited gluconeogenesis.

Besides, Group 4 and 5 cows had lower ($p \leq 0.01$) metabolic potential for the synthesis of adenosylcobalamin from cobinamide in the rumen. Adenosylcobalamin is rumen microbe-produced vitamin B_{12} [87]. Many bacteria lack a complete set of genes required to synthesize adenosylcobalamin de novo; however, they are able to convert cobinamide into cobalamin [88]. To that end, the upper ligand of 5′-deoxyadenosine connects to adenosylcobinamide, which is further phosphorylated to produce adenosylcobinamide phosphate, an intermediate of de novo biosynthesis.

These modifications of the functional potential in Group 4 and 5 cows could be due to metabolic disorders [89]. Previously, Lima et al. [90] showed that the change in the number of B_{12} biosynthesis-associated genes in the rumen microbiota was due to the amount of feed intake. Ogunade et al. [91] studied the functional annotation of the rumen microbiome in young Holstein bulls and found the livestock with symptoms of subacute acidosis to have altered potential of 10 metabolic pathways including carbohydrate, amino acid, energy, vitamin, and cofactor metabolism; the change also affected the formation of bacterial biofilms. In the present research, changes in metabolic potential corresponded to the decrease in the rumen α-biodiversity, as well as to the disappearance of some taxa (the superphylum *Armatimonadota*, the phylum *Chloroflexi*) and a decrease in the population of other taxa (the superphylum *Verrucomicrobiota*, the family *Oscillospiraceae* of the phylum *Firmicutes*, the family *Saccharimonadaceae* of the superphylum *Patescibacteria*, etc.) in the rumen in Groups 4 and 5.

Thus, the most pronounced differences were observed in: the composition of the rumen microbiome between early/mid-lactation and dry cows; the metabolic potential between mid-/late lactation and dry cows. Data suggests that the biodiversity and functions of microorganisms depend on the condition and feeding of cows in different physiological periods. The findings also suggest that the rumen microbiome and its functional potential are more susceptive to negative effects in

early, mid-, and late lactation than shortly after calving, which probably means that common conceptions might need some reviewing [13, 14].

As noted, the production of lactic acid by the rumen microbiota is a key mechanism behind the metabolic disorders in ruminants [92]. The authors compared taxonomic research data and metabolic potential estimates for rumen microorganisms against the analysis of the expression of lactate dehydrogenase synthesis-associated bacterial genes. For analysis, quantitative PCR with reverse transcription was used, a bacterial gene expression assessment method whose reproducibility, high sensitivity, and specificity are commonly recognized [93]. Lactate is produced by lactic acid fermentation from precursors under the influence of two different forms of NAD-bound lactate dehydrogenases: one (EC 1.1.1.27) produces L(+)-lactate and is encoded primarily by the *Ldh-L* gene [51]; the other one (EC 1.1.1.28) produces D(–)-lactate and is encoded by *Ldb 0813* [94]. The authors of [95] believe there is a significant difference between the effects of the two enantiomers on ruminants, although stereoisomers have similar physical and chemical properties. The important difference between the isomers lies in their renal excretion capacity, which is lower for D-lactate and thus determines its primary role in provoking metabolic acidosis [95]. This is why the authors believe the *Ldh-L* and *Ldb 0813* genes are important candidate biomarkers that could indicate the activity of lactic acid synthesis in the bovine rumen.

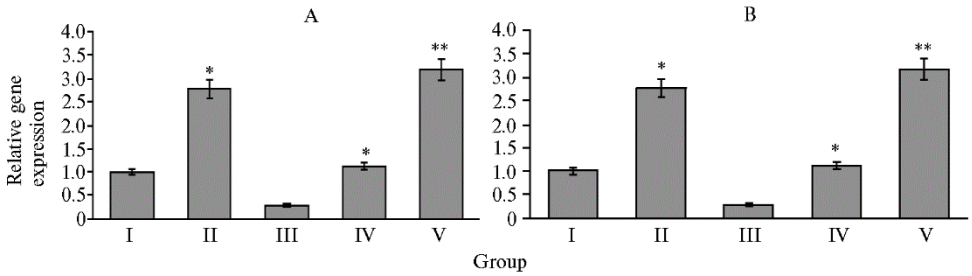

Fig. 6. Relative expression (compared to the gene of cows from Group 1) **of the genes *Ldh-L*** (A) **and *Ldb 0813*** (B) **in the microbial community of the rumen microbiota of black-and-white Holsteinized dairy cows (*Bos taurus*) in different physiological periods:** I — dry cows, II — fresh cows, III — lactating cows, IV — stable lactation, V — late lactation period (JSC Agrofirma Dmitrova Gora, Tver Province, 2020).

*, ** Differences of Groups II, III, IV, and V from Group I are statistically significant at $p \leq 0.01$ and $p \leq 0.001$.

Gene expression was reduced 11.3-fold for Ldh-L and 9.9-fold for Ldb 0813 in Group 3 (early lactation) as compared to newly calved cows (Group 2) ($p \leq 0.001$). That being said, animals in early lactation were able to better resist the negative effects of starch enrichment than their newly calved counterparts. This could be due to the emergence of adaptive mechanisms in the rumen microbiota.

A notably increased expression (10.6-fold for *Ldh-L* at $p \leq 0.001$, 2.8-fold for *Ldb 0813* at $p \leq 0.05$) occurred in the late lactation period (Group 5 vs. Group 4). This could be due to the rising populations of *Asteroleplasma*, *Sharpea*, *Moryella*, *Oribacterium*, *Shuttleworthia*, which produce lactate as one of the major fermentation products.

These variations in gene expression corresponded to the reduction in the α-diversity of the rumen microbial community and to the inhibition of the carbohydrate, protein, energy, and lipid metabolism potential of the microbiota. Based on these findings, one can conclude that in late lactation, animals are, too, at risk of metabolic disorders, which should be borne in mind when devising a strategy for less metabolic diseases in dairy cows. Some changes in the composition and

functions of the rumen microorganisms could be due to the specifics of metabolism, feeding, stress (e.g. postpartum and early lactation stress), immune and hormonal status in different physiological periods.

Thus, we used bioinformatics methods to study in detail the rumen microbiome structure and to predict its functional potential in different physiological periods of dairy cows. 16S metagenomic sequencing showed a reduction in the α-diversity of the bacterial microbiota of the rumen in mid- and late lactation. Twelve superphyla and phyla of microorganisms were found; the superphylum *Bacteroidota* and the phylum *Firmicutes* could be considered the dominant bacteria in the rumen, as they represented up to 59.94±1.86 and 46.82±14.40% of the microbiome, respectively. A relation has been found between the presence of certain taxa of rumen microorganisms and physiological periods in dairy cows. The superphylum *Actinobacteriota* was only found in dry cows' rumens, fully eliminated from lactating cows. The superphylum *Armatimonadota* was absent from the rumen in newly calved cows and cows in mid-lactation; the phylum *Chloroflexi* was absent in early and mid-lactation. Detailed microbiome analysis showed the animals to differ significantly in terms of eight bacterial families (*Muribaculaceae*, *Prevotellaceae*, *Erysipelatoclostridiaceae*, *Oscillospiraceae*, *Ruminococcaceae*, *Saccharimonadaceae*, candidate families WCHB1-41, vadinBE97). The genera *Asteroleplasma*, *Sharpea*, *Moryella*, *Oribacterium*, *Shuttleworthia*, which were absent from dry cows, appeared after calving and persisted through subsequent periods of lactation. The emergence of these bacteria in the microbiome during lactation could be due to metabolic disbalance since lactate is one of their major products. The predicted functional potential of the rumen microbiota differed across 17 metabolic pathways. Changes, namely the inhibition of various types of chyme metabolism, were most pronounced ($p \leq 0.01$) during mid- and late lactation. Increased expression was detected in newly calved cows vs. dry cows for the *Ldh-L* ($p \leq 0.01$) and *Ldb 0813* ($p \leq 0.05$) that are connected to lactate dehydrogenase synthesis. The bacterial community of the rumen exhibited a significant increase in the expression of *Ldh-L* (10.6x at $p \leq 0.001$) and *Ldb 0813* (2.8x at ≤ 0.05) in late lactation versus mid-lactation.

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A POLYMORPHISM ANALYSIS OF DYSFERLIN GENE LOCUS IN CHICKEN GENE POOL

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Abstract

Disferlin refers to proteins involved in the repair of the muscle membrane. It is assumed that some mononucleotide substitutions in the dysferlin gene (*DYSF*) are associated with the formation of muscle mass in poultry. In this work, for the first time in chickens of the Russian White breed, four mononucleotide substitutions have been identified that are in intron 32 on chromosome 4: rs317801013 (G/A) at position 90672849, rs16455118 (C/A) at 90672756, rs318045896 (A/G) at 90672862, and T/G at 90672805. Mononucleotide polymorphism T/G on chromosome 4 at position 90672805 was submitted for registration to the ENSEMBL database, since it was detected for the first time in the species *Gallus gallus*. In addition, for the first time, we assessed the frequency of occurrence of genotypes and the deviation of the observed genotype distribution from the expected Hardy-Weinberg equilibrium in the gene pool chickens of the Russian White breed. The aim of this work was to study single nucleotide polymorphisms (SNPs) of the dysferlin gene in chicken gene pools and to identify possible associations of *DYSF* gene polymorphisms with economically valuable traits. We studied meat (Cornish) chickens, laying hen (Russian White, Rhode Island, Aurora, Black-and-White Austrolorp, Leningrad Calico) and decorative breeds (Russian Crested, Light Brahma, Bare-Necked) from the gene pool population of the Genetic Collection of rare and endangered breeds of chickens (RRIFAGB, St. Petersburg—Pushkin). DNA was isolated from blood collected from the axillary vein by phenol extraction. Illumina Chicken 60K SNP iSelect BeadChip chip (Illumina, Inc., USA) was used to analyze the rs16455118 polymorphism. The observed and expected frequencies of genotypes *AA*, *AC*, *CC* and their deviations from the Hardy-Weiberg equilibrium were analyzed in laying hens based on the replacement of adenine for cytosine in the dysferlin gene (rs16455118). The reliability of the data obtained was assessed using the Pearson χ^2 test. Dysferlin gene polymorphism was analyzed by sequencing a 237 bp *DYSF* gene region on chromosome 4 in 76 Russian White hens. We analyzed the NCBI and ENSEMBL databases to identify the SNPs found. An analysis of the frequency of genotypes and alleles was carried out for four identified substitutions. Genotyping of 185 hens using Illumina Chicken 60K SNP iSelect BeadChip technology revealed a single nucleotide polymorphism SNP rs16455118. It was found that the allele frequencies shifted towards an increase in heterozygous genotypes of *AC* in decorative chickens while the *AA* genotype was present in the minority. In laying hen, the homozygous genotype *AA* had the highest frequency of occurrence, the *CC* genotype was small in number, and it was completely absent in the population of chickens of the Aurora breed. The Cornish beef breed had a more even distribution of genotypes as compared to decorative and laying hens. Sequencing of the 237 bp dysferlin gene region located on chromosome 4 in Russian White chickens identified mononucleotide substitutions in the intron 32. Single nucleotide substitutions G/A (rs317801013), C/A (rs16455118), A/G (rs318045896) corresponded to those in the publicly available chicken genome in the databases NCBI (https://www.ncbi.nlm.nih.gov/SNP) and ENSEMBL (https://www.ensembl.org/index.html). The single nucleotide polymorphism T/G at 90672805 has been detected for the first time. The shift in the genetic balance in the gene pool of Russian White hens indicates the effect of the founder or selection pressure on the region of the SNP rs16455118. The almost complete absence of heterozygotes in laying hen may indicate inbreeding or strong selection pressure. Our findings can be helpful in the future search for SNPs associated with productivity trait in chickens to create a system of molecular markers to accelerate breeding progress.

Keywords: dysferlin gene, SNP, single nucleotide polymorphism, poultry farming, allele, hens

Currently, traditional poultry breeding has reached a plateau, and progress in increasing productivity has declined significantly. The use of molecular genetic markers is becoming the most effective method for accelerating the selection process in farm animal production [1]. This approach is based on the search for single nucleotide polymorphisms (SNPs) associated with various traits in poultry, including using SNP chip panels. Based on several million SNP loci identified through years of scientific research by Illumina, Inc. (USA), chips of medium and high density were created for the main species of farm animals, including chickens, which allows obtaining data on the localization of areas and genes associated with traits. Sequencing makes it possible to study in more detail the regions of candidate genes to identify variants of genetic polymorphism associated with the traits of interest. For example, using the Illumina Chicken 60K SNP iSelect BeadChip chip technology, a reliable association of the 32nd intron region on chromosome 4 in the dysferlin gene (*DYSF*) with white fluff in Russian White chickens was found [1].

The most urgent task in poultry farming is to increase egg and meat productivity. To work in this direction, the authors of this paper selected the gene *DYSF*, presumably affecting the formation of muscle mass and egg production in poultry [2-4].

Dysferlin (DYSF) is a type II transmembrane protein that is localized at the periphery of muscle fibers and serves as a regulator of vesicle fusion in the sarcolemma. Dysferlin plays an important role in vesicle movement, endocytosis, membrane receptor recirculation, muscle regeneration, and T-tubule formation [2]. It can perform additional functions in the vesicular transport of growth factor receptors that promote muscle growth and regeneration. It is assumed that the dysferlin-dependent transport of such signaling molecules modulates the expression of genes and the function of adult muscle stem (or satellite) cells responsible for the growth and regeneration of skeletal muscles in adults [5].

Mutations in the *DYSF* gene cause a number of muscle diseases with various clinical manifestations known as dysferlinopathies, including limb muscular dystrophy type 2B (LGMD2B) and Miyoshi myopathy [6–8]. Ferlins are proteins that affect Ca^{2+}-driven membrane dynamics and belong to the superfamily of proteins with multiple C2 domains (MC2D) that share functions in binding membrane-associated organelles and proteins on cell membranes. These proteins are often described as sensors of calcium ions (Ca^{2+}) for vesicular transport, capable of forming membranes [3, 9, 10]. In vertebrates, there are six ferlin genes; in humans, there are the dysferlin, otoferlin, myoferlin, *Fer1L5*, and *Fer1L6* genes, and a long gene that does not encode RNA, *Fer1L4* [11].

The most studied function of dysferlin is the restoration of damage in the surface membrane of striated muscle fibers — sarcolemma. The contraction of muscle fibers mechanically affects the sarcolemma, which leads to its microdestruction. The repair process is triggered by Ca^{2+} influx into the sarcoplasm, which depends on a number of proteins, including dysferlin as one of the key participants [12-14]. It probably promotes membrane aggregation and fusion during membrane repair through the interaction of Ca^{2+} with negatively charged phospholipids [15-17].

In the world scientific literature, there are no works devoted to the functions of dysferlin in chickens, including the association of dysferlin with poultry productivity has not been studied.

In the present study, four single nucleotide substitutions located in the 32nd intron on chromosome 4 were first identified in Russian White chickens:

rs317801013 (G/A) at 90672849, rs16455118 (C/A) at 90672756, rs318045896 (A/G) at 90672862, and SNP (T/G) at 90672805. Single nucleotide T/G polymorphism on chromosome 4 at position 90672805 was submitted for registration in the ENSEMBL database. Also, for the first time, an analysis of the frequency of occurrence of genotypes and the deviation of the observed distribution of genotypes from that expected at Hardy-Weinberg equilibrium in the gene pool of chickens of the Russian White breed was carried out for all the above substitutions in the dysferlin gene.

The aim of this work was to study the SNPs of the dysferlin gene in gene pool chicken breeds and to identify possible associations of *DYSF* gene polymorphisms with economically valuable characteristics.

Materials and methods. The studies were performed on chickens (*Gallus gallus*) from the gene pool population of the Genetic Collection of Rare and Endangered Chicken Breeds (All-Russian Research Institute of Genetics and Breeding of Agricultural Animals, St. Petersburg—Pushkin).

DNA was isolated by the phenol method from blood taken from the axillary vein into standard tubes with an anticoagulant (EDTA). To analyze the rs16455118 polymorphism, the research team used a database obtained as a result of genotyping using the Illumina Chicken 60K SNP iSelect BeadChip chip technology (Illumina, Inc., USA).

At the first stage, a total of 185 hens were used: meat (Cornish, $n = 39$), laying hen (Russian White, $n = 19$; Rhode Island, $n = 18$; Aurora, $n = 14$; Black-and-White Austrolorp, $n = 20$; Leningrad Calico, $n = 20$) and decorative (Russian Crested, $n = 20$; Light Brahma, $n = 18$; Bare-Necked, $n = 17$). The authors analyzed the frequency of *AA*, *AC*, and *CC* genotypes and the deviation of the observed genotype distribution from that expected under Hardy-Weinberg equilibrium in gene pool hens by the rs16455118 adenine to cytosine substitution in the dysferlin gene. The validity of the data obtained was assessed using Pearson's criterion χ^2.

In addition, the authors analyzed in more detail the polymorphism of the dysferlin gene in 76 chickens of the Russian White egg breed by sequencing a 237-bp section of the *DYSF* gene on chromosome 4. Amplification primers were designed based on the NCBI database (https://www.ncbi.nlm.nih.gov/) using the PRIMER_3 computer program (https://bioinfo.ut.ee/primer3-0.4.0/). The primer sequences were used: Fw — 5′-GGATGCCATAAGGACGTTGC-3′, Rv — 5′-TCCCCACAGCATCCCCTATAC-3′. PCR was performed in 10 rl of reaction mixture containing 67 mM Tris-HCl (pH 8.6), 2.5 mM $MgCl_2$, 16.6 mM NHOH, 0.125 mM dNTP, 0.5 rM primer, 50-100 ng of genomic DNA, and 2.5 units. Taq polymerases (Sibenzyme, Novosibirsk) on a C 1000 Touch amplifier (Bio-Rad, USA). Amplification mode: 5 min at 95 °C (denaturation); 20 s at 95 °C, 20 s at 62 °C, 20 s at 72 °C (40 cycles); 4 min at 72 °C (final elongation). The analysis of PCR products was carried out in a 2% agarose gel.

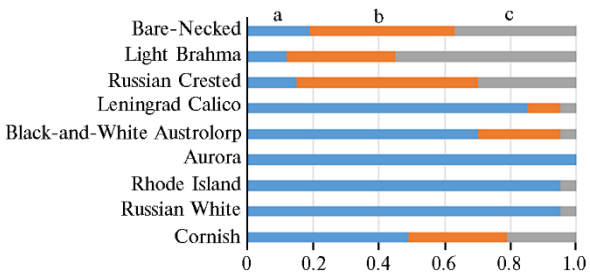
PCR products were purified using a commercial kit ExoSAP-IT Express (Affimetrix, USA) according to the manufacturer's protocol. Sanger sequencing was performed on an Applied Biosystems 3500 genetic analyzer (Thermo Fisher Scientific, Inc., USA) using a commercial BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Inc., USA). Alignment and processing of sequences were performed using the MEGA 6 software (https://www.megasoftware.net/web_help_10/index.htm#t=Citing_MEGA_In_Publications.htm).

To identify the identified substitutions, an analysis of the international genetic databases NCBI (https://www.ncbi.nlm.nih.gov/SNP) and ENSEMBL (https://www.ensembl.org/index.html) was carried out. For four substitutions, the

analysis of the frequency of genotypes and alleles was carried out. The deviation of the observed heterozygosity from the expected heterozygosity in Russian White hens was statistically processed and the validity of the obtained results was calculated using Pearson's criterion χ^2.

Results. Based on the results of genotyping of 185 hens using Illumina Chicken 60K SNP iSelect BeadChip chip technology by the rs16455118 (C/A) replacement, a shift towards an increase in the frequency of the *C* allele in decorative birds was observed. In egg chickens, the homozygous *AA* genotype had the highest frequency, the *CC* genotype was the least frequent, and was completely absent in the Aurora population. The Cornish meat breed had a more even distribution of genotypes in comparison with decorative and laying breeds (Fig.).

Database analysis (https://www.ensembl.org/Gallus_gallus/Variation/Population?db=core;r=4:90672256-90673256;v=rs16455118;vdb=variation;vf=6811490) showed equal distribution of *A* and *C* alleles (C: 0.500; A: 0.500) in red jungle hen, white Plimutrock, white leghorn, and silk hen (native Chinese breed).



Frequency of *AA* (a), *AC* (b), *CC* (c) genotypes by single nucleotide substitution A/C (rs16455118) at position 90672756 on chromosome 4 in the *DYSF* gene in gene pool populations from the Genetic Collection of Rare and Endangered Chicken Breeds (All-Russian Research Institute of Genetics and Breeding of Farm Animals, St. Petersburg—Pushkin).

When analyzing the frequency distribution of genotypes for the replacement rs16455118 among the gene pool breeds, a strong shift of genotypes towards homozygosity was revealed. The deviation of the observed heterozygosity (H_o) from the expected (H_e) turned out to be statistically insignificant for the bare-necked breeds ($\chi^2 = 0.28$, $p = 0.9$), Light Brahma ($\chi^2 = 0.28$, $p = 0.5$), Russian Crested ($\chi^2 = 0.68$, $p = 0.71$), Black-and-White Austrolorp ($\chi^2 = 0.009$, $p = 1$) in accordance with the Hardy-Weinberg law. In other words, the actual frequencies were in good agreement with the theoretically expected ones. The deviation of the observed distribution of genotypes from that expected at Hardy-Weinberg equilibrium was statistically significant for the populations of Cornish ($\chi^2 = 7.38$, $p = 0.025$), Rhode Island ($\chi^2 = 5.18$, $p = 0.05$), Leningrad Calico ($\chi^2 = 7.9$, $p = 0.019$), and Russian White ($\chi^2 = 40$, $p = 0.00001$). The obtained value of criterion χ^2 was greater than the critical one (3.84 with the number of degrees of freedom 1); therefore, the shift in genetic equilibrium in the analyzed populations indicated the founder's effect or selection pressure on the region of the rs16455118 single nucleotide substitution, presumably associated with egg production.

Frequency of occurrence of genotypes and alleles for four SNPs in the dysferlin gene *DYSF* in gene pool chickens of the Russian White breed from the Genetic Collection of Rare and Endangered Chicken Breeds (All-Russian Research Institute of Genetics and Breeding of Farm Animals, St. Petersburg—Pushkin).

SNP	Allele, genotype	Frequency	χ^2	p	H_o	H_e
rs318045896	*A*	0,593	6.004	0.014	13	20.75
	G	0,401				
	AA	0,442				
	AG	0,302				
	GG	0,256				

Continued Table

rs16455118	*A*	0,623	45.1	1.026187e–10	4	28.6557
	C	0,377				
	AA	0,590				
	AC	0,066				
	CC	0,344				
rs317801013	*A*	0.280	4.31	0.037	18	24.52
	G	0.720				
	AA	0.131				
	AG	0.295				
	GG	0.573				
Отсутсвует в базе ENSEMBL	*T*	0.180	2.68	0.1	15	18.8
	G	0.820				
	TT	0.063				
	TG	0.234				
	GG	0.703				

N o t e. H_o — observed heterozygosity, H_e — expected ожидаемая heterozygosity.

The almost complete absence of *AC* heterozygotes in egg chicken breeds and a few *CC* homozygotes also indicated the association of the homozygous *AA* genotype with egg productivity and strong selection pressure on these populations, which resulted in an increase in the degree of inbreeding. Apparently, selection for increasing egg production using closely related crosses led to the founder effect, as a result of which the proportion of heterozygotes and individuals with the *CC* genotype decreased, which, in turn, led to a shift in the distribution of genotypes according to the Hardy-Weinberg law (Table).

Sequencing of a 237 bp region of the dysferlin gene, located on the 4th chromosome in Russian White chickens ($n = 76$), revealed four single nucleotide substitutions in the 31st intron in the following positions: G/A in position 90672849 (rs317801013), C/A in position 90672756 (rs16455118), A/G in position 90672862 (rs318045896), T/G in position 90672805. Single nucleotide substitutions rs317801013, rs16455118, and rs318045896 coincided with known substitutions in the chicken genome (NCBI and ENSEMBL databases). Mononucleotide polymorphism T/G at position 90672805 was detected for the first time and was submitted by the authors for deposition in the ENSEMBL database.

Despite the increase in the sample of egg chickens to 76 individuals, when genotyping using the Illumina Chicken 60K SNP iSelect BeadChip chip technology to replace rs16455118, a deviation of the actual distribution of genotypes from that expected at Hardy-Weinberg equilibrium was also observed ($\chi^2 = 45.1$, $p = 1.026187e{-}10$), the tendency towards homozygosity remained, the *AA* genotype remained the most numerous, but the number of individuals with the *CC* genotype increased.

In a previous study [4], no significant relationship was found between alternative genotypes *AA*, *AC*, *CC* according to the dysferlin gene with live weight, age of first egg-laying, and egg weight in Russian White chickens; however, a relationship was established with egg production. Chickens with the *CC* genotype laid in 180 days on average 10 eggs less (134.06 ± 5.96) than those with the *AA* (145.00 ± 2.35) and *AC* (143.94 ± 2.31) genotypes ($p \leq 0.05$). This work partly confirms the hypothesis about the influence of the *AA* genotype on the egg production of chickens, since the *AA* genotype prevailed in laying breeds. Therefore, the A/C rs16455118 substitution in the dysferlin gene can be considered associated with egg production and used as a molecular marker after a more detailed study in the panel of marker polymorphisms.

Since the A/C mutation is located in the intron and does not lead to an amino acid substitution, let us consider how one can explain its effect on the phenotype. In many eukaryotes, including mammals, plants, insects, and yeast, introns can upregulate gene expression without functioning as a binding site for

transcription factors. This phenomenon is called intron-mediated amplification [18]. Introns can increase the number of transcripts, affecting the rate of transcription, nuclear export, and stability of transcripts, as well as increase the efficiency of mRNA translation [19–21]. Introns proximal to promoters are capable of enhancing transcription in mammalian and plant cells [22–24]. Chromatin immunoprecipitation (ChIP) analysis showed that the number of binding sites for RNA polymerase II (Pol II) on a reporter construct containing an intron was 4 times higher compared to the construct without an intron [25].

Intron-mediated enhancement of transcription correlates with the formation of a loop conformation of genes, which unites their promoter and terminator regions, possibly facilitating recycling and re-initiation of Pol II [26]. According to the results of genome-wide analysis, mRNA stability positively correlated with the number of introns in mice and humans [27–30]. In addition to increasing the mRNA content, the presence of introns increases the efficiency of mRNA translation in yeast, plants, mammals, and other animals [31, 32].

Another hypothesis about the mechanisms of influence of single nucleotide substitution in the intron is to change the pre-mRNA splicing required for the corresponding translation of the protein, which depends on the presence of consensus cis sequences that define the exon-intron boundaries and regulatory sequences [33]. Point mutations in these consensus sequences can cause misrecognition of exons and introns and result in an aberrant transcript of the mutated gene. Typically, such mutations cause errors in the splicing process, lead to improper removal of an intron, and thus cause changes in the open reading frame. Recent studies have highlighted the significant number and importance of splicing mutations in the etiology of inherited diseases.

In the paper by Chinese scientists [34], a reliable association of single nucleotide substitutions in the introns of the *MAGI-1* gene (encodes membrane-associated guanylate kinase 1) and the *ACSF2* gene (encodes acetyl-coA synthetase, an enzyme of the mitochondrial matrix) with egg production in geese was found. Bai *et al.* [35] also showed that polymorphism (A412G) in intron 1 of the *PRL* prolactin gene was significantly associated with egg production in two populations of Chinese domestic ducks. Arango *et al.* [36] showed an association between polymorphism in intron 3 of the bovine growth hormone *BGH* and body weight during the first estrus and first calving.

Therefore, despite the fact that the rs16455118 (A/C) mutation in the dysferlin gene does not change the amino acid sequence, it possibly affects the gene expression and the stability of its transcription, which leads to a change in the DYSF protein content [18–21]. The rs16455118 A/C mutation in the *DYSF* gene may be associated with economically significant traits by affecting mRNA splicing or stability, as well as with linkage disequilibrium with an unidentified missense mutation associated with the trait. The influence of the dysferlin gene on egg production is presumably related to the ability of the ferlins superfamily to trigger the influx of Ca^{2+}, an important macronutrient for oviposition, into the reproductive tract of the laying hen, since these proteins are described as sensors of calcium ions (Ca^{2+}) and key participants in a number of physiological processes [11–16].

The authors plan to continue studying the possible associations of single nucleotide substitutions rs317801013, rs318045896, and the T/G substitution at position 4:90672805 presented in the ENSEMBL database with signs of productivity. The identified associations can be further used in the selection of highly productive lines of domestic chicken breeds.

Thus, the study of SNPs rs16455118 of the dysferlin gene in chickens from

the gene pool of the Genetic Collection of Rare and Endangered Breeds of Chickens (All-Russian Research Institute of Genetics and Breeding of Farm Animals) showed that the homozygous genotype *AA* had the highest frequency of occurrence in poultry of the egg direction, the lowest the *CC* genotype, and the latter was completely absent in the aurora population. The Cornish meat breed was characterized by a more even distribution of genotypes in comparison with decorative and laying breeds. As a result of the sequencing of the dysferlin gene region located on chromosome 4, four single nucleotide substitutions in intron 32 were identified in Russian White chickens: rs317801013 (G/A) in position 90672849, rs16455118 (C/A) in position 90672756, rs318045896 (A/G) in position 90672862, and mononucleotide polymorphism (T/G) in position 90672805. Analysis of the frequency of occurrence of genotypes and the deviation of their observed distribution from the expected by Hardy-Weinberg showed a shift in the genetic equilibrium for all substitutions in the dysferlin gene found in gene pool chickens. The substitution of rs16455118 in the *DYSF* gene is associated with increased egg production and may be a consequence of selection pressure during breed improvement.

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GENETIC DIFFERENTIATION OF TURKEY BREEDS WITH MICROSATELLITE MARKERS

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Abstract

One of the trends of modern industrial agriculture is the reduction of breed genetic recourses in farm animals and poultry. Current programs on maintenance of farm animals breeds are giving great attention to the genetic studies, including the use of microsatellite loci. The microsatellite analysis is one of the informative and accessible methods. During the implementation of the Global Project for the Measurement of Domestic Animal Genetic Diversity (MoDAD), 50 populations of different poultry species were studied using microsatellite markers. The works on biodiversity in turkeys initially involved chicken microsatellite loci (*Gallus gallus*), then informative loci were established for the genome of turkeys (*Meleagris gallopavo*). Data on genetic profiles, similarities, differences, and interbreed differentiation of turkeys breeds bred in the USA, Italy, Hungary and other countries have been accumulated. In the present work, the genetic relationship between the Russian turkey breeds and the turkey gene pool population of the University of Minnesota based on microsatellite markers was established for the first time. The obtained data indicate that the genetic distances between breeds is largely determined by their origin, breeding range, and the contribution of the gene pool of some breeds in creating and improving the productive qualities of other breeds. Our purpose was to study genetic diversity and interbreeding differentiation of turkeys of Russian and foreign breeding using microsatellite loci. The research was performed at the North Caucasus zonal experimental station for poultry farming in 2019. Blood samples were taken from 30 individuals of each of seven turkey breeds (*Meleagris gallopavo*) of the Russian selection (Belaya shirokogrudaya, BSH; Bronzovaya Severokavkazskaya, BrSK; Belaya Severokavkazskaya, BeSK; Serebristaya Severokavkazskaya, SSK; Moscowskaya Belaya, MB; Chernaya Tikhoretskaya, CHT; Uzbekskaya palevaya, UP). DNA was isolated according to the protocol for the commercial AmpliPrime DNA-sorb-B kit (InterLabService, Russia). The amount and quality of isolated DNA were assayed using a standard spectrophotometric method (a NanoDrop 2000 spectrophotometer, Thermo Scientific, USA). Genotyping was performed for 12 microsatellite loci (MNT9-MNT20). The described genotypes of turkeys gene pool farm (AM) (Nicholas Turkey Breeding Farms) of the University of Minnesota were used for comparison with the genotypes of turkeys of Russian breeds. The average number and number of effective alleles per locus (Na, Ne), the degree of observed and expected heterozygosity (N_o, N_e), and Shannon index (I) were determined. The genetic structure of populations was assessed based on the F_{ST} values and genetic distances according to M. Nei. The Neighbor Joining Method was used to construct the phylogenetic tree. It was shown that low genetic diversity is characteristic of both Russian breeds of turkeys and the AM population. The number of identified alleles in the microsatellite loci as a whole in the breed sample varied from 1 to 4, the average number of alleles per locus ranged from 1.0 to 1.83. The least genetic difference occurred between the MB and BSH breeds. The BeSK, SSK, and BrSK breeds formed a separate node, with BrSK exhibiting the greatest genetic distance, forming the largest branch by genetic distance. Separate branches at relatively equal distances formed the breeds CHT, UP, and AM population. Thus, our findings confirm an insignificant genetic diversity of the gene pool of the

studied Russian turkeys' breeds and populations as compared to the gene pool of other species of farm animals.

Keywords: turkey breeds, microsatellites, phylogenetic analysis, genetic diversity

One of the problems of modern industrial agriculture is the reduction of the national genetic resources of breeds of farm animals and poultry, a decrease in their genetic diversity. Sometimes there is a threat not only of reduction but also of complete loss of unique, especially valuable gene pools. The priority of reducing the loss of genetic resources, preserving the diversity of existing local breeds, regional groups, types of animals and poultry capable of producing products in different breeding conditions, and ensuring sustainable development of animal husbandry is confirmed by the international convention on biological diversity [1].

An important aspect in the development of programs for the conservation of breeds of farm animals is the study of their genetic characteristics. Multi- and monolocal DNA markers, or microsatellites and single nucleotide polymorphisms (SNPs), are widely used to assess such features, as well as to certify breeds [2].

Microsatellites are usually highly polymorphic and include many alleles per locus. The FAO (Food and Agriculture Organization) recommendations for the selection of microsatellite loci in the study of various types of farm animals are based on a list (DAD-IS library, http://www.fao.org/dad-is/), developed by the ISAG-FAO group on genetic diversity. Microsatellites are recognized as informative for the analysis of the origin and mapping of quantitative trait loci [3, 4]. At the same time, with the development of molecular testing technologies, the analysis of SNP using microarrays, or chips, is gaining increasing recognition and advantage over them [5].

For the conservation and rational use of genetic resources of agricultural animals and poultry, FAO carried out a large-scale project for the analysis of their genetic diversity (Global Project for the Measurement of Domestic Animal Genetic Diversity, MoDAD) [6]. More than 50 populations of different bird species have been studied for microsatellite loci [7–9].

The study of microsatellite loci in turkeys (*Meleagris gallopavo*) began in the 2000s using microsatellite panels developed for chickens (*Gallus gallus*). Reed *et al.* [10] used 520 chicken microsatellite markers to work on turkeys. In 280 cases (54%), amplification products were obtained, most of which were either close in size to the fragments amplified with chicken DNA, or completely coincided. When assessing the informative value for genetic mapping of turkey, allelic polymorphism was determined in 57 out of 280 amplified regions. In total, 20 out of 57 markers (35%) were found to be polymorphic (on average, 1.4 alleles per locus). It was concluded that about 20% of chicken microsatellite loci could be used to map the turkey genome [10].

Chicken microsatellites were used to study the gene pool of turkeys of the Brianzolo, Colli Euganei, and Italian Black (Brianzolo, Colli Euganei, Nero d'Italia) breeds. Of the 31 loci, 22 were informative. At that, 12 loci (ADL0112, LEI0192, LEI0234, MCW0014, MCW0016, MCW0037, MCW0067, MCW0098, MCW0103, MCW0111, MCW0165, MCW0183) were studied in single PCR, 10 loci were studied using multiplex panels (Multiplex Master Mix 1 — ADL0268, ADL0278, LEI0094, MCW0216, MCW0248; Master Mix 2 — MCW0034, MCW0069, MCW0081, MCW0222, MCW0295). In nine markers (LEI0166, MCW0020, MCW0078, MCW0080, MCW0104, MCW0123, MCW0248, MCW0284, MCW0330), there were no amplified DNA regions [11]. Eight microsatellite markers of chickens *Gallus gallus* (MCW0111, MCW0067, LEI0104, MCW0123, MCW0081, MCW0069, MCW0104, MCW0183), of which seven were polymorphic, were used to study the gene pool of BIG6 and BIG10 BUT turkeys

(British United Turkeys) [12, 13].

Later, the research team used 772 microsatellite markers developed for three species of birds – chicken, quail, and turkey. As a result of screening for the study of allelic polymorphism and construction of a genetic map of turkeys, 410 microsatellite loci (53.1%) were selected. On a specially created genetic model (family), including direct relatives of three generations (224 individuals), genotyping was carried out at the selected loci. Of the 410 markers, 109 (26.6%) were polymorphic (2.3 alleles per marker). Higher polymorphism (61.1%) was found when using turkey-specific markers. When using markers specific for quail and chicken, polymorphism was 33.3 and 22.7%, respectively. The authors concluded that quail and chicken microsatellite loci could be used to construct a comparative genetic map of turkeys [14].

The next stage was the integration of the data obtained at two research centers – the Roslin Institute (Edinburgh, Scotland) and the University of Minnesota (Minneapolis, USA) [15]. Out of 279 microsatellite markers identified and tested at the Rosslyn Institute, 240 were used for screening on turkeys at the University of Minnesota experimental farm. Of these, 89 turned out to be genetically informative and were used for genotyping F_2 offspring. Analysis using the BLAST (Basic Local Alignment Search Tool) software package made it possible to unify 483 nucleotide sequences of microsatellites.

The researchers also performed BLAST alignment of the marker sequences of the turkey and chicken genomes. There were 263 matches and 1700 sequences with high homology [15].

In the joint work of scientists from the United States and Turkey, the search for informative microsatellite markers was carried out to study the biodiversity of turkeys and create a unified panel. Based on the nucleotide data library, primers were designed for 164 fragments of the turkey genome containing microsatellites. One hundred fifty-four informative genetic markers were identified; however, according to the authors, this is not enough for the general panel [16].

Interest in the study of the genetic diversity of turkeys, including for the genetic certification of commercial breeds, is primarily dictated by the fact that the production of turkey meat in the world is constantly growing. It is 6.1 million tons in the global volume of poultry meat, ranking second. Positive dynamics are also observed in Russia: according to Agrifood Strategies, the growth of turkey meat production in 2019 compared to 2007 amounted to 185 thousand tons (from 37 to 289 thousand tons, or 7.8 times), which characterizes this segment market as the most promising and rapidly growing.

Russia has its own genetic resources in turkey breeding: seven breeds, three crosses, and seven lines of domestic breeding turkeys are registered in the register of breeding achievements. The North Caucasian Zonal Experimental Station for poultry farming is not only the owner of the domestic gene pool of turkeys but also the only enterprise in Russia where systematic work is underway to create new breeding forms.

For the first time, the genetic profile of Russian turkey breeds by microsatellite loci was studied in 2017. The number in the samples of the studied breeds ranged from 9 to 15 individuals. The Hunter-Gaston index was used to assess the representativeness of the samples, the cluster analysis was performed using the UPGMA method, and the dendrogram was built using the START 2 computer program. It was found that the Bronzovaya Severokavkazskaya, Belaya Severokavkazskaya, and Belaya shirokogrudaya breeds had the greatest genetic affinity, followed by Chernaya Tikhoretskaya, Serebristaya Severokavkazskaya, and Mos-

cowskaya Belaya. The most distant was the Uzbekskaya palevaya breed [17]. However, no comparison was made between the gene pool of domestic turkey breeds and the gene pool of imported breeding.

In this work, on the basis of microsatellite markers, for the first time, the genetic relationships between the breeds of turkeys of Russian selection and the gene pool of the University of Minnesota were established. It is shown that the value of genetic distances between breeds is largely determined by their origin, breeding area, as well as the contribution of the gene pool of some breeds to the creation and improvement of the productive qualities of others.

The aim of this work is to study the genetic diversity and inter-breed differentiation of turkeys of Russian and foreign selection using microsatellite loci.

Materials and methods. The work was carried out at the North Caucasian Zonal Experimental Station for poultry farming in 2019. Blood samples were taken from the axillary vein from 30 individuals of each of seven breeds of turkeys (*Meleagris gallopavo*) of Russian selection (Belaya shirokogrudaya, BSH; Bronzovaya Severokavkazskaya, BrSK; Belaya Severokavkazskaya, BeSK; Serebristaya Severokavkazskaya, SSK; Moscowskaya Belaya, MB; Chernaya Tikhoretskaya, CHT; Uzbekskaya palevaya, UP).

DNA was isolated in accordance with the protocol for the commercial kit AmpliPrime DNA-Sorb-B (InterLabService, Russia). The amount and quality of isolated DNA were controlled using a NanoDrop 2000 c spectrophotometer (Thermo Scientific, United States) by a standard spectrophotometric method; the calculation and visualization of the result were performed using the NanoDrop 2000 software, version 1.4.2. Reference solution – TE-buffer pH 7.8-8.2 (FBSI Central Research Institute of Epidemiology, Russia).

Genotyping was performed at 12 microsatellite loci MNT9-MNT20 [18, 19]. PCR was carried out on a T 100 amplifier (Bio-Rad Laboratories, Inc., USA) in a mixture of a final volume of 20 rL containing the following reagents per reaction: 1 rL of forward and reverse primers (Federal Government Health Institution Stavropol Plague Control Research Institute of the Federal Service for the Oversight of Consumer Protection and Welfare, Russia), 2 rl of dNTP solution, 4 rl of RNA-eluent, 10 rl of PCR-mixture-2red (InterLabService, Russia) and 2 rl of DNA samples. The amplification mode was as follows: 15 min at 95 °C; 30 s at 95 °C, 30 s at 58 °C (for loci MNT10, MNT11, MNT20 – 56 °C), 30 s at 72 °C (35 cycles); 5 min at 72 °C.

Capillary electrophoresis was performed using an Experion System station (Bio-Rad Laboratories, Inc., USA) and a kit of reagents for visualization of DNA fragments Ex-perion DNA 1K Analysis Kit (Bio-Rad Laboratories, Inc., USA).

For comparison with the genotypes of turkeys of Russian breeds, the described genotypes of turkeys (AM) of the gene pool of the University of Minnesota (Nicholas Turkey Breeding Farms) were used [18, 19].

The average number of alleles and the number of effective alleles per locus (Na, Ne), the degree of observed and expected heterozygosity (H_o, H_e), and Shannon's index (I) were determined using the Microsoft Excel 2007 and GenAIEx v 6.5 software packages [20]. Means (*M*) and standard deviations (± SD) were calculated. The genetic structure of populations was assessed based on the F_{st} values [21] and genetic distances according to Nei [22]. The phylogenetic tree was constructed using the neighbor-joining method using the Structure 2.3.4 software [23].

Results. For the work, the researchers selected microsatellite loci, which were used in the study of turkeys of the gene pool of the University of Minnesota (NTBF) (Table 1).

1. Primers used for microsatellite genotyping of turkeys (*Meleagris gallopavo*)

Locus	Accession number in GenBank	Nucleotide sequence	Primer sequence (5′→3′)		DNA fragment size, bp
			forward	reverse	
MNT9	AF482368	$(CA)_{18}$	TGGGAGTGGAAAGGTGAAAG	TTCTCCTCAGCTCAGCAACC	164, 168
MNT10	AF482369	$(TG)_{10}+(TTTTG)_5$	TTCCCAGTGCACTACCTGAAC	TGAACAGTGATTCCACTGAAGC	67, 78
MNT11	AF482370	$(TG)_{12}$	TTTCTGACACAGGTACAAGGAAAC	GCCCTCGAGTATTAGCCACTC	90
MNT12	AF482371	$(TG)_{14imp}$	AGGTGTTTTTGGGCAGTCTC	TGCAAGCACCATCTGCTAAG	121, 145
MNT13	AF482372	$(TG)_{20}$	TTAGGGGATGCTGAACTGTG	GCGTAATTGGTGCTTTCTCC	183, 185, 187, 235
MNT14	AF482373	$(CA)_{10}$	AAACAGAACAACCTCAAGGACAG	GAATTGGGTTTGCATTTGAG	177, 181
MNT15	AF482374	$(CA)_{12}$	TTGTTGCTGTTGTTTTTGTGG	TTTCTGTGCCTAAGCTTAATGTG	188
MNT16	AF482375	$(TG)_{13}+(TG)_{11}+(TG)_8+(TG)_5$	TGTTTGCCTGCAATAAGCTG	GCACCCTCCCACTGACTG	219, 226, 234
MNT17	AF482376	$(TA)_5+(CA)_{29}$	AGGAGCACCCAGCTCAAAG	GAGTAATACCAAGGAAAAGTGTGC	181
MNT18	AF482377	$(TG)_{13}$	GCAGGCACAGAGAGCTACG	CCAATGTTGAAGCAGGTGAG	158, 159, 161, 162
MNT19	AF482378	$(TG)_{22}$	GCAGGAGGCTCTGAGCTATG	TTATACGGAAGGCGGTTGAG	224, 250
MNT20	AF482379	$(CA)_{15}$	TAACTGTCTGCCAGGTGGTG	GATCTCGGGTGGTGATTGC	192, 195

Analysis of the data obtained made it possible to establish that the turkeys of Russian breeds and the gene pool of the University of Minnesota were characterized by low genetic diversity. The number of identified alleles in microsatellite loci as a whole for the breed sample varied from 1 to 4 (Table 2).

2. The number of alleles of microsatellite loci in turkeys (*Meleagris gallopavo*) of Russian breeds (North Caucasian Zonal Poultry Experimental Station, Stavropol Territory, 2019) **and Gene Pool Populations of Nicholas Turkey Breeding Farms** (University of Minnesota)

Locus	Breed							
	BSH	BrSK	BeSK	SSK	MB	CHT	UP	AM
MNT9	2	2	2	1	2	2	1	2
MNT10	1	1	1	2	2	1	1	1
MNT11	1	1	1	1	1	1	1	3
MNT12	2	2	2	1	2	1	1	1
MNT13	4	3	3	2	2	1	1	2
MNT14	2	1	1	1	2	1	1	2
MNT15	1	1	1	1	1	1	1	2
MNT16	2	2	2	1	1	2	1	2
MNT17	1	1	1	1	1	1	1	2
MNT18	3	2	2	1	3	1	1	1
MNT19	1	1	1	1	2	2	2	1
MNT20	2	1	1	1	2	2	2	2

Note. BSH — Belaya shirokogrudaya, BrSK — Bronzovaya Severokavkazskaya, BeSK — Belaya Severokavkazskaya, SSK — Serebristaya Severokavkazskaya, MB — Moscowskaya Belaya, CHT — Chernaya Tikhoretskaya, UP — Uzbekskaya palevaya, AM — population of a gene pool of the University of Minnesota [18, 19].

The average number of alleles (Na) per locus ranged from 1.0 to 1.83, with the largest number being characterized by the BSH (1.83), MB (1.75) breeds, and the AM population (1.75). One allele per locus was identified in the SSK and UP breeds. Similar patterns were observed in relation to the number of effective alleles (Ne): the highest value of this indicator was in the BSH breed and the AM population (1.58 and 1.55), the minimum − in the SSK and UP breeds (1.0), the Moscowskaya Belaya and Chernaya Tikhoretskaya occupied an intermediate position (1.30 and 1.16). The revealed low number of alleles per microsatellite locus in turkeys of Russian breeding is consistent with the data of foreign researchers, who indicate a low genetic diversity of commercial turkey breeds in comparison with other species of farm animals and poultry, as well as wild turkey populations [24, 25].

3. Genetic diversity of turkeys (*Meleagris gallopavo*) of Russian breeds ($n = 30$, North Caucasian Zonal Poultry Experimental Station, Stavropol Territory, 2019) **and Gene Pool Populations of Nicholas Turkey Breeding Farms** (University of Minnesota) inferred from microsatellite loci ($M \pm SD$)

Breed	Na	Ne	H_o	H_e	$H_o–H_e$	I
BSH	1.83±0.27	1.58±0.19	0.273±0.07	0.279±0.07	–0.006	0.416±0.12
BrSK	1.50±0.19	1.07±0.03	0.063±0.02	0.065±0.02	–0.002	0.122±0.04
BeSK	1.50±0.19	1.43±0.18	0.207±0.07	0.212±0.07	–0.005	0.304±0.11
SSK	1.00±0.01	1.00±0.00	0.000±0.00	0.000±0.00	0.000	0.000±0.00
MB	1.75±0.17	1.30±0.12	0.189±0.05	0.194±0.05	–0.005	0.312±0.08
CHT	1.33±0.14	1.16±0.08	0.106±0.04	0.111±0.04	0.005	0.162±0.07
UP	1.00±0.01	1.00±0.00	0.000±0.00	0.000±0.00	0.000	0.000±0.00
AM	1.75±0.17	1.55±0.15	0.286±0.07	0.291±0.07	0.005	0.419±0.14

Note. BSH — Belaya shirokogrudaya, BrSK — Bronzovaya Severokavkazskaya, BeSK — Belaya Severokavkazskaya, SSK — Serebristaya Severokavkazskaya, MB — Moscowskaya Belaya, CHT — Chernaya Tikhoretskaya, UP — Uzbekskaya palevaya, AM — population of a gene pool of the University of Minnesota [18, 19]. Na and Ne — average and effective numbers of alleles per locus, H_o and H_e — observed and expected heterozygosity, I — Shannon index.

Comparison of the values of expected and observed heterozygosity ($H_o–H_e$) showed a lack of heterozygotes in all studied breeds and populations from

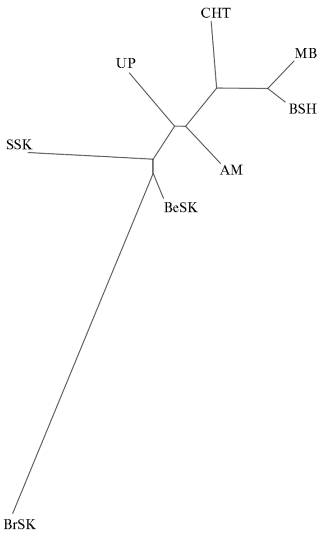
0.2 to 0.6%. The low genetic diversity was also evidenced by the Shannon information index, which did not exceed 0.50, and in the SSK and UP breeds it was equal to zero (Table 3).

Calculation of Nei's genetic distances (Table 4) and cluster analysis using the neighbor-joining tree method made it possible to obtain a graphical display of the phylogenetic relationship (Fig.) between the Russian breeds of turkeys and the population of the gene pool of the University of Minnesota.

4. Genetic differentiation of turkeys (*Meleagris gallopavo*) of Russian breeds (North Caucasian Zonal Poultry Experimental Station, Stavropol Territory, 2019) **and Gene Pool Populations of Nicholas Turkey Breeding Farms** (University of Minnesota) inferred from microsatellite loci

Порода	BSH	BrSK	BeSK	SSK	MB	CHT	UP	AM
BSH	0.000	0.250	0.127	0.594	0.344	0.457	0.162	4.872
BrSK	0.346	0.000	0.046	0.425	0.493	0.430	0.461	–
BeSK	0.180	0.127	0.000	0.579	0.514	0.469	0.212	–
SSK	0.495	0.423	0.514	0.000	0.131	0.363	1.386	–
MB	0.300	0.467	0.443	0.178	0.000	0.228	0.760	–
CHT	0.392	0.431	0.415	0.502	0.267	0.000	0.762	–
UP	0.241	0.612	0.339	0.870	0.672	0.672	0.000	3.684
AM	0.600	0.728	0.643	0.735	0.633	0.705	0.723	0.000

Примечание. BSH — Belaya shirokogrudaya, BrSK — Bronzovaya Severokavkazskaya, BeSK — Belaya Severokavkazskaya, SSK — Serebristaya Severokavkazskaya, MB — Moscowskaya Belaya, CHT — Chernaya Tikhoretskaya, UP — Uzbekskaya palevaya, AM — population of a gene pool of the University of Minnesota [18, 19]. Nei's genetic distance [22] is above the diagonal, Fst values in pairwise comparison is under the diagonal. Dashes indicate no indicators.



Genetic links between turkeys (*Meleagris gallopavo*) of Russian breeds (North Caucasian Zonal Poultry Experimental Station, Stavropol Territory, 2019) **and Gene Pool Populations of Nicholas Turkey Breeding Farms** (University of Minnesota) **inferred from Nei's genetic distance [22]:** BSH — Belaya shirokogrudaya, BrSK — Bronzovaya Severokavkazskaya, BeSK — Belaya Severokavkazskaya, SSK — Serebristaya Severokavkazskaya, MB — Moscowskaya Belaya, CHT — Chernaya Tikhoretskaya, UP — Uzbekskaya palevaya, AM — population of a gene pool of the University of Minnesota [18, 19].

The genetic distance dendrogram showed the smallest genetic difference between the MB and BSH breeds. The BeSK, SSK, and BrSK breeds formed a separate node, while the BrSK breed showed the greatest genetic removal, both in this node and with other breeds, forming the largest branch in terms of genetic distance. CHT, UP, and AM populations formed separate branches at relatively equal distances. The location of turkey breeds on the genetic distance tree, apparently, was due to the history of their creation. The Belaya shirokogrudaya breed is one of the oldest breeds in the world, created in the 1960s in the United States. She is of genetic origin from Dutch white turkeys. The Belaya shirokogrudaya turkey breed, namely four lines of the Hidon cross (A, B, C, D), were brought to the North Caucasian zonal station for poultry in 1980 from Holland. On the basis of lines B and D, the parental forms were selected, which have remained pure to the present day. Their DNA samples were used in the present study. The Moscowskaya Belaya breed was created in the Moscow Region. Initially, local white turkeys and Beltsville turkeys were used, and finally, white Dutch turkeys were used. That is, the gene pool of white turkeys was used for a long time to create breeds of Belaya shirokogrudaya and Moscowskaya Belaya, which led to their close location on the tree of genetic distances.

The first Russian breed of turkeys, the Bronzovaya Severokavkazskaya one,

was bred in the 1950s-1960s by crossing local aboriginal turkeys with producers of the Bronzovaya and Bronzovaya shirokogrudaya breeds. The second domestic breed, the Belaya Severokavkazskaya breed, was created in the 1970s-1980s by crossing native Bronzovaya Severokavkazskaya turkeys with males of the Belaya shirokogrudaya breed of English origin. When breeding the Serebristaya Severokavkazskaya breed released in 2008, the population of Uzbekskaya palevaya turkeys in the first stages was improved by the Belaya shirokogrudaya breed, mainly males of the O4 line of the maternal type, in order to increase reproduction and meat productivity. At the final stage of the creation of the breed, individuals with different shares of the gene pool of the white broad-breasted were bred "in themselves" with strict culling of phenotypes that did not meet the requirements. Consequently, the Belaya Severokavkazskaya and Serebristaya Severokavkazskaya breeds were created using the gene pool of Belaya shirokogrudaya, which, apparently, determined their great genetic proximity to each other and some distance from Bronzovaya Severokavkazskaya, which was created exclusively using the bronze plumage breeds. It is possible that the formation of a common node by the Bronzovaya, Belaya, and Serebristaya Severokavkazskaya breeds was also influenced by the fact that they were all created in the North Caucasian region. Habitat factors of the same type, apparently, contributed to the selection of closely related genotypes.

The genetic remoteness of the Chernaya Tikhoretskaya and Uzbekskaya palevaya breeds is explained by the fact that they were created to a greater extent using populations of local turkeys. The Uzbekskaya palevaya breed was bred on the basis of the Uzbekskaya Bronzovaya turkeys, which were pointwise improved by the Belaya shirokogrudaya breed. The use of the Belaya shirokogrudaya breed in the breeding of Uzbekskaya palevaya should have influenced their genetic affinity. However, the Chernaya Tikhoretskaya breed turned out to be closer to the Belaya shirokogrudaya and Moscowskaya Belaya breeds. It can be assumed that the significant geographical remoteness of the area of creation of the Uzbekskaya palevaya breed determined a greater genetic difference with the Moscowskaya Belaya and Belaya Severokavkazskaya breeds, which, like the Chernaya Tikhoretskaya, were created in the North Caucasian region.

The population of turkeys of the gene pool of the University of Minnesota showed a certain genetic distance from Russian breeds. However, this distance was not as pronounced as expected. Probably, the gene pool of the AM population includes both the gene pool of breeds with bronze plumage and the gene pool of the most widespread white broad-breasted breed in the world, which determines its equal distance from the studied Russian turkey breeds.

The data obtained in this study largely coincide with the results of Fisinin et al. [17]. Constructing a dendrogram based on genetic distances using the same microsatellite loci, but using a smaller sample and using the START 2 program, distributed the studied breeds into two clusters. The first cluster was formed by a part of the genotypes of Belaya shirokogrudaya and all genotypes of the Uzbekskaya Palevaya breed, the second — by two large subclusters. The first subcluster was formed by the genotypes of the Bronzovaya Severokavkazskaya, Belaya Severokavkazskaya, and Belaya shirokogrudaya breeds, the second — by the genotypes of the Chernaya Tikhoretskaya, Serebristaya Severokavkazskaya and Moscowskaya Belaya breeds [17]. In the above and the present study, the closest were the Belaya shirokogrudaya and Moscowskaya Belaya, Belaya Severokavkazskaya and Serebristaya Severokavkazskaya breeds; the Chernaya Tikhoretskaya and Uzbekskaya palevaya were more genetically remoted. The use in the presented work of a larger number of individuals and the method of the nearest neighbor in the Structure 2.3.4 program revealed a more significant genetic differentiation of the Bronzovaya

Severokavkazskaya breed.

In another study, also carried out at the North Caucasian Zonal Experimental Station for Poultry, using the DNA fingerprinting method, it was shown that the most similar breeds were Bronzovaya Severokavkazskaya and Belaya Severokavkazskaya, followed by Serebristaya Severokavkazskaya and Uzbekskaya palevaya. Chernaya Tikhoretskaya showed a significant genetic distance from the breeds of the Belaya shirokogrudaya, Belaya Severokavkazskaya, Serebristaya Severokavkazskaya, Bronzovaya Severokavkazskaya, and Uzbekskaya palevaya [26].

Many authors point out that the main factors affecting the degree of genetic differentiation of domestic breeds and wild populations of turkeys are the use of the gene pool of some breeds when creating others and the geographical area of their breeding. At the same time, scientists are unanimous in the opinion that the genome of turkeys is much less diverse than the genome of other types of farm animals and poultry.

Latch et al. [24] investigated wild oriental (*M. gallopavo silvestris*) and Russian turkey (*M. gallopavo*) using seven microsatellite markers. The number of alleles per locus varied from 5 to 15, while Russian turkeys compared to eastern wild ones were characterized by significantly fewer alleles per locus and general heterozygosity.

Kamara et al. [27] studied the genetic differentiation between commercial and non-commercial turkey breeds — Narra-gansett, Bourbon Red, Blue Slate, Spanish Black, and Royal Palm from the gene pool of the Virginia College farm 10 microsatellite loci (RHT0009, RHT0011, RHT0024, RHT0095, RHT0131, RHT0216, RHT0294, TUM16, TUM20, ADL0023). Using phylogenetic analysis, it was found that the Narra-ganset, Bourbon Red, and Blue Slate breeds had greater genetic similarity to commercial breeds than Spanish Black and Royal Palm [27]. Similar data for these breeds were obtained with other genetic marker systems (SNPs and DNA fragments of random sequences − random amplification of polymorphic DNA, RAPD) [28]. Kusza et al. [25] carried out a clear genetic differentiation between the Hungarian bronze and Belaya shirokogrudaya turkey breeds based on 15 microsatellite loci. The Hungarian Bronze breed was more polymorphic (average number of alleles per locus 3.20) than Belaya shirokogrudaya (average number of alleles per locus 2.77).

Mock et al. [29] used microsatellite markers and mitochondrial DNA in its most variable part to study genetic relationships between wild turkey populations. They studied 24 populations of six subspecies of wild turkeys: seven — Rio Grande (*M. gallopavo intermedia*), six — eastern turkey (*M. gallopavo silvestris*), three — Florida (Florida, *M. gallopavo osceola*), five — Merriam's (*M. gallopavo merriami*), three — Gould's (*M. gallopavo mexicana*). The authors established the correspondence of the modern division of wild turkey subspecies, based on the morphological description, to their genetic characteristics, except for the eastern turkey and Florida, which showed no genetic differences. The populations of Merriam and Rio Grande showed a positive relationship between genetic and geographic distance, while no such relationship was found in populations of eastern turkey.

For deeper information on the genetic diversity of turkey breeds and lines, Aslam et al. [30] used a more modern and informative method — whole-genome sequencing. As a result of scanning the genome of 32 turkeys from different populations, 5.49 million SNPs were identified in relation to the described reference turkey genome (UMD 2.01), which is 1.1 billion bases [31, 32]. The heterozygosity of individuals varied from 0.17 to 2.73 SNP, and throughout the entire sample ranged from 0.73 to 1.64 SNP per thousand base pairs. The authors concluded that the studied commercial breeds and lines of turkeys had a common origin, while the genetic basis for their breeding was wild forms of turkeys, which are

characterized by higher heterozygosity. The authors also emphasize that the genome of turkeys, in comparison with the genome of other species of farm animals and poultry, is characterized by a much greater conservatism.

Thus, the genetic peculiarity and differences in the genotypes of turkey breeds of the breeding and genetic center for breeding and preserving the gene pool of domestic turkey breeds in comparison with the turkey population of the gene pool of the University of Minnesota are shown. The smallest genetic differences were found between the breeds of Moscowskaya Belaya and Belaya shirokogrudaya, Belaya Severokavkazskaya and Serebristaya Severokavkazskaya. The Chernaya Tikhoretskaya and Uzbekskaya palevaya breeds, as well as the population of turkeys of the University of Minnesota gene pool, showed great genetic remoteness both from the above breeds and among themselves. The highest genetic differentiation was demonstrated by the Bronzovaya Severokavkazskaya breed. Intra-breed characteristics and inter-breed differentiation of seven Russian turkey breeds by microsatellite DNA markers largely reflect the history of their creation and improvement. Analysis of the number of alleles per locus made it possible to confirm the regularity revealed by other researchers, according to which the gene pool of breeds and populations of domestic turkeys is characterized by insignificant genetic diversity. For further rational use of the gene pool of domestic turkey breeds, as well as obtaining new information about their genetic characteristics and place in genetic differentiation among other turkey breeds bred in the world, it is advisable to use additional methods and modern markers of genetic analysis, such as SNP and MLST sequencing.

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EFFECT OF T-2 TOXIN ON EXPRESSION OF GENES ASSOCIATED WITH IMMUNITY IN TISSUES OF THE BLIND PROCESSES OF THE INTESTINAL AND PANCREAS OF BROILERS (*Gallus gallus* L.)

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Abstract

A significant proportion of poultry feed is contaminated with T-2 toxin. The bird's immune system is one of the targets of this xenobiotic. However, the results of studying the effect of T-2 toxin on the expression of immunity genes in birds are extremely limited. In the present study, we have shown that contamination of broiler feed with T-2 toxin affects the level of expression of genes associated with the functioning of the immune system in the cecum and pancreas. The aim of the work was to assess the effect of T-2 toxin on the level of expression of genes involved in the immune system responses in the tissues of the intestine and pancreas cecum in broilers. The feeding trials with T-2 toxin added to the feed were carried out on broilers of the Smena 8 cross from 30 to 50 days of age (the vivarium of the Federal Research Center VNITIP RAS, 2020). Broilers were assigned to four treatments. The control group I received a diet with no T-2 toxin added, group II received a diet added with 100 μg/kg T-2 toxin, group III with 200 μg/kg, and group IV with 400 μg/kg. Gene expression was analyzed by quantitative PCR with reverse transcription (RT-qPCR). A reverse transcription reaction was performed to generate cDNA on an RNA template using the iScript™ Reverse Transcription Supermix (Bio-Rad, USA). The following primer pairs were used: for Interleukin 6 (*IL6*) F — 5′-AGGACGAGATGTGCAAGAAGTTC-3′, R — 5′-TTG-GGCAGGTTGAGGTTGTT-3′; for Interleukin 8 (*IL8*) F — 5′-GGAAGAGAGGTGTGCTTGGA-3′, R — 5′-TAAC-ATGAGGCACCGA-TGTG-3′; for Interferon 7 (*IRF7*) F — 5′-ATCCCTTGGAAGCACAACGCC-3′, R — 5′-CTGA-GGCAACCGCGTAGACCTT-3′; for Prostaglandin-endoperoxide synthase 2 (*PTGS2)* F — 5′-TC-GAGATCACACTTGATTGACA-3′, R — 5′-TTTGTGCCTTGTGGGTCAG-3′; for avian beta-defensin 9 (*AvBD9*(*Gal9*)) F — 5′-AACACCGTCAGGCATCTTCACA-3′, R — 5′-CGTCTTCTT-GGCTGTAAGCTGGA-3′, for avian beta-defensin 10 (*AvBD10*(*Gal10*)) F — 5′-GCTCTTCGCT-GTTCTCC-TCT-3′, R — 5′-CCAGAGATGGTGAAGGTG-3′; for Caspase 6 (*Casp6*) F — 5′-CAG-AGGAGACAAGTGCCAGA-3′, R — 5′-CCAGGAGCCGTTTACAGTTT-3′. The beta-actin protein gene was a reference control. Amplification reactions were performed using a SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA). The amplification mode and conditions corresponded to those proposed by the primer developers. The relative expression level was estimated by the $2^{-\Delta\Delta CT}$ method. Biochemical blood profiles of broilers were analyzed (a Sinnowa BS3000P semi-automatic biochemical analyzer, SINNOWA Medical Science & Technology Co., Ltd, China) with a set of

veterinary diagnostic reagents (DIAKON-VET, Russia). Principal component analysis (PCA) was used to compare gene expression levels and blood biochemical parameters. The expression of genes associated with the inflammatory response, apoptosis, antimicrobial and antiviral protection was evaluated. Activation ($p \leq 0.05$) of the expression of the pro-inflammatory genes *IL6* and *PTGS2* occurred in broilers fed T-2 toxin. This can adversely affect the health and productivity of the birds, since the overproduction of proinflammatory cytokines is involved in the pathogenesis of several diseases. An increase (up to 41.7-fold, $p = 0.0005$) in the *PTGS2* gene expression in the pancreas was characteristic of all groups fed T-2 toxin compared to the control. In the tissues of the intestinal cecum, there was a decrease (up to 12.5-fold, $p = 0.02$) in the expression level of the *Casp6* gene of the apoptosis factor regardless of the T-2 toxin dosage. In the pancreas, there was a reverse tendency of a sharp increase in the *Casp6* gene expression as the T-2 toxin concentration increased ($p \leq 0.0008$). In group II, the expression increased 22.4 times ($p = 0.0008$), in group III 715.8 times ($p = 0.0003$), in group IV 31288.3 times ($p = 0.0003$) compared to the control. The expression of *AvBD9* and *AvBD10* genes of avian β-defensins which are associated with a higher bacteriostatic activity against many pathogens decreased 2.1 to 5.3 times ($p \leq 0.05$) in the caecum of broilers fed 200 and 400 200 μg/kg T-2 toxin. In the pancreas, regardless of the T-2 toxin dosage, on the contrary, the expression of these genes significantly increased ($p \leq 0.04$). In the caecum, 100 μg/kg T-2 toxin exposure inhibited the *IRF7* gene expression 3-fold ($p = 0.03$) compared to the control. This can negative affect birds' health, since the *IRF7* gene of the interferon regulatory factor 7 participates in counteraction against many viruses. In general, the pancreas was found to be more sensitive to the effects of the T-2 toxin because the expression of almost all studied genes was significantly increased as compared to that in the cecum tissue. This difference in the immune response may be due to the functional divergence between the intestine and the pancreas. PCA method revealed a close relationship between the expression of the *PTGS2* gene in the pancreas, the *IL6*, *PTGS2*, *IL8*, *IRF7*, *AvBD9*, *AvBD10*, and *Casp6* genes in the cecum and the total blood protein, trypsin, glucose, alkaline phosphatase, triglycerides, and phosphatase/trypsin coefficient. Our findings indicate the effect of feed contamination with T-2 toxin on the immunological functions of the caecum and pancreas of broilers through modulation of the immunity genes expression. Quantitative PCR analysis of the expression of immunity genes can serve as an effective tool for the search for predictive markers of T-2 toxicosis of poultry to monitor the health status of livestock in poultry farms.

Keywords: T-2 toxin, mycotoxicosis, broilers, gene expression, bird immunity, cytokine, interferon, apoptosis, β-defensins

A significant part of poultry feed, primarily feed grains, is contaminated with mycotoxins – by-products of microscopic fungi. Among them, T-2 toxin has been acknowledged as one of the most toxic [1]. This compound belongs to the sesquiterpenoid trichothecene mycotoxins produced by molds of the genera *Fusarium*, *Myrothecium*, *Cephalosporium*, *Verticimonosporium*, and *Stachybotrys*. Micromycetes of *Fusarium* spp. are the main producers of T-2 toxin and the most common pathogens of agricultural crops in the temperate climate of Europe, Asia, and North America [2]. In Russia, a wide prevalence of T-2 toxin has been demonstrated in feed raw materials (grain feed, cake, meal) and in complete feed for poultry [3, 4]; data have been obtained on the high frequency of T-2 toxin contamination of sunflower meal and sunflower meal [5].

The toxicity of *Fusarium* mycotoxins is based on the ability to bind to eukaryotic ribosomes and inhibit protein synthesis, as well as the ability to generate free radicals [6]. As a result of the effect of T-2 toxin on animals, a change in metabolism in the tissues of the spleen, thymus, stomach, and liver was noted [7].

In poultry, when the feed is contaminated with T-2 toxin, feed refusal, deterioration of its conversion rates, bloody diarrhea, a decrease in live weight gain, egg production, and thinning of the eggshell are noted [8]. The action of mycotoxins is most susceptible to the epithelial surface of the digestive tract [9, 10], which manifests itself in ulcerative necrotic inflammation. In other animals, when exposed to T-2 toxin, acinar degeneration and necrosis in the pancreas were also noted [11].

A number of studies [12, 13] have confirmed the hypothesis that the immune system is one of the targets of mycotoxins. Trichothecene mycotoxins act in different directions and with unequal intensity on different links of the immune system, showing both immunostimulating and immunosuppressive

properties depending on the dose and frequency of exposure. The digestive tract is characterized by the function of the regional immune system [14, 15]. The tissues of the mucous membrane lining the gastrointestinal tract contain lymphoid structures, which represent the first line of defense against pathogens and xenobiotics entering the body [15]. In birds, lymphoid tissues in the gastrointestinal tract are well developed and consist of lymphoid cells proper, as well as specialized lymphoid structures – Peyer's patches [16]. These structures play an important role in the induction of immune responses [17]. Epithelial cells of the mucous membrane of the avian digestive system are equipped with TLR receptors associated with the induction of the synthesis of chemokines, cytokines, lysozymes, β-defensins, cathelicidins, and avidin [18–20]. In chickens, the proinflammatory cytokines interleukin-1β (IL1B), interleukin-6 (IL6), interleukin-17 (IL17A), interleukin-22 (IL22) have been described [21]. Among the main groups of antimicrobial defensin peptides in chickens, β-defensins (previously known as gallinacins) AvBD1 (Gal1, Gal1), AvBD2 (Gal2), AvBD4 (Gal4), AvBD10 (Gal10) were identified [22–24]. Due to the conformation, avian β-defensins exhibit more pronounced efficacy against gram-positive bacteria [25]. The enzymes of the caspase group play a key role in apoptosis. In addition to apoptosis, these enzymes are involved in modulating inflammatory responses [26].

Thus, poisoning with mycotoxins, in particular with T-2 toxin, can be accompanied by complications associated with a violation of the biological mechanisms of resistance to infections and other immune functions. The volume of studies of such changes is growing [1, 27]; however, information on the effect of the T-2 toxin on the expression of genes associated with immunity in birds remains extremely limited. The accumulation of these data will allow understanding the patterns of disorders of immune homeostasis, which is necessary for the search for effective immunostimulants or immunosuppressants for the prevention and treatment of post-intoxication infectious complications and diseases and the identification of biomarkers of T-2 toxicosis.

This work has shown for the first time that the expression of some genes associated with immunity serves as an early prognostic marker of T-2 toxicosis in broilers. This concerns the activation in the tissues of the pancreas of the genes of regulatory molecules that provide the initial stages of the development of the inflammatory response, in particular *IL6* and *PTGS2*, as well as genes associated with cell death, for example, *Casp6*. In addition, the activation of genes for antimicrobial factors, primarily *AvBD10*, can be a marker of the pathological process.

The aim of the study was to assess the effect of 20-day exposure to T-2 toxin when introduced with feed on the expression of genes associated with inflammatory response, apoptosis, antimicrobial and antiviral protection.

Materials and methods. Experiments were performed on broiler chickens (*Gallus gallus* L.) of the Smena 8 cross from 30 to 50 days of age (the vivarium of the Federal Research Center VNITIP RAS, 2020) in accordance with the European Convention for the protection of vertebrate animals used for experiments or other scientific purposes (ETS No. 123, Strasburg, 1986). The conditions of feeding and keeping corresponded to the requirements for the cross ("Methodology for conducting scientific and industrial research on feeding poultry. Molecular genetic methods for determining the intestinal microflora". Sergiev Posad, 2013).

The basis of the poultry ration was the PK-6 compound feed (Russia). The feed was mechanically contaminated with standard T-2 toxin (powder with a mass fraction of the main substance of 99.7 ± 0.3%, Romer Labs, Austria, cat. No. 10000310, LOT # S17052T) to the set MPC in compliance with personnel safety requirements. The amount of mycotoxins in the feed before contamination (background content) and after contamination was monitored by tandem high-

performance liquid chromatography-mass spectrometry (HPLC-MS/MS); a chromatographic system Agilent 1260 Infinity LC, (Agilent Technologies, USA), a mass spectrometer ABSCIEX TripleQuad™ 5500 (Sciex, USA), a column for reverse-phase separation Gemini® C18 110A 5 μm 150×4.6 mm (Phenomenex, USA). Chromatographic separation was performed in the binary gradient elution mode. When preparing mobile phases A and B and carrying out HPLC-MS/MS, the researchers were guided by GOST 34140-2017 "Food products, feed, food raw materials. Method for the determination of mycotoxins using high-performance liquid chromatography with mass spectrometric detection" (Moscow, 2020). Separation mode: 1.5 min – 100% eluent A, 1.5 min – a linear increase in the proportion of eluent B to 50%, 9 min – a linear increase in the proportion of eluent B to 100%, 5 min – chromatographic separation at 100% eluent B; column temperature 25 °C, mobile phase flow rate 1 cm^3/min, sample injection temperature 10 °C, sample volume 5 mm^3. The retention time for T-2 toxin is 10.30 min, for NT-2 toxin – 9.79 min. The detection limits are 3.25 rg/kg for the T-2 toxin and 2.70 rg/kg for the HT-2 toxin, the quantitative limits for the T-2 toxin are 5.23–129.20 rg/kg, for the HT-2 toxins – 3.50–129.20 rg/kg. The calculated ion (in the mode of positive ionization by spraying in an electric field) for T-2 toxin is 305.1 m/z, for NT-2 toxin – 345.1 m/z. Standard solutions of T-2 and HT-2-toxin of the Biopure series (Romer Labs, Austria) were used to construct calibration graphs and as internal standards.

The chickens were divided into four groups (5 animals in each): group I received a diet without the introduction of T-2 toxin (control), II — a diet with the addition of T-2 toxin in an amount of 100 rg/kg (1MPC), III — 200 rg/kg (2MPC), IV — 400 rg/kg (4MPC).

To analyze gene expression in broilers, at the end of the experiment, tissues of the caecum and pancreas were taken. IntactRNA fixative was used to stabilize RNA in biological samples (Evrogen, Russia). The tissues were homogenized in liquid nitrogen. To isolate total RNA, the Aurum™ Total RNA mini-kit (Bio-Rad Laboratories, Inc., USA) was used following the manufacturer's instructions. The reverse transcription reaction was performed using an iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Inc., USA) [28].

Seven genes were selected for expression analysis. Specific primer pairs: for *IL6* F — 5′-AGGACGAGATGTGCAAGAAGTTC-3′, R — 5′-TTGGGCAGGTTGAGGTTGTT-3′; for *IL8* F – 5′-GGAAGAGAG-GTGTGCTTGGA-3′, R – 5′-TAACATGAGGCACCGATGTG-3′; for *IRF7* F – 5′-ATCCCTTGGAAGCACAACGCC-3′, R – 5′-CTGAGGCAACCGCG-TAGACCTT-3′; for *PTGS2* F – 5′-TCGAGATCACACTTGATTGACA-3′; R – 5′-TTTGTGCCTTGTGGGTCAG-3′; for *AvBD9 (Gal9)* F – 5′-AACACCGTCAGGCATCTTCACA-3′, R – 5′-CGTCTTCTTGGCTGTAAGCTGGA-3′; for *AvBD10 (Gal10)* F – 5′-GCTCTTCGCTGTTCTCCTCT-3′, R – 5′-CCAGAGATGGTGAAGGTG-3′, for *Casp6* F – 5′-CAGAGGAGACAAGTGCCAGA-3′, R – 5′-CCAGGAGCCGTTTACAGTTT-3′. The housekeeping gene *ACTB* (beta-actin protein) was used as a reference control with primers F – 5′-ATTGTCCACCGCAAATGCTTC-3′, R – 5′-AAATAAA-GCCATGCCAATCTCGTC-3′.

PCR was performed using a DT Lite-4 detecting amplifier (NPO DNA-Tekhnologiya, Russia) and a SsoAdvanced™ Universal SYBR® Green Supermix kit (Bio-Rad Laboratories, Inc., USA) in accordance with the manufacturer's protocol [29]. The amplification mode and conditions corresponded to each primer [30]. The relative expression level was assessed by the $2^{-\Delta\Delta CT}$ method [31].

Blood for biochemical analysis (2-3 ml) was taken at the end of the experiment after 14-hour fasting of birds from the axillary vein on the inner side

of the wing above the ulnar articulation ("General and Special Methods for Studying the Blood of Birds of Industrial Crosses". Yekaterinburg—St. Petersburg, 2009). Sodium citrate was an anticoagulant. The studies were performed on a Sinnowa BS3000P semi-automatic biochemical analyzer (SINNOWA Medical Science & Technology Co., Ltd, China) with a set of veterinary diagnostic reagents (DIAKON-VET, Russia).

To compare the levels of gene expression and blood biochemical parameters, the principal component analysis (PCA) was used with the construction of a correlation matrix. The number of significant principal components was determined on the basis of an eigenvalue criterion equal to 1, with the preservation of any component with an eigenvalue > 1 [32]. The quality of the presentation of the variables on the factorial map and the overall contribution to the principal components of individual traits were assessed based on the calculation of cos^2. Cos^2 visualization for variables in all dimensions was performed using the corrplot package (https://cran.r-project.org/web/packages/corrplot/corr-plot.pdf; https://github.com/taiyun/corrplot).

Mathematical and statistical processing of the results was carried out by multifactor ANOVA in Microsoft Excel XP/2003, R-Studio (Version 1.1.453) (https://rstudio.com). Mean values (*M*) and standard errors of means (±SEM) are shown. Mean values were compared using Tukey's significant difference test using the TukeyHSD function in the R Stats Package (https://cran.r-project.org/web/packages/TukeyC/Tu-keyC.pdf, https://github.com/jcfaria/TukeyC); differences were considered statistically significant at $p \leqslant 0.05$.

Results. Feed consumption by broilers averaged 150 g/day, that is, poultry of groups II-IV received daily toxin with T-2 feed in an amount of 15, 30, and 60 rg/bird, respectively.

Aflatoxins (B_1, G_1), fumonisins (B_1, B_2, B_3), and deoxynivalenol (DON) were not found in the feed used for contamination. The T-2 toxin was present in a background amount of 4.33 μg/kg, which could not significantly affect the results of the experiment, given that the MPC for T-2 toxin is 100 μg/kg. Ochratoxin A (2.27 μg/kg at MPC 50 μg/kg) and zearalenone (2.25 rg/kg at MPC 1000 μg/kg) were also detected in quantities that were much lower than the MPC.

Fig. 1 shows the effect of T-2 toxin on the expression of genes for interleukins *IL6*, *IL8*, as well as prostaglandin endoperoxide synthase *PTGS2*, which are associated with proinflammatory factors. T-2 toxin at a concentration of 100 μg/kg (1MPC) did not affect the expression of the *IL6, IL8,* and *PTGS2* genes in the caecum in group II of broilers. However, in group III (2MPC of T-2 toxin), the expression of the *PTGS2* gene increased by 3.0 times ($p = 0.03$), and in group IV (4MPC of T-2 toxin), the effect was even more pronounced: the level of expression of *PTGS2* increased by 5.9 times ($p = 0.001$); in addition, the level of expression of the *IL6* gene increased in comparison with the control ($p = 0.005$). In the tissues of the pancreas, the effect of exposure to T-2 toxin was more pronounced than in the tissues of the cecum of the intestine. For example, an increase in the expression of the *PTGS2* gene (up to 41.7-fold, $p = 0.0005$) was observed in all experimental groups compared with the control. T-2 toxin had a specific effect on the expression of proinflammatory genes in broilers, since no effect on the *IL8* gene was revealed (in contrast to the *IL6* and *PTGS2* genes) ($p > 0.05$). In general, an obvious dose-dependent effect of T-2 toxin occurred on the expression of proinflammatory genes in the tissues of the cecum of broilers. Previously, a similar effect with respect to *IL6* when exposed to toxic substances in animals was demonstrated by Brown et al. [33].

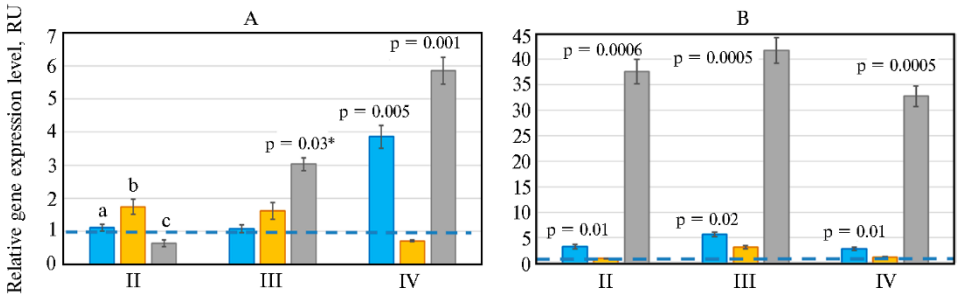

Fig. 1. The expression of genes associated with proinflammatory factors in the tissues of the epithelium of the cecal processes of the intestine (A) **and pancreas** (B) **in Smena 8 cross broiler chickens (*Gallus gallus* L.) under experimental T-2 toxicosis:** II, III, IV — the groups receiving 1MPC, 2MPC and 4MPC of T-2 toxin, respectively (1MPC — 100 μg/kg feed); a, b, c — genes *IL6*, *IL8* and *PTGS2*, respectively. Relative units are the frequency of changes in expression compared to group I (control), where the expression level was taken as 1. The dashed blue line shows the expression level in the control. The results are shown as *M*±SEM (the vivarium of the Federal Research Center VNITIP RAS, 2020).

* Differences with control are statistically significant at $p \leq 0.05$.

The activation of the expression of proinflammatory genes when fed with T-2 toxin may have adverse effects on the health and productivity of broilers. On the one hand, interleukins are important for innate protective immune responses, attracting additional leukocytes to the site of infection, which increases the resistance of epithelial cells [34, 35]. On the other hand, the overproduction of proinflammatory cytokines is involved in the pathogenesis of a number of diseases in animals and humans [36], and is also associated with a decrease in the productivity of farm animals [37]. It has been shown [38-40] that the administration of drugs based on cytokines to healthy animals provoked negative reactions of the organism. The activation of inflammatory cytokines is closely related to the expression of the *PTGS2* gene, since cytokines are able to induce this gene [41]. *PTGS2* is a gene for prostaglandin endoperoxide synthase (cyclooxygenase 2), which is involved in the oxidative conversion of arachidonic acid to prostaglandin, which in these reactions is also metabolized to biologically active prostacyclin and thromboxane A2. Prostaglandin, prostacyclin, and thromboxane A2 are involved in both local and systemic inflammatory reactions [42].

The obtained data indicating the activation of the expression of proinflammatory genes when feeding the T-2 toxin to broilers is consistent with the results of previous studies. It has been shown [43-45] that under the influence of toxic substances in animals, the production of pro-inflammatory factors, in particular, the cytokines *IL4*, *IL10,* and *IL13*, increases. It was noted [46] that exposure to trichothecene mycotoxins was capable of transcriptionally and post-transcriptionally enhancing the expression of genes associated with the inflammatory response.

Fig. 2 shows the level of expression of the *Casp6* gene associated with the apoptosis factor in the epithelial tissues of the digestive system of broilers in response to feeding with T-2 toxin. A decrease (up to 12.5-fold, $p = 0.02$) in the expression of the *Casp6* gene was noted in the tissues of the intestinal cecum in all groups compared with the control (Fig. 2). A dose-dependent effect was revealed: an increase in the concentration of the toxin in the food led to a more pronounced inhibition of expression. The opposite trend was observed in the tissues of the pancreas. With an increase in the concentration of T-2 toxin in feed, the expression of the *Casp6* gene sharply increased ($p \leq 0.0008$): in group II — by 22.4 times ($p = 0.0008$), in group III — by 715.8 times ($p = 0.0003$), in group IV — 31,288.3 times ($p = 0.0003$) compared to the control.

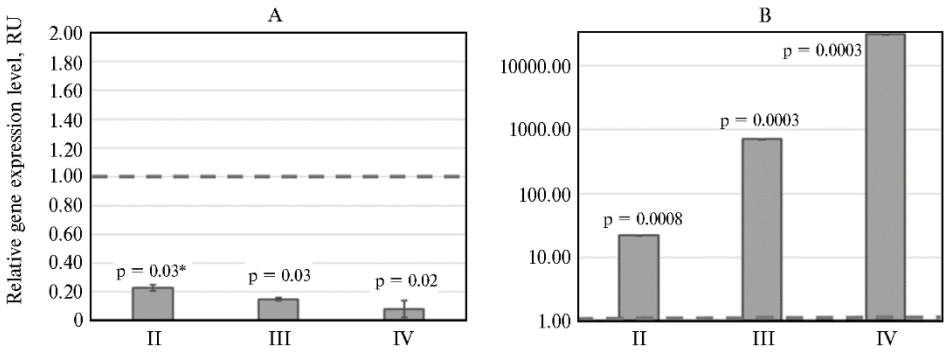

Fig. 2. The expression level of the *Casp6* gene associated with the apoptosis factor in the tissues of the epithelium of the cecal processes of the intestine (A) **and pancreas** (B) **in Smena 8 cross broiler chickens (*Gallus gallus* L.) under experimental T-2 toxicosis:** II, III, IV — the groups receiving 1MPC, 2MPC and 4MPC of T-2 toxin, respectively (1MPC — 100 μg/kg feed). RU is the multiplicity of changes in expression compared to group I (control), where the expression level is taken as 1. The dashed line shows the expression level in the control. The results are shown as *M*±SEM (vivarium of the Federal Scientific Center VNITIP RAS, 2020).

* Differences with control are statistically significant at $p \leq 0.05$.

Casp6 encodes the caspase 6 protein, which belongs to the family of cysteine-aspartate-specific proteases, the activation of which plays a central role in cell apoptosis [47]. On the one hand, apoptosis (genetically programmed cell death) is the most important mechanism of immunoregulation from the moment of maturation and differentiation of immunocompetent cells to the stage of implementation of the mechanisms of innate and adaptive immunity [48, 49]. On the other hand, in the tissues of the digestive system, toxin-induced apoptosis has negative consequences, since it is associated with damage to mitochondria and the release of cytochrome C from them (mitochondria-mediated apoptosis pathway) [50-52]. In addition, toxic substances can have a cytotoxic effect on macrophages and monocytes [52-54]. The activation of members of the caspase family is capable of inducing DNA damage in macrophages through the cleavage of the enzyme poly [ADP-ribose] polymerase 1 (PARP-1) [52, 55]. Studies have shown that certain toxic substances initiate apoptosis of B cells, which can aggravate the state of the immune system [56].

The obtained results, demonstrating the activation of *Casp6* gene expression in the broiler pancreas under the influence of T-2 toxin, coincide with the data published earlier. The activation of several caspases under the influence of trichothecenes has been reported [57-59]. It was shown [47] that high doses of trichothecene mycotoxins provoked apoptosis of leukocytes with concomitant suppression of immunity. In vivo administration of trichothecenes to rodents, including the T-2 toxin, led to apoptosis in the thymus, spleen, and bone marrow [60, 61].

Fig. 3 shows the level of expression of the *AvBD9* and *AvBD10* genes in the caecum and pancreas of broilers when exposed to T-2 toxin. *AvBD9* (*Gal9*) and *AvBD10* (*Gal10*) are avian β-defensin genes [62]. Defensins, selectively recruiting monocytes, T-lymphocytes, immature dendritic and mast cells to the foci of infection, participate in adaptive immunity reactions [63-65]. Defensins are associated with increased bacteriostatic activity against many pathogens, including *Klebsiella typhimurium*, *Streptococcus bovis*, *Enterococcus faecalis*, *Salmonella typhimurium* [24]. In the tissues of the cecum of the intestine in broilers of groups III and IV, the level of expression of the *AvBD9* and *AvBD10* genes decreased by 2.1–5.3 times ($p \leq 0.05$) compared to the control (see Fig. 3). On the contrary, the expression of these genes (especially *AvBD10*) in the pancreas tissues of broilers

from all groups increased significantly compared to the control ($p \leq 0.04$). Thus, in group IV, a 40.8-fold activation of the expression of the *AvBD10* gene ($p = 0.0002$) was noted.

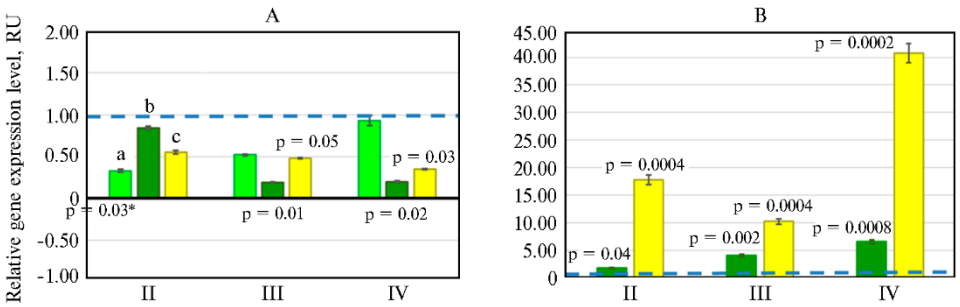

Fig. 3. The expression level of the genes associated with antimicrobial and antiviral factors in the tissues of the epithelium of the cecal processes of the intestine (A) **and pancreas** (B) **in Smena 8 cross broiler chickens (*Gallus gallus* L.) under experimental T-2 toxicosis:** II, III, IV — the groups receiving 1 MPC, 2MPC and 4MPC of T-2 toxin, respectively (1MPC — 100 μg/kg feed); a, b, c — genes *IRF7*, *AvBD9* and *AvBD10*, respectively. RU is the multiplicity of changes in expression compared to group I (control), where the expression level is taken as 1. The dashed line shows the expression level in the control. The results are shown as *M*±SEM (vivarium of the Federal Scientific Center VNITIP RAS, 2020).

* Differences with control are statistically significant at $p \leq 0.05$.

Note that the ability to both reduce and increase the resistance of animals to pathogens has been described by many researchers for various mycotoxins. Broiler chickens fed feed contaminated with ochratoxins were found to be more susceptible to Salmonella contamination [66]. In another study [67], the introduction of ochratoxin A into broiler feed did not affect the abundance of *S. typhimurium* in the caecum; however, the synergistic effect of salmonella and toxin led to an increase in poultry mortality by 13.2% and a decrease in body weight. In pigs with fumonisin B_1 contamination of feed, an increase in intestinal colonization of pathogenic *Escherichia coli* was associated [68], while feeding with T-2 toxin in the intestinal contents and other organs decreased the abundance of *Salmonella typhimurium* as compared to the control (feed without experimental contamination with T-2 toxin) [69].

In addition, in the caecum of the intestine of broilers from group II, the expression of the *IRF7* gene was inhibited by 3.0 times ($p = 0.03$) compared with the control (see Fig. 3). The *IRF7* gene encodes the regulatory factor interferon 7 (a member of the family of regulatory factors for interferon transcription) [70]. Due to its key role in host immunity, *IRF7* is involved in counteracting many viruses using various strategies [71, 72]. Pestka et al. [46] reported similar results regarding the expression of the *IRF7* gene in the tissues of the caecum of the intestine of broiler chickens with experimental T-2 toxicosis. Feeding broiler chickens with a mixture of mycotoxins, including DON, reduced IFN-γ mRNA synthesis. At the same time, the example of pigs [73] showed an increase in the production of interferon-γ upon contamination of feed with DON.

The comparison of the influence of T-2 toxin on the expression of genes associated with immunity in the cecum with the pancreas suggests that the tissues of the pancreas are more sensitive to the T-2 toxin, since the expression of almost all studied genes here was significantly enhanced compared to the tissues of the intestinal cecum. First of all, this concerns the activation in the tissues of the pancreas of the genes of regulatory molecules that provide the initial stages of the development of the inflammatory response, including *IL6* and *PTGS2*, genes associated with cell death, for example *Casp6*, and the synthesis of antimicrobial

factors, especially *AvBD10*. The authors believe that such a difference in the severity of the immune response may be due to functional differences between the intestine and the pancreas. It was shown [74] that the endocrine, as well as the exocrine, parts of the pancreas had enormous functional plasticity, which could also contribute to the enhancement of the expression of the studied genes when exposed to the T-2 toxin. This research confirms the data of Scaglia et al. [75], who described active apoptosis in the islets of Langerhans in the pancreas of 13-17-day-old rats. Autoimmune cytokines, in particular IL1β and TNFa, associated with additional insulin release, induce islet cell apoptosis [76]. Possibly, T-2 toxin may affect the synthesis of exocrine digestive enzymes by acinar cells or the synthesis of peptides by insulocytes of the islets of Langerhans of the pancreas. It was found that somatostatin, produced by delta cells of pancreatic islets, significantly affected the expression of genes involved in inflammatory reactions and leukocyte chemotaxis [77]. T-2 toxin could also cause damage to acinar cells in broilers [11] and, as a consequence, increased expression compared to the caecum. There is evidence that damage to acinar cells is accompanied by an increase in the synthesis of proinflammatory cytokines IL6, IL1β, TNFα, and IL8 [78].

In order to search for factors that influenced the expression of genes associated with immunity in experimental T-2 toxicosis of broilers, the researchers compared the data on *IL6, IL8*, *PTGS2*, *IRF7*, *AvBD9*, *AvBD10,* and *Casp6* in the cecum and pancreas with the biochemical parameters of blood (total protein, trypsin, glucose, alkaline phosphatase, cholesterol, triglycerides, AlPh/T — alkaline phosphatase/trypsin ratio) (Table), using PCA (Fig. 4).

Biochemical blood parameters in Smena 8 cross broiler chickens (*Gallus gallus* L.) under experimental T-2 toxicosis (*M*±SEM, vivarium of the Federal Scientific Center VNITIP RAS, 2020)

Parameter	Group (n = 5)			
	I (control)	II (1MPC)	III (2MPC)	IV (4MPC)
Total protein, g/l	39.5±0.83	36.8±1.94	39.9±0.17	41.5±1.31
	40.1±0.71	40.3±1.23	40.4±0.19	41.4±0.16
	36.3±1.20	36.4±0.13	35.5±0.21	40.1±0.85[b]
Trypsin, U/l	74.2±3.17	60.3±1.52	62.6±0.68	67.5±4.51
	93.3±4.36[a]	82.0±1.58[ab]	82.5±1.12[ab]	72.6±0.31[b]
	77.5±6.43	68.6±3.02	60.4±3.89	66.3±7.65
Glucose, mmol/l	12.1±0.19	11.1±0.32	11.1±0.13	10.9±0.09[b]
	12.4±0.24	11.2±0.33	12.8±0.07	13.0±0.21[a]
	10.4±0.32[a]	9.8±0.16[a]	10.0±0.16[a]	9.0±0.31[a]
Alkaline phosphatase (ALP)	6232±590.7	5456±313.1	7974±621.2	8157±296.5
(U/L)	4179±456.1[a]	4310±609.7	4370±476.2[a]	2987±757.8[ab]
	1298±192.5[a]	754±37.7[ab]	1303±118.8[a]	3695±114.7[ab]
Cholesterol, mmol/l	2.9±0.09	3.0±0.12	3.3±0.23	3.5±0.12
	3.4±0.04	3.1±0.05	3.6±0.25	3.6±0.21
	2.8±0.14	3.1±0.60	3.1±0.26	2.8±0.04
Triglycerides, mmol/l	0.4±0.01	0.3±0.01	0.3±0.02	0.4±0.02
	0.3±0.01	0.3±0.02	0.4±0.02	0.4±0.28
	0.8±0.04[a]	0.4±0.02	0.4±0.15	0.2±0.01[a]
AlPh/T (ALP/trypsin)	83.9	90.5	127.4	120.8
	44.8	52.6	52.9	41.1
	16.7	10.9	21.6	55.7

N o t e. For each parameter, the first row shows values for 33 days of age (before the start of the experiment), the second row for 40 days of age (received T-2 toxin for 7 days), and the third row for 47 days of age (received T-2 toxin for 14 days); the control group T-2 did not receive toxin; 1MPC corresponds to 100 μg/kg T-2 toxin concentration.

[a] Differences between indicators in chickens of different ages are statistically significant at $p < 0.05$.

[b] Differences between the parameters in control chickens and chickens receiving T-2 toxin at different doses are statistically significant at $p < 0.05$.

Fig. 5 characterizes the quality of the presented variables (cos^2) on the factor map (see Fig. 4). Variables including values of *PTGS2* gene expression in

the pancreas, *IL6, PTGS2, IL8*, *IRF7*, *AvBD9*, *AvBD10*, and *Casp6* in the caecum, as well as data on total protein, trypsin, glucose, alkaline phosphatase, triglycerides, FPI in the blood, made a high contribution to PC1 with a $\cos^2$ value of 0.44-0.88 (see Figs. 4, 5).

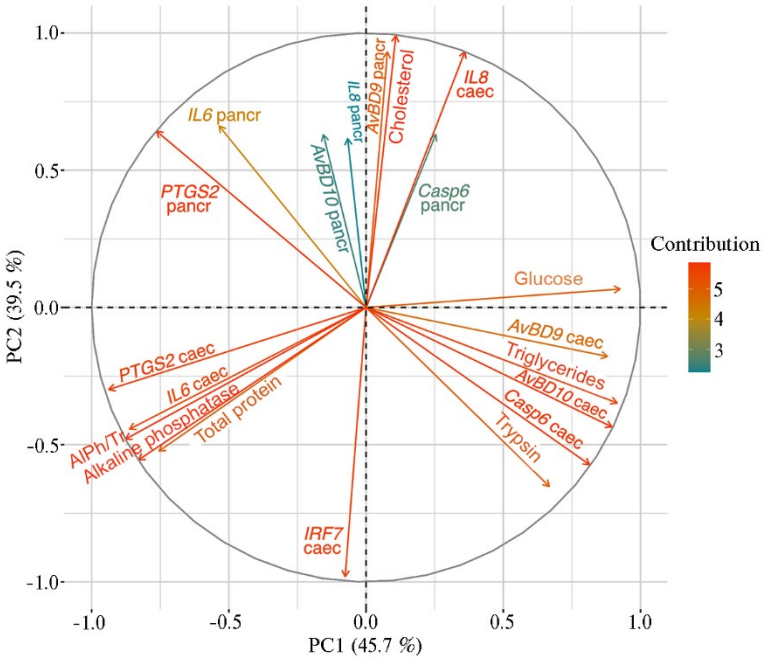

Fig. 4. Factor map (principal component analysis, PCA) **based on the expression levels of genes associated with immunity in tissues** (pancr — pancreas, caec — caecums of the intestine), **and biochemical blood parameters in Smena 8 cross broiler chickens (*Gallus gallus* L.) with experimental T-2 toxicosis.** The color gradient and closeness to the circle of correlations reflect the contribution of variables to the main components: red and close to the circle of correlations means high contribution, blue color and distance from the circle of correlations means low contribution. A variable on one side of another variable has a high value for that variable, and one on the diametrically opposite side of another variable has a low value for that variable. AlPh/T means the alkaline phosphatase/trypsin ratio (vivarium of the Federal Scientific Center VNITIP RAS, 2020).

The results obtained indicate that the studied indicators were in close connection with each other, which seems logical. Thus, the paper [79] showed a change in the activity of digestive enzymes in broilers under heat stress and a related change in the synthesis of mRNA of some genes, in particular *SGLT1*, *GLUT2, FABP1*. In experiments on experimental hyperstimulation of acute pancreatitis in animals [80, 81], it was found that inflammatory diseases of the pancreas were associated with the activation of trypsin synthesis in acinar cells. Therefore, the change in trypsin synthesis upon contamination of broiler feed with T-2 toxin could contribute to the regulation of the expression of proinflammatory genes, in particular *PTGS2*. In addition, it was reported [82] that trypsin was involved in the formation of so-called "fragile" membranes in organelles, through which cathepsin B and other enzymes enter the cytosol. The released cathepsin B induces apoptosis (through activation of caspases) and death of acinar cells of the pancreas [82]. In another study [83], it was shown that proteasomes that provide degradation of proteins in cells contain two trypsin-like and two caspase-like proteolytic regions, for which complex interactions have been proven: substrates of caspase-like sites allosterically inhibit chymotrypsin-like activity. These facts clarify the reason for the revealed relationship between the expression of the *Casp6* gene and the blood trypsin content.

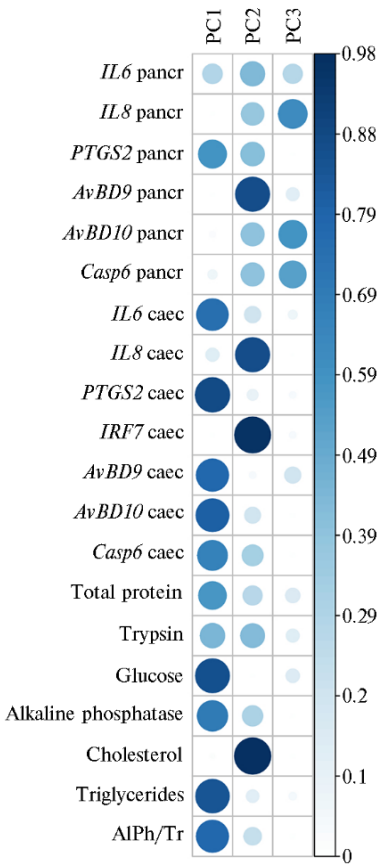

Fig. 5. Visualization of the quality of variables (cos^2) **on the factor map** (see Fig. 4, principal component analysis, PCA) **for the expression levels of genes associated with immunity in tissues** (pancr — pancreas, caec — caecums of the intestine) **and biochemical blood parameters in Smena 8 cross broiler chickens (*Gallus gallus* L.) with experimental T-2 toxicosis** (the corrplot package https://cran.r-project.org/web/packages/corrplot/corrplot.pdf; https://github.com/taiyun/corrplot). The diameter of the circle and the color intensity reflect the contribution of the variables (cos^2) to the principal components: large diameter and dark blue (high cos^2) indicate a high contribution of the variable, no circle and white (low cos^2) — the lowest contribution (vivarium of the Federal Scientific Center VNITIP RAS, 2020).

Expression of the genes *IL6*, *AvBD9* in the pancreas, *IL8*, *IRF7* in the caecum and blood cholesterol in broilers made a high contribution to PC2 with a cos^2 value of 0.44-0.98 (see Figs. 4, 5). The obtained results indicate the mutual influence of these variables. Indeed, it was reported [84] that the *IL8* gene, activating the expression of the *miR-183* gene in macrophages, which in turn inhibits the expression of the *ABCA1* gene, interferes with the *ABCA1*-dependent cholesterol efflux. In another work [85], it was found that a high level of intracellular cholesterol played a decisive role in the transmission of signals initiated by the proinflammatory gene product *IL-17A* in keratinocytes.

The results of the study show that increased levels of expression of genes that control the body's defense reactions and determine the nature of immunological reactions when exposed to T-2 toxin can be considered as early prognostic markers of T-2 toxicosis in broilers. First of all, this is the activation in the tissues of the pancreas of the genes of regulatory molecules that are involved in the initial stages of the development of inflammatory reactions (*IL6* and *PTGS2*), genes associated with cell death (*Casp6*), genes of antimicrobial factors (primarily *AvBD10*).

Therefore, the obtained data indicate the effect of contamination of broiler feed with T-2 toxin on the immune functions of the caecum and pancreas through modulation of the expression of genes associated with immunity. The observed activation ($p \leq 0.05$) of the expression of the proinflammatory genes *IL6* and *PTGS2* can create health risks for poultry and reduce its productivity, since the overproduction of proinflammatory cytokines is involved in the pathogenesis of various diseases. In the pancreas, the expression of the *PTGS2* gene increased even at 1MPC of the T-2 toxin in the food, reaching 41.7-fold values as compared with the control ($p = 0.0005$). In the cecum tissue, the expression of the gene *Casp6* for the apoptosis factor decreased, starting from 1MPC (up to a 12.5-fold decrease, $p = 0.02$), and in the pancreas, on the contrary, the activity of the *Casp6* gene with an increase in the concentration of T-2 toxin in feed increased sharply (by 22.4-31,288.3 times, $p \leq 0.0008$). The expression of the β-defensin genes *AvBD9* and *AvBD10* in the cecum tissues decreased compared to the control (2.1-5.3 times, $p \leq 0.05$) at 2MPC and 4MPC of T-2 toxin, and in the pancreas, regardless of the T-2 toxin dose, on the contrary, increased ($p \leq 0.04$) (to a greater extent, this

concerned *AvBD10*). The expression of the *IRF7* gene associated with the manifestation of antiviral activity was inhibited by 3.0 times in the blind processes of the intestine of birds at 1MPC T-2 toxin ($p = 0.03$). In general, in the tissues of the pancreas, the expression of almost all studied genes associated with immunity was more noticeably activated, and the frequency of apoptosis, which is closely related to the activation of caspase, significantly increased. That is, the pancreas turned out to be more sensitive to the T-2 toxin than the tissues of the cecum of the intestine, which may be due to the functional differences between the intestine and the pancreas. PCA showed that the expression of *PTGS2* genes in the pancreas, *IL6*, *PTGS2*, *IL8*, *IRF7*, *AvBD9*, *AvBD10*, and *Casp6* in the caecum of the intestine, as well as the level of total protein, trypsin, glucose, alkaline phosphatase, triglycerides, and the alkaline phosphatase/trypsin ratios in broiler blood, were closely related. Increased levels of expression of the genes *IL6*, *PTGS2*, *Casp6*, *AvBD10* can be considered as markers of the development of T-2 toxicosis in broilers and used to quantitatively characterize the potential effect of T-2 toxin and assess the sensitivity of poultry to it when monitoring the state of the livestock and carrying out preventive and therapeutic measures in poultry farms.

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THE INTESTINAL T-2 AND HT-2 TOXINS, INTESTINAL AND FECAL DIGESTIVE ENZYMES, MORPHOLOGICAL AND BIOCHEMICAL BLOOD INDICES IN BROILERS (*Gallus gallus* L.) WITH EXPERIMENTALLY INDUCED T-2 TOXICOSIS

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Abstract

At present over 400 mycotoxins have been identified with mutagenic, teratogenic, embryotoxic, allergenic, and immunosuppressing properties, suppressing cellular and humoral immunity. Trichothecene mycotoxins are prevalent on the territory of Russian Federation, the most abundant and hazardous being T-2 and HT-2 toxins inducing gastroenterites, necroses of skin and mucosa of the oral cavity, disturbances in the function of central nervous system. The exposure limit for T-2 toxin in feeds for poultry is established in SanPiN 2.3.2.1078-1 at 100 μg/kg. Mycotoxin induced injuries of the organs are preceded by functional disturbances affecting the hematological status and enzymatic reactions in the digestive tract; the most exact data on these disturbances can be obtained by *in vivo* experiments on fistulated birds. We used this approach in our study in combination with the analyses of mycotoxin concentrations in lyophilized samples of duodenal chyme and feces by high-performance liquid chromatography and tandem mass spectrometry (HPLC MS/MS). The aim of the study was the investigation of the T-2 toxin in the gastrointestinal tract and the effects of the toxin on the intestinal activities of the digestive enzymes and morphological and biochemical blood indices in cross Smena 8 broilers with experimentally induced T-2 toxicosis. Birds with chronic duodenal fistulae were fed the feeds contaminated with T-2 toxin in doses from 100 μg/kg (corresponding to the exposure limit statutorily set for chicken) to 400 μg/kg for 2 weeks. The measurements of the T2 toxin in the duodenal chyme and feces evidenced the transformation of T-2 to HT-2 toxin in the intestine and intense absorption of the latter into the bloodstream since its concentration in feces was significantly lower in compare to the duodenal chyme. All dietary doses of T-2 increased the duodenal activities of the total proteases (by 46.3-96.6 %, $p < 0.05$), lipase (by 16.8-25.5 %, $p < 0.05$), and amylase (by 99.7 %) at T-2 dose 400 μg/kg while activity of alkaline phosphatase decreased by 23.8-27.9 % ($p < 0.05$). In the feces the increase in proteolytic activity by 76.0-169.1 % ($p < 0.05$) and decreases in the activities of lipase (by 23.2 % at T-2 dose 400 μg/kg) and amylase (by 55.1-57.2 %) were found. The activity of trypsin in blood serum decreased by 12.2-22.2 % ($p < 0.05$) while the increases in the activity of alkaline phosphatase varied from 52.4 % to 5-fold. As a result the phosphatase-protease index increased from 31 to 92 in average. At T-2 dose 400 μg/kg the decreases in the concentrations in blood serum of total protein (by 13.2 %, $p < 0.05$), glucose (by 13.5 %, $p < 0.05$), and triglycerides (4-fold) were found. The total leukocyte number in blood decreased by 15.8-16.6 %. At T-2 doses 100 and 200 μg/kg the trend to higher percentage of the lymphocytes was found though the percentage of neutrophils remained unaffected, evidencing the activation of the specific antitoxic protective mechanisms. The inversed correlations between the degree of the transformation of T-2 toxin into HT-2 toxin and tryptic activity in duodenal chyme and phosphatase-protease index in blood serum were found; these correlations can be used in the diagnostic test for T-2 toxicosis.

Keywords: T-2 toxin, HT-2 toxin, T-2 toxicosis, broilers, duodenal chime, digestive enzymes, proteases, lipase, amylase, biochemical blood indices, trypsin, alkaline phosphatase, triglycerides, cell blood composition, leukocytic formula

Foods and feeds often contain mycotoxins, which are secondary metabolites produced by imperfect fungi (formal class *Fungi imperfecti*) of the genera *Fusarium, Aspergillus*, *Myrothecium*, *Trichoderma*, *Trichothecium*, *Penicillium*, etc. Mycotoxins vary in chemical structure, toxicity, and mechanism of action [1, 2]. Today, there are over 400 known mycotoxins; the most common ones that are toxic to humans and animals are aflatoxins (aflatoxin B_1), fumonisins, zearalenone, type B trichothecenes (deoxynivalenol), type A trichothecenes (T-2 toxin), and ochratoxin A [3].

Trichothecenes are synthesized by the genus *Fusarium* fungi; however, *Cephalosporium*, *Myrothecium*, *Stachybotrys*, and *Trichoderma* are also capable of producing these compounds [4]. There are over 150 known structurally related compounds that are classified into four types, A to D, by their chemical structure [5]. The most common toxins are Group A (HT-2 and T-2 toxins) and Group B (deoxynivalenol (DON), 3-acetyl deoxynivalenol, 15-acetyl deoxynivalenol, nivalenol). T-2 and HT-2 toxins, although a small group, are the most toxic trichothecenes [6]. These toxins can cause slower live weight gain, hemorrhagic lesions in mucous membranes, abortions, lower milk productivity and egg production [7].

Monitoring feeds for mycotoxin presence is a major area of focus around the world. Corn [8], compound feed [9], and barley are recognized to have the highest contamination with type A trichothecenes. Type A trichothecene mycotoxins (T-2 toxin, etc.), Type B trichothecenes (DON, etc.), zearalenone, ochratoxin, and fumonisins are the most common trichothecenes in Russia's climate [11, 12].

T-2 contamination is a grave threat to industrial poultry farming, as this toxin has multiple negative effects on birds' physiology due to its ability to inhibit protein synthesis, alter the expression of inflammatory genes [13], which later causes necrosis and desquamation of epithelial cells in the glandular stomach mucosa [14]. Since T-2 toxin has low bioavailability, most of its transforms into metabolites, in particular HT-2 toxin [15].

Large T-2 and HT-2 intake results in lower productivity, alters blood biochemistry [16], restructures hepatocytes [17], splenocytes [18], and the bursa of Fabricius [19]. T-2 toxins also exhibit additive toxicity if the feed contains ochratoxin A [20], fumonisin B and deoxynivalenol [21], cyclopiazonic acid [22], or aflatoxin [23], making it a source of substantial harm to animal husbandry.

Complete compound feeds are the staple diet for farm animals. Feed monitoring studies [24] report T-2 to be prevalent in all Russian regions. It is most commonly found in compound feeds for poultry and pigs (79.1% of the tested samples) [25]. T-2 toxin was also found in 32% of wheat samples, 70.9% of barley samples, and 94% of corn samples [26], which must be borne in mind when making complete compound feeds. Enzyme immunoassay for mycotoxin contamination showed poultry compound feeds [27] to contain all major groups of mycotoxins, with T-2 toxin detected in 88.2% of the tested samples.

For a long time, mycotoxicosis risk evaluations were based on testing the feeds for safety. Biomaterials such as blood, urine, or feces are the best candidate for testing in terms of informativeness with respect to a mycotoxin-affected animal's condition [28]. High-performance liquid chromatography-mass spectrometry (HPLC-MS) accurately detects mycotoxins and their metabolites in blood, heart, liver, spleen, lung, kidney, glandular stomach, muscular stomach, small intestine, muscle, bone, and brain samples [29].

Pathogenesis in poultry fed with T-2 and HT-2-contaminated feeds remains an under-investigated issue; to date, there are no effective methods for diagnosis and prevention of these mycotoxicoses. Before mycotoxins take their toll on organs, the organs develop functional disorders that affect the enzymatic status of the digestive canal and the morphobiochemistry of blood. Once in the body, mycotoxins undergo biochemical transformations. In vivo experiments on fistulated animals with HPLC-MS testing for mycotoxin contamination of samples can provide the most complete insight into the patterns of these processes.

Here, using this approach, we showed the T-2 toxin-contaminated feed to result in both T-2 and HT-2 appearance in broilers' intestines. The latter toxin is intensively absorbed into the blood stream and seems to pass into tissues as well, as it is far less present in feces than in the duodenum. The activity of the enzymes in the duodenal chyme, blood enzyme activity, and WBC differential change, a sign of increased hepatic exposure to toxins and of an emerging pathology. The authors propose the phosphatase-protease index (PPI) as a marker for early diagnosis of mycotoxicosis, with blood PPI > 20 being indicative of digestive disorders [30].

The goal hereof was to quantify the presence of T-2 and HT-2 toxins in the chyme and feces, the activity of digestive enzymes, and the morphological and biochemical blood parameters in broilers affected experimentally with T-2 toxicosis.

Material and methods. The experiment involved cross Smena 8 broiler chicks (*Gallus gallus* L.) in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123, Strasbourg, 1986) [31]. From Day 1 to Day 47 of age, all the birds involved in the experiment were kept in a vivarium (Russian Poultry Research Center of the Russian Academy of Sciences — VNITIP, 2020); their feed and housing were compliant with the requirements applicable to this age and cross [32]. Twenty specimens were fistulated on Days 20 to 25; the researchers used their own method to cannulate the site opposite to the location of pancreatic and bile inflows into the intestines [33]. After a single postoperative no-feed day, the poultry was switched to rationed feeding; chyme passage through the intestines was carefully monitored. Stitches were removed on post-op Day 5. The birds, clinically healthy, were split into four five-specimen groups: Group 1 for control, no T-2 toxin in the diet; Group 2 had its diet T-2 toxin-contaminated at MPC levels (100 μg/kg), Group 3 received 2MPC (200 μg/kg), and Group 4 had 4MPC (400 μg/kg). T-2 toxin was added to feeds mechanically until reaching the required concentration; all personnel safety requirements were complied with. Standard T-2 toxin (powder, mass fraction of the toxin 99.7±0.3%, Romer Labs, Austria. cat. N 10000310, LOT #S17052T). Fresh feed was accessible every day; water access was not restricted.

The preparatory phase lasted from Day 26 to Day 33 of age; the experiment itself was 14 days long (Days 34 to 47 of age). Duodenal chyme and fecal samples for testing for T-2 and HT-2 presence, as well as for enzymatic activity, were sampled every day throughout the experiment (including the preparatory phase) and placed in a freezer at –20 °C. Combined samples were further made for each broiler (combined preparatory-phase sample + combined sample for each week of the experiment, i.e., samples taken up until Day 40 and up until Day 47 of age); they were subject to subsequent preparations and testing. For T-2 and HT-2 quantifications, the research team further merged samples in each group. Chyme samples (1.0 to 2.0 ml) and fecal samples (5.0 g) were taken every morning and placed in a

refrigerator at –20 °C; 5-g samples were dried in a TFD lyophilic drier (ilShinBioBase Cl., Ltd., South Korea) over 34 hours at –77.8 °C and 5 mTorr to remove 97% of moisture from the substrate while preserving bioactive compounds. Two milliliters of blood was sampled before the experiment, then at the end of every week (on Days 33, 40, and 47 of age) from the vena cutanea ulnaris on the inner side of the wing above the ulnar joint. The puncture site was covered with a sterile swam for a few minutes. Samples for biochemical testing were collected into sterile vacuum tubes with lithium heparin (4.0 ml, Shandong Weigao Group Medical Polymer Co., Ltd., China); samples for morphological testing were collected into tubes with the K3-EDTA anticoagulant (2.0 ml, SOYAGREENTEC Co., Ltd., South Korea). The samples were centrifuged at 5000 rpm for 5 minutes to separate the plasma from the corpuscles.

Feed was sampled for testing per GOST 13496.0-2016 Compound feeds, feed raw materials. Methods of sampling (Moscow, 2020) from bags. The arbitration sample was made from an average 1 kg sample. Each feed sample was tested thrice. Samples were prepared per GOST 34140-2017 Food products, feed, food raw materials. Method for determination of mycotoxins by high-performance liquid chromatography – mass spectrometry (Moscow, 2020).

T-2 and HT-2 in the feedstock, the duodenal chyme, and feces was measured twice for each tested sample by HPLC-MS (an Agilent 1260 Infinity chromatographic system, Agilent Technologies, Germany; an AB SCIEX Triple Quad™ 5500 mass spectrometer, Applied Biosystems, USA; a Gemini® C18 5 μm 150×4.6 mm column, a reverse-phase sorbent based on silica gel with an organic polymer, Phenomenex, USA). Standard Biopure series T-2 and HT-2 solutions (Romer Labs, Austria) were used as internal standards, as well as for plotting the calibration curves. Tests were designed per GOST 34140-2017. For testing, the research team used methanol, acetonitrile (HPLC-MS purity, min. 99.9% mass fraction of the substance, Merk, Germany); glacial acetic acid, ammonium acetate (min. 99.0% mass fraction of the substance, Sigma, Germany). Eluent A: 890 cm^3 of deionized water, 100 cm^3 of methanol, 10 cm^3 of acetic acid, and 0.384 g of ammonium acetate (5 mmol/l). Eluent B: 970 cm^3 of methanol, 20 cm^3 of deionized water, 10 cm^3 of acetic acid, and 0.384 g of ammonium acetate (5 mmol/l). Extraction solution: acetonitrile:water:acetic acid at 79:20:1. Chromatographic separation was performed by binary gradient elution: 1.5 min at 100% Eluent A, linear increase in Eluent B up to 50% over 1.5 min, followed by a linear increase in Eluent B up to 100% over 9 minutes. Chromatographic separation at 100% Eluent B for 5 min, followed by column balancing at 100% Eluent A for 3 min. Column temperature: 25 °C; mobile phase velocity: 1 cm^3/min; sample injection temperature: 10 °C, the volume of injected samples: 5 mm^3 each. The retention time was 10.30 min for T-2 toxin, 9.79 min for HT-2 toxin; the detection limit was 3.25 and 2.70 μg/kg, respectively; the quantification limit was 5.23 to 129.20 and 3.5 to 129.20 μg/kg, respectively. The estimated m/z values (positive ionization by spraying in an electric field) were 305.1 for T-2, 345.1 for HT-2.

Amylase was determined by the high-activity modification of the Smith-Roy method [34]; protease activity was determined by hydrolysis of Hammarsten-purified casein (calorimetric control at $\lambda = 450$ nm); lipase was quantified on a SINNOWA BS-3000P semi-automatic biochemical analyzer (SINNOWA Medical Science & Technology Co., Ltd, China) using veterinary diagnostic reagents from DIACON-VET, Russia.

Biochemical blood tests were run on a BS-3000P analyzer (China) using a kit for total protein, alkaline phosphatase, glucose, cholesterol, triglycerides, and lipase (DIACON-VET, Russia). Trypsin activity in plasma was measured on a BS-

3000P analyzer kinetically [35] using Na-benzoyl-DL-arginine-p-nitroanilide as substrate (BAPNA, ACROS ORGANICS, Switzerland).

Blood morphology was tested on a DF-50 automatic hematological analyzer for veterinary applications (Dymind Biotech, China) using the manufacturer's reagents.

Statistical tests were run (JMP Trial 14.1.0 (https://www.jmp.com/en_us/software/data-ana-lysis-software.html). The results are reported as $M \pm SD$, where M is the arithmetic mean, $\pm SD$ is the standard deviation. Significance was tested by Student's t-test with a threshold $p < 0.05$.

Results. Mycotoxins are metabolized in the digestive canal, and the metabolites end up in the blood. T-2 metabolism is known to involve deacetylation, hydroxylation, and conjugation [2, 16]. The authors of this paper earlier showed T-2 toxin to convert into HT-2 toxin in birds and to significantly affect the concentration of nitric oxide and the activity of pancreatic enzymes [36]. In continuation of that effort, this paper complements chemical, toxicological, physiological, and biochemical findings with the results of general and biochemical blood tests as markers of the clinical status in poultry sustaining experimental T-2 toxicosis. Besides, lyophilic drying was used to better prepare the samples.

1. Mycotoxin concentration (μg/kg) **in the duodenal chyme and feces of Smena 8 broiler chicks (*Gallus gallus* L.) upon T-2 toxicosis experiment** ($M \pm SD$, vivarium of the Federal Scientific Center VNITIP RAS, 2020)

Микотоксин	Group (n = 5)			
	I (control without T-2 toxin)	II (T-2 toxin 100 μg/kg feed)	III (T-2 toxin 200 μg/kg feed)	IV (T-2 toxin 400 μg/kg feed)
Duodenal chyme				
T-2 toxin	0.5±0.13	20.2±2.75*	49.6±6.00*	41.9±6.36*
HT-2 toxin	30.0±4.90	47.9±1.60*	57.9±3.20*	53.9±1.45*
Feces				
T-2 toxin	2.5±0.26	2.3±0.40	4.8±0.68*	19.2±6.20*
HT-2 toxin	40.6±8.40	44.6±3.26	53.9±3.60	38.5±5.67

Note. Measured concentrations of toxins in the original non-contaminated feed were 4.3±0.13 μg/kg for T-2, 5.6±0.47 μg/kg for HT-2. Chyme and feces were sampled from each broiler daily throughout the 14-day experiment and combined into weekly samples (one for 40 days of age, one for 47 days of age; the experiment began on Day 34 of age). Weekly samples were further merged into combined lyophilized samples for each group. Analyte concentrations were determined by tandem high-performance liquid chromatography and mass spectrometry (HPLC-MS), each test being run thrice on an Agilent 1260 Infinity system (Agilent Technologies, Germany) and an AB SCIEX Triple Quad™ 5500 mass spectrometer (Applied Biosystems, USA). Means (M) for weekly samples are reported on a group-by-group basis.

* Difference from the control group is statistically significant at $p < 0.05$.

T-2 and HT-2 testing of feed, duodenal chyme, and feces of the fistulated controls (Table 1) showed that prior to contamination, T-2 and HT-2 toxins were present in the feeds in comparable amounts (4.3±0.19 and 5.6±0.47 μg/kg, respectively). Chicks on a non-contaminated diet had these toxins in the chyme and in feces in other ratios. On average, the control group's feces contained 5 times more T-2 toxin than was found in the duodenum, $p < 0.05$, which shows that most of the toxin would be removed unaltered. However, the sixfold presence of HT-2 in the duodenum compared to T-2 concentrations indicated that the digestive canal actively metabolized T-2 toxin and converted it into a blood-absorbable metabolite. HT-2 toxin amounts in feces were the same as in the duodenal chyme except in Group 4. Raising the T-2 toxin presence in the feed to 100 μg/kg (1MPC) drastically increased 40-fold in the chyme compared to the controls; however, its concentrations in feces remained on part with the controls. This could be due to the T-2 to HT-2 conversion and further absorption in blood and tissues, as the HT-2 presence in feces did not change significantly. The dosage increase to 200 μg/kg resulted in a 2.4-fold increase in T-2 toxin in the chyme ($p < 0.05$), 2.1-fold increase in feces compared to the 1MPC group. HT-2 presence increased by 20.9% ($p < 0.05$) and

20.8% ($p > 0.05$), respectively. Therefore, the T-2 to HT-2 ratio in the intestines and feces indicates intensive absorption of HT-2 toxin into blood. When dosed at 400 μg/kg, a 7.7 times greater fecal presence of T-2 toxin was found in comparison with the controls, a sign of intestinal adaptation towards more intensive removal of the toxin, probably by faster chyme movement in the caudal direction. Clinical manifestations included digestive disorders and enteritis with signs of diarrhea.

The authors' earlier experiment showed high mycotoxin doses to negatively affect the activity of digestive enzymes [37]. To complement these findings, the authors further studied the activity of digestive enzymes in lyophilized duodenal contents and in feces for this research, see Table 2. T-2 contamination of the feed affected the proteolytic activity of the duodenal chyme: it rose by 78.9% in Group 2 ($p < 0.05$), by 46.3% in Group 3 ($p < 0.05$), and by 96.6% in Group 4 ($p < 0.05$) compared to the preparatory-phase readings. Higher lipase activity in Groups 2 and 3 constituted a significant difference of 25.5% and 16.8%, respectively ($p < 0.05$). Amylase activity rose by 99.7% ($p < 0.05$) in Group 4. Unlike digestive enzymes, the activity of alkaline phosphatase showed a downward trend, as it dropped by 23.8% in Group 2, by 27.9% in Group 3, did not change in Group 4.

2. Activity of digestive enzymes in duodenal chyme and feces of Smena 8 broiler chicks (*Gallus gallus* L.) upon T-2 toxicosis experiment (*M*±SD, vivarium of the Federal Scientific Center VNITIP RAS, 2020)

Metric	Group (n = 5)					
	II (T-2 toxin 100 μg/kg feed)		III (T-2 toxin 200 μg/kg feed)		IV (T-2 toxin 400 μg/kg feed)	
	preparatory phase	experiment	preparatory phase	experiment	preparatory phase	experiment
Duodenal chyme						
Amylase, mg/(g·min)	6433±446.0	8100±861.2	8550±1670.0	5450±437.5	5033±314.1	10050±875.2*
Lipase, μmol/(g·min)	29.4±2.59	36.9±1.66*	25.6±0.49	29.9±1.29*	22.0±3.43	26.1±0.35
Proteases, mg/(g·min)	190±18.7	340±21.2*	229±22.8	335±27.6*	233±13.5	458±56.3*
Trypsin, μmol/(g·min)	4.92±0.364	6.86±0.354	5.27±0.581	5.84±0.196	6.47±0.589	9.33±2.045
Alkaline phosphatase, μmol/(g·min)	147.5±13.64	112.4±5.19*	134.6±1.88	97.1±7.18*	140.0±2.11	188.8±37.62
Phosphatase-protease index	29.9	16.4	25.5	16.6	21.6	20.2
Feces						
Amylase, mg/(g·min)	1413±111.0	635±67.1*	1890±275.1	810±82.0*	1145±153.1	1140±94.2
Lipase, μmol/(g·min)	1802±54.0	1946±316.0	913±6.6	880±43.0	1225±43.0	941±33.0*
Proteases, mg/(g·min)	77±10.3	182±22.1*	71±10.0	125±6.5*	68±6.3	183±4.6*
Trypsin, μmol/(g·min)	4.38±0.349	2.72±0.175	3.79±0.233	3.32±0.242	3.39±0.313	3.00±0.382
Alkaline phosphatase, μmol/(g·min)	211.6±10.08	140.8±30.98*	104.9±8.55	77.5±4.92*	82.8±4.19	143.1±14.08*
Phosphatase-protease index	48.3	51.8	31.6	23.3	24.2	47.7

Note. Measured concentrations of toxins in the original non-contaminated feed were 4.3±0.13 μg/kg for T-2, 5.6±0.47 μg/kg for HT-2. For the physiological experiment (with breakdown by groups and period), chyme and fecal samples were collected from each broiler on a daily basis before the experiment began (the preparatory phase from Day 26 to Day 33 of age), then throughout the 14-day experiment (from Day 34 to Day 47 of age); the experimental samples were combined into weekly samples (Day 40 and Day 47).
* Difference from the preparatory-phase readings is significant at $p < 0.05$.

Individual changes in the activity of proteases, amylase, trypsin, and lipase in the duodenal chyme sampled from broiler chicks were indicative of their response to different T-2 doses in the feed; data can be found on http://www.agrobiology.ru, Fig. 1. In most specimens, increased proteolytic enzyme activity was the characteristic response to the toxin in the feed.

Feces were tested biochemically (see Table 2) after lyophilic drying that preserved the digestive enzymes in their active state. Amylase activity was found to have decreased in Groups 2 and 3 by 55.1% and 57.2%, respectively, compared to the preparatory-phase readings. Lipase activity dropped by 23.2% ($p < 0.05$) at maximum dosage. Proteolytic activity in feces rose significantly with the T2 toxin presence in the feed: by 136.4%, 76.0%, and 169.1% against the preparatory-phase

readings, respectively.

Thus, protease activity in the duodenal contents was far higher than in feces: 2.5-fold difference in the preparatory phase and 1.9-fold difference during the experiment in Group 2; 3.2-fold and 2.7-fold in Group 3; 3.4-fold and 2.5-fold in Group 4. Enzymes return to the blood as the enzyme recirculation hypothesis stipulated [38, 39], or intestinal microflora destroys them; either suggestion explains why protease activity decreases significantly further down the digestive canal. Notably, mycotoxins stimulate the activity of proteases in the duodenal contents. Besides, fecal matter also shows a high proteolytic activity, a sign of digestive disorders, as elevated intestinal motility removes excess enzymes by defecation. Thus, should the body receive a small dose of the toxin in the feed, its initial adaptation consists in increased proteolytic activity of the intestinal enzymes.

Given that intestinal protease activity correlates with the enzymatic activity of the blood, clinical diagnosis of T-2 toxicosis could measure the change in the trypsin activity in broiler plasma in addition to testing fecal proteolytic activity, see Table 3.

3. Biochemical blood metrics of Smena 8 broiler chicks (*Gallus gallus* L.) upon T-2 toxicosis experiment (*M*±SD, vivarium of the Federal Scientific Center VNITIP RAS, 2020)

Metric	Group (n = 5)			
	I (control without T-2 toxin)	II (T-2 toxin 100 μg/kg feed)	III (T-2 toxin 200 μg/kg feed)	IV (T-2 toxin 400 μg/kg feed)
Trypsin, U/l	93.3±4.36 77.5±6.43	81.9±1.58* 68.6±3.02	82.5±1.12 60.4±3.89*	72.6±0.31* 66.3±7.65
Alkaline phosphatase, U/l	4179±456.1 1298±192.5	4310±609.7 754±37.7	4370±476.2 1304±118.8	6369±757.8* 6487±570.5*
Phosphatase-protease index	45 17	53 11	53 21	87 98
Total protein, g/l	40.1±0.71 36.3±1.20	40.3±1.23 36.4±0.13	40.4±0.19 35.5±0.21	41.4±0.16 40.1±0.85*
Glucose, mmol/l	12.4±0.24 10.4±0.32**	11.3±0.33 9.8±0.16**	12.9±0.07 10.0±0.16**	13.0±0.21 9.0±0.31*. **
Triglycerides, mmol/l	0.3±0.01 0.8±0.04**	0.3±0.02 0.4±0.02	0.4±0.02 0.4±0.15	0.4±0.28 0.2±0.01
Cholesterol, mmol/l	3.4±0.04 2.8±0.14	3.1±0.05* 3.1±0.60	3.6±0.25 3.1±0.26	3.6±0.21 2.8±0.04**

N o t e. Boilers aged 40 days above the line, 47 days below the line.
* Difference from the Group I (control) is statistically significant at $p < 0.05$.
** Difference between 47-day old and 40-day old chickens is statistically significant at $p < 0.05$.

Biochemical blood tests showed experimental T-2 toxicosis to suppress trypsin activity at higher T-2 doses; the effect was most noticeable at 4MPC, see Table 3. Alkaline phosphatase had the opposite dynamics: at 4MPC, its activity pentupled over two weeks, and PPI rose from an average of 31 to 92, a sign of decreased secretory function of the pancreas, lower availability of nutrients, and higher toxic load on the liver [40]. At 4MPC, total blood protein rose by 13.2% but glucose dropped by 13.5% and triglycerides decreased fourfold, a sign of stress response to T-2 toxin intake.

Further morphological test results were indicative of the hematological abnormalities attributable to experimental mycotoxicosis, see Table 4. The leukocyte count decreased by 15.8% on Day 40, 16.6% on Day 47 at 2MPC. However, this was still within the normal range, so it would be too early to diagnose immunity deficiency. WBC differential showed higher lymphocyte counts compared to pseudo-eosinophil counts at 2MPC and 4MPC, which is attributable to specific defenses against infectious and non-infectious pathologies. The eosinophil percentage was lower at 2MPC and higher in other groups compared to the controls. The monocyte percentage was reduced to a third at

1MPC, then stabilized at the same level. At 2MPC, monocyte counts dropped more significantly (6-fold and 2-fold, respectively) compared to the controls. The maximum toxin dosage (4MPC) multiplied monocyte counts by 1.5 on Day 40 compared to the controls. The basophil percentage showed downward trends in Day 47 samples in Groups 2 and 3 (the proportions were halved compared to the controls).

4. Blood leukocytes and WBC differential of Smena 8 broiler chicks (*Gallus gallus* L.) upon T-2 toxicosis experiment (M±SD, vivarium of the Federal Scientific Center VNITIP RAS, 2020)

Metric	Group (n = 5)			
	I (control without T-2 toxin)	II (T-2 toxin 100 μg/kg feed)	III (T-2 toxin 200 μg/kg feed)	IV (T-2 toxin 400 μg/kg feed)
Leukocytes, 10^9/l	32,2±0,90	27,1±0,61*	29,6±0,70	32,7±1,65
	30,8±1,25	25,7±0,23*	28,1±0,90	28,6±2,88
Pseudo-eosinophils, %	44,8±1,49	33,9±1,95*	36,0±1,23*	47,8±3,05
	41,1±2,46	34,2±0,98	45,2±6,24	36,6±1,70
Lymphocytes, %	48,8±1,70	62,3±2,24*	58,9±1,73*	41,0±4,02
	52,4±2,22	59,9±0,41*	47,7±7,56	55,2±0,29
Monocytes, %	0,6±0,08	0,2±0,03*	0,1±0,05*	0,9±0,05*
	0,4±0,07	0,3±0,10	0,2±0,06	0,5±0,09
Eosinophils, %	5,5±0,42	3,1±0,28*	4,7±0,48	9,7±1,05*
	5,7±0,75	5,3±0,50	6,6±1,24	7,2±1,36
Basophils, %	0,4±0,04	0,4±0,05	0,2±0,01*	0,4±0,03
	0,4±0,06	0,2±0,03*	0,2±0,03*	0,3±0,03

N o t e. Boilers aged 40 days above the line, 47 days below the line.
* Difference from the Group I (control) is statistically significant at $p < 0.05$.

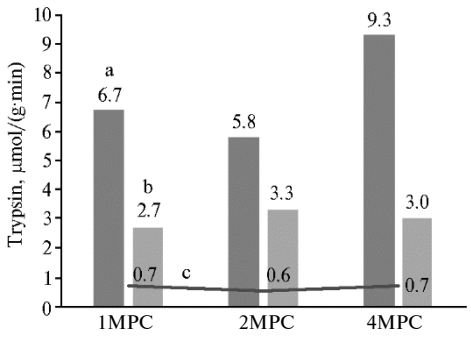

Fig. 2. Trypsin activity in various biological materials of Smena 8 broiler chicks (*Gallus gallus* L.) upon T-2 toxicosis experiment (average values throughout the experiment per doses): a — duodenal chyme, b — feces, c — blood; 1MPC — 100 μg/kg feed, 2MPC — 200 μg/kg feed, 4MPC — 400 μg/kg feed (vivarium of the Federal Scientific Center VNITIP RAS, 2020).

T2 toxin actively converted into a different form as indicated by the above-noted increase in HT-2 amounts in chyme and feces sampled from broiler chicks fed with 2MPC of T-2 toxin, see Table 1. Duodenal chyme contained 2.5 times T-2 and 1.2 times HT-2 amounts found in Group 1 (1MPC). At the same time, the activity of trypsin, which is involved in both protein digestion and metabolism regulation [35], decreased in the duodenum and increased in feces whilst not changing significantly in blood, see Fig. 2. This indicates a protective response of the digestive glands to the toxin, which manifests as increased secretion of pancreatic and intestinal juices to preserve enteral homeostasis when the body is sustaining a large fluid loss. As intestinal chyme moves faster due to adaptation to the toxin, it disrupts enzyme recirculation, and some of the enzymes are excreted with feces [36]. At 4MPC, the T-2 toxin presence was reduced 2.6-fold in the duodenal chyme and increased by a factor of 8.4 in feces. Intestinal adaptation to 4MPC of T-2 differed significantly from adaptation to lower doses: trypsin activity in the duodenal chyme was rising whereas the fecal presence of the enzyme was virtually constant, see Fig. 2.

Co-analysis of T-2 to HT-2 metabolization (HT-2 and T-2 ratio) and chyme trypsin activity revealed an inverse relationship between the two processes, which was more apparent at 4MPC, see Fig. 3. Thus, our research

revealed an inverse relationship between blood PPI and the quantitative ratio of HT-2 to T-2 in the duodenal chyme at 4MPC, which could be useful for diagnosing T-2 mycotoxicosis, see Fig. 3.

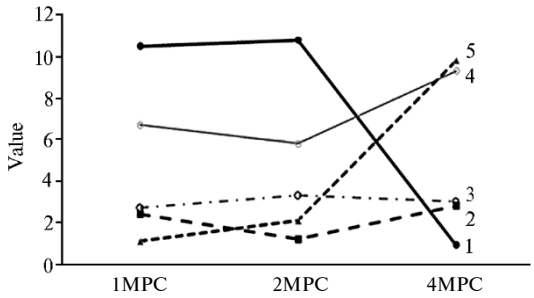

Fig. 3. The HT-2 toxin/T-2 toxin concentration ratio in duodenal chyme (1) **and feces** (2)**, activity of trypsin** (U/ml) **in feces** (3) **and duodenal chyme** (4)**, and phosphatase-protease index** (5) **of Smena 8 broiler chicks (*Gallus gallus* L.) upon T-2 toxicosis experiment:** 1MPC — 100 μg/kg feed, 2MPC — 200 μg/kg feed, 4MPC — 400 μg/kg feed (vivarium of the Federal Scientific Center VNITIP RAS, 2020).

Thus, experimental T-2 toxicosis showed T-2 toxin to convert into HT-2 toxin in the digestive canal of broilers; HT-2 absorbs intensively into the blood and seemingly ends up in tissues, as it is far less present in feces than in the duodenum (by 28.7%). T-2 poisoning of feed (1MPC = 100 μg/kg, 2MPC = 200 μg/kg, 4MPC = 400 μg/kg) increased the proteolytic activity of the duodenal chyme by 78.9% ($p < 0.05$), 46.3% ($p < 0.05$), and 96.6% ($p < 0.05$), respectively; that is, the greatest effect was observed at 4MPC. That concentration also yielded maximum amylase activity (twofold). An increase in lipase activity (less substantial but still significant at 25.5% and 16.8%, $p < 0.05$) was observed at 1MPC and 2MPC dosage. On the contrary, alkaline phosphatase activity either tended to decrease (1MPC and 2MPC) or did not change. Trypsin activity in the blood was 12.2–22.2% lower (Groups 2, 4) on Day 40 of age, 22.1% lower (Group 3) on Day 47, whereas alkaline phosphatase activity was pentupled over the two weeks of the 4MPC diet. The PPI increase indicated a higher toxic load on the liver. Leukocyte counts were 16% lower on average at 1MPC. WBC differential showed a higher percentage of lymphocytes at 1MPC and 2MPC in contrast to pseudoeosinophils, which is attributable to specific antitoxin defenses of the body. The PPI of blood was found to be inversely related to the quantitative ratio of HT-2 and T-2 in the chyme at 4MPC T-2 presence in the feed. PPI was found to increase at high doses of T-2 in the feed. Thus, PPI testing could be used in the diagnosis of T-2 toxicosis in poultry.

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DETECTION AND QUANTITATIVE ASSESSMENT OF VIRAL AND BACTERIAL PATHOGENS IN BOVINE RESPIRATORY DISEASES BY REAL-TIME-qPCR

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Abstract

Bovine respiratory diseases are widespread in all countries with intensive animal husbandry and cause significant economic damage. They are the result of a synergistic interaction of several viruses and bacteria, predominantly of the *Pasteurellaceae* family. Clinical signs and pathological changes in internal organs depend on the presence or absence of a particular pathogen. Mass outbreaks occur when animals from different sources are mixed. The etiological structure of such outbreaks has been sufficiently studied, however, there is insufficient data on the distribution of bacteria and viruses in the respiratory tract and their quantitative determination. The article presents the results of studying the etiological structure of the outbreak of respiratory diseases in the big dairy farm after the import of cattle, during which more than 400 animals of different age and sex groups died. Samples of internal organs of 58 dead animals of different ages were examined. When studying the etiological structure of the outbreak, standard bacteriological methods were used, viral agents were identified by PCR by gel electrophoresis, and real-time PCR was used to quantify all detected infectious agents. In total, 9 viruses and bacteria were identified, of which the respiratory syncytial virus of cattle (BRSV, Bovine Respiratory Syncytial Virus, genus *Pneumovirus*, family *Paramyxoviridae*) and bacteria of the *Pasteurellaceae* family played a leading etiological role. Using quantitative PCR, the concentrations of the virus and bacteria *Pasteurella multocida* and *Mannheimia haemolytica* were determined in the respiratory tract organs of 13 calves of different ages with similar clinical signs, pathological changes and the presence of three pathogens in the respiratory tract organs. The concentration of agents ranged from 0.1±0.03 to 4.8±0.47 $\log_{10}$ genomic equivalents (GE)/ml for BRSV, from 1.3±0.60 to 4.1±0.30 $\log_{10}$ GE/ml for *P. multocida*, and from 1.9±0.03 to 4.9±0.67 $\log_{10}$ GE/ml for *M. haemolytica*. The concentration and distribution of pathogens in the organs of calves of different ages differed. BRSV was detected in a wider range of respiratory organs, both free from bacteria and colonized by them. In the lungs, the concentration of the virus was higher than in the tracheal and bronchial exudate. *P. multocida* was present only in the upper and middle lobes of the lungs of 2.5-4-month-old calves at approximately equal concentrations in acute bronchopneumonia. The degree of colonization of the lungs by this bacterium increased with age and in calves at the age of 6 months its number reached maximum values in the upper and middle lobes of the lungs, pulmonary lymph nodes and washes from the mucous membranes in chronic bronchopneumonia. *M. haemolytica* was detected in acute bronchopneumonia in calves at the age of 2.5 months in a minimum amount in the middle lobes of the lungs, in a maximum amount in tracheal and bronchial exudates. The results showed that the virus and bacteria multiply in different parts of the lungs without suppressing each other, which confirms the effect of their synergistic interaction and leads to an increase in the severity of the course of pneumonia. Quantification of viruses and bacteria by real-time PCR can be a useful tool for studying the pathogenesis of mixed viral-bacterial infections in vivo. The results obtained underline the role of the BRSV in the development of pulmonary pasteurellosis.

Keywords: cattle, respiratory infections, real-time PCR, quantitative analysis, respiratory syncytial virus, *Pasteurella multocida*, *Mannheimia haemolytica*, synergism

Bovine respiratory diseases are widespread in all countries with intensive

animal husbandry and cause significant economic damage [1, 2]. They are usually a result of synergistic interaction of several viruses and bacteria, predominantly of the *Pasteurellaceae* family. Viral pathogens include the infectious bovine rhinotracheitis (IBR) virus (IBRV, BoHV-1 or bovine herpes virus-1, synonymous with the Bovine alphaherpesvirus 1, genus *Varicellovirus*, the *Herpesviridae* family), bovine viral diarrhea virus types 1-3 (BVDV1-3, synonymous with Pestivirus A, B, and H, genus *Pestivirus*, the *Flaviviridae* family), bovine parainfluenza type 3 (BPIV-3 synonymous with bovine respirovirus 3, genus *Respirovirus*, the *Paramyxoviridae* family), and bovine coronavirus (BCoV, genus *Betacoronavirus*, the *Coronaviridae* family), and a few more [3, 4]. In practice, various viral-bacterial associations may occur, where one pathogen is dominant or absent; this affects the severity of the disease, as well as the nature of pathological changes [5]. Often, such outbreaks happen when a farm receives mixed livestock in bulk, including imported livestock.

Bovine respiratory syncytial virus (BRSV, genus *Orthopneumovirus*, the *Pneumoviridae* family) is one of the etiological agents of the bovine respiratory disease complex [6-9]. Animals of all breeds and ages are susceptible [10]. The virus may cause bronchitis, interstitial pneumonia, and pulmonary emphysema on its own; however, its key danger lies in the ability to create a predisposition to bacterial pneumonia [11, 12]. The papers [12-16] describe the synergy of the virus with the bacteria *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*.

Literature reports no data on BRSV accumulation in different compartments of the respiratory tract in naturally infected animals, nor any data on co-infection with *P. multocida*, *M. haemolytica*. Such studies could help significantly expand the knowledge of how BRSV contributes to the occurrence and progression of bovine viral-bacterial infections. The virus could be not just a trigger but also an independent pathogen replicating in organs and tissues in bacterial presence.

The novelty of the obtained results consists in collecting new data on the detection and finding the concentration of BRSV, *P. multocida*, and *M. haemolytica* genomes in animal respiratory tracts should a mass outbreak of respiratory diseases occur at a large dairy farm that has recently imported animals from abroad.

The goal was to study the etiological structure of a mass outbreak of bovine respiratory infection in a limited population and to find the concentration of BRSV genomes, *P. multocida,* and *M. haemolytica* in bovine respiratory tracts using real-time quantitative PCR.

Materials and methods. For testing, the researchers sampled nasal discharge, tracheal and bronchial exudate, mucous membranes of the trachea and large bronchi, and pulmonary lymph nodes, as well as various parts of the lungs from 58 differently aged Holstein animals that had succumbed on Day 7 to Day 14 after the first clinical signs. Samples were collected within 2 hours of death, frozen immediately, then delivered to the laboratory within 12 hours. Four hundred sixty-four samples were made in total. The farm's livestock was subject to 2-year morbidity monitoring.

The sampling technique was detailed earlier in [17, 18]. The samples were immediately frozen and then delivered to the laboratory, where they were split into two parts. The first part would be immediately tested for infectious agents; the second part was stored at –80 °C to further find the concentrations of pathogens.

Upon thawing, homogenized samples were centrifuged at 10,000 rpm for 5 min; 100 μl of clarified supernatant was used to isolate RNA and DNA as described in [17, 18]. Step 1 of studying the etiological structure of the outbreak

consisted in identifying the viral agents in PCR by gel electrophoresis using primer pairs and PCR parameters published earlier for BOHV-1 [17], BVDV-1 and BVDV-2 [18], BRSV [19], BPIV-3 [20], BCoV [21].

Bacteriological studies followed the World Organization for Animal Health (OIE) Guidelines [22]. Bacteria were isolated on artificial agars: blood meat peptone agar (BMPA) and Hottinger agar based on meat digest (10% horse serum added); they were typed in PCR using primers as described in [23-25].

Reverse transcription used the Reverta-L kit (Rospotrebnadzor CRIE, Russia) per the manufacturer's manual.

To quantify BRSV, *P. multocida,* and *M. haemolytica*, the researchers ran RT-PCR on 104 biosamples stored at –80 °C; preliminary studies identified only these three pathogens simultaneously.

Quantitative RT-PCR (*RT-qPCR)* used 30 μl of the mixture containing 5 μl of cDNA or DNA, 0.1 μM of each primer and probe as recommended for the tested pathogens [26–28], and a ready-made reagent mix: BioMaster PCR-RV (Biolabmix, Russia). The amplification program was as follows: 5 min at 95 °C, followed by 45 cycles: 15 s at 95 °C, 1 min at 60 °C. All reactions were run at a Real-time CFX96 Touch amplifier (Bio-Rad, USA).

Positive control samples (PCSs) were obtained using the TA Cloning® Kit with pCR™2.1 vector (In-vitrogen, USA) by cloning fragments of genes (*N* for BRSV, bovine *gapdh*, *kmt1 for P. multocida*, and *sodA for M. haemolytica*) into the pCR™ 2.1 cloning vector. PCS concentration was determined using the Quant-iT™ dsDNA Assay Kit, HS (Invitrogen, USA) and a QUBIT 4 fluorimeter (Invitrogen, USA), then converted into copy counts (see http://molbiol.ru/scripts/01_07.html for the converter software). Five milliliters of the PCS was added per reaction. Gene fragments (PCR specificity controls) were sourced from the strains RSB (BRSV), 1231 *(P. multocida)*, and S1-16 *(M. haemolytica)* obtained from the microorganism collection of the All-Russian Research Institute of Experimental Veterinary Medicine. The same strains were used to evaluate the sensitivity and reproducibility of reactions, and to calculate the coefficient of determination R^2.

To quantify pathogen RNA and DNA in the sample, a standard amplification curve was plotted, PCSs of known concentration were diluted 10-fold. The quantities of BRSV RNA and bacterial RNA in samples were estimated by comparing the cycle threshold of the sample against the standard curve; the quantities were then written in $\log_{10}$ genomic equivalents (GE for viral RNA or bacterial DNA) per 1 ml of the suspension (N $\log_{10}$ GE/ml), then normalized in relation to 10^5 GE of bovine *gapdh* as described in [29].

RT-qPCR sensitivity was tested in three repetitions.

The results were processed statistically in Statistica 8 (StatSoft, Inc., USA). For processing, the researchers calculated the means (*M*) and the standard errors of the mean (±SEM).

Results. The research was carried out at a dairy farm in possession of 1080 Holstein cows producing 7000.00 liters of milk per year on average; it was initiated after the importation of livestock from abroad that caused a respiratory disease outbreak in animals of all ages. As of the time of the study, the animals were not being vaccinated against viral diseases. The animals were stabled all-year-round pursuant to physiological and zootechnical standards.

In 2016, the farm imported 752 high-productivity heifers. These were housed separately from the local livestock; however, strict quarantine was not observed; 40 (5.3%) of the imported animals showed clinical signs of a disease, of which 10 (1.3%) soon succumbed to acute fibrinous pneumonia. Over the course

of 2017, these heifers produced 700 calves, 160 (22.8%) of which died before 30 days of age and had the same diagnosis. One month later, clinical signs of the disease were found in local animals including calves aged 1 to 6 months. Of the 600 calves, 490 (81.6%) contracted the disease, 240 (48.9%) succumbed to it. Beside the calves, the disease was found in 400 local cows; emergency slaughter and death count totaled 30 heads or 7.5%. Calves aged 10 days to 1 month were less susceptible. Clinical examinations revealed suppression, refusal of feed, fever, rapid abdominal breathing (mouth open, tongue out), lowered neck and head, abundant foaming at the mouth. Local calves were diagnosed with acute catarrhal-fibrinous bronchopneumonia postmortem. Their lungs showed lobar pneumonia and affected anteroventral sites: red hepatization in calves aged 2.5 months or younger, gray hepatization in older calves. Interlobular septa were edematous and impregnated with gelatinous exudate; they contained fibrin. Incisions showed lung tissue to be hemorrhagically inflamed and compacted with sites of necrosis. Pulmonary emphysema and edema were reported in some cases.

1. Primer pairs and PCR parameters used to identify viruses in biosamples of fallen Holstein animals of various ages after a mass outbreak of respiratory diseases (Novosibirsk Province, 2017)

Pathogen	Gene	Sequence (5′→3′)	Amplification protocol	DNA fragment size, bp	Reference
BoHV-1	*gB*	ACGTGCTGCTCAACGTGTAC AGGACGAGCTCGCGGATATA	95 °C 5 min; 95 °C 1 min, 54 °C 1 min, 72 °C 1,5 min (35 cycles); 72 °C 5 min	464	[17]
BVDV-1 BVDV-2	*NS5B*	GAGATCTTTCACACAATAGCTG GAACCTAAGAACTAAATCGG TGTTTCACCCAGTTATACATGC	95 °C 5 min; 95 °C 45 c, 60 °C 45 c, 72 °C 1 min (35 cycles); 72 °C 5 min	356 586	[18]
BRSV	*gF*	CATCAATCCAAAGCACCACACTGTC GCTAGTTCTGTGGTGGATTGTTGTC	95 °C 5 min; 95 °C 30 c, 60 °C 30 c, 72 °C 45 c (35 cycles); 72 °C 5 min	371	[19]
BPIV-3	*M* gene	GATCAGGAACTCTTAAAGGC TTTTCCCGACCCCTTCTAT	95 °C 5 min; 95 °C 15 c, 57 °C 25 c, 72 °C 30 c (40 cycles); 72 °C 5 min	739	[20]
BCoV	*N* gene	GCCGATCAGTCCGACCAATC AGAATGTCAGCCGGGGTAT	95 °C 5 min; 95 °C 30 c, 55 °C 30 c, 72 °C 45 c (35 cycles); 72 °C 5 min	407	[21]

Step 1 of studying the etiological structure of the outbreak consisted in identifying the viral agents in PCR with separation by gel electrophoresis using primer pairs and PCR parameters shown in [18-21, 23-25], see Tables 1 and 2.

2. Primer pairs and PCR parameters used to type the bacteria isolated on artificial nutrient agar media from biosamples of fallen Holstein animals of various ages after a mass outbreak of respiratory diseases (Novosibirsk Province, 2017)

Pathogen	Gene	Sequence (5′→3′)	Amplification protocol	DNA fragment size, bp	Reference
Mannheimia haemolytica	*sodA*	GACTACTCGTGTTGGTTCAGGCT CGGATAGCCTGAAACGCCT	95 °C 5 min; 95 °C 15 c, 57 °C 20 c, 72 °C 30 c (45 cycles); 72 °C 5 min	126	[23]
Pasteurella multocida	*kmt1*	ATAAGAAATAACTCAACATGGAAAT GAGTGGGCTTGTCGGTAGTCTT		211	[23]
Mycobacterium bovis	*urvC*	TTACGCAAGAGAATGCTTCA TAGGAAAGCACCCTATTGAT	95 °C 5 min; 95 °C 15 c, 52 °C 20 c, 72 °C 30 c (45 cycles); 72 °C 5 min	1600	[24]
Histophilus somni	*16S rDNA*	GAAGGCGATTAGTTTAAGAG TTCGGGCACCAAGTRTTCA	94 °C 5 min, 95 °C 1 min, 55 °C 1 мин, 72 °C 1 min (35 cycles); 72 °C 5 min	397	[25]

Bacteriological studies isolated and typed *M. haemolytica* (53.4%) and *P. multocida* (60.3%) in most animals, with *H. somni* (12.3%) and *Mycobacterium bovis* (6.9%) being rarer pathogens. Five viruses were found in the organs of dead

animals, mostly BRSV (65.5%), sometimes BCoV (22.4%) and BPIV-3 (20.7%). BoHV-1 and BVDV-1 were rarely detected: 6.9% and 8.6%, respectively. Twenty-seven animals had BRSV and BCoV: monovariant in four calves and in two- or three-virus association in 23 specimens, see Table 3.

3. Detection rates (number/%) **of respiratory pathogens in biosamples of fallen Holstein animals of various ages after a mass outbreak of respiratory diseases** (Novosibirsk Province, 2017)

Age	*n*	BRSV	BHV1	BVDV1	BVDV2	BPIV	BCoV	*Mannheimia haemolytica*	*Pasteurella multocida*	*Histophilus somni*	*Mycobacterium bovis*
Calves aged 30 days or younger	16	11/68.8	1/6.3	5/12.5	0	1/6.3	7/31.2	3/18.75	12/75.0	0	1/6.25
Calves aged 2-3 months	10	10/100.0	0	0	0	1/10.0	4/40.0	7/70.0	7/70.0	2/20.0	0
Calves aged 4-6 months	12	12/100.0	0	0	0	2/16.7	2/16.7	9/75.0	8/66.7	1/8.3	0
Heifers	10	2/20.0	3/30.0	0	0	5/50.0	0	5/50.0	3/30.0	3/30.0	1/10.0
Cows	10	3/30.0	1/10.0	0	0	3/30.0	0	7/70.0	5/50.0	2/20.0	2/20.0
Total	58	38/65.5	4/6.9	5/8.6	0	12/20.7	13/22.4	31/53.4	35/60.3	8/13.8	4/6.9

Pathogen detection rates were age-dependent. Calves aged 30 days or younger were more likely to have *M. haemolytica* (75.0%) and BRSV (68.8%), less likely to have *P. multocida* (18.8%) and BCoV (18.8%). Of calves aged 2 to 3 months, 100% had BRSV, 70% had *P. multocida,* 70% had *M. haemolytica,* 30% had BPIV-3. Of calves aged 4 to 6 months, 100% had BRSV, 75% had *M. haemolytica*, and 65.7% had *P. multocida*. BCoV and BPIV-3 were found in 25.0% and 16.7% of the specimens, respectively. Cows and heifers were 20% more likely to have *M. haemolytica* than *P. multocida*. BRSV in them was found in 20% and 30% of specimens in each group, respectively.

Three pathogens (BRSV, *M. haemolytica*, and *P. multocida*) were most prevalent in organ samples from nearly all respiratory tract compartments. For this reason, qPCR was then used to find the concentrations of these agents in various compartments of the respiratory tract where all the three pathogens had been found. Table 4 shows nucleotide sequences of the primers and probes used to that end.

4. Primer pairs and probes used in RT-qPCR to quantify the concentrations of major infectious agents in biosamples of fallen Holstein animals of various ages after a mass outbreak of respiratory diseases (Novosibirsk Province, 2017)

Target	Gene	Sequence (5′→3′)	Reference
BRSV	*N*	GCAATGCTGCAGGACTAGGTATAAT ACACTGTAATTGATGACCCCATTCT (FAM)ACCAAGACTTGTATGATGCTGCCAAAGCA(BHQ1)	[26]
Bos taurus	*gapdh*	GATGGTGAAGGTCGGAGTGAAC GTCATTGATGGCGACGATGT (ROX)CTGGTCACCAGGGCTGCTT(BHQ2)	[27]
Pasteurella multocida	*kmt1*	ATAAGAAACGTAACTCAACATGGAAATA GAGTGGGCTTGTCGGTAGTCTT (FAM)AAACCGGCAAATAACAATAAGCTGA(BHQ1)	[28]
Mannheimia haemolytica	*sodA*	GACTACTCGTGTTGGTTCAGGCT CGGATAGCCTGAAACGCCT (ROX)CTGGTTAGCGGTTGAAACAACGG(BHQ2)	[28]

RT-qPCR had a detection limit of 12 to 18 GEs per reaction or 1.08 $TCID_{50}$ for BRSV, 0.15 CFU for *M. haemolytica* and *P. multocida*, the coefficient of determination (R^2) ranging from 0.95 to 0.99, see Table 5.

5. Reproducibility and sensitivity of RT-qPCR test for quantification of major infectious agents in biosamples of fallen Holstein animals of various ages after a mass outbreak of respiratory diseases (Novosibirsk Province, 2017)

Pathogen	Sample	Coefficient of determination R^2	Minimum detectable concentration	GE to $TCID_{50}$ or GE to CFU ratios
BRSV	PC	0.98	18 GE	16.6
	Strain RSB	0.95	1.08 $TCID_{50}$	
Mannheimia haemolytica	PC	0.99	12 GE	80.0
	Strain S1-16	0.97	0.15 CFU	
Pasteurella multocida	PC	0.99	13 GE	86.7
	Strain 1231	0.96	0.15 CFU	

N o t e. PC stands for positive control samples, GE stands for genomic equivalents.

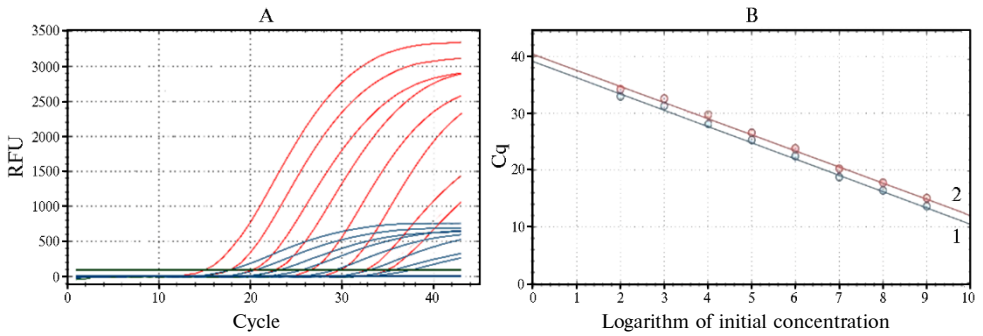

Fig. 1. Multiplex PCR curve (A) **and dynamic range of multiplex PCR readings (standard curve)** (B) **for detection of the genes *N* of BRSV and *gapdh* of *Bos taurus* in biosamples of fallen Holstein animals of various ages after a mass outbreak of respiratory diseases** (Novosibirsk Province, 2017). A: The X-axis shows PCR cycles, the Y-axis shows fluorescence in relative fluorescence units (RFU). B: The X-axis shows the sample quantity, N $\log_{10}$ GE/ml, the Y-axis shows the threshold cycle Cq (○ standard, × unknown, 1: FAM E = 123.4%, R^2 = 0.993, slope = –2.865, y-int = 39.133, 2: ROX E = 125.3%, R^2 = 0.995, slope = –2.835, y-int = 40.377). Blue lines are for the FAM probe (BRSV *N* gene); red lines are for the ROX probe (*Bos taurus gapdh* gene).

Fig. 1 shows the curve of multiplex PCR for the detection of BRSV *N* and *Bos taurus gapdh* genes, as well as the dynamic range of readings; Fig. 2 shows the same metrics for multiplex PCR detecting *P. multocida kmt1* and *M. haemolytica sodA* genes.

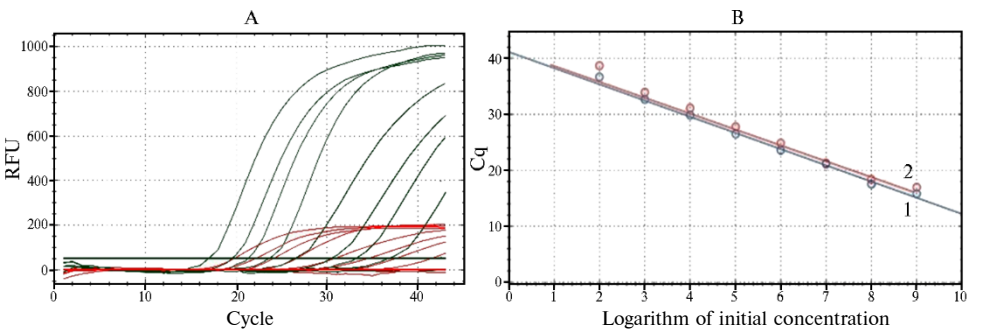

Fig. 2. Multiplex PCR curve (A) **and dynamic range of multiplex PCR readings (standard curve)** (B) **for detection of the genes *kmt1 Pasteurella multocida* and *sodA Mannheimia haemolytica* in biosamples of fallen Holstein animals of various ages after a mass outbreak of respiratory diseases** (Novosibirsk Province, 2017). A: The X-axis shows PCR cycles, the Y-axis shows fluorescence in relative fluorescence units (RFU). B: The X-axis shows the sample quantity, N $\log_{10}$ GE/ml, the Y-axis shows the threshold cycle Cq (○ standard, × unknown, 1: FAM E = 121.3%, R^2 = 0.958, slope = –2.,899, y-int = 41.182, 2: ROX E = 125.0%, R^2 = 0. 993, slope = –2.839, y-int = 41.488). Blue lines are for the FAM probe (gene *kmt1 P. multocida*); red lines are for the ROX probe (*sodA M. haemolytica*).

Table 6 shows the results of quantifying BRSV RNA and P. mul*tocida and M. haemolytica* DNA in biomaterial sampled from dead animals.

6. Concentration (log_{10} GE/ml suspension) of **Bovine Respiratory Syncytial Virus, *Pasteurella multocida*, *Mannheimia haemolytica* in biosamples of fallen Holstein heifers of various ages after a mass outbreak of respiratory diseases at a large livestock farm upon importation of animals** (Novosibirsk Province, 2017)

Biomaterial	Age								
	2.5 months			4 months			6 months		
	BRSV	*P. multocida*	*M. haemolytica*	BRSV	*P. multocida*	*M. haemolytica*	BRSV	*P. multocida*	*M. haemolytica*
Lung:									
cranial lobe	–	3.1±0.22	4.1±0.67	3.3±0.50	3.2±0.60	3.3±0.46	–	3.4±0.31	4.4±0.46
middle lobe	4.8±0.47	1.9±0.39	1.9±0.03	2.9±0.27	2.5±0.09	2.4±0.46	1.5±0.21	3.1±0.32	2.3±0.12
caudal lobe	2.1±0.30	–	2.5±0.13	1.8±0.21	–	3.5±0.31	1.3±0.5	–	3.1±0.27
Bronchial lymph nodes	–	–	2.3±0.21	0.1±0.03	–	2.1±0.12	–	2.1±0.09	2.1±0.55
Nasopharyngeal swab	–	–	2.8±0.35	2.1±0.25	–	4.1±0.30	–	2.2±0.55	1.6±0.30
Bronchotracheal swab	–	–	4.9±0.67	2.5±1.30	–	3.3±0.55	0.3±0.21	1.3±0.60	2.1±0.58
Mucosa:									
tracheal	0.5±0.03	–	–	–	–	–	–	–	–
bronchial	1.3±0.21	–	2.3±0.39	1.2 ±0.29	–	–	0.3±0.21	–	–

N o t e. GE stands for genomic equivalents; «–» means undetectable concentrations of pathogens.

The Table 6 shows BRSV concentration to vary from 0.1±0.03 to 4.8±0.47 $\log_{10}$ GE/ml, *P. multocida* and *M. haemolytica* to vary from 1.3±0.60 to 4.1±0.30 $\log_{10}$ and from 1.9±0.03 to 4.9±0.67 $\log_{10}$ GE/ml, respectively, in calves of various ages. The lungs had higher viral concentrations than tracheal or bronchial exudate, a sign of predominant pulmonary tropism of the virus.

P. multocida was found only in upper and middle lobes in calves aged 2.5 months (3.1±0.22 and 1.9±0.39 $\log_{10}$ GE/ml, respectively) and 4 months (3.2±0.60 and 2.5±0.09 $\log_{10}$ GE/ml, respectively) in approximately equal concentrations. This bacterium's colonization of the lungs correlated positively with age, as 6-month calves had up to 3.4±0.31 and 3.1±0.32 $\log_{10}$ GE/ml in upper and middle lobes. Besides, calves of this age group had *P. multocida* in pulmonary lymph nodes and mucosal swabs. This fact confirms the dominant role *of P. multocida* in the etiology of chronic bronchopneumonia in older calves.

In calves aged 2.5 months, *M. haemolytica* had the lowest concentration (1.9±0.03 $\log_{10}$ GE/ml) in the middle lobes and the highest concentration (4.9±0.67 $\log_{10}$ GE/ml) in bronchial exudate.

Thus, the detection percentages show the RSV and *M. haemolytica*, *P. multocida* to have played a key etiological role in the post-importation outbreak at the dairy farm. These results are consistent with the international researchers' reports on the synergistic interaction of the virus with *Pasteurellaceae* in severe animal bronchopneumonia [1, 4, 14, 16].

The respiratory syncytial virus is believed to have pathogenetic effects only in early stages of infection, thus being undetectable (or rarely detectable) if the lungs are colonized by bacteria, whereby it disappears after a transit infection [3, 6-9]. The data reported herein suggests that BRSV is present in the lungs even when colonized by *P. multocida* and *M. haemolytica*.

Bacteriological diagnosis is a time-consuming procedure, and its effectiveness depends on many factors including the use of antibacterial drugs, compliance with biosampling guidelines, freezing and thawing effects, as well as the presence of other microorganism species in the respiratory organs [1].

Detection and quantification of various pathogens in naturally infected animals by real-time PCR can be a useful tool for studying the pathogenesis of mono or mixed infections in vivo. The researchers did not find any results of similar studies in the available literature. There are reports of quantifying the concentration of the virus in nasal discharge or bronchoalveolar lavage in calves infected experimentally with highly pathogenic virus strains; those, however, concern monoinfection only. No earlier reports were found on finding the virus concentration in naturally infected animals [30–33]. Some reports concern co-infection with BRSV and other respiratory viruses [34–35], BRSV and bacteria (*Histophilus somni* [13], *P. multocida*, *M. haemolytica*, and *Trueperella pyogenes*) as detected by multiplex PCR [36], as well as immunohistochemical detection of *P. multocida* and *M. haemolytica* infections [34]. However, these viral and bacterial agents had not been quantified before this study.

RT-PCR made it possible to identify and quantify the two bacteria in the respiratory tract organs of the tested calves; the quantities were low. It is not clear whether this could be attributable to the limitations of the method or to the weak reproduction of the bacteria in the respiratory tract within the specific timeframe.

The results reported herein show the RSV and the *Pasteurellaceae* bacteria to be well-capable of simultaneously, non-mutually-suppressed reproduction in various lung parts, which confirms their synergy [1, 4, 14, 16], as well as the *M. haemolytica* + *P. multocida* interaction, which makes the clinical signs and the course of pneumonia in animals more severe. Besides, compared to the bacteria, the virus was found in a broader range of biosamples (nasopharyngeal and bronchotracheal

swabs, tracheal and bronchial mucosa, bronchial lymph nodes, cranial, middle, and caudal lobes) taken from animals with signs of pulmonary pasteurellosis. The concentration of the BRSV genome was also determined in naturally infected animals in a broader range of respiratory organs than the researchers did on experimentally infected gnotobiotic calves in [30-33].

Thus, RT-PCR made it possible to evaluate the distribution of three pathogens in the respiratory tract organs in naturally infected calves and to quantify BRSV, *Pasteurella multocida,* and *Mannheimia haemolytica* concentrations, which varied from 0.1±0.03 to 4.8±0.47 $\log_{10}$ GE/ml, 1.3±0.60 to 4.1±0.30 $\log_{10}$ GE/ml, and 1.9±0.03 to 4.9±0.67 $\log_{10}$ GE/ml, respectively. BRSV had maximum concentrations in the lungs, a sign of its predominant pulmonary tropism. RT-PCR could be useful for studying the spread and reproduction dynamics of pathogens in mixed viral-bacterial infections, namely the pathogenesis and synergistic interaction of pathogens from different nosological groups in bovine respiratory diseases including pulmonary pasteurellosis. Besides, these findings highlight the importance of RT-PCR as a diagnostic method, as BRSV replicates weakly in cell cultures, and its infection is transit and short-lived.

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IDENTIFICATION OF NEW ISOLATES OF THE HORSE STRANGLES CAUSATIVE AGENT IN NORTHERN SIBERIA

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Abstract

Infectious and invasive diseases cause significant damage to the economy and decrease the productivity of horse herd farming. Equine strangles (*Streptococcus equi*) is the most economically damaging. Specific prevention of the disease widespread in Asia, Russia and CIS poses a serious problem. In this work, for the first time in the Far North, we isolated and identified three new *Streptococcus equi* strains prospective for the diagnostics and development of strangles vaccines. The study aimed to culture, identify morphologically, culturally, biochemically, and genotypically new isolates of the equine strangles causative agent for the development of vaccines. A survey of 6-10-month old Yakut and Kazakh foals (*Equus ferus caballus*) was performed in the Republic of Sakha (Yakutia) regions (the farms in Namskiy, Khangalasskiy, Amginskiy, Megino-Kangalarskiy districts and in Yakutsk) and in Kazakhstan in 2015-2017. In total, 63 collected biospecimens included 45 nasal discharges (27 from diseased and 18 from healthy foals), 7 submandibular lymph node abscesses, and 11 parenchymal organs. The infectious agents were isolated and identified by 16S rDNA genotyping using PCR and based on biochemical traits. Morphological and cultural properties were studied using meat peptone broth (MPB) added with 1 % glucose and 10 % horse blood serum and on meat peptone agar (MPA) with 1 % glucose and 10 % horse blood serum or 5 % defibrinated horse blood. Pus swabs and preparations of liquid and agar cultures stained by the Gram procedure were investigated with a light microscope. Biochemical properties were studied by plating on MPA with 40 % bile, 6.5% saline MPA, agar with sodium azide, and Giss's medium with glucose, lactose, mannitol, maltose, sucrose, sorbitol, and dulcite. The isolates were biochemically assigned to species using API 20 Step strips (an API test system, bioMerieux, France). The virulence of the isolates (LD_{50}) was assessed on white mice challenged subcutaneously with 0.2-0.5 cm^3 of 1-day suspensions (1×10^3 to 1×10^9 CFU per mouse). Genotyping was performed with specific primers Seel-F 5′-CGGATACGGTGAT-GTTAAAGA-3′ and Seel-R 5′-TTCCTTCCTCAAAGCCAGA-3′. The *Streptococcus equi* 16S rRNA gene was sequenced for six isolates of strangles streptococcus, of which three we suggest for the development of strangles vaccines. Polymerase chain reaction with specific primers serves as the most reliable and fastest method for identifying strangles streptococcus. Based on genotyping data and the cultural, morphological and biochemical properties, the *Streptococcus equi* H-5/1 isolate belongs to the *Streptococcaceae* family, *Streptococcus* genus, *Streptococcus equi* ssp. *equi* and corresponds to the typical characteristics of the species. The nucleotide sequence of the 16S rRNA gene fragment of the isolate H-5/1 after sequencing was deposited in the NCBI GenBank database (MW486609). The *Streptococcus equi* H-5/1 strain was deposited in the All-Russian State Collection of Microorganism Strains Used in Veterinary Medicine and Animal Husbandry (VGNKI, registration number VKSHM-B-141P, certificate of deposit dated May 22, 2018), and patent for invention No. 2703485 ("A strain of bacteria *Streptococcus equi* used for the production a vaccine against strangles") dated 10/17/2019 was received. The new *Streptococcus equi* strains we described here hold promise in the developing strangles vaccines. Note, *Enterococcus faecales*, *Streptococcus piogenes*, toxigenic and mold fungi *Aspergillus* and *Mucor* genera were also iso-

lated from foals with clinical sings of equine strangles. Our findings attract attention to these microorganisms possibly involved in the development of equine strangles in young horses, which should be accounted in diagnostics of this pathology.

Keywords: equine strangles, streptococcus, *Streptococcus equi*, biochemical traits, genotyping, Yakut horses, Kazakh horses, Siberia, the Far North, bacterial infections

Horse breeding is a key sector of animal husbandry in many countries. However, it is hindered by such factors as horse infections, of which equine strangles (caused by *Streptococcus equi*) is most common [1, 2]. The pathogen is believed to have changed only slightly over the past 700 years, although region-specific traits of strains are not denied [3]. In Russia, the disease has been reported in the Novosibirsk Region, Krasnoyarsk Territory, Altai Territory, Republics of Khakassia, Sakha (Yakutia), and Altai, and Irkutsk Region, as well as in Kazakhstan, Kyrgyzstan, and Mongolia [4–6].

In the Republic of Sakha (Yakutia), with its emphasis on horse breeding in herds, the incidence of the disease in follows is 57.8–62.7%; associated mortality rates reach 4.0% to 22.0% depending on how the epizootic process unfolds [6]. The incidence and mortality rates in the Republic of Kazakhstan are 30.1–46.7% and 16.0–28.3%, respectively [7]. In 2017–2020, the disease was registered in 42–59% of all Yakut foals (unpublished in-house data).

The pathogen needs to be studied in different regions if effective methods for diagnosis, prevention, and treatment of equine strangles are to be developed [8]. In all countries where equine strangles occurs, including Kazakhstan [7], Kyrgyzstan [9], the Netherlands [10], the Arab Republic of Egypt [11], Russia [6, 12], Korea [13], and Brazil [14], isolation and identification of *Streptococcus equi* rely on its morphological, cultural, and biochemical properties. With the advancement of molecular genetics, reports have surfaced that the pathogen can be identified by polymerase chain reaction (PCR) using species-specific genes that are potentially involved in forming the virulent phenotype of *Streptococcus equi* [2, 15–18]. Strains thus obtained are used to diagnose the disease and to manufacture vaccines in the United States, Kazakhstan, and the Netherlands [7, 8, 10].

Strangles pathogen has been reported to cause arthritis in goat kids [19], abortions in mares [6]. It can be isolated in clinically healthy horses, too, since they are its carriers [6, 13, 14]. The *Streptococcus equi* strain H-34 was earlier deposited by the authors hereof at the Russian State Center for Animal Feed and Drug Standardization and Quality (VGNKI, Moscow); it was proposed for the production of equine strangles vaccines and diagnostic streptococcal serum, Serogroup C [6]. The strain has since been un-deposited due to loss of specific antigenicity, i.e., the Russian Federation currently has no vaccine against equine strangles. Other countries produce a variety of vaccines, none of which is registered in Russia [7, 8, 10].

For this research, three new isolates of *Streptococcus equi* have been isolated and identified in the Far North for potential use in the diagnosis of, and development of vaccines against, equine strangles.

The goal hereof was to isolate and study new isolates of *Streptococcus equi* and to identify them in terms of morphological, cultural, biochemical, and molecular genetic properties.

Materials and methods. Biological samples were collected in 2015–2017 at farms in the Republic of Sakha (Yakutia: Namskiy, Khangalasskiy, Amginskiy, Megino-Kangalarskiy Districts, and the City of Yakutsk), as well as in Kazakhstan. In total, 63 collected samples taken from 6-10 months old Yakut and Kazakh horses (*Equus ferus caballus*) included 45 nasal discharge samples (27 from diseased and 18 from healthy foals), 7 samples of submandibular lymph node abscess,

and 11 parenchymal organs (sampled from strangles-claimed foals).

Preseeding treatment was applied before bacteriological studies. Discharge samples on swab probes, pieces of organs, and lymph nodes were submersed for 5 minutes in sterile saline, then treated with 70° alcohol and further washed 2-3 times in saline.

The isolates were studied morphologically and culturally by seeding in meat-peptone broth (MPB): 1% glucose and 10% horse serum; and on meat-peptone agar (MPA): 1% and 10% horse serum or 5% defibrinated horse blood. Pus smears and culture preparations were fixed and Gram-stained. The isolates were studied biochemically by seeding on MPA (40% bile) or on 6.5% salt MPA or on sodium azide agar and Hiss medium with glucose, lactose, mannitol, maltose, sucrose, sorbitol, and dulcite. The cultures were incubated in a TS-1/80 SPU thermostat (JSC Smolenskoye SKTB SPU, Russia) at 37 °C over 18-48 h.

The isolated cultures were identified taxonomically per Bergey's Manual of Systematic Bacteriology [20]; the research team also followed guidelines on laboratory diagnosis of strangles, staphylococcosis, and streptococcosis [21, 22]. To identify the species of the isolates by biochemical indicators, API 20 Step strips in the API test system (bioMerieux, France) were used.

Samples of streptococcal isolates were transplanted from semiliquid agar into tryptic soy broth and cultured at 37 °C for 48 hours, then transplanted into dishes with blood agar and onto a dense medium (Colombian agar) with potassium tellurate. The dishes were placed in a thermostat at 37 °C for 24 h. Cultural and morphological properties, the type of hemolysis, the presence of catalase activity were determined, and smears were prepared. Agar cultures were transplanted onto Colombian agar and cultured for 24 h at 37 °C; a suspension in saline was prepared from these cultures. Bacterial suspension in the amount of 0.1-0.2 cm^3 was placed in each well of the plate. Reagents and sterile liquid paraffine were added. The cultures were incubated for 5-24 h at 38 °C. The results were reported in the Reaction Interpretation Table of the test system and processed in Microsoft Excel.

To assess streptococcal virulence, the research team used white outbred mice ($n = 70$) of either gender, 5-8 weeks of age, 18−20 g of weight. The mice were injected subcutaneously with a suspension of live bacterial cells of the streptococci: 0.2-0.5 cm^3 (1×10^3 to 1×10^9 CFUs per specimen). The virulent activity of the LD_{50} isolates was tested by the Kerber method as modified by Ashmarin and Vorobyov [23].

To isolate DNA [24], 1.5 ml of liquid bacterial culture was centrifuged on a multifunctional 5804R unit (Eppendorf, Germany) until a dense precipitate was formed, which was then dissolved in 567 μl of TE buffer; 30 μl of 10 % SDS and 3 μl of proteinase K (20 mg/ml) were added until reaching the final concentration of 100 rg/ml. One-hour incubation at 37 °C would render the solution viscous, a sign of cellular wall destruction. One hundred microliters of 5 M NaCl was added to the solution, followed by thorough stirring and further addition of 80 μl of a CTAB/NaCl solution; 10-min incubation at 65 °C was performed afterwards. Then an approximately equal volume of a 24:1 chloroform-isoamyl alcohol mixture was added to the solution, which was then stirred thoroughly and centrifuged for 5 minutes; the supernatant was transferred into a clean tube, and an equal quantity of a 25:24:1 phenol-chloroform-isoamyl alcohol mixture was admixed; the final mixture was stirred again and centrifuged for 5 minutes.

For DNA precipitation, the supernatant was transferred into a clean tube and isopropanol was added in a quantity equal to ~ 0.6 times the amount of the original supernatant. The DNA precipitate was washed with 70% ethanol, dried

and dissolved in 30 rL of TE buffer. The quality and concentration of the resulting DNA preparation were assessed on a NanoPhotometer™ P330 unit (Implen, Germany).

To genotype the *Streptococcus* isolates, PCR was conducted using strain-specific primers Seel-F 5′-CGGATACGGTGATGTTAAAGA-3′ and Seel-R 5′-TTCCTTCCTCAAAGCCAGA-3′ [17, 18] (CFX-96, amplifier, Bio-Rad, USA) and a qPCRmix-HS LowROX kit (Evrogen, Russia). PCR and reaction medium were configured in accordance with the kit manufacturer's manual. Each reaction mixture (25 μl) contained: 5 μl of qPCRmix-HS LowROX (the master mix), 2 μl of each primer (1.0 μl), and 18 μl of nuclease-free water. PCR parameters: 5 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C. PCR products were visualized by electrophoresis in 1.5% agarose gel containing ethidium bromide as an intercalating dye; electric field strength was 6 V/cm. The sensitivity and reliability of the PCR method for identification of *Streptococcus equi* had been confirmed by studies carried out by the Probiotics Quality and Standardization Department, Russian State Center for Animal Feed and Drug Standardization and Quality (Moscow).

The 16S rRNA gene was sequenced at Genomika (Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, Novosibirsk). A ~ 1500 bps amplicon was produced using specific 16S primers (27F: 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R: 5′-GGTTACCTTGTTACGACTT-3′) for each isolate in a preparative amount, then purified by sorption on magnetic particles (Agencourt AMPure XP, Beckman Coulter, Inc., USA). Sanger sequencing was run on an ABI 3130xl Genetic Analyser (Applied Biosystems, USA) per the manufacturer's standard protocols. Nucleotide sequences were BLAST-analyzed (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify homology with nucleotide sequences deposited in the GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Fig. 1. Mucopurulent nasal discharge of a Yakut breed foal (*Equus ferus caballus*) showing clinical signs of stranglers (Khangalasski District, village Nemyugyuntsy, 2017).

Results. Forty cultures similar to *Streptococcus equi* in cultural, enzymatic, and hemolytic properties were sampled in 2015-2016 from horses showing clinical signs of stranglers, see Fig. 1. Eleven samples were found to be contaminated with toxigenic and mold fungi of the genera *Aspergillus* and *Mucor*.

Pre-treatment was very effective in suppressing the growth of concomitant microflora, resulting in easier and faster isolation of a pure culture. Intravital diagnosis of equine stranglers remains challenging, especially in the Far North. Poor low-quality diets were found to contribute to the fungal infection of the respiratory tract in animals, which is concurrent with equine stranglers.

All isolated cultures were preliminarily classified as *Streptococci*. They grew well in 10% horse serum MPB, as well as on 10% horse serum 1% MPA. Culturing

in 1% glucose serum MPB exhibited uniform clouding of the medium with white sediment that would go up and form pigtail-like pieces, which is typical of *Streptococcus equi*. On 1% glucose serum agar, some cultures produced tiny dewdrop-like colonies; convex shiny white colonies that had smooth edges and matte merged convex colonies were observed as well.

Subsequently, 7 isolates grew on 1% glucose, 40% bovine bile MPA, as well as on 6.5% sodium chloride MPA; they would ferment glucose, lactose, maltose, mannitol, sorbitol, and dulcite, whereby they produced acid without gas. These isolates exhibited near-bottom growth of dense white sediment in broths. On agars, they would form mucous white colonies. Black colonies grew on the potassium tellurate medium. No catalase activity. Optical microscopy revealed short cocci chains in broth cultures, grape cluster-like cocci in Gram-stained preparations of agar-grown cultures.

Identified species of streptococci isolated from equine strangles-affected Yakut and Kazakh foals (*Equus ferus caballus*) (Republic of Sakha—Yakutia, Republic of Kazakhstan, 2015-2017)

Isolate	Identification	
	PCR genotyping	biochemical traits
P1	*Streptococcus equi*	–
N-1 kaz	*Streptococcus equi*	*Streptococcus equi*
N-5/1	*Streptococcus equi*	*Streptococcus equi*
N-12-3	*Streptococcus equi*	*Streptococcus equi*
7-3	*Streptococcus equi*	–
1-3	*Streptococcus equi*	–
H-34	–	*Enterococcus faecalis*
Khatas-3	–	*Streptococcus pyogenes*
ChG	–	*Enterococcus faecalis*
4g	–	*Enterococcus faecalis*
MK 1/1	–	*Enterococcus faecalis*
YuG	–	*Enterococcus faecalis*
SM	–	*Enterococcus faecalis*
M	–	*Enterococcus faecalis*

N o t e. Dashes denote isolates not studied by the corresponding method.

Biochemically, culturally, and morphologically, the isolates 4g, MK 1/1, YuG, SM, H-34, and M were classified as *Enterococcus faecalis*, whereas Khatas-3 was classified as *Streptococcus piogenes*, see Table. At this point, it was impossible to isolate *Streptococcus equi,* as the samples were highly contaminated with toxigenic and mold fungi, as well as with an association of *Streptococci*. These findings bring attention to the role of *Enterococcus faecales* and *Streptococcus piogenes* in the progression of the respiratory infectious process.

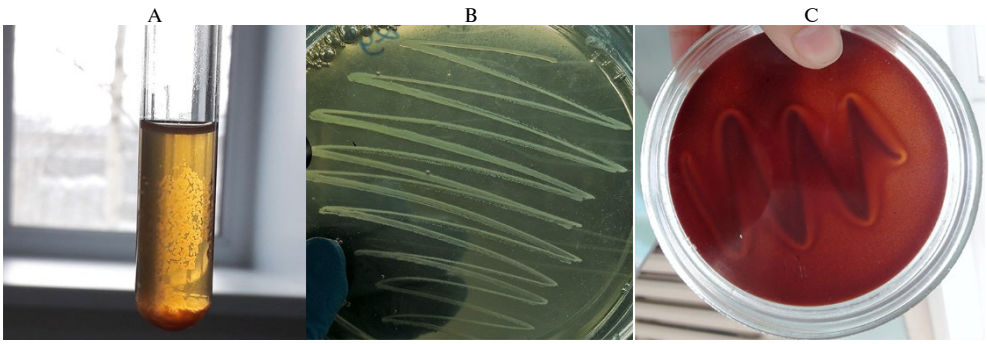

Fig. 2. Growth of *Streptococcus equi* isolated from the nasal cavity of stranglers-affected Yakut foals (*Equus ferus caballus*) in meat-peptone broth (A), **on meat-peptone agar** (B), **on blood agar (β-hemolysis)** (C) (Republic of Sakha—Yakutia, Republic of Kazakhstan, 2017)

In October and November 2017, new streptococcal isolates were sampled

from stranglers-affected foals; in broth, these exhibited near-wall growth and formed white flaky sediment, see Fig. 2, A, whereas on agar, they formed tiny dewdrop-like translucent colonies, see Fig. 2, B. β-hemolysis was observed on blood agar, see Fig. 2, B. Isolates would not grow on media containing bile or sodium chloride; they fermented glucose and lactose, whereby acid would be produced without gas; however, they would not ferment mannitol, sorbitol, or dulcite. Microscopy of Gram-stained preparations revealed long convoluted chains of gram-positive cocci in a one-day broth culture, short chains, paired or singular cocci in agar-grown colonies. Morphologically, culturally, and biochemically, these isolates were identical to *Streptococcus equi*.

Testing of 40 isolates identified the nucleotide sequence of the *Streptococcus equi* rRNA S16 gene in six of them (P1, H-1 kaz, H-5/1, H-12-3, 1-3, 7-3), all sampled from foals with clinical signs of equine stranglers. Three (H-1 kaz, H-5/1, and H-12-3) were further selected for vaccine development. The H-34 strain, deposited earlier as *Streptococcus equi* (All-Russian Collection of Cell Cultures, Virus Strains, Microbes and Micropathogens, VGNKI, Moscow; no longer deposited) was no longer compliant with the requirements and specifications of the test system (see Table) due to multiple repeated inoculations in long-term storage. Molecular genetic typing confirmed the analyzed nucleotide sequence of the H-5/1, H-12-3, H-1 kaz strains to be 100% identical to the nucleotide sequence of a *Streptococcus equi* 16S rRNA gene fragment. *Streptococcus equi* H-5/1, being the most promising strain, passed certification testing at the Probiotics Quality and Standardization Department of VGNKI (All-Russian Collection of Microorganism Strains).

Streptococcus equi H-5/1 produced β-glucosidase, β-glucuronidase, and leucinaryl amidase; it fermented esculin, starch, and glycogen producing acid without gas; it did not ferment ribose, arabinose, mannitol, sorbitol, trehalose, inulin, or raffinose, nor would it produce acetoin or hydrolyzed hippurate.

For white mice, LD_{50} of *Streptococcus equi* H-5/1 was 1×10^2 CFU/specimen in parenteral administration.

Sequencing of the 16S rRNA gene for the H-5/1 isolate yielded the following nucleotide sequence:

TGCAAGTGGAACGCACAGATGATACGTAGCTTGCTACAATTATCTGTGAGTCGCGAACG
GGTGAGTAACGCGTAGGTAACCTAGCTTATAGCGGGGGATAACTATTGGAAACGATAGC
TAATACCGCATAAAAGTGGTTGACCCATGTTAACCATTTAAAAGGAGCAACAGCTCCACT
ATGAGATGGACCTGCGTTGTATTAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGAC
GATACATAGCCGACCTGAGAGGGTGAACGGCCACACTGGGACTGAGACACGGCCCAGA
CTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGGAACCCTGACCGAACA
ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGAGAAGAACAG
TGATGGGAGTGGAAAGTCCATCATGTGACGGTAACTAACCAGAAAGGGACGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGT
AAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCAGTGGCTTAACCATTGTA
TGCTTTGGAAACTGTTAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCG
GTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTA
ACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGCTGAGTGCTAGGTGTTAGGCCCTTTCCGGGGCTTAGTGCCGGAGCT
AACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC
TTACCAGGTCTTGACATCCCGATGCTATTCTTAGAGATAAGAAGTTACTTCGGTACATTG
GAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTAAGTTGGGCACTCTAGCGAGACT
GCCGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACC
TGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTCGCAAGCCGGTGACGGCAAG
CTAATCTCTGAAAGCCAATCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTC
GGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTA
CACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCGTTA
AGGAGCCAGCCGC

BLAST analysis showed this sequence to match the 16S rRNA genes in two *Streptococcus equi* subsp. *equi* strains: NCTC9682 (MW486609) and ATCC 39506 (MW486609).

Therefore, the cultural, morphological, and biochemical properties, as well as the genetic markers identified the H-5/1 isolate as *Streptococcus equi* ssp. *equi* (fam. *Streptococcaceae*, genus *Streptococcus*); the isolate matched the type characteristics of this species. Nucleotide sequence of the 16S rRNA gene fragment, H-5/1 isolate, was deposited in NCBI GenBank after sequencing (MW486609). Based on the findings, the *Streptococcus equi* H-5/1 was deposited in the All-Russian State Collection of Microorganism Strains Used in Veterinary Medicine and Animal Husbandry (VGNKI, registration number VKSHM-B-141P, certificate of deposit dated May 22, 2018); novelty confirmed by Invention Patent No. 2703485 ("A strain of bacteria *Streptococcus equi* used for the production a vaccine against strangles") dated Oct 17, 2019.

Streptococcus equi H-5/1 is now used as a production strain to make immunobiologicals for the prevention of equine strangles in the Russian Federation. *Streptococcus equi* H-1 kaz isolated from a sick Kazakhstani horse could be used to develop a vaccine for use in herd breeding in Kazakhstan. Many researchers note the need to isolate region- or country-specific *Streptococcus equi* strains to make vaccines [1, 2, 8].

The presence of toxigenic and mold fungi in the nasal discharge of respiratory disease-affected fowls could be due to the prevalence of microscopic fungi (genera *Aspergillus and Mucor*) in the vegetation of the winter-grazing pastures in Yakutia, especially in rainy years [25, 26].

The findings presented herein are in line with what Dauvillier *et al.* reported [27], where they noted the presence of various fungi in equine respiratory tracts and pointed out the need to study their role in the etiology of respiratory diseases in horses and humans. Alarming is the fact that *Streptococcus zooepidemicus*, which causes severe illness in humans, is excreted from the nasal cavity in healthy and sick horses alike [28], although the present study did not detect these microorganisms. Any effort to diagnose and prevent strangles must be adjusted for the possibility of the horse contracting rhinopneumonia or influenza. It is crucial to investigate the relations between bacterial, viral, and fungal infections [6, 29, 30], which could be the line of future research.

Our data suggests Sakhabaktisubtil, a probiotic developed by the Yakutsk Agricultural Research Institute, could effectively treat mycotoxicosis [25]. Newly devised stranglers prevention methods must provide room for using antifungals and immunomodulators to improve immunological reactivity.

The findings reported herein confirm that isolating *Streptococcus equi* from sick or infected animals by standard culturing methods (and developing a vaccine) could be challenging due to low sensitivity coupled with the complexity of the process [31], especially under the extreme conditions of herd breeding. PCR is the fastest, most sensitive and specific method for diagnosis of equine stranglers and identifying *Streptococcus equi* [15, 16, 31].

Thus, the research team was able to isolate, identify by their cultural, enzymatic, and hemolytic properties, and genotype new isolates of *Streptococcus equi*, which could be used to diagnose this pathology, as well as to develop a stranglers vaccine. The *Streptococcus equi* H-5/1 strain has been deposited in the All-Russian State Collection of Microorganism Strains Used in Veterinary Medicine and Animal Husbandry (VGNKI, registration *number VKSHM-B-141P*, certificate of deposit dated May 22, 2018). Invention Patent No. 2703485 ("A strain of bacteria *Streptococcus equi* used for the production a vaccine against strangles") dd. Oct

17, 2019 was thereby granted. *Enterococcus faecales*, *Streptococcus piogenes* cultures, toxigenic and mold fungi of the genera *Aspergillus* and *Mucor*. were isolated from foals exhibiting clinical signs of stranglers. Efforts to diagnose and prevent stranglers and other respiratory diseases in foals should be adjusted for the presence of these microorganisms as possible pathoflora.

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EPIZOOTIC MONITORING OF PROTOZOOZES IN THE FUR FARMS OF THE KALININGRAD REGION (2018-2020)

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Abstract

Parasitic diseases are widespread in fur-bearing animals, especially in minks. Coccidiidoses occupy a special place among invasive diseases, as they often occur without any symptoms and in some cases are not timely diagnosed. Despite the mild clinical manifestation of invasion, it causes serious damage to animal health and significant economic damage to fur-bearing animal farms. After analyzing the literature data, it became clear that the distribution of eimerioses of fur-bearing animals in the Kaliningrad region has not been studied well enough. In particular, there is no information about the prevalence rates, invasion intensity, and age dynamics of the mink eimeriosis in the fur farms of the region. The purpose of this work was to study the epizootic situation on mink (*Mustela vison*, Linnaeus, 1761, *Neovison vison* Schreber, 1777) coccidiidoses in fur-bearing animal farms in the Kaliningrad region. Investigations were performed in three fur-bearing animal farms of the Kaliningrad region. The species composition of protozoa in minks was determined by the morphological features of coccidia and by deep sequencing of the 18S rDNA V4 region. OTUs (operational taxonomic units) revealed by the bioinformatic analysis were used to establish the taxonomic affiliation of pathogens which confirmed the results of light microscopy. In analyzing the obtained results and choosing research methods, the age, sex of the animals, as well as housing and feeding conditions were taken into account. Young minks of 5-6 months and adult livestock, 1-2 year-old females and males, were surveyed. In total, 561 animals were examined in three farms, including farm 1 — 273 minks (198 young animals, 75 adults — 33 males and 42 females); farm 2 — 160 minks (68 young animals, 92 adults — 44 males and 48 females), and farm 3 — 128 minks (28 young animals, 100 adults — 44 males and 56 females). In all the farms surveyed, we found the protozoan of the *Eimeriidae* family. The greatest prevalence rates (56 %) and the widest variety of coccidia species occurred in the animals of farm 1 under the invasion of two eimeria, *Eimeria vison* and *E. furonis*, and two isospores, *Isospora laidlawi* and *I. eversmanni* (the *I. eversmanni* we have earlier identified in minks in the Kaliningrad region for the first time). In all fur farms in the Kaliningrad region, eimerioses more often occur as mono invasions. In mixed invasions, 68.55 % were two-parasite invasions, 23.67 % were three-parasite invasions. Young minks are more susceptible to eimeriidoses than adults. In the animals of the current year of birth, *E. vison* (18.36 %), *I. laidlawi* (16.32 %), and *E. vison* + *I. laidlawi* (11.90 %) prevailed.

Keywords: *Mustela vison*, *Neovison vison*, minks, *Eimeria vison*, *Eimeria furonis*, *Isospora laidlawi*, *Isospora eversmanni*, mono invasion, mixed invasion, invasion extensity, invasion intensity, fur farms, Kaliningrad region

Fur animals in fur farms are often exposed to various infectious and invasive diseases [1-3]. Captive carnivores, especially those kept in cages, are often infested with intestinal protozoa. Analysis of research letters shows that in Russia

and other countries, mink are infected mainly with protozoans of the family Eimeriidae Munchin, 1903 [4-6]. Researchers note the most significant vulnerability to eimeriosis of young mink 2-3 months of age, among which the infection reaches 68% [7-9].

Diseases of parasitic etiology cause tangible economic damage to global fur farming [3, 10-12]. In the pathogenesis of eimeriosis, the solution of continuity of the integrity of the intestinal mucosa in sick mink, which is due to the endogenous stage of the parasite (merogony) biological cycle, takes an important place, resulting in pathological changes in the morphology of the small intestine mucosa. The inflammatory dendrite that develops during epithelial desquamation and the protozoa metabolic by-product create conditions for penetration and reproduction of secondary microflora. Due to catarrhal and hemorrhagic enteritis and intestinal necrosis, the body does not absorb nutrients, animals lose weight, which may be lethal [13-15]. *Eimeria* are strictly specific parasites and parasitize in animals of different systematic groups [1, 5, 8].

Studies conducted by a research team on Danish farms between April and October 2016 showed that out of 4,140 animals examined, 108 were infected; hence a prevalence (P) was 2.6%. Morphological analysis of sporulated oocysts ($n = 20$), carried out by light microscopy, allowed establishing their species membership to the genus *Eimeria*. The size of the oocysts was 21.0×13.8 μm with a length-to-width ratio (L/W) of 1.5. Until today, the species membership of *Eimeria* is debatable in some cases. Only molecular genetic studies and phylogenetic analysis of 18S rRNA sequences (1221 bps) of samples obtained from infected minks allowed establishing that *E. vison* is the species with the most significant genetic similarity with *Eimeria* sp. first isolated in the black-striped field mouse (*Apodemus agrarius*) in the Czech Republic. The shorter 18S rRNA site (531 bps) showed that the *E. vison* genome sequences had 97.7% similarity with another species, *E. furonis* [16]. The findings of Petersen et al. [16] may indicate that *E. vison* and *E. ictidea* are probably the same species because they have a high morphological and genetic similarity. Thus, it is possible to solve the question of the taxonomic independence of this or that species of Eimeridae only with a comprehensive molecular genetic study [16, 17].

The epizootic situation of eimeriosis within the regions of Russia is not the same, and in some regions, it requires additional study [7, 18]. Analysis of specialized literature showed that eimeriosis of fur animals within the Kaliningrad Region had not been studied sufficiently; in particular, there is no information on the infection extensiveness and prevalence and intensity, age dynamics of mink eimeriosis in fur farms [19].

This paper presents the epizootic situation monitoring protozoosis in fur farms of the Kaliningrad Region for the first time in many years. The infection extensiveness and prevalence by coccidia in mink of different age groups were determined. Using sequencing and analysis of the small subunit ribosomal RNA gene (SSUrDNA), the parasitic fauna of minks was identified.

The work purpose is to study the epizootic situation of mink eimeriosis in fur farms of the Kaliningrad Region.

Materials and methods. Studies were conducted from 2018 to 2020 in three fur farms of the Kaliningrad Region on mink (*Mustela vison* Linnaeus, 1761, *Neovison vison* Schreber, 1777) (young animals 5-6 months old and adult stock of females and males aged 1 to 2 years). Animals' age, sex, and feeding and housing conditions were considered when selecting research methods and analyzing the data obtained.

A total of 561 mink (*Eimeriidae*-infected and intact), including 267 adult animals (115 males and 152 females) and young animals (294 individuals), were

examined during the observation period. The sampling at fur farm No. 1 was 273 animals, including 198 juveniles and 75 adults (33 males and 42 females); at fur farm No. 2 — 160 minks, including 68 juveniles and 92 adults (44 males and 48 females); at fur farm No. 3 — 128 minks, including 28 juveniles and 100 adults (44 males and 56 females).

Freshly isolated fecal mass samples (10-20 g each) from the examined minks were placed in individual containers, labeled, and transported (at +2-8 °C) to the laboratory for analysis.

Eimeriidae oocysts were isolated from fecal masses by the Darling method using a universal flotation diagnostic fluid [20]. The obtained material was viewed by a light microscope Microton-200M (OOO Petrolaser, Russia), using a Micrometer OMP LOMO (AO LOMO, Russia) tip and a Primo Star (Carl Zeiss, Germany) microscope with visualization at 10×10, 10×20, and 10×40 magnifications. Photorecording was performed using a camera microscope and a Mi MIX 2 smartphone (Xiaomi, China). When determining the parasite species, attention was paid to the oocysts' size, shape, color, shell thickness, the presence or absence of micropyle, clear globule, polar granule, and the shape index was determined (length to width ratio).

Eimeriidae oocysts were cultured according to Arnastauskene's method (1985) using a 2% potassium dichromate solution [3, 20]. After concentrating and washing, the oocysts were placed in advanced Petri dishes and incubated in an incubator at 25-28 °C [21], viewing daily under a microscope under magnification (10×10 and 10×40) [9, 18] to the sporulation timing.

The infestation intensity was assessed by counting oocysts of *Eimeriidae* in 1 g of feces using a VIGIS counting chamber (VIGIS, USSR). The protozoa taxonomic affiliation was determined according to the description of their morphological characteristics in the monograph by Pellerdy [22]. According to Darling, the prevalence was assessed by a flotation test of fecal samples as the percentage of infested samples from the number of animals examined.

The sequencing method was used to confirm the coccidia species composition, and phylogenetic analysis of the small subunit ribosomal RNA gene (SSUrDNA) was performed.

Ten fecal samples from different animals were used for DNA isolation. The washed oocysts were morphometric and subjected to 3-fold freezing in liquid nitrogen (–196 °C) to destroy the walls and release sporocysts. Genomic DNA was extracted and purified as described [23-25]. The concentration of the obtained DNA was evaluated on a spectrophotometer SS2107 (MEDIORA OY, Finland), the preparations were stored at +4 or –20 °C [23].

Each sample was genotyped at two loci, nuclear 18S rDNA (SSUrDNA) [26] and mitochondrial cytochrome oxidase subunit I (mt COI) [27]. Sites from nu 18SSUrDNA and mitochondrial cytochrome oxidase subunit I (mt COI) DNA were amplified using polymerase chain reaction (PCR). For all samples, real-time PCR amplification was performed on a Veriti® Thermal Cycler (Life Technologies, Inc., USA) in a 25 rl volume containing ~100 ng of genomic DNA, 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 1 unit TaqDNA Invitrogen Platinum polymerase (Thermo Fisher Scientific, Canada), and 400 nM of each primer [28, 29]. For sequencing, PCR fragments were obtained in a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, Singapore). Amplification mode: 3 min at 95 °C; 30 s at 94 °C, 30 s at 56–62 °C, 30–75 s at 72 °C (35 cycles); 7 min at 72 °C (final elongation). The obtained amplification products were separated by electrophoresis in 2% agarose gel, stained with ethidium bromide, visualized on a WUV-M10 ultraviolet transilluminator (DAIHAN Scientific, South Korea), and separated by

electrophoresis in agarose gel with fluorescent detection. According to the manufacturer's recommendations, they were then analyzed using a CEQ 8000 automatic sequencer (Beckman Coulter, USA). The instrumental error of the CEQ 8000 was 5% or less [23]. The results were counted by peak size and area using a program unit in the Geneious database (https://www.geneious.com/); taxonomic annotation of *Eimeriidae* was performed by BLAST searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using nucleotide sequences published in GenBank (https://www.ncbi.nlm.nih.gov/gen-bank/) [23-25, 28]. Phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA v. 7.1) program [30, 31].

Statistical processing of the results was performed using Microsoft Excel 2013 and Primer of Biostatistics 4.03 for Windows.

Results. Monitoring the invasion and parasitic fauna of minks is vital to determine their essential role in transmitting parasitic zoonoses [32]. In animal farm No. 1 (Bagrationovskiy District, Kaliningrad Region), *Eimeriidae* infestation was found in 153 minks out of 273 examined (P = 56%). The research team found two species of eimeria, *E. furonis* and *E. vison* (Fig. 1, a), and two species of isospores, *I. laidlawi* and *I. eversmanni* (see Fig. 1, b).

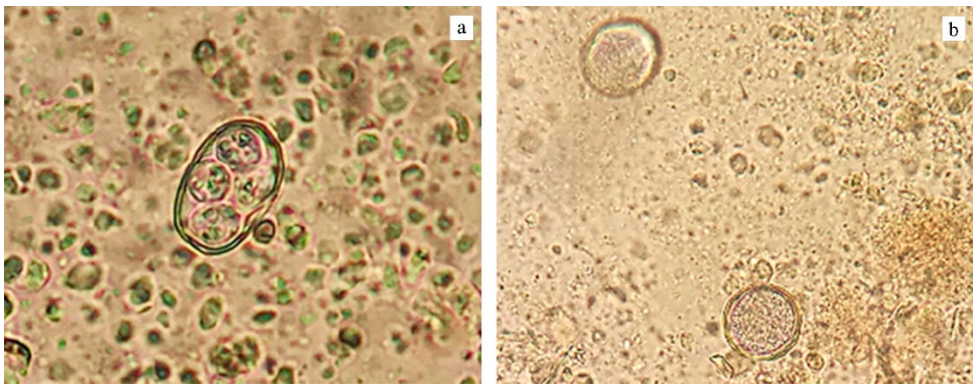

Fig. 1. Light microscopy of oocysts of *Eimeriidae* isolated from minks (*Mustela vison*) in fur farms (Kaliningrad Province, 2018-2020): a — sporulated oocyst of *Eimeria vison* (magnification ×1480), b — non-sporulated oocysts of *Isospora laidlawi* + *I. eversmanni* (magnification ×1280) (Microton-200M (OOO Petrolaser, Russia).

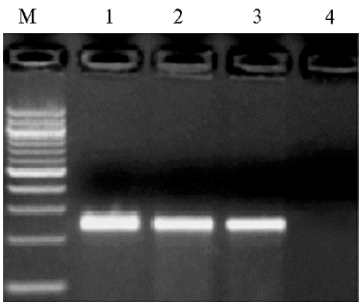

Fig. 2. Electrophoretic analysis of 18S rDNA libraries in genotyping of *Eimeriidae* isolated from minks in fur farms (Kaliningrad Province, 2018) (an example): M — molecular weight-size marker (GeneRuler 1 kb Plus DNA Ladder, Thermo Fisher, USA), 1-3 — Nor1 sample (*Eimeria vison*), different amounts of DNA (1, 2, 3 μl), 4 — Ctrl_NegControl (2% agarose gel).

The species composition of protozoa in the examined minks was confirmed by metagenomic sequencing (Fig. 2) with the following primers (Table 1) [28].

1. Primer pairs for amplification of nuclear and mitochondrial loci [24-26] **used for genotyping of *Eimeriidae* isolated from minks (*Neovison vison*) in fur farms** (Kaliningrad Province, 2018-2020)

Locus	Primer	Sequence (5′→3′)	Author
18S rRNA	V4F	CCAGCASCYGCGGTAATTCC	S. Balzano et all. (2015)
	V4RB	ACTTTCGTTCTTGATYRR	
mt COI	COI_10F	GGWDSWGGWRYWGGWTGGAC	J.D. Ogedengbe et all. (2011)
	COI_500R	CATRTGRTGDGCCCAWAC	

Molecular analysis of the nucleotide sequence of the ribonucleic acid gene of the small ribosomal subunit determined the species identity of the isolated eimeriosis pathogens. Deep sequencing of the V4 site of the 18S rRNA gene and bioinformatic analysis made it possible to identify operational taxonomic units and establish their affiliation. According to the genotyping results, the predominant protozoan species parasitizing minks were *E. vison, E. furonis, and I. laidlawi*, and a rarely encountered species, *I. eversmanni* [25]. The sequence of a 383-bp 18S rDNA fragment detected in the oocysts assigned to the *E. vison* species by light microscopy had the highest (99.48%) similarity to the sequences of *E. ictidea* [25]. The latter species was not previously detected by the authors during coproovoscopy under a light microscope in any of the surveyed fur farms. This result suggests that more detailed molecular genetic studies of Eimeriidae using a more extended nucleotide sequence are required. The high morphological and genetic similarity between *E. vison* and *E. ictidea* raises the question of the possible need to synonymize these two species [9]. At the same time, the conducted phylogenetic analysis and the construction of a combined tree based on the comparison of a 383-bp 18S rDNA fragment sequence of sample OTU 213 indicate that *E. ictidea* is still most likely an independent species of Eimeridae, which differs from *E. vison*, judging by the nucleotide sequences published in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (Fig. 3).

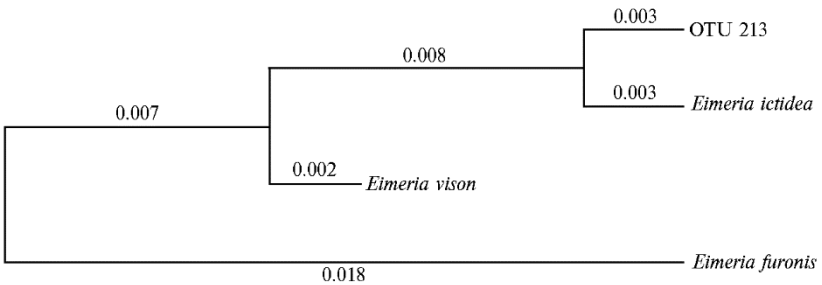

Fig. 3. Phylogenetic analysis (combined tree) **of *Eimeriidae* species based on the nucleotide sequence of a 383-bp 18S rDNA fragment.** OTU 213 was isolated from minks in a fur farm in the Kaliningrad region in 2019; nucleotide sequences of *Eimeria vison*, *E. furonis* and *E. ichdea* are published in Gen-Bank (https://www.ncbi.nlm.nih.gov/genbank/).

The results of genotyping based on the analysis of nuclear 18S rDNA SSUrDNA (with confirmation by mt COI) also indicated the circulation in the surveyed farms of two *Eimeria* species, *E. furonis* and *E. vison* and two isospores species, *I. laidlawi* and *I. eversmanni*, which were detected by light microscopy. It should be noted that in *E. vison* Kingscote, 1934, oocysts have morphological similarity with another species of *Eimeria*, *E. ichdea* Hoare, 1927, that is, it is difficult to differentiate them only by light microscopy (morphometrically). According to the morphometric description we performed in *E. vison* species, the size of the oocysts is 16.3-27.7 μm (length)×11.6-18.54 μm (width), on average 22.0×15.07 μm, and shape index 1.46. There is no micropyle. The zygote is fine-grained, globular-extended, and centered. At one pole, there is a polar granule between the wall and the germ mass. Gerasimchik [3] given a similar description, indicating a maximum oocyst size of 27.72×15.86 μm and a minimum size of 17.71×11.17 μm, with a shape index of 1.18-2.01, averaging 1.59 [3]. Despite the slight variation in measurements, the authors in other studies identified the species they found as *E. vison* by the same morphological characters.

According to Pastor [29], the oocysts identified as the *E. ichdea* species had the following morphometric parameters: length 23.98 μm (18.59-30.57 μm), width 18.55 μm (13.73-23.83 μm), and the shape index 1.01-1.60 (mean 1.30).

The oocysts were elliptic in shape, had a colorless double shell, and contained four sporocysts, each with two sporozoites. The sporocysts were ovoid and contained Stieda bodies and a clear globule [29]. At the same time, Pastor points out a rather large dispersion and variability in the measurements of oocysts (Fig. 3) [29].

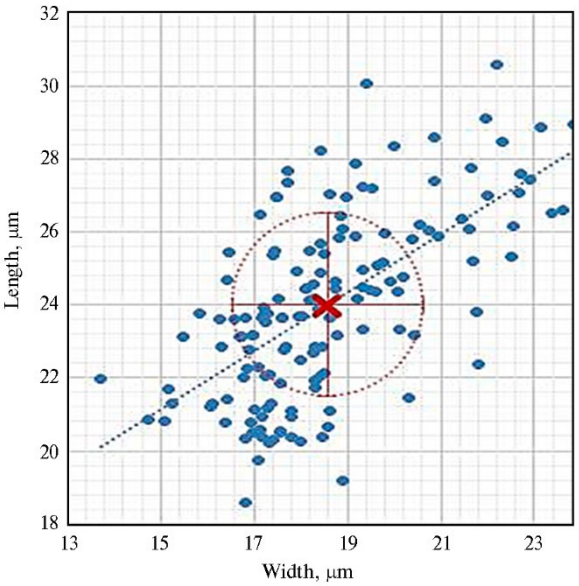

Fig. 4. Measurements of shape, length and width of *Eimeria ictidea* sporulated oocysts in black-footed ferrets (*Mustela nigripes*) [26]. The check (×) marks the mean value, the dashed oval covers one standard deviation from the mean.

Thus, there are two morphologically very similar species with relatively similar shapes, oocyst size, differing only in the presence or absence of the polar granule, which is sometimes difficult to detect under light microscopy. Consequently, molecular and genetic studies are necessary to determine the genus and species of oocysts, especially of unsporulated oocysts. It should be noted that no *E. ictidea* species were detected by light microscopy.

In fur farm No. 1, 47.0% of examined animals wer found to be monoinfested with coccidia. Co-infestation caused by the association of two parasites was noted in 39.0%, three — in 14.0% of the number of infected. *E. vison*, the predominant species of *Eimeridae* causing monoinfestation, was found in 37 minks. At the same time, P among animals infested with this species was 24.20%. The second most common isospore was *I. laidlawi* (17.60%) (Table 2).

2. Protozoa prevalence (P) **in minks of the surveyed fur farms** (Kaliningrad Province)

Protozoa species	Number of animals		P, %
	surveyed	infested	
Fur farm No. 1 (Bagrationovsky District, 2018)			
Eimeria vison	273	37	13.55
E. furonis	273	4	1.47
Isospora laidlawi	273	27	9.89
I. eversmanni	273	3	1.10
Mono invasions in total	273	71	26.01
E. vison + *E. furonis*	273	3	1.10
E. vison + *I. laidlawi*	273	33	12.09
E. vison + *I. eversmanni*	273	2	1.10
E. furonis + *I. laidlawi*	273	10	3.66
E. furonis + *I. eversmanni*	273	1	0.37
I. laidlawi + *I. eversmanni*	273	11	4.03
Associations of two parasites in total	273	60	21.98
E. vison + *I. laidlawi* + *I. eversmanni*	273	4	1.47
E. vison + *E. furonis* + *I. laidlawi*	273	18	6.59
Association of three parasites in total	273	22	8.06
Total number of invasions	273	153	56.04
Fur farm No. 2 (Gur'evsky District, 2019)			
E. vison	160	45	28.13
I. laidlawi	160	33	20.63
Mono invasions in total	160	78	48.75
E. vison + *I. laidlawi*	160	6	3.75
Associations of two parasites in total	160	6	3.75
Total number of invasions	160	84	52.50

Continued Table 2

Fur farm No. 3 (Zelenogradsky District, 2019)

E. vison	128	4	3.13
I. laidlawi	128	41	32.03
Итого моноинвазий:	128	45	35.16
E. vison + *I. laidlawi*	128	1	0.78
Associations of two parasites in total	128	1	0.78
Total number of invasions	128	46	35.94

Monoinvasions caused by *E. furonis* (2.6%) and *I. eversmanni* (2.0%) were less common. Previously, the authors reported that the species *I. eversmanni* was first found in mink within the Kaliningrad Region [25]. According to the literature, Svanbaev in 1956 [6] first discovered and described this species in the steppe polecat in Kazakhstan. Later, Gerasimchik [3] discovered this species in minks in the Republic of Belarus. The *I. eversmanni* species distribution in the Northwestern region has not been studied before.

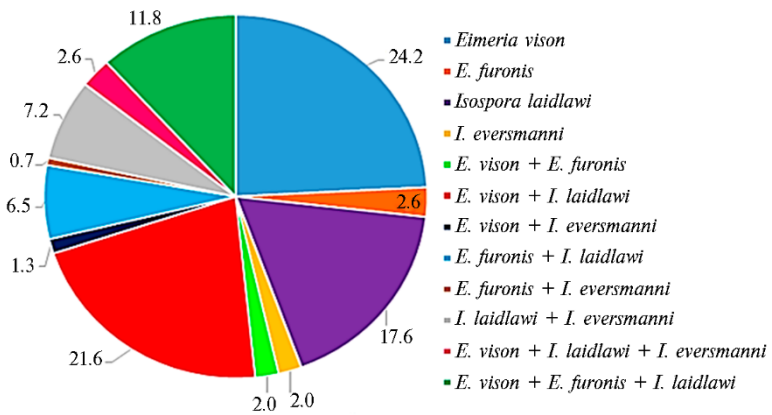

Fig. 5. Parasitic fauna structure in mink infested with *Eimeriidae* in fur farm No. 1 (Kaliningrad Region, 2018).

Co-infestation with two species of parasites in fur farm No. 1 occurred in 39.0% of infected animals and was represented by associations of different protozoan species. Co-infestation with *E. vison* + *I. laidlawi* was found in 33 animals, which amounted to 21.6% of the examined animals. The remaining parasite associations ranged from 0.7% (*E. furonis* + *I. eversmanni*) to 7.2% (*I. laidlawi* + *I. eversmanni*) (see Table 2, Fig. 5). Co-infestation with three species of parasites was recorded in 14.0% of the infected minks. The most frequent association (in 18 animals) of *E. vison* + *E. furonis* + *I. laidlawi* (11.8%) was observed. In 2.6% of infected mink, simultaneous parasitization of *E. vison* + *I. laidlawi* + *I. eversmanni*.

In fur farm No. 1, *Eimeriidae* infestation was found in 23 of 42 examined females (P = 54.76%). Of the 33 males examined, 13, or 39.39%, were infected with protozoa. It was found that in males, as in females, the species *E. vison* was predominant, and to a lesser extent, they were infested by *I. laidlawi*. Co-infestation was less common in adult mink than in juveniles. Juveniles of the current year of birth turned out to be the most infested with *Eimeriidae*, with a P of 76.50%.

In fur farm No. 2, out of 160 examined minks, 84 were infected, which amounted to 52.50% (see Table 2). The coccidia species detected in the examined minks are *E. vison* and *I. laidlawi* (P of 28.13% and 20.63%, respectively). Eimeriosis in this farm was represented mainly by monoinvasions (48.75% of the number of examined minks). Co-infestation with the association of two coccidia species was detected in 3.75% of animals. The ratio of coccidia species within this farm among infected individuals was 53.57% for *E. vison*, 39.29% for *I. laidlawi*, and 7.14% for association of these species.

Within fur farm No. 2, P was 51.85% in females, 44.70% in males, and 57.35% in juveniles of the current year of birth.

Within fur farm No. 3, P for coccidia in minks was 36.0% (see Table 2). In this farm, coccidiosis was predominantly monoinfastation. Among the isospores, the species *I. laidlawi* was found in 41 animals (32.0%). Parasitization of *E. vison* was noted in 3.13% of minks. The association of protozoa was also represented by these two species. Co-infestation was found in one animal (0.78% of the total number of examined animals) (see Table 2). In the parasitofauna structure within this farm, the species *I. laidlawi* (89.13%), *E. vison* (8.70%) was found less frequently in sick animals, and the association of these parasites was rare (2.17% of the number of infested animals).

Note that most of the 128 minks examined by the authors within fur farm No. 3 (see Table 2) were not infested with protozoans (82 animals). Out of 56 adult females, only 6 animals were infested (P = 10.71%), and only one species was detected in them – *I. laidlawi*. Of the 44 males examined, 27, or 61.36%, were infested with protozoa. It was found that in males, as in females, the species *I. laidlawi* was predominant, and to a lesser extent, they were infested by *I. laidlawi*. Co-infestation has not been observed in the adult mink population. Juveniles of the current year of birth were the most infested with coccidia (P = 46.42%). Juveniles were predominantly infested with *I. laidlawi,* and the species *E. vison* was less common. This species structure of the parasitofauna was common for both juvenile and adult males. Co-infestation in juveniles was diagnosed only in one animal (*E. vison* + *I. laidlawi*).

Within all three surveyed farms, P in juveniles was high. Within fur farms No. 1 and No. 2, it was higher than in other groups of animals (76.50% and 46.40%, respectively). Within fur farm No. 3, this characteristic was 28.30% and significantly lower than in adult males with P = 58.70%. Perhaps, the reason for the widespread infestation was the unsatisfactory sanitary condition of the sheds in which the males are kept. In all farms, predominant infestation with eimeriosis in females compared to males was observed.

Infestation intensity in animals in all three farms was low. The infestation intensity ranged from 1 to 50 in females, 1 to 80 in males, and 1 to 180 oocysts in juveniles.

Thus, in all surveyed farms within the Kaliningrad region, protozoans of the family *Eimeriidae* were detected in mink. According to the authors' observations, the widespread eimeriosis is promoted by their asymptomatic course, making timely diagnosis difficult. Molecular genetic studies showed that the sequence of a 383-bp 18S rDNA fragment isolated from samples of oocysts identified by light microscopy as *E. vison* had the most significant (99.48%) similarity with the sequences of *E. ictidea*. However, coproovoscopy did not detect *E. ictidea* in any of the surveyed fur farms. This indicates the need for a more detailed molecular genetic study of Eimeriidae, including the development of methods for their detection and identification to diagnose eimeriosis. The high morphological and genetic similarity between *E. vison* and *E. ictidea* leaves open the discussion of the possible need to synonymize these two species [9].

Within fur farm No. 1, the highest P (56.00%) and the widest variety of coccidia species (*E. vison*, *E. furonis*, *I. laidlawi, I. eversmanni*) were found with the predominance of monoinvastations (47.00% of examined animals). Mink were less infested within farm No. 2 (52.50%), and also with the predominance of monoinvasions (48.75%). In the examined minks, the species of *E. vison* and *I. laidlawi* have been identified. Within fur farm No. 3, the lowest P was set at 35.94%.

In the surveyed fur farms of the Kaliningrad region, differences both in

invasive load and in the species composition of protozoa were revealed. The parasitofauna was more diverse at the breeding fur farm (fur farm No. 1), where the breeding stock is annually renewed by purchasing animals from other farms both in the Russian Federation (Stavropol Territory) and abroad (Denmark). Lack of strict veterinary control at the border or when moving fur animals from one region of Russia to another leads to the spread of protozoosis pathogens. The asymptomatic eimeriosis course makes them difficult to diagnose. Veterinary control is significant when minks come from regions where monitoring for protozoan infestation is not mandatory. The animals were not imported to other examined farms; therefore, in the authors' opinion, two species of parasites, *E. vison* and *I. laidlawi*, can be considered as endemics of mink within the Kaliningrad Province.

Thus, in all surveyed farms, eimeriosis occurs in the form of both mono- and co-infestations. On the whole, monoinfestations account for 68.55% of cases, co-infestations with two parasite species account for 23.67%, and three species account for 7.77%. Two species of *Eimeria* (*Eimeria vison*, *E. furonis*) and two species of isospores (*Isospora laidlawi*, *I. eversmanni*, with *I. laidlawi* dominated) predominate. Young minks are more susceptible to eimeriosis than adult animals. In animals of the current year of birth, there are *E. vison* (18.36%), *I. laidlawi* (16.32%), *E. vison* + *I. laidlawi* (11.90%). These monitoring results allow the development of regulations for treatment and prophylactic measures in the Kaliningrad Region fur farms, taking into account the species composition and biology of *Eimeriidae*.

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METABOLIC PROFILES AND SPERM PRODUCTION IN IMPORTED HOLSTEIN BULL SIRES UNDER DIFFERENT CLIMATIC AND GEOCHEMICAL CONDITIONS OF RUSSIA AND KAZAKHSTAN

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Abstract

Currently, the potential of highly productive animals adapted to industrial farming should be used most effectively and not depend on the geographical location and agro-climatic resources of the region. Our study showed that the observation of required technologies minimizes effects of regional climatic and geochemical factors. Our findings give more understanding on the metabolic peculiarities of the sire bulls in various geo-climatic conditions of the 55.86°N and 51.18°N zone. This may be of interest for the practice of breeding the Holstein breed in countries with similar geo-climatic factors. We compared the influence of climatic and geochemical conditions of the Central Russia (the Head Center for the Reproduction of Farm Animals, Moscow Province), the Middle Urals (JSC Uralplemcenter, Sverdlovsk Province), and the Northern Kazakhstan (RCPZh JSC Asyl-Tulik, Akmola region) on the adaptive status of the imported Holstein 3-9-year-old bull sires (n = 122). Blood levels of bioelements Ca, P, Mg, Ca:P, Fe, chlorides, Se, Cu, and Zn were recorded. To assess protein-lipid metabolism parameters and blood enzyme activity, the total protein, albumin, globulins, urea, creatinine, total bilirubin, urea, triglycerides, cholesterol, alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase were measured. The endogenous hormone levels (testosterone, estradiol, thyroxine, and cortisol) were measured. The volume of ejaculate, the concentration of spermatozoa in the ejaculate, and the number of spermatozoa in the ejaculate were assessed to evaluate sperm productivity. Climatic and geochemical characteristics of the regions were a temperate climate with sod-podzolic soils for the Moscow region, a sharply continental climate with bedrock rocks with sandy-clayey and sod-podzolic soils for the Middle Urals, and sever sharply continental climate with dark chestnut soil for the Northern Kazakhstan. The study revealed that the balance of macroelements was within the permissible limits and did not have significant differences between regions, i.e., 2.34-2.53 mmol/l Ca, 1.47-2.01 mmol/l P (Ca:P 1.20-1.65), and 0.79-0.98 mmol/l Mg. The iron supply in the Moscow region was within the normal range (23.82±6.18 μmol/l), reached the upper limits in the Northern Kazakhstan (30.74±6.97 μmol/l) and exceeded the physiological level (40.32±7.30 μmol/l) in the Middle Urals. The balance of Se (0.72-1.13 μmol/l) and Cu (12.6-16.0 μmol/l) was within allowed limits. On the soils of the Moskvoretsko-Oka geochemical province (the Head Center for the Reproduction of Farm Animals), the bulls were 65.8 % provided with Zn compared to 95.9 % provision (of

that minimum allowed) observed in the dry steppe zone on dark chestnut soils of the Northern Kazakhstan. The enzymatic activity (as per the de Ritis coefficient) increased 2-fold in bulls of the Moscow region and the Northern Kazakhstan. All the sires had a sufficient concentration of both total protein and its fractions. The sires of the Moscow region fed an excessive amount of protein, as evidenced by the urea concentration at the upper limit (7.57±2.82 mmol/l) and creatinine 147.45±37.94 μmol/l). The bulls of the Northern Kazakhstan showed iron overload syndrome of 30.74±6.97 μmol/l with an increased bilirubin of 9.15±3.42 μmol/l. The balance of blood steroid hormones indicates a slight testosterone deficiency (39.17±5.06 nmol/l) and an excess of cortisol (226.75±45.62 nmol/l) in bulls of the Moscow region compared to the Middle Urals (50.36±5.80 and 138.81±21.48 nmol/l) and Kazakhstan (52.79±4.14 and 190.50±50.30 nmol/l); the differences are not statistically significant. The average level of blood thyroxine was within the physiologically permissible values, from 66.65±3.52 nmol/l in the Middle Urals to 91.13±3.35 and 95.39±1.86 nmol/l in the Moscow region and the Northern Kazakhstan, respectively. The level of estradiol varied from 0.197±0.02 nmol/l in the Moscow region to 0.234±0.02 and 0.276±0.04 nmol/l in the Northern Kazakhstan and the Middle Urals, which fit into physiological norms of 0.2-0.4 nmol/l for bull sires. The average ejaculate volume varied from 3.72 to 4.87 ml, with an average sperm concentration of 1.21-1.52 billion/ml. The total number of spermatozoa in the ejaculate was 5.32-6.00 billion; the differences were not statistically significant. Therefore, stable keeping conditions, strict control of the requirements in nutrients and mineral elements, and proper light regime (morning-day solar insolation, darkness at night) make it possible to avoid the negative influence of climatic and geochemical factors on breeding animals.

Keywords: bulls, Holstein breed, spermatozoa, metabolic balance, protein-lipid balance, bio-element balance, endogenous hormones, climatic zones, adaptation

Currently, when milk production is concentrated at large enterprises, the potential of highly productive animals adapted to industrial farming should be used most effectively and not depend on the geographical location and agro-climatic resources of the region [1, 2].

In Russia, the development of dairy farming is determined and limited by a certain set of agro-climatic factors [3–5]. It is known that the breed zoning of cattle is based on the specifics of fodder and climatic features of ecological and biogeochemical provinces, which is largely related to the bio-element status and body functions [6, 7]. Traditionally, dairy cattle breeding tends to areas of intensive agriculture. The territories of the Russian Federation with developed dairy cattle breeding are geographically located between 60 and 50 °N and are bounded by the Sayans from the east. According to the All-Russian Research Institute of Breeding (VNIIplem, Moscow Region) for 2018, 70.0% of the judged dairy livestock of the Russian Federation is concentrated on the East European Plain in the second climatic zone. The southern part of the West Siberian Plain, belonging to the third climatic zone, accounts for only 20.4% of the dairy cattle population. The potential of these territories can be used for the development of dairy cattle breeding in the Russian Federation.

In dairy cattle breeding, in terms of productivity and adaptability to industrial conditions, the world popularity belongs to Holstein cattle [8-11], despite the limited period of production [12-14]. According to VNIIplem data for 2018, in Russia, this period averages 2.0-2.3 lactations.

The spread of Holstein cattle in Russia began in the 1970s when in the advanced farms of the USSR, imported Holstein bulls were used to improve the dairy productivity of black-and-white cattle [15]. Since the 2000s, breeding stock has been actively imported to complete large high-tech agricultural holdings that have replaced inefficient farms [16]. Due to the enlargement of dairy production, the displacement of zoned breeds has accelerated due to the influx of blood, as well as the import of heifers and spermatozoa of highly productive Holstein cattle [17]. As a result, breeding enterprises in various regions of the Russian Federation began to massively abandon the bulls of zoned breeds, replacing them with imported Holstein sires from countries with developed dairy farming – Canada, the USA, France, Germany, the Netherlands; their share in the structure of the herd of bull sires of

the Russian Federation is 60% [18].

However, the exploitation of highly productive Holstein cattle (including bull sires) revealed difficulties associated with the diversity of natural and climatic conditions in Russia and an unusual fodder base for animals [19]. The Russian Federation has accumulated considerable experience in studying the processes of adaptation of productive breeding stock and bull sires in various climatic conditions [20, 21].

When transferring highly productive animals to conditions different from their typical habitat, with a sharp change in climate and time zones, the body experiences adaptive stress, expressed in a shift in metabolic processes [22–26]. During long-term breeding in the conditions of the specific climate of Sakhalin, an adaptive decrease in the productive potential of Holstein cattle was noted [27]. There is evidence that Holstein cattle adapt to hypothermia faster than to hyperthermia [28, 29]. One of the reasons for metabolic adaptation syndrome in highly productive Holstein cattle is an unbalanced diet and feed of inadequate quality [20, 30, 31].

Bull sires of the Holstein breed, obtained from highly productive mothers [32], are also sensitive to changes in environmental conditions, feed quality, farming and operation technology. With an increase in the genetic potential of productivity in cows, adaptive stress is increasingly noted, which develops into a metabolic syndrome in their calves. When acclimatizing bull sires, it is important to minimize the consequences of changes in time zones, climatic, and feeding conditions [33] to avoid prolonged adaptation and persistent decline in spermatozoa quality as a stress response [34].

The quality of the spermatozoa depends on the physiological status of the bull sire and determines its role in the reproduction of offspring, which is not limited to the actual fertilization of the cow. It was found out that the nature of the stress response in the next generation was determined by nine types of miRNAs that are present in spermatozoa [35, 36]. Changes caused by external factors are inherited from fathers through DNA methylation of spermatozoa, modification of proteins involved in DNA packaging, as well as through epigenetic modifications caused by changes in the composition and structure of RNA contained in spermatozoa, therefore, males under chronic stress produce offspring with a significantly weakened stress response in adulthood [37]. The quality of spermatozoa affects both the efficiency of female fertilization and the ability of the embryo to survive throughout pregnancy [38]. Individual differences in gene expression in bull sires and the coherence of such differences with the effectiveness of artificial insemination were determined [39]. The presence of aberrant RNA in defective spermatozoa can affect and even disrupt early embryogenesis [40]. Thus, oxidative stress of spermatozoa, neuropathy, and androgen deficiency induced by the metabolic syndrome are the most significant mechanisms for the realization of its neuroendocrine and reproductive consequences [41].

Deviations from the physiological norm in terms of protein-lipid and bioelement status reflect the functional state of organs and systems of the body, serve as an early marker of metabolic syndrome before its clinical manifestations [42]. Earlier, the authors of this paper analyzed in detail the protein-lipid [43] and trace element [44] status of bull sires in connection with sperm production and spermatozoa quality. Metabolic syndrome is accompanied by dysfunction of the endocrine system, including androgen deficiency in males [45, 46]. The authors' previous studies have described in detail the hormonal status of highly productive bulls of modern breeding [47-50]. The endocrine system is the most important regulatory link that supports homeostasis. In male mammals, the content of hormones in the

peripheral blood varies depending on the region of habitat. This indicator is influenced by many factors, in particular, the length of daylight as the most stable value for a particular latitude in the same periods of the year. The length of daylight determines the cyclicity of processes in the body, including at the level of regulatory systems [51-55]. It changes throughout the year, most clearly it is noticeable with a change in geographical latitude [56]. The direct dependence of the content of thyrotropin, prolactin, cortisol, and insulin in the blood and the reverse – for somatotropin, thyroxine, and triiodothyronine on the duration of daylight has been established [57].

The latitude factor is determined by the angle of sun rays, but their distribution depends on cloudage, surface relief, and the degree of air transparency. Radiation balance is one of the main climate-forming factors. It forms and determines the natural heat turnover, seasonal changes in air and soil temperature, the rate of evaporation, and some other important ecological and climatic characteristics of the area. Thus, geographical points located at the same latitude but different longitudes are in different climatic conditions [58].

Different studies provide data on the effects of ultraviolet radiation, the intensity of which is closely related to the latitude factor. In particular, with the displacement of the place of habitat from the equator to the poles, ultraviolet radiation decreases, a lack of vitamin D occurs in the body [59]. The role of this vitamin is not limited to the regulation of phosphorus-calcium metabolism, and the development of the metabolic syndrome and obesity is associated with a decrease in its amount [60, 61].

Constant exposure to climatic factors (along with seasonal fluctuations in the amount of hormones), daylight duration, and solar insolation can cause changes in the functional reserves of the endocrine system [62].

It should also be taken into account that even within the natural zone, the adaptability of animals of the same breed, but imported from countries with different climatic conditions, may be different and depend on many factors, including the chemical characteristics of water and soil cover [7]. In the work by Krymova *et al.* [63], a pattern of fluctuations in the concentration of trace elements with zonal gradation in tissue samples depending on the place of residence or birth of an individual was found. The content of strontium, lead, copper, and zinc in water, soil and plants increases zonally from humid (wet) to arid (dry) climatic zones. The reverse pattern is typical for manganese: the highest concentrations are observed in wet zones, the lowest in steppe dry zones. An increase in the concentration of lead and copper in geochemical provinces is associated with high man-made pollution. Mineral exchange and its significance for bull sires are described in more detail earlier in the work [44].

Thus, the quantitative and qualitative indicators of sperm are determined not only by the genotype but also by the peculiarities of the individual's metabolism and hormonal status. These features, in turn, depend on natural and climatic factors, which are determined by the geolocation of the individual. However, in the practice of animal husbandry, these circumstances, as a rule, are not considered entirely. In this work, for the first time, the authors conducted a comprehensive (by 33 parameters) study of the influence of climatic and geochemical conditions of Central Russia, Middle Urals, and Northern Kazakhstan on the enzyme status, macro- and microelement balance, protein-lipid metabolism, endogenous hormone production and the formation of the adaptive status of Holstein bull sires of foreign breeding in connection with sperm production. It is established that the negative effect of regional climatic and geochemical factors can be minimized if

all technological regulations are observed. The study results expand the understanding of the peculiarities of the metabolism of bull sires in various geoclimatic conditions of a moderate circulation zone between 55.86 and 51.18 °N, which may be of interest for the practice of breeding Holstein breed in countries with similar natural factors.

The purpose of the study is to compare the influence of climatic and geochemical conditions of Central Russia, Middle Urals, and Northern Kazakhstan on the formation of the adaptive status of imported Holstein bull sires.

Materials and methods. The study was carried out in 2017-2018 on 122 Holstein bull sires of foreign origin aged 3 to 9 years: 20 bulls — JSC Head Center for Reproduction of Farm Animals (JSC HCR, Moscow Region), 56 bulls — JSC Uralplemcenter (Sverdlovsk Region), 46 bulls — JSC RCPZh Asyl-Tulik (Akmola Region, Republic of Kazakhstan). The conditions of feeding and farming the animals corresponded to the requirements of the National Technology of freezing and using the sperm of bull sires [64].

Blood for analysis was taken from the jugular vein (September 2017) in the morning hours after taking the semen. After separating the serum from the formed elements, the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), the concentration of albumin, creatinine, urea, total bilirubin, as well as total protein and cholesterol were determined. Macronutrients (Ca, P, Mg, Fe) were determined in blood serum using a ChemWell 2902 biochemical analyzer (Awareness Technology, Inc., USA), trace elements (Cu, Zn, Se, Mn) — in whole blood using a Quantum-2A atomic absorption spectrometer (CJSC CORTEC, Russia). The concentration of endogenous hormones was measured by enzyme immunoassay in 2-fold repetition using laboratory reagents (CJSC Immunotech, Russia) for estradiol, thyroxine TT4, cortisol, and testosterone on a UNIPLAN device (AFG-01) (CJSC Pikon, Russia). Cholesterol content was evaluated on an automatic analyzer ChemWell 2902 (Awareness Technology, Inc., USA) following the manufacturer's instructions.

Spermological parameters (volume of ejaculate, ml; concentration of sperm in the ejaculate, billion/ml; the total number of sperm in the ejaculate, billion) were studied following the National Technology of freezing and using sperm of bull sires [64] and the instructions for the organization and technology of work with sires of different types of animals in breeding centers of the Republic of Kazakhstan.

Statistical processing was carried out in Microsoft Excel. The tables show the mean values (M) and standard errors of means (±SEM). Statistical significance of the differences was assessed by Student's t-criterion (at significance levels $p < 0.05$; $p < 0.01$; $p < 0.001$) and with the use of IBM® SPSS® Statistics (https://www.ibm.com/ru-ru/analytics/spss-statistics-software).

Results. The observations made over the past 20 years have made it possible to apply previously made preliminary conclusions to the analysis of the obtained data. In this work, carried out within the framework of the EurAsEC, when determining the degree of influence of climatic and geochemical factors on the adaptation and metabolism of imported Holstein bull sires, their bio-element, protein-lipid, enzymatic, and hormonal statuses were taken into account. For the survey, the most typical and well-known breeding centers were selected, located within the middle latitude (deviations of no more than 5.67 °N), but distant from each other in east longitude, which affects the climatic and geochemical conditions of geographical points where breeding centers are geographically located (all of them are in a moderate circulation zone) (Table 1).

1. Geographical, physical and climatic characteristics of the studied regions

Climate zone	Region	Geographic coordinates		ASLE, m	AAP, mm
		° N	° E		
II (East European Plain)	Moscow (JSC Head Center for Reproduction of Farm Animals)	55.42	37.55	162	668
III (West Siberian Plain)	Middle Urals (OJSC Uralplemcenter)	56.85	60.11	255	497
	Northern Kazakhstan (JSC RCPZh Asyl-Tulik)	51.18	71.44	358	308

N o t e. ASLE — above sea level elevation, AAP — average annual precipitation. The data are taken from the Scientific and Applied reference book "Climate of Russia" (http://aisori-m.meteo.ru/climsprn /) and the National Atlas of Russia (https://национальныйатлас.рф/cd2/about.html) .

The Moscow Province is located in the zone of domination of the continental climate of temperate latitudes, characterized by relatively mild winters with rare thaws and warm relatively humid summers, belongs to the zone of sufficient moisture. In the Moscow Region, observations of recent years show an increase in average annual and seasonal air temperatures, increased aridity in summer, and warming in winter [65]. The main climate-forming factor in the Middle Urals is its geographical location. The Ural Mountains are divided by natural synoptic areas; zonal patterns of distribution of various meteorological elements, especially air temperature and precipitation, are violated here, which serves as the basis for the allocation of this territory into a separate climatic region with a sharp change in weather. The climate is close to moderately cold with a significant amount of precipitation. As a result of the rhythmic features of the climate, winter and spring increases in air temperature have been typical for recent years [66]. The territory of Northern Kazakhstan belongs to the continental steppe of the West Siberian climatic region with a flat relief and a sharply continental climate with a shortage of moisture [67]. Despite the low agro-climatic potential, characterized by a short growing season and expressed seasonality of agriculture [68], crop production remains the main specialization of the region, but prospects for the development of milk and cattle meat production are being considered [69]

The biochemical analysis showed (Table 2) that the average values of concentrations of macronutrients in the blood plasma of the examined animals in all regions were mainly within the reference values [70] and had no significant differences in Ca, P, Ca:P, Mg. Macronutrients, in particular calcium, phosphorus, and magnesium, enter the animal's body with the main diet, and their blood concentration in ruminants depends on the initial quality of feed, vitamin D availability, and solar insolation [71]. The obtained results (see Table 2) indicate that the supply of bull sires with the listed macronutrients at all enterprises corresponds to regulatory indicators with a certain zonal variability.

2. The blood content of macroelements in the Holstein bull sires of foreign breeding in regions with different climatic and geochemical conditions (M±SEM, 2017)

Element	Region			Reference values
	Moscow (JSC HCR) (n = 20)	Middle Urals (OJSC Uralplemcenter) (n = 56)	Northern Kazakhstan (JSC RCPZh Asyl-Tulik) (n = 46)	
Ca, mmol/l	2.43±1.17	2.34±0.14	2.53±0.23	2.06-3.16
P, mmol/l	1.47±0.21	2.01±0.36	1.98±0.33	1.13-2.91
Ca:P	1.65±0.26	1.20±0.23	1.31±0.23	0.82-2.39
Mg, mmol/l	0.79±0.12	0.92±0.15	0.98±0.35	0.75-1.34
Fe, μmol/l	23.82±6.18	40.32±7.30	30.74±6.97	12.90-37.10
Chlorides, mmol/l	100.45±2.86	97.61±4.28	95.69±9.28	90-108

N o t e. JSC HCR stands for JSC Head Center for Reproduction of Farm Animals.

When harvesting coarse fodder in the Southern Moscow Region, it is necessary to take into account the peculiarity of loamy soils on low-carbonate covering

deposits of the Podolsk-Kolomna high plains with low natural fertility and insufficient content of available magnesium (less than 120 mg/kg). Acidic and strongly acidic sod-podzolic soils are characterized by the leaching of magnesium, which, with sufficient moisture, moves to the lower horizons of the soil, creating a deficit in the root zone of plants [72]. Soil deficiency of Mg (0.79±0.12 mmol/l) indirectly affected the availability of bull sires at the lower limit of the norm for this element. In the Middle Urals, the main part of agricultural land (69.9%) falls on sod-podzolic soils formed on rocks rich in calcium and magnesium, which differ from the same soils of Central Russia by an increased content of humus, increased capacity of cation exchange, and the entire absorbing complex [73]. As can be seen from Table 2, Mg content in the blood serum of bull sires from JSC Ural-plemcenter is close to the average normative indicators and is 0.92±0.15 mmol/l with values for phosphorus and calcium 2.01± 0.36 and 2.34±0.14 mmol/l, respectively. Fodder crops in the North Kazakhstan region can accumulate significant amounts of Ca and Mg carbonates from upper arable horizons with low mobility of these compounds along the soil profile since they are cultivated in moderately arid areas on chernozems and dark chestnut soils [74]. Bull sires in this region are well provided with Mg due to the basic diet (the concentration of Mg and Ca in the blood serum was 0.98±0.35 and 2.53±0.23 mmol/l, respectively) with a relatively reduced supply, in particular, with phosphorus (1.98±0.33 mmol/l).

Even though the concentration of available iron in soil solutions depends on the composition of the parent rock and increases with increasing acidity of the soil [75], the bull sires in all the studied regions did not lack Fe. The concentration of iron in the blood of bulls in the Moscow Region did not exceed the norm, in Northern Kazakhstan corresponded to its upper limit, and in the Middle Urals, exceeded the norm. In the technogenic territories of the Urals and Northern Kazakhstan, Fe as a heavy metal accumulates in the soil-plants-animals system on dark gray forest, chernozems, and dark chestnut clay-loamy soils with an alkaline reaction of the medium [76]. When a large amount of iron is supplied with feed and water, ferritin accumulates in the villi of the mucous membrane and the so-called mucosal blockade develops, iron absorption stops [77]. In the blood serum of bull sires from the Moscow Region, the concentration of iron (23.82 ± 6.18 mmol/l) did not exceed the regulatory limits, despite the contamination of water with iron (its concentration in some sources can reach 10 mg/l) compared with water of deeper occurrence [78].

Thus, no significant differences have been revealed in the availability of macronutrients for bull sires, despite the diversity of geochemical zones and agroclimatic conditions in the regions where the surveyed breeding centers are located.

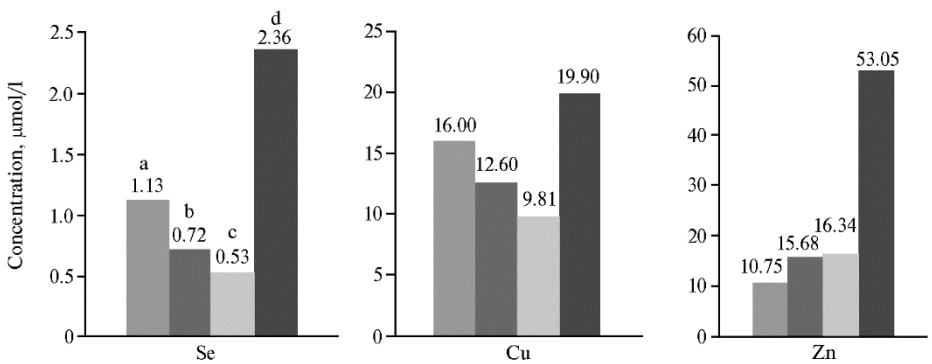

Fig. 1. Blood concentration of trace elements in the Holstein bull sires of foreign breeding in regions with different climatic and geochemical conditions (2017): a — Moscow Province (JSC Head Center for Reproduction of Farm Animals, n = 20), b — Northern Kazakhstan (JSC RCPZh Asyl-Tulik, n = 46), c — lower limit of the norm, d — upper limit of the norm.

Biogenic trace elements are necessary for the formation of full-fledged spermatozoa and to increase the efficiency of the use of bull sires. Trace elements are part of enzymes, vitamins, hormones, respiratory pigments, cell structures and significantly affect the qualitative and quantitative characteristics of ejaculate and sperm fertility [44, 79–82]. The availability of microelements for animals (Fig. 1) depends on the feed set of the main diet and the geochemical conditions of the area.

The change in the concentration of trace elements in the blood of Holstein bull sires of foreign breeding is demonstrated in the example of the Moscow Region and Northern Kazakhstan. For the analyzed regions, the average values for Se and Cu in the blood serum of bull sires were in the range of reference values, exceeding the minimum threshold concentration (see Fig. 1), for Zn – were below the permissible minimum. It is known that the amount of zinc in soils increases from tundra landscapes to steppe arid soils [63]. The same pattern can be traced by the concentration of this element in the blood serum of bull sires. Thus, in the Moscow Region (JSC HCR) at the border of the distribution of sod-podzolic and gray forest soils, the concentration of zinc in the blood serum of bulls was 65.2% of the threshold reference value that ensures normal metabolism. In the dry steppe zone on dark chestnut soils (Northern Kazakhstan, JSC RCPZh Asyl-Tulik) zinc content in the blood of bull sires was 95.7% of the minimum threshold value, which is significantly higher than in animals in the central part of the East European (Russian) plain (JSC HCR). Copper concentration in the blood of bulls, while remaining within the reference values, was 21% higher in the Moscow Region than in Northern Kazakhstan. It is shown that for copper, the distribution in soils is similar to that for zinc, strontium, and lead [63]. However, due to industrial pressure, Cu content in the soil, water, and plants may increase, the consequences of which was noted in the south of Moscow suburbs. Selenium belongs to trace elements, its content in soils is mainly due to the parent rock and climatic features of the region. The accumulation of Se in plants is determined by its quantity and forms in the soil, its type, acidity, precipitation, and ambient temperature, the stage of growth of the plant itself [83]. Currently, there are no systematic data on the content of selenium in various types of soils [84]. The concentration of selenium in the blood serum of bulls in the south of the Moscow Region was 63.7% higher than in Northern Kazakhstan, although, according to biogeochemical surveys, the soils of the Moscow Region can be attributed to selenium deficiency [85].

Trace elements as cofactors are involved in all vital metabolic processes. The activity of many enzymes is manifested only in the presence of metal ions (Zn^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, K^{+}, Na^{+}) [86].

3. Cellular enzyme activity (IU/л, *M*±SEM) **in blood of the Holstein bull sires of foreign breeding in regions with different climatic and geochemical conditions** (*M*±SEM, 2017)

Enzyme	Norm	Region		
		Moscow (JSC HCR) (n = 20)	Middle Urals (OJSC Uralplemcenter) (n = 56)	Northern Kazakhstan (JSC RCPZh Asyl-Tulik) (n = 46)
Alanine aminotransferase (ALT)	10-36	40.15±15.34	68.45±16.38	37.47±11.96
Aspartate aminotransferase (AST)	41-107	132.55±74.61	104.00±15.95	111.35±14.09
AST ALT	1.3-1.5	3.03	1.51	2.97
Alkaline phosphatase	31-163	72.7±75.66	125.25±45.43	232.02±119.77

The presence of cellular enzymes — AST, ALT, and alkaline phosphatase in the blood above the norm is a sign of cytolysis and a marker of some diseases [87-89]. As a rule, an increase in activity for AST often coincides with a decrease for ALT and vice versa. ALT is more specific for the diagnosis of hepatic

pathologies, an increase in its activity in plasma indicates more damage to cells than a violation of liver function in general. In the current survey, the highest ALT index — 68.45±16.38 IU/l, which is 2 times higher than the regulatory activity, was found in bull sires in the Middle Urals (JSC Ural-plemcenter), in animals from the Moscow Region (JSC HCR) and Northern Kazakhstan (JSC RCPZh Asyl-Tulik), ALT activity (40.15±15.34 and 37.47±11.96 IU/l, respectively) was at the upper limit of the norm (Table 3). According to AST, indicators corresponding to the upper limit of the norm were recorded in bulls in JSC Ural-plemcenter, excess of the norm in JSC RCPZh Asyl-Tulik and JSC HCR (see Table 3). The value of the De Ritis ratio in these animals was 2 times higher than the standard value (see Table 3), which indicates the predominance of the tricarboxylic acid cycle in the metabolism [90, 91]. The activity of alkaline phosphatase in bulls in Northern Kazakhstan (JSC RCPZh Asyl-Tulik) exceeded the upper limit of reference values by 30%, which, with normal calcium-phosphorus metabolism, serves as a marker of high functional loads on the liver and heart and the active work of young bone cells [92, 93].

An increase in the content of total and direct bilirubin, high activity of AST and ALT can serve as a diagnostic test of liver diseases of alimentary etiology [94] and characterize protein metabolism [81]. At the same time, Shkuratova et al. [95] believe that a high level of inorganic phosphorus and alkaline phosphatase can be attributed to the peculiarities of the metabolic profile of bull sires. In addition, the formation of high-quality spermatozoa capable of enduring a "cold shock" is impossible without the inclusion of animal feed rich in protein and phospholipids in the diet of bulls [96, 97]. The scarcity of protein in the diet of bull sires leads to hormonal imbalance, an increase in secondary immunodeficiency, and a decrease in the quantitative and qualitative characteristics of the ejaculate [43].

4. Parameters of protein-lipid metabolism in the Holstein bull sires of foreign breeding in regions with different climatic and geochemical conditions (*M*±SEM, 2017)

Parameter	Region			Reference values
	Moscow (JSC HCR) (n = 20)	Middle Urals (OJSC Uralplemcenter) (n = 56)	Northern Kazakhstan (JSC RCPZh Asyl-Tulik) (n = 46)	
Total protein, g/l	90.44±6.14	84.83±4.05	88.81±6.72	70-92
Albumin (A), g/l	32.40±2.77	33.20±1.56	29.22±3.40	25-36
Globulin (G), g/l	59.08±6.13	51.18±4.45	59.60±7.55	40-63
A/G	0.55	0.64	0.50	0.4-0.9
Urea, mmol/l	7.57±2.82	3.90±1.39	3.40±0.65	2.4-7.5
Creatinine, μmol/л	147.45±37.94	121.04±30.90	146.66±34.01	62-163
Bilirubin, μmol/l	2.26±0.51	2.36±1.30	9.15±3.42	1.16-8.15
Triglycerides, mmol/l	0.55±0.07	0.14±0.09	0.35±0.11	0.09-0.37
Cholesterol, mmol/l	2.37±0.49	3.46±0.61	2.93±0.50	2.1-8.2

In bull sires, the main indicators of protein-lipid metabolism were mainly within the reference values (Table 4). Some differences are caused by small feeding errors. The content of total protein and protein fractions in the blood of animals was sufficient to maintain normal metabolism and the formation of full-fledged ejaculates. The concentration of urea and creatinine in the blood serum at the upper limit of the norm (7.57±2.82 mmol/l and 147.45±37.94 mmol/l respectively) was a consequence of the fact that bulls from the Moscow region received an excessive amount of protein with feed. In males, an increase in the concentration of triglycerides is due to the active secretion of testosterone [98], but their excess can lead to metabolic syndrome [99]. In bull sires from the Moscow Region (JSC HCR), this indicator was 0.55±0.07 mmol/l, which exceeded the upper limits of the norm by 32%. Bulls from Northern Kazakhstan (JSC RCPZh Asyl-Tulik) have a high bilirubin index (9.15±3.42 mmol/l) which in combination with

an increased iron content (30.74±6.97 mmol/l) (see Table 2) may indicate additional hemoglobin release with excessive destruction of red blood cells. As a consequence, iron overload syndrome [100-102] may form, which occurs when erythropoiesis is impaired, in the case of liver diseases, hereditary defects and serves as the main factor predisposing to the accumulation of iron in liver cells.

Optimal indicators of protein-lipid metabolism were typical for bull sires in the Ural region, which indicates the balance of their diet.

Cholesterol concentration in the blood serum of bull sires in all studied regions was at the lower limit of the reference values and amounted to 2.37−3.46 mmol/l. Since cholesterol is a precursor of steroid hormones (including sex hormones), its low level may be a signal of hormonal status disorders. It is known that hormones of the pituitary-thyroid gland and pituitary-adrenal cortex systems represent a key link in the hormonal regulation of adaptive metabolic processes [102, 103]. It has been established that the aromatase enzyme is present in adipose tissue, under the influence of which androgens are converted into estrogens [104]. Metabolic changes associated with the size of fat cells serve as a trigger for a decrease in testosterone levels [105, 106].

Tables 5 and 6 show the average and maximum concentrations of endogenous hormones characterizing reproductive and adaptive function in Holstein bull sires kept in two different geographical locations in Russia and Northern Kazakhstan.

5. Blood concentration of endogenous hormones in the Holstein bull sires of foreign breeding in regions with different climatic and geochemical conditions (*M*±SEM, 2017)

Parameter	Region			Average
	Moscow (JSC HCR) (n = 19)	Middle Urals (OJSC Uralplemcenter (n = 9)	Northern Kazakhstan (JSC RCPZh Asyl-Tulik) (n = 17)	
Testosterone, nmol/l	39.17±5.06	50.36±5.80	52.79±4.14	47.10±2.39
Cortisol, nmol/l	226.75±45.62	138.81±21.48	190.5±50.31	195.48±27.35
Thyroxine, nmol/l	91.13±3.35	66.65±3.52	95.39±1.86	81.95±2.26
Estradiol, nmol/l	0.197±0.02	0.276±0.04	0.234±0.02	0.339±0.02

6. Variability of blood concentration of endogenous hormones in the Holstein bull sires of foreign breeding in regions with different climatic and geochemical conditions (min-max, 2017)

Parameter	Region			Over the total sample (n = 73)
	Moscow (JSC HCR) (n = 19)	Middle Urals (OJSC Uralplemcenter (n = 9)	Northern Kazakhstan (JSC RCPZh Asyl-Tulik) (n = 17)	
Testosterone, nmol/l	10.46-81.14	28.12-79.12	26.27-74.45	10.46-81.14
Cortisol, nmol/l	21.27-728.21	43.69-238.41	25.60-725.32	21.27-728.21
Thyroxine, nmol/l	53.25-109.43	50.20-81.20	85.63-109.05	32.96-126.92
Estradiol, nmol/l	0.093-0.350	0.100-0.426	0.073-0.374	0.073-0.909

Despite the slight difference in latitude, Moscow (JSC HCR), Yekaterinburg (JSC Ural-plemcenter), and Nursultan (JSC RCPZh Asyl-Tulik) are located in different climatic zones and differ in average annual temperatures and solar radiation (Fig. 2), under the influence of which up to 70% of vitamin D affecting testosterone production [59] is synthesized in the body. Thus, in Northern Kazakhstan, the average annual cross-section of solar radiation exceeded that in the Southern Moscow Region by 24.0%, and the testosterone level in bull sires — by 25.8% (see Table 5) [107]. In males, the participation of testosterone at the molecular level in maintaining bone mineral density (BMD) has been established [108].

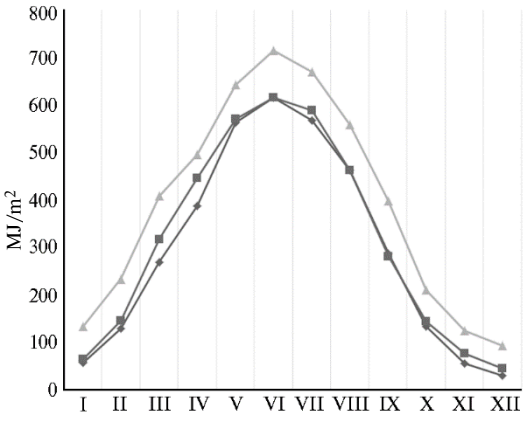

Fig. 2. The power of total (direct and diffuse) solar radiation on the horizontal surface of the Earth by months during the year under actual cloud conditions in Moscow (♦), Ekaterinburg (□) and Nursultan (▲) (http://meteo.ru/pogoda-i-klimat/197-nauchno-prikladnoj-spravochnik/).

Testosterone production, as noted, is also characterized by circadian rhythmicity [109]. The highest testosterone peak occurs between 6^{00}-8^{00}, the anti-peak is from 21^{00} to 23^{00}. This physiological cycle is the basis of the technological regime for sperm production. The annual rhythm of the maximum testosterone level falls in August-September with a decrease in the winter months and is also associated with the latitudinal duration of daylight and solar insolation [110, 111]. In the authors' previous studies [47], in the blood of bull sires from the Ural region in December, an average testosterone level of 20.5 nmol/l was detected with variability from 4.8 to 50.6 nmol/l by groups, and low indicators (up to 10 nmol/l) were recorded in 50% of the animals in the herd. In this paper (see Table 5) in September, the average value in bulls from the same region was 50.36±5.80 nmol/l. The obtained results correspond to the annual cycle of changes in the concentration of testosterone. Among bulls in the studied territories, the minimum average level of this hormone (39.17±5.06 nmol/l) was observed in producers from the Moscow region, the maximum (52.79±4.14 nmol/l) — in animals in Northern Kazakhstan (the difference is statistically insignificant).

Cortisol is also characterized by a circadian rhythm with an increase in the level in the morning [112-114]. In the present work, blood was taken for examination in the morning. High (226.75±45.62 nmol/l) cortisol content was registered in bull sires from the Moscow region, in animals in Northern Kazakhstan and the Middle Urals, the average values were slightly lower (by 16 and 39%, respectively, the difference is not statistically significant) (see Table 5). An increase in cortisol concentration is associated with hypertriglyceridemia, increased protein, fat, and carbohydrate metabolism [115, 116]. In stressful situations, the hormone promotes the rapid release of glucose. Excess glucose in the body leads to some metabolic complications: increased blood osmolarity, increased production of carbon dioxide and lactate, as well as fatty liver infiltration due to the conversion of excess glucose into fatty acids [117]. Significant variability of cortisol concentration in the blood of bulls (see Table 6) shows that in the Moscow region and Northern Kazakhstan in the studied groups, there are animals in a state of metabolic stress. Previously, the authors of this paper showed that after a doublet cage, the cortisol level in bull sires significantly increases over the next two days, especially in adult animals [47].

The effect of cortisol on the strengthening or weakening of one or another enzymatic activity complements thyroxine since the presence of another hormone is necessary for the manifestation of the action of one hormone [23]. The average thyroxine content in the examined bulls was within the physiologically permissible norm (51-141 nmol/l) (see Table 5): in the Middle Urals — 66.65±3.52 nmol/l, in the Moscow region and Northern Kazakhstan — slightly higher (91.13±3.35 and 95.39±1.86 nmol/l, respectively). This gives reason to believe that the thyroid gland in these males functions normally and the hormone (thyroxine) secreted by

it adequately affects physiological processes, even though the surveyed farms are located in territories that are deficient and moderately deficient in iodine [118].

To identify significant differences in the hormonal background between animals from different regions, the authors conducted pairwise comparisons of indicators using the Mann-Whitney U-test method. The analysis showed a highly significant influence of climatic and geochemical conditions (territorial factor) on the thyroxine content ($p < 0.001$) in the blood serum of the examined bull sires in the compared regional pairs of enterprises: the Southern Moscow suburbs–the Middle Urals, the Middle Urals–Northern Kazakhstan (no significant differences were found in other indicators).

The previous work [44] found that the content of endogenous estradiol in the blood of bulls depended on the time of year ($p < 0.001$). In spring, the average concentration of estradiol was minimal, in autumn a significant increase in the amount of hormone ($p < 0.001$) was observed in 94% of animals. In this study, estradiol content varied (see Table 5) — from 0.197±0.02 nmol/l in the Moscow region to 0.234±0.02 and 0.276±0.04 nmol/l, respectively, in Northern Kazakhstan and the Middle Urals, which is within the physiological norm (0.2-0.4 nmol/l).

The results of the analysis of the state of bull sires according to the characteristics of native sperm are presented in Table 7 (the qualitative characteristics of cryopreserved sperm are not given since they met GOST requirements).

7. Characteristics of native semen of the Holstein bull sires of foreign breeding in regions with different climatic and geochemical conditions (*M*±SEM, 2017)

Parameter	Region		
	Moscow (JSC HCR) ($n = 20$)	Middle Urals (OJSC Uralplemcenter) ($n = 56$)	Northern Kazakhstan (JSC RCPZh Asyl-Tulik) ($n = 46$)
Ejaculate volume, ml	4.87±1.8	3.72±0.08	4.4±0.13
Concentration of spermatozoa, $\times 10^9$/ml	1.21±0.20	1.52±0.02	1.29±0.01
Total number of spermatozoa per ejaculate, $\times 10^9$	6.00±0.64	5.60±0.16	5.32±0.27

The difference between the indicators shown in Table 7 is not statistically significant.

Thus, the absence of a significant difference in the main indicators characterizing metabolism between animals from different regions that received a balanced diet taking into account the geochemical conditions of the terrain with a similar operating mode according to the regulations provided for by the technology of farming and use indicates minimization of the influence of climatic and geochemical factors and sufficient adaptive ability of Holstein bulls in a moderate circulation zone between 55.86° and 51.18°N. This is evidenced by the comprehensive comparison of the state of protein-lipid, macro- and microelement, enzymatic and hormonal status carried out by the authors for the first time, taking into account the reproductive ability of Holstein bull sires farmed in various geoclimatic conditions.

The results obtained give grounds to conclude that protein-lipid and macronutrient metabolism in producers under the conditions of typical feeding technology and maintenance has no significant geographical differences and mostly depends on the quality of the main feed of the diet.

The deficiency of essential trace elements, in particular Zn, is typical for most of the territory of Russia. Mammals can have Zn from food only. The average daily requirement for a mature bull sire is 300-600 mg, depending on body weight

(norms and diets). During the period of intense sexual activity, metabolism of bull sires increases significantly, therefore, the consumption and content of zinc in the body increases [20, 44]. Zinc is mainly secreted by the prostate gland, it is contained in a significant amount in maturing spermatozoa, its concentration correlates with oxygen consumption and the stability of nuclear chromatin [119, 120]. Zinc content in the male reproductive system significantly exceeds that in other organs and tissues; its deficiency can cause disorders of spermatogenesis [121]. Even though iron is irreplaceable in the processes of hematopoiesis, respiration, and cellular metabolism, its excess can initiate lipid peroxidation, toxic damage to proteins and nucleic acids [122], which negatively affects the quality of native sperm [123]. To determine the need of bull sires for essential elements, they should be monitored in the blood serum, taking into account the deficiency or excess intake into the body according to the results of chemical analysis of water, soils, and basic feeds, compensated with targeted premixes. To provide animals with water in regions with high levels of soil iron, it is necessary to use deep-lying wells and water de-ironing stations.

The identified features should be taken into account when farming and using breeding bull sires in various geochemical provinces of Russia, Kazakhstan, and other countries that are members of the EurAsEC. The composition of the diets should be developed taking into account not only the geochemical and climatic conditions of the place of farming but also possible deficits of intrauterine development and postnatal nursery of bull sires.

Taking into account modern studies of neuroendocrine regulation of male sexual development [124, 125], in conditions of large-scale breeding for high productivity with an index evaluation of ancestors, it is necessary to pay attention to prenatal and early postnatal development, which determines the future reproductive health of the animal. A bull with a high genomic prognosis, but sperm not suitable for cryopreservation [126], has no breeding value.

Comparative studies have shown that hormones of the hypothalamic-pituitary-gonadal and thyroid axis of endocrine regulation in bull sires kept at mid-latitude in various climatic and geochemical conditions serve as a key factor in long-term adaptation to external conditions [23].

In the authors' opinion, one of the reasons for the decrease in testosterone status in bull sires from the Moscow region compared to animals in the Middle Urals and Northern Kazakhstan is solar insolation (see Fig. 2). The deficiency of solar insolation and vitamin D synthesis, on the one hand, and the inevitable influence of technological effects, on the other hand, lead to the formation of and increase in metabolic stress [59, 127]. On-duty night lighting in livestock premises disrupts circadian rhythms. It has been shown that the blue component of the spectrum, including artificial lighting, suppresses the production of melatonin hormone significantly, the secretion of which affects the quality of animal sleep [128]. In turn, melatonin indirectly has a regulating effect on the functions of the testicles [129, 130]. Its excess, as well as its deficiency, leads to an imbalance in the secretion of steroid hormones, which is subject not only to circadian but also to infradian rhythms [130]. A complex relationship between circadian rhythm failure and metabolism has been proven, a change in the sleep-wake cycle can lead to the formation of metabolic syndrome and cardiovascular diseases [131]. During sexual activity, testosterone and cortisol levels increase simultaneously in males, which is presumably associated with rivalry, territorial defense, as well as courtship and mating behavior [132].

The authors did not note a significant influence of various climatic and geochemical conditions of the regions on the main indicators of sperm production

in bull sires. However, constant exposure to such factors in combination with seasonal fluctuations in the amount of hormones, the influence of daylight hours, and solar insolation can affect the functional reserves of the endocrine system [133, 134]. In this case, the hormonal profile of bull sires can serve as a marker of body plasticity.

So, with the stabilization of farming conditions and strict rationing of the needs of bull sires in nutrients and minerals, as well as the regulation of the light regime (morning-daytime solar insolation, darkness at night), it is possible to minimize the negative impact of climatic and geochemical factors on the body of breeding animals. The obtained data on the total number of spermatozoa in the ejaculate indicate the adaptability of Holstein bull sires in various climatic conditions with the used technologies.

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SEXUAL ACTIVITY AND SPERM PRODUCTION OF CHAROLAIS AND ILE-DE-FRANCE RAMS IN DIFFERENT SEASONS OF THE YEAR

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Abstract

Sheep (*Ovis aries*) are polyestrous animals with a distinct breeding season. Long artificial selection has minimized the impact of seasonal environmental factors on the sheep reproductive function. The seasonality of reproduction is practically not characteristic of sheep bred near the equator but is well pronounced in the middle and high latitudes. The basic strategy of sheep breeding in Russia is production of young mutton and lamb. As Russia does not have its own gene pool of highly productive specialized meat breeds, the use of the best alien breeds is inevitable. However, experimental data on the reproductive performance of alien meat rams under the conditions of the Central zone of the Stavropol territory is practically absent. Our study aimed to examine sexual activity and sperm production in meat sheep in different seasons in the Central zone of the Stavropol territory. During the breeding (autumn) and non-breeding (winter, spring, and summer) seasons, sperm samples were taken from Charolais rams ($n = 8$) and Ile-de-France rams ($n = 5$) using an artificial vagina. The rams of both breeds showed significant seasonal variation in their sexual activity and sperm production. In winter, the sexual activity of the rams decreased and the time required for the expression of the entire complex of sexual reflexes increased. Compared to autumn, the time of receiving ejaculate from Charolais sheep increased 1.70 times in winter, 2.56 times in spring, and 2.62 times in summer. The Ile-de-France rams showed similar dynamics, but a decrease in sexual activity was less pronounced compared to the Charolais rams. Ile-de-France rams took longer to ejaculate (1.09 times in winter, 1.62 times in spring, and 1.78 times in summer compared to breeding season). Sperm motility in Charolais rams varied from 9.1 points in breeding season to 7.4 points in spring. In winter and summer, the motility was 8.5 and 8.1 points, respectively. The volume of ejaculate and sperm cell concentrations showed similar patterns, being the highest in autumn, the lowest in spring, and intermediate in summer and winter. In autumn, Ile-de-France rams produced the largest volume of ejaculate with maximum concentration and the highest motility of sperm cells, i.e., 1.15 ml, 3.75 billion/ml, 8.57 points vs. 0.98 ml, 3.38 billion/ml, 7.95 points in winter, 0.88 ml, 2.85 billion/ml, 7.55 points in spring, and 0.96 ml, 3.15 billion/ml, 8.19 points in summer. Microstructural analysis detected sperm cells with an intact acrosome ranging from 65 % in summer in Charolais rams to 82 % in autumn in Ile-de-France rams. Therefore, despite significant seasonal variations, Charolais and Ile-de-France rams can produce high-quality semen both in breeding and non-breeding seasons under the climatic conditions of the Central zone of the Stavropol territory.

Keywords: rams, breeding and non-breeding season, sexual activity, quality of semen

Sheep breeding is one of the most important branches of the agricultural sector of the Russian economy. In the pre-reform period (until 1990), industry profitability in the country was 30-50% and was ensured by the state policy regarding the production of fine wool. However, termination of state support in market conditions led to the unprofitability of this production. Since 2001, it has become clear that the future of the industry depends on the development of meat

and partly dairy sheep breeding. In sheep-breeding countries, due to long-term market traditions, the breeding area of existing meat breeds of sheep has rapidly expanded over the past decades, and new breeds of meat and combined productivity have also been bred [1].

The main strategy for the development of sheep breeding in Russia in the medium term will also be based on the economic feasibility of the production of young mutton and lamb. However, currently, Russia does not have a gene pool of highly productive specialized meat breeds that fully meet such modern requirements as polycyclicity, multiple fertility, earliness, high growth energy, and excellent meat qualities. In this regard, it is inevitable to use the best meat breeds of foreign breeding for breeding purposes and create arrays of meat sheep in the regions of the country by industrial crossing with sheep of Russian breeds. The effectiveness of wide replication of the gene pool of sheep of the improving breed for a short time will largely depend on the completeness of the reproductive function of final rams, their high sexual activity, and sperm production. The optimal methodology will be an algorithm that provides for the maximum use of rams in the autumn sexual season for insemination of sheep with freshly obtained and cooled transported sperm and the accumulation of sperm in frozen form in other seasons of the year [2-4].

It is known that long-term artificial selection helped to minimize the impact of the season on the reproductive activity of sheep. The seasonality of reproduction is poorly expressed or does not manifest itself at all in breeds bred near the equator, but is common in middle and high latitudes. The breeding season of females is significantly shorter than that of males and is limited to the period from late summer to January. Rams also show seasonal fluctuations in sexual activity and the quality of sperm production, but their physiological volatility is less expressed. Many factors influence the libido and sperm characteristics of rams: breed, age, conditions of managing and feeding, environmental conditions (photoperiod, insolation, temperature, atmospheric pressure, humidity, etc.) [5–8]. In addition, reproductive management, the professionalism of the technician for obtaining sperm, the method and frequency of sperm selection are of some importance [9-11].

Sheep breeds are characterized by different photoreactivity [12, 13]. Interesting data on seasonality and circadian variations of sexual behavior of rams in tropical latitudes have been obtained [14]. Thus, reducing the length of daylight increases the rate of spermatogenesis due to the production of melatonin, which stimulates the release of gonadotropin-implementing hormones [15, 16].

Seasonal fluctuations in sperm production (ejaculate volume and sperm concentration), testosterone content in the blood, and testicular diameter were studied in dry and rainy seasons on 10 sheep of the Ouled Djellal breed in the Shlef area [17]. It was found that the diameter of the testicles, ejaculate volume, and sperm concentration change in the same way as the level of testosterone in the blood, the amount of which is characterized by high values in the autumn-winter period and low in the spring-summer season. These results reflect the presence of a special seasonal rhythm of functioning of the pituitary gland of rams of the studied breed.

Bravo et al. [18] revealed the presence of sperm subpopulations in Ile-de-France rams with specific movement characteristics in freshly obtained ejaculates and determined changes in the structure of motile sperm subpopulations in different seasons of the year. Malejane et al. [19] report on the seasonal fertility of rams of the most common breeds in South Africa (including the Dorper breed), but information on this issue is very limited.

In Russia, there is very little experimental data on the implementation of

the reproductive function in meat rams of foreign breeding. Moreover, the fragmentary nature and locality of the experiments raise doubts about their reliability and exclude the extrapolation of such results on a larger scale.

In this work, in the conditions of the central zone of the Stavropol Territory, the range of variability of sexual activity and sperm production in Charolais and Ile-de-France meat rams, depending on the season of the year, was established for the first time. The greatest reproductive activity was revealed in the autumn period.

The goal of the work was to study seasonal fluctuations in sexual activity and parameters of sperm production in rams (*Ovis aries*) of meat breeds of foreign selection of Charolais and Ile-de-France in the conditions of the central zone of the Stavropol Territory.

Materials and methods. The research was carried out from October 2019 to September 2020 at the experimental station of the All-Russian Research Institute of Sheep and Goat Breeding – a branch of the North Caucasus Federal Research Agrarian Center on breeding rams of meat breeds of foreign selection Charolais ($n = 8$) and Ile-de-France ($n = 5$).

The animals were imported in October 2019 from the UK. After a 30-day quarantine, their sexual activity and sperm production were investigated comprehensively. All rams were provided with the same conditions of management and care. Feeding was carried out according to the rations of the breeding period.

Sperm was obtained using an artificial vagina. The sexual activity shown by the same rams was taken into account by the time spent on the allocation of one ejaculate, i.e., by the duration of the manifestation of the complex of all sexual reflexes – from the entry of the ram into the exercising lot with the female fixed in the machine to the ejaculation reflex.

Each ejaculate was evaluated by volume, motility, and concentration [20]. Ejaculate volume was determined using a graduated pipette (up to 0.1 ml) or in a graduated single-wall ejaculator. Sperm motility was established under a Nikon Ts2R microscope (Nikon, Japan) at a magnification of ×200-×400 in several fields of view in a drop of sperm. The motility index was evaluated on a 10-point scale. The concentration of sperms was determined on an Accucell photometer (IMV Technologies, France) or calculated in a Goryaev counting chamber under a Nikon Ts2R microscope at a magnification of ×400, for which the sperm was prediluted 200 times with a 3% sodium chloride solution in an erythrocyte blood-count mixing pipette (GEN LLC, Russia).

The length of daylight hours (h) was calculated as the difference between the official sunrise and sunset times on each day of the experiment. Precipitation (mm) and air temperature fluctuations (°C) were registered daily. For each parameter, the average values for the season of the year were calculated.

Statistical processing was carried out in Microsoft Excel 2016. The results were expressed as means and standard deviations ($M \pm SD$). To determine the statistical significance of the differences in the mean values, Student's t-criterion was used under three conditions of p probability and different numbers of degrees of freedom.

Results. A significant part of the territory of the experimental station where the experiments were conducted is located within the Stavropol upland at an altitude of 350-600 m above sea level (Stavropol Territory, Shpakovsky District). According to the long-term data of the Stavropol meteorological station, the area belongs to the zone of moderate humidification. An important feature of the climate is a rapid increase in temperature in spring and a slow decrease in autumn. From mid-April, a frost-free period is established, which lasts 175-180 days. Summer is quite hot, with an average monthly temperature of +20...+25 °C.

Atmospheric droughts are often repeated, and during the summer period, their duration is about 60 days. Only three months of the year have an average air temperature below 0 °C, but even during this period, thaws are often observed. Snow cover appears in late November—early December. Snowfall usually occurs in late March—early April, the period with snow cover and air temperature below 0 °C counts 90-95 days. In some years, no snow cover is observed even in winter. The annual amount of precipitation is 550-600 mm, mainly in summer. A significant part of precipitation quickly evaporates due to high temperatures and exposure to dry winds.

Table 1 shows the average parameters of climatic data for the seasons of the year for the experimental period.

1. Climatic data in experiments on seasonal fluctuations in sexual activity and sperm production of Charolais and Ile-de-France rams (*Ovis aries*) (Stavropol Territory, Shpakovsky District, September 2019—August 2020)

Season	Air temperature, °C		Precipitations, mm	Average day length, hours
	min	max		
Осень	–3	+29	97	10.8
Зима	–12	+18	109	9.2
Весна	–9	+22	77	13.8
Лето	+15	+35	46	14.7

Ile de France is one of the most popular French-bred meat breeds in the world, leading in improving both the maternal characters of sheep and the paternal characters of rams. It is positioned as a polycyclic breed; therefore, it is widely used for intensive off-season production of lambs and pasture lambs from early spring lambing. The live weight of rams used in the experiment ranged from 100 to 125 kg. They were characterized by well-developed meat forms: they had a broad head, a short, wide neck, rounded ribs, well-muscled thighs.

Charolais is a meat-wool breed of French breeding. Experimental rams had a live weight from 105 to 140 kg and expressed meat forms: a wide head, a short, muscled neck, a barrel-shaped body with rounded ribs, perfectly muscled thighs.

Numerous observations and experiments conducted in the middle latitudes have proved that gametogenesis (ovogenesis) in sheep practically stops in the period from January to July. Accordingly, animals lack unconditional sexual reflexes (chase, estrus, ovulation). This season is commonly called asexual, or anestrous. With all conventionality of this name, it determines the physiological state of sheep accurately [21].

On the contrary, in rams of most breeds, spermiogenesis and secretion of male sex hormone are carried out continuously throughout the year. This theoretically allows them to be used to obtain sperm or breed all year round. Nevertheless, practical observations and numerous studies show that seasonal environmental and climatic factors can have a noticeable effect on both the sexual activity of rams and the quality of sperm they secrete [15, 22, 23].

In total, in different seasons of the year, 432 ejaculates from Charolais and Ile-de-France ram were received (Table 2). Sexual activity of animals in different seasons remained high. All rams had a well-expressed locomotor reaction to the female fixed in the machine, a courtship complex, embracing reflex, and ejaculation reflex. However, significant variations in the speed and intensity of these processes were observed depending on the season of the year. The tendency to decrease sexual activity was manifested in winter, then intensified in spring and summer, and was expressed primarily by an increase in the time for the manifestation of the entire complex of sexual reflexes.

2. Activity of sexual reflexes in Charolais (n = 8) and Ile-de-France (n = 5) rams (*Ovis aries*) as depends on the season (M±SD, Stavropol Territory, Shpakovsky District, September 2019—August 2020)

Season	Breed	Investigated ejaculates, n	Time to receive one ejaculate, s
Autumn	Charolais	88	48.6±0.15
	Ile-de-France	40	88.5±0.19
Winter	Charolais	72	83.9±0.28*
	Ile-de-France	36	96.5±0.35*
Spring	Charolais	68	124.6±0.24**
	Ile-de-France	34	143.4±0.38**
Summer	Charolais	56	127.5±0.33**
	Ile-de-France	38	157.9±0.15**

*, ** Differences with indicators in the autumn period are statistically significant at $p < 0.01$ and $p < 0.001$, respectively.

For example, in Charolais rams, the time to receive ejaculate in the winter months increased 1.7-fold compared to autumn months, and in spring and summer months 2.56-fold and 2.62-fold, respectively ($p < 0.001$). Ile-de-France rams showed similar dynamics of the activity of sexual reflexes. At the same time, the decrease in libido intensity was significantly less expressed compared to Charolais rams. In winter, Ile-de-France rams spent 1.09 times more time on the release of one ejaculate than in the sexual season, in spring and summer periods — 1.62 and 1.78 times more time, respectively ($p < 0.01$). Consequently, the sexual activity of Ile-de-France rams was less affected by seasonal factors, unlike the rams of Charolais breed, who needed a significantly ($p < 0.001$) longer time to manifest the full complex of ejaculate release reflexes in spring and summer periods.

Sexual activity of rams is important, however, to obtain high efficiency of insemination, the quality of sperm production is a priority. The researchers examined the volume, motility, and concentration of freshly obtained sperm from 284 ejaculates of Charolais rams and 148 ejaculates of Ile-de-France rams. The sperm motility index was evaluated on a 10-point scale (Table 3).

3. Evaluation scale of sperm motility in Charolais and Ile-de-France rams (*Ovis aries*)

Grade class	Scores	Movement description
Excellent	9-10	Dense, very fast-moving waves; 90% or more of the sperm are active
Good	7-8	Vigorous wave movements, but not as fast as at 9-10 points; 70-85% of sperms are active
Satisfactory	5-6	Only small, slow-moving waves; 45-65% of sperm are active
Unsatisfactory	3-4	Waves do not form, some movement of sperms is visible; 20-40% of sperms are alive, but with poor motility
Badly	1-2	About 10% of sperms show signs of weak movement
Dead	0	No movement of sperm

4. Characteristics of sperm production in Charolais (n = 8) and Ile-de-France (n = 5) rams (*Ovis aries*) as depends on the season (M±SD, Stavropol Territory, Shpakovsky District, September 2019—August 2020)

Indicator	Season	Charolais	Ile-de-France
Volume, ml	Autumn	1.2±0.15	1.1±0.14
	Winter	1.0±0.11	1.0±0.19
	Spring	0.9±0.17	0.9±0.12
	Summer	1.0±0.19	1.0±0.14
Concentration, $\times 10^9$/ml	Autumn	3.8±0.15	3.7±0.20
	Winter	3.3±0.18	3.4±0.27
	Spring	2.9±0.12	2.8±0.39
	Summer	2.9±0.19	3.1±.022
Motility of fresh sperm, score	Autumn	9.1±0.53	8.6±0.56
	Winter	8.5±0.46	8.0±0.73
	Spring	7.4±0.59	7.5±0.54
	Summer	8.1±0.63	8.2±0.62
Number of sperms with an intact acrosome, %	Autumn	78.0±2.29	82.0±3.84
	Winter	75.0±3.43	78.0±2.56
	Spring	68.0±2.18	75.0±3.17
	Summer	65.0±2.26	72.0±2.89

The parameters of sperm production of rams of both breeds in all seasons of the year remained quite high and met the requirements (Table 4). At the same time, some variations were identified.

In rams of the Charolais breed, sperm motility varied from 9.1 in the sexual season to 7.4 points in the spring period, when the lowest value of this indicator was observed. In winter and summer, motility was 8.5 and 8.1 points. Sperm volume and sperm concentration also had the lowest values in spring, while in winter and summer, they occupied an intermediate position.

The quality and quantity of sperm production in Ile-de-France rams had high variability depending on the season of sperm production. The highest indicators of volume, concentration, and motility were in the autumn period (1.15 ml, 3.75 billion/ml, and 8.57 points, respectively), whereas in winter, these parameters were 0.98 ml, 3.38 billion/ml, and 7.95 points, in spring — 0.88 ml, 2.85 billion/ml, and 7.55 points, in summer — 0.96 ml, 3.15 billion/ml, and 8.19 points.

The decrease in sperm quality led to an increase in microstructural damage and abnormalities of sperm. The number of sperms with intact acrosome ranged from 65% in summer in Charolais rams to 82% in autumn in Ile-de-France rams.

The analysis of the data obtained allows making a preliminary conclusion that, despite the significant volatility, rams of the Charolais and Ile-de-France breeds in the climatic conditions of the central zone of the Stavropol Territory can be used to obtain high-quality sperm in all seasons of the year. The highest rates in were observed in the autumn sexual season, which was a predictable natural result. At the same time, low parameters in the spring period turned out to be quite an unexpected fact. In the spring season, the photoperiod (length of daylight) is approximately the same as in autumn; the average ambient temperature, duration of insolation, and humidity are similar to the average autumn indicators. On the contrary, in summer, all these climatic indicators, except humidity, reach the highest values. However, in summer, sperm production in rams turned out to be significantly higher than in spring. Winter and summer periods are transitional, and spring, which differs from autumn only in one natural indicator, serves as an antagonist to the sexual season. If the assumption is correct, then in the realization of sexual function, the main role is played not by absolute climatic indicators (photoperiod, temperature, insolation), but by the positive dynamics of daylight, i.e., an increasing photoperiod. Indirect confirmation of these conclusions can be found in the works of foreign colleagues [22-25]; however, additional research is needed for a more substantiated statement.

The authors consider the obtained data on the dynamics of the main parameters of sperm production in the context of breeds to be extremely important for the adoption of an optimal algorithm for working with rams of the Charolais and Ide-de-France breeds to obtain crossbreed young stock. At the same time, it seemed interesting to study the individual differences of rams in sperm production within the same breed, which can provide theoretical and practical material on the limiting parameters of the main indicators of sperm quantity and quality.

The authors considered a representative sample of rams sufficient to identify a pattern or at least a trend (Table 5).

According to the results of the experiment, both intrabreed individual differences between rams and interbreed variations were found. In animals of the Charolais breed, sperm motility in average annual terms was 8.16 points, with the variability of the indicator between rams from 7.60 to 9.10 points. The same high volatility was observed in terms of sperm volume and concentration: with averages of 1.02 ml and 3.03 billion/ml, the variability ranged from 0.85 to 1.12 ml and from 2.75 to 3.22 billion/ml, respectively. One of the key parameters, the total

number of sperm with rectilinear motion (RM) in the ejaculate with an average value of 2.53 billion varied with more than 50% amplitude: from 2.09 to 3.18 billion.

5. Individual parameters of sperm production in Charolais and Ile-de-France rams (*Ovis aries*) (*M*±SD, Stavropol Territory, Shpakovsky District, September 2019—August 2020)

Breed	Ram No.	Studied ejaculates, *n*	Characteristics of sperm production			The total number of spermatozoa with RM in the ejaculate
			motility, score	volume, ml	concentration, $\times 10^9$/ml	
Charolais	06894	35	9.1±0.73	1.1±0.13	3.1±0.32	3,2±0,18
	06943	30	8.2±0.83	1.0±0.22	3.2±0.30	2,8±0,21
	01181	38	7.8±0.70	0.9±0.09	3.0±0.10	2,1±0,09
	06947	34	8.3±0.44	1.1±0.14	3.1±0.13	2,8±0,16
	06860	36	7.7±0.77	1.0±0.21	3.2±0.32	2,5±0,12
	06917	40	8.5±0.53	1.1±0.19	2.8±0.23	2,6±0,14
	01011	36	8.1±0.49	1.0±0.17	2.9±0.36	2,3±0,11
	01029	35	7.6±0.87	0.9±0.22	3.1±0.22	2,1±0,18
Total for the breed (n = 8):		284	8,2±0,45	1.0±0.08	3.0±0.12	2.5±0.20
Ile-de-France	01121	32	9.0±0.53	1.2±0.23	3.4±0.31	3,7±0,21
	01126	27	8.8±0.68	1.2±0.13	3.5±0.37	3,6±0,26
	01132	29	7.9±0.88	1.1±0.22	3.1±0.41	2,6±0,12
	01111	26	7.8±0.61	1.1±0.21	3.4±0.30	2,8±0,14
	04511	34	8.4±0.77	1.1±0.19	3.3±0.33	3,0±0,21
Total for the breed (n = 5):		148	8,4±0,33	1.1±0.09	3.4±0.16	3.1±0.19

N o t e. RM — rectilinear motion.

Ile de France rams had motility volatility ranging from 7.8 to 9.0 points with an average score of 8.38 points. Sperm volume varied from 1.06 to 1.2 ml with an average of 1.12 ml, concentration − from 3.10 to 3.52 billion/ml with an average of 3.34 billion/ml. As an integral consequence of these three parameters, the total number of sperm with RT averaged 3.14 billion with a limit of 2.62 to 3.67 billion.

The decrease in sperm quality in the hot season is to some extent explained by an increase in the temperature of the scrotum and, as a consequence, degeneration of the testes under the influence of high ambient temperatures [26, 27]. It can also lead to infertility or subfertility [28, 29]. There are studies [30, 31], proving that an increase in external temperature affects the absolute temperature in various areas of the testes, thereby determining sperm quality and fertility of animals raised on pastures.

This study revealed that Charolais and Ile-de-France rams had a decrease in the concentration and total number of sperm during the anestrous period. Some foreign studies [32-36] demonstrated differences in sexual activity and parameters of sperm production depending on the season of the year in different climatic zones.

For example, Ibrahim [32] examined a total of 900 sperm ejaculates from 10 rams. The highest quality sperm was obtained in winter (volume — 0.77 ml, pH — 6.95, motility — 4.53 points on a 5-point scale, sperm concentration — 4932.72×10^{6}/ml); however, the authors assess sperm quality as good in all seasons of the year. At the same time, the season of the year had a significant ($p < 0.01$) effect on the sexual behavior of rams: the highest sexual activity was recorded in autumn, the lowest in summer. Benmoula et al. [33] evaluated sperm from five adult rams INRA180 (age 2-3 years). Scrotum circumference, sperm quality, and total protein concentration in the seminal plasma remained relatively constant throughout the year ($p > 0.05$). There were no differences in total sperm motility ($p > 0.05$), while the only parameters showing seasonal fluctuations were the content of cholesterol, total lipids in sperm, and progressive sperm motility. Azawi et al. [34] examined sperm samples from six sexually mature rams of the Avassi breed.

Larger ($p < 0.05$) sperm volume was observed in August (1.55±0.08 ml) and March (1.27±0.15 ml). Sperm concentration was the highest ($p < 0.05$) in the breeding season in September ($4.21±0.86×10^9$ sperm/ml). Individual sperm motility and percentage of live gametes observed in August and May had the highest values and differed ($p < 0.05$) from those in December and January.

On the contrary, the research by Tomkins et al. [35] showed that in autumn ejaculate volume and percentage of live sperm decreased, and the percentage of abnormal sperm increased, while sperm concentration in the ejaculate showed a greater decrease in summer. Belkadi et al. [36] revealed that the sexual activity of rams was high during the mating seasons (spring and autumn), which was determined by high testosterone content (4.89±2.06 and 3.09±1.35 ng/ml, respectively). The mean values of ejaculate volume, total motility, the number of live sperm, and scrotal circumference were higher in spring ($p < 0.05$) – 1.23±0.26 ml, 3.39±1.07 points, 79.16±15.82% and 36.29±1.91 cm, respectively, sperm concentration in autumn ($1.19±0.56×10^9$ sperm/ml compared to $0.46±0.13×10^9$ sperm/ml in spring).

Thus, libido and gametogenesis in rams of foreign selection of Charolais and Ile-de-France in the conditions of the central zone of the Stavropol Territory remain high in all seasons of the year. At the same time, wide seasonal variations in indicators of sexual activity and sperm production were noted for both breeds. Rams produced the best sperm in terms of quality and volume in the autumn (sexual) season. In other seasons of the year, sperm production in the main parameters also met the instructional requirements, although a steady trend towards a decrease in the volume, concentration, and mobility of sperm was observed. The decrease in these indicators in winter compared to autumn was the greatest (by 1.5-2.0 times). Consequently, in rams of these breeds, spermatogenesis occurs continuously in all seasons of the year and their sperm can be obtained year-round and used either for insemination of sheep or for preservation in frozen form for long-term storage.

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ANTICLASTOGENIC ACTIVITY OF AMINOSELETON UNDER THE EFFECT OF CYCLOPHOSPHAMIDE ON THE BONE MARROW OF MICE

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Abstract

The tissue drug aminoseleton, designed at the All-Russian Veterinary Research Institute of Pathology, Pharmacology and Therapy, was obtained from the spleen of cattle by cryogenic fractionation. Adaptogenic, membrane stabilizing, stress-protective, antioxidant and immunomodulatory properties of aminoseleton have been already shown. In this work, the anticlastogenic effect of the tissue drug aminoseleton on the bone marrow cells of mice exposed to the experimental mutagen was revealed for the first time. In addition, the preservation of cytogenetic stability and mitotic activity in bone marrow cells of healthy animals was shown when using the study drug. The objective of this work was to assess the effect of aminoseleton on the cytogenetic stability of bone marrow cells in healthy mice and mice exposed to the experimental mutagen, as well as to identify the antimutagenic properties of the drug in relation to the genotoxic effect of cyclophosphamide (CP) using a micronucleus test. The experiments were carried out on outbred white mice (*Mus albus officinarum*), which were divided into six groups subjected to the following treatments: i) intramuscular administration of sterile isotonic sodium chloride solution in a volume of 0.2 ml (negative control, $n = 12$); ii) intraperitoneal injection of 0.2 ml of CP (Baxter Oncology GmbH, Germany) at a dose of 20.0 mg/kg of body weight (positive control, $n = 12$); iii) intramuscular single injection of 0.2 ml of aminoseleton at a therapeutic dose of 0.5 ml/kg ($n = 12$); iv) intramuscular single injection of 0.2 ml of aminoseleton at a tenfold therapeutic dose of 5.0 ml/kg ($n = 12$); v) intramuscular single injection of 0.2 ml of aminoseleton at a dose of 0.5 ml/kg with intraperitoneal injection of 0.2 ml of CP at a dose of 20.0 mg/kg in 72 h ($n = 6$); vi) intramuscular fivefold injection of 0.2 ml of aminoseleton at a dose of 0.5 ml/kg with 24 h intervals and intraperitoneal administration of CP is similar to animals of other groups 72 h after the fifth injection ($n = 6$). To determine the amount of chromosomal aberrations in the bone marrow, 2.5 h before euthanasia, mice were injected intraperitoneally with 0.025 % colchicine (PanEco, Russia). Bone marrow cells were washed out of the femurs using Hanks' buffer solution (pH 7.4), the cell suspension was incubated in 0.075 molar hypotonic KCl solution, then the cells were fixed with acetoalcohol cooled to 4 °C and stained by Romanowsky-Giemsa procedure. The mitotic index (MI) was assessed by the number of dividing cells per 1000 bone marrow cells. The number of cells with chromosomal aberrations was counted in 100 metaphase plates per animal. Single and paired fragments, exchanges and achromatic gaps, as well as cells with multiple pathologies were counted. To study the frequency of micronuclei (micronucleus test) of polychromatophilic erythrocytes (PCE), the obtained bone marrow cells were added to 1 % albumin solution in Hanks' buffer solution (pH 7.4) and applied to glass slides, then the samples were dried, fixed with methanol and stained by Romanowsky-Giemsa protocol. The frequency of micronuclei per 1000 PCE was determined; a total of 2000 PCE per animal was studied. The proportion of PCE per 500 normochromic erythrocytes (NE) and PCE was also calculated. The frequency of chromosomal aberrations and micronuclei when administering the drug at the studied doses did not statistically significantly differ from that in animals of their negative control group that was 1.0±0.40 and 0.2±0.06 %, respectively. The administration of aminoseleton also had no effect on the mitotic index of bone marrow cells in experimental animals.

The course administration of aminoseleton reduced the clastogenic effect of cyclophosphamide, assessed by the number of micronuclei in polychromatophilic erythrocytes of the bone marrow, from 2.3±0.21 % in mice from the positive control group to 1.0±0.40 % in animals after a course of aminoseleton injections. Thus, the clastogenic activity of cyclophosphamide decreased by 51.3 % that was probably due to the correction of the prooxidant-antioxidant system of the animal body with the studied drug. A decrease in the number of micronuclei induced by cyclophosphamide in polychromatophilic erythrocytes of the bone marrow indicates the presence of an anticlastogenic potential in aminoseleton.

Keywords: aminoseleton, cyclophosphamide, mutagenicity, anticlastogenic properties, micronuclei, chromosomal aberrations, bone marrow, white mice, polychromatophilic erythrocytes

Reducing the negative impact of stress factors on the animal body through the use of adaptogen preparations is one of the areas of research in veterinary medicine [1]. Among the drugs of this class, a group of drugs can be distinguished that contain extracts of animal organs and tissues as part of the extract. Since the spleen serves as a source of a significant amount of cytokines of various types, it became the basis for several Russian and foreign organic preparations [2]. For many of them, immunomodulatory and adaptogenic properties have been shown [3, 4]. However, the species and age parameters of the animals from which the organs were obtained, as well as the technological features of the processing of raw materials during the production of the drug, can to some extent change the final chemical composition of the drug and, therefore, affect its biological activity and therapeutic efficacy [4].

At the All-Russian Veterinary Research Institute of Pathology, Pharmacology and Therapy, a new tissue drug aminoseleton was developed, obtained by cryogenic fractionation of the spleen of cattle. Previously, the adaptogenic, stress-protective, antioxidant, and immunomodulatory properties of aminoseleton were shown [5, 6].

Assessment of the genotoxic properties of new drugs is one of the mandatory stages of preclinical studies in their development [7]. Chemical mutagens are widespread in the environment and can cause hereditary and congenital diseases, carcinogenesis, aging, and mitochondrial diseases [8, 9]. The mutagenic and carcinogenic effects of various genotoxic substances also include the formation of free radicals that overload the endogenous antioxidant defense systems that inhibit oxidative stress, one of the causes of DNA damage. In general, all antioxidant agents can be considered as potential inhibitors of mutagenesis and carcinogenesis [10].

Potentially, anti-mutagenic substances include preparations of plant or animal origin, the use of which is associated with their lower toxicity, the affinity of biologically active substances that make up the drugs and are present in the body of animals, and their availability from an economic point of view. However, the effect of natural remedies on the hereditary apparatus of cells has not been sufficiently studied. The accumulation of data on the assessment of the clastogenic and antimutagenic properties of drugs used in veterinary practice is of scientific interest and of great practical importance. According to the authors' data, aminoseleton induced a decrease in the amount of malondialdehyde and nitric oxide metabolites, as well as an increase in the total antioxidant activity of the blood serum of animals. The drug modulated the enzymatic and non-enzymatic links of the antioxidant defense, for example, increased the activity of glutathione peroxidase and catalase, the concentration of vitamins A and E [11]. In this regard, it is advisable to evaluate not only the safety of aminoseleton but also its potential antimutagenic properties in a model of cyclophosphamide-induced (CP-induced) mutagenic action in mouse bone marrow cells [7]. CP is an alkylating drug used in oncology. This substance is activated by cytochrome P-450 in the liver, forming nitrogen mustard, which through a chain of reactions has an alkylating effect on DNA and causes the formation of cross-links between DNA strands at the guanine nitrogenous base,

which can cause mutations and cell death [12].

In the present work, the anticlastogenic effect of a tissue drug aminoseleton on the bone marrow cells of mice exposed to an experimental mutagen was revealed for the first time. In addition, the preservation of cytogenetic stability and mitotic activity in bone marrow cells of healthy animals was shown when using the study drug.

The aim of this work is to assess the effect of aminoseleton on the cytogenetic stability of bone marrow cells in healthy and exposed mice, as well as to identify the antimutagenic properties of the drug in relation to the genotoxic effect of cyclophosphamide using a micronucleus test.

Materials and methods. The experiments were carried out on outbred white mice (*Mus albus officinarum*) (n = 60 divided into six groups) according to the guidelines for preclinical trials of drugs [7]. The experimental animals were kept under standard vivarium conditions (air temperature 18-23 °C, relative humidity 45-60%). Access to water and feed was free. Experimental manipulations were carried out in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental or other Scientific Purposes (Strasbourg, 1986, reaffirmed in 2006). Aminoseleton was obtained by cryofractionation in the form of a liquid suspension.

Group I (negative control, n = 12) was single administered intramuscularly with 0.2 ml sterile isotonic sodium chloride solution, Group II (positive control, n = 12) was single injected intraperitoneally with 0.2 ml CF (Baxter Oncology GmbH, Germany) at a dose of 20.0 mg/kg of body weight [15]. Group III (n = 12) was single injected intramuscularly with 0.2 ml aminoseleton at 0.5 ml/kg (a therapeutic dose). Group IV (n = 12) was single injected intramuscularly with 0.2 ml aminoseleton at 5.0 ml/kg (a 10-fold therapeutic dose). Group V (n = 6) was single injected intramuscularly with of 0.2 ml aminoseleton (0.5 ml/kg) followed with intraperitoneal injection of 0.2 ml CP (20.0 mg/kg) in 72 hours. Group VI (n = 6) was injected intramuscularly with 0.2 ml aminoseleton (0.5 ml/kg), five times with a 24-hour intervals, followed by intraperitoneal injection of 0.2 ml CP (20.0 mg/kg) in 72 hours after the fifth injection of aminoseleton.

To determine the amount of chromosomal aberrations in the bone marrow, 2.5 hours before euthanasia, mice were injected intraperitoneally with 0.025% colchicine (PanEco, Russia). Then, bone marrow cells were washed out of the femurs using Hanks buffer solution (pH 7.4) with a 5 ml syringe, the cell suspension was incubated in 0.075 molar hypotonic KCl solution (25 min at 37 °C), then the cells were fixed using cooled to 4 °C acetoalcohol (methanol: acetic acid − 3: 1) and stained according to Romanovsky-Giemsa. The mitotic index (MI) was assessed by the number of dividing cells per 1000 bone marrow cells in the studied samples. The number of cells with chromosomal aberrations in 100 metaphase plates was counted for each animal [7, 14]. The samples were examined at a magnification of ×800 and ×1000 using a Bioskop-1 microscope (LOMO-Microanalysis LLC, Russia). Single and paired fragments, interchanges and achromatic gaps, as well as cells with multiple pathologies were taken into account [7]. Six animals from each group were examined, except for Groups V and VI.

To determine the frequency of micronuclei (micronucleus test) of polychromatophilic erythrocytes (PCEs) [15], the obtained bone marrow cells were added to a 1% albumin solution in Hanks buffer solution (pH 7.4) [16] and applied to glass slides, then the preparations were dried and fixed with methanol and stained according to Romanovsky-Giemsa [15]. Bone marrow preparations were examined at ×800 and ×1000 magnifications. The frequency of micronuclei was set at 1000 PCE; a total of 2000 PCE per animal was studied. The proportion of

PCE per 500 normochromic erythrocytes (NE) and PCE was also taken into account [7]. The study was carried out on 6 animals from each group.

Statistical processing of the obtained results was carried out in the STADIA 8.0 program (NGO Informatika and Computers, Russia). Mean values (*M*) and standard errors of means (± SEM) were calculated. The nonparametric van der Waerden test was used. Differences were considered statistically significant at $p \leq 0.05$.

Results. The metaphase plates of the bone marrow cells of the mice of the studied groups were analyzed (Table 1).

1. Frequency of chromosomal aberrations in bone marrow cells in outbred white mice (*Mus albus officinarum*) exposed to various doses of aminoseleton (n = 12, M±SEM)

Group	Number of metaphases	Number of abnormalities per 100 examined cells, %					Total number of cells with abnormalities, %
		gaps	single fragments	paired fragments	interchanges	MP	
I	600	0.5±0.37	0.2±0.18	0	0.2±0.18	0.2±0.18	1.0±0.40
II	600	0.5±0.24	12.7±0.92*	2.8±0.34*	2.0±0.49*	0.3±0.23	11.2±0.52*
III	600	0.7±0.23	0.2±0.18	0	0.8±0.44	0	1.7±0.46
IV	600	1.0±0.40	0	0	0.5±0.24	0.5±0.24	2.2±0.59

N o t e. MP — multiple abnormalities (more than five per cell). For a description of the groups, see the "Materials and methods" section.
* The difference with the negative control (Group I) is statistically significant at $p \leq 0.05$.

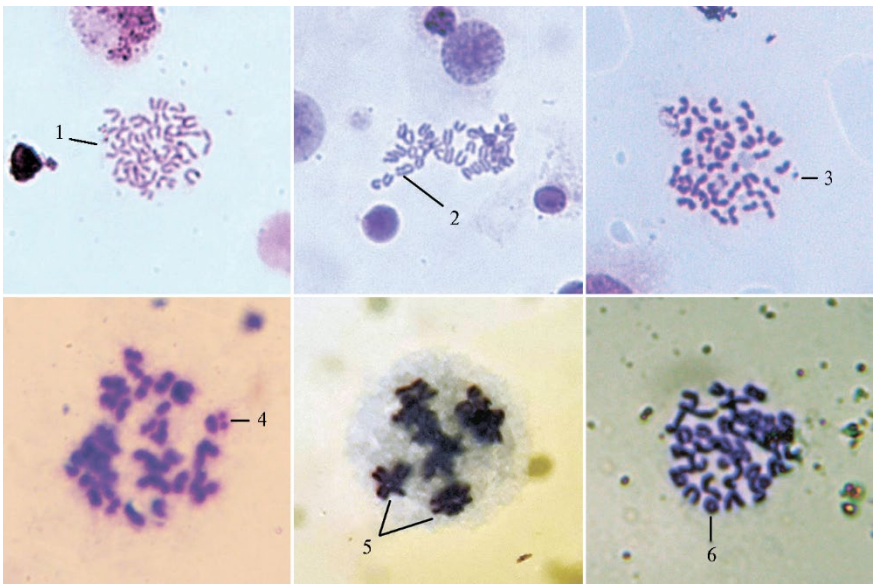

Fig. 1. Micrographs of metaphase bone marrow plates of outbred white mice (*Mus albus officinarum*) with the introduction of intramuscularly sterile isotonic sodium chloride solution in a volume of 0.2 ml (negative control): 1 — chromosomes without abnormalities, 2 — achromatic gap, 3 — single fragment, 4 — paired fragment, 5 — chromosomal and chromatid interchanges, 6 — ring chromosome (1-3 — magnification ×800, 4-6 — ×1000; a Bioskop-1 microscope, LOMO-Microanalysis LLC, Russia).

A single intramuscular injection of aminoseleton both at a therapeutic (0.5 ml/kg) and 10-fold (5 ml/kg) dose did not induce a statistically significant increase in the proportion of cells with chromosomal aberrations (Fig. 1) relative to the negative control group (Group I) after 24 hours (see Table 1). The clastogenic effect of CP (20 mg/kg), on the contrary, led to a 10-fold increase in the frequency of cells with pathologies. The introduction of aminoseleton also had no effect on MI in bone marrow preparations: in Groups I-IV, it was 4.0 ± 0.73, respectively; 3.1 ± 0.41; 3.5 ± 0.79 and 3.6 ± 0.75%.

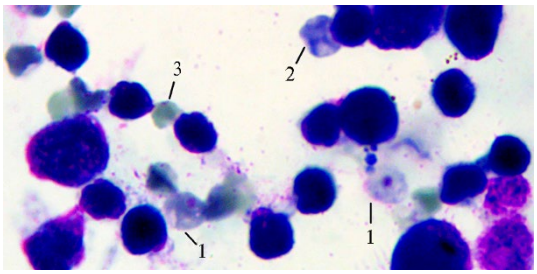

Fig. 2. Micrograph of a bone marrow preparation of an outbred white mouse (*Mus albus officinarum*) with the introduction of intramuscularly sterile isotonic sodium chloride solution in a volume of 0.2 ml (negative control): 1 — polychromatophilic erythrocyte with a micronucleus, 2 — polychromatophilic erythrocyte, 3 — normochromic erythrocyte (magnification ×1000, a Bioskop-1 microscope, LOMO-Microanalysis LLC, Russia).

The administration of aminoseleton at d 0.5 and 5 ml/kg of body weight in mice did not lead to a change in the frequency of PCE micronuclei (Fig. 2) as compared to the negative control (0.23±0.06%) (Table 2). CP injection induced a statistically significant increase in the frequency of micronuclei in PCE in mice from Groups II, V, and VI to 2.2±0.21, 2.1±0.26, and 1.1±0.09%, respectively. We found the anticlastogenic effect of aminoseleton, which was used, before CP administration, five times at 0.5 ml/kg (Group VI). Thus, the frequency of micronuclei in PCE in mice from Group VI was 51.3% ($p \leq 0.003$) lower than in animals from Group II, which received only CP. A single administration of aminoseleton (Group V) did not lead to a statistically significant decrease in the frequency of micronuclei. The research team also estimated the proportion of PCE in the bone marrow of mice, which can be an indicator of the toxic effect of xenobiotics [7]. No statistically significant difference occurred in this indicator between animals of all study groups, including the difference with the positive control.

2. Frequency of polychromatophilic erythrocytes (PCE) **with micronuclei in the bone marrow of healthy outbred white mice (*Mus albus officinarum*) and animals that received an injection of cyclophosphamide, with the preliminary administration of the drug aminoseleton in various doses** (*M*±SEM)

Group	PCE with micronuclei/1000 PCE, %	PCE/(NE + PCE), %
I (*n* = 12)	0.2±0.06	49.9±0.93
II (*n* = 12)	2.3±0.21[a]	49.0±1.57
III (*n* = 12)	0.2±0.07	48.9±0.65
IV (*n* = 12)	0.3±0.11	49.5±1.43
V (*n* = 6)	2.1±0.26[a]	48.2±1.79
VI (*n* = 6)	1.1±0.09[a, b]	50.9±1.86

N o t e. NE – normochromic erythrocytes. For a description of the groups, see the "Materials and methods" section. [a], [b] Differences with negative (Group I) and positive (Group II) controls, respectively, are statistically significant at $p \leq 0.05$.

A number of drugs containing spleen extract are used in clinical practice as immunomodulators or drugs to reduce the side effects of anticancer therapy [4, 17]. The obtained data are consistent with the information on the absence of mutagenic properties in other drugs based on the spleen of animals. For example, the Polyerga™ drug recommended for clinical use (HorFerVit Pharma GmbH, Germany) showed no genotoxic effect in the Ames test [18]. In contrast to our data on the absence of the effect of aminoseleton on the proportion of PCEs in the bone marrow of mice, the results of Lu et al. [17] testify to the protective effect of the spleen extract of newborn calves in relation to mice with cyclophosphamide-induced inhibition of hematopoietic activity in the bone marrow. This can be explained by the significantly higher dose of CP (100 mg/kg body weight) used by the authors in the model of cyclophosphamide-induced hematopoiesis suppression [17].

The anticlastogenic effect of aminoseleton discovered by the authors and the absence of mutagenic properties in it is to some extent confirmed by the studies of Dychko et al. [19], who demonstrated a decrease in the number of micronuclei

in erythrocytes when spleen extract was administered to mice exposed to X-rays. The authors believe that the radioprotective effects of the spleen extract were due to its membrane-stabilizing effect on mouse cells, which was also shown by us for aminoseleton [20].

Currently, various mechanisms of antimutagenic action have been identified [21]. CP-induced genotoxicity and cytotoxicity towards bone marrow cells can be partially leveled out due to antioxidant activity [22]. Probably, the anticlastogenic effect of aminoseleton is due to the complex of biologically active substances included in the drug (amino acids, phospholipids, vitamins, oligopeptides, trace elements, nucleic acids) [20], which have an antioxidant effect [21]. Thus, ascorbic acid reduces the frequency of PCE with micronuclei induced by CP in bone marrow cells [23]. In addition, it has been shown that the introduction of aminoseleton increases the activity of glutathione peroxidase and leads to an increase in the content of reduced glutathione, the amount of which decreases with the introduction of CP and plays an important role in the antioxidant defense of the body [11, 24]. However, the antimutagenic effect of the same substances can occur through different mechanisms [25].

Since in the present studies, aminoseleton manifested itself as an immunomodulator of the cellular and humoral links of the immune system [6], its antimutagenic effect could be due to the induction of the synthesis of endogenous cytokines with a gene-protective effect, such as interferon [8]. Thus, a number of studies have shown the anticlastogenic and anticarcinogenic effects of interferons and their inducers, apparently due to the activation of post-replicative DNA repair [26, 27]. In a study by Jia et al. [28], the administration of an extract of spleen of newborn calves to mice with CP-induced immunosuppression led to an increase in the content of INF-α and INF-γ in the blood serum, which may testify in favor of the proposed hypothesis.

The sensitivity of cells to the action of genotoxicants depends on the intake of B vitamins with antioxidant properties, micronutrients, and other trace elements (such as magnesium or zinc) involved in reparative processes, maintenance of cell homeostasis, and antioxidant protection [8]. Their replenishment can have a systemic effect on the antioxidant defenses of the body. The prolonged anticlastogenic effect of aminoseleton even 72 h after the final administration is due to the prolonged activation of the antioxidant system upon administration of the drug [11].

Thus, the tissue drug aminoseleton obtained by cryofractionation from the bovine spleen in therapeutic (0.5 ml/kg) and high (5.0 ml/kg) doses had no destabilizing effect on the cytogenetic characteristics of cells, which was assessed by the number of PCEs with micronuclei and chromosome aberrations in bone marrow cells of mongrel mice. The drug did not affect the activity of cell division, assessed by the mitotic index. At the same time, aminoseleton showed an anticlastogenic effect when combined with the genotoxicant cyclophosphamide, reducing the frequency of occurrence of PCEs with micronuclei in the bone marrow cells of mice by 51.3% relative to the positive control group.

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METABOLIC STATUS AND MILK PRODUCTIVITY OF COWS INJECTED WITH A TISSUE BIOSTIMULANT DERIVED FROM REINDEER HUSBANDRY WASTE

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Abstract

The metabolism intensity and pathways during lactation largely determine cow milk production. The use of tissue bio-stimulants optimizes metabolic processes in animal body by activating neurohumoral regulation mechanisms and has a positive effect on lactogenesis and lactopoesis. Tissue biostimulants are derived from various raw materials under various technologies. Therefore, understanding the mode of their action on physiological status and productive qualities of farm animals is relevant. This paper is the first experimental conformation that fourfold administration of the new tissue biostimulant to cows during the dry period and the first 100 days of lactation promotes protein and lipid metabolism in lactating cows. It was proven that the administration of the tissue bio-stimulant according to the developed scheme increases daily milk yields and milk protein and butterfat yields during the first 60 days of lactation. Our goal was to evaluate the effect of the new tissue bio-stimulant derived from reindeer waste on metabolic indices of cows and their milk production. The study was conducted in the herd of Black-Pied cows of the Priobskiy type during dry period and early lactation (the farm of the AO Uchkhoz Prigorodnoye, Barnaul, the Altai Region, 2019). Dry cows at the 3rd lactation were assigned to two groups (n = 10) 55-60 days before the expected calving. In the control group, saline solution was injected during the dry period (55-60 days before the expected calving fourfold, 22.5 ml per head two weeks apart) and during the first 100 days of lactation — from day 15 at the same dose and frequency. In the trial group, the tissue bio-stimulant was administered according to the same scheme. The batch of the tissue bio-stimulant (Russian Patent No. 2682641) was obtained from mesenteric lymph nodes and mediastinums, spleen, liver, uteri with 2-3 month old fetuses, and placentae collected under aseptic conditions from healthy animals at slaughtering. Before the experiment and on days 15 and 60 of lactation, blood samples were collected from the tail veins into clot activator tubes. The levels of total blood protein, albumin, cholesterol, triglycerides, and glucose were measured (an automated ChemWell Combi 2910 Analyzer, Awareness Technology Inc., USA), the amount of blood globulins, the albumin to globulin ratio and the cholesterol to triglyceride ratio were calculated. The daily milk yields were recorded on days 15, 30, and 60 of lactation by control milking; the milk yield over 60 days of lactation was calculated. On days 15 and 60 of lactation, the concentrations of protein and butterfat in milk were determined (a MilkoScan FT 120 analyzer, Foss Electric, Denmark), the yields of milk protein and butterfat over 60 days of lactation were calculated according to the common formula. The administration of the tissue bio-stimulant had a promoting effect on metabolism activation and increased the indices of constructive and energy metabolism. The serum levels of total protein, albumin, and cholesterol increased by 3.0-6.4 % ($p < 0.01$), 9.2-6.9 % ($p < 0.001$), and 18.3-26.0 % ($p < 0.01$), respectively, albumin to globulin ratio by 9.0-11.1 % ($p < 0.01$) as compared to the control. On day 60 of lactation vs. day 15, the total serum protein increased by 9.7 % ($p < 0.001$) and 6.1 % ($p < 0.001$) in control group and trial group, respectively, serum globulin by 20.5 % ($p < 0.001$) and 16.2 % ($p < 0.001$), and cholesterol by 2.0 % and 8.6 % ($p < 0.01$). The albumin to globulin ratio decreased by 18.2 % ($p < 0.001$) and 16.7 % ($p < 0.001$). In the trial group, milk production for

60 days of lactation increased by 19.3 % ($p < 0.01$) and the yields of milk protein and butterfat by 26.7 % ($p < 0.05$) and 22.1 % ($p < 0.05$).

Keywords: tissue stimulant, cows, metabolism, milk quality, lactation performance

Lactation induces a strong functional tension of the body [1]. The intensity and direction of metabolism in lactating animals are associated with milk production and active sorption of blood metabolites by the mammary gland, activation of enzymes involved in the processes of lactogenesis, redistribution of substrate flows to ensure lactation function [2]. Lactation process development should be ensured by the high activity of the endocrine glands directly involved in the synthesis of precursors of milk components [3, 4].

Bio-stimulants are biologically active drugs that can increase natural resistance, stress resistance, affect enzymatic and other processes, thereby increasing quantitative and qualitative indicators of production [5-8]. In the previous decade, a huge demand for the use of bio-stimulants both in crop production and animal husbandry was observed since they are harmless, do not cause addiction, and create favorable conditions for the manifestation of the body's own protective reactions [5, 7, 9, 10]. Bio-stimulants can be obtained from a variety of bio-organic raw materials, therefore, no single mechanism of their effect on the body was revealed [7, 11-13].

Tissue preparations belong to the class of bio-stimulants of natural origin [14-17]. They affect metabolism favorably, in particular, the amount of total protein, albumins, bilirubin, urea, cholesterol increases, the activity of alanine aminotransferase, and aspartate aminotransferase transamination enzymes increases, by having antioxidant properties [16], prevent lipid peroxidation [15, 18-20]. When using bio-stimulants, natural resistance increases significantly due to an increase in the lysozyme and bactericidal activity of blood serum, functional activity of neutrophils, and an increase in the content of T- and B-lymphocytes in blood [21-23]. The use of tissue preparations contributes to the intensive growth of young farm animals during fattening [19, 24], milk production, and reproductive qualities of cows, the safety of young animals [9].

In the presented work, it was established for the first time that the use of a new tissue bio-stimulant according to the developed scheme (4-fold administration of a new tissue bio-stimulant to cows in the dry period and during calving) activated protein and lipid metabolism, leading to an increase in daily milk yields and yield of milk protein and fat for the first 60 days of lactation.

The work objective was to evaluate the effect of tissue bio-stimulant derived from reindeer waste on metabolic indices of cows and their milk production.

Materials and methods. The study was conducted on cows (*Bos taurus taurus*) of the Priobsky type of the Black-Pied breed during the dry period and early lactation (AO Uchkhoz Prigorodnoye, Barnaul, the Altai Region, 2019). Two groups ($n = 10$ in each) of dry cows at the age of the third lactation and older were formed 55–60 days before the expected calving. The animals were selected taking into account their milk productivity, which preceded the dry period (the average daily milk yield for the last control milking before launch was 22.5 liters) and live weight (550 kg). The fatness of the animals at the beginning of the experiment was 3.2 points.

Cows from the control group were injected with saline solution in two stages: during dry period — 55-60 days before the expected calving 4-fold at a dose of 22.5 ml/head with an interval of 14 days; during calving — from day 15 of lactation at the same dose and multiplicity. The animals of the experimental group were injected with a tissue bio-stimulant according to a similar scheme.

An experimental batch of tissue bio-stimulant was made from slaughterhouse by-products of reindeer (Shanshin, N.V., Evseeva, T.P. Method for production of biogenic medical preparations Russian Patent No. 2682641(RF) MKI A 61K 35/12. Federal Altai Scientific Center of Agrobiotechnologies (RF). No. 2698707 C1. 2019). The mesenteric lymph nodes and mediastina, spleen, liver, uteri with 2-3-month-old fetuses, and placentae collected under aseptic conditions from healthy animals at slaughtering served as the material for the preparation of the drug. The resulting native material was placed in the refrigerator for 6 days at 2-4 °C. At the end of the specified period, all material was crushed in equal parts and placed in the Elmsonic P ultrasonic unit (Elma Schmidbauer GmbH, Germany). Quality control for toxicity and reactogenicity was carried out on white mice (GOST 31926-2013. Medicine remedies for veterinary use. Methods of safety identification. Moscow, 2014).

Before the experiment and on days 15 and 60 of lactation, blood samples were collected from the tail veins into vacuum tubes (with clot activator). The levels of total blood protein, albumin, cholesterol, triglycerides, and glucose were measured with an automated biochemical analyzer ChemWell Combi 2910 (Awareness Technology Inc., USA); the amount of blood globulins, the albumin to globulin ratio, and the cholesterol to triglyceride ratio were calculated.

Daily milk yields were recorded on days 15, 30, and 60 of lactation by control milking; the milk yield over 60 days of lactation was calculated. Milk samples for laboratory studies were taken on days 15 and 60 of lactation. The content of protein and fat in milk was determined using the MilkoScan FT 120 device (Foss Electric, Denmark); the yield of milk protein and fat over 60 days of lactation was calculated according to the common formula.

The obtained data were biometrically processed using the Microsoft Excel 2016 software package; arithmetic mean values (*M*), mean square errors (±MSE), and reliability criterion (p) were calculated. The reliability of the experimental results concerning the control group was calculated by the Student's *t*-criterion for independent samples, differences were considered statistically significant at $p < 0.05$; $p < 0.01$; $p < 0.001$. The values of biochemical parameters of the blood of cows on day 60 of lactation in comparison with day 15 according to the Student's *t*-test for dependent samples were considered statistically significant at $p < 0.05$, $p < 0.01$, $p < 0.001$.

Results. Biochemical blood parameters of cows before the drug administration corresponded to physiological values in pregnant cows (during the dry period) and lactating animals (immediately after calving) and did not differ significantly in the control and experimental groups (Table 1).

1. Blood bochemical parameters of the Priobsky type of the Black-Pied cows (*Bos taurus taurus*) on days 15 and 60 of lactation when using a tissue bio-stimulant from the slaughterhouse by-products of reindeer ($n = 5$, M±MSE; AO Uchkhoz Prigorodnoye, Barnaul, the Altai Region, 2019)

Parameter	Group				Norm [25-27]
	control	background	test	background	
Total protein, g/l	72.9±0.83 80.0±0.54$^{\Delta\Delta\Delta}$	73.7±3.35	77.6±0.62** 82.4±0.81$^{*\Delta\Delta\Delta}$	76.3±1.62	72.0-86.0
Albumins, g/l	38.9±0.56 39.0±0.41	39.9±0.70	42.5±0.22*** 41.7±0.41***	37.9±2.15	38.0-50.0
Globulins, g/l	34.0±0.49 41.0±0.34$^{\Delta\Delta\Delta}$	33.9±2.38	35.1±0.59 40.8±0.75$^{\Delta\Delta\Delta}$	38.4±2.31	34.0-36.0
A/G	1.1±0.02 0.9±0.01$^{\Delta\Delta\Delta}$	1.2±0.09	1.2±0.02* 1.0±0.02$^{**\Delta\Delta\Delta}$	1.0±0.11	0.6-1.3
Cholesterol, mmol/l	4.9±0.13 5.0±0.24	5.6±0.15	5.8±0.20** 6.3±0.21$^{**\Delta\Delta}$	5.1±0.44	2.3-6.6

Continued Table 1

Triglycerides, mmol/l	0.34±0.015 0.41±0.021	0.32±0.011	0.34±0.006 0.36±0.028	0.28±0.025	0.22-0.55
Ch/T, U	17.0±1.64 12.3±0.77	17.7±0.05	17.6±0.53 18.2±1.77*	18.1±0.13	17.0-37.0
Glucose, mmol/l	3.0±0.26 3.4±0.11	2.7±0.20	2.6±0.21 3.2±0.09$^{\Delta}$	2.4±0.72	1.32-4.89

N o t e. A/G — albumin to globulin ratio, Ch/T — cholesterol to triglycerides ratio. Values on day 15 of lactation are above the line, on day 60 of lactation below the line. Background stands for values at the beginning of the dry period before drug administration. See the description of groups in the "Materials and methods" section.
*, **, *** Differences with control are statistically significant at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively.
$^{\Delta}$, $^{\Delta\Delta}$, $^{\Delta\Delta\Delta}$ Differences in indicators on day 60 of lactation compared to day 15 are statistically significant at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively.

A four-fold subcutaneous administration of a tissue bio-stimulant to cows during the dry period contributed to an increase in the amount of total protein and albumins in blood serum by 6.4 ($p < 0.01$) and 9.2% ($p < 0.001$), respectively, and an increase in the albumin to globulin ratio by 9.0% ($p < 0.05$) on day 15 after calving in comparison with control. An increase in protein metabolism indicators, including an increase in the albumin to globulin ratio, indicates an increase in the intensity of colloidal osmotic processes and maintenance of acid-base balance [28].

The increase in milk productivity of cows is accompanied by an increase in metabolism intensity [29], which in the present experiment was manifested by an increase in total protein content in blood serum in the control and experimental groups, respectively, by 9.7 ($p < 0.001$) and 6.1% ($p < 0.001$) on day 60 of lactation. The use of tissue bio-stimulant enhanced anabolic processes and increased the amount of total protein in cows of the experimental group by 3.0% ($p < 0.05$) in comparison with the control group.

As a result of increased protein-synthetic liver function in cows from the experimental group, the amount of albumins in the blood was 6.9% ($p < 0.001$) higher than in other cows from the control group. An increase in the concentration of albumins in the blood [30–32] contributes to the maintenance of oncotic pressure, transportation of various biologically active substances, including hormones, vitamins, and fatty acids, which secretory cells of the glandular tissue of the udder of lactating cows use for the synthesis of milk components [30].

The concentration changes of globulin protein fractions in the control and experimental groups were similar. The increase in milk productivity of cows by day 60 of lactation was accompanied by an increase in the number of globulins by 20.5 ($p < 0.001$) and 16.2%, respectively ($p < 0.001$) in the blood of cows from the control and experimental groups. According to the content of globulins in the blood serum, the animals of the experimental group on both days 15 and 60 of lactation had no statistically significant differences in comparison with the control. The ratio of the total amount of albumins and globulins in cows from the experimental group on day 60 of lactation was 11.1% higher ($p < 0.001$) than in the control group. The dynamics of the albumin to globulin ratio was characterized by its decrease by 18.2 ($p < 0.001$) and 16.7% ($p < 0.001$) in the control and experimental groups, respectively, in comparison with indicators on day 15.

Some authors have also found that an increase in milk productivity is combined with an increase in the total protein content due to an increase in the content of globulins and a decrease in albumins. The albumin to globulin ratio decreases during this period in comparison with the beginning of lactation [33]. Metabolic changes of this nature may be since, during early lactation, the need for energy in animals increases sharply, the body fat reserves decrease, the load on the liver increases, and its functional activity decreases [34].

Placenta-based tissue bio-stimulants contain hepatocyte growth factors

[35, 36], in which proteins are synthesized from amino acids, which may explain the more intensive protein metabolism in cows from the experimental group. The tissues of the liver, spleen, lymph nodes, and uterus with fetuses, which are part of the bio-stimulant, contain a native combination of vitamins, amino acids, and minerals that provide optimal conditions for improving metabolic processes in the liver [37]. In particular, vitamins have a hepatoprotective effect, promote hepatocyte regeneration, prevent excessive collagen production by stellate cells, reduce the oxidative stress of the liver, and prevent fibrosis development [38]. Trace elements increase the functional activity of the liver, its adaptive properties, prevent the destruction of hepatocytes, contribute to the accumulation of glycogen in them [39]. Such essential amino acids as cysteine and methionine serve as precursors of the lipotropic substance choline, which prevents fatty degeneration of the liver [40]. Studies of other authors indicate an increase in regenerative and metabolic processes in the liver, as well as immune functions when using biogenic stimulants [35, 41–43]. An increase in the amount of total protein and albumins in the blood serum of lactating cows with the use of tissue preparations was noted in similar studies [44, 45].

It is known that cholesterol content in the blood of healthy animals is in direct correlation with the indicators of their milk productivity [29, 46]. The use of a tissue bio-stimulator led to an increase in the amount of cholesterol in the serum of cows of the experimental group on days 15 and 60 of lactation by 18.3 and 26.0% ($p < 0.01$) in comparison with the control group. The obtained data are consistent with the results presented in previously published papers [47]. Higher cholesterol content in lactating animals from the experimental group contributed to an increase in their milk productivity since it is known about the participation of cholesterol in the processes of renewal of mammary membrane lipids, which leads to an increase in the amount of glandular tissue in the mammary gland [29, 48]. Some authors have found that the activation of fat, carbohydrate, and protein metabolism during lactation, which promotes the proliferation of breast glandular tissue cells [49] and its growth [48, 50], can occur due to biologically active substances contained in placenta extract [48].

The use of a tissue preparation did not have a significant effect on the amount of triglycerides in animals in the experimental group. At the same time, the ratio of cholesterol to triglycerides in the blood serum of cows of the experimental group on days 15 and 60 of lactation was 3.5 and 47.9%, respectively ($p < 0.05$) higher than in the control group which indicates an increase in energy costs, including the use of glucose as an energy source [26]. In the first days of lactation, the mammary gland becomes the main consumer of glucose, and therefore its amount in the blood serum decreases [51]. In the present experiment, lower glucose values were also found in the blood of animals from the control and experimental groups on days 15 and 60 of lactation; they increased by 13.3 and 23.0% ($p < 0.05$) by day 60.

The degree and direction of metabolism had a significant impact on the indicators of milk productivity of animals (Table 2). With subcutaneous administration of a tissue bio-stimulant at a dose of 22.5 ml/head, daily milk yields increased on days 15, 30, and 60 of lactation, respectively, by 21.0 ($p < 0.01$), 18.9 ($p < 0.05$) and 18.6% ($p < 0.05$) in comparison with the control group. During 60 days of lactation, animals from the experimental group produced 32.0% more milk ($p < 0.01$) than in the control.

An increase in daily milk yields in the experimental group was accompanied by a slight decrease in protein and fat content — by 0.1 and 0.4% ($p < 0.05$) relative to the control group. However, the yield of milk protein and fat for 60 days of lactation due to quantitative indicators of milk yield in cows from the

experimental group increased by 26.7 ($p < 0.05$) and 22.1% ($p < 0.05$).

2. Milk production of the Priobsky type of the Black-Pied cows (*Bos taurus taurus*) when using a tissue bio-stimulant from the slaughterhouse by-products of reindeer ($n = 10$, $M \pm$MSE; AO Uchkhoz Prigorodnoye, Barnaul, the Altai Region, 2019)

Paramter	Group	
	control	test
Daily milk yield, l:		
day 15	33,3±0,75	40,3±1,99**
day 30	34,9±1,67	41,5±2,05*
day 60	35,3±0,89	41,9±2,03*
Milk yield over 60 days, l	2002,0±113,95	2644,0±57,76**
Protein content per 1 liter, %	3,1±0,07	3,0±0,17
Milk protein yield over 60 days of lactation, kg	63,5±4,28	80,5±3,58*
Fat content per 1 l, %	4,5±0,09	4,1±0,08*
Milk fat yield over 60 days of lactation, kg	92,0±4,95	112,4±4,32*

N o t e. See the description of groups in the "Materials and methods" section.
*, ** Differences with control are statistically significant at $p < 0.05$ and $p < 0.01$.

The increase in dairy productivity of farm animals with the use of tissue preparations has also been established by other authors [14, 52, 53]. The active principle of tissue bio-stimulants, according to academician Filatov, are substances produced by cells in the process of vital activity in extremely unfavorable conditions, which he called biogenic [54]. These include a complex of organic carboxylic acids, compounds such as albumins and peptones – large protein fragments of incomplete hydrolysis of proteins that have a general stimulating effect on the body, actively participate in physiological processes in tissues and organs. In the mechanism of action of tissue preparations, the leading role is assigned to the neuro-humoral and humoral systems, the basis of which is the central nervous system and the hypothalamic-pituitary complex. It has been established that the main role in changing the body's resistance to external influences belongs to the nervous system, its adaptive-trophic function. The hypothalamic-pituitary complex regulates neuroendocrine activity and supports homeostasis [14].

During the early lactation of cows, the catabolic nature of metabolism leads to a change and redistribution of the main metabolic flows to the processes of lactogenesis and lactopoiesis [55]. A need to activate metabolism, which can be achieved through the use of tissue bio-stimulants, is observed. Signals from mechanical, chemical, and other stimuli are transformed into signals directly related to the central nervous system and all links of the neurohumoral apparatus, which causes a variety of effects of tissue bio-stimulants on various physiological systems of the body [56].

In the present experiment, the use of tissue bio-stimulant led to an increase in the concentration of total protein, albumins in the blood serum of cows, an increase in the albumin to globulin ratio, and cholesterol level. Activation of protein and energy metabolism leads to an increase in the secretory function of the mammary epithelium, which is expressed in an increase in milk productivity [57].

Thus, the administration of tissue bio-stimulant derived from reindeer waste at a dose of 22.5 ml/head 4-fold with an interval of 14 days contributed to the activation of metabolism and an increase in the indicators of plastic and energy metabolism in lactating cows of the Priobsky type of the Black-Pied breed. The serum content of total protein, albumins and cholesterol increased relative to the control by 3.0-6.4 ($p < 0.01$), 9.2-6.9 ($p < 0.001$), and 18.3-26.0% ($p < 0.01$), respectively, with an increase in the albumin to globulin ratio by 9.0-11.1% ($p < 0.01$). Milk productivity over 60 days of lactation increased by 19.3% ($p < 0.01$), the yield of milk protein and fat by 26.7 ($p < 0.05$) and 22.1% ($p < 0.05$).

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HISTOSTRUCTURE OF THE TRACHEAL WALL OF BROILER CHICKENS DEPENDING ON AIR CIRCULATION CONDITIONS IN CLOSED POULTRY HOUSES

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Abstract

Currently, optimization of indoor microclimatic conditions in poultry houses is attracting considerable interest due to the intensification of broiler meat production. However, given the increase in flock sizes of broilers, little research has focused on the effect of microclimate parameters in poultry houses on the bird's respiratory system. Insufficient air exchange in the premises can cause functional respiratory disorders in broiler chickens. This paper is the first to report that air circulation in closed poultry houses contributes to maintaining productivity and improves the histostructure and histochemical properties of the tracheal wall in broiler chicks (*Gallus gallus domesticus*). Our work aimed to study the influence of different air circulation regimes in closed poultry houses on histostructure and histochemical characteristic of the trachea in Ross 308 broiler chicks and their productive performance. The study was conducted in 2020-2021 at the LLC Chelny-Broiler poultry farm (Republic of Tatarstan). Ross 308 cross broiler chicks were raised until 39 days of age in five closed premises under different airflow distribution and air circulation (five groups of 35 birds each). For morphometry, 525 preparations of 175 trachea specimens from of all broilers (2500 g bodyweight) were measured. Trachea sections were stained by hematoxylin and eosin procedure. For histochemical studies of acidic and neutral mucins, sections were stained by a combined method for detecting polysaccharides using the Schiff-iodic acid (PAS-reaction) and alcian blue according to the manufacturer's recommended (LLC Labico, Russia). In the control groups 2, 3, and 4, there was no air circulation; in the experimental groups I and V, circulation was provided by forced ventilation, capacity of 8.5 thousand m^3/h (SF-550-02, AgroKurs, Russia). Ventilation was run at the 10 day-age of the broiler chicks. Insufficient air circulation in the poultry rearing rooms caused destructive changes in the tracheal mucous membrane, i.e., its own lamina proliferation, edema, a decrease in the height of the epithelium, and destruction of cilia. This led to metaplasia of the epithelium and disruption of mucociliary transport. The thickness of the mucous membrane and its own lamina was minimum in the experimental group 1 (147.2±3.3 μm and 129.1±3.1 μm, respectively) and maximum in the control group 3 (404.7±9.4 μm and 395.7±9.4 μm) ($p \leq 0.01$). The thickness of the tracheal epithelial layer significantly increased in the experimental groups 1 and 5 (by 14 % on average) compared to the control groups 1, 2, and 3 ($p \leq 0.01$). The lack of indoor air circulation led to a significant decrease in the height of cilia in the control groups 2, 3, and 4 (by 39.5, 58.1, and 67.5 %, respectively) as compared to the experimental groups 1 and 5. The increase in birds' bodyweight at 5 weeks of age in the experimental group 1 increased compared to the control groups 2, 3, and 4 by 6.5, 3.2, and 7.1 %, respectively ($p \leq 0.05$). The histochemical characteristics suggests the presence of simple multicellular endoepithelial glands in the

tracheal epithelium layer of birds. Thus, with the provision of proper air circulation in an enclosed space, the thickness of the mucous membrane and its own lamina decreases, and the thickness of the epithelial layer and the height of the tracheal cilia increases. These characteristics are indicative of proper airexchange in the poultry houses.

Keywords: *Gallus gallus*, trachea, histostructure, tracheal mucosa, ciliated epithelium, microclimate, air circulation, ventilation system, respiratory tract, histochemistry, PAS-reaction, alcian blue

The morphofunctional structure of mammalian respiratory systems has been studied well, in particular the morphogenesis of ciliary and ciliated epithelium [1-3], the mechanisms of the mucociliary apparatus [4, 5], whereas the respiratory anatomy of birds remains relatively understudied [6, 7]. Earlier research has covered the histological structure of respiratory tracts in various avian species [8, 9], including tracheal and laryngeal morphology of quails [10], broilers [11], turkeys [12], and guinea fowls [13, 14], as well as the lungs and air sacs of geese, turkeys, chickens, and ducks [15].

Avian respiratory systems maintain gas exchange and temperature homeostasis in the body. The trachea carries air from the larynx to the lungs, moistens and warms up such air while also removing mechanical particles, bacteria, and viruses from it. Like mammals, birds' trachea consists of a mucous membrane, submucosa, fibrocartilage, and adventitia [9, 16]. The mucous membrane is lined with multirow ciliated epithelium that consists of several cell types. Those are mainly tall ciliated cells that reach the edge of the layer and bear cilia on the apical pole. Epithelial cells also include goblet cells, which are simple unicellular glands that produce a mucous secretion [1].

The ratio of cell types depends on the species, age, health status, and environment. Matveev et al. [11] studied the trachea in Ross 308 broiler chicks and found the goblet cells to be in a 1:10 ratio to the rest of the epithelial cells. Lamina propria, made of loose connective tissue, underlies the epithelium. It contains collagen, elastic, and reticular fibers with the amorphous ground substance in-between [12]. Then goes the submucosa, also of loose connective tissue. Some authors refer to lamina propria and the submucosa as dense connective tissue [9]. Then follows fibrocartilage consisting of hyaline rings with dense connective tissue in-between. Birds have closed overlapping tracheal cartilages that can ossify in some species [12, 16]. The mucus secreted by goblet cells and tracheal glands is viscous and contains PAS-positive glycoproteins and glycosaminoglycans that are stained with Alcian blue [1]. Glycosaminoglycans are highly sulfated polysaccharides that form proteoglycans with protein molecules [17]. This mucus is on top of the cilia and catches mechanical particles and bacteria carried by the inhaled air. The ciliated apparatus, goblet cells, and tracheal glands operate in sync and make the mucociliary transport system in the trachea and in the bronchi, thus completing the defense system. Abnormalities in this system cause inflammation [1].

As a defensive mechanism, the trachea is the first to suffer the effects of environmentally induced changes in, or damage to, the histological structure. Thus, the ciliated epithelium has been found to be able to convert into typical multilayered epithelium when exposed to aggressive external effects (toxic vapors, mechanical or thermal effects) [1]. There are reports on the suppressive effects of radiation on all tracheal wall elements [7], as well as on the negative carbon dioxide effects that cause mucosal edema [2]. Some papers note the effects of low temperatures on the anatomy and histology of the trachea [4, 18, 19].

In most cases, failure to comply with the physiological standards of poultry farming contributes to non-infectious respiratory diseases in broilers [20]. Respiratory allergies in birds might be caused not only by pathogenic microflora in the air-dust bioaerosol [21, 22] but also by insufficient air circulation in enclosed

facilities [23, 24].

As of today, the histological structure of the respiratory system in poultry has been studied well in the context of resistance to respiratory diseases [25], infectious bronchitis in chickens [26, 27], and laryngotracheitis virus [28, 29], air contamination [20], microclimate uniformity [30, 31], harmful gas concentrations [32, 33]; however, the histological and histochemical structure of tracheal walls in birds grown in enclosed facilities with different air circulation rates has not been studied yet.

This paper is the first to report that air circulation in enclosed poultry houses contributes to maintaining productivity and improves the histological structure and histochemical properties of the tracheal wall in broiler chicks.

The objective hereof was to determine the histological and histochemical structure of the tracheal wall, as well as the dynamics of live weight in Ross 308 broiler chicks (*Gallus gallus domesticus*) kept in ventilated vs. non-ventilated facilities.

Materials and methods. The research was carried out in 2020-2021 at Chelny-Broiler LLC (Republic of Tatarstan), which has a good epizootic status. Ross 308 cross broiler chicks were raised until 39 days of age in five enclosed facilities under different airflow distribution and air circulation conditions (five groups of 35 birds each, sampled by the analog-pair method). The poultry was kept on a deep litter. It was fed in seven phases with an all-in-one formula. Live weight was recorded specimen-by-specimen during Weeks 3, 4, and 5.

Tracheae were sampled from all 35 broilers (2500 g live weight on average) in all of the five groups, a total of 175 samples. The samples were fixed in 10% formalin, then washed and poured into paraffin. Five-micrometer-thick tissue sections (three for each sample, a total of 525 preparations) were made per the standard guidelines [17], stained with hematoxylin and eosin to make review preparations. For histochemical studies of acidic and neutral mucins, sections were stained by a combined method for detecting polysaccharides using the Schiff-iodic acid (PAS-reaction) and Alcian blue staining (acidic glycosaminoglycans are stained blue whereas PAS-positive glycoproteins are stained purple) [17]. For more accurate differentiation of glycoproteins and acidic glycosaminoglycan-containing proteoglycans, sections taken from the same samples were stained with the PAS reaction only. The researchers used reagent kits from Labico LLC and followed the manufacturer's manuals.

The preparations were then inspected with a Biolam M-3 optical microscope (LOMO JSC, Russia) using different magnifications (15×8, 15×20, 15×40), photographed, and described. Epithelial thickness and tracheal cilia were counted using an ocular micrometer; relative values were then converted into absolute values using an object micrometer.

In all facilities where poultry was kept, air exchange was provided by a supply-and-exhaust ventilation system using negative pressure. SF-550-02 circulation axial fans (AgroKurs, Russia), each rated at 8.5 thousand m^3/h (total capacity of 42.5 thousand m^3/h) in Chambers 1 (Group 1) and 5 (Group 5) were installed at the same height as gas generators and placed 10.8 m away from the gas generator outlets, sloped at 5° down towards the poultry. Circulation fans were powered together with the gas generators so that air heating and circulation would start simultaneously. In Chamber 1, air flow from the fans went towards the exhaust vents whereas in Chamber 5, it went from the exhaust vents. Circulation fans were started once the broilers reached 10 days of age. Facilities with the control groups (2, 3, and 4) had no air circulation. Control groups differed in the age of parent flocks that the broilers were brooded by: 28 weeks in Group 2, 47 weeks in

Group 3, and 38 weeks in Group 4.

The experiments were in line with the Guide for the Care and Use of Agricultural Animals in Research and Teaching, 3rd edition (Federation of Animal Science Societies, 2010). Everything was done to minimize the birds' suffering and the number of euthanized specimens.

Data were processed by variational statistics using Student's *t*-test in Microsoft Excel 2010. For processing, the researchers calculated the means (*M*) and standard errors of the mean (±SEM). Significance thresholds were $p \leq 0.01$ for biological values, $p \leq 0.05$ for zootechnical values.

Results. In Ross 308 chicks, tracheal morphogenesis ends on Day 35 of postnatal ontogenesis [11]; thus, none of the histological structure changes observed were age-related. Tissue samples taken from Groups 1 and 5 differed from those of Groups 2, 3, and 4: they had thinner mucous membranes and laminae propriae.

1. Thickness of tracheal mucosa layers (μm) in Ross 308 broilers (*Gallus gallus domesticus*) as a function of air circulation in the facility (*M*±SEM, poultry house conditions, Chelny-Broiler LLC, Republic of Tatarstan, 2020-2021)

Tracheal wall	Group (n = 105)				
	1	2	3	4	5
Mucosa	147.2±3.3	267.5±4.1*	404.7±9.4*	298.1±10.5*	161.2±2.9*
Lamina propria	129.1±3.1	253.9±4.2*	395.7±9.4*	285.3±10.4*	144.8±2.9*
Epithelial layer	16.1±0.4	16.2±0.3	13.2±0.3*	15.1±0.3*	18.4±0.4*
Cilia	4.3±0.1	2.6±0.1*	1.8±0.1*	1.4±0.1*	4.3±0.1*

N o t e. See the description of groups in the "Materials and methods" section.
* Differs statistically significantly from Group I at $p \leq 0.01$.

Group 1 birds had minimum mucous membrane and lamina propria thicknesses (147.2±3.3 and 129.1±3.1 μm, respectively), whilst Group 3 had the maximum values (404.7±9.4 μm and 395.7±9.4 μm, respectively) ($p \leq 0.01$). The epithelial layer was significantly thicker in Groups 1 and 5 by an average of 14% than in Groups 2, 3, and 4 ($p \leq 0.01$). Lack of air circulation in the facilities resulted in a statistically significant reduction in cilia height in Groups 2, 3, and 4 by 39.5%, 58.1%, and 67.5%, respectively, against Groups 1 and 5 ($p \leq 0.01$), see Table 1.

The thickness of the epithelium and height of the cilia correlate positively with the ability of the mucosa to retain particles of inhaled air. As organelles of movement, cilia have an important role to play in protecting the respiratory tract from exogenous particles; they are also involved in nonspecific immune responses [1]. A statistically significant increase in cilia size in the air-circulated groups was a sign of better growth conditions. The cilia size did not correlate with the thickness of the epithelium.

Micrographs of the tracheal wall histostructure are shown for Groups 1, 5, and 3 based on the minimum and maximum values.

Histological testing confirms the existing data on the tracheal wall structure (mucous membrane, submucosa, fibrocartilage, and adventitia [9, 16, 34]. The mucosa, in turn, consisted of multilayer ciliated epithelium and lamina propria of loose connective tissue. Beside the fibrous component and the amorphous ground substance, it also contained cellular forms characteristic of this tissue type, as well as leukocytes. The submucosa consisted of loose connective tissue, the fibrous component being predominant, which is also in line with earlier reports [9, 11-13]. Fibrocartilage consisted of closed hyaline cartilage rings and dense connective tissue in-between. Due to the anatomical overlapping of tracheal rings, sections (cuts) of two adjacent rings were visible in the preparation. Adventitia consisted of loose connective tissue, see Fig. 1.

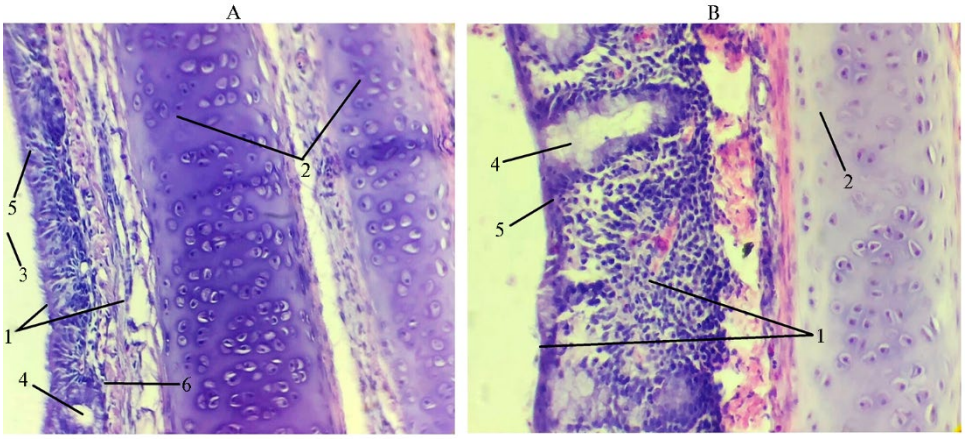

Fig. 1. Histological structure of the tracheal wall in Ross 308 chicks (*Gallus gallus domesticus*) of Group 1 kept in a ventilated facility (A)**, and Group 3 kept in a non-ventilated facility** (B)**:** 1 — the mucous membrane; 2 — the fibrocartilage; 3 — the tracheal lumen; 4 — mucous glands; 5 — epithelium; 6 — lamina propria (poultry house conditions, Chelny-Broiler LLC, Republic of Tatarstan, 2020-2021). Staining with hematoxylin and eosin, magnification ×15×20, a Biolam M-3 microscope (AO LOMO, Russia).

Complex PAS reaction and Alcian blue staining of sections made it possible to trace the distribution of acidic glycosaminoglycans and PAS-positive glycoproteins in the structures of the organ wall, see Fig. 2.

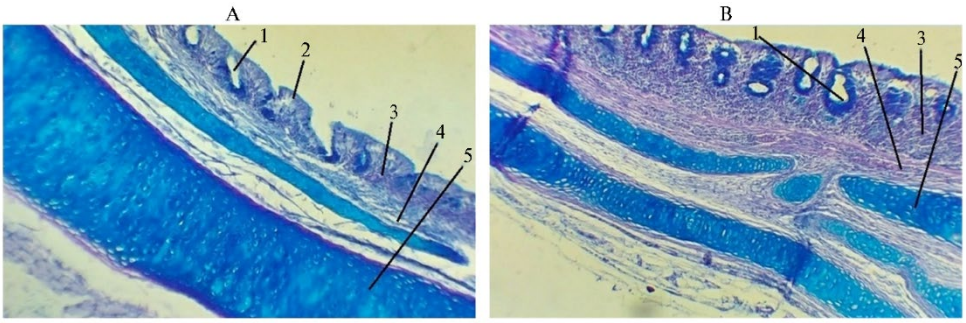

Fig. 2. Histological structure of the tracheal wall in Ross 308 chicks (*Gallus gallus domesticus*) of Group 5 kept in a ventilated facility (A) **and Group 3 kept in a non-ventilated facility** (B)**:** 1 — the glands; 2 — epithelium; 3 — lamina propria; 4 — the submucosa; 5 — the fibrocartilage (poultry house conditions, Chelny-Broiler LLC, Republic of Tatarstan, 2020-2021). Staining with Alcian blue + Periodic Acid — Schiff (PAS) reaction, magnification ×15×8, a Biolam M-3 microscope (AO LOMO, Russia).

An additional PAS reaction confirmed the localization of neutral mucopolysaccharides, as they would sometimes be overlapped by a brighter Alcian blue stain. Thus, acidic mucopolysaccharides containing sulfated glycosaminoglycans and stained bright blue in the preparation were mainly localized in the cartilage and were part of the mucus secreted by both unicellular and multicellular glands. Additional PAS-enabled identification showed that purple-stained neutral glycoproteins were present in the maximum concentration in the gland secretion yet were much less stained in the cartilage region. In the fibrocartilage, they were mainly localized in the perichondrium and in the young cartilage region. The observed distribution of chemical groups showed the chondromucoid to consist more of sulfated (acidic) mucopolysaccharides rather than their neutral counterparts. Gland-secreted mucus was composed of acidic and neutral mucopolysaccharides in an even distribution. The distribution of these substance categories in

other structures of the wall showed that both acidic glycosaminoglycans and neutral glycoproteins were present in the intercellular substance of loose connective tissue in moderate amounts.

Group 5 birds had no pathological changes in the ciliated epithelium. Lamina propria had visible fibrocyte nuclei, a fibrous component, and an amorphous ground component. Single-layered multirow ciliated epithelium had tall cylindrical tells with cilia on the apical pole, as well as cambial cells that were located near the basal membrane without reaching the edge of the layer. The apical pole of the cells was not destroyed, see Fig. 1. In some places, the epithelial layer had mucus-secreting goblet cells. Beside goblet cells, the epithelial layer also contained simple endoepithelial glands (see Fig. 3) as noted in [9, 12, 13]. Their contents had both a PAS-positive reaction and Alcian blue staining. Goblet cells were found as single cells and clusters alike, see Fig. 4; some studies report the same [4, 12].

Besides, lamina propria had simple tracheal mucous glands that had the same staining. The cilia region had more intense Alcian blue staining whereas the gland-secreted mucus was equally well-stained by both methods. Perhaps the near-cilia mucus, being a more liquid fraction [4], contained more acidic mucopolysaccharides. Mucosal and submucosal connective tissue consisted mainly of acidic mucopolysaccharides, see Fig. 2. Histostructural examination of the trachea in Group 1 birds made findings similar to those of Group 5.

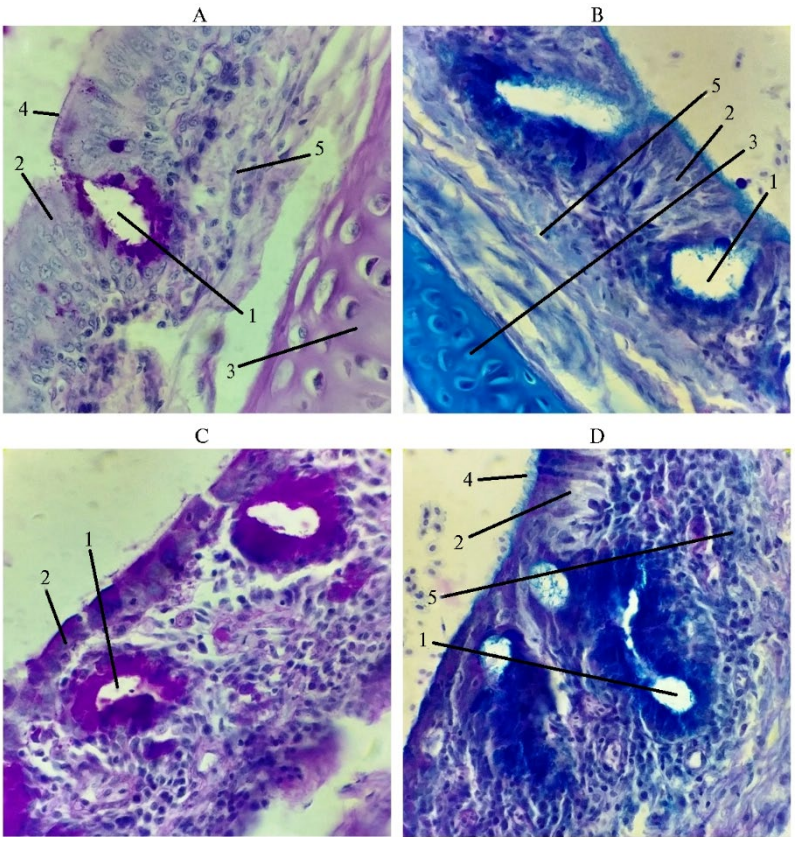

Fig. 3. Histological structure of the tracheal wall in Ross 308 chicks (*Gallus gallus domesticus*) of Group 5 kept in a ventilated facility (A, B) **and Group 3 kept in a non-ventilated facility** (C, D)**:** 1 — the glands; 2 — epithelium; 3 — the cartilage; 4 — the cilia; 5 — lamina propria (poultry house conditions, Chelny-Broiler LLC, Republic of Tatarstan, 2020-2021). PAS reaction to the left, Alcian blue staining + PAS reaction to the right, magnification ×15×40, a Biolam M-3 microscope (AO LOMO, Russia).

Group 3 birds had noticeably thinner tracheal epithelium, see Fig. 1. The epithelium had local signs of metaplasia; elsewhere, it was significantly lower ($p \leq 0.01$) than in Groups 1 and 5, with local sites of the near-complete absence of cilia. The tracheal lumen sometimes contained cell clusters or singular cells including erythrocytes, macrophages, and leukocytes that had migrated from lamina propria to the epithelial surface. This is in line with the reports on congestion in the avian respiratory systems due to insufficient air circulation [4]. Partial deficit of air exchange results in an excess concentration of harmful gases, as well as in increased dustiness, which may cause inflammation of the tracheal wall. A similar effect was observed when growing broilers from 42 days of age in a poultry house where the air contained 100 ppm NH_3/l (the experiment lasted 1 week) [32], as well as in an experiment where turkeys were kept in a poultry house where the air contained 10 ppm NH_3/l [33]. The experimenters did not report on damage to the ciliated epithelium or goblet cells in the trachea; however, the trachea had suppressed mucociliary apparatus in turkey: the cilia were tangled, and some sites were deciliated [32, 33].

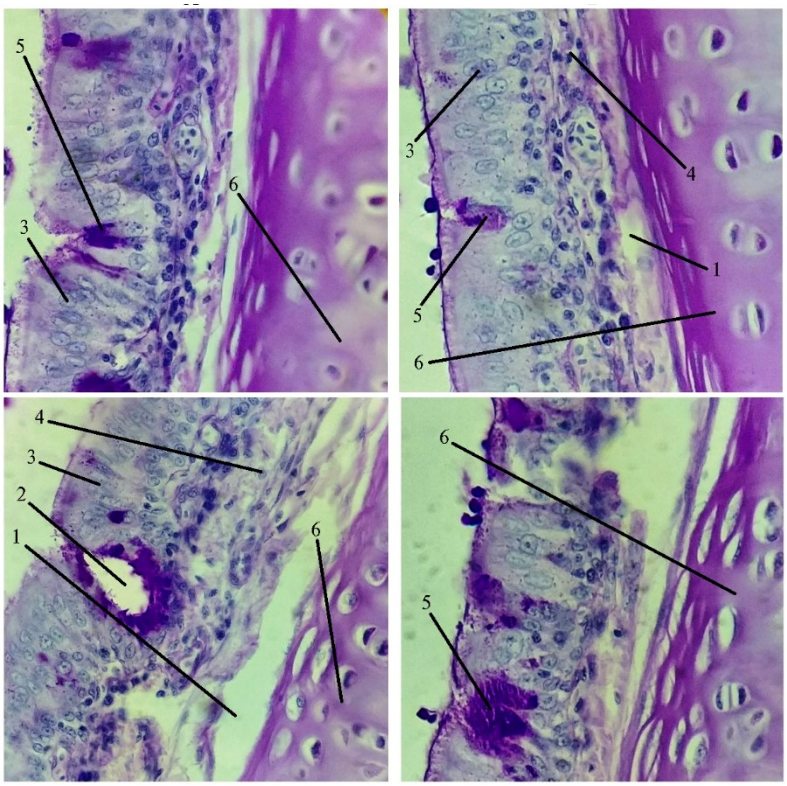

Fig. 4. Endoepithelial glands and goblet cells in trachea of Ross 308 chicks (*Gallus gallus domesticus*) of Group 5 kept in a ventilated facility: 1 — the submucosa; 2 — endoepithelial glands; **3** — epithelium; 4 — lamina propria; 5 — goblet cells; 6 — the cartilage (poultry house conditions, Chelny-Broiler LLC, Republic of Tatarstan, 2020-2021). PAS reaction, magnification ×15×40, a Biolam M-3 microscope (AO LOMO, Russia).

The data collected in this experiment is partly consistent with the reports on the pathologies that are sometimes encountered in infected chicks. Thus, restructuring of the ciliated epithelium, catarrhal inflammation, and epithelial edema in the mucous membrane due to lymphocyte infiltration are observed in birds affected with an avian influenza virus isolate or with laryngotracheitis [28, 29]. Deciliation, desquamation, and alteration of ciliated epithelium cells in the trachea are observed in chickens infected with the infectious bronchitis virus [26, 27, 35].

Intense PAS reaction and Alcian blue staining of the epithelium were observed when trying to identify mucopolysaccharides in Group 3, which did not happen in Groups 1 or 5. Lamina propria of the tracheal mucosa in Group 3 consisted mainly of the cellular component; fibers were few. Leukocyte (mostly lymphocyte) infiltration of the mucous membrane was observed. The loose connective tissue of the mucosa and submucosa had more glycoproteins in Group 3 than in either Group 1 or 5, see Fig. 2. The epithelium was not intensely multirow; deciliation and metaplasia sites were observed. Groups 2 and 4 had a similar tracheal histostructure to Group 3.

Increased concentrations of carbon dioxide in inhaled air may cause mucosal edema as confirmed earlier in [2]. Mucous membrane layers in the tracheae of chickens infected with infection bronchitis virus strains of various serotypes have moderate lamina propria edema and delamination of tracheal cells with an increased quantity of goblet cells [26, 27].

Goblet cells and tracheal glands are believed to secrete viscous mucus when exposed to local irritants; they are not innervated with adrenergic and cholinergic receptors [35]. Besides, goblet cells are short-lived (2 to 4 days [1]), which causes increased sensitivity to external effects. Thus, various local effects on the mucosa may cause qualitative and qualitative changes in these glands.

The research team discovered the presence of both tracheal glands and goblet cells in the initial segment of the trachea in Groups 1 and 5; however, the balance was shifted towards the glands. These findings are in line with the earlier reports [9, 12, 13] that the glands are dominant in the initial sections whereas the goblet cells dominate the caudal trachea. The goblet cells were found as both isolated and clustered cells. Hystochemical staining revealed cells fully filled with mucous secretion; some rare goblet cells had weak staining, presumably due to generalized production of granule secretion, see Fig. 4. Human goblet cells have been described similarly [1]. Some authors note an increase in goblet cells when the tracheal epithelium is exposed to adverse environmental factors [4, 7, 26, 27].

In this research, Groups 2, 3, and 4, which had insufficient ventilation, had less goblet cells, perhaps due to structural epithelial changes; however, they had more tracheal glands. This was confirmed by the increased amounts of PAS-positive mucopolysaccharides in the loose connective tissue around the glands. Notably, the control groups had significantly more acidic and neutral mucopolysaccharides than the experimental groups. Such histochemical and morphological changes in the tracheal wall of birds whose facilities were not sufficiently ventilated could be a compensatory mechanism for the recovery of mucociliary transport.

2. Live weight (g) **dynamics in Ross 308 broilers (*Gallus gallus domesticus*) as a function of air circulation in the facility** ($M\pm$SEM, poultry house conditions, Chelny-Broiler LLC, Republic of Tatarstan, 2020-2021)

Group (n = 35)	Week 3	Week 4	Week 5
1 test	1047.6±10.6	1712.2±12.4	2366.9±19.2
2 control	1080.9±10.2	1602.0±19.2*	2214.1±29.1*
3 control	1149.3±11.7	1688.2±15.5	2290.7±18.5*
4 control	1110.4±10.3	1591.1±10.4*	2198.2±21.0*
5 test	1159.6±11.2	1690.1±16.5	2317.6±20.0

N o t e. See the description of groups in the "Materials and methods" section.
* Differs statistically significant from Group I at $p \le 0.05$.

Live weight gain in broilers as affected by different air circulation conditions was also studied, see Table 2. At five weeks of age, Group 1 had gained more weight than the birds in Groups 2, 3, and 4 by 6.5%, 3.2%, and 7.1%, respectively ($p \le 0.05$). At four weeks of age, Group 1 differed from Groups 2 and 4 by 6.4% and 7.1% ($p \le 0.05$), whereas no statistically significant differences

had been observed until 4 weeks of age.

These findings confirm the fact that air circulation in enclosed facilities affects the histostructure and the histochemical status of the tracheal wall in Ross 308 broilers. Insufficient air circulation in growing chambers causes destructive changes in the tracheal mucosa, which manifest as the growth of lamina propria, edema, reduced epithelium height, destruction of cilia, and lower productivity. This causes epithelial metaplasia and disrupts mucociliary transport. Air circulation in enclosed facilities reduces the thickness of the mucous membrane and increases the height of the epithelial layer and tracheal cilia. The benefits of air circulation in enclosed poultry houses are reliably evidenced by a 2.6x reduction in tracheal mucosa thickness (by 61.9%), a 2.9x reduction in the lamina propria thickness (by 65.4%), a 1.3x thickening of the epithelial layer (by 23.3%), and a 3.1x increase in cilia height (by 67.5%), as well as an up to 7.1% increase in live weight gain. The obtained histological preparations suggest that the epithelial layer in birds contains simple multicellular endoepithelial glands. The described changes in the tracheal wall histostructure could indicate unfavorable poultry house conditions. Broiler crosses do not take long to grow; thus, investigations of longer-term effects of high gas and dust concentrations in the air need to involve other poultry groups, e.g., the parent flock.

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QUALITY PARAMETERS IN EGGS OF LAYER CROSS Hisex Brown AS AFFECTED BY THE OVIPOSITION TIME

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Abstract

Chicken eggs are valuable and chip source of the nutrients in human diets; this fact has propelled the interest toward the availability of this commodity and modification of its chemical composition in desirable directions. The morphology of the eggs is closely correlated with certain parameters of nutritive value and shelf life. The formation of eggs is a long process: the maturation of large yellow follicles in the ovarian hierarchy (until the ovulation) lasts for 7-10 days; the formation of egg in the oviduct (since ovulation to oviposition) takes 22.5-26.1 hours, depending on age and productivity level in parental hen. The quality of eggs is affected by multiple factors acting before the oviposition (breed and cross of chicken, individual physiological peculiarities, live bodyweight, laying rate, oviposition time, age, the regimes of management and nutrition, artificially induced moult, stresses, health status) and after the oviposition (conditions of collection, transportation, storage, washing and sanitary treatments, the effects of these factors being also depend on the initial egg quality formed before the oviposition. The optimization of egg quality requires the thorough knowledge on mechanisms and factors involved. In the study presented it was found out that oviposition can affect different parameters of egg quality though insignificantly with the exception of B2 content. The effects of the oviposition time on egg quality was studied on five treatments of commercial Hisex Brown layers (Hendrix Genetics BV, the Netherlands) since 210 to 450 days of age housed in cage batteries mounted in standard windowless poultry house (7 birds per cage) with constant lighting regime 14L: 10D with the onset of lighting at 5 am, setout at 7 pm. The eggs were collected during 5 periods of a day: 5-8 am (treatments 1), 8-10 am (treatment 2), 10-12 am (treatment 3). 0-2 pm Treatment 4), and 2-4 pm. Average weight of the "earliest" eggs (laid until 8 am) was significantly higher (by 2.1-2.5 g or 3.6-4.4 %, $p < 0.001$) as compared to later periods; absolute yolk weight was higher by 7.2-8.7 %, relative yolk weight by 1.0-1.4 % ($p < 0.001$); the resulting albumen/yolk ratio was lower by 4.2-8.0 % ($p < 0.01$-0.001). The late eggs (laid between 0 and 4 pm) featured better eggshell quality as indicated by increased average eggshell weight (by 3.2-6.7 %), eggshell thickness (by 1.8-5.7 %), and egg density (by 0.18-0.46 %). The shape index varied from 78.9 % (0-2 pm) to 77.7 % (2-4 pm). Chemical composition of the eggs was not significantly affected by oviposition time with the exception of the significantly higher concentrations of vitamin B_2 in yolk at early hours (5-8 am) as compared to later hours ($p < 0.05$-0.001). Earlier eggs (5-12 am) featured higher percentages of cracks and crackles while "evening" eggs featured the absence of extra-heavy eggs (>75 g); other external parameters of egg quality were generally similar in all treatments; however, egg weight tended to increase with hen age. The relative weight of the albumen was similar at 420 and 450 days of age, with the minimal index shape eggs laid in the interval 0-2 pm being found (with the exception of 210 and 450 days of age; $p < 0.05$-0.001). The increases in the concentration of total cholesterol in yolk was found at 270 and 420 days of hens' age (with general trend to increase with age). The age-related increase in the concentration of carotenoids in yolk was found at 360 days of age. As well as the trend to higher concentration of vitamin A in yolk.

Keywords: *Gallus gallus* L., laying hens, laying time, egg weight, yolk weight, shell weight, shell thickness, egg shape index, quality defects, chemical composition, vitamin B_2

Chicken eggs are traditionally considered an important and cheap source of nutrients for humans. The formation of large yellow follicles in the ovarian hierarchy (until the ovulation) lasts for 7-10 days, the formation of an egg in the

oviduct (since ovulation to oviposition) takes 22.5-26.1 hours, depending on age and productivity level in the parental hen [1].

The quality of chicken eggs, including external (weight and egg shape; purity, thickness, weight, and strength of shell) and internal (weight of albumen and yolk, height and density of egg albumen, yolk color, Haugh units, chemical composition of albumen and yolk) characteristics [2], is influenced by a complex of various factors. The main part of them (breed, cross, individual characteristics, live weight, egg-laying capacity, egg-laying time, age, feeding and management conditions, forced molting, various stresses, the state of the bird's health) acts during egg formation, other factors (conditions of collection, transportation, storage, washing and processing of eggs) affect the already laid egg [3-7].

Many studies have found that the time of egg-laying affects the quality of eggs and the productivity of laying hens [8–10]. For example, in some works, it is shown [11-13] that the weight of eggs collected at 6^{00} was the largest. According to Harms [14], in a commercial herd of laying hens, the egg weight steadily decreased between 7^{45} and 15^{45}, after which it increased. In the experiments of Patterson [15], the largest weight was typical for eggs laid early in the morning, then (from 5^{00} to 18^{00}) it decreased. The work by Aksoy *et al.* [16] reported that eggs laid at 9^{00} had the largest weight, and after 15^{00} it was the smallest. However, in the experiment of Tůmová *et al.* [17], eggs laid between 10^{00} and 14^{00} were heavier than those laid at 6^{00}.

The time of laying plays an important role in the formation of the quality of the eggshell, since it is known that its weight linearly depends on the time the egg is in the oviduct (shell gland) after ovulation and, consequently, on the thickness and strength of the shell [17].

Most studies have noted that the quality of the shell is higher in eggs laid in the middle of the day [11-13, 18, 19]. According to some authors [13], the higher shell quality of "day" eggs is associated with a greater shell thickness and less elastic deformations. In such eggs, the proportion of the shell in the egg weight is slightly higher — 10.33 and 10.31% in eggs laid at 14^{00} and 10^{00}, respectively, compared with 10.03% in eggs laid at 6^{00} [18]. Similar results were also obtained in the work by Tůmová *et al.* [12]: in studies on three lines of the Dominant cross (Czech Republic), all shell quality parameters (absolute and relative weight, thickness, and strength) were significantly higher in eggs laid in the middle of the day (14^{00}). According to Harms [14], the shell weight of eggs laid before 7^{45} was significantly higher than in eggs laid between 7^{45} and 11^{45}. Then shell weight significantly increased by 12^{45} and remained high until the end of the day, excluding only the interval between 14^{45} and 16^{45}. Other authors [19], on the contrary, observed a tendency to decrease shell weight during the day, and especially strongly in ISA-Brown cross birds (Hendrix Genetics BV, the Netherlands).

When comparing the morphological characteristics of eggs from laying hens of three breeds — Brown Leghorn, Oravka, and Brahma from 20 to 64 weeks of age with deep-litter housing (egg collection at 6^{00}, 10^{00}, and 14^{00} in the beginning, the middle, and the end of the productive period). The maximum weight of eggs was in the Brahma chicken breed at 6^{00}, and the relative weight, strength, and eggshell thickness was significantly higher at 14^{00} [20]. In brown Leghorns, egg-laying time did not have a significant effect on egg weight, relative weight, and the yolk index; the greatest shell thickness was noted at 6^{00}, and the maximum albumen index and Haugh units were at 14^{00}. In Oravka chickens, egg-laying time had no significant effect on egg weight, the relative weight of the albumen, yolk, and shell, the albumen and yolk index, Haugh units. At the same time, the formation of a thicker shell was registered at 14^{00} [20]. In another study [21], when eggs were collected at 10^{00} and 14^{00} in the first case, egg weight (65.25 vs. 63.94 g), absolute and relative albumen

weight (40.91 g and 62.65% vs. 39.94 g and 62.42%), absolute yolk weight (16.56 vs. 16.35 g) and shell weight (7.78 vs. 7.64 g), egg shape index (76.72 vs 76.70%) and shell color (12.18 vs. 12.16 points) were higher, and the relative weight of yolk (25.40 vs. 25.60%) and shell (11.93 vs. 11.98%), Haugh units (76.60 vs. 76.70), shell thickness (0.51 vs. 0.54 mm) were lower.

Recent studies [22] have reported a significant effect of egg-laying time on the mineral content in the shell. Thus, the maximum calcium content (352 g/kg) was observed in eggs laid at 7^{30}, whereas at 15^{30}, this indicator was 2.84% lower. The content of phosphorus and magnesium in the shell increased with increasing egg-laying time: for 7^{30}, these indicators were 1.20 and 3.56 g/kg, respectively, for 15^{30} – 1.43 and 3.88 g/kg. Some authors [23] suggest that higher calcium content in the shell of morning eggs is associated with increased calcium deposition in the medullary bone at night.

According to some researchers [21, 24], the time of egg-laying affects the intensity of the shell color of colored eggs. Chickens lay the most colored eggs in the morning, less colored – in the late afternoon. In another report [24], the intensity of shell coloring decreased with the age of chickens, and the sequence number of an egg in the cycle (the number of eggs laid in a row) slightly affected shell color. Here, in the authors' opinion, there is a certain contradiction, since, as is known, the first egg in the cycle is laid by chickens early in the morning, and with an increase in the serial number, the laying is postponed to a later time of day [1]. In addition, according to many authors [25–28], the color of eggshell positively correlates with its thickness and strength, and these indicators, as already noted [12, 19], are highest in eggs collected at noon or the end of the day, but not in the morning.

There are data about the effect of egg-laying time on the cholesterol level in the egg, while some authors note an increase in its content in morning eggs [17], and others, on the contrary, in noon [29].

Thus, the analysis of scientific publications indicates that the time of egg-laying significantly affects the characteristics of egg quality. However, according to some indicators, the data are ambiguous and often contradictory, which is since when studying the effect of egg-laying time on egg quality characteristics, the authors do not always pay attention to the duration of the light or "subjective" day, and the time of the first light switch on in the lighting mode, which has a significant impact on the time of egg-laying by chickens [1]. In addition, it should be noted a certain genetically determined specificity of the effect of egg-laying time on some indicators of egg quality. This necessitates an in-depth study of morphological and biochemical parameters characterizing the quality of eggs, the mechanisms of their formation, and the conditions affecting these processes in different breeds.

In the presented work, it was found that morphological indicators of egg quality in Haisex Brown chickens were associated with the time of egg-laying, however, the observed effect was not the same for different traits. On average, over the entire observation period, eggs laid before 8^{00} had a higher egg weight, absolute and relative yolk weight. Eggs laid before noon had a greater number of shell defects, and those laid from 12^{00} to 16^{00} had a higher shell quality. No significant changes were detected in the chemical composition of the egg depending on the time of laying (except for vitamin B_2 content).

The work objective is to determine the quality indicators of eggs in the Haisex Brown egg cross hens depending on the time of egg-laying, starting from the peak productivity.

Materials and methods. The study was carried out on chickens of the commercial herd of Haisex Brown cross in the conditions of a typical windowless

poultry house (vivarium of the Selection and Genetic Center Zagorskoe Experimental Breeding Farm ARRTPI RAS, 2020). Among 210-day-old chickens of the same age, kept in KP-112LM cage batteries (Pyatigorskselmash, Russia), 7 birds per cage (cage area – 531 cm^2/bird) with a constant lighting mode of 14 h (light)/10 h (darkness) (turning on the light at 5^{00}, turning off at 19^{00}), a group of 490 laying hens was selected. The feeding and management conditions, which are not the subject of study in this experiment, were maintained following generally accepted and valid recommendations for the experimental period [30]. Eggs were collected at 5^{00}-8^{00}, 8^{00}-10^{00}, 10^{00}-12^{00}, 12^{00}-14^{00}, 14^{00}-16^{00} (groups 1, 2, 3, 4, 5 respectively). Accounting data were recorded in the age periods of 210 days, 270 days, 360 days, 420 days, and 450 days (in each group, 30 eggs were collected 3 days in a row — on days 210-212, days 270-272, days 360-362, days 420-422, and days 450-452).

The weight of eggs (g) was determined by individual weighing on laboratory scales with an accuracy of 0.1 g; the yield of eggs by categories (%) was determined by the results of weighing and inspection of eggs laid by chickens for 3 consecutive days, according to the current GOST ("Interstate standard GOST 31654-2012 "Food chicken eggs. Specifications". Moscow, 2013). Egg shape index (%) was measured using an indexometer during weighing; egg density (D, g/cm^3) — by weighing in distilled water and air in a sample of 30 eggs from each group (10 eggs, 3 days in a row) on the same scales (accuracy up to 0.01 g). D value was calculated as $D = W/(W - W1)$, where W is the weight of an egg in air (g), W1 is the weight of an egg in water (g). The weight of the albumen, yolk, shell, albumen/yolk weight ratio were estimated when separating and individually weighing the components in a sample of 30 eggs from each group (10 eggs, 3 days in a row) (laboratory scales, measuring accuracy up to 0.1 g). The albumen index (AI) was evaluated by the ratio of the height of the outer layer of dense albumen (measured with a micrometer with an accuracy of 0.01 mm) to the average diameter of its spreading. The formula for calculation is $AI = 2h/(d + D) \times 100\%$, where h is the height of dense albumen (mm), d and D are small and large diameters of the poured albumen (mm). Yolk index (YI) was evaluated by the ratio of the height of the yolk poured onto the horizontal surface (measured with a micrometer with an accuracy of 0.01 mm) to the average the diameter of its spreading. YI was calculated as $YI = 2h/(d_1 + d_2) \times 100\%$, where h is the height of the yolk (mm), d_1 and d_2 are small and large diameters of the poured yolk (mm). Haugh units were assessed according to a special table [32] based on the height of the albumen and the weight of the egg. The thickness of the shell (μm) was measured using a micrometer (accuracy up to 0.01 mm) in three parts of the egg (in the middle, at the blunt and point ends) with calculation of the average value for a sample of 30 eggs from each group (10 eggs, 3 days in a row). The intensity of the yolk coloring was individually assessed on the BASF color chart.

Total cholesterol, carotenoids, vitamins A, E and B_2 (μg/g) in the yolk, vitamin B_2 (μg/g) in the albumen, calcium (%) in the shell were determined in the sample of 5 eggs (combined sample) from each group 3 days in a row in the middle of each month during the observation period.

When measuring carotenoids (sum) and vitamins in egg yolk, single sample preparation was used (saponification of samples with 50% potassium hydroxide solution followed by extraction with diethyl ether) [32]. The mass fraction of vitamins A and E was determined by the normal-phase high-performance liquid

chromatography (chromatographic system Knauer Advanced Scientific Instruments, Knauer Engineering GmbH Industrieanlagen & Co., Germany) following P 4.1.1672-03 “Guidelines for quality control and safety of biologically active food additives” (Moscow, 2003). Carotenoids were measured colorimetrically (photometer KFK-3-01, ZOMZ, Russia) using potassium bichromate for constructing a calibration graph with OD_{450} measurement (blue light filter). To quantify vitamin A and carotene, the 292 and 450 nm wavelengths were used where the absorption spectra of these substances practically do not overlap [33]. The components were separated on a column Luna 5 μ m Silica(2) 100 A New Column 150×4.6 mm (Phenomenex, USA), eluted with a mixture of hexane:isopropyl alcohol (98:2), the detection of vitamins A and E was performed at 292 and 324 nm, respectively, preparations Retinol Sigma of the category No. R 7632 were used as standards (Sigma-Aldrich, USA) and (+/–)-α-Tocopherol Fluka category No. 95240 (Fluka, Germany).

Water-soluble vitamin B_2 (riboflavin) in egg yolk and egg albumen was determined fluorometrically using a Fluorat-02-3M liquid analyzer (NPFNP Lumex, Russia). Sample preparation consisted of alcohol extraction (96% ethanol from albumen, 55% ethanol from yolk) followed by filtration through a paper mesoporous filter (“yellow stripe”). The fluorescence intensity of the obtained solutions was measured in ultraviolet rays, the concentration of riboflavin was calculated relative to the working standard solution of vitamin B_2.

To determine total cholesterol, the albumen was separated from the yolk. The yolk membrane was pierced, the yolk was poured into a glass container and frozen for 24 hours in the freezer at –20 °C. The frozen yolk was placed in a TFD series freeze dryer (Ilshinbiobase Co., Ltd., Korea) for 48 hours at –77.8°C and at a pressure of 5 mTorr, which made it possible to dry yolk, removing up to 97% of moisture, preserving biologically active substances. The dried yolk was crushed in a laboratory mortar, a subsample (100 mg) was mixed with Ringer's solution in a ratio of 1:100 and homogenized (B. Braun Melsungen AG, Germany) for 1 minute at 1500 rpm. The resulting liquid was centrifuged at 4000 rpm for 3 minutes. Cholesterol concentration was measured in the supernatant by the endpoint method on a BS-3000P semi-automatic biochemical analyzer (Sinnowa Medical Science & Technology Co., Ltd., China) with a flow cell, using the appropriate set of reagents of the company DIAKON-VET (Russia).

Calcium was determined following the adapted method (GOST 26570-95 "Fodder, mixed fodder and mixed fodder raw material. Methods for determination of calcium". Moscow, 2003) in an air-acetylene flame on an atomic absorption spectrophotometer (VARIAN, USA). The samples were prepared by the dry ashing technique. To prepare the standards, the state standard sample was used (Calcium ion GSO 7772-2000, EAA Eco-Analytica, Russia).

The data were processed by methods of variational statistics in Microsoft Excel. The tables show the means (M) and their standard errors (±SEM). The statistical significance of the differences between the groups was assessed by Student's t-criterion at $p < 0.05$.

Results. Weight is one of the main indicators of the quality of incubation and food eggs. With the change in the weight of the egg, its quality also changes in many ways [3]. The results of the performed study showed (Table 1) that egg-laying time affected such an important indicator of egg quality as weight. For example, in all age periods and on average during the experiment, the weight of eggs laid from 5^{00} (the moment the light was turned on) to 8^{00} h (group 1) was

significantly ($p < 0.01$-0.001) higher than later during the day. The exception was eggs from 450-day-old laying hens: the difference was insignificant, but the trend remained. On average, during the experiment, group 1 of eggs exceeded the others by weight by 2.1-2.5 g, or by 3.6-4.4% ($p < 0.001$). The obtained data are consistent with the results of other authors [11, 34-36], in whose studies eggs laid early in the morning had a higher weight than those laid during the rest of the day. In the present experiment, eggs collected at 8^{00}-10^{00}, 10^{00}-12^{00}, 12^{00}-14^{00}, and 14^{00}-16^{00} (groups 1, 2, 3, 4, 5) did not differ significantly in weight. However, a certain tendency to decrease the weight for egg collection from 8^{00} to 12^{00} and to increase after 12^{00} was observed. This contradicts the data of Patterson [15], according to which from 5^{00} to 18^{00} the weight of eggs decreased by 2–9 g. According to other data [16], eggs laid after 15^{00} also had the lowest weight.

1. Weight (g) **and categories of eggs of different laying times in Haisex Brown cross chickens of the commercial herd** ($n = 90$, $M \pm$SEM; vivarium of the Selection and Genetic Center Zagorskoe Experimental Breeding Farm ARRTPI RAS, 2020)

Age of hens, days	Laying time				
	5^{00}-8^{00} (group 1)	8^{00}-10^{00} (group 2)	10^{00}-12^{00} (group 3)	12^{00}-14^{00} (group 4)	14^{00}-16^{00} (group 5)
	Egg weight, g				
210	56.6±0.33[a]	54.2±0.37	53.4±0.39	54.4±0.40	53.9±0.39
270	58.8±0.27[a]	56.1±0.35	55.7±0.66	56.3±0.40	55.6±0.37
360	61.4±0.42[ab]	59.4±0.42	58.8±0.39	59.6±0.42	58.8±0.38
420	61.0±0.47[a]	58.3±0.49	57.6±0.43	57.3±0.44	58.4±0.41
450	61.4±0.47	60.7±0.58	60.5±0.47	60.8±0.44	61.3±0.42
On average	59.8±0.20[a]	57.7±0.23	57.3±0.22	57.7±0.22	57.6±0.21
	Egg yield by category for 210-450 days, %				
Higher	0.2	0.5	0.2	0	0
Selected	11.8	6.4	5.3	6.0	8.0
First	76.0	62.7	63.1	64.4	60.5
Second	10.7	29.1	29.6	28.9	30.4
Third	0	0.2	0.5	0	0.2
Breakage and check	1.3	1.1	1.3	0.7	0.9

[a, b] Differences between group 1 and other groups (for each age and on average by the experiment) are statistically significant, respectively, at $p < 0.001$ and $p < 0.01$.

The yield of eggs by category directly depended on the weight of eggs. For example, in group 1 (5^{00}-8^{00}), the proportion of eggs of the selected and first category for the period of 210-450 days was higher by 3.8-6.5 and 11.6-15.5%, in group 2 lower by 18.2-19.7%, respectively, than in other groups. Other groups in the yield of eggs of different categories did not differ much.

In groups 4 and 5, where eggs were laid between 12^{00}-14^{00} and 14^{00}-16^{00}, the number of damaged eggs was less by 0.4-0.6 and 0.2-0.4%. This is due to the better quality of the eggshells laid after 12^{00}, as evidenced by such indicators of the quality of the eggshells as relative weight, thickness, and density of eggs (Table 2). Both in all age periods and on average during the experiment in groups 4 and 5, these indicators of eggshell quality were significantly higher ($p < 0.001$-0.05) than in the groups of eggs laid before 12^{00}. The worst indicators of shell quality were observed in groups 1 and 2 (5^{00}-8^{00} and 8^{00}-10^{00}). The obtained data are consistent with the results of studies [12, 18, 20], where it was noted that eggs laid at 14^{00} had a higher relative weight of the shell and its thickness in comparison with eggs obtained at the beginning of daylight (6^{00}). The authors suggested that the weight of the shell tended to increase towards the last egg in the cycle. A later study by Tůmová et al. [37] indicates the dependence of the weight of the shell and, consequently, its strength on the duration of the egg in the eggshell gland of the oviduct.

2. Morphological parameters of eggs of different laying times in Haisex Brown cross chickens of the commercial herd (n = 30, for D n = 90, M±SEM; vivarium of the Selection and Genetic Center Zagorskoe Experimental Breeding Farm ARRTPI RAS, 2020)

Parameter	Laying time				
	5^{00}-8^{00} (group 1)	8^{00}-10^{00} (group 2)	10^{00}-12^{00} (group 3)	12^{00}-14^{00} (group 4)	14^{00}-16^{00} (group 5)
Aged 210 days					
Weight:					
albumen[A]					
g	37.1±0.47	36.5±0.50	36.0±0.37	36.7±0.42	35.3±0.46
%	64.2	65.7	65.3	65.5	64.8
yolk[B]					
r	14.6±0.16	13.3±0.18	13.1±0.13	13.1±0.16	13.0±0.16
%	25.3	23.9	23.8	23.4	23.9
shell[C]					
g	6.1±0.07	6.0±0.10	6.0±0.08	6.2±0.09	6.2±0.11
%	10.5	10.4	10.9	11.1	11.3
Egg shape index, %[D]	79.6±0.24	78.6±0.24	79.3±0.29	79.4±0.22	77.9±0.26
Egg density, g/cm^3[E]	1.091±0.001	1.089±0.001	1.091±0.001	1.094±0.001	1.097±0.001
Albumen index, %[F]	12.5±0.36	13.4±0.28	13.5±0.32	13.8±0.34	13.2±0.36
Yolk index, %[G]	47.9±0.42	48.7±0.50	48.2±0.41	49.7±0.47	48.4±0.56
Shell thickness, rm [H]	378±4.4	365±4.7	377±4.9	386±5.2	391±6.6
Albumen/yolk weight ratio[I]	2.5±0.05	2.8±0.06	2.7±0.04	2.8±0.05	2.73±0.05
Yolk color intensity, score[J]	2.7±0.12	2.5±0.11	2.8±0.15	2.9±0.17	2.5±0.12
Haugh units[K]	94.0±0.76	95.8±0.65	96.1±0.66	96.1±0.63	94.6±0.84
Aged 270 days					
Weight:					
albumen[A]					
g	36.4±0.30	35.7±0.25	35.7±0.31	35.5±0.32	34.9±0.40
%	62.2	63.6	63.5	63.3	61.6
yolk[B]					
g	16.0±0.27	14.6±0.16	14.5±0.17	14.5±0.16	15.6±0.38
%	27.4	26.0	25.8	25.8	27.5
shell[C]					
g	6.1±0.05	5.8±0.09	6.0±0.16	6.1±0.09	6.2±0.11
%	10.4	10.4	10.7	10.9	10.9
Egg shape index, %[D]	79.7±0.27	79.6±0.24	79.7±0.24	80.4±0.27	79.0±0.27
Egg density, g/cm^3[E]	1.088±0.0001	1.087±0.0010	1.090±0.0020	1.093±0.0010	1.093±0.0010
Albumen index, %[F]	12.5±0.34	13.4±0.37	13.5±0.45	13.1±0.46	12.4±0.36
Yolk index, %[G]	46.2±0.43	47.1±0.52	45.5±0.54	46.7±0.53	46.3±0.47
Shell thickness, rm [H]	372±4.0	365±5.7	383±7.3	388±6.1	391±6.7
Albumen/yolk weight ratio[I]	2.3±0.05	2.5±0.03	2.5±0.04	2.5±0.04	2.4±0.04
Yolk color intensity, score[J]	3.7±0.13	3.3±0.12	3.4±0.12	3.1±0.09	3.0±0.10
Haugh units[K]	94.3±0.93	96.6±0.75	95.1±0.58	95.0±0.93	94.1±0.85
Aged 360 days					
Weight:					
albumen[A]					
g	38.2±0.31	37.3±0.22	37.0±0.32	37.5±0.27	37.6±0.28
%	62.1	62.8	62.6	62.7	63.1
yolk[B]					
g	17.0±0.25	16.0±0.15	15.7±0.16	15.6±0.16	15.3±0.20
%	27.6	26.9	26.6	26.1	25.7
shell[C]					
g	6.3±0.09	6.1±0.07	6.4±0.11	6.7±0.09	6.7±0.09
%	10.3	10.3	10.8	11.2	11.2
Egg shape index, %[D]	78.5±0.24	78.7±0.25	78.5±0.25	79.5±0.29	77.7±0.23
Egg density, g/cm^3[E]	1.086±0.001	1.089±0.001	1.095±0.001	1.097±0.001	1.096±0.001
Albumen index, %[F]	11.3±0.28	10.6±0.37	11.5±0.45	10.4±0.35	11.7±0.36
Yolk index, %[G]	47.0±0.52	46.4±0.46	46.7±0.36	46.2±0.43	46.6±0.37
Shell thickness, rm [H]	372±4.4	372±3.4	386±7.4	403±5.1	399±4.9
Albumen/yolk weight ratio[I]	2.3±0.06	2.3±0.03	2.4±0.04	2.4±0.04	2.5±0.05
Yolk color intensity, score[J]	3.3±0.10	3.4±0.10	3.6±0.15	3.3±0.12	3.3±0.11
Haugh units[K]	92.1±0.90	90.3±1.13	92.5±1.21	89.7±1.11	93.1±0.98
Aged 420 days					
Weight:					
albumen[A]					
g	37.8±0.29	36.4±0.27	35.8±0.31	35.9±0.25	36.7±0.29
%	62.0	62.2	62.0	62.2	62.5
yolk[B]					
g	17.0±0.18	16.0±0.19	15.7±0.16	15.4±0.20	15.6±0.18
%	27.9	27.4	27.1	26.7	26.5

Continued Table 2

shell[C]					
g	6.2±0.09	6.1±0.09	6.3±0.12	6.4±0.09	6.4±0.07
%	10.1	10.4	10.9	11.0	11.0
Egg shape index, %[D]	77.4±0.24	77.5±0.25	77.6±0.26	78.0±0.27	77.1±0.26
Egg density, g/cm^3[E]	1.089±0.001	1.089±0.001	1.092±0.001	1.094±0.001	1.089±0.001
Albumen index, %[F]	11.4±0.37	11.5±0.37	11.1±0.40	11.4±0.46	11.3±0.40
Yolk index, %[G]	46.6±0.55	46.4±0.40	46.5±0.51	46.5±0.44	46.0±0.37
Shell thickness, rm [H]	370±5.5	368±5.1	385±7.1	393±5.2	383±4.4
Albumen/yolk weight ratio[I]	2.2±0.03	2.3±0.03	2.3±0.03	2.3±0.04	2.4±0.04
Yolk color intensity, score[J]	3.3±0.10	3.3±0.10	3.3±0.10	3.1±0.12	3.2±0.08
Haugh units[K]	92.8±1.10	92.5±0.99	91.0±1.18	91.2±1.22	88.5±2.93
		Aged 450 days			
Weight:					
albumen[A]					
g	38.1±0.34	38.1±0.35	37.8±0.41	37.9±0.30	38.5±0.27
%	62.3	62.9	62.6	62.4	62.9
yolk[B]					
gr	16.9±0.18	16.3±0.12	16.3±0.22	16.3±0.26	16.2±0.14
%	27.6	26.9	27.0	26.9	26.5
shell[C]					
g	6.2±0.09	6.2±0.08	6.3±0.09	6.5±0.08	6.5±0.11
%	10.1	10.2	10.4	10.7	10.6
Egg shape index, %[D]	76.7±0.26	76.2±0.25	77.0±0.26	77.3±0.27	77.0±0.30
Egg density, g/cm^3[E]	1.089±0.001	1.090±0.001	1.086±0.001	1.091±0.002	1.092±0.001
Albumen index, %[F]	10.1±0.32	9.7±0.32	9.7±0.40	9.1±0.44	9.8±0.27
Yolk index, %[G]	46.7±0.39	45.9±0.34	46.0±0.5	45.9±0.5	47.1±0.41
Shell thickness, rm [H]	364±4.4	376±4.3	376±4.5	380±7.6	379±6.8
Albumen/yolk weight ratio[I]	2.3±0.04	2.3±0.03	2.3±0.05	2.3±0.05	2.4±0.03
Yolk color intensity, score[J]	3.3±0.09	3.4±0.10	3.4±0.10	3.3±0.10	3.5±0.10
Haugh units[K]	88.7±1.05	87.4±1.12	87.1±1.32	84.9±1.89	88.4±0.85
		On average			
Weight:					
albumen[A]					
g	37.5±0.16	36.8±0.16	36.5±0.17	36.7±0.16	36.6±0.19
%	62.5	63.4	63.3	63.2	63.0
yolk[B]					
g	16.3±0.12	15.2±0.12	15.0±0.12	15.0±0.12	15.1±0.14
%	27.2	26.2	26.0	25.8	26.0
shell[C]					
g	6.2±0.03	6.0±0.04	6.2±0.05	6.4±0.04	6.4±0.05
%	10.3	10.4	10.7	11.0	11.0
Egg shape index, %[D]	78.4±0.12	78.1±0.12	78.4±0.13	78.9±0.13	77.7±0.12
Egg density, g/cm^3[E]	1.089±0.0004	1.089±0.0004	1.091±0.0006	1.094±0.0006	1.093±0.0006
Albumen index, %[F]	11.6±0.17	11.7±0.19	11.9±0.22	11.5±0.23	11.7±0.18
Yolk index, %[G]	46.9±0.21	46.9±0.21	47.0±0.22	47.0±0.23	46.9±0.21
Shell thickness, rm [H]	371±2.0	369±2.1	382±2.8	390±2.7	389±2.7
Albumen/yolk weight ratio[I]	2.3±0.02	2.4±0.02	2.5±0.02	2.5±0.02	2.5±0.02
Yolk color intensity, score[J]	3.2±0.05	3.2±0.06	3.3±0.06	3.2±0.06	3.1±0.05
Haugh units[K]	92.3±0.45	92.5±0.51	92.4±0.60	91.4±0.63	91.7±0.71

N o t e. A-K — parameters, a-e — groups.

*, **, *** Differences between groups are statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Significant differences in indicators between groups:

for 210 days: A — *ab, de; B — ***ab, ac, ad, ae; D — *bd, **ab, ce, ***ae, de; E — *ad, cd, de, **bd, ***ae, be, ce; F — *ab, ac, **ad; G — *dc, **cd; H — **bd, be; I — *ab, ae, **ac, ***ad; K — *ac, ad;

for 270 days: A — *ad, **ae; B — *be, ce, de, ***ab, ac, ad; C — *bd, **ab, cd; D — *ae, bd, be, ce, ***de; E — *bc, cd, ce, **ad, ae, ***bd, be; G — *bc; H — *ad, ae, **bd, be; I — **ab, ac, ad; J — *ab, ce, ***ad, ae; K — *be;

for 360 days: A — *ab, ac; B — **ab, be, ***ac, ad, ae; C — *bc, cd, ce, **ad, ae, ***bd, be; D — *ad, ae, bd, cd, ce, **be, ***de; E — *ab, ***ac, ad, ae, bc, bd, be; F — *be, de; H — ***ad, ae, bd, be; I — *ae, **be; K — *de;

for 420 days: A — *ae, ce, de, **ab, ***ac, ad; B — *bd, ***ab, ac, ad, ae; C — *bd, be; D — *de; E — *ac, bc, ce, **ad, bd, de; H — *be, **ad, bd; I — *be, **ad, bd;

for 450 days: C — *ad, ae, bd, be; D — *bc, be, **bd; E — *ac, ae, cd, **bc, ***ce; G — *be; I — *be; J — differences between groups are not significant; differences between groups in egg weight are not significant.

on average: A — **ab, ad, ae, ***ac; B — ***ab, ac, ad, ae; C — **ae, bc, cd, ce, ***ab, ad, bd, be; D — *be, cd, **ad, ***ae, bd, ce, de; E — *ce, **ac, bc, cd, ***ad, ae, bd, be; H — *cd, **ac, ***ad, ae, bc, bd, be; I — **ab, bc, bd, be, ***ac, ad, ae; J — *ce.

The albumen and yolk content in eggs also depended on the time of laying. In eggs laid before 8^{00}, the absolute weight of the albumen and yolk on average was the highest; in the other periods of the day, it significantly ($p < 0.001$-0.01)

decreased. However, the relative weight of the albumen in eggs collected from 8^{00} to 14^{00} increased on average from 62.5 to 63.4%, and at the end of the day (14^{00}-16^{00}) decreased slightly to 63.0%. In 420- and 450-day-old laying hens, the relative weight of the albumen does not change depending on the time of egg-laying. The relative weight of the yolk decreased on average by the end of the day (14^{00}-16^{00}) from 27.2 to 25.8%. A change in the weight of the albumen and yolk, depending on the time of egg-laying, entails a significant ($p < 0.01-0.001$) increase in the ratio of albumen and yolk weight (on average from 2.3 to 2.5). Thus, eggs laid early in the morning have more yolk and less albumen than in other periods of the day. Consequently, the nutritional value and yield of dry matter of eggs laid in the period from 5^{00} to 8^{00} are higher than those laid in the rest of the day, which serves as a positive factor for deep processing of eggs.

The increase in the absolute weight of the albumen, yolk, and, consequently, the weight of eggs laid before 8^{00}, in the authors' opinion, can be explained by the fact that the first eggs in the cycle after a pause in oviposition (from one to several days) are laid by chickens early in the morning.

On average, throughout the experiment, the egg-laying time did not affect such indicators of egg quality as the albumen index and Haugh units, but in young birds (210-day-old), the albumen in eggs laid before 8^{00} is more liquid than in other batches. In this group, the albumen index was significantly lower ($p < 0.01$-0.05) than in eggs of groups 2, 3, 4 by 0.9, 1.0, and 1.3%, respectively and Haugh units — by 1.8, 2.1, and 2.1 units, respectively, which is important during egg incubation since dense albumen affects the embryonic development of poultry and incubation results deteriorate by 1-5% [38]. However, from the point of view of consumer qualities, eggs with a high albumen density have a better amino acid composition and taste, as well as foaming ability [39].

Regardless of the age of laying hens (except for 210- and 450-day-olds), eggs laid from 12^{00} to 14^{00} had the maximum value of the shape index. On average, during the observation period in this group, this indicator was significantly higher ($p < 0.05$-0.001) than in other groups. In all age periods and on average during the experiment, the lowest shape index was observed in eggs laid from 14^{00} to 16^{00}.

According to the chemical analysis (Table 3), on average, during the experiment period, no significant differences were found between the egg groups in terms of moisture content, calcium in the shell, carotenoids, vitamins A and E in the yolk, and vitamin B_2 in the albumen. However, in terms of vitamin B_2 content in the yolk, eggs laid from 5^{00} to 8^{00} in all age periods (except for 450 day-olds, when a slight superiority was in favor of eggs laid from 8^{00} to 10^{00}) and on average during the experiment period significantly ($p < 0.05-0.001$) exceeded eggs laid in other periods of the day. Tůmova et al. [2] report a significant effect of the time of egg-laying on the content of calcium in the shell: in eggs laid at 7^{30}, its content was 35.2% and laid at 15^{30} – only 34.2%. In the present study, this pattern was not confirmed.

The authors' observations revealed a tendency to an insignificant increase in total cholesterol content in the yolk of eggs laid from 5^{00} to 8^{00}, on average during the observation period. Tůmova *et al.* [17] also recorded an unreliably higher cholesterol content in the yolk of early morning eggs compared to daytime eggs. At the same time, this contradicts the results of Abdalla *et al.* [29], who, on the contrary, noted a significantly lower amount of total cholesterol in morning eggs than in daytime eggs, calculated both per unit yolk weight (12.53 vs 16.23 mg/g) and per egg (176.63 vs. 221.14 mg/egg).

3. Chemical composition of eggs of different laying times in Haisex Brown cross chickens of the commercial herd (by $n = 15$ in 3 combined samples for each period, $M \pm$SEM; vivarium of the Selection and Genetic Center Zagorskoe Experimental Breeding Farm ARRTPI RAS, 2020)

Parameter	Laying time				
	5^{00}-8^{00} (group 1)	8^{00}-10^{00} (group 2)	10^{00}-12^{00} (group 3)	12^{00}-14^{00} (group 4)	14^{00}-16^{00} (group 5)
	Aged 210 days				
Content of:					
moisture, %	75.76	75.43	75.75	76.18	76.09
calcium in the shell, %[A]	36.91±0.30	36.91±0.33	37.66±0.10	37.27±0.18	36.34±0.07
in the yolk, μg/g:					
carotenoids [B]	6.29±0.03	6.52±0.10	6.28±0.02	6.08±0.09	6.28±0.01
vitamin A [C]	4.68±0.14	4.78±0.09	4.78±0.30	4.75±0.11	4.68±0.03
vitamin E [D]	52.91±2.21	52.82±1.91	54.65±1.98	53.62±3.01	53.79±1.68
vitamin B_2 [E]	5.33±0.07	4.43±0.16	3.63±0.15	4.20±0.15	3.98±0.01
cholesterol, mmol/l [F]	42.1±0.10	43.1±1.25	40.1±1.24	38.7±0.94	42.8±1.12
in the albumen of vitamin B_2, μg/g[G]	3.33±0.08	3.76±0.01	3.37±0.20	3.61±0.17	3.73±0.08
	Aged 270 days				
Content of:					
moisture, %	75.90	75.48	75.98	75.37	74.79
calcium in the shell, %[A]	35.92±0.35	36.75±0.20	36.51±0.13	36.47±0.23	36.15±0.46
in the yolk, μg/g:					
carotenoids [B]	2.60±0.03	2.54±0.07	2.54±0.07	2.45±0.06	2.86±0.03
vitamin A [C]	5.45±0.14	5.39±0.08	5.17±0.05	4.88±0.15	5.36±0.12
vitamin E [D]	110.26±2.25	107.47±2.65	108.98±2.10	105.32±1.01	105.29±1.21
vitamin B_2 [E]	5.58±0.26	5.49±0.02	4.50±0.18	4.72±0.06	5.16±0.03
cholesterol, mmol/l [F]	82.9±2.42	74.4±3.10	78.1±1.76	75.2±2.71	74.7±2.14
in the albumen of vitamin B_2, μg/g[G]	3.64±0.16	4.11±0.11	4.07±0.05	4.16±0.17	3.87±0.19
	Aged 360 days				
Content of:					
moisture, %	75.76	75.43	75.75	76.18	76.09
calcium in the shell, %[A]	36.77±0.36	37.14±0.23	37.14±0.21	36.59±0.42	36.96±0.13
in the yolk, μg/g:					
carotenoids [B]	9.43±0.71	9.37±0.09	10.2±0.27	9.63±0.09	10.09±0.32
vitamin A [C]	5.16±0.04	5.20±0.13	5.44±0.12	5.70±0.35	5.16±0.21
vitamin E [D]	49.23±2.39	47.27±2.86	48.53±0.93	51.78±0.76	55.74±1.55
vitamin B_2 [E]	5.04±0.32	4.91±0.08	4.58±0.19	4.51±0.28	4.19±0.18
cholesterol, mmol/l [F]	63.7±4.72	69.7±7.56	52.8±4.85	56.6±5.47	51.3±4.77
in the albumen of vitamin B_2, μg/g[G]	3.42±0.24	3.18±0.16	3.30±0.41	3.32±0.30	3.18±0.24
	Aged 420 days				
Content of:					
moisture, %	74.52	76.07	75.53	75.43	74.35
calcium in the shell, %[A]	35.82±0.16	36.67±0.19	36.48±0.18	36.20±0.26	36.60±0.44
in the yolk, μg/g:					
carotenoids [B]	4.44±0.15	4.66±0.23	4.34±0.20	4.50±0.06	4.31±0.06
vitamin A [C]	5.38±0.35	5.75±0.08	5.70±0.20	5.42±0.31	5.46±0.12
vitamin E [D]	68.97±3.32	71.81±0.71	76.15±1.53	73.23±3.23	71.43±8.55
vitamin B_2 [E]	5.67±0.12	4.70±0.19	5.55±0.12	4.57±0.24	4.77±0.07
cholesterol, mmol/l [F]	83.3±8.38	76.9±9.98	61.9±4.52	67.1±4.49	68.2±5.54
in the albumen of vitamin B_2, μg/g[G]	3.91±0.28	4.38±0.18	4.51±0.15	4.19±0.22	4.32±0.13
	Aged 450 days				
Content of:					
moisture, %	74.83	75.65	75.25	76.06	75.66
calcium in the shell, %[A]	37.26±0.05	37.04±0.20	36.44±0.32	37.18±0.11	36.79±0.35
in the yolk, μg/g:					
carotenoids [B]	3.47±0.33	3.11±0.32	3.84±0.29	3.52±0.23	3.60±0.13
vitamin A [C]	4.64±0.06	5.20±0.20	5.29±0.10	5.21±0.23	5.40±0.18
vitamin E [D]	64.23±1.74	69.39±3.64	76.30±0.64	63.54±0.60	64.34±1.22
vitamin B_2 [E]	5.07±0.21	5.13±0.09	4.81±0.46	4.21±0.08	4.71±0.22
cholesterol, mmol/l [F]	30.7±0.61	31.9±1.00	30.2±0.88	28.5±0.69	30.5±2.5
in the albumen of vitamin B_2, μg/g[G]	4.15±0.41	4.19±0.44	3.61±0.31	3.80±0.29	3.43±0.24

Continued Table 3

	On average				
Content of:					
moisture, %	75.35	75.61	75.65	75.84	75.40
calcium in the shell, %[A]	36.54±0.18	36.90±0.10	36.85±0.15	36.74±0.15	36.57±0.15
in the yolk, μg/g:					
carotenoids [B]	5.24±0.66	5.24±0.67	5.44±0.72	5.24±0.67	5.43±0.70
vitamin A [C]	5.06±0.11	5.26±0.10	5.28±0.11	5.19±0.13	5.21±0.09
vitamin E [D]	69.12±5.90	69.19±5.42	72.92±5.70	69.50±5.27	70.06±5.23
vitamin B_2 [E]	5.34±0.11	4.93±0.11	4.61±0.19	4.44±0.09	4.56±0.12
cholesterol, mmol/l [F]	60.54±3.20	59.20±3.30	52.60±2.40	53.22±2.50	53.50±2.50
in the albumen of vitamin B_2, μg/g[G]	3.69±0.13	3.92±0.14	3.77±0.16	3.82±0.12	3.70±0.13

Notie. A-E — parameters, a-e — groups.

*, **, *** Differences between groups are statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Significant differences in indicators between groups:

for 210 cyr: A — *ab, ac, **cd, ce; B — *ab, ad, bc, be, cd, de, **bd; E — *be, cd, ce, **bc, ***ab, ac, ad, ae; F — *bd, de, **ad; G — ***ab, be;

for 270 cyr: B — ***ae, be, ce, de; C — *ad, bc, de, **bd; E — **ac, ad, ***bc, bd, be, de; F — *ab, ae; G — *ab, ac, ad;

for 360 cyr: B — *bc, ce; C — *ac; D — *ae, be, de, cd, **ce; E — *ae, **be, ***de;

for 420 cyr: A — *ac, **ab; B — *de; D — *bc; E — *cd, **bc, ***ab, ad, ae, ce; F — *ac;

for 450 cyr: A — *ac, cd, de, **be, ***ab, ae; C — *ab, ad, **ae, ***ac; D — ***ac, ad, ae; E — *de, **ad, ***bd; F — *ad, cd;

on average: E — *ab, be, **ac, bd, ***ad, ae.

So, in laying hens of the Haisex Brown cross, with a constant lighting mode (turning on the light at 5^{00}, turning off at 19^{00}) and cellular content, the egg-laying time significantly affects the morphological indicators of egg quality. In eggs laid up to 8^{00}, on average during the observation period, the average weight is 2.1-2.5 g or 3.6-4.4% higher, the absolute weight is 7.2-8.7%, and relative yolk weight is 1.0-1.4% and, consequently, the albumen/yolk weight ratio is 4.2-8.0% lower. Eggs laid from 12^{00} to 16^{00} have better shell quality, as evidenced by higher shell weight (by 3.2-6.7%), its greater thickness (by 1.8-5.7%), and higher egg density (by 0.18-0.46%). The egg shape index varied curvilinearly, its maximum value (78.9%) was in eggs laid from 12^{00} to 14^{00}, the minimum (77.7%) — in eggs laid at the end of the day (from 14^{00} to 16^{00}). Eggs laid before noon had a greater number of shell defects (breakage and check), and in the evening batches, there were no eggs of the highest category. Within each age, the indicators between the groups differed little. However, the egg weight in each group generally increased with age (for example, in the group of eggs collected at 5^{00}-8^{00} from 56.6±0.33 to 61.4±0.47). In 420- and 450-day-old laying hens, the relative weight of the albumen does not change depending on the time of egg-laying. In a young (210-day-old) bird, the albumen of eggs laid before 8^{00} is more liquid. Regardless of age (except for 210- and 450-day-olds), eggs laid from 12^{00} to 14^{00} had the maximum value of the shape index. In all age periods and on average during the experiment, the lowest shape index was observed in eggs laid from 14^{00} to 16^{00}. The time of oviposition did not affect the chemical composition of eggs, except for vitamin B_2 in the yolk; in eggs, collected from 5^{00} to 8^{00}, this indicator significantly exceeded that of eggs collected at a later time (except for the 450-day-old laying hens that have a slight advantage in favor of eggs laid from 8^{00} to 10^{00}). The increased cholesterol content in the yolk was noted in eggs of 270- and 420-day-old birds (in general, it increased with the age of the bird), carotenoids — in 360-day-old laying hens. The content of vitamin A in the yolk also tended to increase depending on the age of chickens. The experimental data obtained will allow optimizing the egg collection schedule, selection and use of packaging containers to reduce damage to marketable products. In breeding programs, early egg-laying can serve as a sign that ensures the best quality of food and incubation eggs. In further experiments, the authors plan to study the effect of egg-laying time on the growth,

development, and homogeneity of embryos, the results of incubation, and the subsequent productivity of poultry.

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RELATIONSHIP OF DIELECTRIC PROPERTIES OF THE HAIR COVER WITH ITS MORPHOPHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS IN FARMED FUR-BEARING ANIMALS

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Abstract

The hair cover of furbearing animals is a dielectric material able to electrify with accumulation of static electricity charges. Electrically charged surface attracts dust particles causing loss of fur shine, accelerated aging, destruction and a decrease in strength characteristics and the quality of raw materials, as well as the transfer of static electricity charges when in contact, for example, with the human body. In this paper, for the first time, we propose an empirical equation describing the dielectric properties of natural fur and the interrelations between the composition and quality of the skin and hair covers with its electrophysical characteristics for different species of furbearing farm animals. The work aimed to study the influence of morphophysiological characteristics, biochemical composition (mineral and amino acid), and the state of the skin and hair on dielectric properties and to reveal relationships to more correctly assess the quality of fur raw materials. In the work, we used fur raw materials obtained from physiologically healthy silver-black foxes (*Vulpes vulpes*), silver foxes (*Alopex lagopus*), and standard minks (*Mustela vison*) (Zverosovkhoz Saltykovsky, Moscow Province). Hair density per 1 cm^2 rump, the guard hair density per 1 cm^2 rump, the linear dimensions of various types of hair, their thickness, the thickness of the rump skin, and the pelt area were measured and Na, Ni, B, V, Se, Al, Fe, K, I, Ca, Co, Mg, Mn, Cu, P, Cr, Zn, Si contents were determined. Amino acid analysis of biosubstrate hydrolysates was carried out for 17 amino acids. The pelts were tested for dielectric properties. The discharge kinetics equations were obtained at 20° C, 62 % relative humidity and voltage of 10.0, 18.5, and 28.5 kV (a HT-705 5kVA 50KV AC/DC high-voltage charge generator, Wuhan Huatian Electric Power Automation Co., Ltd., China). When assessing the quality of fur raw materials, a voltage of 9.5, 12.5, 15.5 and 18.5 kV was used. The charge leakage time constant and the change in the magnitude of the static electric field strength at certain time intervals were determined. A comparison of the morphophysiological parameters of the skin and hair in different species of furbearing animals with the data on electrizability allowed us to derive an empirical equation reflecting the dielectric properties of the skin and hair — $E = E_0 \cdot e^{-t/\tau}$, where E_0 is the maximum value of the tension and τ is the charge leakage time constant. Correlation analysis showed close interrelations between the rate of charge runoff and the length and thickness of the guard hairs (r = 0.83-0.90 at $p < 0.05$), the density of the guard and down hair (r = 0.92-0.98 at $p < 0.001$), and the length of the down hairs (r = 0.94 at $p < 0.001$). The charge leakage closely correlated with the total mass of chemical elements in the hair and skin (r = 0.97; r = 0.97 at $p < 0.05$) and the total amino acid composition of the hair cover (r = 0.95 at $p < 0.05$). The E_0 value closely correlated with the total mass of elements in the hair and skin, and with the amino acid composition of the hair cover (r = 0.90; r = 0.86; r = 0.99 at $p < 0.05$). Therefore, the dielectric properties of the skin and hair of furbearing animals depend on both the morphophysiological characteristics and biochemical composition. The skin and hair cover defects were established to affect the electrophysical parameters (τ and E_0). Decreased fur density, haircut, broken awn, fur mattedness reduce the E_0 index by 25-90 % and change the charge leakage time constant τ by 15-70 %

compared to defect-free skins. The dielectric parameters E_0 and τ provide more accurate fur quality estimates in silver-black fox, silver fox, and standard mink. The technology uses electrophysical measurements instead of not subjective organoleptic analysis.

Keywords: furbearing animals, *Vulpes vulpes*, silver fox, *Alopex lagopus*, Arctic fox, *Mustela vison*m, mink, skin cover, hair cover, dielectric properties, electrizability, mineral composition, amino acid composition, fur defects

At fur auctions and enterprises, the quality, integrity, and condition of the skin and hair covering of fur animals, as well as their physical and mechanical characteristics, as a rule, are determined by outdated methods – manually and organoleptically [1, 2]. Assessing the quality of fur raw materials and semi-finished products by this method is time-consuming, subjective, often controversial, and inaccurate. It depends on the experience and professionalism of the sorter, his/her well-being, fatigue.

The skin and hair of fur animals are dielectric materials, accumulating a significant static charge [2-4]. Electrification of fur raw materials and semi-finished products leads to their rapid dust pollution, which deteriorates the quality of fur products e.g., reduces luster, accelerates aging and destruction of the hair, increases its fragility [2].

Studying the static characteristics of the skin and hair covering and their causes is also necessary to establish factors that would reduce it to safe values for humans. Unfortunately, in Russia and abroad, such works are not carried out to date. There are no methods or devices that would make it possible to study electrification processes. Unlike textile materials, animal hair has a heterogeneous and uneven surface, consisting of different hair categories with unequal structure, linear parameters, and geometric shapes. Developing a physical and mathematical model of the static characteristics of hair covering is necessary to take into account all the many nuances associated with its complex architectonics.

The hair cover of fur animals, differing in height, density, hair color, and skin of animals, has different mineral and biochemical compositions [5-7]. Hair covering quality depends on complete feeding: balance of proteins, fats, carbohydrates, vitamins, minerals [8-10], and other feed ingredients [11-13]. The influence of the above factors on fur electrification (tribocharging or surface charging by friction) has so far been impossible.

The present work first revealed correlations between biochemical, mineral composition, state, morphophysiological characteristics, and dielectric properties of the skin and hair covering in caged fur animals. Based on these data, the decrease in the electrostatic field strength on the electrified pelt surface with time occurring according to the exponential law was found and described by the formula $E = E_0 \cdot e^{-t/\tau}$.

The work aimed to study the influence of morphophysiological characteristics, biochemical composition (mineral and amino acid), and the state of the skin and hair covering on its dielectric properties, furthermore, establish the relationships that can be used for a more accurate and objective assessment of the quality of raw and semi-finished fur raw materials.

Materials and methods. In this work, the research team used fur raw materials obtained from physiologically healthy animals: silver-black foxes (*Vulpes vulpes*), silver arctic foxes (*Alopex lagopus*), and standard minks (*Mustela vison*) (JSC Saltykovsky fur farm, Moscow Region). The animals were kept in standard sheds under conditions accepted by the farm for each species, complying with zootechnical and veterinary requirements. Diets were generally accepted, provided for the corresponding physiological condition and age of animals, a season of the year. The animals received meat and fish feeding under the recommendations of

Afanasyev NIIPZK. The mineral composition of the diet was analyzed according to the recommendations [14].

Skin and hair covering characteristics, the hair density per 1 cm^2 (averaged values of the total number of hairs per 1 cm^2 of the rump, thousands), hair density per 1 cm^2 of the rump, linear dimensions of different hair categories, their thickness, and the skin tissue thickness of the rump and pelt area were determined according to the methodical recommendations [15]. Mineralization of hair and skin samples, decomposition of prepared biomaterial, and determination of mineral composition (total concentration, mg%) of 18 elements were performed (Na, Ni, B, V, Se, Al, Fe, K, I, Ca, Co, Mg, Mn, Cu, P, Cr, Zn, Si) (15). The total mass of elements in the hair and skin biosubstrates per 1 cm^2 of the rump (rg/cm^2) was also determined.

Acid hydrolysis of hair covering and skin biosubstrates was performed according to the method of Balakirev et al. [15]. Hydrolysates were analyzed for 17 amino acids: cysteine, threonine, serine, asparagic and glutamic acids, glycine, proline, alanine, methionine, isoleucine, leucine, arginine, valine, tyrosine, phenylalanine, histidine, and lysine [15]. Total concentrations of amino acids (mol%) present in hydrolysates of biosubstrates were determined.

Defect-free belts of standard mink (12 pieces), silver arctic fox (12 pieces), and silver-black fox (12 pieces), and belts of grades I and II with different types of defects (2 pieces each), obtained after slaughter of animals at 7 months of age and subjected to standard primary processing, were used to study dielectric properties [2, 16]. Discharge kinetics equations for hairs of different species of fur animals were obtained at 20 °C, 62% RH and voltage 10.0; 18.5, and 28.5 kV on an HT-705 5kVA 50KV AC/DC high voltage charge generator (Wuhan Huatian Electric Power Automation Co., Ltd., China). When assessing the quality of fur raw materials, voltage of 9.5, 12.5, 15.5, and 18.5 kV was used. Based on the experimental data, the time dependence of the static electric field (SEF) strength (discharge kinetics) was plotted, which was used to determine the leakage time constant and the rate of charge leakage, and the maximum value of SEF strength [16]. The charge and SEF strength were measured until the charge on the sample completely disappeared. Repeated measurements were performed five times.

The results were processed according to GOST 8.207-76 (Moscow, 1986). Statistical analysis was performed in the Microsoft Excel package. The arithmetic mean of the measured value (M) and the standard error of the mean (±SEM) were determined. Student's t-test ($p \leq 0.05$) was used to assess the reliability of differences between the compared averages. Correlations between the electrophysical quantities influencing the static characteristics of the skin and hair covering and its morphophysiological characteristics, and biochemical composition were established using correlation analysis. The correlation relationship was considered weak positive or negative at $r < 0.3$ or $r > -0.3$, strong positive or negative at $r > 0.69$ or $r < -0.69$, medium positive or negative at $0.3 < r < 0.69$ and $-0.69 < r < -0.3$. For the medium and strong relationship, the correlation coefficient values were significant at $p < 0.0$, $p < 0.01$, $p < 0.001$.

Results. It is known that the dielectric properties of materials depend on their chemical composition [2, 21]. Since the static characteristic of various dielectrics, including natural fur, refers to contact electricity and is a surface phenomenon, the research team determined the morphophysiological parameters of the skin and hair covering (Table 1) and the surface concentration of macro- and microelements (Table 2), i.e., the total masses of elements in the hair and skin coverings biosubstrates per 1 cm^2 of the rump, and the amino acid composition of biosubstrates (see Table 2).

1. Morphophysiological characteristics of the skin and hair covering in silver-black foxes (*Vulpes vulpes*), silver arctic foxes (*Alopex lagopus*), and standard minks (*Mustela vison*) (n = 12, M±SEM; JSC Saltykovsky fur farm, Moscow Region, 2014)

Parameter	Silver-black foxes	Silver arctic foxes	Standard minks
Hair density, thousand/cm^2	20.5±0.5	35±1	9.5±0.3
Guard hair density, hairs/cm^2	420±20	540±30	240±20
Length of guard hairs, mm	69.1±0.3	66.7±0.3	29.7±0.2
Length of fur hairs, mm	42.7±0.2	43.5±0.1	13.7±0.2
Thickness of guard hairs, mm	45.9±0.4	47.6±0.3	60.1±0.4
Thickness of fur hairs, μm	10.5±0.3	10.6±0.1	9.3±0.2
Leather thickness, mm	0.63±0.04	0.72±0.01	0.82±0.01

2. Mineral and amino acid composition of the skin and hair covering in silver-black foxes (*Vulpes vulpes*), silver arctic foxes (*Alopex lagopus*), and standard minks (*Mustela vison*) (M±SEM; JSC Saltykovsky fur farm, Moscow Region, 2014)

Parameter	Silver-black foxes	Silver arctic foxes	Standard minks
Mineral composition (n = 20)			
Total concentration of macro- and microelements in the hair covering, mg%	107.8±1.9	99.1±1.7	59.1±1.5
Total concentration of macro- and microelements in the skin covering, mg%	148.0±2.0	183.0±3.0	116.3±1.9
Total weight of elements in the hair covering from 1 cm^2 of the rump, μg/cm^2	106.09±0.70	161.41±0.15	25.02±0.14
Total weight of elements in the skin covering per 1 cm^2 of the rump, μg/cm^2	75.83±0.05	108.78±0.11	77.16±0.03
Amino acid composition (n = 12)			
Amino acid composition of hair, mol.%	90.76±0.18	94.50±0.20	86.67±0.19
Amino acid composition of skin, mol.%	88.40±0.10	89.50±0.10	89.30±0.10

The electrified samples of fur raw materials showed the charge leakage from the surface into the surrounding space, i.e., the value of the charge decreased over time. It led to a SEF weakening and, consequently, to a decrease in this field strength (E) compared to the original value (E_0).

For the convenience of comparing materials of different composition and dielectric properties, the value of volume resistance (R) under the same atmospheric conditions (relative humidity and temperature) is used. Fibrous natural protein materials (wool, natural fur, silk) have R = 10^9-10^{13} Ohm · m, whereas synthetic fibers used for artificial fur production have R = 10^{10}-10^{18} Ohm · m [2]. Therefore, products made of artificial fur accumulate on the surface a bigger charge, that is, are more electrified than natural fur, and require additional antistatic treatment. In addition, the safe disposal of artificial fur, which is not subject to natural decomposition, requires additional costs. It is also important to note that materials made of natural fibers (as opposed to synthetic) in the tribocharging contribute to the accumulation of negative charges on human skin, a beneficial effect on health [2]. Consequently, natural fur is aesthetically, hygienically, and environmentally preferable to artificial fur.

We compared the research data with those known from the literature on the effect of the material nature (polytetrafluoroethylene, polyurethane, stainless steel, and latex in contact with one another) [17], its structure (fibrous or granular) [18, 19], and the surface shape [20, 21] on dielectric properties. This fact made it possible to derive the discharge kinetics equation describing the change in the SEF strength of the skin and hair coverings of fur animals with time. It turned out that the change occurred according to an exponential law: $E = E_0 \cdot e^{-t/\tau}$. After taking logarithms, the equation becomes $\lg E = \lg E_0 - t/\tau \cdot \lg e$, or $\lg(E/E_0) = -0{,}434t/\tau$, where E is the magnitude of the SEF at time t; E_0 is the magnitude of the SEF at time t = 0 (parameter b, Table 3). is the time constant of the charge sink, or the charge retention time, defined as a time of the electric field strength magnitude

decrease by half. The charge sink rate was determined by the slope value of the linear dependence (parameter a, see Table 3), equivalent to the change in lgE from time to the time axis. At the same time, for all types of fur raw materials, there was a general tendency: with an increasing degree of electrification of the hair cover (E_0), the charge leakage rate decreased (see Table 3). It should be noted that the above papers discuss different mathematical models for the charging (tribocharging) of materials depending on the composition, nature, and shape of the contacting materials [17-21] and provide techniques for obtaining reproducible data [20, 21]. However, all these works consider simpler objects in structure and composition compared to the hair covering of fur animals.

3. Analytical view of the discharge kinetics equations y = lgE at different voltages on the charge generator (U) for hair covering of silver-black foxes (*Vulpes vulpes*), silver arctic foxes (*Alopex lagopus*), and standard minks (*Mustela vison*) (n = 12, M±SEM; JSC Saltykovsky fur farm, Moscow Region, 2014)

Animal species	U, kV	y = –at + b where b = lgE_0	E_0, kV/m
Silver-black foxes	10.0	y = –0.0064t + 5.34	219±11
	18.5	y = –0.0049t + 5.58	380±20
	28.5	y = –0.0014t + 5.77	590±30
Silver arctic foxes	10.0	y = –0.0031t + 5.14	138±11
	18.5	y = –0.0026t + 5.37	234±15
	28.5	y = –0.0008t + 5.57	370±20
Standard minks	10.0	y = –0.0081t + 5.37	234±15
	18.5	y = –0.0061t + 5.57	372±19
	28.5	y = –0.0040t + 5.82	660±20

N o t e. E_0 is the maximum value of the static electric field strength; E is the value of the static electric field strength at time t. The R^2 values characterizing the approximation reliability were 0.83-0.98. Repeated measurements were performed five times.

Since skin and hair covering of different species of fur animals differ markedly in the set of morphophysiological and biochemical characteristics, a correlation analysis was conducted to identify the main parameters that have the greatest effect on the dielectric properties of raw fur. A strong correlation was observed between the charge sink rate and the guard hair length and thickness (r = 0.83-0.90 at $p < 0.05$), the fur hair length (r = 0.94 at $p < 0.001$), and the density of protective and fur hair (r = 0.92-0.98 at $p < 0.001$). Strong positive and negative correlations occurred between the charge sink rate and the total mass of elements in the skin and hair, the total amino acid composition of the hair covering (r = 0.97; r = 0.97; r = 0.95 at $p < 0.05$), and between the E_0 value and the parameters mentioned above for skin and hair (r = –0,90; r = –0.86; r = –0.99 at $p < 0.05$). Medium to weak correlations were noted between the mineral composition of the hair covering (total concentration of macro-, microelements in the hair coat), the total amino acid composition of the skin coat, and the rate of flow of the charge (r = 0.64; r = 0.29 at $p < 0.05$) and E_0 (r = –0.43; r = –0.54 at $p < 0.05$). Consequently, the dielectric properties of the skin and hair covering, its morphophysiological characteristics, and its biochemical composition are interrelated.

Defect-free belts of fur animals can be obtained only with balanced and adequately organized animal feeding and housing. Otherwise, the lack of nutrients (proteins, fats, carbohydrates) [22], minerals [23-25], and vitamins [26] in feed leads to the disease development (mineral, immune deficiency, various avitaminosis) associated with the hair covering growth damage [22, 27]. Usually, intravital defects that reduce the quality and value of fur raw materials arise due to disturbed molting, mechanical damage, excessive contamination of the hair covering, skin diseases, malnutrition, and mismanagement of fur animals.

Thus, the sparsity of hair covering in which the belts have areas of reduced hair density compared with the standard is caused by a lack of vitamins B, B_9,

folic acid [22, 28, 29], and vitamin C [22, 30, 31]. As a result of scabies, ringworm, and mechanical damage due to inconvenient for the animals' access holes in the sheds, areas free of hair (hairless spots) appear on the skin and hair covering [22]. In many farms, the frequency of hair biting from the neck, back, and tail in animals is up to 30%. This behavior (so-called, haircut) is usually observed in minks and arctic foxes, less often in foxes. The cause is considered violations in the technology of feed preparation, which contains oxidized by-products, undernutrition of animals, use of feed mixtures with an imbalance of amino acids, biotin deficiency, and feeding large amounts of bones to fur animals [15, 22]. Lack of sulfur-containing amino acids, B vitamins, sulfur, magnesium, copper, and cobalt in fur animals' diets leads to the occurrence of growth damages such as guard hair breakage or cutting (breakage of the upper part of the outer-coat fiber stem of the hair covering). Previously, the authors of this paper showed that the mineral composition of the hair covering in sick foxes differed significantly in the elements mentioned above from that in healthy animals [32]. These elements may affect the processes of hair keratinization. Due to defective keratinization of the hair shaft, hair loses elasticity, accompanied by spontaneous brittleness, reducing the quality of the down and the value of fur raw materials [22]. The guard hair cutting, as a rule, subsequently leads to felting hair, i.e. the appearance of areas with tangled felt-like hair and the disturbed ratio of hair of certain categories. This disease is caused by poor housing conditions and poor nutrition of fur animals [22].

The listed lifetime defects of fur raw materials are only a tiny part of the defects associated with poor feeding and housing of animals and errors in the storage and processing of skins after slaughter and receipt of fur raw materials. In the authors' opinion, an objective assessment of the quality of fur raw materials (huge volumes) on the whole set of characteristics is convenient to carry out with the electrophysical equipment.

4. Influence of skin and hair covering defects on its electrophysical parameters in silver-black foxes (*Vulpes vulpes*), silver arctic foxes (*Alopex lagopus*), and standard minks (*Mustela vison*) (*M*±SEM; JSC Saltykovsky fur farm, Moscow Region, 2014)

Animal species	U, kV	Grade, fault group	y = –at + b, where y = lgE, b = lgE$_0$	E$_0$, kV/m	τ, s
Silver-black foxes	9.5	Grade I, fault-free	y = –0.0015t + 5.00	97±7	289±7
	12.5	Grade I, fault-free	y = –0.0019t + 5.13	140±10	219±10
	15.5	Grade I, fault-free	y = –0.0024t + 5.21	160±10	180±10
	18.5	Grade I, fault-free	y = –0.0031t + 5.28	190±10	140±7
	9.5	Grade I, Group 1	y = –0.0013t + 4.47[г]	31±7	340±20
	12.5	Grade II, Group 2	y = –0.0009t + 4.57[в]	35±9	340±20
	15.5	Grade I, Group 1	y = –0.0020t + 4.44[а]	31±8	220±30
	18.5	Grade I, Group 2	y = –0.0023t + 5.08[д]	120±10	190±20
Silver arctic foxes	9.5	Grade I, fault-free	y = –0.0029t + 5.23	180±20	144±8
	12.5	Grade I, fault-free	y = –0.0024t + 5.27	197±19	185±9
	15.5	Grade I, fault-free	y = –0.0019t + 5.31	201±19	210±10
	18.5	Grade I, fault-free	y = –0.0014t + 5.34	230±20	324±17
	9.5	Grade I, Group 1	y = –0.0024t + 4.41[г]	32±9	181±17
	12.5	Grade II, Group 2	y = –0.0012t + 4.64[в]	38±9	360±20
	15.5	Grade I, Group 1	y = –0.0017t + 4.86[а]	77±9	255±15
Standard minks	18.5	Grade I, Group 2	y = –0.0012t + 5.11[д]	130±15	378±19
Standard minks	9.5	Grade I, fault-free	y = –0.0037t + 5.22	164±8	126±6
	12.5	Grade I, fault-free	y = –0.0028t + 5.30	200±9	159±8
	15.5	Grade I, fault-free	y = –0.0026t + 5.28	178±10	171±8
	18.5	Grade I, fault-free	y = –0.0017t + 5.45	290±10	271±11
	9.5	Grade I, Group 1	y = –0.0018t + 4.18[а]	18±3	250±50
	12.5	Grade II, Group 1	y = –0.0008t + 5.40[б]	263±15	531±19
	15.5	Grade II, Group 2	y = –0.0017t + 4.72[в]	57±11	275±18
	18.5	Grade I, Group 1	y = –0.0013t + 5.12[г]	143±15	319±16

N o t e. For defect-free belts n = 12, for belts with each type of defect n = 2; [a] — sparsity of hair covering, [b] — hairless spot, [c] — "haircut", [d] — broken hair cutting, [e] — felted hair. U — voltage at the charge generator. E0 is the maximum value of the static electric field strength; E is the value of the static electric field strength at time t, τ is the charge leakage time constant. The R^2 values characterizing the approximation reliability were 0.77-0.99. Repeated measurements were performed five times.

The dielectric properties of fur raw materials (belts of silver-black fox, silver arctic fox, and standard mink) turned out to be very sensitive to the integrity of the skin and hair covering and the presence of various defects on it. Samples with different types of defects (sparsity, felted, haircut, sparsity, hairless spots, guard hair cutting) and defect-free samples were exposed to static electricity. The results showed that the presence of these defects affected the static characteristics of fur raw (Table 4). In this case, there was a change in both the analytical form of the discharge kinetics equations ($y = -at + b$, where $y = lgE$, $b = lgE_0$) and electrophysical quantities characterizing dielectric properties (maximum value of strength E_0 and time constant of charge leakage τ).

For all types of fur raw materials, the sparsity of hair covering reduced the values of E0 by 60-90% and τ by 20-50% in comparison with defect-free belts, guard hair cutting by 50-80 and 15-20%, accordingly, haircut by 70-80 and 40-50%, hairless spots by 25-70% for minks, felted hair covering by 40 and 15-25% for arctic foxes and foxes (see Table 4).

Thus, the morphophysiological and biochemical characteristics of the skin and hair covering of fur animals have a strong influence on the dielectric properties of fur raw material. Correlation analysis showed a close dependence of the charge leakage and the maximum strength of the static electric field (E_0) of the skin and hair covering on the density, length, thickness of the protective and fur hair and on its mineral and amino acid composition. Dielectric properties also depend on the quality of fur products and defects. Intrinsic defects (sparsity, haircut, guard hair cutting, felted hair covering) reduce E0 by 25-90% and change the charge leakage time constant τ by 15-70% compared to defect-free belts. Hairless spots, on the contrary, increase the charge leakage time and the maximum value of strength E0. Based on the results presented, one can recommend the dielectric values E_0 and τ for quality control of furry raw materials obtained from silver-black foxes, silver arctic foxes, and standard minks. This approach is based on electrophysical equipment and (in contrast to the subjective organoleptic determination) allows an objective and more accurate assessment of the marketable properties of fur products.

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