ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

AGRICULTURAL BIOLOGY

Since January, 1966

ANIMAL BIOLOGY

Vol. 58, Issue 2
March-April

2023 Moscow

A peer-reviewed academic journal for delivering current original research results and reviews on classic and modern biology of agricultural plants, animals and microorganisms
Covered in Scopus, Web of Science (BIOSIS Previews, Biological Abstracts, CAB Abstracts, Russian Science Citation Index), Agris

Science editors: E.V. Karaseva, L.M. Fedorova

Publisher: Agricultural Biology Editorial Office NPO

Address: build. 16/1, office 36, pr. Polesskii, Moscow, 125367 Russia
Tel: + 7 (916) 027-09-12
E-mail: felami@mail.ru, elein-k@yandex.ru **Internet:** http://www.agrobiology.ru

For citation: Agricultural Biology,
Сельскохозяйственная биология, Sel'skokhozyaistvennaya biologiya

ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)
ISSN 2412-0324 (English ed. Online)

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AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 2, pp. 205-222
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Reviews, challenges

UDC 636.52/.58:577.2

doi: 10.15389/agrobiology.2023.2.205eng
doi: 10.15389/agrobiology.2023.2.205rus

ASSOCIATION OF SINGLE NUCLEOTIDE POLYMORPHISMS IN CANDIDATE GENES WITH ECONOMICALLY USEFUL TRAITS IN CHICKENS (*Gallus gallus domesticus* L.)

(review)

L.G. KORSHUNOVA✉, R.V. KARAPETYAN, A.S. KOMARCHEV, E.I. KULIKOV

Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail lg@vnitip.ru (✉ corresponding author), ruben@vnitip.ru, kulikovegor33@yandex.ru, kas1380@bk.ru
ORCID:
Korshunova L.G. orcid.org/0000-0002-4393-7499
Karapetyan R.V. orcid.org/0000-0001-6610-7749
Komarchev A.S. orcid.org/0000-0003-0919-3905
Kulikov E.I. orcid.org/0000-0001-5553-4454
The authors declare no conflict of interests
Acknowledgements:
The article was prepared according to the state task of the Ministry of Science and Higher Education of the Russian Federation on the topic "Development of breeding and genetic methods to increase the yield of poultry pedigree stocks and commercial products" (State. reg. No. 121030100022-8)
Final revision received November 29, 2022
Accepted December 20, 2022

Abstract

Economically useful traits of chickens associated with productivity are inherited polygenically. With the discovery of numerous DNA regions characterized by single nucleotide polymorphism (SNP) and the development of modern genomic technologies, a detailed assessment of the results of breeding in poultry farming has become possible to successfully predict the effect of breeding (L. Wang et al., 2011; C.M. Seabury et al., 2017). This review summarizes data on genes and SNP markers used in domestic chicken breeding and describes new polymorphic allelic variants in genes that are associated with integrated productivity indicators in chickens from the world gene pool. In Russia, domestic meat, egg and dual-purpose chicken breeds are currently subjected to thorough genotyping. Polymorphic variants of key genes *LCORL* (*ligand dependent nuclear receptor core-pressor-like*) and *NCAPG* (*non-SMC condensin I complex, subunit G*) that affect egg-laying performance has been found. Differences in SNP between egg and meat and egg and decorative chickens were revealed (T.A. Larkina et al., 2021). For the *NCAPG* gene, a significant association of rs14991030 alleles with shell weight, percentage of shell weight to egg weight, and shell thickness was identified (O.Yu. Barkova et al., 2016). In Russian White chickens, single nucleotide polymorphisms of the dysferlin gene (*DYSF*) were identified and their association with economically valuable traits was studied (O.Yu. Barkova et al., 2021). For safe breeding and selection of chicken populations and breeds, it important to prevent the spreading of genetic diseases and to ensure the maintenance of heterozygosity of the domestic gene pool. In the Smena 8 broiler meat cross line B5, typing SNPs in the *DMA*, *RACK1*, and *CD1B* genes responsible for a higher IgY titer revealed the fixation of an allele of a lower IgY titer at the Gga_rs15788237 locus and the predominance of an unfavorable allele at the Gga_rs15788101 locus and a favorable allele at the Gga17_rs160 locus. Changes in the Gga_rs16057130 and Gga_rs15788101 loci in the B5 broiler line bred at Smena State Breeding Center (Moscow Province) are most likely associated with selection for productivity traits (A.M. Borodin et al., 2020). Poultry genome studies are currently focused on analyzing large datasets across several generations to find associations (GWAS, genome-wide association studies) between SNPs and economically important traits such as growth rate, egg quantity and quality, meat and fat deposition. (A. Wolc, 2014; S.K. Zhu et al., 2014; J.H. Ouyang et al., 2008). Genome-wide genotyping using a high-density SNP array revealed candidate genes *GRB14* and *GALNT1* whose single nucleotide polymorphisms had statistically significant associations with egg production and egg quality parameters, including egg weight, eggshell weight, yolk weight, eggshell thickness and strength, albumen height and Haugh value for hens aged 40-60 weeks (W. Liu et al., 2011). GWAS analysis identified candidate genes *ZAR1*, *STARD13*, *ACER1b*, *ACSBG2*, and *DHRS12* which were associated with the weight of yolk, follicles, and ovaries of hens from the beginning of oviposition to 72 weeks of age. As estimated by SNP analysis, the heritability was moderate for yolk weight (h^2 of 0.25-0.38) and relatively low for follicle weight (h^2 = 0.16) and ovary weight (h^2 = 0.20) (C. Sun et al., 2015). Two genes, *MSX2* and *DRD1* are associated with embryonic

and ovarian development and contain significant SNPs associated with egg quality, i,e,, height of albumen and Haugh value. Three genes, the *RHOA*, *SDF4*, and *TNFRSF4* have been identified as candidate genes for eggshell coloration (Z. Liu et al., 2018). It has been reported (S.A. Azmal et al., 2019) that in the Chinese chicken breed Jing Hong, SNPs in the *RAPGEF6* gene are associated with the egg laying rate during late oviposition. Several studies support the notion of dopamine involvement in the regulation of egg production in birds. Four SNPs (G+123A, T+198C, G+1065A, C+1107T) in dopamine receptor gene (*DRD1*) were found which significantly affect the age of the first oviposition (it characterizes the rate of puberty of hens), the weight of the first egg and the yield of standard eggs (H. Xu et al., 2010). The *VIP* (receptor for vasoactive intestinal peptide-1) gene polymorphisms are associated with brooding instinct and egg production rate (M. Zhou et al., 2010). X. Li et al., (2019) found five polymorphisms in the promoter region of the *FSHR* (follicle-stimulating hormone receptor) gene and determined their association with the total egg production for 43 weeks of life and with the age of laying the first egg. H. Zhou et al. (2005) found significant associations of single nucleotide polymorphism in the *IGF1* (insulin-like growth factor 1) gene promoter with growth rate, body composition, skeletal condition and physiological parameters of chickens. Meat quality is due to a complex of quantitative traits and is controlled by multiple genes such as *FABP* (fatty acid binding protein) (K.H. Cho et al., 2011), *CAPN1* (micromolar calcium activated neutral protease gene) (J.T. Shu et al., 2015), *PRKAG3* (*protein kinase AMP-activated non-catalytic subunit gamma 3*) (Y. Yang et al., 2016). The identified statistically significant associations of single nucleotide polymorphisms with economically important traits can be used in poultry breeding and selection programs.

Keywords: gene, SNP, single nucleotide polymorphism, allele, chickens, meat productivity, egg productivity, full genome associations, GWAS

Over the past two decades, knowledge about the genomes of farm poultry of different breeds and species has deepened significantly. The development of molecular genetics methods has made it possible to study both genes and entire genomes. The genomes of *Gallus gallus* L., *Taeniopygia guttata* (Vieillot, 1817), *Meleagris gallopavo* L., and another 45 bird species have been sequenced [1]. The principles of using molecular genetic markers to improve the accuracy of predicting the breeding value of an animal have been proposed and largely implemented. Computer technologies have created the prerequisites for the rapid development of complex systems for such forecasting [2, 3]. Significant success in predicting breeding value is associated with DNA marking based on genome-wide genotyping of candidate animals for several thousand single nucleotide polymorphisms (SNP, single nucleotide polymorphism) and analysis of their association with breeding qualities [4].

Single nucleotide polymorphisms (SNPs) are the most common markers of genetic variation (there is approximately one SNP for every 200 nucleotides), followed by short (≤ 100 nucleotides) insertions and deletions (InDels, insertions and deletions). Approximately 20 million SNPs have been identified in chickens. Not only the presence, but also the characteristics of these polymorphisms are important for association with physiological processes in the body.

Single nucleotide polymorphisms cause changes in gene expression, which directly affects the formation of certain traits. With the discovery of numerous DNA sites characterized by single nucleotide polymorphism and the advent of SNP chip technology, it has become possible to evaluate the results of selection in detail at the genomic level for subsequent successful prediction of the selection effect in poultry farming [5, 6].

The purpose of this review is to summarize data on genetic variants of candidate genes and associations of single nucleotide polymorphisms with economically significant traits in chickens, to discuss the practical use of SNPs as an additional criterion in assessing reproductive characteristics and predicting egg productivity in the early stages of puberty of industrial and local breeds of chickens. The search for scientific sources was carried out mainly in the databases eLIBRARY.RU (https://www.elibrary.ru/defaultx.asp) and PubMed® (NCBI, The National Center for Biotechnology Information, https://pubmed.ncbi.nlm. nih.gov/), as well as using lists of citations in the retrieved publications.

Economically useful traits of chickens associated with productivity are

characterized by polygenic inheritance. Preventing the spread of genetic diseases in the domestic poultry gene pool and maintaining the required level of heterozygosity are factors that ensure the safety of Russian populations and breeds during breeding work and in practical poultry farming.

Currently, domestic scientists are actively conducting research on genotyping chicken breeds for meat [7-9], meat-egg [10, 11] and egg [12, 13] productivity areas.

Egg production is an important economic characteristic of poultry and a current subject of breeding and molecular genetic research. Egg production and the quality of hatching eggs are traits that exhibit a polygenic mode of inheritance. Key genes and functional SNPs affecting egg production rates have been studied [14, 15]. On the chicken chromosome GGA4, a region including the *LCORL* (*ligand dependent nuclear receptor corepressor-like*) and *NCAPG* (*non-SMC condensin I complex*, *subunit G*) genes is associated with growth traits. In an area close to this region, single nucleotide polymorphism sites associated with egg production traits have been identified. When studying the genetic variability of the *NCAPG-LCORL* locus in chickens of 49 gene pool breeds and hybrid forms from the Common Use Center Genetic Collection of Rare and Endangered Breeds of Chickens (VNIIGRZh, St. Petersburg; http://www.biores.cytogen.ru/rrifagb_anm) using SNP analysis identified five statistically significant SNPs: GGaluGA265966, GGaluGA265969, rs15619223, rs14491017 and rs14491028 [10]. The resulting characteristics of genetic variations and the genetic structure of populations based on SNPs of key genes for chicken productivity make it possible to determine the characteristics of local populations and can be used in breeding. Among chickens of different productivity directions, the authors identified differences in SNPs located in the locus that covers the NCAPG-LCORL genes. Egg-laying chickens differed significantly from chickens of other productivity types. Thus, significant differences between the egg-meat and egg-decorative groups were identified in the substitution GGaluGA265969. Presumably, putative association of this SNP with chicken body weight may explain such differences [10].

A series of works is devoted to identifying SNP markers for quantitative traits of egg quality and studying the relationship between alleles of markers and traits of laying hen eggs. Associations of the SNP marker rs14991030 in the condensin gene *NCAPG* were studied (the *NCAPG* gene encodes the non-SMC condensing I complex subunit G) with egg production traits in chickens [13, 16]. A significant relationship was found between the rs14991030 allele of the *NCAPG* gene and the shell weight, the percentage of egg to shell weight, and the shell thickness. The studies were carried out on chickens of two lines of the Russian cross UK Kuban 7 with a brown shell, derived from the gene pool of the Rhode Island breed. Also the experiments involved a two-line CD hybrid of the parent form of the Lohmann Brown cross. Analysis of variance based on the SNP marker rs14991030 data (the line UK 72) revealed significant differences between genotypes AG, AA and GG for the shell weight and shell percentage (i.e., the average proportion of shell weight to the weight of the entire egg). In the CD cross, of all the traits, only the eggshell thickness had a significant difference. A significant effect of the rs14991030 polymorphism was described for traits that differed significantly between three genotypes of the UK 72 line. When replacing the G allele with the A allele, an effect was noted in shell weight and shell percentage. In the CD cross, the replacement of the G allele with the A allele led to a change in shell thickness. Chickens with the GG genotype had thicker shells, indicating the additivity of allele replacement. The presence of the A allele was manifested by an increase in shell weight, shell thickness, and shell percentage (as a percentage) of egg weight [13]. Analysis of the expression of a region of chromosome 4 in the immediate environment

of the microsatellite MCW0114 in the tissues of the chicken oviduct (transcript CR523443, the ChEST985k21clone, was detected for this region) made it possible to determine the relationship with shell thickness. Six SNPs were found in the immediate vicinity of the CR523443 sequence, three of which were associated with eggshell thickness. A genetic analysis of the association of single nucleotide substitutions with other economically useful egg traits was carried out [12]. A significant relationship between SNP2_1 alleles and eggshell thickness in chickens of the UK 72 line was established. The statistical significance of the effect of C/T substitution in SNP2_1 was assessed. For this trait, the dominance of the T allele was revealed. Associations of SNP2_1 with shell weight, egg production, and egg weight were shown [17).

In order to identify possible associations with economically valuable characteristics, single-nucleotide polymorphisms of the dysferlin gene were studied in the Russian White chickens from the gene pool population of the Genetic Collection of Rare and Endangered Chicken Breeds (All-Russian Research Institute of Genetics and Breeding of Farm Animals, St. Petersburg-Pushkin) [18]. Genotyping 185 chickens using the Illumina Chicken 60K SNP iSelect BeadChip technology (Illumina, USA) identified the single nucleotide polymorphism rs16455118 for the first time. Four single-nucleotide substitutions found were located in the intron 32 on the chromosome 4. These were rs317801013 (G/A) at position 90672849, rs16455118 (C/A) at position 90672756, rs318045896 (A/G) at position 90672862 and a mononucleotide polymorphism (T/G) at position 90672805. The T/G mononucleotide polymorphism on chromosome 4 at position 90672805 was submitted for registration to the ENSEMBL database (https://www.ensembl.org). The results obtained may be useful for creating a system of molecular genetic markers [18].

The results obtained in studying the diversity of four original lines (B5, B6, B7, B9) of the Smena 8 broiler meat cross allowed us to conclude that they are highly genetically conservative [7].

A quantitative real-time PCR-based test and an algorithm have been developed for identifying the homo- and heterozygous state of the *K* and *k* alleles in 1-day-old chicks. The *K* and *k* alleles are sex-linked and responsible for the growth rate of wing feathers. Using this test, the percentage of genotypes *KK*, *Kk* and *kk* was determined among 145 roosters of the original lines B5, B6, B7 and B9 of the domestic meat cross Smena 8. Further breeding work involves traditional and molecular genetic assessment of the birds in order to exclude roosters of the line B7 with genotypes *KK* and *Kk*, roosters of the line B9 with genotypes *Kk* and *kk* and their descendants as not corresponding to the target breeding parameters [8]. A single nucleotide substitution rs317093289 of the gene *FSHR* (*follicle stimulating hormone receptor*) was analyzed in the original line CM9 of the Smena 9 cross. Among the studied chickens of this line, the TA genotype was most frequent (42%), the TT genotype had a frequency of 24%, the AA genotype 34 %. At 210 days of age, the bird with the TA genotype exceeded the bird with the AA genotype by 2.4% in egg weight, the group with the TT genotype was close to the TA group (the differences between the TT and TA groups are not significant). The studied SNP had a significant effect on egg production. The TA genotype exceeded the TT genotype in the number of eggs laid for 210 and 308 days by 15.0 and 2.8%, respectively [9].

It is known that selection of animals for high productivity leads to a weakening of their immunity, fertility, and a decrease in the ability to withstand stress [19, 20]. These negative effects may result from either pleiotropy of genes during breeding for increased productivity, or a combination of unfavorable alleles with alleles subject to selection, or genetic drift. Understanding the nature of the adaptive mechanisms acting on the chicken genome provides insight into the complex relationship, while simultaneously opening up new directions for improving

this commercial species, which is so important for food security [21]. Until recently, poultry selection and breeding methods were aimed at improving production and reproductive traits without taking into account health-related traits due to the lack of appropriate genetic markers that could be integrated into breeding programs. New opportunities have arisen due to significant advances in genomics and related technologies. Currently, the research strategy is aimed at identifying genes, gene structures and regulatory regions that can be used in breeding. In addition, there is growing interest in deciphering the genetic parameters underlying the immune response. More and more data is accumulating on the negative impact of selection for economic traits on the immune system of chickens due to a decrease in the variability of genes encoding elements of the immune system [22, 23]. SNP typing of three genes was carried out, the *DMA* (*major histocompatibility complex, class II, DM alpha*), *RACK1* (*receptor for activated C kinase 1*) and *CD1B* (*CD1b molecule*) responsible for an increased IgY titer in line B5 of broiler meat cross Smena 8. All three SNPs are localized within the corresponding genes. Fixation of the allele that determines a lower IgY titer was detected in the Gga_rs15788237 locus, the unfavorable SNP allele in the Gga_rs15788101 locus, and the predominance of the favorable SNP allele in the Gga_rs16057130 locus. Changes in the Gga_rs16057130 and Gga_rs15788101 loci in chickens of the meat cross Smena B5 line are most likely associated with the selection for productivity traits, which in the future can lead to the fixation of alleles in these loci. Studying the negative impact of selection for economic traits on immunity should help reduce negative consequences and find ways to obtain disease-resistant animals [22].

Technical advances in genotyping (GWAS, genome-wide association studies) allow researchers to analyze large amounts of information obtained over a number of generations in order to search for associations between SNPs and economically significant traits in birds, such as growth rate, quantitative and qualitative indicators of eggs, meat and fat deposition [24-26]. The accuracy of genotyping depends, among other things, on the density of SNPs on the chips [27-30]. Despite the great interest in SNP arrays, the cost of genotyping is still too high for large-scale population studies. Genotyping coverage using the commercial Chicken 600K Affymetrix® Axiom® SNP chip varies among different chicken populations of egg-type. This chip consists of approximately 560 thousand tested SNPs for commercial lines and crosses of egg- and meat-type chickens, of which about 14 thousand SNPs are associated with economically important traits of egg-type chickens [31-33].

Z. Liu et al. [34] used the high-density Affymetrix 600 K chicken SNP chip (Affymetrix, Inc., USA) for GWAS of a population of 1078 chickens from the age of first egg laying to 80 weeks of age to identify genomic variations associated with egg mass. The results showed that a 90 kb genome region (169.42~169.51 Mb) in GGA1 is significantly associated with egg weight in 36-week-old hens and is also potentially associated with egg weight in hens at 28, 56, and 66 weeks of age. The rs13972129 locus on GGA1, most significantly associated with egg weight in hens at 36 weeks of age (EW36), was associated with 3.66% (SE = 0.04) of phenotypic variation. Two candidate genes, *DLEU7* (*deleted in lymphocytic leukemia 7*) and *MIR15A Mir-15* (*microRNA precursor family*), may map to this narrow significant region and pleiotropically influence egg mass. In addition, the *CECR2* (*Histone acetyl-lysine reader*) gene on GGA1 and two genes, *MEIS1* (*Meis homeobox 1*) and *SPRED2* (*Sprouty-related, EVH1 domain-containing protein 2*) on GGA3 which are involved in embryogenesis and organogenesis are also classified as candidate genes associated with first egg weight and egg weight in hens at 56 weeks of age. According to the authors, the results may provide a theoretical basis for obtaining eggs of ideal size based on selective breeding based on the studied markers [34].

A genome-wide scan with a high-density SNP chip containing 57636 markers

allowed the authors [35] to discover new loci associated with egg production and quality in White Leghorns and Brown Dwarf chickens. Eight SNPs were identified that correlated with egg production and egg quality parameters, including egg, eggshell and yolk weight, eggshell thickness and strength, albumen height and number of Haugh units determined at 40 and 60 weeks of age for laying hens. Some significant SNPs are located in known genes, including *GRB14* (*growth factor receptor bound protein 14*) and *GALNT1* (polypeptide N-acetylgalactosaminyl transferase 1), which may influence ovarian development and function, but a larger number are located in genes with unclear functions. Further study is required to confirm the functional significance of these newly identified SNPs [35].

GWAS analysis identified loci and genes associated with egg yolk, follicle and ovary weight in chicks (n = 1534) from laying onset to 72 weeks of age [36]. For all ages studied (11 age points), moderate SNP-based heritability estimates for yolk mass were shown (h^2 = 0.25-0.38), while estimates for follicle mass (h^2 = 0.16) and ovary mass (h^2 = 0.20) were relatively low. Independent univariate genome-wide screens for each character studied identified 12, 3, and 31 new significant substitutions associated with yolk, follicle, and ovary weight, respectively. The candidate genes *ZAR1* (*Zygote arrest 1*), *STARD13* (*StAR related lipid transfer domain containing 13*), *ACER1b* (*alkaline ceramidase 1*), *ACSBG2* (*acyl-CoA synthetase bubblegum family member 2*) and *DHRS12* (*dehydrogenase/reductase 12*) were identified as having a probable function in yolk and follicle development [36].

With an increase in the duration of laying period in chickens, the problem of a decrease in the quality of eggs at the end of the laying period has emerged. Thus, external characteristics consist of the color of the eggshell, the egg shape index, the thickness and strength of the eggshell, while internal characteristics include the height of the albumen, the color of the egg yolk, and Haugh units. Basically, these are all quantitative characteristics [37].

With the development of molecular genetics, many studies have been carried out to identify the genetic encoding of egg quality [38-40]. GWAS analysis discovered genomic associations with egg quality at later stages of laying, which have important theoretical and practical significance. A population of 1078 chickens aged 72 and 80 weeks was subjected to GWAS analysis with the high-density Affymetrix 600 K chicken SNP chip. The analysis showed that the genome region at positions 8.95 to 9.31 Mb (~ 0.36 Mb) on GGA13 was significantly associated with egg albumen height and Hau units, and the two most significant SNPs accounted for 3.12 5.75% of the phenotypic variance. Two important genes, *MSX2* (*msh homeobox 2*) and *DRD1* (*dopamine receptor D1*), which are associated with embryonic and ovarian development, have also been found to influence egg quality. Three genesб the *RHOA* (*ras homolog family member A*), *SDF4* (*stromal cell derived factor 4*), and *TNFRSF4* (*TNF receptor superfamily member 4*) have been identified as candidate eggshell color genes [41].

Maintaining high egg production of chickens throughout the entire laying period is of decisive importance for ensuring optimal production performance in industrial poultry farming. Extension of the laying cycle and, therefore, a decrease in egg production rates is one of the problems of modern poultry farming [37, 42, 43]. SNPs in the *RAPGEF6* (*Rap guanine nucleotide exchange factor 6*) gene associated with the intensity of egg laying in the Chinese Jing Hong chicken were studied [44]. The authors assessed the intensity of egg laying in hens of the parent flock at the age of 61-69 weeks both by phenotype and genotype, using a high-density SNP chip (600K Affymetrix Axiom HD SNP-array, Aviagen Ltd., UK). The results of GWAS analysis showed that the egg production trait is significantly associated with five SNPs (AX-75745366, AX-75745380, AX-75745340, AX-75745388 and AX-75745341) located in the *RAPGEF6* gene on chromosome 13. A total of 1676 Jing

Hong laying hens were genotyped, including 858 hens of the 1st generation and 818 hens of the 2nd generation. Three of the five polymorphisms (AX-75745366, AX-75745340 and AX-75745341), which significantly affected egg production at a later stage of laying, are proposed as molecular genetic markers in breeding chickens [44].

The study of polymorphism of prolactin genes and its dopamine receptor may be of practical importance for chicken breeding. It is known that the hormone prolactin in birds takes part in the regulation of the reproductive cycle. Thus, an increase in the blood prolactin levels leads to a decrease or cessation of egg production [45]. Dopamine actively influences the secretion of prolactin [46-48]. Five dopamine receptor subtypes have been identified and are divided into two classes called D1-like (DRD1, DRD5) and D2-like (DRD2, DRD3, DRD4). In birds, dopamine is involved in both stimulation and inhibition of prolactin secretion [49]. Activation of DRD1 stimulates prolactin secretion, and through DRD2, secretion is inhibited [50]. These and other studies confirm the regulatory role of dopamine in egg production in birds. The authors assessed the relationship of the *DRD1* gene with egg production and hatchability in 644 chickens [51]. In the *DRD1* gene, 29 single nucleotide polymorphisms were identified. Of these, 7 SNPs were selected to analyze their association with egg production traits in chickens. A significant effect was shown of four SNPs (G+123A, T+198C, G+1065A, C+1107T) on the age of laying the first egg (it characterizes the rate of sexual maturation), the weight of the first egg, and the yield of conditioned eggs [51].

Vasoactive intestinal peptide (VIP), a releasing factor of the hormone prolactin in birds, stimulates the secretion of prolactin and is involved in the regulation of the activity of the prolactin gene. Associations have been found between the polymorphism of the chicken *VIP* gene, brooding instinct and egg production [52]. Sequencing revealed 69 single nucleotide substitutions in a 9305 bp region of chicken *VIP* gene. Five polymorphisms, the C 3134T, "AGG" indel from –2648 to –2650, C+338T, G+780T and A+4691G were used to evaluate their effect on egg production and brooding traits in 644 Ningdu Sanhuang chickens. Analysis of the association of the marker showed that "AGG" indel is associated with the total number of eggs and the number of quality eggs in hens aged from 90 to 300 days. The C+338T polymorphism was found to be associated with egg hatchability [52].

Follicle stimulating hormone (FSH) and its receptor (FSHR) play an important physiological role in animal reproductive function [53, 54]. FSH is a glycoprotein synthesized and secreted by the cells of the anterior pituitary gland. When it enters the bloodstream and binds to a specific transmembrane receptor (FSHR) located on target cells, this hormone and receptor play a vital role in gonadal function and fertility [55]. The nucleotide sequence of the chicken *FSHR* gene was determined in 2005. A number of studies have been carried out on the regulation of *FSHR* transcription in mammals, but its mechanism in chicken is not fully understood [56]. Differences in the *FSHR* gene expression among different chicken breeds may lead to variations in egg production parameters, including age at first egg (AFE), total number of eggs, and egg weight. In addition, polymorphisms in the *FSHR* gene promoter may affect *FSHR* transcription and egg production. A study was conducted [57] on two breeds, a local Chinese breed Dongxiang with black plumage and skin, laying blue-shelled eggs [58] and reduced growth rate and egg production [59]; a Chinese breed Suken with yellow plumage, beak and claws [60] and laying cycle of approximately 268 days with a peak within 40 days. The PCR-RFLP method detected five nucleotide polymorphisms in the *FSHR* gene promoter, including 200 bp indel at –869, C 1684T, C 1608T, G 368A and T 238A associated with egg production traits in both the Dongxiang and Suken breeds. The age at which the first egg was laid in Suken chickens differed significantly ($p < 0.01$) depending on the genotype for indel –869. In poultry farming, the number of eggs laid

during 43 weeks of life is usually an effective indicator of overall egg production [61]. For SNP C 1684T, in Dongxiang chickens with the CC genotype, the number of eggs laid at the age of 43 weeks was greater than in individuals with the TC genotype ($p < 0.05$), while in Suken chickens, on the contrary, for the TC genotype, the AFE indicator was higher than for the CC genotype ($p < 0.05$). For AFE in Suken breed, the CC genotype for SNP C 1608T was superior to the TC genotype ($p < 0.05$), and the AG genotype for SNP G 368A was superior to the GG genotype ($p < 0.05$). In total, this study [57] identified five polymorphisms in the *FSHR* promoter region and rebealed their association with egg production at the age of 43 weeks and of the first egg laying.

Growth and reproduction, which are controlled by multiple genes, are the two most economically important traits for poultry production. The integration of emerging technologies, the identification of related genes, and the unraveling of the molecular mechanisms governing their activity provides the opportunity for more effective selection of chicks for growth rate and reproductive performance [62-64]. QTL (quantitative trait loci) associated with chick growth and reproductive performance (body weight and age at first egg laying) have been investigated in recent decades [65, 66]. The identification and use of potential candidate genes and QTLs that have significant effects on economically important traits are becoming increasingly important in poultry breeding programs [67-71].

Selection has led to increased growth rates and breast muscle yield in broilers. However, physiological disturbances arose, leading to increased fat deposition and deterioration of the bird's skeleton [72]. To simultaneously improve performance and physiological traits, molecular markers associated with one or both sets of traits may be useful. Insulin like growth factors (IGFs) are a family of polypeptide hormones that are structurally similar to insulin and have multiple metabolic and anabolic functions [73]. IGF-I and IGF-II stimulate the proliferation, differentiation, and metabolism of myogenic cell lineages in different species [74, 75]. IGF genes have been reported to influence growth rate and regulate muscle tissue growth in chickens [76-78].

Further, data [79-82] indicate that IGF1 is a candidate positional gene involved in the control of growth and fat deposition in chickens. A study of 392 egg cross chickens and 321 meat cross chickens revealed statistically significant associations of single-nucleotide polymorphism in the *IGF1* gene promoter with the majority of the studied traits (growth rate, body composition, skeletal condition and physiological properties) [83].

Boning condition is becoming increasingly important for both broilers and layers. Genotype has been shown to play a key role in bone integrity, but little is known yet about the architecture of the genetic basis of this trait [84]. S. Jansen et al. [85] based on a study of genes associated with bone tensile strength and mineralization in laying hens, selected 16 candidate genes and assessed the effects of 490,745 SNP markers [85]. The identified genes and SNPs were associated with bone strength. The authors also identified genes that are critical for bone integrity. The mechanisms of participation of these genes in the formation of the skeletal system require further study with a view to using them to reduce bone disorders in laying hens [85].

Meat quality indicators are economically significant. In meat and poultry products, a high content of nutrients is combined with a relatively low calorie content, but the consumer properties of the resulting products depend on the conditions at all stages of poultry farming, from the fertilized egg to processing of raw materials [86-88].

Basic meat quality characteristics that are of interest and quantifiable (e.g., pH, water-holding capacity, meat color) have collectively become selection criteria

in chicken selection and breeding programs [89). However, improving these quality parameters using traditional breeding methods is difficult because the measurements are time-consuming and require slaughter of the bird, which significantly increases the intergenerational interval and slows overall genetic progress [90]. However, estimates of the heritability of meat quality traits (h^2 ranging from 0.35 to 0.81) indicate that genetic selection is the most effective tool for improving broiler meat quality [91]. Therefore, it is important to understand the genetic background of traits associated with poultry meat quality. They are considered complex quantitative traits and are controlled by many genes. Research has shown that the fatty acid binding protein *FABP* gene plays an important role in improving overall meat quality [92]. The *CAPN1* (*micromolar calcium-activated neutral protease*) gene was found to be significantly associated with tenderness and other quality traits of meat [93], and SNP V315M in the *PRKAG3* (*protein kinase AMP-activated non-catalytic subunit gamma 3*) gene was significantly associated with the water-holding capacity of meat (94). In several studies for different animals, including poultry, it has been shown that *PHKG1* (*phosphorylase kinase catalytic subunit gamma 1*) is a candidate gene influencing meat quality characteristics [95-97]. In local Chinese Ningdu yellow chickens, the effect of single nucleotide polymorphisms in the *PHKG1* gene on traits associated with meat quality was studied, and the associations between polymorphisms of the *PHKG1* gene, meat quality and carcass parameters were analyzed. Significant associations of the SNP marker rs15845448 with 17 studied traits were identified, and this marker can be used in breeding programs for the Chinese Ningdu yellow breed [98].

The Table summarizes the information about candidate genes, single nucleotide polymorphisms in which are statistically significantly associated with economically valuable traits in chickens.

Candidate genes and SNPs associated with economically valuable traits in chickens

Gene	Function	Economically valuable traits associated with SNP in candidate genes
NCAPG (Non-SMC condensin I complex, subunit G)	Condensins are subunit protein complexes that play a fundamental role in the structural and functional organization of chromosomes; condensins I and II are involved in the regulation of gene expression, recombination and repair	Egg productivity [17], egg shell weight and thickness [12, 13, 16, 17]
LCORL Ligand dependent nuclear receptor corepressor-like	One of the key genes that determines the characteristics of body weight in vertebrates	Live weight of chickens [10]
GRB14 growth receptor binding 14	A gene that encodes a protein that binds the growth factor receptor. In humans and mammals, *GRB14* mRNA is expressed at high levels in the ovaries, liver, kidneys, skeletal muscles, etc.	Development and function of the ovaries [35]
GALNT1 UDP-N-acetyl-alpha-D-galactosamine: a polypeptide of N-acetylgalactosaminyltransferase 1	Ensures normal ovarian functions. The characteristics of this gene in chickens are still unclear, and the mentioned study is the first report that its polymorphism is associated with the quality characteristics of eggs	Development and function of the ovaries, quality characteristics of eggs [35]
MSX2 msh homeobox 2, a member of the msh homeobox family	Expressed in many embryonic tissues. In the chicken, it is expressed in the apical ectodermal ridge and ectoderm of the genital tubercle, plays a decisive role in the growth and formation of limb mesoderm	Embryonic development, ovarian development, egg quality [41]
RHOA Small GTPase of the ras homologue (Rho) family	Molecular switches that control a wide range of cellular functions — cytoskeletal reorganization, cell motility and gene expression. The *RHOA* signaling system plays a role in the modulation of actin stress fibers and chondrogenesis	Body growth, eggshell color, egg quality at later stages of laying [41]

Continued Table

SDF4 Stromal cell derived factor 4	Its human ortholog is known as *Cab45*. Regulates cell migration through various molecular mechanisms	Eggshell color [41]
TNFRSF4 Tumor Necrosis Factor Receptor Superfamily, Member 4	*TNFRSF4* can be used to specifically modulate the expression of other genes that directly stimulate effector T cell activity	Eggshell color [41]
RAPGEF6 Rap guanine nucleotide exchange factor 6	Plays a fundamental role in spermatogenesis, indicating that RAPGEF6 is required for reproductive development	Egg production at late stage of laying [44]
PRL Prolactin	One of the hormones of acidophilic cells of the anterior pituitary gland. Almost all known functions are related to reproduction	Regulation of oviposition. Plays a decisive role in the emergence and maintenance of the brooding instinct [41, 45]
DRD1 Dopamine D1 receptor gene Dopamine D5 receptor gene	In birds, dopamine is involved in both stimulation and inhibition of prolactin secretion	Egg production, egg hatchability [49-51]
VIPR1 Vasoactive intestinal peptide receptor-1	Vasoactive intestinal peptide (VIP)-releasing factor of the hormone prolactin in birds	Brooding instinct, egg hatchability, egg production [52]
FSH Follicle-stimulating hormone gene *FSHR* Follicle-stimulating hormone receptor gene	Gonadal and fertility functions	Egg production of chickens, age of laying the first egg [55, 57]
IGF1, IGF2 Insulin-like growth factors I and II	Proliferation, differentiation, metabolism of myogenic cell lines	Growth of muscle tissue in chickens [76-78], growth and fat deposition in chickens [79-82], height, body composition, skeletal condition [83]
FABP Fatty acid binding protein gene	Participation in lipid metabolism, transport of fatty acids at intermediate stages of adipogenesis and fat deposition	Meat quality [92]
CAPN1 Micromolar calcium-activated neutral protease gene	Calpains (intracellular Ca2+-dependent cysteine proteases) (EC 3.4.22.17) are involved in muscle growth and development. CAPN1 degrades myofibrillar proteins and appears to be the main enzyme in the process of post-mortem softening	Qualitative characteristics of meat, mainly tenderness [93]
PRKAG3 5'-AMP-Activated Protein Kinase Gamma 3 Subunit gene	Controlling the energy balance of the cell, the AMPK 3-gamma subunit can bind 3 AMP molecules, one of them is constantly bound to the protein, regardless of the energy status of the cell. Has an affinity for the nucleus	Qualitative characteristics of meat, its moisture-holding capacity [94]
PHKG1 Phosphorylase Kinase Catalytic Subunit Gamma 1 gene	A member of the Ser/Thr protein kinase gene family, it encodes a protein with one protein kinase domain and two calmodulin-binding domains. The protein is a catalytic element of a 16-subunit protein kinase complex, consisting of four types of subunits in equimolar ratios	Meat quality, meat color [98]

Thus, genomic approaches in the selection of commercial and local breeds of chickens can significantly accelerate breeding progress both in the breeding of heavy crosses focused on the yield of meat products, and in maintaining high levels of egg productivity in parent flocks. Sequencing chicken genome has meant enormous advances in poultry genetics and breeding research [99, 100], but a more complete and in-depth research are necessary to understand the mechanisms that control desirable traits.

So, due to the improvement of genomic methods, researchers can analyze poultry genomes to directly confirm bird's genetic potential and reveal its effect on economically significant traits. It is necessary to protect the domestic gene pool of chickens from the spread of genetic diseases and maintain the level of individual and group heterozygosity during breeding and selection of Russian populations and breeds. Currently, genotyping of chicken breeds for meat, egg and combined productivity is being actively carried out. A significant amount of information has been accumulated on single nucleotide polymorphisms (SNPs) associated with productivity in chickens of local and some commercial breeds at different periods

of the productive cycle. According to available data, single nucleotide polymorphism affects economically significant traits of chickens, and SNP markers can be used in the development of breeding and genetic programs in poultry farming.

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AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 2, pp. 223-248
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636:575.174.015.3

doi: 10.15389/agrobiology.2023.2.223eng
doi: 10.15389/agrobiology.2023.2.223rus

DNA MARKERS AND MICROSATELLITE CODE
(review)

V.I. GLAZKO, G.Yu. KOSOVSKY, T.T. GLAZKO✉, L.M. FEDOROVA

Afanas'ev Research Institute of Fur-Bearing Animal Breeding and Rabbit Breeding, 6, ul. Trudovaya, pos. Rodniki, Ramenskii Region, Moscow Province, 140143 Russia, e-mail vigvalery@gmail.com, gkosovsky@mail.ru, tglazko@rambler.ru (✉ corresponding author), felami@mail.ru
ORCID:
Glazko V.I. orcid.org/0000-0003-4242-2239
Kosovsky G.Yu. orcid.org/0000-0003-1889-6063
Glazko T.T. orcid.org/0000-0002-0520-7022
Fedorova L.M. orcid.org/0000-0002-1514-3050
The authors declare no conflict of interests
Final revision received January 10, 2023
Accepted February 6, 2023

Abstract

The search for genetic markers that simplify the selection of animals for crosses, increasing the likelihood of offspring obtaining with the desired manifestation of economically valuable traits is a central problem in modern animal husbandry. Here, we discuss the most successful applications of various types of DNA markers of genomic element polymorphisms for solving specific breeding problems. Microsatellites are used to exclude errors of origin, single nucleotide polymorphisms (SNPs) to create maps of genomic regions in which polymorphism is associated with the variability of phenotypic characteristics (D.J. Rigden, X.M. Fernández, 2023) and to identify the localization of key genes of adaptation to natural selection factors at the natural habitat edges and in areas of animal husbandry risky (E.K. Cheruiyot et al., 2022; L. Buggiotti et al.., 2021, 2022). The loci of increased variability in the copyicity of genome regions (CNV) are used to assess their involvement in responses to natural and artificial selection factors of such polygenic systems as sensory, immune, and transporter (Y. Huang et al., 2021; P. Davoudi et al., 2022). The predominant involvement of regulatory networks including dispersed and tandem repeats, in particular microsatellite repeats, in epigenetic and phenotypic variability is discussed (R.P. Kumar et al., 2010). The structural and functional complexity of microsatellite loci, individual features of variability of specific loci and their participation in evolutionary, recombination, transcription processes are considered. Their involvement in the organization of secondary DNA structures, participation in the formation and variability of the architectonics of the interphase nucleus and regulation of gene expression profiles is noted (R.P. Kumar et al., 2010; X. Tang et al., 2022). The study of regulatory networks is of particular importance, since there is evidence that the size of the genome in animals of different taxa, as well as the distribution and composition of mobile genetic elements (sources of components of regulatory networks) differ significantly, in contrast to the similarity in the number of genes encoding proteins (V.I. Glazko et al., 2022). Accumulating evidence suggests that polylocus genotyping of individual microsatellites and dispersed repeats can contribute to solving practical problems, such as information on the specific features of the population-genetic structure, consolidation and differences between closely related groups of animals.

Keywords: DNA markers, microsatellites, short tandem repeat, STR, single nucleotide polymorphism, SNP, copy number variations, CNV, genome-wide association studies, GWAS

The success of breeding work is determined by the quality of selection and selection of animals for crossing, which directly depends on the ability to predict the manifestation of desirable economically valuable traits in specific environmental conditions. The idea of selection using markers was first put forward by the Soviet geneticist A.S. Serebrovsky who introduced the concept of a signal gene. According to these ideas, the so-called signalia are alternative genes convenient for Mendelistic observations with a more or less known chromosomal localization, which, without directly affecting the transgressive trait being studied and influencing in a fairly specific way, facilitate the genetic analysis of this trait, allowing one

to monitor the inheritance of that trait the region of the chromosome in which these signalia are located [1]. Further, as markers, antibodies to protein antigens of biological objects were considered in animals, then polymorphism of protein products of the same genes, detected by the electrical charge of molecules (biochemical markers) in both plants and animals [2].

The next stage was the development of DNA markers of various types. According to the general definition, DNA markers are markers of polymorphism of genomic regions the variability of which can potentially be associated with its manifestations at the phenotypic level. However, with in-depth study of some DNA markers of various genomic elements, their involvement in complex networks of relationships between them, in ensuring the stability, variability and evolution of biological systems, becomes obvious.

In this review, we presented examples of the effectiveness of different generations of DNA markers as tools in applied research and analyzed data on microsatellite (short tandem repeat, STR) DNA markers. The variety of biological functions of STRs is considered, including, in particular, participation in the formation of secondary DNA structures, the architectonics of the interphase nucleus, elements of gene expression regulatory networks, which, in our opinion, changes the understanding of their biological role and the possibilities of practical application.

Practical application of DNA markers. Types of DNA markers and their significance for genetic and genomic research. Since the 1980s, polymorphism of genomic regions began to be used for genetic marking. In the 1980s, the most common DNA markers were those based on restriction fragment length polymorphism (RFLP); in the 1990s, markers detected using polymerase chain reaction (marker-assisted selection) selection, MAS), after 2000, DNA chips for detecting single nucleotide polymorphisms (SNPs) during whole-genome sequencing became widespread.

Polylocus genotyping using DNA markers (genomic scanning) in agricultural species is widely used i) to determine the parameters of variability within and between breeds; ii) to identify population genetic characteristics in geographically separated groups and/or when mixing animals of different origins in groups; iii) to study evolutionary relationships and search for centers of origin and migration routes; iv) to map the main genes with polymorphism is associated with variability in phenotypic characteristics (including the identification of known alleles for genetically determined diseases and the identification of their carriers, and v) to detect alleles associated with increased resistance to infectious and non-infectious diseases). For multilocus genotyping, microsatellite markers are widely used - tandem repeats, the elementary (repeating) unit of which can be from 2 to 6 bp in length. (simple, or short tandem repeat, STR) [3].

In agreement with the Food and Agricultural Organization (FAO) and the International Society of Animal Genetics (ISAG), genotyping panels for farm animals were initially developed for blood groups, then for biochemical markers, and currently, by microsatellite polymorphism. These panels are specific for each species, include a couple of dozen loci and are designed in such a way that they allow PCR to be carried out simultaneously for several markers and solve a number of current problems (identification of origin, identification of breed population genetic characteristics of animals). For more than two decades, genetic certification of animals, necessary to exclude errors of origin, was carried out using such panels of highly polymorphic markers. The degree of their heterozygosity, despite frequent inbreeding in farm animals, is often 75% or more. Typically, dinucleotide and trinucleotide repeats are used for certification. Along with them, due to the expansion of whole-genome sequencing of representatives of different species, new

generations of DNA markers appear, usually based on SNPs.

To date, more than 30,000 Holstein bulls have been genotyped using BovineSNP50 BeadChip DNA microarrays (Illumina, USA), which allows simultaneous analysis of 54,001 SNPs (approximately one SNP per 50,000 bp). Using such microarrays, maps of SNP distribution across genomes have been constructed for different species [4] and genome-wide association maps of SNP localization sites with variability in phenotypic characteristics (genome-wide association study, GWAS) have been created [5, 6].

DNA microarrays (chips) make it possible to detect not only SNP-linked markers, but also copy number variations (CNVs), including deletions, duplications, translocations and inversions [7]. CNV is attracting increasing attention because it is often associated with variability in phenotypic traits in agricultural animal species [8-13] and with unfavorable phenotypic manifestations in humans [7]. Detailed chromosomal maps of the distribution of CNV markers were created. Specifically, in humans, CNV loci cover 12% of the genome (Database of Genomic Variants, http://projects.tcag.ca/variation/), meaning CNVs involve more nucleotides per genome than SNPs [14]. Spontaneous CNVs are predicted to occur at an average frequency of 10^{-4} bp [15]. The high level of variability gives reason to believe that the use of CNV markers of polymorphism in genomic DNA regions will increase the accuracy of mapping the main genes of such economically important animal characteristics as resistance to biotic and abiotic environmental factors and productive qualities [16-18].

Thus, it is worth noting the successful development of methods for sequencing biomolecules, especially using fourth-generation technologies (nanopore-based sequencing) [19], as well as complete reading of genomes in most agricultural animal species, including mammals [20]. However, the directions for practical application of the huge volume of results of these gene and genomic studies are still not sufficiently developed. It is necessary to correlate the data accumulated over decades on the genotypes of representatives of different breeds by microsatellites (STR) and the results of genotyping using SNP panels, which is currently (as previously STR analysis) carried out to control origin, breed identification, and identify relationships between genotypes and variability of phenotypic traits [21-24]. It turned out that, among other complexities, the STR panel recommended by ISAG for genotyping farm animals includes microsatellites that differ significantly from each other in polymorphism and efficiency in differentiating animals within and between breeds [21]. A study of the colocalization of STRs and SNPs in the cattle genome showed that only 57.1% of STRs are in linkage disequilibrium (LD), while the remaining 42.9% are located outside such blocks [25]. In other words, in the cattle genome, a significant number of STRs are not linked to SNPs (probably due to the specific mechanisms of mutations and genomic distribution of STRs, as well as their increased polymorphism). It follows from the fact that each microsatellite has its own characteristics of polymorphism and mutability, and combining different microsatellites into a common panel can lead to erroneous conclusions.

The widespread replacement of whole genome sequencing (WGS) with a relatively limited number of SNPs on DNA chips in order to identify SNPs associated with variability in phenotypic characteristics also often carries sources of significant errors [26]. As in the case of STR, the possibility of using a limited number of SNPs to predict the variability of phenotypic traits will depend on the localization of SNPs in various genomic elements and their structural and functional features [26].

The development of a new direction, the pangenomics (in particular, pangenomics of cattle), due to the accumulation of WGS data for many genomes,

makes it possible to add to the reference genome of cattle presented in GenBank (*Bos taurus*, https://www.ncbi.nlm.nih.gov /data-hub/taxonomy/9913/), appx. 4% of nucleotide sequences [27]. This may influence the shift in the positions of the analyzed SNP allelic variants. Thus, in cattle, in addition to the reference genome, 83,250 SNPs were identified, for which polymorphism is observed both within breeds and between breeds [27].

Search for DNA markers of economically valuable traits. In the last decade, the development of GWAS has allowed advances in understanding the genetic basis of complex traits and diseases in both humans and livestock [28]. An interesting fact is that most SNPs associated with phenotypic variability of properties are localized in non-coding sequences of the genome [28]. In many cases, such sequences are closely related to numerous regulatory elements (RE) influencing gene expression profiles [29]. However, their evolutionary preservation, variability, and involvement in the manifestation of complex polygenic traits still remain insufficiently studied.

STR or SNP polymorphism can be used to map genomic regions of the localization of genomic elements, the variability of which makes a significant contribution to the manifestation of quantitative economically valuable traits in animals of agricultural species, and to detect key genes and/or RE of such traits. As a rule, these genomic elements are detected in animals living in extreme environmental conditions. A striking example of successful searches are studies of the resistance of milk productivity of Australian Holstein cows to high temperatures [30, 31], and of Kholmogory and Yakut cattle to low temperatures [32, 33].

The composition and functional organization of genomic elements involved in the genetically determined control of milk production in cattle may be an example of the complex genetic basis of quantitative economically valuable traits. To date, a chromosomal map of genes involved in the formation of the udder and milk production in cattle (the so-called lactome map) has been created, which includes 197 milk protein genes and more than 6000 genes involved in the development and functioning of the mammary gland [34]. It turned out that these genes are scattered across all 30 chromosomes of cattle. Comparison of the genomes of the platypus, opossum, placental mammals (cattle, dog, human, mouse, and rat) [34] for the genes of milk proteins and mammary gland formation revealed losses and duplications, phylogenetic relationships, conservatism of the sequences of these genes and their evolution. Evidence has been obtained that in cattle, the genes for milk and mammary gland proteins evolve more slowly than in other studied placental species. It was found that in cattle, in comparison with other listed species, the genes of milk proteins that determine its nutritional and immune properties were the most divergent; the genes associated with the processes of milk secretion turned out to be the most conservative.

Analysis of the transcriptome at different stages of lactation showed that 16,892 genes are expressed during the intermediate period of the lactation cycle, 19,094 at the peak of lactation, and 18,070 during the decline of lactation. The expression level of genes encoding caseins, whey proteins and enzymes of the metabolic pathway for lactose synthesis was increased at the beginning of lactation, and most genes of the metabolic pathways of lipid metabolism were increased in the intermediate period and at the peak of lactation [35].

It is obvious that milk production is influenced by the genetic characteristics of the individual, epigenetic processes, nutrition, pathogens, climatic conditions and other external factors. This is especially clearly revealed by the example of differences in selection indices calculated for the same Holstein bulls based on the milk productivity of their daughters born in different ecological and geographical regions, in Luxenburg and Tunisia [36].

Over the past decade, there has been a significant increase in the number of studies assessing the impact of epigenetic variability associated with regulatory networks, which are represented, in particular, by microRNAs (miRNAs, small non-coding RNAs with transcriptional and post-transcriptional effects). The direct involvement of microRNAs in the control of gene expression profiles and in the regulation of the development and functioning of the mammary gland is increasingly being revealed [37, 38]. MicroRNAs have been found to play an important role in many processes associated with breast development and disease, as well as milk secretion. Hundreds of miRNAs have been identified in the mammary gland, but the number of miRNAs whose functions are fully known is very small. The problem is that one miRNA can be involved in the control of hundreds of genes, so functional validation of each target gene for this miRNA is difficult. The situation is further complicated by the fact that the response to the same environmental factors can be provided by different microRNAs, not only in closely related species [39], but also in breeds [40].

The diversity of microRNA spectra is very wide, as is their connection with the regulation of gene expression of different metabolic pathways and their intersection points. Of the 19,994 protein-coding orthologous gene pairs between *Bos taurus* and the extinct species *B. primigenius*, 1,620 genes differ in microRNA binding site polymorphism in the 3′-UTR [41]. These 1,620 genes are primarily involved in the control of pigmentation, reproduction, neural function, general metabolism, immune responses, and variation in animal performance traits, including milk quality and feed efficiency.

Applicability of DNA markers for practical selection. One of the first conclusions that can be drawn based on data from genetic and genomic studies of farm animals is apparently the following. There is no need to look for a universal method to solve all breeding problems. If STR panels have shown success in eliminating origin errors, there is no point in replacing them with more complex and expensive SNP-based test systems. Moreover, a search is already underway for universal STR panels, orthologous in different species, with the desired level of polymorphism [42], which will make it possible to differentiate species and intraspecific variability of biological objects [43].

Obviously, SNP maps are indispensable when searching for genomic regions in which genes for resistance to critical biotic and abiotic external factors are localized. Based on their refinements and expansion of mapping volumes, environmental genomics will be formed as a continuation of environmental genetics - a scientific direction laid down by A.S. Serebrovsky [1].

SNP analysis is important for searching for candidate genes for economically valuable traits when their expression differs significantly in the animals being studied, since in this case it is possible to search for a small number of genes that cause such differences. It is difficult to overestimate the success of using SNPs to reconstruct the genetic dynamics of populations based on the analysis of runs of homozygosity (ROH) [44] or when searching for mutations in genes critical for animal reproduction (loss-of-function or fertility haplotypes) [45].

A significant amount of data on the distribution of SNPs has allowed genome-wide association analyzes (GWAS) of SNPs with various traits in plant and animal species to be performed [46]. The results are presented on the resources of the National Genomics Data Center, China National Center for Bioinformation [47] and in the database collection of the journal Nucleic Acids Research [47]. For such a complex trait as milk production, it is difficult to expect obvious and reliable success [49]. L. Flori et al. [49) reported associations between SNP haplotypes (in linkage disequilibrium) with variability in milk production parameters in the three main dairy breeds of France — Holsteins, Normans, and Montbeliards. In areas of

increased density of such haplotypes in three breeds, a total of 40 genes were identified, mainly differing in the studied breeds. Perhaps the observed contradictions are due to epistatic interactions between gene ensembles involved in the manifestation of such economically valuable traits as the amount of total milk yield, the percentage and amount of milk fat and protein. In a GWAS analysis using 76,109 SNPs in 294,079 Holstein cows of the first lactation, the effect of pairwise epistasis on indicators of milk productivity and reproduction (total milk yield, yield of milk fat, protein, percentage of milk fat and protein, pregnancy rate of daughters) was assessed [50]. Of the top 50,000 identified pairwise epistasis effects for each trait, five involve large chromosomal regions with intrachromosomal epistasis [50]. In fact, this can explain the well-known undesirable correlations between total milk yield and milk protein content, milk fat content and reproduction in cows. A clear demonstration of the difficulty of identifying gene elements whose polymorphism is associated with variability in an economically valuable trait is the quantitative trait locus on chromosome 18 (BTA18), associated with ease of calving and stillbirth in Holstein-Friesian cattle and its crosses (51). This fact has been known for more than 20 years, but its genetic basis has not yet been identified. To identify it, based on genotyping of 2697 Holstein Friesians, a detailed analysis of the corresponding BTA18 region was performed and an assessment of linkage disequilibrium in this region was performed. As a result, the connection of the polymorphism with the described pathology was confirmed, 4 SNPs with almost perfect linkage disequilibrium were identified, but not a single candidate gene associated with the specified pathology was identified. An abundance of segmental duplications was found within and around the region [51].

The method of genotyping using CNV markers appears to be very effective in identifying physiological systems involved in the direction of selection, primarily at the interspecific level. Indirect support for this assumption is the fact that in farm animals, CNVs are most often detected in the case of genes involved in various functions of the immune system [52-55].

MicroRNAs as elements of regulatory networks have attracted attention due to their involvement in the control and modulation of the functional activity of genes [37-40]. However, it must be taken into account that the degree of variability in the expression level of different genes is not the same. Our analysis of gene expression profiles in the liver of pigs [56] revealed two groups of genes, with and without individual differences between the animals studied. In pigs, using the KEGG Metabolic pathway database (https://www.genome.jp/kegg/path-way.html), we assessed the involvement in different metabolic pathways for 17 genes with similar expression values in individuals (these genes conditionally designated as a group with constitutive expression) and 18 genes that make up the variable part of gene expression profiles with pronounced individual differences between animals. In our experiment, the products of 17 constitutively expressed genes were involved in 25 metabolic pathways, or an average of 1.5 pathways per gene, and each of the 18 genes with variable expression levels accounted for 3 metabolic pathways [56]. That is, the greater the number of metabolic pathways in which the gene product is involved, the more complex the potential regulatory elements that unite and control them, the higher the individual variability of gene expression. Thus, although the role of microRNAs in regulatory networks is indisputable (as is the role of the regulatory networks themselves in the formation of traits and changes in their manifestations under the influence of influencing factors), the promise of microRNAs as molecular markers is ambiguous. As noted above, one microRNA can affect the transcripts of several dozen genes, and different microRNAs can affect the activity of one gene [37-40]. Thousands of transcription regulatory factors also change it by interacting with non-coding nucleotide sequences of the

gene [40, 41]. In addition, the interaction of gene products of the same metabolic pathway and/or different metabolic pathways can also activate processes leading to changes in gene activity profiles [40, 41].

Microsatellites, structural and functional diversity. *Structural and functional features of STR markers.* Microsatellites belong to the first generation of DNA markers, which have been widely used in the genetics and genomics of humans, farm animals and plants for more than 30 years [42]. The experimental data accumulated over this period made it possible to obtain a fairly complete pattern of the complex structural and functional organization of DNA markers of this type. To date, species-specific features of the number and genomic distribution of microsatellites have already been studied. Maps of the distribution of microsatellites in the genomes of different species have been created [57].

Using the example of the distribution of STRs in the genome of the domestic rabbit (*Oryctolagus cuniculus*), one can note a pronounced difference in the frequency of occurrence of STRs due to the length of the elementary repeat: 579097 mono-, 927755 di-, 122482 tri-, 767458 tetra-, 614173 penta- and 1739144 hexanucleotide repeats [57]. The large number of hexanucleotide repeats is explained by the fact that in mammals the conserved telomeric repeat TTAGGG is duplicated several thousand times [58]. It is interesting that in total in the genome of both the rabbit and a number of other mammalian species, including humans, there are significantly fewer trinucleotide repeats than di-, tetra- and pentanucleotide repeats, that is, this difference is not related to the length of the elementary repeat unit [57]. Probably because the structure of trinucleotide repeats corresponds to the triplet principle of the genetic code, they are under selection pressure.

There are pronounced differences between the frequency of occurrence of microsatellites with the same length of the elementary unit, but with different core motifs, for example AG and AC. In the rabbit, the AG motif occurs approximately 5 times more often than the AC motif and more often than all other microsatellites, while in humans, on the contrary, there are more microsatellites with the AC core motif than with the AG motif [57, 59].

STRs also occur in prokaryotes, but at low frequency [56). Trinucleotide STRs are common in nematodes and insects, and dinucleotide STRs are common in fish, a relative deficiency of which is observed in birds [60]. Closely related species can differ significantly in the number of microsatellite loci [60]. We have already noted that, in general, in many mammalian species, di- and tetranucleotide STR motifs are more frequent than trinucleotide ones, but the frequency of the latter varies significantly depending on the microsatellite core motifs, and these differences may be species-specific [61].

Different STRs are generally found most frequently in intergenic spaces, followed by introns, promoter regions, and least frequently in exons [56]. The microsatellite database of different species [56] shows that trinucleotide STRs are found more often in exons than di- and tetranucleotide STRs. Moreover, the frequency of occurrence of trinucleotide STRs in exons can be quite high.

Despite the relatively reduced frequency of occurrence of STRs in exons, their contribution to the polymorphism of encoded proteins can be significant. Thus, recently, by comparing the results of whole-genome sequencing in laboratory mice (71 lines), STR alleles present in the coding regions of 562 genes were identified and evidence was provided that these alleles can change the folding of the encoded protein and thus have a significant impact on its function [62].

Expansion of microsatellites during pathologies and adaptations. It is known that many human pathologies (most often neurocognitive and neurodegenerative) are associated with polymorphism in the copy number (length) of trinucleotide microsatellites [63-66]. In many studies of the genetic basis of diseases in humans,

amplification of triplets $(CCG)_n$, $(CGG)_n$, $(GCC)_n$, $(GCG)_n$ and $(CAG)_n$ was found in the coding regions of the genome, which determine the synthesis of polyproline, polyarginine, polyalanine and polyglutamine [67]. The development of a number of diseases is also associated with changes in the lengths of dinucleotide microsatellites with the GA core motif at their specific genomic localization [67].

Variability in the lengths of trinucleotide STRs is also found in some examples of adaptations. Thus, a connection between an increase in the copy number of microsatellites and an increase in adaptive potential was found in the giant panda during adaptation to food rich in carbohydrates, and in polar and brown bears - to the temperature of the habitat [68]. Variation in STR associated with these adaptations has mainly been identified in regulatory genes (e.g., transcription regulatory factor genes, insulin-like growth factor receptor signaling pathway). These genes are mainly involved in two metabolic pathways involved in key physiological processes (cardiovascular function and regulation of energy metabolism).

The presence of STRs in coding sequences can affect protein folding and change its flexibility, which allows it to bind to various substrates, be it nucleotides, lipids or proteins. Such proteins, containing amino acid repeats encoded by STRs, are expected to participate in the regulation of gene expression, are often multifunctional and have pleiotropic effects, increasing the resistance of cells and multicellular organisms to variability in environmental factors, which seems to justify the complexity associated with the potential high mutability of microsatellites [69, 70].

Non-coding STRs are also capable of significantly influencing phenotypic variation. In humans, it has been reported [71] that 10-15% of heritable variation in gene expression is associated with the presence of STRs. STRs were identified alleles of which differed significantly between ethnic groups. Fifteen STRs were found in which the repeat length correlates with the level of gene expression, two of these genes (*Glutathione Peroxidase 7* and *Glutathione S-Transferase Mu 3*) are involved in glutathione metabolism [72].

The complexity of the mechanisms of influence on transcription and translation with increasing STR lengths is discussed. An increase in the copy number of trinucleotide STRs in coding sequences can lead to pathologies due to the appearance of polyglutamine or polyalanine regions incorrectly localized in proteins, which leads, in particular, to disruption of protein-protein interactions, many of which are involved in the regulation of transcription, DNA repair and/or interfere formation of molecular condensates [73]. The formation of a condensation of a certain transcript with transcription regulatory factors affects the expression of a number of genes, and the accumulation of amino acid repeats can lead to disruption of such condensation and changes in transcription regulation. For example, the polyglutamine protein TBP (TATA-box Binding Protein) binds to the TATA box of gene promoters to initiate transcription, but when the length of the polyglutamine repeats are enlarged, its ability to condense with transcriptional activators is altered, leading to the transcriptional dysregulation observed in many polyglutamine diseases [73]. In other cases, such as with Ataxin-2, the RNA Binding Protein (RBP), involved in the assembly of condensates and stress granules and in RNA processing, carries a polyglutamine sequence. An increase in its copy number leads to neurodegenerative diseases (Spinocerebellar ataxia type 2, SCA2) [74, 75]. Let us recall that RNA-binding proteins also play a significant role in nuclear-cytoplasmic transport, the disruption of which also leads to neurodegenerative diseases [76].

For the studied cases, as noted above [57], expansion of trinucleotide repeats is typical for exons, while for introns, promoters, 3'- and 5'-UTRs, variability in the lengths of not only trinucleotides, but also tetra-, penta-, hexa- and decanucleotide

repeats occurs [77]. When an STR is amplified, the methylation of the corresponding DNA region may change; the STR elongation itself can change the distance between regulatory motifs in promoters, which will significantly affect expression [78]. Transcription of long STRs leads to the formation of RNA aggregates that serve as a trap for proteins and to multimolecular interactions with other transcripts, which, in turn, can affect splicing, gene expression profiles, and RNA interference [79-81].

STRs can be transcribed in the sense and antisense directions. This leads to the appearance of toxic peptides due to translation from non-ATG initiated triplets (repeat-associated non-ATG translation, RAN) transcribed from the STR [82].

Some studies note that many pathologies associated with STR amplification are detected in genes encoding transcription regulatory factors, which is accompanied by impaired formation of multimolecular condensates and interferes with their interaction with RNA polymerase II [77].

Features of the occurrence of mutations in STR. It has been found [83] that the localization of STRs and recombination hotspots in meiosis, which are usually located in gene promoters, often coincide. STRs can influence recombination processes at such points. This has been shown for STRs with core motifs GA, CA, GT, CT due to their high affinity for recombination enzymes (84). STR mutation rates vary widely, from 10^{-2} to 10^{-8} per locus per generation, but vary widely from locus to locus [85-87]. The dependence of the frequency of STR mutations on the action of environmental factors was described in model objects (*Caenorhabditis elegans*) when comparing mutability in laboratory and natural conditions [88].

In some cases, mechanisms have been discovered which compensate the adverse effects of STR mutations. Thus, there is a close relationship between the mutability of STRs and the polymorphism of the chromatin proteins that package them, which mitigate the adverse effects of changes in the length and organization of STRs on the processes involved in the suppression of transposon expression, accurate transmission of chromosomes, and ensuring their integrity [89].

Of particular structural and functional significance, including for mutability, is the ability of STR to form secondary DNA structures. The formation of G4 quadruplexes in tracks enriched with G/C, triplexes in purine-pyrimidine tracks, R-loops (DNA-RNA duplexes with displacement of the second DNA strand, which is not complementary to RNA), and other loop and hairpin structures affect gene expression profiles and enzyme function repairs, DNA polymerase function, STR instability [90-92].

STRs can result in non-canonical DNA structures that differ from the classical dextrorotatory B-helix, as determined by the primary nucleotide sequence. For example, levorotatory Z-DNA contains alternating GC-rich purine-pyrimidine sequences, and supercoiling is critical for the formation and stabilization of the Z-form of DNA. Z-DNA is thought to regulate the level of supercoiling and thus plays important roles in transcription, gene expression, recombination, translocation, and deletion [93]. Thus, Z-DNA formation induces instability in the region of trinucleotide repeats (CAG, CGG, and GAC), which are associated with various neurodegenerative diseases [93].

The emergence of non-canonical multistranded structures in the regions of purine-pyrimidine tracks consisting of microsatellites, for example AG/TC, GAG/CTC, includes DNA-DNA interactions with the release of one DNA strand, DNA-RNA interactions with the same effect, and interactions of double-stranded RNA with RNA [94-96]. The interaction of the third strand with duplex DNA or RNA in a double-stranded sequence-specific manner, leading to the formation of an intermolecular triplex, has a significant impact on transcription, post-transcriptional modifications, and mutagenesis [95].

It should be emphasized that all secondary structures are dynamic in nature, appear under certain conditions, and disappear when they change [93].

Special attention is attracted by STR sequences predisposed to the formation of cruciform structures in the regions of localization of inverted repeats, since many proteins involved in the control of cell division, for example, topoisomerases, p53, Rif1 (Replication Timing Regulatory Factor 1), can induce the formation of such structures [97]. Cross-shaped DNA forms play an important role in the regulation of replication and gene expression, are involved in the formation of nucleosome structure and recombination [98], and serve as targets for many architectural and regulatory proteins, e.g., histones H1 and H5, topoisomerase IIβ, proteins HMG, HU, p53, proto-oncogenic protein DEK [97, 98]. A number of DNA-binding proteins (eg, members of the HMGB-box family, Rad54, the BRCA1 protein, and the polymerase PARP-1) preferentially bind to cruciform structures [97]. It is assumed that, according to their function, proteins that interact with cruciform structures are mainly divided into four main groups: topoisomerases; DNA repair proteins and transcription regulatory factors; proteins involved in replication; chromatin-associated proteins [98]. The prevalence of cruciform structures formed by inverted STR repeats and their role in epigenetic regulation and maintenance of cellular homeostasis allow us to consider inverted repeats as essential components of regulatory systems [98].

In mammals, sex differences in the frequency of STR mutations have been described. Since oocytes, unlike spermatozoa, in particular in mammals, are in a state of rest for a long time, mutations that arise in STR during homologous recombinations, unequal crossing over, and double-strand breaks can accumulate and have more pronounced manifestations [99]. In general, STR mutations inherited through the maternal germline have a slightly higher frequency than those inherited from the father [100]. However, with age, the number of STR mutations in oocytes remains virtually unchanged, while in sperm it increases 2-fold (studies were conducted in a group of men from 20 to 58 years old) [101].

Complete sequencing of the genomes of 544 people from 29 families in three generations (database of the Center for the Study of Human Polymorphism (Center d'Etude du Polymorphisme Humain, CEPH, https://uofuhealth.utah.edu/center-genomic-medicine/research/ceph-resources) showed a high diversity of new types of STR mutations occurring in different families and at different STR loci [102]. A relationship was found between repeat length and mutation frequency (with the exception of mononucleotide STRs). The lowest frequency of mutations was detected in exons (only two in trinucleotide STRs), the largest part of them (53.38%) occurs in intergenic regions, slightly less than half (44.87%) are located in introns, apprx. 1.6% in 5′ - and 3′-UTR. The average calculated mutation rate (5.24×10^{-5}) is consistent with that for other types of mutations, including averages for SNPs, but in this family-based analysis using data from CEPH there was no correlation between the occurrence of new alleles at STRs and SNPs. It turned out that approximately 30% of STR mutations occur in Alu elements (short interspersed element, SINE), comprising 11% of the genome, while LINE-1 (long interspersed element-1, LINE-1, or L1) insertions, covering 17% of the human genome, only 10% of STR mutations are found [102]. That is, a family analysis of three generations revealed that a fifth of all new STR mutations occur in retrotransposon sequences.

Involvement of microsatellites in genomic variability and organization of the interphase nucleus. Relationship between microsatellites and mobile genetic elements. Close relationships between STRs and mobile genetic elements—transposons (TEs) have been identified quite a long time ago [103]. Many microsatellites arose from genomic TE insertions. It is assumed that

this may be the result of a number of events: tandem insertions of TEs into certain regions of the host genome (duplicated target sites, target site duplication, TSD), the presence of direct and inverted repeats in TEs and interactions between them, captures of host genome sequences on the flanks of TEs , recombinations between different TEs. The extensive interactions of TEs with each other lead to the conclusion that they can be reconstructed into repeated noncoding or coding sequences. This suggests an evolutionary relationship between such DNA sequences and that the evolution of genomes involved frequent shuffling of repetitive sequences, a process referred to as DNA remodeling [103-105]. Multiple TE insertions are caused by the presence of preferred sites for such integration in target genomes, resulting in the appearance of new TE recombination products formed at a high rate during periods of active transposition. In other words, the TE transpositions itself regularly generates sequences from which new microsatellites can arise [58, 105].

Thus, non-autonomous short dispersed Alu retrotransposons (SINEs), containing a poly(A) tail and a central linker region rich in adenines, are widespread in the human genome. Significant connections are shown between the 3' ends of Alu sequences not only with mononucleotide repeats $(A)_n$, but also with $(AAC)_n$, $(AAT)_n$ and tetra- and hexanucleotide repeats enriched in A-nucleotides, while for dinucleotide repeats $(AT)_n$ such a connection is significantly weaker [106]. The localization of dinucleotide repeats $(AC)_n$ is also preferentially associated with Alu elements, with 75% of them identified at the 3′-end of the element, while the rest are in the central region. Interestingly, $(GAA)_n$, a trinucleotide repeat whose amplification is observed in Friedreich's ataxia, may have arisen with the participation of the Alu element. Of the 788 loci in the human genome containing $(GAA)_n$ repeats, 63% (501 loci) have homology with Alu of at least 25 bp. Among them, 94% are associated with the poly(A) tail, and the rest are associated with either the 5′-end of the element or the central region [106]. In Carnivore species studied, several hundred tRNALys-derived SINEs have been identified that contain microsatellite repeats predominantly enriched in AG and A [107]. In a number of species, including fur seal (*Phoca vitulina concolour*), cattle (Bos taurus), CA/GT microsatellite sequences of varying lengths are found flanking the most common autonomous TE, the L1 [108].

It is known that in genomes TEs form areas of preferential localization, so-called nests [109, 110], and both TEs themselves and their integration sites contain microsatellite sequences, due to which recombination occurs here and new TE variants arise.

It is important to highlight that the ability for horizontal transfer between different taxa has been described for some TEs (e.g., L1 and BovB). They are especially common in mammals, and their representation in the genome can vary significantly even among groups of animals that are relatively close in origin [111].

Some STRs show a direct relationship between their sequence and TE. For example, microsatellite $(AGC)_n$ is more common in cattle and sheep than in other mammalian species. In particular, in cattle, the representation of microsatellite loci with the AGC core is 90 and 142 times higher, respectively, than in humans and dogs [112]. Moreover, in the cattle genome, 39% of such microsatellite loci are directly associated with the Bov-A2 retrotransposon (part of BovB) [112, 113], the evolutionarily young and species-specific for cattle. Interestingly, in cattle, Bov-A2 functions as an enhancer of type II interferon gene expression [113]. The close association of $(AGC)_n$ with Bov-A2 turned out to be specific for this STR and TE; in approximately 60% of other STRs, no genetic linkage with TE was detected [112].

Thus, both the TEs themselves and their flanks contain STR sequences,

and in some cases they are closely related to each other. The variability of STR and TE is determined by various events and mechanisms (recombination processes, the prevalence of the nesting principle of TE localization in each other), but each time the STR and TE loci have a pronounced individuality in the speed and features of evolution, which is apparently due to structural and functional characteristics and the action of selection factors.

Microsatellites, sites of increased chromosome fragility — karyotype evolution. In humans, approximately 230 sites of potential increased fragility containing STR have been described [114]. Ten fragile regions of human chromosomes have been identified, for which expansions of gene-specific tandem repeats with core motifs CGG and CCG are shown. The authors of this study suggest that increasing STR lengths may lead to the emergence of new sites of increased fragility [114].

The tuco-tuco genus (*Ctenomys*) of subterranean rodents (*Rodentia*: *Ctenomyidae*) contains about 65 species, which exhibit the most significant chromosomal variations among mammals (from $2n = 10$ to $2n = 70$). Moreover, karyotypic variability is possible even within a species, for example, $2n$ in *C. minutus* can vary from 42 to 50, in *C. talarum* from 44 to 48, and in *C. lami* from 54 to 58 [115]. Among them, *C. minutus* stands out, with 45 different cytotypes already identified, of which seven are believed to be the original ones (they are common in the coastal plains of Southern Brazil). In tuco-tuco, repeating DNA regions, including microsatellites and LINE-1, were mapped, and a direct connection was revealed between the localization of STRs with different core motifs and LINE-1, on the one hand, and karyotypic variability (formation of cytotypes) on the other in different populations within species [115]. It is important to emphasize that the described cytotypes included not only Robertsonian translocations, chromosome fusions and splits, but also tandem repeats, paracentric and pericentric inversions. The involvement of centromeric and telomeric repeats in intraspecific variability and the formation of intraspecific chromosomal races is well known in a number of shrew species [116]; three variants of intraspecific chromosomal races ("Robertsonian fans") were described by N.N. Vorontsov [117] in mice of the genus *Leggada*, house mice *Mus domesticus* of the superspecies *Mus musculus* and mole voles of the group *Ellobius tancrei*, belonging to the superspecies *Ellobius talpinus*. The involvement of STR and TE in karyotypic variation in fish has been described [118]. Thus, a comparative analysis of the sequenced genomes of three fish species showed that the commercial species *Solea senegalensis* has undergone extensive chromosomal evolution associated with the localization of STR and TE in areas with an increased density of chromosomal rearrangements.

Bursts of rapid karyotype evolution, often referred to as karyotypic megaevolution or chromosomal tachythely, have been found across taxa [119]. Apparently, the most obvious example is provided by two species of deer, the karyotypes of which differ sharply, these are the Indian muntjac *Muntiacus muntjak* ($2n = 6$) and the Chinese muntjac *M. reevesi* ($2n = 46$). Comparative analysis of the sequenced genomes of muntjac, red deer (*Cervus elaphus)* and cattle (*Bos taurus*) confirms the evolutionary sequence of chromosome divisions and fusions described cytogenetically. It was found that since the divergence of deer and cattle species (apprx. 20 million years ago), the rapid evolution of the Indian muntjac karyotype has not been accompanied by major inversions or other internal rearrangements (except for discrete events of splitting and fusion of chromosomes) [119].

Chromosome-level genome comparisons made for *Hydropotes inermi*s (water deer, $2n = 70$), *Muntiacus reevesi* ($2n = 46$), female and male *M. crinifrons* (black mujac, $2n = 8$ or 9) and *M. gongshanensis* (Gongshan Mountains deer, $2n = 8$ or 9) [120], led the authors to conclusion that unique centromeric satellite

repeats, including STRs, telomeric STRs, and palindromic repeats, could be responsible for repeated chromosome fusions in deer of these species [120].

In order to reconstruct the karyotypes of 16 phylogenetic nodes of mammals, including the karyotype of their common ancestor, large-scale studies of genomic sequences were carried out in 32 species belonging to eutherians (19 orders), marsupials and monotremes (3 orders each) as representatives of three superorders of mammals, the *Euarchonta*, *Laurasiatheria* and *Xenarthra*, respectively [121]. Modern species in which the number of chromosomal rearrangements have been estimated relative to the putative common ancestor of mammals are humans, sloths (*Choloepus didactylus*), and cattle. The findings suggest that the common ancestor of mammals probably had 19 pairs of autosomes. However, nine of the smallest chromosomes were shared by the common ancestor of mammals and the common ancestor of all amniotes (of which three chromosomes are still conserved in extant mammals) [121]. The number and types of chromosomal rearrangements for transitions between the karyotype of mammalian ancestors, descendant ancestors and existing species were determined. Common regions of increased rates of evolutionary transformations of chromosomes (evolutionary breakpoint regions, EBRs) and evolutionarily conserved blocks (homologous synteny blocks, HSBs) have been identified [121]. It turned out that EBR regions differ from HSB regions in the increased density of actively transcribed genes and repeating elements. There is a non-random distribution of EBRs in genomes and their association with fragile sites during tumorigenesis [121]. The high density of expressed genes detected in the EBR, as the authors suggest, may explain the increased tendency for DNA double strand breaks (DNA in open transcriptionally active regions of chromatin is more sensitive to damage). Analysis showed that the EBR regions are enriched in genes whose products are involved in sensory perception and transcriptional regulation, whereas the HSB blocks have an increased density of genes involved in the formation of anatomical characteristics and the development of the central nervous system [121]. EBR has a significantly higher density of repeats of all types, segmental duplications, SINE (SINE; all SINE and Alu), LINE (LINE; L1), and long terminal repeats (LTR; all LTR and endogenous retrovirus 1, ERV1) than HSB [121].

According to the same authors [121], the evolutionary history of chromosomes of ancestral mammals (mammalian ancestor chromosomes, MAM) varied significantly depending on the size of the chromosomes. Large long MAMs were more often involved in chromosomal rearrangements than short ones, and some extant species (e.g., *Mus musculus*, *Equus caballus*, *Canis familiaris*, *Bos taurus*, and *Capra hircus*) maintained gene synteny at the chromosome level. Nine of the 14 small MAMs in the listed mammalian species turned out to be orthologous in gene synteny with the chromosomes of *Gallus gallus* and the reconstructed chromosomes of the ancestors of birds and amniotes. Some MAMs were conserved as individual chromosomes or as closed units (i.e., entire chromosomes fused to one or more chromosomes without breaking synteny) in mammalian genomes. For example, MAM7 was conserved as an entire chromosome in *Oryctolagus cuniculus*, *Rhinolophus luctus*, and *Procavia capensis*, which represent three orders of mammals [122]. MAM13 and MAM14 are present as distinct chromosomal units in more than 15 extant mammalian species [121].

Taken together, these results demonstrate striking conservation of synteny over the approximately 320 million years of vertebrate evolution since the common ancestor of all amniotes. The reconstructed genome of the ancestors of mammals showed that the existing genomes of mammals are a mosaic obtained as a result of the evolutionary shuffling of 2557 syntenic segments, which is from 69 to 94% of the genome size in the analyzed species, and EBR sequences enriched in TE

act as links between such segments and STR [121]. Analyzing the data obtained, the authors suggested [121] that evolutionarily conserved syntenic segments, the HSBs serve as the main building blocks (genomic elements similar to elements of the periodic system of chemical elements) for the genomes of all mammals, preserving, along with synteny, biological functions [121]. A similar assumption was formulated in studies of the characteristics of the organization and distribution of microchromosomes in reptiles, birds and mammals [122]. It has been shown that the genomes of birds and reptiles, but not mammals, consist of several large macrochromosomes from 3 to 6 microns in length and many tiny microchromosomes, less than 0.5 μm. Microchromosomes have centromeric and telomeric regions, carry a large number of genes, are enriched in GC nucleotides, are highly conserved among birds and reptiles, and have homology with one or more tiny chromosomes of invertebrates that diverged from vertebrates more than 680 million years ago. Microchromosomes associate with each other, are TE-poor and cluster together in the center of interphase nuclei, which, according to the authors, indicates functional coherence. In turtles, snakes and lizards, many microchromosomes disappeared due to fusion into macrochromosomes; in most mammals, microchromosomes disappeared completely, but some platypus chromosomes coincide with several previously described microchromosomes of reptiles and birds. This suggests that such chromosomes represent the building blocks of mammalian chromosomes, the connection between which is formed by the participation of TE and STR [121, 122].

STR and TE are directly involved in the architecture of the interphase nucleus. In the interphase nucleus, chromatin is organized in the form of a hierarchy from nucleosomes to chromatin domains (CD), then to the formation of topologically associated domains (TADs) and to higher-level compartments; The top of the hierarchy is the so-called chromosomal territories (CT) [123]. According to modern concepts, chromatin organization is a critical factor regulating gene expression [123-125]. Enhancers interact with target genes almost exclusively within the TAD, the distally located co-expressed genes are recruited into common protein clusters upon activation, and compact domains exhibit movement and configurational changes in vivo [124, 125].

The non-random radial positioning of CTs in the nucleus indicates the possibility of preferential patterns of interaction between chromosomal territories. Their ability to form specific interchromosomal networks has been discovered, which change during the cell cycle, during cell differentiation, and during neoplastic transformation [125]. It is assumed that the dynamics of these networks correlate with the global control of structural changes and regulation of the functional activity of the genome. The tendency to various translocations in pathologies can be explained by the close and normally demonstrating specificity of the location of certain chromosomal regions during the co-expression of genes localized in them. It is possible that genomic regions from the EBR are characterized by predominantly open euchromatin, which promotes epigenetic modifications due to the availability of DNA for regulation and active gene function and/or their interactions [124].

The participation of STR and TE in the regulation of changes in gene expression programs through dynamic changes in the architecture of the interphase nucleus has been reported in many studies [123-125]. In our opinion, the most striking example is a study performed on the interphase nuclei of columnar photoreceptor cells in nocturnal mammals [125]. The authors revealed an inversion in the localization of heterochromatin and euchromatin compared to the nuclei of ganglion cells. The heterochromatin was located inside while the euchromatin on the periphery of the nucleus under the lamina [125]. Typically, SINEs are

associated with actively transcribed regions, LINEs with heterochromatized ones, localized at the periphery of the interphase nucleus under the nuclear envelope, and only in the case of cylindrical photoreceptor cells of nocturnal mammals is LINE, together with heterochomatin, concentrated in the center of the interphase nucleus, SINEs at the periphery. According to the authors of the study [125], this arrangement of hetero- and euchromatin is largely due to contacts between homologous dispersed repeats, which are localized in different regions of chromatin.

It has been suggested that STRs can be considered as components of the so-called "genome packaging code" which determined the features of its condensation depending on the cell type [126]. It has been noted that in some organisms STRs are grouped in certain regions of chromosomes, for example, in pericentromeric or subtelomeric regions; in complex genomes, STRs are distributed throughout its entire length in a non-random manner, located predominantly in intergenic spaces [57, 126]. Several proteins are known that specifically bind to STR [125, 126]. Therefore, STRs can be "anchors" for the involvement of groups of loci with which they are linked in the processes of intra- and interchromosomal interactions, the formation of TAD and CD. The involvement of STR in intercellular interactions in complex organs through its putative influence on the architecture of chromatin packaging and, as a consequence, on gene expression programs, has been discussed in a number of works, in particular in the case of microsatellites with the GATA core motif in animals and plants [126, 128]. It has been shown that GAGA repeats, which are abundantly present in the eukaryotic genome, are recognized by the GAGA-associated factor GAF, which influences chromatin packaging (GAGA pioneer factor, GAF) in Drosophila, BBR proteins (barley B recombinant protein) in barley, GBP (GAGA- binding protein) in soybean and rice [126, 128].

The hypothesis of the existence of such a "microsatellite code" of gene expression programs [126] is also important for understanding the molecular genetic mechanisms of the formation of convergent characters in evolutionarily distant taxa. There have been numerous attempts to find common structural genes for such traits [129]. In particular, a comparison was made of transcriptomes in eight vertebrate species (lizards, mammals, sharks) that carry embryos in the uterus [129]. It turned out that in all viviparous groups the basic set of physiological functions of the uterus does not differ, but in the same set of genes, none is expressed specifically for all viviparous lineages or even in all lineages of viviparous amniotes that form the embryonic membranes [129]. Thus, the morphological and physiological traits necessary for successful pregnancy in distantly related vertebrates turn out to be controlled by different genes. Apparently, evolutionary changes in viviparity as a mode of procreation occurred multiple times, involving different genes due to cooperation and collaterality of metabolic pathways, but the set of such genes was still initially limited by their composition in the ancestors of each lineage [129].

The example of viviparity can clearly explain the relatively low efficiency of marker-assisted selection (MAS), where the selection and selection of animals for crosses is based on genotypes for a small number of protein-coding genes [130]. That is why the search for DNA markers of regulatory networks, the variability of which underlies the organization of gene expression profiles, is of particular importance. Accumulated data indicate that, despite chromosomal rearrangements and structural transformations due to the interaction between macro- and microchromosomes, fairly high evolutionary conservation in the gene composition of chromosomal regions in TAD A (actively transcribed domains of interphase chromatin) and TAD B (heterochromatized domains) remains [121, 123-125]. This suggests the presence of a spectrum of regulatory elements involved in such a

division. One of these elements is STR involved in the structural interactions of macro- and microchromosomes and in the formation of the architecture of the interphase nucleus, which, in turn, is closely related to the modulation of gene expression profiles.

Summarizing the discussion of the use of DNA markers, it is important to note that many issues that are significant for breeding are already being resolved using modern molecular genetic methods. The key stages of the breeding process are the selection and selection of animals for crossing and the assessment of the breeding value of the parents based on the characteristics of the offspring. DNA markers make it possible to exclude errors of origin and facilitate the identification of mutations associated with phenotypic and reproductive defects, resistance to biotic factors and environmental stress. If the desired development of economically valuable traits is controlled by a small number of key genes, DNA marking is applicable to search for the corresponding allelic variants, usually associated with variability in the quality of the final product. Attempts to detect associations between sets of SNP genotypes and variability of phenotypic traits are not always successful due to the complex design of gene networks, competitive relationships between molecular genetic structures that serve as targets for natural and artificial selection factors, and variability in the contribution of elements of regulatory networks to interactions between metabolic pathways depending on the genotypic environment and the influence of external factors. "Elusive master genes" of quantitative traits and differences in the genetic control of similar phenotypic characteristics are also examples of the fact that DNA marking of genes and loci associated with a desired trait is not always sufficient for successful selection.

Note that among animal species, the number of protein-coding genes varies relatively little, while variations in genome size are significant and are due to differences in the prevalence of dispersed and tandem repeats in them [110, 126]. Tandem repeats (particularly microsatellites) account for more nucleotides in the mammalian genome than protein-coding genes [126]. The division of the genomes of various animal taxa into evolutionarily conserved (HSB) and evolutionarily unstable (EBR) gene blocks, and the enrichment of the latter with dispersed repeats [121], suggests the direct involvement of dispersed repeats and their derivatives, such as STRs, in the regulation of gene networks. The wide representation of microsatellite repeats in genomes and the diversity of their biological effects distinguish these DNA markers as elements of regulatory networks and, possibly, independent targets of variability and selection. Polylocus genotyping of these particular genomic elements will make it possible to analyze the population genetic structure of animal groups with high resolution, assess the degree of their consolidation and identify differences from closely related groups.

So, one of the central problems in modern animal husbandry remains the search for genetic markers that would simplify the selection and selection of animals for crossing and increase the likelihood of obtaining offspring with desirable economically valuable traits. Developed DNA markers of different types and generations are successfully used to solve a number of issues that are significant for breeding. Microsatellites (STR) are used to exclude errors of origin, mononucleotide polymorphisms (SNP) for mapping genomic regions associated with phenotypic characteristics and adaptation to the pressure of natural selection on edges of habitats and in areas of risky livestock farming. Areas with increased copy number variation (CNV) are involved in analysis of polygenic system responses to the factors of natural and artificial selection. With a small number of main genes that determine the manifestation of a trait, DNA marking is applicable for searching for allelic variants of structural genes and analyzing the variability of elements of regulatory networks and the relationships between metabolic pathways. Long-term

studies have revealed the multiple involvement of STR in basic processes (replication, repair, transcription, translation, adaptation and morphogenesis, epigenetic effects) that determine the stability, variability and evolution of biological systems. In this regard, STRs can be considered as elements of regulatory networks, being the main targets of natural and artificial selection. Polylocus genotyping based on microsatellite and dispersed repeats seems promising for analyzing the population genetic structure and consolidation of animal groups and their differences from closely related groups.

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AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 2, pp. 249-259
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.1:612.616.2:577.1

doi: 10.15389/agrobiology.2023.2.249eng
doi: 10.15389/agrobiology.2023.2.249rus

BIOCHEMICAL MARKERS OF STALLION SPERM QUALITY
(review)

M.M. ATROSHCHENKO✉, D.V. MEDVEDEV

All-Russian Research Institute for Horse Breeding, Divovo, Rybnoe District, Ryazan Province, 391105 Russia, e-mail atromiks-77@mail.ru (✉ corresponding author), meddmit@mail.ru
ORCID:
Atroshchenko M.M. orcid.org/0000-0001-6023-0332 Medvedev D.V. orcid.org/0000-0002-0627-7348
The authors declare no conflict of interests
Acknowledgements:
Supported financially from the Russian Science Foundation (grant No. 20-16-00101)
Final revision received September 7, 2022,
Accepted October 6, 2022

Abstract

Seminal plasma is a multicomponent fluid that serves as a vehicle for delivering spermatozoa to the oocyte. This fluid transports male gametes and provides their protection and nutrition during further movement in the female genital tract (T.R. Talluri et al., 2017). Thereby, understanding the effect of the components of seminal plasma on reproductive cells, as well as the search for markers of cryo-resistance and sperm fertility is undoubtedly interesting for researchers. Because stallion sperm lifespan, capacitation capacity, and fertility vary widely between individuals, it is important for horse breeding to investigate the factors that influence these parameters. The purpose of our review is to analyze current publications on the study of biochemical markers that characterize sperm quality and to consider methods for determining reactive oxygen species and oxidative stress products in spermatozoa and seminal plasma. Metabolites of seminal plasma, enzymes activity in it, indicators of oxidative stress and antioxidant defense system can serve as biochemical markers of sperm quality (S. Pesch et al., 2006). To ensure motility, spermatozoa need a large amount of ATP. Monosaccharides and organic acids such as lactate, pyruvate, citrate, and succinate are good energy substrates for these cells. This gives rise to interest in them as markers of fertility (C.R. Darr et al., 2016; E.B. Menezes et al., 2019; M.F. Lay et al., 2001). The concentration of nitric oxide (II) metabolites is another promising indicator for assessing the quality of stallions sperm, since it plays an important role in the regulation of sperm motility and capacitation and the fertilization process (M.B. Herrero et al., 2000; P.T. Goud et al., 2008; F. Francavilla et al., 2000). Among enzymes of seminal plasma, lactate dehydrogenase, alanine and aspartate aminosferases, γ-glutamyl transpeptidase are of interest. While semen analysis is considered the gold standard for diagnosing male fertility, it cannot detect the molecular abnormalities that are responsible for unexplained cases of male infertility. Currently, oxidative stress is considered one of the main causes of such phenomena. It damages sperm proteins, lipids and DNA, which in turn leads to poor embryo implantation and a decrease in pregnancy rates. In this review, the main producers of free radicals in sperm and the antioxidant defense system of male gametes as well as methods for the determination of reactive oxygen species and end products of oxidative stress in spermatozoa and seminal plasma are considered (A. Agarwal et al., 2003; S. Bisht et al., 2017; H. Sies, 2018). Based on the literature data, it was concluded that biochemical markers, such as seminal plasma metabolites, enzyme activity in it, and indicators of oxidative stress, have significant potential for characterizing stallion sperm quality.

Keywords: stallions, fertility, spermatozoa, seminal plasma, oxidative stress, enzymes, metabolites

Sperm consists of spermatozoa suspended in a liquid medium called seminal plasma which is secreted by the epididymis and accessory gonads before and during ejaculation. Seminal plasma is a complex fluid that serves as a vehicle for the delivery of ejaculated sperm on the way from the testes to their target, the oocyte. Seminal plasma not only transports spermatozoa, but also provides them with protection and nutrition during further movement in the female reproductive tract [1]. It consists of various biochemical components

such as proteins (including antioxidant and intracellular enzymes), metabolites, mineral elements that are important for the functioning of sperm [2]. These parameters have been recommended as markers of sperm quality because they indicate function and characterize sperm damage [3].

The anatomy of the accessory sex glands, the composition of their secretions, and the chemical composition of seminal plasma differ not only between animal species, but also between individuals within a breed [3]. Since the lifespan of spermatozoa, their ability to capacitate and the fertility of stallions varies from one individual to another, for a comprehensive assessment of the reproductive status of sires, it is necessary to study the factors influencing these indicators.

The purpose of our review is to analyze current publications on the study of biochemical markers characterizing the quality of stallion sperm, and to consider methods for determining reactive oxygen species and oxidative stress products in sperm and seminal plasma.

Sperm plasma metabolites. Of significant interest is the study of seminal plasma metabolites as markers of stallion fertility. Determination of metabolite concentrations is quite simple and can provide information about animal fertility and disease. Seminal plasma contains a large number of organic compounds. Thus, a study of the metabolome of bull sperm plasma using gas chromatography with mass spectrometric detection revealed 63 metabolites [4]. Of the low-molecular organic compounds, it contained the most fructose, citric, lactic, phosphoric acids and urea. At the same time, stallion sperm has a number of features, one of which is a low concentration of fructose and low fructolytic activity of sperm under anaerobic conditions [5].

Sperm motility is one of the most important properties determining male fertility. The movement of sperm along the female genital tract occurs independently and against the movement of the fluid. A significant amount of energy is required to ensure mobility. In this regard, sperm use different methods for producing ATP, the glycolysis in the cytoplasm (including through the involvement of fructose) and oxidative phosphorylation in mitochondria. It is believed that pyruvate is an important substrate for the production of energy by male reproductive cells, including stallions, under aerobic conditions [6]. In addition to oxidative decarboxylation of pyruvate and the Krebs cycle, β-oxidation of fatty acids may occur in sperm as a source of reduced coenzymes for the respiratory chain [7]. There is also evidence that sperm mitochondria contain a lactate-oxidizing complex, which allows them to actively utilize lactate as a source of pyruvate under aerobic conditions [7, 8]. Consequently, the content of lactic acid in seminal plasma is an important indicator of sperm energy metabolism.

Citrate, another essential component of seminal plasma, enters the spermoplasm mainly with the secretion of the prostate gland. Despite the presence of mitochondria in spermatozoa, it is believed that the use of citrate in the tricarboxylic acid cycle does not have a significant effect on its concentration in the sperm plasma, since carbohydrates are the main source of energy for sperm movement. The main function of citrate is considered to be the binding of spermoplasm cations and maintaining osmotic balance [8]. There is evidence that the concentration of citrate in the sperm plasma reflects the androgen content in mammals [9].

Succinate is also of interest as an energy substrate for sperm. One recent study demonstrated that succinic acid is a good substrate for oxidation in mitochondria during sperm capacitation, providing an increase in their proton potential [10]. The authors suggest that succinate is transported into mitochondria using a dicarboxylate transporter.

Research on nitric oxide metabolites can provide information about fertility. NO is involved in many physiological processes, including the regulation of reproductive function [11]. It is important for spermatogenesis, penile erection, folliculogenesis, and ovulation, among other things [12]. In sperm, NO plays an important role in the regulation of spermatozoa motility and capacitation [13]. However, the effect of NO on mammalian sperm is dose-dependent. Low amounts of NO are beneficial, while high amounts appear to be harmful [14]. Spermatozoa are capable of producing NO, and its synthesis is critical for motility, capacitation, and fertilization [15-17]. NO has been shown to stimulate human sperm motility through activation of soluble guanylate cyclase, subsequent cGMP synthesis, and activation of cGMP-dependent protein kinases [18]. Information about the role of the NO system in stallion reproduction is rather limited [19]. However, immunohistochemical studies have demonstrated the presence of all three isoforms of nitric oxide synthase NOS (eNOS, nNOS, and iNOS) in the stallion reproductive system, with their expression varying between different cell types [20]. In stallions, there is a positive correlation between NO production, on the one hand, and the motility and speed of sperm after thawing, on the other [21]. In old individuals, the concentration of NO metabolites in the seminal plasma is lower than in young individuals [21].

Seminal plasma enzymes. Assessment of enzyme activity in seminal plasma can be recommended as a biological marker of seminal fluid quality, since their content characterizes the function and reflects the integrity of spermatozoa [22, 23]. Changes in the activity of seminal plasma enzymes must be interpreted, taking into account where the enzyme is localized and how it enters the spermoplasm, whether it is actively secreted or appears in it due to a violation of the integrity and increased permeability of membranes. Thus, alkaline phosphatase is mainly derived from the testes and epididymis and can be used as a marker to differentiate azoospermia or oligospermia from ejaculatory failure in clinical cases [24]. In addition, this enzyme is secreted from the plasma membrane of spermatozoa [25].

Aspartate aminotransferase (AsAT) and alanine aminotransferase (AlAT) are intracellular enzymes found in the cytoplasmic droplets of sperm [26]. Increased activity of AsAT and AlAT in the spermoplasm of stallions may be associated with damage to the spermatozoa membrane [23]. Lactate dehydrogenase (LDH) is found in the cytosol, mitochondria, and plasma membrane of spermatozoa [27]. The enzyme gamma-glutamyl transpeptidase (GGT) is known to be located in the external region of spermatozoa [28]. GGT is an enzyme involved in the gamma-glutamyl cycle, which transports amino acids across membranes. In cells, the enzyme is present not only in the cytoplasmic membrane, but also in lysosomes and cytoplasm.

In a study by S. Pesch et al. [23], LDH activity was significantly correlated with motility, live-to-dead sperm ratio of stallions, and sperm pathology. The authors concluded that LDH can be considered the most prognostically significant enzyme for determining sperm quality.

Indicators of oxidative stress and fertility of stallions. Although semen analysis is considered the gold standard for diagnosing male fertility, it cannot detect abnormalities at the molecular level that are responsible for unexplained cases of infertility [29, 30]. Currently, oxidative stress (OS) is considered one of the main causes of unexplained cases of male infertility [31]. OS leads to damage to proteins, lipids and DNA of sperm, which, in turn, causes poor embryo implantation and a decrease in pregnancy rates. In addition, it also affects the health of the offspring and can cause mutations

in the germline, leading to severe pathologies, polygenic disorders, and even cancer [32]).

Oxidative stress is an imbalance between oxidants and antioxidants in favor of oxidants, leading to disruption of redox signaling and/or molecular damage [33]. Cell damage in OS occurs mainly due to the action of reactive oxygen species (ROS), which are represented by free radicals, the compounds that have unpaired electrons. These are hydroxyl radical ($OH^{\bullet}$), superoxide anion radical ($O_2^{\bullet -}$), peroxide radicals ($RO_2^{\bullet -}$), as well as non-radical molecules with oxidizing properties, e.g., the singlet oxygen, hydrogen peroxide (H_2O_2), hypochlorous acid (HOCL), lipid peroxides (LOOH), ozone (O_3) [34]. In addition to ROS, active forms of nitrogen, the nitric oxide (NO) and peroxynitrite ($ONOO^-$) play an important role in molecular damage during OS, although in some cases the name nitrosative stress is used for this process.

Of the ROS, superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) are the most important for spermatozoa [35]. These compounds are sequentially formed during the reduction of oxygen in the respiratory chain of mitochondria:

$+e^- + e^- + 2H^+ + e^- + H^+ + e^- + H^+$,

$O_2 \rightarrow O_2^{\bullet -} \rightarrow H_2O_2 \rightarrow H_2O + OH^{\bullet} \rightarrow 2H_2O$.

Superoxide and hydroxyl radicals are unstable compounds with half-lives of milli- and nanoseconds, respectively, causing these radicals to react at the site of their formation [36]. Hydrogen peroxide can be a source of formation of hydroxyl radical, the most reactive ROS:

$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^{\bullet} + Fe^{3+}$ (Fenton reaction);

$O_2^{\bullet -} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + O_2$ (Haber-Weiss reaction).

At physiological concentrations, ROS are involved in capacitation by stimulating the synthesis of adenylate cyclase [37], in the acrosomal reaction by regulating the activity of protein kinases [27], and in sperm-oocyte fusion by inhibiting protein tyrosine phosphatase and thus maintaining phospholipase A2 activity [38].

The two most important sources of ROS for sperm are leukocytes and immature sperm [39]. Leukocytes, especially neutrophils and macrophages, are closely associated with excessive ROS production leading to male gamete dysfunction [32]. They produce approximately 1000 times more ROS than sperm. They play a significant role in genital tract infections, inflammation, and cellular defense mechanisms [40, 41]. One recent study found a positive correlation between leukocyte counts, ROS, and the number of sperm with fragmented DNA, and a negative correlation between leukocyte counts, sperm concentration, and spermatozoa_motility [42].

Stallion spermatozoa are called “professional” producers of ROS (mainly superoxide and H_2O_2) due to their high mitochondrial activity. They are characterized by a complex system for controlling redox homeostasis [43]. Since ROS damage the cellular structures near which they are formed, the study of ROS production in sperm is of significant interest. Mitochondria and the cytoplasmic membrane are most important for the production of ROS in sperm. The appearance of immature sperm, especially with excess residual cytoplasm, as well as various sperm abnormalities (residual cytoplasm or cytoplasmic droplets) are associated with excessive ROS production [44, 45]]. Typically, such sperm are removed by Sertoli cells during spermatogenesis. Excessive ROS formation in cytoplasmic droplets is associated with the presence of the enzyme glucose-6-phosphate dehydrogenase, which produces NADPH, which is then oxidized by two oxidoreductases located in the cytoplasmic and mitochondrial membranes, respectively [46].

Antioxidant protection of sperm. The specific cellular structure of sperm and their plasma membrane, the large number of mitochondria, the small volume of the cytoplasm and the low amount of antioxidants in it make male gametes quite vulnerable to damage by free radicals [47]. Enzymatic and non-enzymatic antioxidants are present in sperm. Enzymatic antioxidants are proteins that neutralize excess ROS and prevent damage to cellular structure. These include superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase [48]. Currently, these enzymes are considered as possible markers of cryotolerance in stallion spermatozoa [49, 50].

SOD (EC 1.15.1.1) catalyzes the reaction $2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$. The main task of superoxide dismutases is the removal of superoxide anion radicals. Superoxide is constantly formed during cell life as the first intermediate product in O2 reduction reactions. Its main producer is the mitochondrial respiratory chain [51]. SOD is a critical enzyme for the bioavailability of NO by preventing its reaction with superoxide and conversion to peroxynitrite [52]. In boar seminal plasma, extracellular SOD is detected, containing Cu and Zn as cofactors [53]. The results of the study by M. Kowalowka et al. [53] show that in boar, extracellular SOD serves as a seminal plasma antioxidant enzyme that plays an important physiological role in counteracting oxidative stress in sperm. It has been demonstrated that in stallions whose ejaculates have good freezing ability, SOD activity is higher than in the group with low cryoresistance, which is associated with the preservation of the integrity of the acrosomal membrane [49, 50].

Catalase (EC 1.11.1.6) is an enzyme that catalyzes the disproportionation reaction of hydrogen peroxide $2H_2O_2 \rightarrow H_2O + O_2$. This heme protein is also capable of oxidizing low molecular weight alcohols and nitrites in the presence of hydrogen peroxide. The presence of catalase in sperm has been demonstrated in sheep and cattle. It potentially plays a role in the aging process and OS control in cells [54]. Along with SOD and glutathione peroxidase, catalase serves as the main endogenous antioxidant enzyme in the seminal plasma of stallions [55].

Glutathione peroxidases (EC 1.11.1.9 and EC 1.11.1.12) catalyze the reduction of H2O2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione: $2GSH + H_2O_2 \rightarrow GS\text{-}SG + 2H_2O$. Some glutathione peroxidase isoenzymes contain selenium as a cofactor, which is linked to cysteine in their active site [56]. The level of reduced glutathione inside cells is maintained thanks to glutathione reductase, which reduces glutathione, and GGT, which transports glutathione into the cell.

Non-enzymatic antioxidants include vitamin C, vitamin E, zinc, selenium, taurine, hypotaurine, and glutathione [34]. Many of them exhibit their properties by being cofactors (selenium for glutathione peroxidase, zinc for SOD) or coenzymes (glutathione for glutathione peroxidase) of antioxidant enzymes. Vitamin E is able to react directly with peroxide radicals, sequentially oxidizing into a fairly stable tocopheryl radical, and then into tocopheryl quinone, interrupting the chain reactions of free radical oxidation. A large number of studies are currently devoted to studying the effect of externally added antioxidants on sperm quality and safety [34, 54, 55].

Methods for determining ROS and final OS products in spermatozoa and seminal plasma. To study OS, it is of interest to determine the generation of both ROS and end products of free radical oxidation.

One of the fairly stable products of lipid peroxidation is malondialdehyde, which is usually determined by reaction with thiobarbituric acid [57]. In addition to malondialdehyde, some other compounds also react; together they

are called thiobarbituric acid reactive substances (TBARS). There is evidence that TBARS content is increased in frozen but not in chilled bull semen [58]. However, there were no differences in malondialdehyde concentrations in fresh and frozen semen in healthy men [59].

Oxidation of proteins under the influence of free radicals leads to the formation of carbonyl groups, fragmentation and aggregation of protein molecules. These modifications can affect the functions of proteins. Carbonyl derivatives of amino acid residues in proteins are stable end products of protein oxidation, which makes their determination an informative method for studying the oxidative modification of proteins [60]. They can be assessed quantitatively by reaction with 2,4-dinitrophenylhydrazine. The resulting 2,4-dinitrophenylhydrazones have a specific absorption spectrum in the visible and ultraviolet parts of the spectrum [61].

A chemiluminescence assay can be used to directly measure sperm ROS generation: special reagents interact with the oxidation end products, resulting in an electrical signal that can be measured as photons per minute using a luminometer [62]. This assay is effective for measuring both intracellular and extracellular ROS.

Another test for measuring intracellular sperm ROS is flow cytometry using fluorescent and chemiluminescent probes [63]. The most common technique is with 2′-7′-dichlorodihydrofluorescein diacetate, which allows direct assessment of the redox status of the cell [64]. Another simple and cost-effective test for measuring ROS using a light microscope is the nitroblue tetrazolium reaction. The principle of the method is based on the transformation of nitroblue tetrazolium into the blue pigment diformazan upon interaction with superoxide produced by sperm or leukocytes [62].

Thus, at present, studying the effects of seminal plasma components on germ cells, as well as the search for markers of cryostability and fertility of stallion sperm are of undoubted interest. Seminal plasma contains a large number of components (metabolites, enzymes, antioxidants) that affect the structural integrity, progressive motility and fertilizing ability of sperm. Among the metabolites, attention is focused on energy substrates, thr pyruvate, lactate, citrate, succinate. Stallion sperm are very vulnerable to damaging factors during cryopreservation, in particular to oxidative stress due to the significant content of polyunsaturated fatty acids in the phospholipids of the plasma membrane. In this regard, methods for studying the redox status are promising for assessing ejaculates: determination of substances that react with thiobarbituric acid, products of oxidative modification of proteins, NO metabolites, chemiluminescent analysis, flow cytometry. Active attempts are being made to detect markers of resistance of stallion sperm to freezing and subsequent thawing among antioxidant enzymes, i.e., SOD, catalase, and glutathione peroxidase. Biochemical markers, such as seminal plasma metabolite concentrations, seminal plasma enzyme activity, and indicators of oxidative stress, have significant potential for characterizing the quality and cryostability of stallion sperm.

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AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 2, pp. 260-273
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.4:619:578

doi: 10.15389/agrobiology.2023.2.260eng
doi: 10.15389/agrobiology.2023.2.260rus

ATYPICAL PORCINE PESTIVIRUS (*Pestivirus K*) — A NEW CHALLENGE FOR PIG FARMING

(review)

A.M. ANOYATBEKOVA✉, A.G. YUZHAKOV, M. ANOYATBEKOV, T.I. ALIPER, A.M. GULYUKIN

Federal Scientific Centre Skryabin and Kovalenko All-Russian Research Institute of Experimental Veterinary RAS, 24/1, Ryazanskii Prosp., Moscow, 109428 Russia, e-mail aafshona@mail.ru (✉ corresponding author), anton_oskol@mail.ru, anoyatbekov@list.ru, aliper@narvac.com, admin@viev.ru
ORCID:
Anoyatbekova A.M. orcid.org/0000-0002-6579-779X
Yuzhakov A.G. orcid.org/0000-0002-9354-6824
Anoyatbekov M. orcid.org/0000-0002-0180-0574
Aliper T.I. orcid.org/0000-0003-2696-1363
Gulyukin A.M. orcid.org/0000-0003-2160-4770
The authors declare no conflict of interests
Acknowledgements:
Carried out according to the State assignment No. FGUG-2022-0009
Final revision received August 22, 2022
Accepted September 7, 2022

Abstract

Pestiviruses are highly variable RNA viruses of the genus *Pestivirus*, family *Flaviviridae*. The genus *Pestivirus* includes 11 species, from *Pestivirus A* to *Pestivirus K* (B.G. Orlyankin et al., 2020; D.B. Smith et al., 2017; A.M.Q. King et al., 2018). In the infectious pathologies of pigs, pestiviruses are highly important due to significant economic losses. Over the past two decades, many previously undescribed pestiviruses have been found in domestic pigs and wild boars. Due to the tendency to rapid spread, they can cause a serious threat to pig production. In 2015, in the framework of the Porcine Reproductive and Respiratory Syndrome Virus genetic diversity project in the US, an atypical porcine pestivirus (*Pestivirus K*) was first identified in blood serum by metagenomic sequencing. Initially, it was assumed that pigs infected with atypical pestivirus did not show clinical signs of the disease. However, the experiments on the study of atypical pestivirus infectious properties showed that the pestivirus causes congenital tremor (CT) type A-II in piglets (B.L. Arruda et al., 2016; A. de Groof et al., 2016; A. Postel et al., 2017). Adult domestic pigs and wild boars are also susceptible to the virus. Atypical porcine pestivirus is transmitted vertically and horizontally and is widespread in many countries of Europe, America and Asia. In Russia, atypical porcine pestivirus has not been diagnosed in pigs. The phylogenetic analysis of the genome of all known isolates revealed 3 genetic groups (1st-3^{d}) and seven subgenotypes within the 1st genetic group (1.1-1.7) of the virus (F. Yuan et al., 2021). The first genetic group includes all isolates identified in the USA, Europe and several isolates from China. The second and third genetic groups are isolates from China only. The circulation of atypical porcine pestivirus in herds can complicate the differential diagnosis of classical swine fever, due to some similarity of symptoms, the congenital tremor particularly. Consequently, the knowledge of the epidemiology of the atypical porcine pestivirus in different geographic regions will help optimization of its control and prevention of spreading. The review covers current data of the etiology, distribution, clinical manifestations, diagnosis and prevention of the atypical porcine pestivirus infection.

Keywords: atypical porcine pestivirus, identification, differential diagnosis, congenital tremor, pigs, classical swine fever, diseases prevention

Pestiviruses (genus *Pestivirus*, family *Flaviviridae*) belong to RNA viruses with a single positive strand of RNA (+ssRNA). Previously, only three representatives of this genus were known, including bovine viral diarrhea virus (BVDV), border disease virus (BDV) and classical swine fever virus (CSF). swine fever virus, CSFV) [1]. It was assumed that pestivirus infection in pigs is caused only by the classical swine fever virus, a transboundary, highly contagious and economically significant disease for industrial pig production in many countries [2, 3]. However, given the close relationship of pestiviruses and their ability for interspecific

transmission, infection of pigs by other representatives of this genus could not be ruled out. Thus, back in 1964, the bovine viral diarrhea virus was identified in pigs in Australia [4, 5]. Infection of pigs with VDV under natural conditions has also been described in the USA [6], the Netherlands [7], China [8] and Brazil [9]. During routine serological monitoring of CSF in Spain [10] and Japan [11], sheep border disease virus was detected in pigs. Infection with a HoBi-like pestivirus has been experimentally reproduced [12, 13]. Infection of pigs with ruminant pestiviruses does not cause obvious clinical signs, but the circulation of such pestiviruses among livestock makes the differential diagnosis of classical swine fever difficult [9]. In recent years, outbreaks of emerging viral infections in pigs have become more frequent, posing a serious threat to pig production. Thus, in 2003, a severe outbreak of a disease of unknown etiology occurred in Australia, which was characterized by stillbirths, pre-weaning mortality and the birth of mummified and weak fetuses with porcine myocarditis syndrome. The causative agent of the disease was identified only in 2007 and was named Bungowannah virus [14, 15]. Another pestivirus, *Linda virus* (lateral-shaking inducing neuro-degenerative agent, a neurodegenerative agent that causes lateral tremor), was discovered in southeastern Austria in Styria in piglets with congenital tremor [16, 17]. Congenital tremor (CT) (Myoclonia Congenita) is a neurological disease of newborn piglets, which manifests itself in the form of tremor of the skeletal muscles of the head and trunk. It manifests itself both locally and generally [18-21]. The first report of congenital tremor in piglets dates back to 1922 [18]. Those born with this trait were known as dancing piglets or shaker piglets [18, 22]. Based on the nature of pathological damage to the central nervous system, two types of congenital tremor, A and B are distinguished. With CT type A, histopathological changes in the brain and spinal cord are observed, with CT type B no such changes occurred [18-22]. CT type A, in turn, is divided into five subtypes (I-V). Subtype A-I is caused by the CSF virus [18], subtype A-III is considered a genetic defect in male Landrace pigs, CT A-IV is also a hereditary type of pathology, which is manifested by hypomyelination brain and spinal cord of British Saddleback pigs [18, 19, 22]. The cause of CT subtype A-V is poisoning of pregnant sows with trichlorfon, which was previously used to treat pigs from ectoparasites [18]. For many years, the cause of CT A-II remained unknown. It was believed that the pathology could be caused by a virus [20]. In 2015, during experimental infection of pigs, it was found that type A-II CT develops during transplacental infection of sows with a new atypical porcine pestivirus (APV) [23, 24].

This review summarizes current information on the distribution of atypical porcine pestivirus (*Pestivirus K*), characteristics of the pathogen, clinical manifestations, diagnosis and prevention of infection.

Etiology and modern classification of pestiviruses. In 2015 in the USA, as part of a project to study the genetic diversity of the porcine reproductive and respiratory syndrome (PRRS) virus, B.M. Hause et al. [23] examined 182 porcine serum samples using metagenomic sequencing. In analyzing their results, the authors determined that the nucleotide sequences found in five sera obtained in 2014 from Nebraska, Arizona, North Carolina, Minnesota, and Kansas had 68% similarity to the genome sequences of the bat pestivirus *Rhinolophus affinis* (*RaPV*) [25] and 25-28% similarity with the VD, PB and CSF viruses. The authors found that the virus they identified belongs to the genus Pestivirus and is widespread in the United States [23]. Attempts to isolate it in cell cultures at that time were unsuccessful. The pathogenicity of the new virus remained unknown. In 2016, B.L. Arruda et al. [24] reproduced APS infection and showed that infection of individuals during pregnancy leads to the birth of piglets with congenital tremor type A-II. Since then, APS has been associated with

A-II CT.

In 2018, the taxonomy of the genus *Pestivirus* was revised and ratified by the International Committee on Taxonomy of Viruses (ICTV) [27, 28]. An atypical pestivirus of pigs was isolated into a separate nosological unit and named *Pestivirus K*. Other representatives of the genus *Pestivirus* of the *Flaviviridae* family are *Pestivirus A* (viral diarrhea virus type 1), *Pestivirus B* (viral diarrhea virus type 2), *Pestivirus C* (classical diarrhea virus swine fever), *Pestivirus D* (sheep border disease virus), *Pestivirus E* (pronghorn pestivirus), *Pestivirus F* (Bangowanna virus), *Pestivirus G* (giraffe pestivirus), *Pestivirus H* (viral diarrhea virus type 3, HoBi-like pestivirus), *Pestivirus I* (Aydin-like pestivirus) and *Pestivirus J* (rat pestivirus) [26-30].

Due to the discovery of new pestiviruses in both pigs and other animals, A. Postel et al. [30], having studied the genetic relationship of pestiviruses, propose expanding the number of their species by including in the taxonomy *Linda virus* (*Pestivirus L*), Phocoena pestivirus (*Pestivirus M*), sheep pestivirus isolated in Tunisia (*Pestivirus N*), sheep pestiviruses isolated in Italy (*Pestivirus O*), pangolin pestivirus (*Pestivirus P*), rodent pestiviruses (*Pestivirus Q*, *Pestivirus R*) and bat pestiviruses (*Pestivirus S*) [30].

Distribution of atypical porcine pestivirus. APS was first discovered in 2015 in the United States [23]. Since then, its circulation in domestic pig herds has been reported in the Netherlands [31], various states in the USA [32-34], Austria [35], China [36-40], Spain [41], South Korea [42], Brazil [43-45], Great Britain [46, 47], Taiwan [47], Canada [48, 49], Hungary [50], Japan [51], Italy [52], Serbia [47], Sweden [53], Switzerland [54], Denmark [55], and Germany [47, 56-58]. In Sweden, South Korea, Italy, Spain and Germany, atypical porcine pestivirus was also found in wild boars [52, 59-62]. In Russia, infection caused by atypical porcine pestivirus has not been described.

In Germany, in 2016, APS was first identified in naturally infected piglets born with CT A-II and in clinically healthy adult pigs. The APS genome was found in blood sera, in various parenchymal organs, as well as in the cerebellum and peripheral nerves of newborn piglets with CT [56].

In the Netherlands, on one of the farms in 2012, A. de Groof et al. [31] observed severe flare of CT types A-I—A-V. The mortality rate of piglets was 60%. Long before the outbreak (in November 2009 and December 2010), several piglets on the same farm were diagnosed with CT A-II. Single outbreaks of CT were noted until 2016. The nucleotide sequences of the viral genome, which were found in the blood sera of piglets born with CT, were studied in 2012 using the VIDISCA-454 method (Virus discovery cDNA-AFLP) and showed little similarity with the genome of pestiviruses. After the nucleotide sequences of the genome of the American strain of the APS virus became available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/), it became clear that the cause of CT outbreaks on the farm was APS. In addition, to determine the relationship between CT and APS, the authors of the cited study conducted an experimental infection of pigs. This led them to conclude that transplacental infection of sows results in the birth of infected piglets with CT symptoms that shed the virus in their feces [31].

In China, APS was first discovered in 2017 on a pig farm in Guangdong province during a CT study of blood serum and organs of piglets. J. Yuan et al. [37] reported that APS mainly accumulates in the submandibular lymph nodes of newborn animals. Currently, APS infection is considered widespread in China and is found in almost all provinces [63-66].

In 2017, Austria reported circulating APS in piglets with CT. The research was carried out in 2015-2016. Infection with the virus resulted in a 10% increase

in piglet mortality. ELISA (enzyme-linked immunosorbent assay) revealed antibodies against APS in both piglets and adult pigs. Quantitative reverse transcription PCR (RT-qPCR) analysis showed that large amounts of viral RNA were present in the saliva and semen of adult pigs. In addition, in a study of archival samples obtained in 2013 from pig farms in Lower and Upper Austria from animals with similar symptoms, APS was identified, indicating the circulation of this virus since 2013 [35].

In Spain, the virus was detected in a 2-day-old pig with CT symptoms in 2017, as well as in a retrospective analysis of pig blood sera obtained back in 1997 [41].

In 2018, scientists from Canada also reported the discovery of APS in an outbreak of the disease with symptoms of congenital tremor of subtype A-II in 2-day-old Yorkshire-Landrace piglets. A-II CT in piglets had not previously been reported in Canada. Infected piglets showed severe clinical signs but no stillbirths were observed. Mortality of piglets in litters with CT symptoms reached an average of 24.6% (15 of 61), ranging from 13.3% (2 of 15) to 41.2% (7 of 17), compared with an average mortality of 12 piglets, 7% for litters from other sows on the farm [48].

L. Denes et al. [50] suggest that APS has been circulating in Hungary since 2005. The authors examined collected formalin-fixed organs from piglets with CT symptoms and determined the etiology of disease outbreaks on different farms in Hungary in 2005, 2007, 2010 and 2016-2018. According to an epidemiological study, the seroprevalence of pigs for APS was 37% in Germany, 17.5% in Italy, 7.0% in Switzerland, and 2.3% in the UK. The prevalence of APS among wild boars in Germany, Spain and Italy varies from 0.23% to 52% [57, 59, 67].

Genetic and antigenic characteristics of atypical porcine pestivirus. The genome of the atypical porcine pestivirus is represented by a single-stranded RNA molecule of positive polarity (length 11-12 kb), has one reading frame flanked by 5′- and 3′-untranslated regions (UTR). The open reading frame encodes four structural (C-Erns-E1-E2) and eight nonstructural (Npro-p7-NS2, NS3-NS4A-NS4B-NS5A-NS5B) proteins [21, 22, 25, 26, 29]. The genome organization of APC and all members of the *Pestivirus* genus is similar (with the exception of the pestivirus *Phocoena* found in the guinea pig, which lacks the gene encoding the Npro protein in its genome) [68].

In all pestiviruses, the 5′-UTR begins with a sequence that is capable of forming a stable stem-loop structure [26, 69]. With a thorough genetic analysis of the detected atypical porcine pestivirus using NGS technology, B.M. Hause et al. [23] determined that the 5′-UTR of APS contains only 125 nt and significantly shorter than those of other pestiviruses (370-498 nt). Using the rapid amplification of cDNA ends (RACE) method for APS-positive samples, the authors were still unable to determine the reason for the short size of this region. The length of the 3′-UTR was 245 nt. and corresponded to those of other pestiviruses (200-500 nt) [23].

A feature of the genus *Pestivirus* that distinguishes it from other genera of the *Flaviviridae* family is the presence of the Npro protein, which suppresses the production of antiviral interferon IFN-α/β, disrupting the functioning of the interferon regulatory factor IRF3 and IRF7 signaling [26, 69, 70]. The Npro protein of CSFV and VD viruses is known to be involved in the suppression of IFN-β responses [70, 72]. C. Mou et al. [73] examined the effect of APS Npro protein on the regulation of IFN-β production and found that APS Npro reduced IFN-β production mainly by blocking IRF3 activation. The N-terminal amino acids 31-51 of Npro APS are associated with suppression of the IFN-β response [73]. The nonstructural protein Npro is the very first synthesized protein, which is released

from the formed polypeptide chain during its autoproteolytic cleavage between amino acid residues Cys168 and Ser169. According to B.M. Hause et al. [23] who used the pairwise protein alignment method, a conserved triad of amino acid residues characteristic of Npro pestiviruses (Glu22, His49, Cys69) was identified in APS at positions Glu20, His69, and Cys89 [23, 71]. Despite the conservation of sites of catalytic activity and cleavage sites, the Npro protein of APS did not have significant similarity to the Npro of other pestiviruses (only 9-18% pairwise amino acid identity) [23].

Nucleocapsid protein C has RNA chaperone activity and packages RNA into virions. In APS, compared to other pestiviruses, protein C is longer (111 aa vs. 97-102 aa). The isoelectric point of protein C of APS is similar to that of other pestiviruses (pI in the range of 10.0-10.4) [23, 71].

The Erns protein is another unique protein found only in pestiviruses. In contrast to Npro, Erns is second only to NS3 in terms of conservation among all pestiviral proteins, indicating its important role in the pestivirus life cycle and resistance to modification [70-74]. It is known that Erns has ribonuclease activity against single-stranded and double-stranded RNA, while in the structure of the domain that determines the catalytic activity, Erns is similar to T2 ribonucleases of plants and fungi [75]. T2-RNases are mainly monomeric glycosylated proteins (20-40 kDa) without strict substrate specificity, maximally active in an acidic environment (pH 3.5-6.5). Several biological functions of T2 RNases (both dependent and independent of catalytic activity) have been identified, including cleavage of self-RNA and modulation of the host immune system [75]. The active domain contains the amino acid residues His321, His364, Glu365, Lys368, and His369 [71]. The ectodomain contains nine conserved cysteine residues forming four conserved intrachain disulfide bridges. Using the example of the NADL strain of the VD virus lacking Cys171, it was shown that the interchain disulfide bridge is not essential for the viability of the virus. In the APS genome the T2-RNase domain was identified in the Erns region from aa 319 to 373; the similarity to the Erns proteins of other pestiviruses was 32.9-39.0% [23].

E1 is a structural protein the independent function of which is unknown [26, 69]. Most often, the properties of E1 were analyzed through its interaction with two other envelope proteins, E2 and Erns. E1 is a 25-33 kDa protein (depending on the pestivirus species) with a transmembrane anchor [26]. Together with the E2 protein, it forms heterodimers with a disulfide bond, which play an important role in the penetration of pestivirus into the cell. Heterodimer formation is proposed to occur through interactions between the C-terminal transmembrane domains of the E1 and E2 proteins [26, 69, 71].

Structural glycoprotein E2 is an immunodominant protein of pestiviruses and is important for the formation of protective immunity [26, 71, 78]. The E2 glycoprotein APS plays a major role in this, since it causes the formation of the largest amount of virus-neutralizing antibodies during natural infection of pigs and, possibly, during vaccination [71, 78, 79]. When developing a subunit vaccine based on the APS E2 protein and testing it on laboratory mice, H. Zhang et al. [79] showed that immunized mice mount cellular (Th2-dominant) and humoral immune responses to APS, but further studies are needed to determine the effectiveness of this vaccine in protecting pigs against infection. According to B.M. Hause et al. [23], E2 of APS is 54% identical to E2 of pestivirus RaPV. The size of E2 APS is 241 and 244 aa, which is smaller than that of other pestiviruses (373-378 aa). A deletion in the region encoding the N terminus of APS and pestivirus RaPV E2 results in the loss of the immunoglobulin domains previously described in VVD [80, 81]. Penetration of pestiviruses into cells occurs through receptor-mediated endocytosis. It is known that the VD virus attaches to cells through the

interaction of the E2 glycoprotein with the membrane protein CD46, which acts as an APS receptor [82]. Previously, C. Drager et al. [83] suggested that the penetration of CSFV into cells also occurs with the participation of the cellular receptor CD46 and the glycosaminoglycan heparan sulfate, which acts as an additional receptor. However, G.N. Cagatay et al. [84] refuted this assumption. In their opinion, the CSF and Bangovanna viruses use a different method of entry into the cell, and CD46 does not function as a cellular receptor common to porcine pestiviruses [84] and serves as the main cellular receptor only for APS.

The p7 protein is a small hydrophobic peptide 61-62 aa long, which functions as a viroporin necessary for APS replication in vitro and virulence in vivo. The amino acid similarity of the p7 protein in APS and the pestivirus RaPV is 67% [23].

NS2 is a cysteine autoprotease responsible for proteolysis at the interface of the NS2 and NS3 proteins. NS2 APS has significant amino acid similarity (60%) only with NS2 RaPV; for other pestiviruses this value is only 10-15% [23, 71]. In VD virus, the protease activity of NS2 is due to the presence of the catalytic triad His1447, Glu1462, and Cys1512 (26). Two members of this triad, His1447 and Glu1462, can be identified in NS2 APS (His1237 and Glu1253) [71]. In NS2 APS there is no cysteine residue, but can be compensated by a cysteine residue at position 1280, which shortens the amino acid segment between the Glu and Cys 23 residues [23, 71]. The autoprotease function of NS2 depends on the cellular cofactor Jiv, which is required for the replication of noncytopathogenic pestiviruses [26, 69, 71]. However, this function for NS2 APS has not yet been studied.

NS3 is a chymotrypsin-like serine protease that catalyzes cleavage in both cis and trans. The NS3 protein is identical in APS and RaPV (74%). The similarity of NS4a in APS and RaPV is 61%, in APS and other pestiviruses it is 29-33%. The amino acid identity of NS4b and NS5b of APS and other pestiviruses is 36-45%, whereas NS5a is less conserved (12-17% similarity with other pestiviruses) [23].

In epidemiological studies and in the development of means for the prevention and control of pathology caused by atypical swine pestivirus, it is important to classify the antigenic and genetic groups of isolates from different geographical areas. Phylogenetic analysis of partial and complete genome sequences provides more detailed information about isolates than serological methods and allows detailed discrimination between virus genotypes and subgenotypes. To date, based on the analysis of 76 complete genomes and 16 partial genome sections of APS isolates discovered from 2015 to 2021, three main genetic groups (genotype 1-3) and 7 subgenotypes within genotype 1 (subgenotype 1.1-1.7.) have been identified [67]. Phylogenetic analysis by F. Yuan and L. Wang [67] shows that different genotypes and subgenotypes of APS circulate in the same country. The first genetic group includes all isolates found in the USA, Europe and several isolates from China. The second and third genetic groups are represented only by isolates from China [67].

Clinical signs and modes of transmission of atypical porcine pestivirus. Horizontal and vertical transmission routes of APS have been described [22, 24, 60, 84]. Research by B.L. Arruda et al. [24] and A. de Groof et al. [31] showed that the vertical pathway is responsible for the occurrence of congenital tremor in newborn piglets. Some piglets born with tremors resolve symptoms by 3-14 weeks, but as noted, these piglets shed the virus through feces and saliva [24, 31, 35, 60]. When sick piglets are kept with healthy ones, the latter become infected. It is assumed that the virus can also be transmitted through care items, but this issue has not been sufficiently studied [24, 35, 56] and further research into the routes of transmission of the infectious agent is necessary.

The main clinical sign of naturally infected piglets is congenital tremor. In adult pigs, infection is usually subclinical [23, 24, 56]. Data on postnatal and transplacental infection are insufficient. Experimental infection of pregnant sows was carried out on the 32nd, 45th and 62nd days of gestation [24, 31]. In sows, the infection occurred without clinical signs, but viremia was detected [24, 31, 56], piglets were born with congenital tremors [22, 24, 31, 56], and in some cases abortions and stillbirths occurred [22, 31].

In newborn piglets, clinical signs manifest differently. Both apparently healthy and seriously ill piglets can be born, dying in the first days of life. Tremors can range from mild shaking movements of the head and limbs to severe shaking throughout the body [22, 24]. Sick animals are unable to move independently and suck colostrum, despite the strong manifestation of the sucking reflex, so most die of starvation [56]. In some cases, young animals are born with anatomical abnormalities of the limbs. There may also be temporary dysfunction of the hind limbs, which occurs soon after birth and leads to difficulty walking. The survival rate of piglets born with CT is significantly reduced, but still depends on the conditions of detention and care. Over time, CT signs disappear completely [24, 52, 56]. A. Postel et al. [56] reported that, unlike other pestiviruses, APS most often accumulates in the inner layer of cerebellar granule cells of infected animals. This may explain the resolution of the CT sign over time, since the loss of inner granule cells can be compensated for by migration of cells from the outer granule cell layer during the first weeks after birth [56].

In piglets with CT, the virus can be detected in all organs, feces, saliva, blood serum [24, 31, 32, 35, 36], as well as in the central nervous system (cerebellum) and lymphoid tissue (inguinal, submandibular lymph nodes) [24, 36, 56]. It is assumed that the use of infected semen during artificial insemination leads to the birth of infected offspring, since boars already at the age of 6 months spread the virus through seminal fluid [24, 31, 33]. In utero infected piglets can become persistently infected and spread the virus throughout their lives [31, 35]. However, these mechanisms are not well understood.

Postmortem examination of naturally infected piglets with type A-II CT revealed varying degrees of brain hypomyelination, and a moderate decrease in the amount of myelin was observed in the spinal cord [56].

Diagnostics and prevention. *Methods.* Diagnosis of infection caused by APS involves the use of a set of methods. The primary diagnosis is made when symptoms of congenital tremor appear in piglets of any age. The absence of obvious clinical signs does not serve as a basis for establishing a negative farm status for this infection. For diagnosis, molecular genetic, virological and serological research methods are used.

The use of metagenomic sequencing in veterinary practice has made it possible to identify APS in a number of countries [23, 31, 32]. Based on the study of various regions of the virus genome, several systems for PCR analysis have been developed (quantitative real-time PCR, multiplex PCR test system, semi-quantitative duplex RT-PCR analysis, etc.) [34, 35, 39, 41].

APS were isolated in cell cultures of various species of origin [23, 60, 62]; clinical and pathological material from infected, suspected of infection, and forcedly killed animals were used as a source of the virus. For the first time M. Beer et al. [58] were able to isolate a non-cytopathogenic virus in a culture of pig embryonic kidney cells (SPEV, swine embryo kidney). Virus replication in cell culture was confirmed by RT-PCR and high-throughput sequencing [58]. Research by L. Schwarz et al. [35] showed that APS also multiplies (albeit at low titers) in continuous cell cultures of pig kidneys PK-15 (porcine kidney) and SK-6 (swine kidney). The presence of noncytopathogenic virus was determined by

immunofluorescence and PCR methods [35].

Enzyme immunoassay allows one to reliably and with high sensitivity diagnose APS infection. Various ELISA test systems have been developed based on the NS3, E2 and Erns proteins [35, 47, 57, 62], which can be used to conduct regular serological monitoring in different age groups of pigs. Immunohistochemistry, in situ hybridization [36, 56], neutralization reaction, and immunofluorescence are also widely used to diagnose infection [57, 85, 86].

Differential diagnosis. Based on the history, clinical picture and observed pathological changes, it is necessary to first exclude classical swine fever, since infection of pregnant sows with a low-virulent strain of the CSF virus leads to the birth of piglets with congenital tremor (87). APS must be differentiated from *Linda virus*, *Pestvirus F*, *Porcine teschovirus* (PTV), *Porcine astrovirus* (PAstV) and porcine circoviruses, which can also cause neurological disorders in pigs. Treatment of animals infected with APS has not been reported.

Prevention and control. Currently, no vaccine has been developed to prevent infection caused by APS in pigs. Two vaccines have been reported and are effective in preventing infection in BALB/c mice [77, 79]. As with other viral infections, it is important to prevent the pathogen from being introduced into the herd. Animals entering the herd must be quarantined and tested for the presence of pestiviruses. It is also necessary to examine boar sperm used for artificial insemination for the presence of APS, since it can serve as a source of infection for sows.

So, today atypical porcine pestivirus (APV) is widespread in Europe and Asia. APS infection in pigs is mainly associated with symptoms of congenital tremor (CT). Taking into account the fact that CT symptoms in pigs were described long before the identification of APS, it can be assumed that the virus has been circulating in pig herds for several decades. The use of next-generation sequencing in veterinary practice has made it possible to identify not only APS, but also completely new pestiviruses in both pigs and other animal species. The circulation of new pestiviruses in domestic and wild pig populations may affect the effectiveness of anti-epizootic measures against known pestiviruses, such as classical swine fever and bovine viral diarrhea viruses. It is necessary to constantly monitor the epizootic situation and improve test systems to identify these pathogens and carry out differential diagnosis. To date, information has already been accumulated on the genetic diversity of APS, but the antigenic structure of some proteins of this virus has not been sufficiently studied. An analysis of literature data showed that antibodies to the E2, Erns and NS3 proteins are detected in the body of pigs infected with an atypical pestivirus. In response to E2 and Erns, the largest number of virus-neutralizing antibodies are formed both in naturally infected pigs and in experimentally vaccinated laboratory animals. Glycoprotein E2 of APS is considered immunodominant, since it has antigenic determinants (epitopes) that are recognized in the cellular and humoral immune response to the virus. Therefore, it is necessary to study the antigenic structure of E2 in APS, since this protein can be used as an antigenic marker in the creation of a subunit vaccine against APS for pigs. In addition, to understand the peculiarities of the interaction of APS with the pig immune system, an analysis of epitopes of the E2 protein is necessary. The available data on cross-protection between APS and other pestviruses is also insufficient. The wide distribution of APS in different countries requires studying the ecology, pathogenesis, transmission routes, and persistence of the virus in the host body. There is insufficient knowledge about the role of feral pigs in the spread of APS. In addition, it is important to evaluate the impact of this virus on pig productivity.

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AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 2, pp. 274-287
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 638.14/.15:579.62

doi: 10.15389/agrobiology.2023.2.274eng
doi: 10.15389/agrobiology.2023.2.274rus

NOSEMOSIS TYPE C OF BEES CAUSED BY MICROSPORIDIA *Nosema* (*Vairimorpha*) *ceranae*: CURRENT VIEWS, PATHOGENESIS, PREVENTION, DIAGNOSIS AND TREATMENT

(review)

S.A. TIMOFEEV ✉, A.N. IGNATIEVA, V.V. DOLGIKH

All-Russian Research Institute of Plant Protection, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail ts-bio@ya.ru (✉ corresponding author), edino4estvo@mail.ru, dol1slav@yahoo.com
ORCID:
Timofeev S.A. orcid.org/0000-0001-6664-3971
Ignatieva A.N. orcid.org/0000-0001-7288-4077
Dolgikh V.V. orcid.org/0000-0002-2362-2633
The authors declare no conflict of interests
Acknowledgements:
The authors are grateful to Yury S. Tokarev (All-Russian Research Institute of Plant Protection) for critical evaluation and revision of the manuscript.
Supported financially from the Russian Science Foundation (grant No. 18-16-00054)
Final revision received June 15, 2022
Accepted July 28, 2022

Abstract

Nosemosis type C is a parasitic disease of honey bees caused by the obligate intracellular parasite microsporidia *Nosema (Vairimorpha) cerana*. This disease is widespread worldwide and can lead to a decrease in honey production, a sharp reduction in the population of adults in bee families and their final death (M. Higes et al., 2007; P.J. Marín-García et al., 2022). The purpose of this review is to present up-to-date data on this disease and its causative agent, as well as on modern methods of diagnosis, prevention and treatment in beekeeping. The parasite is mainly transmitted between bees by the fecal-oral route and infects the cells of the middle intestine of insects (R. Galajda et al., 2021).. Vertical transmission of the parasite is also possible, as *N. ceranae* spores have been found in ovarian cells of infected queens (C. Alaux et al., 2011). The pathogenesis of *N. ceranae* is associated with the destruction of infected cells, the restructuring of the host's metabolic processes to meet the needs of the parasite, the shortage of spare resources and vital metabolites in sick bees. hormonal imbalance; negative consequences of part of the immune responses to the pathogen invasion, such as oxidative stress (L. Paris et al., 2017). Ability of *N. ceranae* specifically inhibits such protective reactions of bees as activation of apoptosis of infected cells and production of antimicrobial peptides can enhance the pathogenic nature of nosemosis type C (K. Antunez et al., 2009; C. Kurze et al., 2015). The method of diagnosis of infection includes the primary detection of the parasite using light microscopy, including with the use of various dyes (N.J. Ryan et al., 1993), and further determination of the species of microsporidia using molecular methods such as standard polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP). The most effective drug for the treatment of nosemosis of bees for a long time remained the antibiotic fumagillin, despite the fact that *N. ceranae* can acquire resistance to this drug (W.-F. Huang et al., 2013; I. Tlak Gajger et al., 2018). However, the discovery of residues of this drug in honey produced by bees after treatment and its toxicity to humans led to the prohibition of this drug in a number of countries and the cessation of its production in 2018 (I. Tlak Gajger et al., 2018). In this regard, many studies have been conducted in recent years aimed at finding new ways to treat nosemosis. For example, extracts from various fungi and plants, probiotics such as eugenol, chitosan, naringenin, proteksin, proteasome function inhibitors ixazomib, and ixazomib citrate are considered as agents for the treatment of this disease (V. Chaimanee et al., 2021; S.S. Klassen et al., 2021; E.M. Huntsman et al., 2021). Despite the fact that many of the tested methods have shown encouraging results, a safe analogue of fumagillin, similar to it in terms of the effectiveness of the fight against nosemosis, has not yet been found. The article also provides recommendations for the care of beehives for the prevention of nosemosis type C in beekeeping.

Keywords: *Nosema ceranae*, *Vairimorpha ceranae*, *Apis mellifera*, nosemosis, microsporidia, bee diseases

Pollination of plants by insects is of key importance for agriculture and the Earth biosphere as a whole. Bees are one of the main pollinators [1], and the proportion of crops pollinated by them can be as high as 53% in some countries [2]. In recent years, the European continent has seen a downward trend in pollinator populations, in particular the honey bee *Apis mellifera* Linnaeus, 1758 [3-6]. The reason for this trend may be a number of abiotic and biotic factors, e.g., the intensification of agriculture and the widespread use of chemical insecticides, the spread of transgenic plants and their pollen resistant to phytophagous insects, global climate change, etc. Diseases caused by various pathogens and parasites have been cited as a possible reason for the decline in honey bee populations [6, 7].

Nosema ceranae is an obligate intracellular parasite of bees, belonging to microsporidians (a group of unicellular organisms related to fungi). The species was first described in 1996 in the Chinese wax bee *Apis cerana* [8] and is now considered to be infectious in the taxa of stingless bees (Meliponini), true wasps (Vespidae), and some species of bumblebees and bees, including all subspecies of the honey bee *A. mellifera* [9-12]. A 2020 phylogenetic revision of the genera *Nosema* and *Vairimorpha* showed that *N. ceranae* is correctly classified in the second genus [13]. However, since the vast majority of modern works continue to use the traditional name of the parasite *Nosema ceranae*, we will also stick to this name for the purposes of this review.

N. ceranae is widespread in beekeeping in most countries. It is considered the dominant microsporidia species infecting honey bees and has displaced N. apis, the natural pathogen of *A. mellifera* [14-16]. The disease caused by this parasite, Asian nosematosis, or type C nosemosis, has been associated with sharp declines in the adult population of bee colonies, decreased honey production, and even colony collapse [12, 17].

The purpose of this review is to present current data on Asian nosematosis and its causative agent, as well as modern methods of its diagnosis, treatment and prevention in beekeeping.

Biology and pathogenesis of *Nosema ceranae*. The infectious stage of the life cycle of *N. ceranae*, capable of existing in the external environment, like all microsporidia, is spores, the size of which is about 4.7×2.7 μm [8]. The spores enter the bee's body with food, and probably through contact with other infected bees, for example during grooming [18]. During this process, the bees groom each other, which helps them get rid of other parasites such as *Varroa destructor*, but grooming can also lead to the spread of nosematosis. In the midgut of the insect, protected only by the peritrophic membrane, the embryo (sporoplasm) of the parasite is introduced into the epithelial cell using a complex spore extrusion apparatus. Inside the host cell, the sporoplasm transforms into a meront, and after several cycles of division, the process of formation of new spores begins, which are released from the infected cells into the intestinal lumen when enterocytes are destroyed and serve as a source of infection of other cells of the same insect or other individuals. The discovery of empty shells of *N. ceranae* spores that do not contain the parasite embryo in the intestinal epithelial cells of bees may also indicate that extrusion of spores and infection of neighboring insect intestinal cells takes place inside the infected cell [19, 20].

Vertical transmission of the parasite is also possible, as *N. ceranae* spores have been found in ovarian cells of infected queens [21, 22]. Infection of these cells usually occurs through contact with infected worker bees [23], and drones are infected in the same way [24]. It is believed that in natural and commercial hives only adult bees are infected, however, in laboratory conditions, the development of nosematosis has been shown in prepupae infected with *N. ceranae* at the 3-day larval stage [25].

Although *N. ceranae*, in addition to the intestines, has been found in various organs of worker bees [26, 27], their infection has never been proven histologically. The authors [28] of a recent detailed study of *N. ceranae* tropism in the honey bee suggest that the results described above were due to contamination of tissue samples with parasite spores due to imperfect dissection techniques or destruction of the insect intestine at a late stage of infection. That is, most likely, the intestine serves as the only organ in which *N. ceranae* develops [28]. In this case, the development of the parasite occurs only in enterocytes, but not in the stem cells necessary for the renewal of the intestinal epithelium [29].

Both under natural conditions and during artificial infection with *N. ceranae*, a pathology with similar symptoms occurs in the intestine of the infected insect [30]. Infected epithelial cells show signs of degradation, e.g., the appearance of vacuoles in the cytoplasm, disruption of the integrity of cell membranes, condensation and a decrease in the size of the cell nucleus, usually accompanied by hyperchromatosis (excessive increase in chromatin content) and pyknosis (shrinkage of the cell nucleus during chromatin condensation). The peritrophic membrane disappears completely or becomes significantly fragmented. In the underlying region of the brush border, rupture of the cell plasma membrane is sometimes observed. In the most infected epithelial cells, the nucleus is displaced apically. In some host cells, immature and mature stages of *N. ceranae* can be found in invaginations of the nuclear envelope. Lytic processes are actively occurring in infected cells, as evidenced by numerous vacuoles and aggregates of ribosomes and lysosomes, as well as the loss of glycogen particles [19, 30]. Loss of glycogen and loose aggregation of ribosomes are possible consequences of mitochondrial damage [31]. The utilization of glycogen stores may indicate that the infected cell has switched from a more efficient mode of energy production (e.g., oxidative phosphorylation) to a less efficient anoxybiont glycolysis, perhaps to compensate for the depletion of ATP that is taken up by the parasite from infected cells [32]. ATP deficiency leads to disruption of ion transport across the membrane of the infected cell, which leads to the accumulation of excess sodium in the cell, detachment of ribosomes from the rough endoplasmic reticulum and, ultimately, to necrosis [14, 31].

Impact on the host cell metabolism and its specific reconfiguration to increase the availability of nutrients and energy resources for the parasite is a characteristic feature of microsporidia parasitism [32]. When intestinal cells are infected with *N. ceranae*, *A. mellifera* exhibits upregulation of the alpha-glucosidase gene and three genes involved in trehalose transport, as well as downregulation of genes encoding trehalase and glucose-methanol-choline oxidoreductase [33, 34]. These metabolic changes result in increased availability of trehalose to the parasite, which is considered the main source of glucose for microsporidians [32].

N. ceranae also specifically affects host defense responses at the cellular level. Infected intestinal cells may undergo apoptosis before the parasite has time to complete its full developmental cycle. *N. ceranae* appears to be able to block this process, as evidenced by increased transcription of various apoptosis inhibitors in infected cells [33-35]. This assumption is supported by the fact that in a nosematosis-resistant line of bees, no inhibition of apoptosis was observed during experimental infection [36]. Another cellular mechanism of protection against microsporidia infection is oxidative stress, i.e., the production of reactive oxygen species by infected cells that can destroy intracellular parasites. This mechanism appears to be ineffective against N. ceranae infection because the parasite thrives, despite observed oxidative stress and damage to intestinal cells, by producing catalase, glutathione peroxidase, and glutathione S -transferase [4, 37]. However, it is likely that *N. ceranae* still affects infected bee cells and suppresses oxidative stress responses, since when the latter was artificially induced by pesticides, infection

with *N. ceranae* reduced the amount of reactive oxygen species and reduced damage to intestinal cells by *A. mellifera* [38].

At the organismal level, Asian nosematosis in bees can be asymptomatic or cause significant harm. This largely depends on the external conditions in which bees exist, on fluctuations in temperature and humidity [16, 39]. In the laboratory, infection of bees with a dose of 10^5 spores per individual led to 100% mortality of insects by day 8 after infection [19]. One of the important consequences of nosematosis in bees is energy stress caused by disruption of the damaged intestine and changes in the carbohydrate metabolism of infected cells. Although infected bees consume more food than uninfected bees, they do not appear to be able to utilize the excess amounts of carbohydrates consumed, most of which are used by the pathogen to complete the life cycle [12, 17, 40]. Energy stress is associated with high mortality of worker bees during nectar collection, which requires significant energy expenditure [40, 41]. Infected bees collect nectar less efficiently than healthy bees and more often do not return to the hive due to impaired orientation in space [42]. Infected worker bees also spend more time outside the hive, engaging in risky behaviors such as robbing [43]. Interestingly, similar effects were not observed when a line of bees resistant to nosematosis was artificially infected [44]. Energy stress due to nosematosis is associated with the degradation of the hypopharyngeal glands in nurse bees, which produce secretions for feeding larvae, and the depletion of the secretion itself [40, 41]. Despite the fact that the death rate of drones is usually lower than that of worker bees, the effects of nosematosis and energy stress are usually more pronounced in them, and their mortality from this infection is higher [45].

A number of other changes in the body of infected bees and the consequences of nosematosis are associated with the immune response to the infection. The oxidative stress described above directly affects the reduced lifespan of infected bees [40]. *N. ceranae* appears to suppress other insect defense responses at the organismal level. Infected bees have reduced expression of many genes associated with the immune response, including those encoding antimicrobial peptides and hormones. Suppression of expression was observed for proteins such as abaecin, defensin, hymenopthecin, glucose dehydrogenase, vitellogenin, serine protease 40, and catalase [34, 46]. In addition, suppression of the expression of components of the immune response activation pathways Toll and Imd and pattern recognition receptors was noted [47]. Similar suppression of the immune response in infected bees was observed not only in laboratory experiments and agricultural hives, but also in natural populations [48]. However, in other studies, on the contrary, upon infection with *N. ceranae*, the expression of various components of the immune response of bees increased, including many of the genes mentioned above [49, 50]. This finding suggests that the immune response may be influenced by many factors, such as infectious load, which varies significantly between studies; duration of tests; biomaterial in which gene expression was studied (whole bees, abdominal cavity, ventricles, etc.); age of bees at infection and during the study. The mechanisms of interaction of *N. ceranae* with the host immune system and their role in pathogenesis require further study.

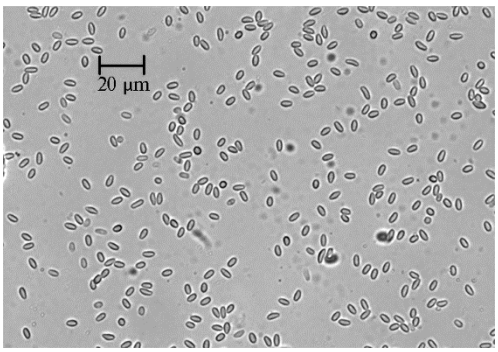
Depending on their age, bees perform different functions in the hive. For example, young bees clean, build, and feed brood within the colony, whereas external tasks are reserved for older bees [51]. *N. ceranae* infection causes hormonal imbalance, leads to behavioral disturbances and accelerates the development of infected bees. The distribution of functions between individuals of different ages is primarily regulated by the ratio of the production of juvenile hormone and vitellogenin in the bee's body. Worker bees infected with *N. ceranae* had increased levels of juvenile hormone, which led to a premature transition from foraging to

foraging outside the hive [51, 52].

Infection with *N. ceranae* may be one of the factors influencing the social demography of a colony. Infected bees die due to destruction of body tissue, are unable to return to the hive due to energy stress, or leave the hive to limit transmission of infection to healthy worker bees. Young bees start searching for nectar earlier due to hormonal imbalance, and the feeding process of larvae and other individuals in the hive is disrupted. The loss of infected worker bees over time can cause accelerated aging and premature foraging in uninfected young foraging insects. Young foragers are less efficient at obtaining food than normal-aged foragers, which becomes a threat to the food security of the colony. The culmination of these effects can ultimately lead to sudden colony death [17, 19, 53].

Detection of *Nosema ceranae* and diagnosis of Asian nosematosis in bees. In a bee colony, clinical and subclinical manifestations of Asian nosematosis are expressed in a longer breeding period in cold months, an increase in the proportion of brood frames relative to the number of nurse bees in warm months, and a decrease in honey production. Infected colonies weaken, the proportion of adult bees decreases, which leads to the death of the colony within 1.5-2 years [12, 54]. None of these manifestations is specific to *N. ceranae* infection and may be caused by other diseases, requiring a differential diagnosis to determine nosematosis [16].

The first step for detecting *N. ceranae* in a hive is microscopic analysis of feces, smears from opened dead bodies, and intestinal homogenates for the presence of spores. Their characteristic ovoid shape (Fig.) makes it easy to identify microsporidia infestations. To facilitate the detection of the parasite, specific dyes, the trichrome and calcofluor white are used in the preparation that bind to the chitin of the shell of microsporidia spores, as well as the nonspecific dye toluidine blue [55, 56].



Microphotograph of *Nosema ceranae* spores from the intestinal homogenate of an infected bee *Apis mellifera* (light transmission microscopy, Axio Imager M1, Carl Zeiss, Germany, magnification ×100; photograph taken by A.N. Ignatieva).

Recently, a method for primary microscopic detection of *N. ceranae* in the field without laboratory equipment was proposed. Using a regular smartphone, ultraviolet LEDs, and a set of simple lenses, the authors developed a device weighing 374 g that allows one to effectively detect *N. ceranae* spores in samples when stained with a modern modification of calcofluor white [57]. Spores of *N. ceranae* differ slightly from spores of *N. apis* morphologically (the latter have a more rounded shape), therefore, molecular detection methods are necessary for reliable differentiation of these species [12, 16].

The main method for detecting nosematosis in bees and establishing the specific species of the parasite is standard polymerase chain reaction (PCR) or real-time PCR (qPCR) [58, 59]. To do this, it is necessary to use primers specific for *N. ceranae*, *N. apis*, and also for both species simultaneously. A complete list of primers that are used in the diagnosis of nosematosis can be found in the work of R. Galajda et al. [18]. Detection using PCR makes it possible to establish the fact of infection of a hive by examining not only infected bees or their excrement, but also the produced honey, in which parasite spores that remain infective are

also found [60, 61]. A recently developed ultra-fast protocol for detecting *N. ceranae* using real-time PCR allows detection of bee infection at the stage of 24 parasite cells in the entire insect body, whereas microscopic detection is usually effective at the stage of mass sporogony, when tens of thousands of *N. ceranae* cells are formed [62]. In addition, molecular diagnostics using PCR makes it possible to assess the degree of infection 8 times more accurately than counting spores during microcopying. Recently, another method for diagnosing nosematosis has been developed, similar to PCR, but not requiring the use of stationary laboratory equipment, which is based on loop-mediated iso-thermal amplification (LAMP) [63].

Prevention and treatment of Asian nosematosis in beekeeping. Recent works by scientists from Italy and Spain have formulated the basic principles of optimal beekeeping to minimize the risks of various diseases and maintain the health of bees in apiaries [64, 65]. In our opinion, these works present the most relevant recommendations to date.

Preventive measures against nosematosis include purchasing queens from bee families not infected with *N. ceranae*, collecting forager bees or debris from the hive in early autumn or spring to diagnose nosematosis microscopically and by PCR analysis. In autumn and spring, bees should be fed stimulants or feed additives to improve their health. Feeding honey and pollen from colonies infected with *N. ceranae* should be avoided. Disinfection of equipment before use is obligatory. The metal tools are sterilized by burning. Fumigation with glacial acetic acid, 5% sodium hydroxide (caustic soda), 0.5% sodium hypochlorite (bleach) and 1.65% ammonia solution can be used to disinfect hives. Queens must be replaced at least every 2 years, except for those that have high genetic value. New families should be kept separately for at least 1 month to control for diseases and infestations and prevent their transmission. Annual renewal of 30% of the cells in the hive is recommended. Beekeepers must minimize stress in bees, winter inspections of hives should be avoided, the use of a smoker should be limited, proper feeding of bees is necessary, etc. IN case of a bee colony death, the hive must be immediately removed from the apiary.

The most effective treatment for both classical and Asian nosematosis is the antibiotic fumagillin, isolated from the fungus Aspergillus fumigatus and which can significantly reduce the infection of the colony and the risk of its destruction [66-68]. Depending on the geographic location and condition of the colony, it is recommended to treat infected colonies from once (in the fall during feeding) to twice a year (in the fall and spring, in case of severe infections). While the fall treatment is aimed at keeping the colony alive during the cold season, the spring treatment is done to improve the health of adult bees, which will be able to properly care for the next generation of individuals raised in the spring. Nevertheless, there are cases where, with an extremely high degree of infection of a bee colony with Asian nosematosis, the use of fumagillin did not stop the spread of infection and did not increase the survival of colonies in winter, regardless of the dose or method of treatment [69, 70]. Cases of resistance to this antibiotic have also been described for *N. ceranae* [68, 71]. In addition, it is important to note that fumagillin cannot be considered completely safe for humans, and the discovery of its residues in honey after processing hives led to a ban on the use of the drug in Europe, and in 2018 the Canadian company Medivet Pharmaceuticals Ltd. stopped its production [72].

Recently, a significant number of studies have been devoted to the search for treatments for nosematosis in bees (Table).

Studies aimed at finding new treatments for Asian nosematosis caused by the microsporidia *Nosema ceranae* in *Apis mellifera* bees (2021)

Substance, method	Effect	References
Dietary supplement containing wheat bran, essential oils, cinnamon, dextrose, brewer's yeast, lecithin, saturated and unsaturated fatty acids, vegetable proteins, essential amino acids, lipids and a vitamin-mineral complex based on vitamin B	Statistically significant reduction in colony infection with nosematosis by approximately 10%	[73]
Various combinations of extracts from 7 types of medicinal plants	A mixture of extracts of 20% blueberry, 40% wormwood, 10% oakmoss, 10% oregano, 10% hops, 5% bay leaf and 5% anise hyssop was most effective against N. ceranae and resulted in more than 2 -fold reduction in infectious load in infected bees	[74]
12 various medicinal extracts plants	9 out of 12 extracts suppressed the development of infection, the production of N. ceranae spores in infected bees decreased by 4-6 times	[75]
Extract of *Agaricus bisporus*	Addition of the extract increased the survival rate of infected bees by approximately 10% and reduced spore production in infected bees by a third. The immunostimulating effect of the extract has been shown to increase the expression of genes encoding abecin, hymenoptaecin, apidecin and vitellogenin in infected bees.	[76]
Propolis produced by *A. mellifera* and *Tetrigona apicalis*	Propolis of both types of reduced bee mortality by more than 2 times, infection by 20-40%, infectivity by 70-80% compared to the indicators in untreated bees and led to a significantly higher protein content in the hypopharyngeal glands and hemolymph in treated bees than in untreated bees	[77]
Prebiotics from dietary fiber, acacia gum, inulin and fructo-oligosaccharide, as well as commercial probiotics Vetapharm, protexin concentrate with one bacterial strain (*Enterococcus faecium*) and protectin concentrate with several strains of bacteria (*Lactobacillus acidophilus, L. plantarum*, *L. rhamnosus*, *L. delbrueckii*, *Bifidobacterium bifidum*, *Streptococcus salivarius* and *E. faecium*)	Acacia gum caused the greatest reduction in *N. ceranae* spore counts (67%) but also significantly increased bee mortality (62.2%). The *E. faecium* strain produced a similar reduction in spore counts (59%) without affecting mortality. The use of a single strain appears promising as it may reduce the proliferation of *N. ceranae* and increase the survival of infected bees even compared to healthy, uninfected bees	[78]
Probiotics eugenol, chitosan, naringenin, protexin	Treatments with eugenol, naringenin and protectin significantly reduced N. ceranae infestation and increased honey production. Protek-sin also increased the number of adult bees, but chitosan was ineffective	[79]
Meal from *Brassica nigra* and *Eruca sativa* seeds, containing a fixed quantity various glucosinolates	More than 2-fold reduction in bee infestation in laboratory conditions	[80]
Meal from *Brassica nigra* and *Eruca sativa* seeds, containing a fixed quantity various glucosinolates	Reduced infestation of bees compared to control in field conditions, manifested to a lesser extent than when using this technique in the laboratory	[81]
Chitosan and peptidoglycan	Stimulation of bee immune responses, increased expression of antimicrobial peptide genes, more than 2-fold reduction in microsporidia infection	[82]
Screening of some plant extracts, microbial fermentation products, organic acids, food chain wastes, bacteriocins and fungi	Some of the introduced ingredients such as high concentration acetic acid, p-coumaric acid and Saccharomyces sp. strain KIA1, showed relative effectiveness in the fight against nosematosis	[83]
The A. mellifera gene encoding the iron ion transporter transferrin was knocked down	Reduced transcriptional activity in N. ceranae cells, reduced iron loss, enhanced immunity and improved survival of infected bees	[84]
Double-stranded RNA, complementary to regions of N. ceranae genes encoding spore coat proteins	More than 2-fold reduction in infestation and increased survival of bees in laboratory conditions	[85]
Proteasome inhibitors - ixazomib and ixazomib citrate	Significant reduction in bee infestation and increase in their survival, comparable in effectiveness to the effect of fumagillin	[86]

N o t e. Unless otherwise stated, the experimental procedure consisted of adding the active substance when feeding infected bees.

Thus, Asian nosematosis of honey bees, caused by an obligate intracellular parasite, the microsporidia Nosema ceranae, is distributed throughout the world.

The infection leads to intestinal dysfunction, hormonal imbalance and energy stress. These factors can change the behavior of infected insects, disrupting the natural division of responsibilities between bees of different ages in the hive. The culmination of such effects can ultimately lead to the sudden death of the colony. Diagnosis of Asian nosematosis usually involves microscopic analysis of insect intestinal preparations and subsequent molecular analysis using PCR or its analogues. The most effective treatment for the disease is the antibiotic fumagillin, which, however, is toxic to humans and is banned in many countries. Special attention is currently being paid to the search for new methods of treating nosematosis, but so far, no safe and effective alternative to fumagillin has been found.

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AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 2, pp. 288-301
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

100th Anniversary of the Zhitkov Russian Game Management and Fur Farming Research Institute: Bioresources and environment management

UDC 639.111.16:612.11/.12

doi: 10.15389/agrobiology.2023.2.288eng
doi: 10.15389/agrobiology.2023.2.288rus

SERUM CHEMISTRY PARAMETERS OF FREE-RANGING MOOSE (*Alces alces* Linnaeus, 1758) OF DIFFERENT SEX AND AGE GROUPS

M.A. KOSHURNIKOVA✉, I.A. DOMSKY, Y.A. BEREZINA, A.V. ECONOMOV

Zhitkov Russian Game Management and Fur Farming Research Institute, 79, ul. Preobrazhenskaia, Kirov, 610000, Russia, e-mail: mperevozchikova@mail.ru (✉ corresponding author), vniioz43@mail.ru, uliya180775@bk.ru, aconom86@mail.ru
ORCID:
Koshurnikova M.A. orcid.org/0000-0003-3638-3712
Domsky I.A. orcid.org/0000-0003-1633-1341
Berezina Y.A. orcid.org/0000-0001-5082-716X
Economov A.V. orcid.org/0000-0002-0242-8954
The authors declare no conflict of interests
Acknowledgements:
The authors thank the hunting team of the Zhitkov *Russian Game Management and Fur Farming Research Institute* for help in collecting biomaterials.
Supported financially by the FSR Program of the State Academies of Sciences FSZZ-2019-0001 (AAAA19-119020190132-5)
Final revision received June 10, 2022
Accepted July 17, 2022

Abstract

Moose (*Alces alces,* Linnaeus 1758) is a perspective species for game farming, characterized by a high growth rate, as well as a wide potential for economic use for meat and milk production. In Russia and the world, there are few studies of the biochemical parameters of moose blood. (S.A. Becker et al., 2010; A.W. Franzmann et al., 1978; M.A. Keech et al., 1998; V. Reshetnyak et al., 2021; M.K. Rostal et al., 2012). The reference range of biochemical blood parameters is necessary for assessing the physical condition of animals, nutrition quality, habitat, stressors, the impact of infectious and invasive diseases (A.A. Cunningham et al., 1998; P. Daszak et al., 1999; W.F. Frick et al., 2010). The article presents for the first time the biochemical parameters of the blood of moose from different sex and age groups. The goal of the work is to determine the biochemical parameters of blood in moose of different sex and age living in the Kirov region. Whole blood was taken from animals (n = 90) bagged during the hunting seasons of 2006-2020. Samples were collected from October to December in the experimental hunting ground of Zhitkov Russian Game Management and Fur Farming Research Institute (Kirov region). All animals were wild and moved freely, feeding on local vegetation. Whole blood was studied from the following sex and age groups: 20 young females aged 6-7 months; 20 adult females aged 2.5-7 years; 20 young males aged 6-7 months; 30 adult males aged 2.5-7 years. All animals were considered clinically healthy. Blood samples were taken by cutting the jugular vein immediately after the animal was shot. Blood serum studies were performed on a Biochem SA semi-automatic biochemical analyzer (High Technology Inc., USA). The analysis included the determination of the activity of aspartate aminotransferase (AsAT), alanine aminotransferase (AlAT), alkaline phosphatase, lactate dehydrogenase (LDH), alpha-amylase, total protein, albumin, total bilirubin, direct bilirubin, creatinine, urea, cholesterol, glucose. In the blood serum of adult moose, high activity of AsAT was noted (in females and males, respectively, 253.8±52.38 and 250.9±47.52 U/l), alpha-amylase (29.2±7.20 and 32 0±8.77 U/l), creatinine content (183.9±18.59 and 182.1±23.66 mmol/l), urea (6.2±0.82 mmol/l and 5.9±0.87 mmol/l). In young individuals, AsAT (161.2±28.30 and 160.0±30.92 U/l), alpha-amylase (24.6±4.91 and 23.2±5.46 U/l), the content creatinine (152.8±20.32 and 149.1±23.78 mmol/l), urea (2.4±0.63 and 2.6±0.98 mmol/l) were significantly lower. In young animals, on the contrary, high activity of AlAT (60.8±6.42 and 58.7±6.74 U/l), alkaline phosphatase (230.4±40.79 and 222.2±31.14 U/l) /l), LDH (805.2±185.57 and 822.9±237.13 U/l), glucose content (6.3±1.01 and 6.3±1.03 mmol/l). Adult animals were characterized by lower levels of AlAT (43.6±7.35 and 41.9±6.33 U/l), alkaline phosphatase (69.3±12.62 U/l and 69.9±11,31 U/l), LDH (614.1±98.11 U/l and 598.2±129.37 U/l), glucose (5.3±1.02 and 5.3±1.14 mmol/ l). Statistically significant ($p < 0.05$) differences in the content of total protein in blood serum between adult females (57.4±7.48 g/l) and males (68.1±4.93) were established. In young females, an average correlation was found between the

content of total and direct bilirubin ($r = 0.57$, $p = 0.01$), in adult females — between the activity of AsAT and alkaline phosphatase ($r = 0.50$, $p = 0.02$) and between AsAT and LDH activity ($r = 0.66$, $p = 0.00$). A statistically significant ($p < 0.05$) effect of age on the content of AsAT, AlAT, alkaline phosphatase, LDH, alpha-amylase, direct bilirubin, creatinine, urea and glucose, as well as the sex of animals on the content of total protein was shown. The dependence of the AsAT, AlAT, alkaline phosphatase, LDH, alpha-amylase, total protein, direct bilirubin, creatinine, urea and glucose levels on body weight was revealed. The obtained biochemical parameters of moose blood have a similar trend in most parameters with the results obtained on other artiodactyls in the wild. Differences are due to species of animals, living conditions, nutrition, age, sex, as well as the method used in blood sampling.

Keywords: moose, females, males, age groups, biochemical blood parameters, blood serum

Farm hunting has emerged in the last decade as a separate area, which involves keeping animals in semi-free conditions and in an artificial habitat, which requires hunting and veterinary measures. In this regard, elk (*Alces alces* Linnaeus, 1758) is a promising species characterized by a high growth rate and wide possibilities for economic use for the production of dietary meat and medicinal milk [1].

The biochemical factor influences the adaptability of the animal body to environmental conditions. Changes in enzymatic reactions make it possible to successfully cope with unfavorable factors or their fluctuations. The biochemical features identified in various organisms became understandable and accessible to study only against the backdrop of modern advances in biochemistry. It is biochemical adaptation that allows species living in different natural conditions to maintain external similarities [2].

Basic values of biochemical blood parameters are necessary to assess the physical condition (function of internal organs, metabolism) and health of animals both at the population and individual levels, the quality of nutrition and habitat, the impact of anthropogenic and environmental stressors, the potential impact of existing and emerging diseases infectious and invasive nature, as well as to determine appropriate strategies to combat them [3-7]. In addition, biochemical blood parameters make it possible to assess the metabolic patterns of elk of different ages depending on environmental conditions [8].

Even in a healthy animal in relatively good body condition, nutrient deficiencies can create physiological imbalances that affect population performance [9, 10]. It has been proven that nutrient limitation and deficiency for moose, especially in winter, leads to decreased reproductive function and survival, especially among young animals [11-14].

When developing a reference range, it is important to consider the presence of diseases in the animals being studied, which may affect the results of the study, especially in individuals with infectious or invasive diseases, iron deficiency anemia, some types of cancer, and other systemic diseases [15-17]. Various conditions of the body will also affect the biochemical parameters of the blood. For example, in emaciated ruminants, there is a decrease in albumin, lactate dehydrogenase (LDH), gamma-glutamine transferase (GGT) and creatinine phosphokinase (CPK), an increase in creatinine and urea (due to muscle catabolism), as well as a change in the amount of beta-hydrobutyric acid. Obesity causes increased glucose and triglyceride levels [18].

Although sampling from animals in the wild is difficult and sometimes virtually impossible, basic blood chemistry ranges have been established for some wild species [19].

Studies of biochemical parameters in moose in Russia and the world are rare [20-25]. The values of chemical indicators were derived from a small number of samples with no age-sex differences, obtained by different methods from hunted, caught, immobilized individuals or from animals kept in captivity, and therefore cannot be fully representative, but nevertheless represent scientific value, and similar

studies should be continued.

In this work, for the first time, biochemical blood parameters of moose from different sex and age groups were established.

The purpose of the work is to study biochemical blood parameters in moose of different sexes and ages living in the Kirov region.

Materials and methods. Whole blood was taken from animals (n = 90) hunted during the 2006-2020 hunting seasons. Sampling was carried out from October to December in the scientific experimental hunting farm of the Professor Zhitkov All-Russian Research Institute of Hunting and Fur Farming (Kirov Province), located in the northern part of the moose habitat (northeast of the European part of Russia; 58°33′04″N, 50°43′42″E). The total area of the farm is 66,250 hectares. The climate is continental with moderately cold winters and warm summers. All animals were wild and moved freely, feeding on local vegetation.

Whole blood was studied in 20 young females aged 6-7 months; 20 adult females aged 2.5-7 years; 20 young males aged 6-7 months; 30 adult males aged 2.5-7 years. All animals were considered clinically healthy (at the time of sampling there were no signs of disease; the health of the animals was assessed by a veterinarian who is part of the hunting team). Body weight was 127.0-185.0 kg (158.6±23.97 kg) in young females, 363.0-432.0 kg (391.8±29.04 kg) in adult females, 178.0 -201.5 kg (191.2±10.58 kg) in young males, and 280.0-420.5 (340.9±53.36 kg) in adult males.

Blood for the study was taken from the jugular vein (*venae jugularis*), which was cut immediately after the animal was shot; no medications were used. The death of an animal from a gunshot wound occurred at lightning speed, or in most cases the agonal period did not exceed several minutes (this corresponded to the lightning-fast rate of dying, in which the agonal period is no more than 15-30 minutes). Blood was collected into 4 ml UNIVAC vacuum tubes (Eiliton, Russia) with a coagulation activator. Before sending to the laboratory (16-24 hours), the blood was stored in a refrigerator at 4 °C. Hemolyzed samples were discarded to avoid analytical errors.

In the laboratory, the blood was centrifuged at 1500 rpm for 20 minutes (Liston C 2204 centrifuge, Liston, Russia). Blood serum studies were carried out immediately after delivery (a semi-automatic biochemical analyzer Biochem SA, High Technology, Inc., USA) with a set of reagents (Eco-Service, Russia) to determine the activity of aspartate aminotransferase (AsAT), alanine aminotransferase (AlAT), alkaline phosphatase, lactate dehydrogenase (LDH), alpha-amylase, total protein content, albumin, total bilirubin, direct bilirubin, creatinine, urea, cholesterol, glucose.

Statistical analysis was performed using Microsoft Excel 2019 and Statgraphics (19-X64) software using standard methods [26]. To describe the samples, means (M), standard deviations (±SD), medians (Me), 25% and 75% percentiles were determined. When comparing indicators between groups, the nonparametric Wilcoxon-Mann-Whitney test (U) was used. Relationships between characteristics were assessed using Spearman rank correlation. To assess the influence of three factors (age, sex, body weight) on biochemical blood parameters, single- and multivariate analysis of variance (ANOVA, MANOVA) was used. The influence of the factor was considered statistically significant at $p < 0.05$.

Results. The results of biochemical studies of blood serum of moose of various sex and age groups are given in the Table.

In young females, an average correlation was established between the content of total and direct bilirubin (r = 0.57, p = 0.01), in adult females between the activity of AsAT and alkaline phosphatase (r = 0.50, p = 0.02) and between AsAT and LDH activity (r = 0.66, p = 0.00).

Blood biochemical parameters in moose (*Alces alces* Linnaeus, 1758) of different sex and age groups (Kirov Province, 2006-2020)

Parameter	Youngsters up to 1 year old, ♀ (n = 20)	Adults, ♀ (n = 20)	Youngsters up to 1 year olf,, ♂ (n = 20)	Adults, ♂ (n = 30)
Aspartate aminotransferase, U/l:				
Min-max	117.5-219.1	157.1-341.6	120.2-207.5	162.3-342.5
M±SD	161.2±28.30	253.8±52.38	160.0±30.92	250.9±47.5
Me	155.6[a]	244.1[a]	154.7[a]	243.3[a]
25 %-75 %	146.9-178.9	219.6-296.0	131.6-193.5	208.4-299.4
Alanine aminotransferase, U/l:				
Min-max	47.2-72.0	29.4-55.4	48.0-71.2	30.4-53.2
M±SD	60.8±6.42	43.6±7.35	58.7±6.74	41.9±6.33
Me	61.9[a]	44.2[a]	57.2[a]	40.6[a]
25 %-75 %	56.4-63.9	39.1-49.7	54.8-64.7	38.3-46.9
Alkaline phosphatase, U/l:				
Min-max	165.2-296.0	49.0-89.1	169.2-270.8	46.6-88.3
M±SD	230.4±40.79	69.3±12.62	222.2±31.14	69.9±11.31
Me	232.0[a]	69.0[a]	219.1[a]	70.4[a]
25 %-75 %	197.7-269.2	57.5-79.3	200.9-253.8	61.4-79.2
Lactate dehydrogenase, U/l:				
Min-max	502.9-1118.6	417.0-803.0	504.9-1222.0	380.5-864.1
M±SD	805.2±185.57	614.9±98.11	822.9±237.13	598.2±129.37
Me	847.5[a]	622.9[a]	778.2[a]	594.4[a]
25 %-75 %	700.4-926.2	558.3-672.1	659.3-1062.7	515.3-699.6
Alpha amylase, U/l:				
Min-max	16.9-35.9	17.1-41.6	15.3-34.6	18.9-47.6
M±SD	24.6±4.91	29.2±7.20	23.2±5.46	32.0±8.77
Me	24.3[c]	27.3[c]	23.4[a]	32.2[a]
25 %-75 %	21.3-27.0	24.7-35.2	19.4-25.9	31.3-35.1
Total protein, g/l:				
Min-max	46.5-83.0	45.4-70.1	45.5-86.1	59.4-79.4
M±SD	61.6±9.91	57.4±7.48	66.4±13.53	68.1±4.93
Me	62.0	57.8[d]	70.5	68.7[d]
25 %-75 %	53.75-67.0	52.4-62.0	53.2-77.8	63.4-71.4
Albumen, g/l:				
Min-max	33.0-49.1	30.9-53.8	32.2-53.3	33.4-53.2
M±SD	40.9±4.48	43.7±5.62	42.9±6.94	42.3±5.65
Me	41.8	44.9	42.9	41.5
25 %-75 %	38.2-43.7	40.2-46.5	36.0-50.0	38.8-46.6
Total bilirubin, mmol/l:				
Min-max	5.6-10.9	6.7-10.7	5.6-10.1	6.5-11.5
M±SD	7.9±1.54	8.6±1.02	8.4±1.11	9.1±1.43
Me	8.0	8.5	8.4	9.2
25 %-75 %	6.3-9.1	8.1-9.3	7.6-9.3	8.3-10.1
Direct bilirubin, mmol/l:				
Min-max	1.45-3.7	1.6-3.7	0.9-3.8	1.3-4.0
M±SD	2.6±0.60	2.6±0.55	2.4±0.90	2.7±0.79
Me	2.6	2.7	2.4	2.8
25 %-75 %	2.3-3.1	2.2-2.9	1.8-2.8	2.1-3.3
Creatinine, mmol/l:				
Min-max	117.7-180.7	156.4-225.7	109.3-180.4	126.3-223.2
M±SD	152.8±20.32	183.9±18.59	149.1±23.78	182.1±23.66
Me	155.1[c]	185.7[c]	142.95	181.2[c]
25 %-75 %	139.8-169.5	169.6-195.3	133.3-173.0	167.5-200.1
Urea, mmol/l:				
Min-max	1.3-3.7	4.8-7.4	1.0-4.0	4.2-7.3
M±SD	2.4±0.63	6.2±0.82	2.6±0.98	5.9±0.87
Me	2.2[c]	6.4[c]	2.2[c]	6.0[c]
25 %-75 %	2.0-2.8	5.5-7.0	1.9-3.6	5.3-6.7
Cholesterol, mmol/l:				
Min-max	0.5-0.8	0.2-0.9	0.3-0.7	0.2-0.8
M±SD	0.6±0.09	0.5±0.18	0.5±0.10	0.5±0.14
Me	0.6	0.5	0.5	0.5
25 %-75 %	0.5-0.7	0.4-0.7	0.4-0.6	0.4-0.6
Glucose, mmol/l:				
Min-max	4.2-7.6	3.6-7.2	4.9-7.7	3.3-7.9
M±SD	6.2±1.01	5.3±1.02	6.3±1.03	5.3±1.14
Me	6.3[b]	5.2[b]	5.9[b]	5.3[b]
25 %-75 %	5.7-7.2	4.5-6.0	5.5-7.5	4.8-6.0

[a, b, c] Dfferences between young animals and adults are statistically significant, at p = 0.000, p = 0.003, p = 0.03, respectively; [d] differences between females and males are statistically significant at p = 0.000.

It should be noted that biochemical blood parameters can be influenced by

various factors. The conducted single- and multifactorial analysis (ANOVA, MANOVA) made it possible to establish the influence of physiological factors (age, gender, weight). According to the results of MANOVA testing, a significant effect of age on the activity of AsAT ($p = 0.00$), AlAT ($p = 0.00$), alkaline phosphatase ($p = 0.00$), LDH ($p = 0.00$), alpha amylase ($p = 0.00$), direct bilirubin ($p = 0.01$), creatinine ($p = 0.00$), urea ($p = 0.00$) and glucose ($p = 0.00$). A statistically significant effect of gender on the amount of total protein was proven ($p = 0.00$).

The individuals we studied from different sex and age groups differed significantly in body weight. Using ANOVA, the dependence of the following biochemical parameters on body weight was established: activity of AsAT ($p = 0.00$), AlAT ($p = 0.00$), alkaline phosphatase ($p = 0.00$), LDH ($p = 0.00$), alpha-amylase ($p = 0.00$), total protein ($p = 0.00$), direct bilirubin ($p = 0.01$), creatinine ($p = 0.00$), urea ($p = 0.00$) and glucose ($p = 0.00$).

Due to the few publications of information on the biochemical parameters of the blood of moose and the lack of sex and age distinctions in them, we considered it possible to compare our data with those obtained for other species of the suborder *Ruminantia*. When compared with the results of foreign and domestic researchers, some differences were identified, but the trend for adult animals and young animals under 1 year of age was similar in most indicators.

AsAT and AlAT play an important role in amino acid metabolism, and their greatest activity is observed in skeletal muscle, myocardium and liver. Our studies established significant age-related differences in the content of AsAT, AlAT, alkaline phosphatase, and LDH.

M.K. Rostal et al. [25] who studied blood biochemical parameters in moose in Norway, described a trend in blood biochemistry similar to our results, but in their studies, the animals were not differentiated by sex. Thus, AsAT activity in young animals and adults averaged 130.0 U/l, which is lower than our values by 16.2 and 46.7%, respectively. The AlAT content in young animals and adults was also lower by 44.5% (33.0 U/l) and 36.3% (27.0 U/l). From the work of S.A. Becker et al. [21] who studied blood parameters in adult female Shirasi elk (*A. a. Shirasi* Nelson, 1914) in the northwestern state of Wyoming (USA), it is known that the AsAT content in adult females was lower than the values in our study by 57.5% (103.7 U/l). A.L. Miller et al. [27] studied the biochemical composition of the blood serum of free-ranging adult Norwegian wild reindeer (*Rangifer tarandus* Linnaeus, 1758) in southwestern Norway. Their results are also presented without taking into account the sex of the animals. The concentrations of AsAT and AlAT were lower than in our study by 60.7 (96.0 U/L) and 23.1% (34.0 U/L), respectively.

For the differential diagnosis of various pathologies, the de Ritis coefficient is of great importance. Having calculated the ratio of the activity of AsAT and AlAT enzymes, we found that it increased unidirectionally with the age of the animals. In young animals under 1 year of age, this indicator was at the upper limit of the norm for domestic cows (*Bos taurus taurus* Linnaeus, 1758) [28] (2.5 for females, 2.7 for males). In adult animals, it was 5.5 and 6.0, respectively, which can be explained by an increase in the metabolic load on skeletal muscles and myocardium when pursuing animals during hunting.

A.E. Weber et al. [29] found that in the blood serum of moose, the activity of the transamination enzymes AsAT and AlAT varies with the seasons. It was maximum in spring, minimum in autumn. The spring rise is due to increased intake and utilization of ammonia, as well as high activity of protein metabolism. In autumn, the intensity of transamination decreases significantly which is apparently due to the attenuation of synthetic processes in the body. In addition, according to M. Koseoglu et al. [30], hemolysis causes a significant increase in AsaT levels. Obviously, the parameters must be interpreted taking this factor into account.

The total activity of alkaline phosphatase in the circulating blood of healthy animals consists of the activity of liver and bone isoenzymes, which is greatest in growing animals [31]. Alkaline phosphatase is involved in the formation of the skeleton during ontogenetic development. Our data are consistent with these statements. The values in young and adult moose from Norway exceeded our values of alkaline phosphatase activity by 20.0%]25], and in adult females of Shiras elk]21] and Norwegian reindeer]27] by more than 70.0%. According to E.V. Gromyko]32], in cows the physiological norm for alkaline phosphatase levels is 55.0-80.0 U/l.

It should be noted that the development of the skeleton and musculoskeletal system is of utmost importance in the formation of the respiratory function of the blood in onto- and phylogenesis [33]. In newborn mammals, the entire red bone marrow is active, and it is in it that the hematopoiesis occurs, while in adult animals a certain part of it is replaced by yellow adipose bone marrow [34].

According to researchers studying the physiology of moose in the Pechora-Ilychsky Nature Reserve (Komi Republic), newborn moose calves grow very quickly. A comparison of the growth rate of elk calves and young cattle shows that the ratio of body weight gain to body weight in the 1st month of life in moose calves is 50.8 [35], in calves 31.3 [36]. According to A.E. Knorre [37], the relative increase in body weight of elk calves is 2 times higher than that of calves. That is, doubling body weight occurs faster in those species whose milk contains a higher concentration of protein and ash elements (calcium, phosphorus), necessary for the formation of the skeleton and muscles. Among ungulates, these are elk and reindeer [38]. Relative growth rate peaks at 3 months of age. In the fall, when switching to twig food, the growth of moose calves slows down sharply. The growth rate during the first half of life in elk calves reaches 1500%, and in cattle 400-407.0% [39]. High growth rates and active metabolism, the formation of physiological maturity of organs and systems of elk calves in the summer determine the success of their survival in winter, when nutrition is limited to twig-poor protein food, and serve as evidence of the adaptive plasticity of the elk body. In addition, the rapid development of the musculoskeletal system is important for more efficient respiratory function of the blood.

Lactate dehydrogenase is an enzyme that catalyzes the reversible conversion of lactate to pyruvate during glycolysis. High LDH activity is inherent in many tissues, primarily the liver, skeletal muscles, myocardium, as well as lung tissue, kidneys, pancreas and stomach. A.W. Franzmann et al. [22] showed that LDH content is significantly higher in young animals. The same trend was observed in our studies, as well as in other works [21, 25]. In addition, significant changes in LDH content were recorded in bovine blood during storage. Thus, this indicator in the blood serum of cattle increases after 24 hours of storage in the refrigerator [25]. Studies of human blood have shown a similar increase in the amount of LDH in refrigerated sera stored for 7 days [40]. In these studies, other biochemical blood parameters did not change significantly.

Alpha-amylase is a hydrolytic enzyme that breaks down complex carbohydrates into maltose and glucose. In our studies, alpha-amylase activity increased statistically significantly ($p < 0.05$) with the age of the animals. In adult moose, it turned out to be higher by 11.1% in females and 27.3% in males than in young animals, which indicates a more intense carbohydrate metabolism in adults compared to young animals. In the studies of M.K. Rostal et al. [25] such a trend was not identified; the concentration of alpha-amylase in young animals and adults did not have statistically significant differences with the indicators in adult moose from the Kirov region. The activity of alpha-amylase in Norwegian reindeer exceeded our values by 33.8% (45.0 U/l).

The correspondence of protein nutrition to the biological needs of the body

is assessed by the concentration of total protein and its fractions in the blood serum. The significantly higher content of total protein in the blood of adult males ($p < 0.05$) that we established was apparently associated with stimulation of its synthesis by the male steroid hormone testosterone. In addition, a high concentration of albumin in the blood indicates the activation of the processes of creating energy and plastic reserves of the body in the summer and autumn periods [41]. Differences in diet and sampling season may also have been reflected in the total protein values in our experiments (32.0% lower) compared to those reported by S.A. Becker et al. [21]. In the autumn and winter periods, the concentration of total protein in the blood serum decreases, which indicates a lower supply of nitrogen to the body when feeding on twig food than in summer [29]. In addition, the causes of hypoproteinonemia may be protein starvation, poor absorption of proteins from food, as well as the mobilization of proteins as energy sources [42]. In Norway moose [25] and reindeer [27], the concentration of total protein in young animals and adults was practically no different from our results. According to E.V. Gromyko [32], the physiological norm for total protein content in cows was 70.0-80.0 g/l, which is probably due to the inclusion of additional protein in the animals' diet through feed additives.

The pigment bilirubin is the end product of the breakdown of hemoglobin. The total bilirubin indicator includes the total content of direct and indirect bilirubin. Direct bilirubin is indirect bilirubin processed by the liver, which is subsequently excreted from the body of animals with bile. In our studies, the content of total and direct bilirubin did not have statistically significant differences and differed by more than 60.0% from the concentration of total bilirubin in the works of other researchers [25]. These differences may indicate the extent of hemoglobin breakdown processes in the animal body.

Creatinine is an end product of metabolism that diffuses into the bloodstream and is then freely filtered by the glomeruli of the kidneys. We noted changes in the amount of creatinine depending on the age of the animals. A decrease in creatinine levels was found in young animals because they had less muscle mass compared to older animals [43]. Creatinine concentrations in our studies were comparable to values obtained in moose [25] and reindeer [27] from Norway.

Urea is the main end product of protein metabolism, synthesized by the liver from amino acids in the Krebs cycle with the participation of enzyme systems. In young animals, due to increased protein synthesis, the amount of urea is slightly reduced compared to the norm for adults [44]. Differences in urea concentrations between adult animals and young animals may also be explained by differences in diet, since calves and adult animals eat different plants. Therefore, reducing protein intake may lead to lower urea levels [25]. Nutritional deficiencies and fasting are important factors affecting blood urea levels, particularly as reported by A.L. Miller et al. [27], low rates were observed in semi-domesticated reindeer under poor nutritional conditions.

Comparison with the results of other studies showed that the concentration of urea in the blood serum of adult moose from the Kirov region is 40% higher than that of moose from Norway [25], and 26.8% higher than that of reindeer [27]. In cows, the physiological norm for urea content has been established to be 3.0-5.6 mmol/l [32]. According to A.E. Weber et al. [29], a high urea content in the blood of moose indicates the intensity of protein metabolism; in contrast to domestic cows, in the fall the amount of urea in moose does not decrease, which is associated with increased rumen-hepatic circulation of nitrogen.

In general, it is believed that an increase in the amount of protein in the blood and a decrease in urea indicate an improvement in nitrogen metabolism. The deterioration of nitrogen metabolism is accompanied by an increase in urea content and a decrease in the concentration of total protein [29].

Cholesterol is an amphipathic lipid synthesized by all cells of the body, but the bulk of production occurs in liver cells and is excreted in bile. In all age and sex groups of moose we studied, we did not establish statistically significant differences in the intensity of lipid metabolism. M.K. Rostal et al. [25] came to the same conclusions, but in their studies, as in the work of A.L. Miller et al. [27], the concentration of cholesterol in the blood averaged 1.3 and 1.5 mmol/l, respectively.

Glucose is the leading diagnostic indicator of the state of carbohydrate metabolism. The concentration of glucose in the blood is a derivative of the activity of the processes of glycogenesis, glycogenolysis, gluconeogenesis and glycolysis. It should be noted that the glucose content in the blood serum of elk in our work was 2.5 times higher than that of domestic cows [32]. According to A.E. Weber et al. [29], the peculiarity of carbohydrate-energy metabolism in moose compared to domestic ruminants is the high concentration of glucose in the blood and the fact that this indicator varies from month to month. So, in May-June it is 4.6 mmol/l, in July it reaches 5.3 mmol/l, and by October, due to the lower intensity of metabolic processes, it becomes a third lower, 3.4 mmol/l. The high blood glucose levels in moose are probably due to the increased role of the glycolytic energy supply system of these animals. In winter, this is associated with a high energy requirement against the background of slow aerobic oxidation, and in summer, with biochemical adaptations of muscles to rapid transitions from rest to movement. As a result, carbohydrate metabolism in moose is characterized by a high intensity of gluconeogenesis not only due to nutritional glucogenic compounds, but also due to hormonal regulation.

It has also been established that the heat source during thermogenesis activated by thyroid hormones is the terminal energy-rich ATP bond. The cleavage of this bond is catalyzed by the Na^+K^+-ATPase system of membranes of calorigenic tissues - skeletal muscles, liver and kidneys. For example, after the administration of thyroid hormones, the activity of the ATPase system increases and, along with this, heat production in the body increases [2].

It is known that the concentration of glucose in the blood increases in response to sympathetic stimulation. The sympathetic nervous system constricts blood vessels and increases blood pressure, thereby diverting blood from organs whose functions in a stressful situation are not necessary for the survival of the body, and, on the contrary, increasing blood flow to the vital skeletal muscles necessary during stress. Also, the increased serum glucose values in our work and in adult female and male Norwegian wild reindeer [27] may be due to the hyperglycemic effects of catecholamines and glucocorticoids released when pursuing animals during a hunt. Other researchers have come to similar conclusions [45-47]. In addition, the high glucose content of moose from the Kirov region and reindeer from Norway, compared with domestic ruminants, facilitates survival in harsh winter conditions [19, 27, 48].

According to A.Yu. Kovtunenko [49], when domestic cows were exposed to negative temperatures of 20 °C, significant changes in biochemical blood parameters were noted. In particular, the glucose content increased by 36.2%, bilirubin by 76.7%, AlAT activity by 79.3%, AsAT by 221.0% compared to the control.

The harvest method of wild animals determines changes in serum biochemical parameters and should be taken into account when assessing or comparing serum composition between animals from different groups or between different studies [19]. In our case, the death of the animal from a gunshot wound occurred at lightning speed or the agonal period did not exceed several minutes. A number of authors [50-53] report that in this case, pulmonary and cerebral edema is absent or slightly expressed. Congestion of the capillary bed of the internal organs and hemorrhages in the tissue without reactive changes were noted. There are completely no signs of

disseminated intravascular coagulation and manifestations of respiratory distress syndrome. Signs of shock changes in hemodynamics are not recorded. The specialized closing arteries of the lungs and brain are spasmed. E.K. Perry et al. [54] indicate that with an increase in the rate of death, a significant decrease in the activity of glutamate decarboxylase, phosphofructokinase, and pH is observed. At the same time, the content of phenylalanine, lysine, leucine and tryptophan increases in brain tissue.

It should be noted that medical workers and expert tanologists mainly use clinical medicine data, which are adapted to forensic practice [55-57]. "Normal" values of laboratory parameters are determined in the process of clinical trials based on the results of measurements of the test analyte in a large population of healthy individuals or other biological objects, selected and grouped by age, sex, biological and other indicators. The obtained data lead to an average value, taking into account statistically possible standard deviations and obtaining a range of values in which the reference values are located. The reference interval gives an idea of the lower and upper limits of the norm of the indicator. We also recommend following this framework to evaluate your results.

Differences in serum biochemical parameters between physically and chemically captured animals have been documented in several species of wild ungulates, including red deer (*Cervus elaphus* Linnaeus, 1758) [58] and white-tailed deer (*Odocoileus virginianus* Zimmermann, 1780) [59]. According to M.K. Rostal et al. [25], when moose were immobilized using etorphine from a helicopter, the average AsAT concentration in the blood serum was 130.0 U/L in young and adult animals, and AlAT was 27.0 and 33.0 U/L, respectively. In the work of S.A. Becker et al. [21] adult female Shiras elk were immobilized using the drugs thiafentanil or carfentanil. At the same time, AsAT activity in their blood averaged 103.7 U/l. A.L. Miller et al. [27] who also used helicopter immobilization with a combination of medetomidine and ketamine to collect blood from adult female and male Norwegian reindeer, obtained an average AsAT value of 104.0 U/L.

I. Marco et al. [58] and M.J. Fettman et al. [16] reported significant increases in serum AlAT, total protein, albumin, sodium, and chloride in physically captured red deer compared with values in chemically immobilized animals. J.M. Arnemo et al. [45] also indicate that AsAT activity and glucose levels increase under stress and exercise, caused, as in our case, by hunting pursuit. A.L. Miller et al. [27] found that stress-induced reindeer had increased values of AAT, glucose, alkaline phosphatase, and urea.

Although we provide data on the serum biochemistry of moose living in the Kirov region, there are some limiting factors that should be taken into account when conducting comparative studies. These are primarily differences in blood collection methods, biochemical analyzers, laboratory diagnostic methods and animal habitats. Obviously, parameter ranges must be interpreted in light of the factors given.

Many issues have not received sufficient discussion in the presented article due to their poor study. Research on moose metabolism will be continued and expanded, and will also be compared with data on other ungulates. We believe that the biochemical parameters of blood can be analyzed in connection with environmental and anthropogenic factors (environment, diet, nutrition, lifestyle, physical activity, etc.).

Thus, it is now important to establish baseline values for significant blood biochemical parameters in wild animals, particularly moose. This will provide veterinarians and game biologists with reference data that can be used to assess population status, as markers of various pathologies and potential reproductive efficiency, and ultimately to assess habitat quality. In the blood serum of adult moose living in the Kirov region, we found high activity of aspartate aminotransferase (in females

and males 253.8±52.38 and 250.9±47.52 U/l, respectively), alpha-amylase (29.2±7.20 and 32.0±8.77 U/l), high levels of creatinine (183.9±18.59 and 182.1±23.66 mmol/l), urea (6.2±0.82 mmol/l and 5.9±0.87 mmol/l). In young individuals, indicators for AsAT (161.2±28.30 and 160.0±30.92 U/l), alpha-amylase (24.6±4.91 and 23.2±5.46 U/l), creatinine (152.8±20.32 and 149.1±23.78 mmol/l), urea (2.4±0.63 and 2.6±0.98 mmol/l) levels were significantly lower. In young animals, on the contrary, high activity of AlAT (60.8±6.42 and 58.7±6.74 U/l), alkaline phosphatase (230.4±40.79 and 222.2±31.14 U/l), lactate dehydrogenase (805.2±185.57 and 822.9±237.13 U/l), high glucose content (6.3±1.01 and 6.3±1.03 mmol/l) were noted. Adult animals were characterized by a decrease in the activity of alanine aminotransferase (43.6±7.35 and 41.9±6.33 U/l), alkaline phosphatase (69.3±12.62 and 69.9±11.31 U/l). l), LDH (614.1±98.11 U/l and 598.2±129.37 U/l), glucose content (5.3±1.02 and 5.3±1.14 mmol/l). Statistically significant differences in the content of total protein in blood serum were found between adult females (57.4±7.48 g/l) and males (68.1±4.93). A significant effect of the age of animals on the content of AsAT, AlAT, alkaline phosphatase, LDH, alpha-amylase, direct bilirubin, creatinine, urea and glucose was shown. A significant influence of the sex of animals on the content of total protein has been established. The dependence of the activity of AsAT, AlAT, alkaline phosphatase, LDH, alpha-amylase, total protein content, direct bilirubin, creatinine, urea and glucose on body weight was revealed. The biochemical values presented in the work have a similar trend for most indicators with the results obtained on other artiodactyls in the wild. The differences are due to the type of animal, living conditions, nutrition, age, gender, as well as the method used to collect blood. Further comparative studies, inclusion of other age categories, and studies of blood chemistry in more species will expand our knowledge of the biology of wild animals. The results obtained can be useful in monitoring the environment, assessing the influence of anthropogenic factors on the parameters of the blood system and the condition of the body as a whole.

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AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 2, pp. 302-312
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 639.111.16:591.2:616-093/-098

doi: 10.15389/agrobiology.2023.2.302eng
doi: 10.15389/agrobiology.2023.2.302rus

CIRCULATION FEATURES OF *Parafasciolopsis fasciolaemorpha* (Ejsmont, 1932) ON THE TERRITORY OF THE VYATKA PRIKAMIE UNDER WEATHER ANOMALIES

T.G. SHIKHOVA[1] ✉, O.V. MASLENNIKOVA[2], A.P. PANKRATOV[1]

[1]*Zhitkov Russian Research Institute of Game Management and Fur Farming*, 79, ul. Preobrazhenskaya, Kirov, 610020 Russia, e-mail biota.vniioz@mail.ru (✉ corresponding author), pankratov-a@bk.ru;
[2]*Vyatka State Agrotechnological University*, 133, Oktyabr'skiy Pr., Kirov, 610017 Russia, e-mail olgamaslen@yandex.ru
ORCID:
Shikhova T.G. orcid.org/0000-0002-4584-4675
Pankratov A.P. orcid.org/0009-0006-1271-8126
Maslennikova O.V. orcid.org/0000-0003-1859-4655
The authors declare no conflict of interests
Final revision received July 18, 2022
Accepted August 23, 2022

Abstract

The parasite fauna of ungulates is a permanent component of natural biocenoses. Species-specific and most pathogenic moose biohelminth *Parafasciolopsis fasciolaemorpha* Ejsmont, 1932 in the forest zone has epizootic significance, forms stable natural foci. The study of this trematode remains insufficient throughout the entire range of the species. There are few scientific publications on the influence of abiotic factors on the helminth life cycle. The presented work is based on monitoring data of parafasciolopsosis in the Kirov Region. There was statistically confirmed for the first time that moose infection depends on the amount of summer precipitation, and to a lesser extent depends on temperature. The purpose of our work is to establish the features of the incidence of parafasciolopsosis in moose with significant deviations in the temperature and humidity regime of summer seasons. The studies were carried out in the floodplain Cheptsa River (a large tributary of the Vyatka River) within the southern taiga zone of the east of the Russian Plain in 2009-2021. Liver samples of 189 moose were processed by helminthological autopsy. A quantitative account of sexually mature specimens *P. fasciolaemorpha* was carried out with subsequent extrapolation of data. The prevalence of invasion and intensity of invasion in moose were calculated. The infestation of the intermediate host, the freshwater snail *Planorbarius corneus* (L., 1758), was determined by the hepatopancreas compression method with counting the number of trematode larvae. To assess the meteorological conditions of the summer seasons, we used the average air temperature (°C) and the average amount of precipitation (mm) for June-August from open source data of Internet resources for the city of Kirov. Weather anomalies of summer seasons (average air temperature, average amount of precipitation) are expressed as a percentage of the average values for the reference period 1961-1990. With an increase in the abundance of the local population of moose (from 3.1 to 16.7 individuals/1000 ha of forest land), a consistent increase in the incidence of parafasciolopsosis is recorded (from 33.3 % to 76.5 %). A high positive non-linear relationship was established between the abundance of the local moose group and the prevalence of invasion ($r = 0.76$, $R^2 = 0.86$, $n = 9$, $p \leq 0.05$). During the study period, there were summer seasons with extreme temperature and humidity regimes: three abnormally rainy, four dry, four cool and five hot years. In dry years, the concentration of the invasiveness in floodplain water bodies increases significantly. An inverse non-linear dependence between the infestation of moose with the trematode *P. fasciolaemorpha* and anomalies in the amount of precipitation during the summer was revealed. The precipitation deficiency provokes an increase prevalence of invasion in the definitive host ($r = -0.60$, $R^2 = 0.89$, $n = 8$, $p \leq 0.05$). The temperature factor has a weak effect on the *Parafasciolopsis* infection of the moose: the correlation with the prevalence of invasion is medium positive ($r = 0.31$, $R^2 = 0.75$, $n = 8$, $p \leq 0.05$), with the intensity of invasion is medium negative ($r = -0.46$, $R^2 = 0.24$, $n = 8$, $p \leq 0.05$). A decrease in the average intensity of invasion was found together with a general high incidence of this trematodosis in moose due to the development of concomitant immunity. To normalize the situation in the parafasciolopsosis focus, it is advisable to selectively shoot weakened male moose during the rutting season. In floodplain lands, it is required to increase the shooting of calves, as the most infected age group, serving as a source of environmental pollution with parasite eggs. In dry seasons, a single application of anthelmintic preparations into licks is necessary.

Keywords: moose, parafasciolopsosis, prevalence of invasion, intensity of invasion, meteorological anomalies, Cheptsa River, Kirov region

In recent decades, weather and climatic conditions are characterized by record anomalies of meteorological parameters during growing seasons (droughts, excessive precipitation, etc.), which causes deviations in the development of plant and animal diseases, imbalance in the dynamic "parasite-host" system, and an increase in the frequency and intensity of parasitic diseases [1, 2].

Among representatives of the deer *Cervidae*, the elk *Alces alces* L., 1758 [3, 4] which has a species-specific parasite in its helminth fauna *Parafasciolopsis fasciolaemorpha* Ejsmont, 1932, is especially susceptible to trematode diseases. The dixenic development cycle of the trematode includes an intermediate host, the aquatic mollusk *Planorbarius corneus* (L., 1758).

The greatest pathogenicity of the disease is manifested in Central and Eastern Europe [5], but throughout the entire range of the species, *P. fasciolaemorpha* is relatively poorly studied. Research on this trematodosis is being conducted in Belarus [6, 7], Poland [8-10] and Latvia [11, 12]. It is known that elk are infected with *Parafasciolopsis* on the territory of Russia in some central regions of the European part (Moscow, Voronezh and other regions) and in the east of the Russian Plain (Kirov region) [13-15]. These works mainly provide data on the extensiveness of invasion (EI), less often on the intensity of invasion (II) and the abundance index (AI). Isolated cases of significant infestation of moose in Central Europe, leading to the death of the animals, have been described, and the histological features of moose livers affected by *Parafasciolopsis* have been described [5, 12, 16].

Modern molecular genetic methods in the study of helminthiases in moose based on the study of fecal samples provide assessment of *P. fasciolaemorpha* prevalence during the growing season and identification of the infection degree in the definitive host depending on sex (the extent and intensity of invasion was higher in males) [10, 17].

Analysis of factors influencing the spread of parafasciolopsosis is mainly limited to assessing changes in the density of moose groups and the abundance of the intermediate host, the composition and quality of natural biocenoses [18]. An increase in the infestation of the main and intermediate host, as well as the fecundity of maritas during the growing season due to seasonal changes in abiotic factors is discussed [10, 19]. Low temperatures in the winter months have a suppressive effect on the fertility of trematodes, reducing it to minimum values [19).

The influence of weather conditions on the circulation of *P. fasciolaemorpha* in natural ecosystems was first characterized by A.S. Rykovsky [20] who established a high probability of an outbreak of parafasciolopsosis in dry seasons due to the movement of moose to water bodies and the accumulation of mollusks in shallow water. Using the example of the central regions of the European part of Russia, there is an increase in the incidence of parafasciolopsosis in moose in dry years by 10-20% [13, 21]. However, in these works there are no statically significant correlation data on the dependence of the infestation of the definitive host on meteorological factors. In the floodplain of the river Cheptsa (Vyatka River basin) a stable focus of parafasciolopsosis is recorded where *P. fasciolaemorpha* represents the core of the elk helminthocenosis, dominating in frequency of occurrence and abundance [4, 18]. Regular observations in the river basin. We have been conducting Vyatka since 2009, but the information received earlier was fragmentary and was not systematic in terms of spatiotemporal parameters.

In the presented work, based on monitoring data on the epizootic situation regarding parafasciolopsosis caused by *P. fasciolaemorpha*, in the Kirov region, for the first time, the high dependence of elk infestation (intensity and extensiveness

of invasion) on the amount of precipitation in the warm season and, to a lesser extent, on temperature factor.

The purpose of our work is to reveal the characteristics of the incidence of parafasciolopsosis in moose with significant deviations in the temperature and humidity regime of the summer seasons.

Materials and methods. The research was carried out in 2009-2021 in the central part of the Kirov Proince on the territory of the scientific and experimental hunting farm of the Professor Zhitkova All-Russian Research Institute of Hunting and Fur Farming (EHF VNIIOZ) with an area of more than 66 thousand hectares, where floodplain lands accounted for 6.5%, forest lands for 65%. The model site was located in the lower reaches of the river Cheptsa (the largest left tributary of the Vyatka River) and covered a wide (up to 4 km) floodplain of the river with a system of oxbow lakes, shallow permanent and temporary reservoirs. The density of the local moose population within the territory under consideration was determined by the method of winter route census of individuals per 1000 hectares of forest land (individual/1000 hectares).

The helminthological dissection method [22] was used to process 189 liver samples from moose killed in the autumn-winter period. A quantitative census of *P. fasciolaemorpha* was carried out, followed by extrapolation of the data. We calculated the extent of invasion (EI, %), the proportion of infected moose in the sample, the intensity of invasion (II, min-max, specimen/individual) is the arithmetic mean number of trematodes in infested individuals, indicating the range of variation in the sample.

The infestation of the mollusks *Planorbarius corneus* was determined by the hepatopancreas compression method with counting the number of trematode larvae [23]. The abundance of mollusks is presented as the arithmetic mean (*M*) with a confidence interval (standard deviation, ±SD).

To assess the meteorological conditions of the summer seasons, an average air temperature (°C) and average amount of precipitation (mm) for June-August, we used data from open access Internet resources [24] for the city of Kirov (60 km west of EHF VNIIOZ). Anomalies of meteorological indicators were expressed as a percentage of the average values for the reference period 1961-1990 (climate norm).

Correlation and regression analysis of the obtained data was carried out using statistical software packages Microsoft Excel and Statistica 12 (StatSoft, Inc., USA). The sample mean and standard deviation of the mean (±SD) for the number of years of observation (*n*) were calculated. The Pearson correlation index (*r*) was used. To assess the quality of linear and polynomial regression models, the coefficient of determination (R^2) was used. The reliability of the obtained data was assessed at the level of statistical significance $p \leq 0.05$.

Results. Modern climatic trends with an increased frequency of extreme weather anomalies are making adjustments to the circulation of trematodes in natural biocenoses. The possibility of a surge in parafasciolopsosis during dry seasons has been considered previously [13, 20] and is confirmed by our studies [3, 25]. This work focuses on the circulation features of the trematode *P. fasciolaemorpha* not only depending on the amount of precipitation, but also on the temperature factor during the alternation of extremely hot and abnormally cold summer seasons.

During the period 2009-2021, there were three abnormally rainy and four dry summer seasons (Fig. 1), as well as four cool (2009, 2015, 2017, 2019) and five hot years (2010, 2011, 2013, 2016, 2021). Four growing seasons were characterized by extreme temperature and humidity regimes in different combinations: a very hot, dry summer of 2010, a cold, very rainy summer of 2017, a cold summer

without precipitation deficit in 2019, and a hot, precipitation-deficient summer in 2021. Dry summer seasons recurred every 1-2 years, while the norm for the Kirov region was once every 3-4 years [26].

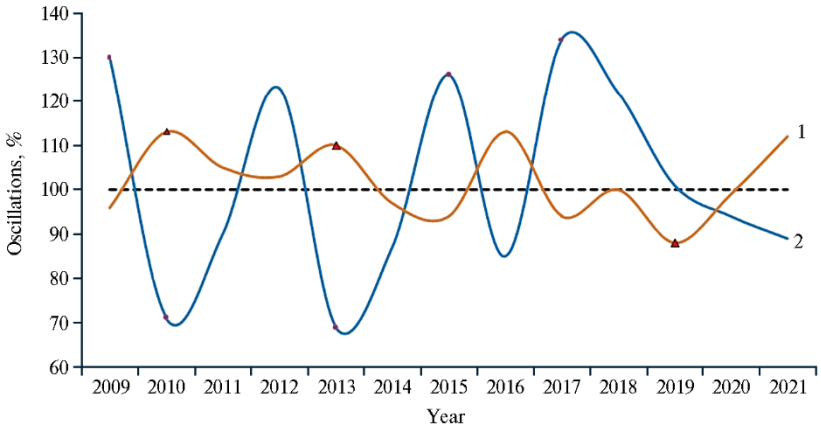

FIg. 1. Anomalies of average air temperature (1) and amount of precipitation (2) for June-August in different years in the central part of the Kirov region (Kirov). The dotted line indicates the norm of the indicator (100%).

An increase in the frequency of weather anomalies with significant changes in temperature and humidity conditions [26] has a significant impact on the quantitative and qualitative indicators of biota development [27], in particular on the behavioral reactions of elk during hot periods [28]. Since 2011, with the relative stabilization of the moose population density in the territory of the EHF VNIIOZ, it has become possible to analyze the cause-and-effect relationships between meteorological parameters and fluctuations in the parafasciolopsis infestation of the definitive host in a short time interval.

In 1996-2000, in the territory of the EHF VNIIOZ, the situation with parafasciolopsosis was favorable with a low density of elk of 3.1±1.1 individuals/1000 hectares of forest land (n = 5). The average EI of *P. fasciolaemorpha* was 16% with an average EI of 1038 (134-2087) per individual.

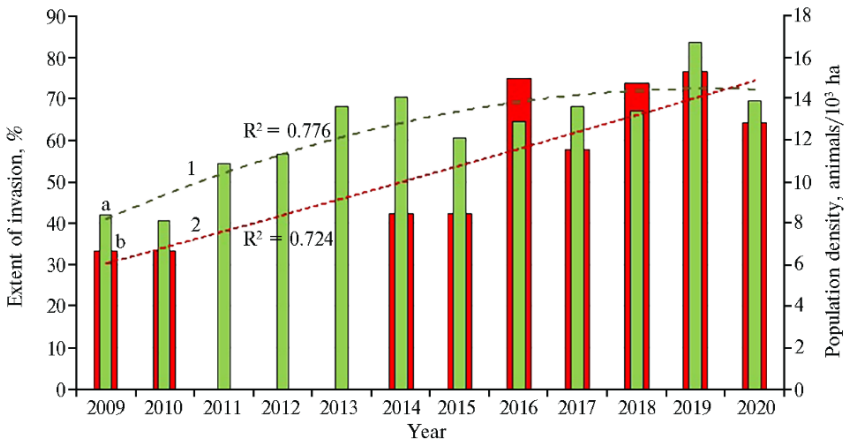

Fig. 2. Absolute values (a) **and polynomial function** (1) **of the local population density of the elk *Alces alces* L., 1758, as well as absolute values** (b) **and polynomial function** (2) **of the intensity of invasion of *Parafasciolopsis fasciolaemorpha* Ejsmont, 1932 over years (NOOH Zhitkov All-Russian Research Institute of Hunting and Fur Farming, Kirov Province).**

After the depression of the 1990s, the number of moose in the territory under consideration steadily increased (by 2014 the increase was 286%), and

subsequently stabilized with small multidirectional deviations under the influence of a number of biotic and abiotic factors [3]. The density of the local moose population has increased 5-fold since the end of the 20th century, reaching 16.7 individuals/1000 hectares in 2019. According to a number of researchers [13, 21], different natural zones with certain landscape, hydrological and climatic features are characterized by different values of the optimal density of elk, the excess of which provokes an increase in the incidence of parasitic diseases. In the conditions of the southern taiga of the European Russia, with an increase in the density of the moose group to more than 9-10 individuals/1000 ha, the risk of an outbreak of parafasciolopsosis significantly increases [13]. This is confirmed by the results of our studies [3, 15]. From 2011 to 2020, in the territory of EHF VNIIOZ, a density of elk grouping formed above the critical one, the 13.3±1.6 (10.1-16.7) individuals/1000 ha (n = 10) (Fig. 2).

With an increase in the abundance of the definitive host, there was a consistent increase in the incidence of parafasciolopsosis in animals. A high positive relationship was established between moose population density and the extent of parafasciolops infestation, $r = 0.76$ ($n = 9$, $p \leq 0.05$) (Fig. 3).

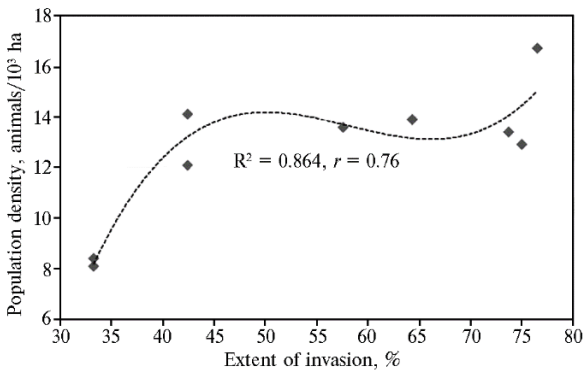

Fig. 3. Correlation field of the abundance of the local population of moose *Alces alces* L., 1758 and the extent of invasion of *Parafasciolopsis fasciolaemorpha* Ejsmont, 1932 (2009-2021, NOOO Zhitkov All-Russian Research Institute of Hunting and Fur Farming, Kirov Province).

The average EI of *P. fasciolaemorpha* for 2009-2020 was 55.39±17.9%, gradually increasing from 33.3% (2009-2010) to 76.5% (2019). The average II for this period is 4305±2694 (17-48984) per individual.

A similar trend is observed in Poland and Latvia, where an increase in the infestation of moose with *Parafasciolopsis* was noted [4, 29]. In Latvia, the infection rate of moose with *P. fasciolaemorpha* ranges from 24 to 42% [12, 29]. In Poland, the incidence reaches 70-100%, and a case of very high intensity of invasion was recorded (more than 10 thousand specimens/individual), which led to irreversible histological damage to the liver and death of the elk [5].

The circulation of trematodes in natural ecosystems is influenced by a complex of abiotic factors (temperature, humidity, lighting, etc.), the synergistic effect of which is ambiguous and in different proportions can stimulate or suppress the development of helminths [30, 31]. In this work, we analyzed the influence of temperature and humidity anomalies in the summer seasons on the circulation of parafasciolopsosis in the southern taiga forests of the Middle Volga region.

On the territory of the Vyatka Prikamye region, during the dry summer seasons of 2010, 2013, 2014, 2016, precipitation fell 15-31% less than the climatic norm, and in the rainy seasons of 2015, 2017, 2018 - 22-40% more. For example, in 2010, there was an abnormally hot, dry summer. In 2013, there was a dry summer with short periods of heat. In the rainy year of 2015, there was a summer flood on all rivers of the Kirov region, and on the river. In Chepetsa, the water rise in August reached 1.4 m. In the cold and rainy summer of 2017, the amount of precipitation exceeded the norm by 35-40%.

The temperature factor influences the behavior of the definitive host of

the marite *P. fasciolaemorpha*. Moose are sensitive to heat stress; on hot days they take refuge under the canopy of trees, and during the cool twilight hours they increase their activity and movement. With further climate warming, changes in the fertility and survival rate of moose, as well as modifications in their behavioral reactions in response to unfavorable weather conditions are possible [28]. Our research has shown that during periods of drought and elevated temperatures, moose more often visit large bodies of water to quench their thirst, cool off, escape from midges and in search of wetland plant food, including the poisonous plant *Menyanthes trifoliata* L. (1753), which promotes circulation bile (moose do not have a gallbladder), pain relief from bites, wound healing. It is in such large bodies of water that the intermediate host of *P. fasciolaemorpha*, the *Planorbarius corneus*, lives. The shallowing of lakes contributes to the accumulation of mollusks in shallow water in the zone of macrophyte thickets. Therefore, in dry years, the concentration of invasive pathogens in the coastal strip of large oxbow lakes increases significantly.

The influence of abnormal seasons in terms of precipitation affected the infection rate of elk after 1-2 years [4]. Thus, the peak intensity of *P. fasciolaemorpha* invasion, the 8610 (19-48984) per individual was recorded in the hunting season of 2014-2015, after the dry years of 2010 and 2013. After the abnormally rainy and cool summer of 2015, during the 2016-2017 hunting season, a minimum intensity of invasion was observed, the 915 (17-3490) per individual, with a decrease in the overall infestation of elk to 57.6%. The cold summer and rainy autumn of 2019 affected the quantitative indicators of parafasciolops infection of elk during the 2019-2020 hunting season. Compared to the previous season, there was a decrease in the occurrence of infected moose to 64.3% with an invasion intensity of up to 3.5 thousand individuals/individual.

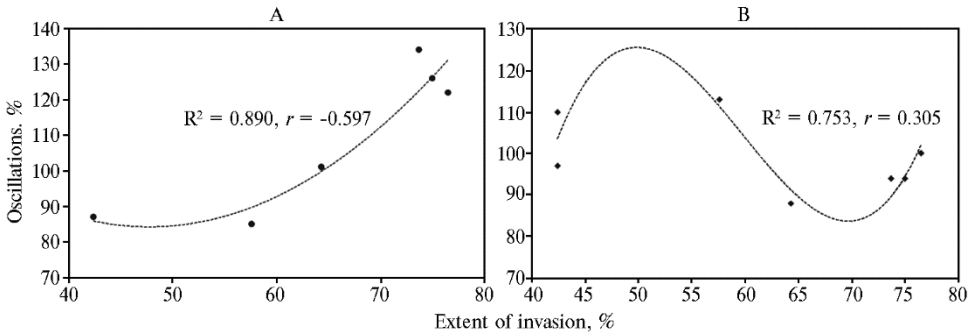

Fig. 4. Correlation fields of the extent of parafasciolopsis infestation of elk *Alces alces* L., 1758 with meteorological anomalies of the summer seasons: A — precipitation, B — average air temperature (2009-2021, NOOO Zhitkov All-Russian Research Institute of Hunting and Fur Farming, Kirov Province).

Based on the results of a conjugate analysis of fluctuations in elk infestation with Parafasciolopsis under the influence of significant deviations from the norm in summer precipitation, an inverse nonlinear relationship was revealed. Deficiency of precipitation provoked an increase in the extent of invasion ($r = 0.60$, $n = 8$, $p \leq 0.05$) (Fig. 4, A). The infection of elk with *Parafasciolopsis* showed an average nonlinear dependence on the temperature factor (see Fig. 4, B). The correlation between temperature anomalies in the summer season and the extent of invasion was averagely positive ($r = 0.31$, $n = 8$, $p \leq 0.05$), with the intensity of invasion - average negative ($r = -0.45$, $n = 8$, $p \leq 0.05$). The weak degree of correlation with the temperature factor is probably explained by the ecological feature of the intermediate host, which inhabits large standing or low-flowing water bodies, where the temperature is not subject to significant fluctuations and the

development of parthenitis occurs in relatively stable interannual conditions. This is consistent with the opinion of UK researchers [32] that temperature within the optimal range does not have a significant effect on the rate of cercariae development in mollusc hosts. However, American scientists [33] have identified changes in the parasite-host system with climate warming: the larval stages of the parasite develop faster in mollusks at elevated temperatures, but there are certain limitations: at temperatures above 30 °C in mid-latitudes, the development of parthenite parasites in mollusks slows down [32].

The fertility of marites *P. fasciolaemorph*a also naturally changes throughout the year depending on the ambient temperature: it is higher in the warm months compared to the winter period. The number of eggs released by the parasite increases during the growing season [10, 19].

Among the many environmental factors that cause changes in parasitic infestations, climatic variables have the greatest influence. Trematodes that have larval stages, living freely in the environment or parasitizing invertebrates (*Arthropoda* and *Mollusca*), are more susceptible to the influence of climatic factors than those helminths in whose life cycle there are no such phases. Abrupt climate changes can impact helminth (fluke) populations and even lead to outbreaks of parasitic zoonoses [1, 34].

Transmission of parasites occurs under conditions of many fluctuating environmental factors. The stage of searching for a host is especially vulnerable when trematode cercariae are exposed to direct external influences of the aquatic environment. According to experimental data (30), cercariae of the littoral trematode Maritrema novaezealandensis died faster at higher temperatures, increased salinity, and when exposed to ultraviolet radiation. Cercariae of *P. fasciolaemorpha* encyst within a few hours after leaving the mollusk and transform into adolescaria, which are more resistant to unfavorable abiotic factors.

The stable circulation of parafasciolopsosis infestation in the territory we are considering was probably caused by the increased population density of the definitive host, which causes the accumulation of invasive material in water bodies, and a combination of abnormal weather conditions - the frequent recurrence of dry seasons: over 12 years of research, 6 years were noted with a deficit of precipitation, of which 4 - abnormally dry. The infection of shellfish by *P. corneus* with parthenites (redia, cercariae) during the study period remained high (EI up to 71%). The average population density of the intermediate host in oxbow lakes was 4.6±3.3 indibiduals/m^2. In years with a deficit of precipitation, the concentration of horny coil in the coastal zone of water areas increased to 15 indibiduals/m^2, and in rainy seasons it decreased to 3 indibiduals/m^2.

The species-specific elk parasite *P. fasciolaemorpha* in the southern taiga forests of the Middle Volga region is a permanent component of the biocenosis, which serves as one of the mechanisms of its ecological balance. Impaired balance can cause an outbreak of parafasciolopsosis. In the context of current climate trends with increasing weather anomalies, it is necessary to take measures to prevent a possible outbreak of this zoonosis. In farms with excessively high densities of elk in limited areas, selective shooting of weakened males during the rutting period, as well as animals with morphological abnormalities and developmental delays, is required. When developing moose quotas, it is advisable to shoot mainly in floodplain lands and mainly young of the year as they are the most infected and serve as a source of environmental pollution with parasite eggs [19, 25, 35].

The organization of anthelmintic activities is especially necessary during dry seasons. According to the recommendations of Belarusian scientists [36], the veterinary drug Triclamizole at a dose of 75 mg/kg can be used once with the laying out of salt licks, the effectiveness of which in moose parafasciolopsosis is

95-100%. To constantly attract moose, it is advisable to locate salt licks near small watercourses and at more than 1.5 km distance from deep oxbow lakes suitable for intermediate host habitat.

Thus, the ability of adult moose to neutralize the impact of *Parafasciolopsis fasciolaemorpha* Ejsmont, 1932 parasites through the development of concomitant immunity, which is maintained through constant contact with the invasive principle, has been established; its interruption even for a short time leads to loss of immune status. This explains the decrease in the intensity of invasion against the background of the overall high extent of invasion by parafasciolopsis. On the territory of the Kirov region, a statistically confirmed high direct dependence of the extensiveness of parafasciolopsis infestation of moose on population density and a high inverse dependence on the amount of precipitation that fell during the summer were revealed. The temperature has a weak effect on the infection of elk with *Parafasciolopsis*.

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AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 2, pp. 313-332
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Genetics and genomics

UDC 636.52/.58:591.1:579.2:577.2

doi: 10.15389/agrobiology.2023.2.313eng
doi: 10.15389/agrobiology.2023.2.313rus

EXPRESSION OF GENES OF IMMUNE RESPONSE AND ADAPTATION AND CECAL MICROBIOME COMPOSITION IN MALES AND FEMALES OF CHICKENS (*Gallus gallus* L.) IN CM5 AND CM9 PREPARENTAL LINES OF SMENA 9 CROSS

G.Yu. LAPTEV[1], E.A. YILDIRIM[1, 2] ✉, L.A. ILYINA[1, 2], V.A. FILIPPOVA[1, 2], K.A. KALITKINA[1], E.S. PONOMAREVA[1], A.V. DUBROVIN[1], D.G. TYURINA[1], V.I. FISININ[3], I.A. EGOROV[3], T.A. EGOROVA[3], V.A. MANUKYAN[3], T.N. LENKOVA[3]

[1]*JSC Biotrof+,* 19, korp. 1, Zagrebskii bulv., St. Petersburg, 192284 Russia, e-mail laptev@biotrof.ru, kseniya.k.a.@biotrof.ru, dubrovin@biotrof.ru, tiurina@biotrof.ru;
[2]*Saint Petersburg State Agrarian University,* 2, lit. A, Peterburgskoye sh., St. Petersburg, 196601 Russia, e-mail deniz@biotrof.ru (✉ corresponding author), ilina@biotrof.ru;
[3]*Federal Scientific Center All-Russian Research and Technological Institute of Poultry RAS,* 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail fisinin@vnitip.ru, olga@vnitip.ru, eta164@yandex.ru, vard13@yandex.ru, dissovet@vnitip.ru

Laptev G.Yu. orcid.org/0000-0002-8795-6659
Yildirim E.A. orcid.org/0000-0002-5846-4844
Ilyina L.A. orcid.org/0000-0003-2490-6942
Filippova V.A. orcid.org/0000-0001-8789-9837
Kalitkina K.A. orcid.org/0000-0002-9541-6839
Ponomareva E.S. orcid.org/0000-0002-4336-8273
Dubrovin A.V. orcid.org/0000-0001-8424-4114
Tyurina D.G. orcid.org/0000-0001-9001-2432
Fisinin V.I. orcid.org/0000-0003-0081-6336
Egorov I.A. orcid.org/0000-0001-9122-9553
Egorova T.A. orcid.org/0000-0002-5102-2248
Manukyan V.A. orcid.org/0000-0003-4564-4427
Lenkova T.N. orcid.org/0000-0001-8026-3983

The authors declare no conflict of interests
Acknowledgements:
Supported financially by the Russian Science Foundation, grant No 22-66-00061 "Expression of productivity and resistibility related genes in chickens of new Russian broiler cross Smena-9 and its effects on immunity and realization of genetic productivity potential with different energy and amino acid nutrition"
Final revision received November 21, 2022
Accepted July 18, 2022

Abstract

The Cornish and Plymouth Rock breeds form the basis of modern specialized meat crosses of chickens. The selection of the paternal line of the Cornish CM5 breed of the new Russian cross of meat chickens Smena 9 is carried out mainly on the basis of meat productivity, while the maternal line of the Plymouth Rock CM9 breed is primarily for reproductive efficiency and viability at a lower live growth rate than that of the CM5 line masses. In the present study, we revealed for the first time that hens and roosters of the parent stock of lines CM5 and CM9 of the novel cross Smena 9 differ in the expression of some immunity and adaptation genes, as well as in the composition of the microbiome and its putative metabolic pathways. Differences are related to genotype and sex. The aim of the work was to compare the level of expression of immunity genes and genes associated with adaptive potential, respectively, in the tissues of the bursa in the liver, as well as the composition and functions of the microbiome of the caecum of the intestine in chickens and roosters of the CM5 and CM9 lines. The experiments were carried out in the vivarium of the Zagorsk EPH (Moscow Province, 2022) on the parent stock of chickens and roosters of the CM5 and CM9 lines of 39 weeks of age, kept under identical conditions and receiving the same diet. From each line and gender, tissue samples were taken from five individuals with a close live weight. Analysis of gene expression in tissue samples was performed using quantitative reverse transcription PCR (RT-qPCR). Total RNA was isolated using the Aurum™ Total RNA mini kit (Bio-Rad, USA). PCR amplification was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA) and a detecting amplifier DTlight (DNA-Technology, Russia). In the liver tissues, we analyzed the expression of genes associated with the adaptive potential: genes *CAT1* of the transporter of cationic amino acids 1, *HSF1* and *HSF2* — transcription factors of heat shock proteins 1 and 2, *SOD* — superoxide dismutase, *Gpx1* — glutathione peroxidase, *HO-1* — heme oxygenase-1. In the tissues of the bursa, the expression of genes associated with immunity was analyzed: genes *IL8* — interleukin-8, *IRF7* — regulatory factor interferon 7,

PTGS2 — prostaglandin endoperoxide synthase, *AvBD1*, *AvBD2, AvBD9* and *AvBD10* — β-defensins 1, 2, 9 and 10, *Casp6* — caspase 6. A primer for the β-actin gene (ACTB) was used as a reference control. The relative level of expression was assessed by the $2^{-\Delta\Delta CT}$ method. Total DNA for analysis of the composition of the microbiome was isolated using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc., USA). The caecal bacterial community was assessed by NGS sequencing on the MiSeq platform (Illumina, Inc., USA) with primers for the V3-V4 region of the 16S rRNA gene. The reconstruction and prediction of the functional content of the metagenome, gene families, and enzymes was carried out using the PICRUSt2 software package (v. 2.3.0). Mathematical and statistical processing of the results was carried out by the method of multivariate analysis of variance (ANOVA) in Microsoft Excel XP/2003 and R-Studio (v. 1.1.453). The results obtained showed an increase in the expression of the *HSF1* and *HSF2* genes in CM5 cocks compared to other groups ($p \leq 0.05$), in particular, the difference with CM9 cocks was 68 and 218 %, respectively ($p \leq 0.05$). The expression of the *HSF1* and *HSF2* genes within the CM5 line in roosters was 1.6 and 3.0 times higher, respectively, than in hens ($p \leq 0.05$). Significant activation of the expression of antimicrobial peptides and pro-inflammatory genes occurred in CM9 cocks compared to CM5 cocks and hens ($p \leq 0.05$). The expression of *AvBD2, AvBD9, AvBD10, IL8* and *PTGS2* genes in CM9 cocks increased 7.6-, 5.3-, 2.1-, 6.3- and 1.5-fold ($p \leq 0.05$), respectively, compared to CM5 cocks. NGS sequencing showed that the microbiome of the caecum of the CM9 hens and roosters contained bacteria of the superphylum *Elusimicrobiota* (0.32±0.11 and 0.49±0.19 %, respectively). These microorganisms did not occur in CM5 roosters while in the SM5 hens, their proportion was 0.04±0.01 %. Significant ($p \leq 0.05$) differences were found between the groups in 25 genera, in some genera, it depends on the genotype, in others — on the sex of the bird. For example, in cocks of the CM5 line, the abundance of microorganisms of the genera *Barnesiella*, Clostridia_UCG-014 and *Frisingicoccus* was 17.2, 2.0 and 4.9 times higher ($p \leq 0.05$), respectively, than in males of the CM9 line. Members of the genus *Desulfovibrio* were present in the intestines of CM5 and CM9 cocks (0.25±0.08 and 0.73±5.6 %). However, we did not find these microorganisms in the intestines of hens of both lines. Based on the results of bioinformatics reconstruction and functional annotation of NGS sequencing data, we identified 357 putative metabolic pathways in the gut microbial community, 65 of which differed ($p \leq 0.05$) between test groups. Genotype- and sex-specific modifications in gene expression, as well as in the structure and function of the gut microbiome, may provide adaptation of a macroorganism under changing conditions.

Keywords: broiler cross, Smena 9, caecum, microbiome, NGS sequencing, predicted metabolic pathways, gene expression, immunity, adaptations, bursa, liver

Test results [1] showed that poultry of the new domestic cross Smena 9 can be successfully used in broiler production. The Cornish and Plymouth Rock breeds form the basis of modern specialized meat crosses of chickens [2]. To date, little is known about the characteristics of the lines SM5 and SM9 of the Smena 9 cross; there are only data relating mainly to zootechnical parameters. The main selection characteristics of the paternal line Cornish CM5 are live weight, muscularity of the chest and legs [3]. However, because of a significant increase in live weight and changes in the exterior of Cornish birds, a decrease in the fertilization of eggs and hatching of chickens is observed. The low fertility of hatching eggs from chickens of selective grandparent and parent flocks is the main reason for the decrease in the yield of broilers from each parent pair. On the contrary, the main selection characteristics of the maternal line of Plymouth Rock SM9 in comparison with the paternal line of Cornish SM5 are superiority in egg production, egg weight, chick yield, timing of puberty, viability at a lower rate of increase in live weight of young animals and worse feed conversion [4]. Let us recall that the Plymouth Rock breed was originally created for dual use (meat and egg production) [5].

However, it is known that different chicken phenotypes are determined by complex traits that are controlled by many genes. Gene expression studies in poultry have shown that some zootechnical parameters, including productivity, may be associated with various cellular mechanisms, including mitochondrial oxidative stress, inflammatory response, protein degradation, stress responses, growth hormone signaling, cell cycle and apoptosis, fatty acid transport [6]. The bursa is a central and unique organ of humoral immunity in birds, in which the expression

of many genes occurs, primarily those associated with immunity [7], as well as a target organ for a number of pathogenic microorganisms. Liver tissue plays a central role in the adaptive response to stresses [8] to which birds are often exposed in intensive poultry farming environments. It has been proven that the liver expresses many genes that help the body deal with changes in environmental conditions, for example, temperature, and oxygen content in the air [9].

The search for candidate genes in chicken breeding often focuses on selection for growth rate [10] and conformation [11], while resistance to disease and stress has received much less attention. Thanks to the functions of the immune and digestive systems (in particular, thanks to the expression of genes associated with immunity and the formation of adaptive potential occurring in the bursa and liver), the bird's genotype interacts with the environment, which ensures resistance to diseases and changes in conditions feeding, etc. Ultimately, this affects productivity and reproduction rates. A number of studies have determined the functional activity of genes in the organs and tissues of broilers of various genotypes [6], but the expression of genes associated with immunity and adaptation in the tissues of the bursa and liver of the lines of the new cross Smena 9 has not been studied previously.

There are also indications that the host genetic background may also influence the variability of some species in the gut microbiome [12]. The chicken gut microbiome includes a huge taxonomic diversity of species as well as the functional potential of their genomes and is known to have a significant impact on the health and productivity of animals and birds [13, 14]. There are also works demonstrating differences in the composition of the microbiome and its predicted functions in animals depending on gender [15]. However, an analysis of changes in the predicted functional potential of the microbiome of animals and birds depending on the genotype using bioinformatics software systems, such as PICRUSt2 and the like, has not previously been carried out.

In previous studies, we studied gene expression in the tissues of the pancreas and intestinal epithelium in broilers of the Smena 8 cross with experimental T-2 toxicosis [16], using T-RFLP (terminal restriction fragment length polymorphism) we analyzed the composition of the intestinal microbiota in two lines of meat chickens B5 (Cornish breed) and B9 (Plymouth Rock breed) [17]. Using NGS sequencing and bioinformatics methods, we studied the composition of the microbiome and its predicted metabolic potential in Smena 8 broilers exposed to T-2 toxin and the use of feed additives [18]. In chickens of different lines of the new Smena 9 cross, the composition and predicted functions of the intestinal microbiota and gene expression have not been previously studied.

As in mammals, in birds males are characterized by a higher growth rate than females. In part, sex differences in growth rate may be due to differences between sexes in the microbiome of the digestive system, since its composition has a significant effect on the digestion, absorption and metabolism of nutrients in the host, and is also closely related to its immune system and health. A number of researchers have observed a pronounced manifestation of sexual dimorphism in the composition of microbiomes in animals [19], as well as in the pattern of gene expression [20], which suggests conducting similar studies in birds.

Finding connections between a host's genetic background, its microbiome composition, and gene expression levels may help uncover new biological mechanisms associated with high productivity and reproductive function, as well as contribute to the development of more efficient and therefore sustainable poultry production systems [21].

In this study, we for the first time identified in hens and roosters of the parent stock, the lines CM5 and CM9 of the new cross Smena 9 differences

associated with genotype and sex in the expression of some immunity genes and genes associated with adaptive potential, as well as in the composition and pro-predicted functional potential of the microbiome.

The purpose of our study was to assess differences in the expression levels of a set of immunity genes in the bursa and genes associated with adaptive potential in the liver, as well as the composition and functions of the microbiome in the caeca of the intestine in hens and roosters of the parent flock of different cross lines Smena 9, namely the paternal line CM5 of the Cornish breed and the maternal line CM9 of the Plymouth Rock breed, which have genetic differences in growth rate, feed efficiency and reproductive function.

Materials and methods. Experiments were carried out in the vivarium of the SGC Zagorskoe EPH (Moscow Province, 2022) on two lines of parent stock of chickens (*Gallus gallus* L.) of the domestic cross Smena 9 selection of the SGC Smena (Moscow Province): on the paternal line CM5 of the Cornish breed and the maternal line CM9 of the Plymouth Rock breed. Experimental conditions complied with the requirements of the European Convention for the Protection of Vertebrate Animals Used for Experimental or Other Scientific Purposes (ETS No. 123, Strasbourg, 1986) [22]. Feeding and maintenance regimes met the requirements for cross-breeding [23] and were identical for all experimental birds. At the age of 30 weeks, the birds were assignrd to 4 groups, each of 5 birds with a similar live weight. Group I was hens of the paternal line CM5 of the Cornish breed, group II was roosters of the paternal line of the CM5 of the Cornish breed, group III was hens of the maternal line of the CM9 of Plymouth Rock breed, group IV was roosters of the maternal line of the CM9 of Plymouth Rock breed. The bird was decapitated at 39 weeks of age and necropsied.

After decapitation, bursa and liver tissues were collected for gene expression analysis. The samples were immediately stabilized using the RNAlater reagent (Thermo Fisher Scientific, Inc., USA) and immediately sent to the laboratory (BIOTROF+ LLC) for RNA isolation.

Gene expression analysis was performed using quantitative reverse transcription PCR (RT-qPCR). Tissue samples were homogenized after adding liquid nitrogen. Total RNA was isolated using the Aurum™ Total RNA Mini Kit (Bio-Rad, USA) following the manufacturer's instructions. The reverse transcription reaction to obtain cDNA on an RNA template was carried out using the iScript™ Reverse Transcription Supermix kit (Bio-Rad, USA) [24].

To analyze the expression of mRNA in the liver and bursa, specific primers were selected [25]. As a reference control, primers were used to amplify the housekeeping gene encoding the ACTB beta-actin protein: F - 5′-CTGTGCCCATCTATGAAGGCTA-3′, R - 5′-ATTTCTCTCTCGGCTGTGGTG-3′. Amplification was carried out using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA) in accordance with the manufacturer's protocol [26] (DTlight detection amplifier, NPO DNA-Technology, Russia). The amplification mode and conditions for the analysis of liver and bursa tissues were as follows: 5 min at 95 °C (pre-denaturation); 30 s at 95 °C, 30 s at 60 °C, 30 s at 70 °C (40 cycles) [27]. The relative expression level was assessed using the $2^{-\Delta\Delta CT}$ method [28].

To analyze the composition of the microbiome, at the end of the experiment, chyme samples were manually taken from the cecum of the intestines of three birds from each group, observing aseptic conditions as much as possible. The collected samples were immediately placed into sterile plastic centrifuge tubes. All samples were frozen at –20 °C and transported in dry ice to the laboratory (BIOTROF+ LLC) for DNA extraction.

Total DNA for analysis of the composition of the intestinal microbiome was isolated using the Genomic DNA Purification Kit (Thermo Fisher Scientific,

Inc., USA) according to the attached instructions using a method based on selective detergent-mediated precipitation of DNA from the substrate using solutions of 1.2 M sodium chloride and chloroform to lyse cell walls and precipitate DNA.

The cecum bacterial community composition was assessed using next generation sequencing (NGS) on the MiSeq platform (Illumina, Inc., USA) with primers for V3-V4 region of the 16S rRNA gene. The forward primer was 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGGNGGCWGCAG-3′, the reverse primer 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′; the PCR protocol 3 min at 95 °C; 30 s at 95°C, 30 s at 55°C, 30 s at 72 °C (necessary to lengthen the sequence) (25 cycles); 5 min at 72 °C (final elongation). Sequencing was carried out with reagents for library preparation Nextera® XT IndexKit (Illumina, Inc., USA), for purification of PCR products Agencourt AMPure XP (Beckman Coulter, Inc., USA) and for sequencing MiSeq® ReagentKit v.2 (500 cycle) (Illumina, Inc., USA) were used. The maximum length of the obtained sequences was 2×250 bp.

Bioinformatics data analysis was performed using QIIME2 v.2020.8 software (https://docs.qiime2.org/2020.8/). After importing the sequences in .fastq format from the sequencing instrument and creating the necessary mapping files containing metadata of the files being studied, paired strings of reads were aligned. Next, the sequences were filtered by quality using default settings. Filtering of noise sequences was carried out using the DADA2 method built into the QIIME2 package, which includes quality information in its error model, providing the algorithm robust to sequences of lower quality. A maximum trimming sequence length was 250 bp (https://benjjneb.github.io/dada2/tutorial.html). To construct a de novo phylogeny, multiple sequence alignment was performed using the MAFFT software package (https://mafft.cbrc.jp/alignment/software/), followed by masked sequence alignment to remove positions that were significantly different. The Silva 138.1 reference database (https://www.arb-silva.de/documentation/release-138.1/) was applied for taxonomy analysis.

Based on the resulting table of operational taxonomic units (OTUs), using plugins of the QIIME2 software package, biodiversity indices were calculated, and a graph of the dependence of the OTUs number on the number of reads was constructed. When statistically analyzing diversity indices, no additional transformation was performed.

Reconstruction and prediction of the functional content of the metagenome, gene families, and enzymes was carried out (the PICRUSt2 v.2.3.0 software package, https://github.com/picrust/picrust2). We worked with the program according to the recommended action scenario, all settings were used by default. The OTUs of each sample were ranked according to their abundance, from highest to lowest, and the values were transformed using the Log2 logarithmic transformation. MetaCyc database (https://metacyc.org/) was used to analyze metabolic pathways and enzymes. Predicted MetaCyc metabolic pathway profiles were assessed by ASV (Amplicon Sequence Variants) abundance. Data and calculation of statistical indicators were visualized using the Phantasus v.1.11.0 web application (https://artyomovlab.wustl.edu/phantasus/), which, in addition to basic visualization and filtering methods, supports methods based on R such as k-means clustering, principal component analysis, or differential expression analysis with the limma package.

Mathematical and statistical data processing was carried out using the method of multivariate analysis of variance (ANOVA) in Microsoft Excel XP/2003, R-Studio v.1.1.453 (https://rstudio.com). The results are presented as means (*M*) and standard errors of the means (±SEM). The significance of differences was determined using

Student's *t*-test; differences were considered statistically significant at $p \leq 0.05$. Means were compared using Tukey's honestly significant difference (HSD) test and the TukeyHSD function in the R Stats Package.

Results. The primers used to analyze the expression of the studied genes are presented in Table 1.

1. Primers used to study gene expression in chickens and roosters (*Gallus gallus* L.) of CM5 and CM9 lines of the new meat cross Smena 9 (vivarium of the SGC Zagorskoe EPH, Moscow Province, 2022)

Gene, its product	Nucleotide sequence (5′→3′)
Genes associated with adaptive potential (in the liver)	
CAT1, cationic amino acid transporter 1	F: ACCAAGTACTGCAAGGCGAA, R: TGAGGGTTCCTCTTCTGGCT
HSF1, heat shock protein transcription factor 1	F: CAGGGAAGCAGTTGGTTCACTACACG, R: CCTTGGGTTTGGGTTGCTCAGTC
HSF2, heat shock protein transcription factor 2	F: CGCTGCTCGCATTCCT, R: TGTGGCCTCACTTGCTTCT
SOD, superoxide dismutase	F: CGGGCCAGTAAAGGTTACTGGAA, R: TGTTGTCTCCAAATTCATGCACATG
Gpx1, glutathione peroxidase 1	F: GCATCCGCTTCCACGACTTCCT, R: CCGCTCATCCGGGTCCAACAT
HO-1, heme oxygenase 1	F: GGTCCCGAATGAATGCCCTTG, R: ACCGTTCTCCTGGCTCTTGG
Immunity-related genes (in bursa of Fabricius)	
IL8, interleukin 8	F: GGAAGAGAGGTGTGCTTGGA, R: TAACATGAGGCACCGATGTG
IRF7, interferon regulatory factor 7	F: ATCCCTTGGAAGCACAACGCC, R: CTGAGGCAACCGCGTAGACCTT
PTGS2, prostaglandin endoperoxide synthase	F: TCGAGATCACACTTGATTGACA, R: TTTGTGCCTTGTGGGTCAG
AvBD1, β-defensin 1	F: CCGTTTCTGTCACCGTCA, R: CCTTTGCTAAAAATCCCTTC
AvBD2, β-defensin 2	F: GCACTCCAGGTTTCTCCA, R: GGCGTCCGACTTTGATTA
AvBD9 (*Gal9*), β-defensin 9	F: AACACCGTCAGGCATCTTCACA, R: CGTCTTCTTGGCTGTAAGCTGGA
AvBD10 (*Gal10*), β-defensin 10	F: GCTCTTCGCTGTTCTCCTCT, R: CCAGAGATGGTGAAGGTG
Casp6, caspase 6	F: CAGAGGAGACAAGTGCCAGA, R: CCAGGAGCCGTTTACAGTTT

Figure 1 shows expression of genes associated with adaptive potential in the liver tissues of hens and roosters of the CM5 and CM9 lines of the Smena 9 cross. Noteworthy is the increase in the expression of the *HSF1* and *HSF2* genes in the paternal Cornish CM5 roosters (group II) compared to groups I, III and IV ($p \leq 0.05$). The difference in the expression level of the *HSF1* and *HSF2* genes with the maternal Plymouth Rock CM9 roosters (IV group) was 68 and 218%, respectively ($p \leq 0.05$). The *HSF1* gene, an important paralogue of which is *HSF2*, encodes a stress-inducible transcription factor and plays a central role in the activation of the heat shock response, which leads to the expression of a large class of molecular chaperones, heat shock proteins (HSPs), that protect cells from damage [29]. Previously, a team of scientists [30] analyzed the expression of the *HSF1*, *HSF3*, *HSP70* and *HSP90* genes in two local Brazilian chicken breeds (Peloco and Caneluda) and a commercial broiler line Cobb 500 in response to heat stress (39±1 °C). It was found out that the expression levels of some heat shock genes (*HSP70* and *HSP90*) during heat stress varied significantly between breeds. Increased gene expression was detected in local breeds compared to Cobb 500 cross, which was associated with behavioral responses and productivity under heat stress.

The main selection characteristics of the Cornish CM5 paternal line are live weight, muscularity of the chest and legs [3]. It is known that in most cases, phenotypic differences between individuals are caused by genetic changes, and

these genetic differences are closely related to gene expression and function [31]. As in other species, body weight in chickens is a polygenic trait and can be influenced by variants at many loci. In addition, in poultry, especially broilers, body weight is subject to direct selection [32]. In mice, body weight has been reported to be a quantitative trait driven by regulatory variation at the *Glypican 3* locus [33] the effect of which is expressed through changes in gene expression in liver tissue. By analogy, genetic variants that affect body mass in birds may indirectly affect, for example, resistance to heat stress. In addition, the liver, in the tissues of which we observed changes in the expression of the *HSF1* and *HSF2* genes, is a key organ involved in the metabolism of carbohydrates, proteins and fats; it produces and breaks down hormones. These facts indicate that changes in the expression of the *HSF1* and *HSF2* genes may be associated with different levels of productivity in the studied lines. Interestingly, these genes also play a role in the regulation of lifespan [34] and, accordingly, may be valuable as a marker associated with productive longevity in poultry.

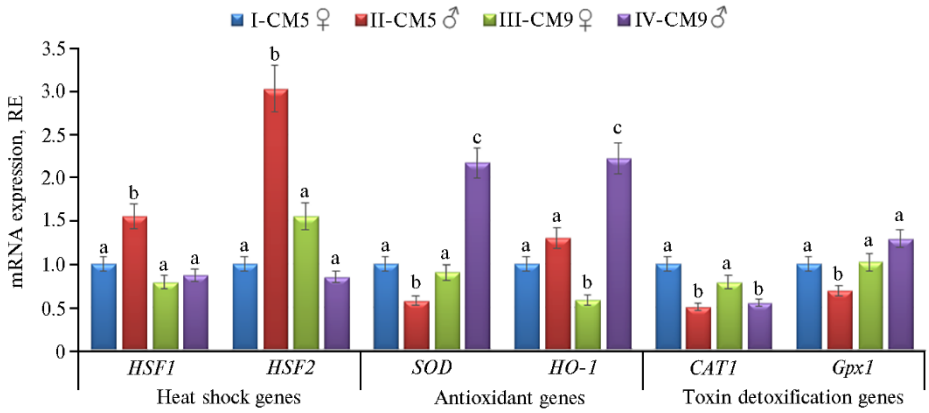

Fig. 1. Expression of genes associated with adaptive potential in the liver of chickens and roosters (*Gallus gallus* L.) of the cross Smena 9 CM5 and CM9 lines (vivarium of the SGC Zagorskoe EPH, Moscow Province, 2022). All groups were kept under identical conditions and received identical diets (data for 39 wk of age). RE is the fold change in the expression level compared to that in group I taken as 1; a-c — differences between values without a common letter designation are statistically significant at $p \leq 0.05$. Results are presented as the mean with standard error of the mean ($M \pm$SEM) for mRNA expression.

In addition, an increase in the expression of the *SOD* and *HO-1* genes was observed (see Fig. 1) in maternal Plymouth Rock CM9 roosters from group IV of the *SOD* and *HO-1* genes with paternal Cornish CM5 roosters (group II) was 160 and 92%, respectively ($p \leq 0.05$). The *SOD* and *HO-1* genes belong to the antioxidant enzyme genes. The superoxide dismutase (SOD) catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide and thus plays a critical role in the antioxidant defense of virtually all cells that are somehow in contact with oxygen [35]. The main function of HO-1 is to catabolize heme to produce biliverdin, free iron, and carbon monoxide. Under stress conditions, the activity of heme oxygenase-1 can increase more than 10 times. Heme oxygenase and heme degradation products exhibit pronounced cytoprotective properties [36]. As noted above, the main selective characteristics of the line of the maternal form CM9 (Plymouth Rock), in contrast to the paternal line CM5 (Cornish), are egg production, egg weight, chick yield, timing of puberty, and viability [4]). Among the various nutrients in the mother's diet that can significantly influence embryonic development and chick viability in the early post-hatching period, natural antioxidants are hypothesized to be critical. The accumulation of endogenous antioxidants in the egg and embryonic tissues appears to serve as a major adaptive mechanism

for protection against oxidative stress experienced at hatching, with SOD as a key element of the antioxidant network playing a major role here [37]. In our opinion, this adaptive mechanism may be related to different levels of expression of the antioxidant genes *SOD* and *HO-1*.

It is worth noting that differences between the sexes occurred in the level of gene expression in the liver of birds within the studied lines. Thus, the expression of the *HSF1* and *HSF2* genes in the CM5 line was 1.6 and 3.0 times higher, respectively, in roosters compared to hens ($p \leq 0.05$). In the CM9 line, the expression of the *SOD* and *HO-1* genes in roosters was higher ($p \leq 0.05$) than in hens. Therefore, in poultry liver, the expression profile of genes associated with adaptive potential varies depending on sex. Given the role of the liver in energy balance and the difference between males and females in body size and physiology, the findings seem logical. Wide sexual dimorphism in gene expression in the liver has previously been demonstrated in mice [20]. It has been shown [38] that approximately 72% of the genes functionally active in mouse liver are sexually dimorphic in expression.

Data from the analysis of the expression of genes associated with immunity in the bursa of chickens and roosters of different lines of the Smena 9 cross are shown in Figure 2. There was a significant activation of the expression of genes for antimicrobial peptides and pro-inflammatory genes in roosters of the CM9 line (group IV) compared to roosters and hens of the CM5 line (groups I and II, $p \leq 0.05$). Thus, compared to group II, the expression of the *AvBD2*, *AvBD9*, *AvBD10*, *IL8* and *PTGS2* genes increased 7.6, 5.3, 2.1, 6.3 and 1.5 times ($p \leq 0.05$), respectively. Expression of the *AvBD2* and *PTGS2* genes was also increased in hens of the CM9 line compared to hens of the CM5 line ($p \leq 0.05$). These data are in accordance with the results presented above of increased expression of the *HO-1* gene in CM9 line roosters compared to CM5 line roosters.

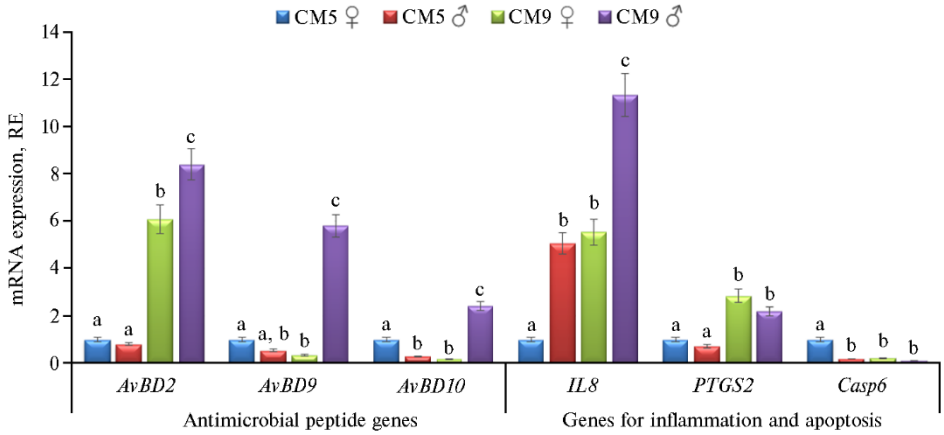

Fig. 2. Expression of genes associated with immunity in the bursa of chickens and roosters (*Gallus gallus* L.) of the cross Smena 9 lines CM5 and CM9 (vivarium of the SGC Zagorskoe EPH, Moscow Province, 2022). All groups were kept under identical conditions and received identical diets (data for 39 wk of age). RE is the fold change in the expression level compared to that in group I taken as 1; a-c — differences between values without a common letter designation are statistically significant at $p \leq 0.05$. Results are presented as the mean with standard error of the mean ($M \pm$SEM) for mRNA expression.

It is known that induction of HO-1 by cells provides a reduced intensity of oxidative processes and also stimulates the production of anti-inflammatory cytokines [36]. AvBD2, AvBD9, and AvBD10 are defensins that have antimicrobial activity against a variety of pathogens, including gram-negative and gram-positive bacteria, viruses, and fungi [39]. In birds, 14 β-defensins have been identified, from

AvBD1 to AvBD14 [40]. Antimicrobial activity of AvBD2, AvBD3, AvBD4, AvBD6, AvBD7, AvBD11 and AvBD13 proteins against *Escherichia coli* has been reported [41]. Proinflammatory cytokines, such as interleukins, play important roles in immunomodulation and inflammation [42]. IL-8 is a chemokine that recruits leukocytes [43]. The activation of proinflammatory cytokines is closely related to the expression of the *PTGS2* gene, since cytokines are able to induce transcription of this gene [44]. The *PTGS2* gene is associated with the synthesis of prostaglandin endoperoxide synthase (cyclooxygenase 2), which catalyzes the oxidative conversion of arachidonic acid to prostaglandin. Prostaglandin is subsequently metabolized to various biologically active metabolites, prostacyclin and thromboxane A2, participating in both local and systemic inflammatory responses [45]. A higher level of expression of immunity genes in bursa tissues in the CM9 line, in contrast to the CM5 line, may be associated with improved reproductive qualities. Defense against infection and reproduction are key traits throughout an individual's life, and selection must therefore ensure optimal regulation of both processes [46]. The consequences of damage from infection with pathogens caused by immunopathologies can lead to deterioration in reproductive qualities. That is, the reproductive health of birds depends on an immune response that can prevent the development of the disease by blocking the penetration of pathogens. However, C.H. Chao and Y.P. Lee [47], in contrast, showed that in Taiwanese chickens, high plasma β-globulin levels were genetically associated with low fertility.

Changes in host gene expression, which are directly related to host metabolism, can influence both the composition of the gut microbiome and the predicted functional profile of microbial communities, with a possible counter-effect (influence of microbiota on expression) [48]. Given the identified differences in gene expression in the liver and bursa of poultry, we further studied the composition and function of the microbiota in hens and roosters of the CM5 and CM9 lines.

In our study, NGS sequencing of the microbiome of the intestinal cecum in birds of the CM5 and CM9 lines generated a total of 109,690 sequenced 16S rRNA gene sequences with a median (*Me*) of reads of 9,601 (min = 4013; max = 13541) (Fig. 3).

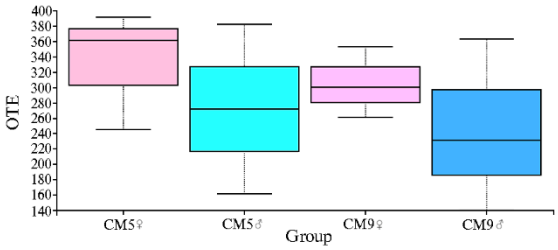

Fig. 3. The number of operational taxonomic units (OTUs) based on NGS sequencing of the intestinal microbiome of chickens and roosters (*Gallus gallus* L.) of cross Smena 9 lines CM5 and CM9 (the vivarium of the SGC Zagorskoe EPH", Moscow region, 2022). All groups were kept under identical conditions and received identical diets (data for 39 wk of age).

When comparing the Chao1, Shannon and Simpson biodiversity indices (Fig. 4), we were unable to identify significant differences between the groups.

The intestinal microbiome of birds from all groups contained 15 bacterial phyla and superphyla (Fig. 5). Of these taxons, *Bacteroidota*, *Bacillota* and *Verrucomicrobiota* dominated, with the phylum *Bacillota* being the most numerous (from 48.7±8.5 to 54.0±13.0%). The dominance of the phylum *Bacillota* bacteria in the poultry gut microbiome was previously reported by other researchers [49, 50].

At the phylum level, the main difference between the CM9 line and the CM5 line in terms of microbiome composition was the presence of bacteria of the superphylum *Elusimicrobiota* in the cecum of the intestines of hens and roosters (groups III and IV) (0.32±0.11 and 0.49±0.19%, respectively; $p \leq 0.05$). These microorganisms were not found in the intestines of roosters of the CM5 line (group

II), while in the intestines of hens of the CM5 line (group I), their content averaged 0.04±0.01%. It is possible that bacteria from the superphylum *Elusimicrobiota* may be associated with a phenotype of improved egg production and reproductive performance. Representatives of this phylum are known to be permanent inhabitants of the intestines of the beetle Pachnoda ephippiata, which feeds mainly on humus, and its intestinal chyme contains high concentrations of glucose, peptides and amino acids [51]. Members of the superphylum *Elusimicrobiota* are thought to promote better digestion in *P. ephippiata* [52].

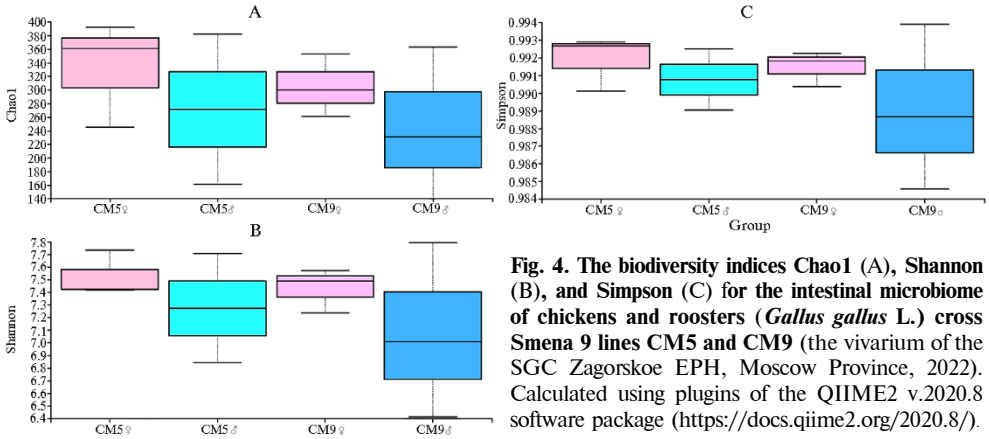

Fig. 4. The biodiversity indices Chao1 (A)**, Shannon** (B)**, and Simpson** (C) **for the intestinal microbiome of chickens and roosters (*Gallus gallus* L.) cross Smena 9 lines CM5 and CM9** (the vivarium of the SGC Zagorskoe EPH, Moscow Province, 2022). Calculated using plugins of the QIIME2 v.2020.8 software package (https://docs.qiime2.org/2020.8/).

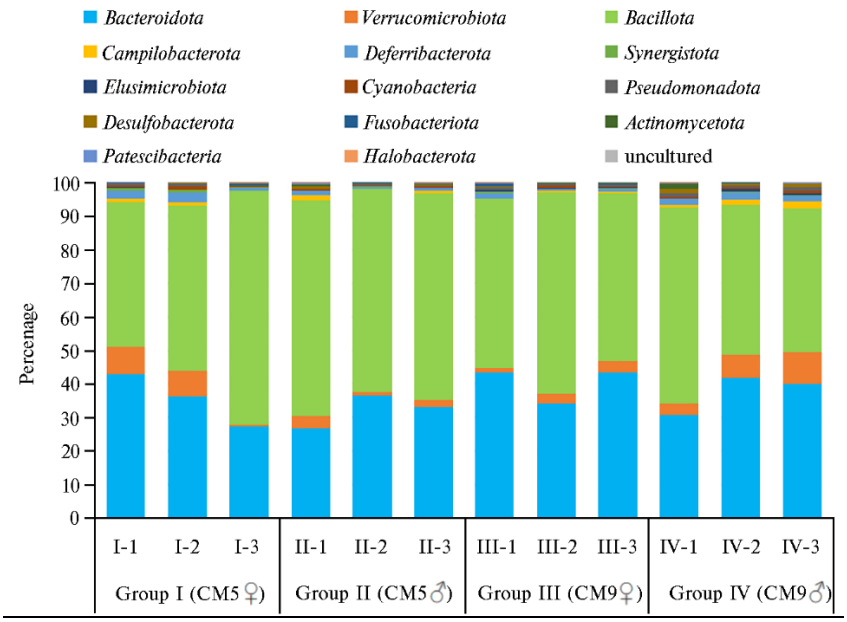

Fig. 5. The intestinal microbiome composition in chickens and roosters (*Gallus gallus* L.) of the Smena 9 cross CM5 and CM9 lines at the level of bacterial phyla (based on NGS sequencing of 16S rRNA gene amplicons; the vivarium of the SGC Zagorskoye EPH, Moscow Provine, 2022).

At the level of bacterial genera, differences between groups ($p \leq 0.05$) were found in 25 genera (Fig. 6), in some of which the representation varied depending on the genotype of the bird, and in some on its sex. Thus, in roosters of the CM5 line (group II) compared to roosters of the CM9 line (group IV), in the cecum, the abundance of the genera *Barnesiella* (superphylum *Bacteroidota*), Clostridia_UCG-014 and *Frisingicoccus* (phylum *Bacillota*) was higher ($p \leq 0.05$) 17.2-fold, 2.0-fold and 4.9-fold, respectively. In addition, in hens and roosters of the

CM5 line (groups I and II) compared to hens and roosters of the CM9 line (groups III and IV), the abundance of the genus *Colidextribacter* (phylum *Bacillota*) in the intestine was 4.7 and 7.5 times higher ($p \leq 0.05$).

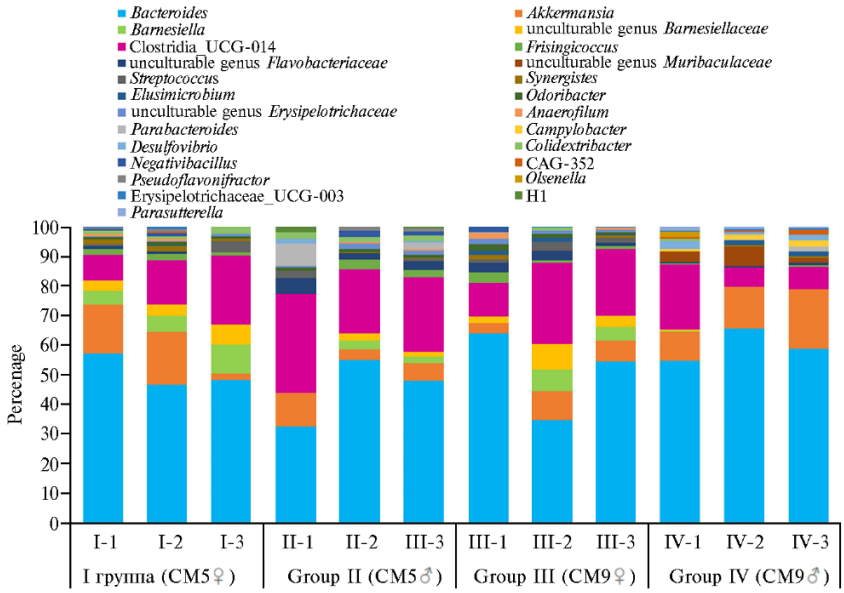

Fig. 6. The intestinal microbiome composition in chickens and roosters (*Gallus gallus* L.) of the Smena 9 cross CM5 and CM9 lines at the level of bacterial genera (based on NGS sequencing of 16S rRNA gene amplicons; the vivarium of the SGC Zagorskoye EPH, Moscow Provine, 2022).

Representatives of the genus *Pseudoflavonifractor* (phylum *Bacillota*) were completely absent in the intestines of hens and roosters of the CM9 line (groups III and IV), while in the chyme of hens and roosters of the CM5 line (groups I and II) these microorganisms accounted for 0.22±0.09 and 0.26±0.05%, respectively. It is likely that representatives of these genera most of which belong to the phylum *Bacillota* may be associated with the phenotype of higher meat productivity observed in the CM5 line compared to the CM9 line. The fact is that an important function of *Bacillota* is the ability to decompose complex polysaccharides with the subsequent formation of short-chain fatty acids [53] for the host's energy metabolism (and, as a result, in the formation of productivity). These substrates promote the growth and normal functioning of intestinal cells which is important for nutrient absorption [54]. Representatives of *Barnesiella* spp. superphylum *Bacteroidota* are involved in regulation of the intestinal microbiota composition, limiting the proliferation of oxygen-tolerant pathogens, in particular those carrying genes for multiple antibiotic resistance, which can affect the health and productivity of the host [55]. Previously, using the example of two lines of chickens, it was shown that a representative of the phylum *Bacillota*, the genus *Lactobacillus*, plays a key role in increasing live weight [12]. In our opinion, the host genotype can affect the structure of the microbial community with the help of genotypically determined factors (these include the composition of the secretion of the glands of the intestinal mucosa, features of peristalsis, modification of the surface of epithelial cells), and as was shown in our study, through changes in gene expression.

Interestingly, at the genus level, there were also differences between sexes ($p \leq 0.05$) in the composition of the intestinal microbiota. Thus, members of the genus *Desulfovibrio* (superphylum *Desulfovibrionia*) were present in the intestines of roosters of both the CM5 and CM9 lines (groups II and IV) in the amount of 0.25 ± 0.08 and 0.73±5.6%. At the same time, these microorganisms were not found in the intestines of chickens of both lines (groups I and III). The abundance

of representatives of the genera *Barnesiella* (superphylum *Bacteroidia*) and *Synergistes* (superphylum *Synergistia*), on the contrary, decreased ($p \leq 0.05$) in roosters of the lines CM5 and CM9 (groups II and IV) compared to chickens (groups I and III). Previously, it was also shown in chickens [56] that the composition of the cecal microbiota differed depending on sex: in cockerels, an enrichment of the intestinal microbiota with representatives of *Bacteroidetes* was noted, and in chickens, an increase in the number of clostridia and Shigella was noted. When trying to identify the reasons for such changes in mice, it turned out that a decrease in testosterone levels during castration of animals during puberty eliminated sex differences in the composition of the intestinal microbiota in adults. This indicates the importance of pubertal testosterone in the formation of sexually dimorphic microbial communities that persist in males into adulthood [57]. However, the mechanism by which testosterone influences the composition of such communities is currently not understood.

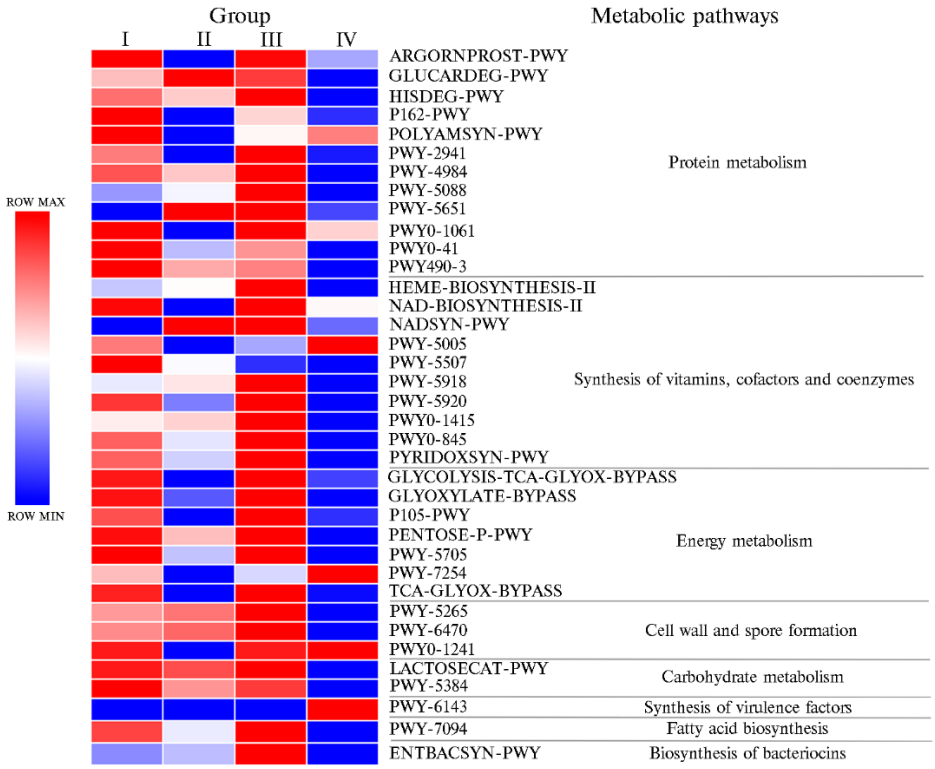

FIg. 7. Functional annotation of predicted metabolic pathways in the cecum microbiome of chickens and roosters (*Gallus gallus* L.) of the cross Smena 9 lines CM5 and CM9 (based on NGS sequencing of 16S rRNA gene amplicons; the vivarium of the SGC Zagorskoye EPH, Moscow Province, 2022). The data were obtained using the PICRUSt2 v.2.3.0 software package (https://github.com/picrust/picrust2). To analyze metabolic pathways and enzymes, the MetaCyc database (https://metacyc.org/) was used. The scale reflects the intensity of potential metabolic pathways of the microbiome: blue color is the lowest (minimum) intensity, red is the highest (maximum). For a description of the groups, see the Materials and methods section.

Based on the results of bioinformatics reconstruction and functional annotation of NGS sequencing data in the intestinal microbial community of the studied birds, we found 357 predicted metabolic pathways, 65 of which showed differences ($p \leq 0.05$) between experimental groups (Fig. 7). These pathways related to protein metabolism (biosynthesis and breakdown of amino acids, transformation of nitrogenous compounds), carbohydrate metabolism (biosynthesis and degradation of sugars), energy metabolism (Krebs cycle, glycolysis, glyoxylate

cycle), to the synthesis of fatty acids, vitamins, cofactors and coenzymes (biotin, adenosylcobalamin, pyridoxal phosphate, heme), to the formation of a cell wall and sporulation (synthesis of peptidoglycan, ADP-L-glycero-beta-D-manno-heptose), to the synthesis of virulence factors (pseudamic acid), bacteriocins. The majority of predicted metabolic pathways that differed between experimental groups ($p \leq 0.05$) were associated with protein and energy metabolism, as well as the synthesis of vitamins, cofactors and coenzymes. The most significant ($p \leq 0.05$) quantitative differences between lines CM5 and CM9 in predicted metabolic pathways concerned the formation of cell walls and bacterial spores (up to 4.9-fold when comparing groups II and IV), protein metabolism (up to 3.4-fold) multiples for groups II and IV), synthesis of adenosylcobalamin (3.2 times when comparing groups I and III). It is likely that the increased activity of these metabolic pathways in the CM5 line may be associated with high meat productivity. This seems natural, since the main component in poultry feed is protein, the hydrolysis products of which are absorbed from the intestines into the blood and used by the body for plastic purposes. Due to the intensification of protein metabolism, additionally synthesized proteins can be directed to the construction of new tissues in a growing organism. The stimulating effect of adenosylcobalamin on poultry meat productivity has also long been established [58].

Within the CM9 line, we identified significant differences ($p \leq 0.05$) in a number of predicted metabolic pathways between chickens (group III) and roosters (group IV). The activity of the putative biological functions of the microbial community, affecting the metabolism of proteins, carbohydrates, energy metabolism, biosynthesis of fatty acids, bacteriocins, vitamins, cofactors and coenzymes, the formation of cell walls and bacterial spores, was higher in chickens (group III) than in roosters (group IV) (differences up to 5.9-fold). Moreover, in roosters of the CM9 line, compared with chickens (group IV vs. group III), the synthesis pathway of CMP-pseudamic acid (PWY-6143), a pathogen virulence factor, was increased 5.8 times ($p \leq 0.05$) [59]. Previously, similar studies in mice revealed that the activity of predicted fatty acid and lipid metabolic pathways was more common in males than in females [15]. The increase in protein metabolism observed in our study in hens of the CM5 line compared to the CM9 line may be associated with an increase in the abundance of representatives of the genus *Synergistes* in the intestine (their main function is the utilization of amino acids) [60]. The enhancement of the CMP-pseudamic acid synthesis pathway in CM9 roosters compared to hens may be influenced by a decrease in the number of bacteria of the genus *Barnesiella*, which, as already noted, limits the proliferation of oxygen-resistant pathogens [55]. An increase in the pathogenicity of the intestinal microbiota in CM9 roosters could induce the expression of genes for antimicrobial peptides and IL8 in the bursa.

So, on the example of the new cross Smena 9 paternal line Cornish CM5 and maternal line Plymouth Rock CM9 with different phenotypes (patterns) in meat productivity and reproductive efficiency [3-5], we demonstrated a fairly high dependence of the transcriptional activity of genes associated in hens with adaptive potential and immunity [35, 39] on genotype and sex. The data obtained may be valuable for identifying new candidate genes affecting meat productivity and egg production. We also obtained results demonstrating the relationship between the quantitative composition of the microbiota and its predicted metabolic pathways with the genotype and sex of the bird. Genotype- and sex-specific changes in gene expression, as well as in the structure and function of the intestinal microbiome, may form the basis of physiological adaptations of the macroorganism to various conditions. We believe that the mechanism that determines differences in the composition of the intestinal microbiota depending on sex and genotype in birds

of the CM5 and CM9 lines of the Smena 9 cross may lie in the subtle harmonization by the transcriptional activity of genes of adaptation and immunity, on the one hand, and, apparently, hormonal background, nutritional needs and provision of nutrients and energy, on the other hand. Understanding these relationships is important for maintaining health, improving reproductive performance and enhancing poultry productivity, and further research is required to establish cause-and-effect relationships between them. In particular, it is of interest to compare the expression of genes for egg and meat productivity in the CM5 and CM9 lines in combination with an analysis of the composition and functional annotation of the intestinal microbiota.

Thus, we assessed differences in the expression of the spectrum of immunity genes and genes associated with adaptive potential, respectively, in the bursa and liver, and determined the composition and functions of the microbiome of the intestinal cecum in hens and roosters of the CM5 and CM9 lines of the new cross Smena 9 (parent stock). The lines differ in live weight gain and egg production. The results showed an increase in the transcriptional activity of the *HSF1* and *HSF2* genes in CM5 line roosters (group II) compared to groups I, III and IV (maternal line CM5 hens of the Cornish breed, maternal line CM9 hens of the Plymouth Rock breed, maternal line CM9 roosters of the Plymouth Rock breed, respectively). The difference in the expression of the *HSF1* and *HSF2* genes compared to roosters of the CM9 line (group IV) was 68 and 218%, respectively. The expression of the *HSF1* and *HSF2* genes in the CM5 line in roosters (group II) was 1.6 and 3.0 times higher compared to hens (group I). There was a significant activation of the expression of genes for antimicrobial peptides and pro-inflammatory genes in roosters of the CM9 line (group IV) compared to roosters and hens of the CM5 line (groups I and II). Thus, compared to group II, the expression of the *AvBD2*, *AvBD9*, *AvBD10*, *IL8* and *PTGS2* genes increased by 7.6, 5.3, 2.1, 6.3, and 1.5 times, respectively. NGS sequencing of the microbiome of the cecum in hens and roosters of the CM9 line (groups III and IV) identified bacteria of the superphylum *Elusimicrobiota* (0.32±0.11 and 0.49±0.19%, respectively). These microorganisms were not found in the intestines of roosters of the CM5 line (group II), and in hens of the CM5 line (group I) their share averaged 0.04±0.01%. Differences were found between the groups in 25 bacterial genera. In some genera, the abundance varied depending on the genotype, in some on the sex of the birds. Thus, in roosters of the CM5 line (group II), the abundance of the genera *Barnesiella*, Clostridia_UCG-014 and *Frisingicoccus* in the cecum was higher (17.2, 2.0 and 4.9 times, respectively) than in roosters of the CM9 line (group IV). Representatives of the genus *Desulfovibrio* were present in the intestines of roosters of both lines CM5 and line CM9 (groups II and IV, 0.25±0.08 and 0.73±5.6%), but in the intestines of hens of both lines (groups I and III), these microorganisms were not detected. Based on the results of bioinformatics reconstruction and functional annotation of NGS sequencing data, we identified 357 predicted metabolic pathways in the intestinal microbial community of the studied birds, 65 of which showed differences between groups. Genotype- and sex-specific modulations in gene expression, as well as in the structure and function of the intestinal microbiome, may provide adaptation to changing conditions.

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AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 2, pp. 333-344
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Reproduction and reproductive technologies

UDC 636.3.035:57.017.53:619

doi: 10.15389/agrobiology.2023.2.333eng
doi: 10.15389/agrobiology.2023.2.333rus

THE EFFECT OF HORMONAL STIMULATION SCHEME AND SEASON ON THE EFFICIENCY OF ESTRUS SYNCHRONIZATION IN ROMANOV EWES (*Ovis aries* L.)

V.A. LUKANINA ✉, R.Yu. CHINAROV, G.N. SINGINA

Ernst Federal Research Center for Animal Husbandry, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail kristybatle@gmail.com (✉ corresponding author), roman_chinarov@mail.ru, g_singina@mail.ru
ORCID:
Lukanina V.A. orcid.org/0000-0003-4744-7873
Singina G.N. orcid.org/0000-0003-0198-9757
Chinarov R.Yu. orcid.org/0000-0001-6511-5341
The authors declare no conflict of interests
Acknowledgements:
Supported financially from the Ministry of Science and Higher Education of the Russian Federation (theme 0445-2021-0004)
Final revision received January 27, 2023
Accepted March 20, 2023

Abstract

Estrus synchronization in sheep is an important element in the organization of reproductive programs, including those based on assisted reproductive technologies. The diversity of sheep breeds, the differences in environmental conditions of breeding zones and the specificities in hormonal status of animals not allow the finding a universal protocol for the management of reproductive cycles. The aim of our work was to identify the conditions of estrus synchronization in Romanov ewes in different seasons of the year. For the first time, a comparative analysis of two schemes of hormonal stimulation of estrus was carried out, involving either two consecutive injections of prostaglandin F2α (Scheme 1), or initially injection of gonadotropin releasing hormone followed by prostaglandin F2α treatment (Scheme 2). The study was carried out on mature Romanov ewes (n = 160). The first group (n = 121) underwent hormonal treatment using the Scheme 1, with two injections of cloprostenol (125 μg per injection) 13 and 2 days before the expected day of estrus (day 0). In the second group of animals (n = 39), the Scheme 2 was used, which included injections of 15 μg of lyuliberin acetate and 125 μg of cloprostenol at days 9 and 2 before expected day of estrus, respectively. In the first group, the results of estrus synchronization were analyzed by seasons, the autumn—winter period (n = 73) and spring—summer period (n = 48). For detail analysis, the data were studied by two-month periods: Sept-Oct (n = 24), Nov-Dec (n = 26), Jan-Feb (n = 23), Mar-Apr (n = 32), and May-June (n = 16). The efficiency of hormonal treatment was evaluated in all experimental animals based on appearance of estrus in 24 and 48 hours after the last injection. In some of the animals that showed estrus response (n = 80), visual assessment of the ovaries for the presence of corpora luteum (CL) was performed using laparotomy or laparoscopy 96 hours after estrus detection. The variance analysis showed a reliable effect of the hormonal treatment scheme (F = 5.21, p = 0.024) as well as the season (F = 13.82, p = 0.0003) on the estrus response. The average number of CL was subjected to greater variability by the year of experimental studies ($p \leq 0.05$) and the season ($p \leq 0.01$), without significant effect of the treatment scheme. Using the Scheme 1 revealed the trend of higher estrus response comparing to Scheme 2, 80.17 % vs 66.67 %. Along with that, in the second group the CL were found in all ewes with the estrus signs, while in the first group the CL were found in 90.77 % of animals ($p \leq 0.05$). Synchronization results for ewes in the autumn-winter period were better than in the spring-summer period: the estrus response was 92.50 % compared to 64.58 % ($p \leq 0.01$), the average number of CL for ewes in the estrus stage was 2.02 vs 1.28 ($p \leq 0.01$), and the average number of CL in ewes with identified CL was 2.18 vs 1.62 ($p \leq 0.05$). A more detailed analysis by the two-month periods showed that the values of the above-mentioned indices were relatively stable during the autumn-winter period, after which they decreased in Mar-Apr and reached minimum values in May-June. Thus, for stimulating the estrus in Romanov ewes we recommend two consecutive injections of cloprostenol (13 and 2 days before the expected estrus). The efficiency of the method is higher in autumn-winter season, after which a decrease occurs in the response of animals to hormonal treatment.

Keywords: ewes, Romanov breed, hormones, prostaglandin F2α, estrus stimulation, ovarian function

Synchronization of the sexual cycle (SC) in sheep is a ways to increase the efficiency of realization of the reproductive potential of females and optimize reproduction programs [1]. Since the effectiveness of synchronization is mostly assessed by the onset of heat, it is often designated as "hunt synchronization". Heat synchronization can neutralize the influence of seasonal factors on sheep reproduction to extend the breeding season, to plan lambing of ewes at a predetermined period of time throughout the year [2-4] and to obtain up to 2-3 lambings in 2 years, which increases the economic efficiency of sheep farming [1, 5].

Synchronization of the reproductive cycle is of particular relevance in connection with the development of assisted reproductive technologies [6], including artificial insemination, in vitro production of embryos, cloning, and embryo transplantation [7-9]. Biological, hormonal and combined methods provide SC synchronization.

Multisensory contact between ewes and rams, called the "male effect", is a biological method for stimulating estrus outside the breeding season [10]. Such multifactorial stimulation involves the olfactory, tactile and visual receptors of females. Stimulation of estrus is based on changing pulsations of the gonadotropic releasing hormone (GnRH) secretion and increasing the secretion of luteinizing hormone (LH). During the first ovulation which occurs 2-3 days after contact of ewes with rams, heat manifestation is often absent ("silent heat"), and the fertility of ewes remains low. The main factor limiting the use of this biological method is the reduced fertility of sheep in the first cycle and a decrease in the effectiveness of synchronization in subsequent cycles. It was noted that the effectiveness of this method increases with the artificial extension of the photoperiod during the previous two months, eventually, estrus occurred in 99% of females [11]. Another technique that increases the effectiveness of the "male effect" is treating females with 20 mg of progesterone when introducing rams into the herd [12]. Biological stimulation of the reproductive cycle in sheep are of significant interest since it does not require treatment with hormonal drugs.

Hormonal drugs, when used optimally, can provide higher efficiency in synchronizing the sexual cycle. There are various schemes based on the use of progestogen drugs, prostaglandin F2α (PGF2α) and its structural analogues. The choice of regimen depends on the breed of sheep, season, and physiological state of the animal [13]. When synchronizing during the breeding season, prostaglandins are usually applied, for which the target is the functional corpus luteum; outside the breeding season, complex progesterone-based regimens are used, followed by the administration of equine chorionic gonadotropin (eCG) and gonadotropin-releasing hormone GnRH [1, 14, 15].

Synthetic progestogens which act as prolongators of the luteal phase of the sexual cycle, can be administered orally, intravaginally, or subcutaneously as boluses. The oral method is the least labor-intensive. The drug is mixed with salt or dissolved in ethyl alcohol and added to the feed. Animals are fed the drug for 8-10 days. However, this method does not guarantee that all animals will consume the required dose [16].

Regardless of the season, progestogen intravaginal sponges impregnated with alcohol or propylene glycol solutions of drugs based on progesterone (P4), fluorogestone acetate (FGA) and medroxyprogesterone acetate (MPA) have become widespread, regardless of the season [17]. The sponge is placed in the vagina for 10-14 days followed by subcutaneous injected of eCG. Signs of heat appear within 24-48 hours in 90% of individuals [18-21]. A study of the possibility of

increasing the effectiveness of intravaginal sponges containing progestogens in combination with subcutaneous melatonin implants showed [22] that in the group receiving melatonin, the fertility of females was 60.4% vs. 32.6% in the control group. The positive effect of melatonin in combination with progestogens and eCG was also noted in Awassi sheep when stimulating estrus during the aestrous period [23]. It has been established that melatonin exhibits a positive effect together with progestogens and eCG, but without other drugs it does not stimulate estrus [24].

Progestogen preparations in the form of boluses are effective and safe for animal health [25]. Using a special applicator, a bolus containing the active substance is injected subcutaneously into the ear for 8-12 days. At the same time, an injection of a synthetic analogue of progesterone - norgestamet (1.5 mg) and estradiol (1.9 mg) is given. After 8-12 days, the implant is removed and an LHC injection is given. With this scheme, within 24 hours, 95-100% of the animals showed signs of heat, and the fertilization rate with fresh sperm was 85-95% [26]. In studies by Z. Mekuriaw et al. [27] boluses were combined with an injection of 300-400 IU eCG and 50 μg cloprostenol 48 hours before implant removal. The onset of heat occurred in 65-95% of animals with a fertility rate of 50-90% [27].

To synchronize hunting during the breeding season (autumn-winter; the duration of the period depends on the breed of sheep), drugs containing prostaglandin F2 and its synthetic analogues are used. Exogenous prostaglandin F2α has no effect on cyclicity in sheep in the absence of the corpus luteum, that is, during the luteal phase of the reproductive cycle. Therefore, preparations based on prostaglandin F2α are more effective during the sexual season, during which at least some of the sheep in the herd have corpora lutea. To synchronize the sexual cycle, animals are injected with a drug based on prostaglandin F2α twice with an interval of 8-12 days [28]. Hunting occurs 48-60 hours after the last injection. The effectiveness of this method can reach 100%. In our earlier studies on a small sample of Romanov breed sheep, we showed greater effectiveness of a regimen based on duble injection of prostaglandin F2α, compared to the combined use of gonadotropic releasing hormone and prostaglandin F2α, the proportion of animals that came into heat, was 100 and 50%, respectively [29].

The variety of sheep breeds and differences in natural and climatic conditions in breeding areas do not allow us to select a universal synchronization protocol. In addition, the effect of hormonal drugs on the reproductive system of females can manifest itself differently depending on the initial functional state of the reproductive organs and hormonal status. Thus, the effectiveness of hormonal stimulation of reproductive cycles in sheep at the beginning of the breeding season is significantly lower than at its end. In lactating females, the effectiveness of stimulation is lower than in non-lactating animals. If hormonal drugs are used incorrectly or the dosage is poorly selected, negative consequences can be observed [30, 31]. Therefore, the choice of the optimal hormonal treatment regimen is the most important element of assisted reproductive technologies in sheep breeding.

In this work, for the first time, a comparative analysis of the effectiveness of two schemes of hormonal stimulation of oestrus, involving either two successive injections of prostaglandin F2α, or an initial injection of gonadotropic releasing hormone followed by treatment of sheep with prostaglandin F2α, was carried out. The use of the first scheme provided the best response to synchronization in relation to the proportion of animals that came into heat. A higher efficiency of synchronization of the sexual cycle was established in the autumn-winter period compared to the spring-summer period when using both schemes.

The purpose of the work was to determine the effectiveness of different schemes for synchronizing the sexual cycle in Romanov breed sheep according to the seasons of the year.

Materials and methods. The study was carried out on sexually mature Romanov breed females aged 1.5-2 years (n = 160, Federal Research Center for Animal Husbandry - Ernst VIZh, Moscow Province, from January 2021 to December 2022). Each animal was used only once during the experimental period. Young ewes were kept in pens under sheds in groups of 15-20 animals. separately from rams. In winter, young ewes received a hay-concentrated diet in accordance with the breed's requirements. In the summer, the animals were on pasture and additionally received concentrates. All animals had unlimited access to mineral salt and water.

The animals were divided into two groups. In group 1 (n = 121), the ewes were subjected to a 2-fold injection of a prostaglandin analogue cloprostenol (Estrophan, Bioveta, Czech Republic), 125 μg per injection over 13 days (–13 days) and 2 days (–2 days) before the expected date of coming into heat (0 days) (scheme 1). Animals of group 2 (n = 39) were injected with 15 μg of gonadotropin luliberin acetate (Surfagon, Mosagrogen, Russia) on day –9 and an injection of 125 μg of cloprostenol on day –2.

The effectiveness of hormonal stimulation schemes was assessed in all females based on the coming into heat, as well as the visual detection of corpora lutea (CL) in the ovaries of some individuals with signs of heat (n = 67 and n = 13 for groups 1 and 2, respectively). Coming into heat was determined using a vasectomized probe ram 24 and 48 hours after the last injection. Ovarian function (presence and number of CLs in the ovaries) was assessed by laparotomy or laparoscopy using endoscopic equipment (Karl Storz SE & Co. KG, Germany) 96 hours after the last injection.

The effectiveness of synchronization of the sexual cycle was determined by the following criteria: the proportion of females that came into heat from the total number of those subjected to hormonal treatment; the proportion of females with CLs out of the total number that showed signs of heat; average number of fatty acids per animal with signs of heat; the average number of CLs per animal with CLs. For each group of animals, these indicators were calculated for the entire test period. The influence of the seasons autumn-winter (September-February) and spring-summer (March-July) were studied in ewes of group 1 (n = 73 and n = 48, respectively). For a more detailed analysis, indicators were compared for five time periods: September-October (n = 24), November-December (n = 26), January-February (n = 23), March-April (n = 32) and May-June (n = 16).

To determine the statistical significance of the influence of the studied factors (hormonal treatment regimen, season and period of the year) on the variability of PC synchronization performance indicators, two-factor analysis of variance without interaction (due to the incomplete rank of the model) was used using the STATISTICA 10 program (StatSoft, Inc., USA) according to the following equation:

$$y_{jilk} = \mu + year_j + scheme_i + season_l + animal_k + e_{jilk},$$

where $year_j$ is the fixed effect of the year of research (j is 2020-2023); $scheme_i$ is the fixed effect of the i-th hormonal treatment scheme (i is 1 and 2); $season_l$ is fixed effect of the l-th season (l is autumn—winter, spring—summer); $animal_k$ is the randomized effect of the k-th animal; e_{jilk} is the residual (randomized) effect of the equation model. To calculate the independent distribution of events according to the proportion of females that came into heat, in connection with the influence of factors, a nonparametric $\chi 2$ test according to Pearson was used. To assess the strength of the influence of factors on the synchronization performance

of sheep in the experimental groups, arithmetic mean values (*M*) and the error of the mean (±SEM) were determined. The statistical significance of differences in arithmetic mean values was assessed by Student's *t*-test. The results were considered highly reliable at $p \leq 0.001$, significant at $p \leq 0.01$ and $p \leq 0.05$.

Results. Figure 1 illustrates the experimental design:

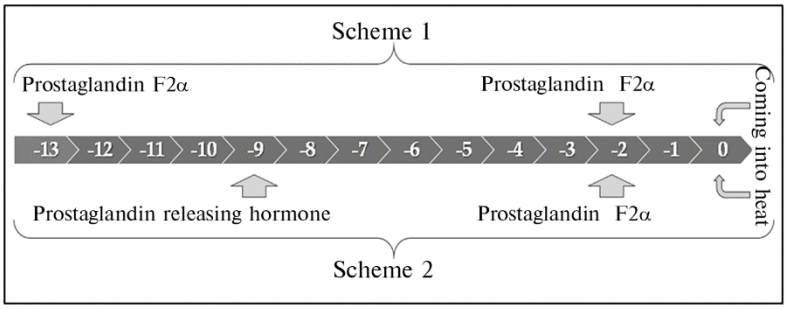

Fig. 1. Schemes of hormonal synchronization of the reproductive cycle in the Romanov 1.5-2-month-old ewes (*n* = 160, Ernst Federal Research Center of Animal Husbandry — VIZh, Moscow Province, from January 2021 to December 2022). Timeline referes to a day before going into the hunt

1. Results of a two-factor analysis of variance for synchronization of the sexual cycle in the Romanov 1.5-2-month-old ewes depending on the year, hormonal treatment scheme and season (*n* = 160, Federal Research Center of Animal Husbandry — Ernst VIZh, Moscow Province, from January 2021 to December 2022)

Parameter	Significance test	Factor			R^2, %
		year	scheme	season	
Proportion of yearling ewe that came into heat	*F*-test	0.18[na]	5.21*	13.82***	10.3
	(p-value)	(0.910)	(0.024)	(0.0003)	
	χ^2 test	0.49[na]	3.02[t]	11.22**	
	((p-value)	(0.921)	(0.082)	(0.008)	
Average number of corpora lutea	*F*-test	3.49*	0.11	10.81**	23.6
	(p-value)	(0.020)	(0.736)	(0.011)	

[na] The influence of the factor is unreliable.
[t] The influence of the factor is statistically significant at $p \leq 0.10$.
*, ** and *** The influence of the factor is statistically significantat $p \leq 0{,}05$, $p \leq 0{,}01$ and $p \leq 0{,}001$, respectively.

For the proportion of females that came into heat (Table 1), a significant influence of the applied hormonal treatment regimen ($F = 5.21$; $p = 0.024$), as well as the season of the year ($F = 13.82$; $p = 0.0003$) was established (see Table 1). The coefficient of determination of the model, which explained the amount of variability in the proportion of bright spots between the compared groups of factors, was 0.103, or 10.3%. Pearson's $\chi 2$ test showed that the difference in this indicator also depended on the synchronization scheme ($p \leq 0.05$) and the season of the year ($p \leq 0.01$), which indicates the contingency of the distribution of events of coming into heat (yes/no) and the inapplicability of zero hypotheses about the mutual independence of the factors under study and the results of observations. The year of study factor did not influence the distribution of threshold characteristics ($F = 0.18$; $p = 0.910$). The average number of CLs was subject to greater variability due to the calendar year of the studies ($p \leq 0.05$) and the season of the year ($p \leq 0.01$), while the treatment scheme did not significantly affect this indicator. An increase in the determination coefficient R^2 (23.6% vs. 10.3%) indicated that the value characterizing the distribution of variations within groups in comparison with the overall group variability for the average number of CLs was higher than for females that came into heat (see Table 1).

As follows from the data presented in Table 2, scheme 1 (group 1) turned out to be more effective than scheme 2 (group 2) when comparing the proportion of animals that came into heat (80.17 vs. 66.67%), however, the differences found

can only be considered as a trend. When using scheme 2 (group 2), CLs were detected in all females with signs of heat, while the use of scheme 1 led to the formation of CLs in 90.77% of females ($p \leq 0.05$). Fot scheme 2, there was a tendency for the average number of CLs to increase compared to that for scheme 1 (+1.17) due to CLs detection in all females with signs of heat. When determining the average number of CLs in females with CLs, the differences between the groups were leveled out.

2. Effectiveness of the sexual cycle synchronization in the Romanov 1.5-2-month-old ewes as influenced by the hormonal treatment sheme (*M*±SEM, Federal Research Center of Animal Husbandry — Ernst VIZh, Moscow Province, from January 2021 to December 2022)

Parameter	Group	
	1	2
Number of animals, n_1	121	39
Proportion of females that came into heat, %	80.17±3.64	66.67±7.65
Number of animals, n_2	67	13
Proportion of females with CL[1], %	90.77±3.59*	100
Average CL number[1]	1.75±0.13	1.92±0.35
Average CL number in the responded animals[2]	1.98±0.11	1.92±0.35

N o t e. n_1 is the number of animals that have undergone synchronization of the reproductive cycle, n_2 is the number of animals that have undergone synchronization of the reproductive cycle and were tested for the presence of corpus luteum (CL); [1] is indicator based on the number of females coming into heat; [2] is an indicator based on the number of females that come into heat and have CL.
* Differences between groups are statistically significant at $p \leq 0.05$.

3. Eeffectiveness of the sexual cycle synchronization scheme 1 in the Romanov 1.5-2-month-old ewes as influenced by the season (*M*±SEM, Federal Research Center of Animal Husbandry — Ernst VIZh, Moscow Province, from January 2021 to December 2022)

Parameter	Season	
	autumn-winter	spring-summer
Number of animals, n_1	73	48
Proportion of females that came into heat, %	90,41±3,45**	64,58±6,90**
Number of animals, n_2	42	25
Proportion of females with CL[1], %	92,50±3,97	80,00±10,69
Average CL number[1]	2,02±0,15**	1,28±0,19**
Average CL number in the responded animals[2]	2,18±0,16*	1,62±0,15*

N o t e. See the description of scheme 1 in Figure 1. The autumn-winter season included the period September-February, spring-summer include March-July; n_1 is the number of animals that have undergone synchronization of the reproductive cycle, n_2 is the number of animals that have undergone synchronization of the reproductive cycle and were tested for the presence of corpus luteum (CL); [1] is indicator based on the number of female ducks that came into heat; [2] is an indicator based on the number of females that come into heat and have CL.
*, ** Differences between groups are statistically significant at $p \leq 0,05$ и $p \leq 0,01$, resptctively.

The data in Table 3 show a significant influence of the season of the year on the effectiveness of the sexual cycle synchronization when assessed by the proportion of females that come into heat. In the autumn-winter period, the value of this indicator was 25.83% higher than in the spring-summer period (92.50 vs. 64.58%, $p \leq 0.01$). A similar seasonal dependence occurred for the proportion of females with CLs from the total number of those showed signs of estrus (+12.5%), however, the differences identified were just a trend. A more detailed analysis carried out for 2-month periods showed that the proportion of animals going into heat was relatively high in the period from September to February (88.5-91.7%), followed by a decrease to 71.9% in March to April. In May-June, the proportion of females that came into heat after hormonal treatment was 50.0% (Fig. 2). An interesting pattern emerged in relation to the corpora lutea. If in September-December 100% of females that showed signs of heat had visualized CLs in the ovaries followed by a decrease in January-April to 81.3-89.5%, in May-June only up to 50 %.

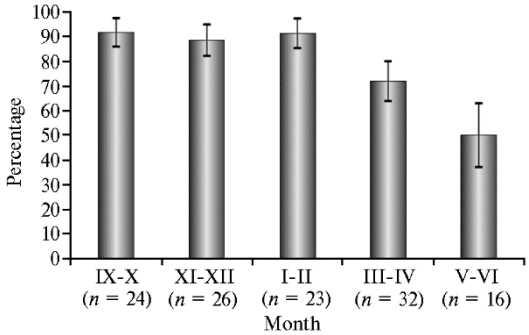

Fig. 2. Effectiveness of the sexual cycle synchronization in Romanov 1.5-2-month-old ewes under hormonal treatment scheme 1: the proportion of females that came into heat from the total number of those subjected to hormonal treatment, % (*M*±SEM; data recorded at a 2-month intervals, Ernst Federal Research Center of Animal Husbandry — VIZh, Moscow Province, from January 2021 to December 2022). See Figure 1 for the description of scheme 1.

We revealed a significantly higher number of CLs in females that underwent synchronization of the reproductive cycle in the autumn-winter period, compared to that in the summer-spring period (2.02 vs. 1.28, $p \leq 0.01$), and the identified differences remained the same when calculating this indicator for females with CLs (2.18 vs. 1.62, $p \leq 0.05$) (see Table 3). Analysis of changes over 2-month periods showed that the mean number of CLs per female that came into heat was greater from September to February, 1.83-2.17. In March-April, the number of identified CLs decreased to 1.47, in May-June it dropped to a minimum for the entire period of the experiment and amounted to 0.67 (Fig. 3). In females that came into heat and had yellow bodies for the above periods of the year the number of CLs was 1.83-2.38, 1.65 and 1.33, respectively.

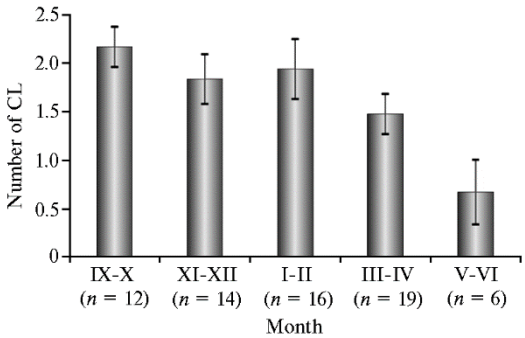

Fig. 3. Effectiveness of the sexual cycle synchronization in Romanov 1.5-2-month-old ewes under hormonal treatment scheme 1 when counting CLs per female: average CL number per all females that came into heat (*M*±SEM; data recorded at a 2-month intervals, Federal Research Center of Animal Husbandry — Ernst VIZh, Moscow Province, from January 2021 to December 2022). See Figure 1 for the description of scheme 1.

Classic protocols for synchronizing SC in sheep include intravaginal insertion of a progesterone-containing sponge or intravaginal device (CIDR) impregnated with fluorogestone acetate or medroxyprogesterone, combined with an intramuscular injection of pregnant mare serum gonadotropin (PMSG) at the date of estrus [20, 32]. Various variants of this protocol are also used (Table 4), but in the vast majority of cases, hormonal synchronization of estrus does not provide 100% effectiveness in terms of the number of animals that come into heat. With only one scheme out of 25 analyzed by us (see Table 4), all treated animals showed signs of heat [20]. In other variants, the effectiveness of synchronization, depending on the scheme and breed, varied from 59.1 to 96.0%. Overcoming seasonality through hormonal stimulation of estrus in this species also remains an unresolved problem [26].

In contrast to the approaches described above, in the present study, to stimulate the estrus, either a 2-fold injection of PGF2α was used 13 and 2 days before the expected date of coming into heat (scheme 1), or injections of GnRH 9 days before and PGF2α 2 days days before the estrus (scheme 2). Both hormonal treatment regimens (albeit to varying degrees) showed effectiveness in stimulating estrus in mature females (see Tables 2, 3). However, during the year (except for the period July-August), the average performance for scheme 1 was higher than for scheme 2, amounting to 80.17%, which is comparable to traditional protocols

(see Table 4). In addition, from September to December, 2-fold administration of PGF2α provided stimulation of estrus in 100% of treated mature females, which was consistent with the result for CIDR in combination with PGF2α, PMSG and estradiol benzoate [20]. It should be noted that the effectiveness of scheme 1 decreased only in the summer.

4. Effectiveness of the reproductive cycle synchronization in different sheep breeds depending on the hormonal treatment scheme

Scheme — drug/device (days to the predicted date of entry into heat)	Animals that came into heat, % of total treated	Breed	References
PGF2α (–13, –2)	80.17	Romanovskaya	This work
GnRH (–9), PGF2α (–2)	66.67		
CIDR (c –7 to 0)	89	Southdown, Ramboulier, Columbian, Suffolk × Hampshire, Romanovskaya × White Dorper, Romanovskaya × Katahdin	[19]
CIDR (c –7 to 0), PGF2α (0)	93		
CIDR (c –14 to 0)	93		
CIDR (from –10 to 0)	68	Burgskaya	[19]
CIDR (from –19 to 0)	72		
CIDR (from –6 to 0), PGF2α (–6), PMSG (0), EB (+1)	100	Dorper	[20]
PG-sponge (from –14 to 0), PMSG (0)	76.7	Lakon, segureno, segureno × Romanovskaya	[21]
PG-sponge (c from –7 to 0), PGF2α (–7), PMSG (0)	80		
PG-sponge (from –7 to 0), PMSG + PGF2α (0)	90		
PG-sponge (from –14 to 0), PMSG (0)	80		
PG-sponge (from –7 to 0) + PGF2α (–7), PMSG (0)	79.2		
PG-sponge (from –7 to 0), PMSG (0), PGF2α (0)	59.1		
PG-sponge (from –13 to –2), PGF2α (–4), GnRH (0)	70	Chinese Hu Sheep	[15]
PG-sponge (from –13 to –2), PGF2α + PMSG) (–4)	83.3		
PG-sponge (from –13 to –2), PGF2α + PMSG (–4), GnRH (0)	86.7		
PG-sponge (from –13 to 0), PGF2α (–1), GnRH (0)	80		
PG-sponge (from –13 to 0), GnRH (0)	76.7		
CIDR (from –5 to 0)	89	Columbian, Hampshire	[33]
CIDR (from –5 to 0), PGF2α (0)	77		
GnRH (–5), CIDR (from –5 to 0), PGF2α (0)	75		
CIDR (from –5 to 0)	78	Horned Dorset, Katahdin	
CIDR (from –5 to 0), PGF2α (0)	90		
GnRH (–5), CIDR (from –5 to 0), PGF2α (0)	96		

N o t e. GnRH — gonadotropin releasing hormone, PMSG — pregnant mare serum gonadotropin, EB — estradiol benzoate, PGF2α — prostaglandin F2α, PG-sponge — progestogen intravaginal sponge, CIDR — device for controlled internal release of the drug.

Thus, our studies on the sexual cycle synchronization in Romanov young ewes revealed a significant influence of the hormonal treatment scheme ($F = 5.21$; $p = 0.024$) and the season of the year ($F = 13.82$; $p = 0.0003$) on the proportion of females who came into heat. A 2-fold injection of prostaglandin F2α on days –13 and –2 before the expected date of estrus (day 0) ensures higher performance compared to the administration of gonadotropin releasing hormone on day –9 and prostaglandin F2α on day –2, 80.17 vs. 66.67%. For the second scheme, all the females that showed signs of heat had corpus luteum (CLs) in their ovaries, whereas with the first scheme their share was 90.77% ($p \leq 0.05$). The best response to hormonal treatment occurs during the sexual season. In September-February, the proportion of females that came into heat, the average number of CLs in females that came into heat, and the average number of CLs in females that had CLs were significantly higher than in March-July, 90.41 vs. 64.58% ($p \leq 0.01$); 2.02 vs. 1.28 ($p \leq 0.01$), and 2.18 vs.1.62 ($p \leq 0.05$) , respectively. To synchronize

the sexual cycle in Romanov ewea, we recommend a 2-fold injection of prostaglandin F2α on days –13 and –2 before the expected date of estrus. In terms of the proportion of females that come into heat, the effectiveness of the scheme we propose is comparable to that described by other reserachers, and in some cases exceeds it.

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AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 2, pp. 345-354
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 638.12:57.017.53:612.616.2

doi: 10.15389/agrobiology.2023.2.345eng
doi: 10.15389/agrobiology.2023.2.345rus

EFFECT OF ACARICIDE TREATMENT ON BODY WEIGHT AND REPRODUCTIVE CHARACTERISTICS OF DRONES OF THE PRIOKSKY BREED TYPE OF CENTRAL RUSSIAN HONEYBEES (*Apis mellifera* Linnaeus, 1758)

A.Z. BRANDORF ✉, A.I. SHESTAKOVA, E.O. LARKINA, E.P. LAPYNINA

Federal Beekeeping Research Centre, 22, ul, Pochtovaya, Rybnoye, Ryazan Province, 391110 Russia, e-mail gordenchuk@mail.ru (✉ corresponding author), anastasiya_07.83@mail.ru, alenaelena98@yandex.ru, elena.p56@yandex.ru
ORCID:
Brandorf A.Z. orcid.org/0000-0003-0535-6578
Shestakova A.I. orcid.org/0000-0002-7311-9307
Larkina E.O. orcid.org/0000-0002-5405-8721
Lapynina E.P. orcid.org/0000-0002-6473-1393
The authors declare no conflict of interests
Acknowledgements:
Supported by the Ministry of Science and Higher Education of the Russian Federation (state order No. 0470-2019-0001)
Final revision received August 18, 2022
Accepted

Abstract

In modern beekeeping, there is a mass death of bee colonies, one of the causes of which is varroosis. To reduce the negative impact of varroosis on the life of a bee colony, acaricides are widely used. However, the acaricidal preparations negatively affect the reproductive performance of drones. Various reports note their ambiguous effect on some the development of individuals in a bee family, including drones, and, consequently, economically useful traits (honey and wax productivity, queen egg production, resistance to diseases). In this work, for the first time, we obtained data that acaricidal preparations of amitraz, fluvalinate and thymol + oxalic acid negatively affect the reproductive performance of honeybee drones of the Central Russian breed and cause a deterioration in sperm quality. The aim of the work was to study the effect of acaricidal preparations on the fertility, deformation and concentration of spermatozoa of honey bee drones of the Prioksky breed type of the Central Russian breed, as well as on their body weight. The work was carried out at the experimental apiary of the Federal Beekeeping Research Centre (Rybnoye, Ryazan Province, spring-summer 2021). Colonies of Central Russian bees (*Apis mellifera* Linnaeus, 1758) of the Prioksky breed type were assigned to four groups, three bee colonies each: group I was not subjected to treatments (control), groups II-IV were treated. The degree of *Varroa destructor* infestation was 1-2 % in all groups. After the appearance of one-day-old drone brood, each test group was treated with one of three acaricidal drugs to combat varroosis. An amitraz-based drug («Sichuan Wangshi Animal Health Co., Ltd.», China; hazard class 3, SanPiN 1.2.25.84-10) was used in group II, a fluvalinate-based drug («Shanxi Zhenxing Fish & Bees Medicine Industry Co., Ltd.», China; hazard class 3, SanPin 1.2.25.84-10) in group III, and a drug containing oxalic acid («Shandong Deshang Chemical Co., Ltd.», China) and thymol («Hunan Insen Biotech Co., Ltd.», China) (hazard class 4, SanPin 1.2.25.84-10) in group IV. Preparations containing the active ingredients amitraz and thymol + oxalic acid were sprayed onto drone brood in 2 repetitions with 7-day interval. The fluvalinate-containing strips were placed on both sides of the drone brood frame. The impact of antivarroal drugs was assessed based on sperm quality parameters and body weight of drones at the age of 26-30 days. Sperm was collected by artificially stimulating endophallus eversion in mature drones aged 26-30 days. The concentration of spermatozoa, motility, viability, morphology (defects and abnormalities) were assessed. When assessing the viability of spermatozoa by fluorescent microscopy, fluorochromes Hoechst 33258 (Pan-Eco, Russia) and PI (Khimmed, Russia) were used with a biological luminescent light-emitting diode microscope MICROMED 3LYUM LED (OOO Observational Instruments, Russia) with 400× magnification. To determine the deformation of the heads of spermatozoa, rapid differentiated staining with a set of reagents Diahim-Diff-Quick (OOO "ABRIS + NPF", Russia) was used. Our results show that the treatment of bee colonies with acaricides affects the weight of drones. The decrease in body weight was significant when using fluvalinate and oxalic acids + thymol preparations, where the maximum weight of drones was 10-20 mg less ($p < 0.05$). It was found that sperm quality parameters decrease after treatment with acaricides. The viability of spermatozoa decreased by 1.3 % on average, sperm concentration decreased 2.2 times, and the number of spermatozoa with abnormal head morphology increased 1.3 times. Therefore, acaricides

should be used only for medicinal purposes when varroasis is confirmed. The frequency of use should depend on the degree of invasion in order to reduce the negative impact of drugs on the reproductive function of drones.

Keywords: *Apis mellifera*, honey bee, drone, sperm quality, spermatozoa viability, spermatozoa morphology, acaricidal preparations, amitraz, fluvalinate, thymol, oxalic acid

Beekeeping is an important element of the agro-industrial complex. Honey bees as pollinators account for about 80% of entomophilous plants. Bee breeding is important for increasing the biodiversity of pollinators in the ecosystem, increasing the yield of entomophilous agricultural crops, obtaining dietary foods, medicinal preparations for apitherapy, and various raw materials for processing [1, 2]. In apimonitoring, on the basis of which the state of environmental pollution is assessed and monitored, honey bees and their products are used as a bioindicator [3, 4].

In Russia, as well as throughout the world, there is a mass death of bee colonies, known as colony collapse disorder (CCD) [5-7]. The causes of CCD are massive use of pesticides, including neonicotinoids; uncontrolled breeding of bees, which leads to mass hybridization; varroosis caused by the ectoparasite *Varroa destructor* which also is a carrier of viral diseases [8, 9].

A serious factor damaging beekeeping is the massive use of pesticides, which causes the death of bee colonies, and residues of harmful substances are found in hives, in beekeeping products, as well as in adult bees and bee brood [10, 11]. The accumulation of pesticide residues in the nests of bee colonies can lead to deterioration in their health and development [12]. Honey bees exposed to pesticides become susceptible to infection by the microsporidia *Nosema ceranae* and other diseases [13, 14]. Treatment of bee colonies infected with *Nosema ceranae* with fipronil has been shown to have a negative effect on drone fertility [15].

Varroosis is widespread throughout the world. The consequences of a high degree of invasion in a bee colony may be a reduction in the number of drones, a delay in their development, and a decrease in weight. With a high degree of invasion, drones develop pathologies in the form of underdeveloped wings or their complete absence, and life expectancy is reduced, i.e., the most drones do not survive to sexual maturity [16]. The main method of combating and preventing varicose veins is the use of organic substances of natural origin (formic acid, oxalic acid, thymol), synthetic compounds (acaricides based on amitraz, fluvalinate, coumaphos, etc.), as well as raw materials of medicinal plants [17, 18].

It has been established that in the case of high infestation, when families are treated with a drug containing the active ingredient fluvalinate (concentration 10%, in strips), drones are significantly reduced in number [19-21]. Also shown is high mortality when treated with drugs containing fluvalinate (20.4 mg/100 ml of acetone) in drones aged 12 to 18 days (66.9% death). In surviving drones after treatment with this drug, a decrease in body weight of approximately 5-10% and in the length and width of the right forewing occurs [21]. It has been established that the sperm concentration in drones treated with drugs containing the active ingredients fluvalinate and amitraz decreases compared to untreated drones [22].

Bees treated with 30% formic acid remove drone brood, development of drones is delayed and their survival rate is reduced, but the organic acid does not have a negative effect on the mass of seminal vesicles and mucous glands. It has been hypothesized that thymol treatment may reduce drone flight activity [23].

The use of coumaphos has a negative effect on bee colonies: high concentrations impair memory, affect movement, as well as the behavior of nurse bees, and reduce trophallaxis of honey bees. Coumaphos (2-5 μg) has also been shown to negatively affect uterine development, including leading to a decrease in body weight [24-26]. Coumaphos caused a decrease in sperm viability and concentration

in semen immediately after collection, as well as in samples stored for up to 6 weeks [21]. A decrease in the mitochondrial activity of sperm in drones and, accordingly, their viability under the influence of imidacloprid (200 μg) has been detected [27]. Sublethal doses of fipronil during puberty resulted in decreased sperm concentration, lower sperm viability, while increased metabolic rate [28, 29].

The pesticides listed above are most effective in combating the *Varroa destructo*r mite, but they all have a negative impact on the life of the bee colony. Any such effects of treatment with one of the acaricides may affect the reproductive function of the queen and drones. Exposure to acaricides during drone development reduces drone viability and body weight, including reproductive gland weight and sperm concentration [30]. It is important to note that drones, by fertilizing the queen bee, take part in the transfer of genetic material to the next generation. A decrease in sperm viability due to treatment with anti-borroosis drugs can negatively affect the overall development of the bee colony.

Currently, there is insufficient data on the effect of acaricides on the physiological state of bees, and especially queen bees and drones, including their reproductive functions [31].

In the presented work, we for the first time obtained data that acaricidal preparations, which include amitraz, fluvalinate and thymol with oxalic acid, negatively affect the reproductive performance of drones of honey bees of the Central Russian breed and cause a deterioration in the quality of sperm.

The purpose of the work was to study the effect of acaricidal drugs on fertility, deformation and concentration of spermatozoa of drones of honey bees of the Prioksky breed type of the Central Russian breed, as well as on their body weight.

Materisl and methods. The work was carried out at the experimental apiary of the Federal Scientific Center for Beekeeping (Ryazan Province, Rybnoye) in the spring-summer period of 2021. From bees (*Apis mellifera* Linnaeus, 1758) of the Prioksky type of the Central Russian breed, 4 groups of bee families were formed, identical in economically useful traits, group I for control (not subjected to treatment), group II-IV for test treatments. Each group had three bee families (8 bee colonies, 3-5 frames with brood, 10 kg of honey, 2 kg of bee bread per family). The infestation by the *Varroa destructor* mite was 1-2%. After the appearance of 1-day-old drone brood, each experimental group was treated with one of three acaricidal drugs to combat varroosis, in group II, with an amitraz-based drug (hazard class 3, SanPin 1.2.25.84-10; Sichuan Wangshi Animal Health Co., Ltd., China); in group III, wirh a fluvalinate drug (hazard class 3,, SanPin 1.2 .25.84-10; Shanxi Zhenxing Fish & Bees Medicine Industry Co., Ltd., China); in group IV with a drug with oxalic acid (Shandong Deshang Chemical Co., Ltd., China) and thymol (Hunan Insen Biotech Co., Ltd., China) (hazard class 4, SanPin 1.2.25.84-10).

Preparations containing the active ingredients amitraz and thymol with oxalic acid were sprayed onto the drone brood in duplicate after 7 days. Strips with the fluvalinate-based drug were placed on both sides of the drone brood frame. In all groups, frames with drone brood were placed in isolators 2-3 days before the drones emerged; after the drones emerged, each was marked with permanent markers of different colors depending on the group.

The body weight and sperm quality indicators of drones were determined at the age of 26-30 days. To determine the mass of drones, laboratory analytical balances AND GR-200 (A&D Co., Ltd., Japan) were used. The measurements were carried out in triplicate, $n = 30$ from each test group. Sperm was collected by artificially stimulating endophallus eversion from sexually mature drones aged 26-30 days.

The concentration [32] and motility of spermatozoa [33] were determined using a Goryaev camera (MiniMed, Russia), viability was determined by membrane integrity [34] using fluorescence microscopy, and the morphology of spermatozoa (defects, anomalies) was also examined [35]. To assess sperm viability, fluorochrome Hoechst 33258 (Pan-Eco, Russia) and PI (Khimmed, Russia) were used. Working solutions of fluorochromes were prepared in Tris buffer (pH 8.8); the final concentration of Hoechst 33258 is 5 μg/ml, of PI is 10 μg/ml. A suspension from the sperm sample was prepared in Tris buffer (pH 8.8) at a ratio of 1:400. The studies were carried out on a biological luminescent LED microscope MICROMED 3 LUME LED (Observational Instruments LLC, Russia) at a magnification of ×400. A total of 200 spermatozoa were counted. To determine the deformation of sperm heads, we used the rapid differentiated staining with Diachim-Diff-Quick reagents (NPF ABRIS+ LLC, Russia).

The results obtained were processed using Statistica software for Windows version 13 (StatSoft Russia, Russia) and Microsoft Excel 2010 by common methods of variation statistics and assessment of the significance of differences by the Student's *t*-test. For each series of data, the arithmetic mean (*M*), standard deviation (±SD) and coefficients of variation (*Cv*) were calculated. Differences between indicators were considered statistically significant at $p < 0.05$.

Results. In honey bees, body weight is an important physiological indicator on which many characteristics depend, including the ability to overwinter for a long time and the maximum supply of nectar to the nest. In a queen bee, body weight characterizes her ability to produce high eggs; in drones, it is an indicator of viability and fertilizing ability.

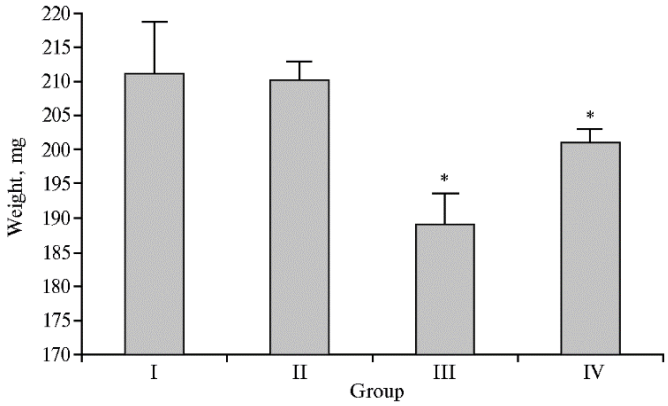

Fig. 1. Body weight of the Central Russian breed (Prioksky type) drones (*Apis mellifera* Linnaeus, 1758) after drone brood treatment with acaricidal preparations ($N = 3$, $n = 30$, M±SD; Rybnoye, Ryazan Province, 2021). For a description of the groups, see the Materials and methods section.
* Differences from control are statistically significant at $p < 0.05$.

In the studied groups, the body weight of drones varied significantly (Fig. 1). In the test groups it was 1-22 mg less than in the control. Statistically significant differences of 5-10% ($p < 0.05$) were established for drones from groups III and IV. Therefore, the negative effects of amitraz on the weight of drones, in contrast to fluvalinate and oxalic acid with thymol, was not observed.

To obtain freshly selected sperm and determine its quality indicators, 100 drones were selected into cages and the ratio of sexually mature drones to their total number was determined (Fig. 2). The largest proportion of sexually mature drones was in the control group. In the experimental groups this indicator decreased. The most noticeable decrease occurred in group III, by 10% vs. control.

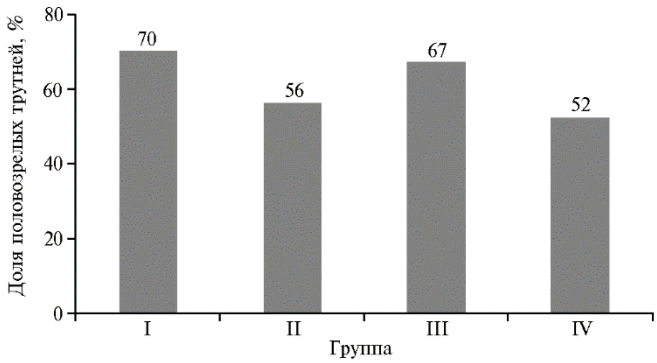

Fig. 2. Percentage of mature Central Russian breed (Prioksky type) drones (*Apis mellifera* Linnaeus, 1758) from the total number of drones after drone brood treatment with acaricidal preparations (n = 100; Rybnoye, Ryazan Province, 2021). For a description of the groups, see the Materials and methods section.

Quality parameters of freshly collected sperm of Central Russian breed (Prioksky type) drones (*Apis mellifera* Linnaeus, 1758) after drone brood treatment with acaricidal preparations (N = 3, n = 200, M±SD; Rybnoye, Ryazan Province, 2021)

Parameter		Group			
		I (control)	II	III	IV
Viability, %	M±SD	99.1±0.52	97.9±0.94	99.4±0.19	97.6±0.48
	Lim	98.3-100.0	96.8-99.7	99.2-99.7	96.7-98.2
	Cv, %	0.9	1.6	0.3	0.8
Deformation of sperm heads, %	M±SD	52.0±0.63	72.9±2.86	52.3±12.64	68.7±0.88
	Lim	51.0-53.1	69.5-78.7	28.8-71.7	67.1-70.2
	Cv, %	2.0	6.8	41.6	2.4
	td		7.06*	0.02	15.5*
Concentration, $\times 10^6$/μl	M±SD	1.9±0.12	1.1±0.21	1.8±0.63	0.7±0.05
	Lim	1.7-2.1	0.7-1.5	0.6-2.8	0.6-0.8
	Cv, %	11.2	38.7	61.7	13.6
	td		3.7*	0.2	9.2*

N o t e. For a description of the groups, see the Materials and methods section.
* The changes vs. control are statistically significant at $p < 0.05$.

The qualitative indicators of sperm, which determine its fertilizing ability, change depending on external factors. Important criteria in assessing the quality of drone sperm are viability, concentration, and the morphological structure of the sperm head (Table).

Sperm viability in drones did not have statistically significant differences vs. control. A slight decrease in sperm viability, by 1.2-1.5%, was detected in groups II and IV where minimal values of this indicator were also noted.

In groups II and IV, we found a statistically significant increase in the proportion of damaged sperm with deformed heads. By 1.4-1.3 times ($p < 0.05$) compared to the control group. It should be noted that the minimum number of damaged sperm in drone sperm was recorded in group I (control), which is significantly ($p < 0.05$) less than in the experimental groups, and is confirmed by the low value of the trait variability coefficient.

With rapid differential staining with a set of Diachim-Diff-Quick reagents, we discovered various anomalies of sperm heads in drones (Fig. 3).

In group II and group IV, the sperm concentration statistically significant ($p < 0.05$) decreased 1.8-fold and 2.7-fold vs. control, respectively, therefore, this significantly reduced sperm quality. It should be noted that lower sperm concentrations were noted in all experimental groups and, compared with group I, this indicator was on average 2.7 times lower, which confirms the negative impact of acaricides on the quality indicator of drone sperm.

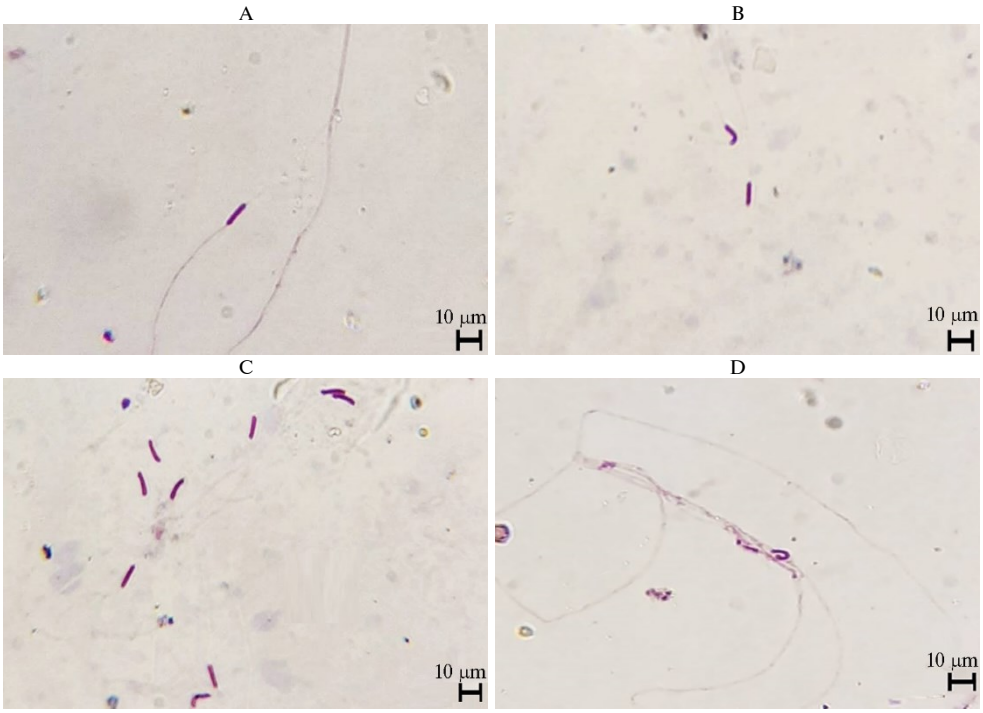

Fig. 3. Deformed heads of spermatozoa in the Central Russian breed (Prioksky type) drones (*Apis mellifera* Linnaeus, 1758) after drone brood treatment with acaricidal preparations: A — normal sperm head without deformation (control), B, C — pathologies of sperm heads in group II, D — deformations of sperm heads in group III (rapid differential staining with a set of reagents Diahim-Diff-Quick, OOO NPF ABRIS+, Russia; microscope MICROMED 3 LUME LED, OOO Observational devices, Russia, magnification ×400; Rybnoye, Ryazan Province, 2021). For a description of the groups, see the Materials and methods section.

Until now, the effect of anti-varroa drugs on honey bee drones has not been fully studied. Although detailed preparations have found fairly widespread use among beekeepers, information about their effect on the quality and reproductive properties of drones is extremely limited [31]. In the experiments of F.B. Abdelkader et al. [36] it was found that in the case of the drug with the active substance amitraz Rulamit-VA (TEKNOVET İLAÇ SANAYİ VE TİCARET ANONİM ŞİRKETİ, Turkey), used according to the manufacturer's instructions, high spermatozoa mortality and a high percentage of spermatozoa with impaired membrane integrity and acrosome defects were detected. Treatment with oxalic acid (spraying 5 ml per frame space) led to a decrease in the concentration and motility of spermatozoa and to violation of the acrosome integrity. A review article by J. Rangel and A. Fisher [30] provides information on the effect of acaricides on the parameters of drone sperm. Thus, in drones from bee colonies exposed to fluvalinate (20.4 mg/100 ml acetone), thymol (at concentrations below LD_{10}, the norm for worker bees) and amitraz (4.3 mg/100 ml acetone), the sperm concentration was lower than in other experimental groups and in the control. Drone mortality was higher in fluvalinate-treated colonies (66.9%) compared to untreated colonies (62.5%).

In our work, we also monitored the effect of acaricidal drugs on the fertility of drones, however, unlike foreign researchers, we sprayed 1-day-old drone larvae and evaluated the effect of the drugs at the larval stage of development.

Thus, the negative impact of acaricidal drugs, which include amitraz, fluvalinate and thymol with oxalic acid, on the development of drones of honey bees of the Central Russian breed and the quality of their sperm has been shown. The

body weight of drones significantly decreased, by 10-20 mg, afther treatment with fluvalinate and oxalic acid + thymol. Acaricides decrease sperm viability on average by 1.3%, sperm concentrations decreases 2.2-fold and the number of spermatozoa with deformed heads in the spermatheca is 1.3 times higher. All tested substances had an equally negative effect on drones. Therefore, acaricides should be used only for medicinal purposes when mite infestation is confirmed. The frequency of tretments should depend on the degree of invasion. To reduce the negative impact of drugs on the reproductive function of drones, do not exceed the doses indicated in the instructions.

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AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 2, pp. 355-372
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Intensive animal farming

UDC 636.4:591.473.31:59.086

doi: 10.15389/agrobiology.2023.2.355eng
doi: 10.15389/agrobiology.2023.2.355rus

EFFECT OF ADAPTOGENS ON MUSCLE TISSUE MICROSTRUCTURE OF HYBRID PIGS (*Sus scrofa domesticus* L.) DURING INTENSIVE FATTENING

A.A. SEMENOVA[1], T.G. KUZNETSOVA[1], V.A. PCHELKINA[1], V.V. NASONOVA[1], S.I. LOSKUTOV[1], N.V. BOGOLYUBOVA[2], R.V. NEKRASOV[2] ✉

[1]*Gorbatov Federal Research Center for Food Systems,* 26, ul. Talalikhina, Moscow, 109316 Russia, e-mail a.semenova@fncps.ru, labsens@mail.ru, v.pchelkina@fncps.ru, v.nasonova@fncps.ru, spbsl21@gmail.com;
[2]*Ernst Federal Research Center for Animal Husbandry,* 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail nek_roman@mail.ru (✉ corresponding author), 652202@mail.ru
ORCID:
Semenova A.A. orcid.org/0000-0002-4372-6448
Kuznetsova T.G. orcid.org/0000-0002-5164-1807
Phelkina V.A. orcid.org/0000-0001-8923-8661
Nasonova V.V. orcid.org/0000-0001-7625-3838
Loskutov S.I.orcid.org/0000-0002-8102-2900
Bogolyubova N.V. orcid.org/0000-0002-0520-7022
Nekrasov R.V. orcid.org/0000-0003-4242-2239
The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation, project No. 19-16-00068-П
Final revision received August 25, 2022
Accepted October 10, 2022

Abstract

At present, improving the quality of pork while increasing the production of pork is of generally recognized economic importance. Diets have been shown to affect the characteristics of muscle fibers. Adaptogens (vitamins and bioflavonoids) that animals need during active growth period can prevent myopathic transformation in meat. This paper for the first time reports on the improved microstructure of the *musculus longissimus dorsi* in crossbred pigs fed a complex of adaptogens (dihydroquercetin and vitamins E, C) during the fattening period. Our aim was to evaluate the effect of the complex of dihydroquercetin + vitamins E, C on the microstructure of meat muscle tissue in hybrid pigs. The experiments were carried out in 2021-2022 at Gorbatov Federal Research Center of Food Systems, Ernst Federal Research Center for Animal Husbandry and BMPK (Bryansk Province). Crossbred piglets (n = 108) (*Sus scrofa domesticus*) F2 [(Large White × Landrace) × Duroc] were randomly selected and assigned to two groups, n = 54 each, for a 58-day fattening. The control group were fed only a complex feed (SK-6, Russia), The test group was additionally fed an experimental dietary complex of adaptogens (DEC) that contains dihydroquercetin (DHQ, Ecostimul-2, JSC Ametis, Russia; 72-73 % DHQ, 32 mg/kg of feed), vitamin E (INNOVIT E60, GC MEGAMIX, Russia; 10 mg/kg feed) and vitamin C (Tiger C 35, Anhui Tiger Biotech Co. Ltd., China; 35 mg/kg feed). Young animals were weighed twice (on day 0 and day 58) by group weighing and individual weighing of 10 animals from each group. After slaughter, the paired carcass weight and the slaughter yield were assessed. To study the microstructure of muscle tissue, 45 min after slaughter, samples (3×3×3 cm) of the longest back muscle (*musculus longissimus dorsi*) were collected and fixed in 10 % neutral buffered formalin solution for 72 h at room temperature. For further study, two fragments (1.5×1.5×0.5 cm) of each sample with longitudinal and transverse orientations were washed with cold running water for 4 h, then compacted at 37 °C in gelatin (AppliChem GmbH, Germany) of ascending concentrations (12.5 and 25 %, for 8 h in each). Serial 16 μm sections were prepared using a MIKROM-HM525 cryostat (Thermo Scientific, USA), mounted on Menzel-Glaser glasses (Thermo Scientific, USA) and stained with Ehrlich's hematoxylin and 1 % eosin water-alcohol solution (BioVitrum, Russia). The histological preparations were examined and photographed using an AxioImaiger A1 light microscope (Carl Zeiss, Germany) with a connected AxioCam MRc 5 video camera (Carl Zeiss, Germany). Morphometric studies were performed using the AxioVision 4.7.1.0 image analysis program (Carl Zeiss, Germany). Muscle fiber diameter, sarcomere length, and giant fiber cross-sectional area were measured online. On cross sections, the shape of muscle fibers, their density, the state of the nuclei, the thickness and state of the connective tissue layers were investigated, and giant fibers were identified. On longitudinal sections, the state and shape of muscle fibers, the state of the sarcolemma, the presence of striation (transverse, longitudinal) and destructive changes (ruptures, cracks, fragmentation), hypercontraction nodes were identified. Despite the absence of statistically significant differences in live weight and

slaughter traits between groups during fattening, histological studies revealed significant differences in average values of muscle fiber density ($p = 0.02$) and sarcomere length ($p = 0.000007$). Almost a 2-fold decrease in the number of giant fibers in test group (11.60 vs. 21.30 pcs/cm^2) indicates a significant improvement in the tissue microstructure compared to the control animals that did not receive DEC. The less pronounced destructive changes in the sarcolemma in the test animals also indicate an increase in the animal stress resistance. Given a significant variability of morphometric parameters in both groups, we applied a scoring procedure which allows us to classify carcasses according to the severity of myopathic changes in muscle tissues based on the results of the muscle fiber microstructure study. In control, there was only one carcass that had no signs of myopathy; four carcasses showed signs of moderate myopathy and five carcasses showed signs of severe myopathy. On the contrary, in the test group, there were four carcasses without signs of myopathy and six carcasses with signs of moderate myopathy. There were no cases of severe myopathy in the study group. The groups differed statistically significantly ($p = 0.004$) in scores characterizing the severity of myopathic changes in muscle tissue. Our findings show that the dietary adaptogen complex DEC can provide the improvement of the microstructure of the muscle tissue and, therefore, has a positive effect on animal stress resistance and the degree of glycolysis in meat.

Keywords: adaptogen, dihydroquercetin, stress, young pigs, muscle tissue, giant fibers, contractile nodes, microstructure, histology

The growth of domestic pork production from 799.9 thousand tons in 2010 to 3254.92 thousand tons in 2021 was due to the industry's transition to intensive pig rearing, which was accompanied by a qualitative change in the livestock - a refusal to use traditional purebred animals. Modern intensive pig farming is characterized by the widespread distribution of hybrid fast-growing individuals, combined with a radical change in the conditions of their keeping and feeding. The main parameters of intensive production include a decrease in the age at which animals reach a live weight of 100 kg, and a high average daily weight gain [1-3]. At the same time, the quality of slaughter products, primarily pork, has changed.

Genetic progress has increased the stress on the body of fast-growing slaughter animals and led to morphological and biochemical modifications of muscle tissue, worsening the consumer and technological characteristics of meat [4)] In the carcasses of modern hybrid pigs, the content of muscle tissue exceeds 50% (wt). As the main and most nutritionally valuable part of the carcass, muscle tissue is considered the main component determining the quality of meat, which is formed during muscle metabolism before and after the death of the animal [5, 6].

Advances in the field of intensive feeding of hybrid pigs have led to the emergence of pathological features in the microstructure of muscle tissue, reducing the value of pig carcasses. The increased stress inherent in hybrid animals during transportation and lairage was considered to be the main reason for these changes [7]. The development of porcine stress syndrome leads to the fact that post-mortem redox processes in pork are characterized by increased glycolysis of muscle tissue, producing meat with signs of PSE (pale, soft and exudative)/myopathy [8]. Such meat is characterized by histopathological abnormalities in the muscles and the appearance of destructive changes (ruptures of the sarcolemma), changes in the shape of muscle fibers, the appearance of atrophied, as well as hypertrophied and giant fibers [9, 10].

In this regard, in recent years there has been renewed interest in studying the microstructural characteristics of muscle tissue of slaughter animals, including the types and properties of muscle fibers, their density and size [11-13]. It has been proven that the characteristics of muscle fibers are directly influenced by both genetic factors (breeds, lines, hybridization), as well as the growth rate of the animal and the final mass fraction of muscle tissue in the carcass [14, 15]. In turn, the characteristics of muscle tissue determine the technological and consumer quality of pork, including tenderness, juiciness and color [16]. A relationship has been shown between the rapid growth of hybrid animals and the high incidence of myopathic changes in muscle tissue, which reduce the quality of pork [17].

Muscle fibers are key components of skeletal muscle, the characteristics of which significantly influence the quality of meat [18, 19]. Histological changes in muscle tissue lead to changes in meat quality [20]. One of the signs of changes in the microstructure of muscle tissue is the appearance of giant fibers. It has been established that giant fibers are found exclusively in postmortem muscles, and most researchers believe that they arise because of overcompression of a portion of the muscle fibers [21-23]. The cause of the appearance of giant fibers is the depletion of some fibers even before slaughter and the very rapid development of rigor mortis in them, while neighboring fibers continue to remain in a relaxed state [24, 25].

Giant fibers in cross-sections of muscles are characterized by a rounded shape and a large cross-sectional area. They are most often located at the edge and, with less frequency, within the primary muscle bundles [22, 26, 27]. Giant fibers stain more intensely with eosin compared to surrounding normal fibers. In longitudinal sections, giant fibers exhibit partial to complete loss of myofibril structure as a result of hypercontraction and fiber disintegration [16, 28]. Increased giant fibers are associated with inhumane handling of animals prior to slaughter [29-31], genetic profile, and breed [31, 32]. The frequency and size of giant fibers determine the decline in pork quality [33, 34].

However, it is believed that giant fibers may not be evident in muscle tissue. For example, surrounding fibers can passively hold the affected fibers in a stretched state, preventing them from transforming into giant ones. Many authors note muscle fiber density/total number of fibers per unit cut area as an important factor determining meat quality [18, 19, 34]. This parameter is related to the diameter of the muscle fibers. On the one hand, fibers of smaller diameter are especially desirable, since they have a beneficial effect on the quality of meat and are considered an indicator of its tender structure [35], on the other hand, the presence of fibers of small diameter may be a sign of muscular dystrophy [36]. Obviously, taking into account significant deviations from the average muscle fiber diameter can be critical for objective assessment of meat quality based on histological studies [37]. Therefore, analysis of not only giant fibers, but also other morphological characteristics of muscle tissue is important [22, 25, 26].

The economic importance of improving pork quality while continuing to increase pork production is now recognized [4]. It has been shown that the choice of diet affects the characteristics of muscle fibers [38], and in preventing the appearance and development of myopathic changes in meat, an important role is played by adaptogen substances, the vitamins and bioflavonoids that animals need during the period of active growth [39-42]. Reducing the risk of receiving low-quality meat can be achieved through the use of adaptogenic drugs in vivo, which increase the stress resistance of animals and ensure optimal glycolysis in meat [34]. The search for adaptogens that act as regulators of the directional development of muscle tissue can become the main way to ensure the quality of meat while intensifying its production.

This paper is the first to report, on the example of hybrid pigs, the positive effect of a complex of adaptogens dihydroquercetin and vitamins E and C fed during the fattening period on the microstructure of *musculus longissimus dorsi*.

The purpose of the work is to assess the influence of the complex of adaptogens dihydroquercetin and vitamins E, C on the microstructural characteristics of muscle tissue of meat obtained from hybrid pigs.

Materials and methods. The experiments were carried out in 2021-2022 at Gorbatov Federal Research Center for Food Systems RAS, Ernst Federal Research Center VIZh and OOO Bryansk Meat Processing Plant (Bryansk Province).

For the experiment, 108 crossbred piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] were assigned. Live weight at the beginning of the experiment was 60-65 kg, age was 120 days. The fattening took 58 days. Experiments were performed in accordance with the principles of good laboratory practice [43-47].

All animals were kept in the same zoohygienic conditions [48] and had free access to feed and water throughout the entire study period. Feeding was carried out from group self-feeders with regard to current standards [49].

The piglets were randomly divided into two groups (control and experimental, 54 pigs in each). The control group received only SK-6 feed, balanced in nutrients and energy as recommended [49]. The experimental group was additionally fed with an experimental complex of adaptogens (DEC), containing dihydroquercetin (DHQ, Ecostimul-2, JSC Ametis, Russia; 72-73 % DHQ, 32 mg/kg of feed), vitamin E (INNOVIT E60, GC MEGAMIX, Russia; 10 mg/kg feed) and vitamin C (Tiger C 35, Anhui Tiger Biotech Co. Ltd., China; 35 mg/kg feed. The DKVES mixture was prepared in laboratory conditions and mixed with crushed wheat grain.

At a feed mill (LLC Bryansk Meat Processing Plant, Bryansk Province), a pilot batch of feed added with the DEC was produced in compliance with the approved recipes. To prepare feed with an adaptogen, the calculated amount of the complex DHQ + vitamins was weighed and mixed with the dry ingredients of the feed in a mixer, ensuring its uniform application. The addition of DEC in the amount of 0.25 kg/t of SK-6 feed did not affect the consumption of energy and nutrients. The choice of dosages was based on previously obtained own data and a generalization of current information on the problem (for more complete information, see the RF patent).

The young animals were weighed twice, before the start of the experiment and on day 58. Individual and group weighing was carried out. For comparative analysis, control slaughter and sampling, control 10 fattening pigs from each group were used (Nos. 1-10). Individual weighing was carried out during fattening and before slaughter. The control slaughter was carried out at an industrial enterprise (OOO Bryansk Meat Processing Plant).

Immediately before slaughter, the live weight (LW) of pigs after fasting was determined. After the slaughter, the weight of the carcass was determined, for treatment 1, the weight of the carcass with the head, legs and tail; for treatment 2, the weight of the carcass without the head, legs and tail). The slaughter yield was calculated as the ratio of the carcass weight to the live weight before slaughter.

For the microstructure study, 3×3×3 cm fragments of the *musculus longissimus dorsi* were taken 45 minutes after slaughter. The samples were fixed in a 10% neutral buffered formalin solution for 72 hours at 22 °C. For the study, two 1.5×1.5×0.5 cm fragments with longitudinal and transverse orientation of muscle fibers from each sample were washed with cold running water for 4 hours and compacted in 12.5% and 25% gelatin solutions (AppliChem GmbH, Germany) at 37 °C, for 8 hours in each concentration.

Serial sections with a thickness of 16 μm were prepared on a MIKROM-HM525 cryostat (Thermo Scientific, USA). Three sections were prepared from each fragment. The resulting sections were mounted on Menzel-Glaser glasses (Thermo Scientific, USA) and stained with Ehrlich hematoxylin and 1% aqueous-alcoholic eosin solution (BioVitrum, Russia) as described [50]. Histological preparations were examined and photographed (an AxioImaiger A1 light microscope with a connected AxioCam MRc 5 video camera, Carl Zeiss, Germany) [51].

For morphometric studies, the image analysis program AxioVision 4.7.1.0 (Carl Zeiss, Germany) was used. Muscle fiber diameter, sarcomere length, and

giant fiber cross-sectional area were measured. At least 100 objects were analyzed for each section. The fiber diameter was determined with an accuracy of ±1.0 μm. The length of sarcomeres was measured with an accuracy of ±0.1 μm. The number of giant fibers per 1 cm^2 section and the density of muscle fibers per 1 mm^2 were calculated.

On cross sections, the shape of muscle fibers, the density of their arrangement, the state of the nuclei, the thickness and condition of the connective tissue layers were determined, and giant fibers were identified. On longitudinal sections, the condition and shape of muscle fibers, the state of the sarcolemma, the presence of striations (transverse, longitudinal), the presence of destructive changes (ruptures, cracks, fragmentation) were determined, and hypercontraction nodes were identified.

Statistical analysis was carried out using R software (version 4.2.1). Quantitative data are presented as arithmetic means (*M*), standard errors of the mean (±SEM), standard deviations (±SD), minimum and maximum values (min/max), confidence intervals (CI) and median (*Me*). The normality of the distribution of parameters of quantitative variables was assessed using the Kolmogorov-Smirnov test. Identification of the relationship between the studied factor and morphometric parameters of muscle tissue was carried by one-way analysis of variance (ANOVA) and Dunnett's test. The differences were considered statistically significant and a relationship between the indicators was accepted at a p-level not exceeding 0.05.

Results. Animals from the control and experimental groups did not differ in either growth dynamics or slaughter indices ($p > 0.05$) (Tables 1, 2).

1. Average bodyweight of pigs (*Sus scrofa domesticus*) F2 [(Large White × Landrace) × Duroc] fed a complex of adaptogens dihydroquercetin and vitamins E, C (*M*±SEM, OOO Bryansk Meat Processing Plant, Bryansk Province, 2021-2022)

Group	Average bodyweight, kg	
	group weighing (*n* = 54)	individual weighing (*n* = 10)
	Day 0	
Control	62,41	65,7±1,4
Test	61,93	63,5±1,1
	Day 58	
Control	113,26	112,6±1,6
Test	114,33	114,1±2,1

Note. For a description of the groups, see the Materials and methods section.

2. Slaughter prameters of pigs (*Sus scrofa domesticus*) F2 [(Large White × Landrace) × Duroc] fed a complex of adaptogens dihydroquercetin and vitamins E, C (*M*±SEM, OOO Bryansk Meat Processing Plant, Bryansk Province, 2021-2022)

Group	Average carcass weight, kg		Average slaughter weight, kg	
	treatment 1 (*n* = 10)	treatment 2 (*n* = 10)	treatment 1 (*n* = 10)	treatment 2 (*n* = 10)
Control	90.7±5.1	83.0±4.7	80.5±1.3	73.6±1.3
Test	90.7±5.0	83.2±4.6	79.5±1.2	73.0±1.3

Note. For a description of the groups and treatments, see the Materials and methods section.

Nevertheless, piglets from the experimental group grew more intensively than their peers in the control group (by 6.8%, or 929.7 versus 870.7 g/day). Of the control group, 98.1% aminals remained alive vs. 100% of the test group, which confirmed the positive role of the studied nutritional factor. In both groups, carcasses did not have statistically significant differences in slaughter yield regardless of calculation mode. This indicated insignificant differences in the dynamics of animal development during the fattening period and similar mass of the resulting carcasses, both with and without by-products (head, legs, and tail).

Figure 1 shows the steps of morphometric analysis of muscle tissue in piglets.

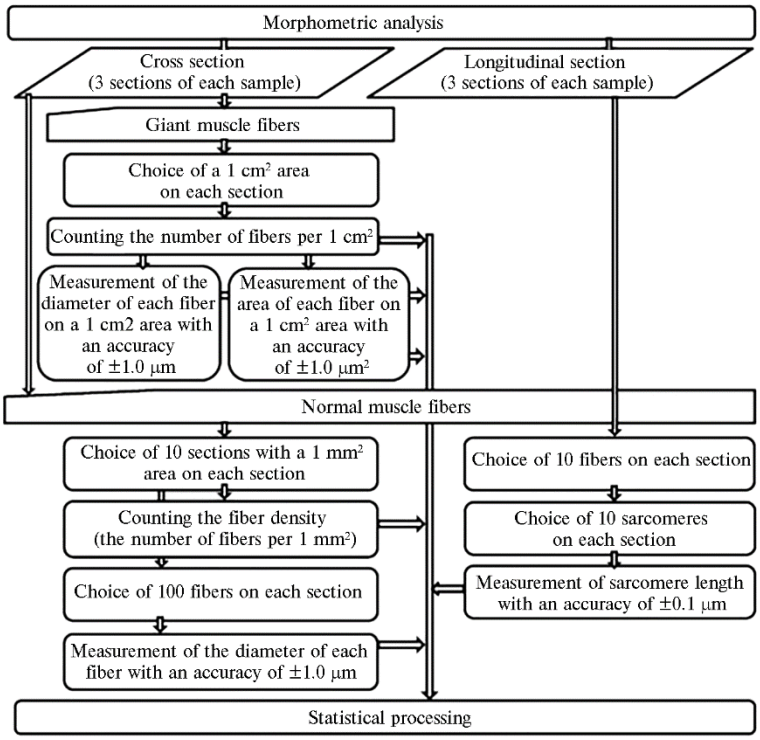
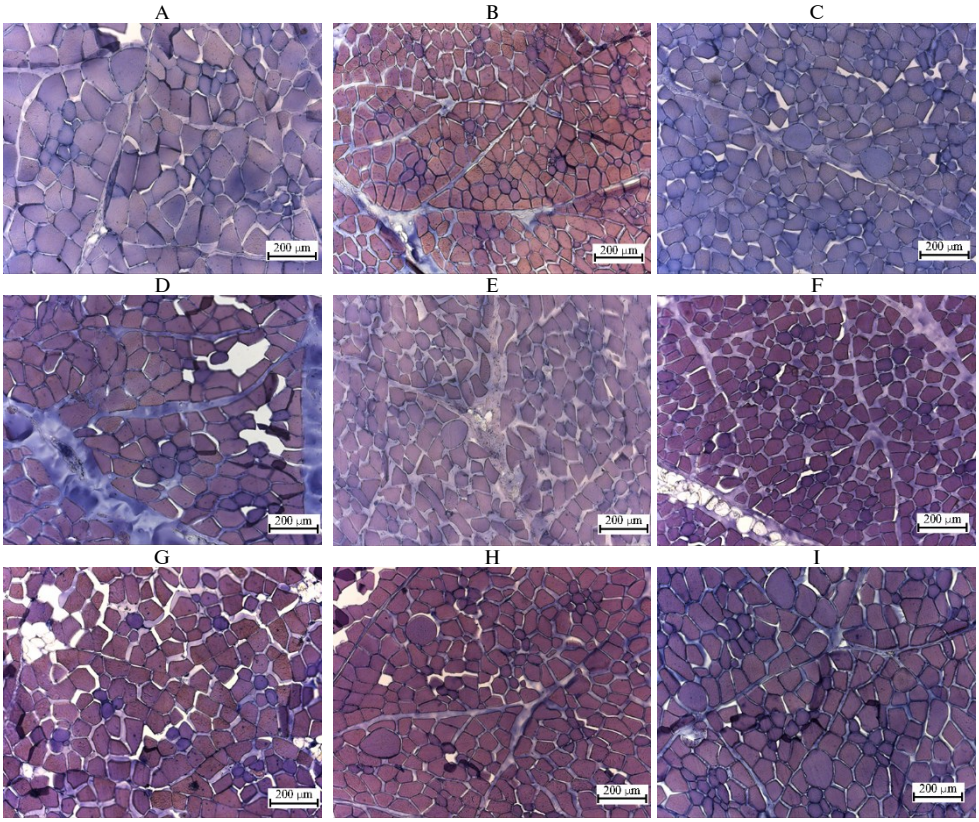


Fig. 1. The sheme of morphometric studies of muscle tissue in pigs (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] fed a complex of adaptogens dihydroquercetin and vitamins E, C (Gorbatov Federal Research Center for Food Systems RAS, 2022).



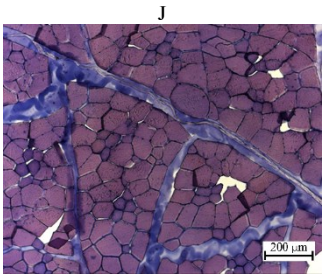


Fig. 2. Cross sections of *musculus longissimus dorsi* of crossbred piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] from the control group fed SK-6 feed: A-J — samples from carcasses Nos. 1-10 (hematoxylin and eosin staining, objective magnification ×10, eyepiece ×10, microscope AxioImaiger A1, Carl Zeiss, Germany; Gorbatov Federal Research Center for Food Systems RAS, 2022).

Despite the fact that all animals were raised, fattened and slaughtered under the same conditions, differences in the muscle tissue were observed in piglets from the control and experimental groups. Figures 2 and 3 show representative photographs of the microstructure of each of the 10 samples in the control and test groups. Figure 4 shows the structure of muscle tissue with giant fibers.

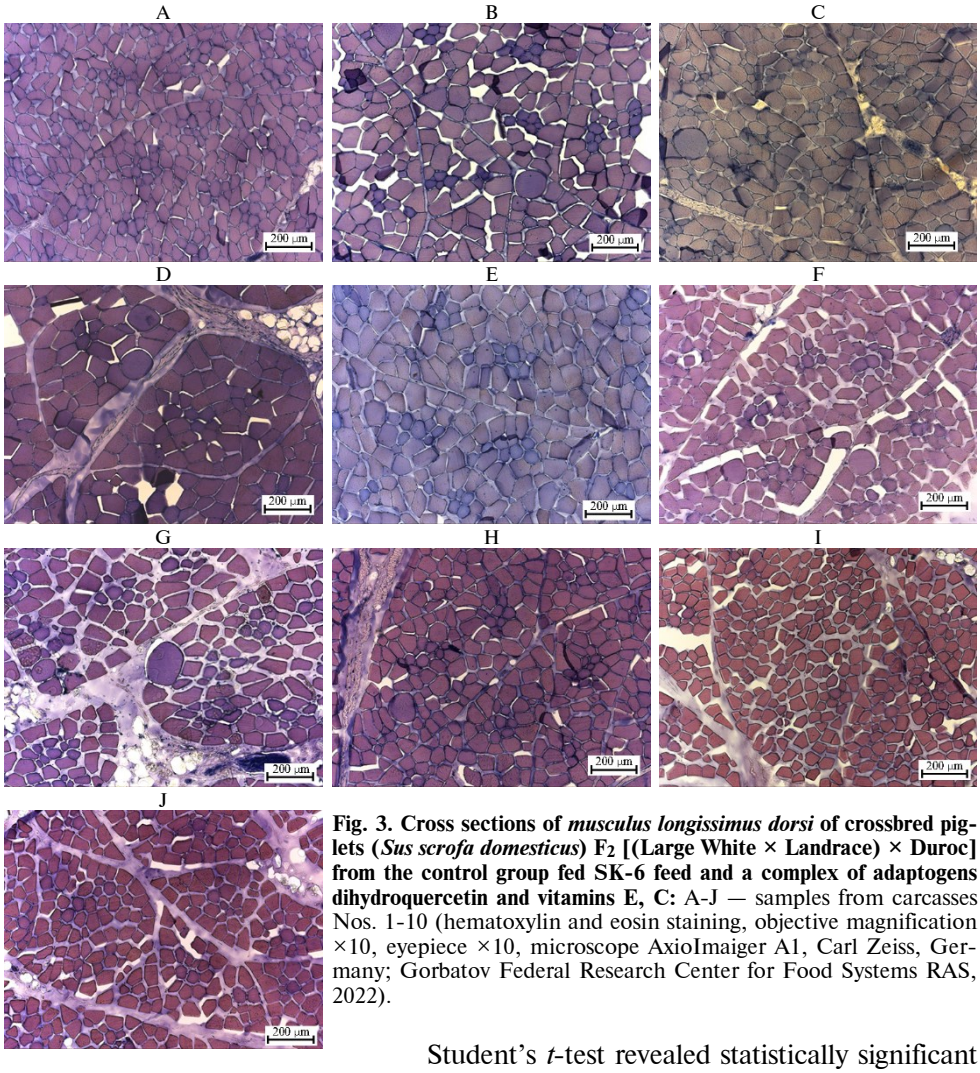


Fig. 3. Cross sections of *musculus longissimus dorsi* of crossbred piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] from the control group fed SK-6 feed and a complex of adaptogens dihydroquercetin and vitamins E, C: A-J — samples from carcasses Nos. 1-10 (hematoxylin and eosin staining, objective magnification ×10, eyepiece ×10, microscope AxioImaiger A1, Carl Zeiss, Germany; Gorbatov Federal Research Center for Food Systems RAS, 2022).

Student's *t*-test revealed statistically significant differences between the control and test groups in the average length of sarcomeres ($p = 0.000007$) and in the density of muscle fibers ($p = 0.02$) (Table 3). The medians of these prameter in the test group were greater (2.1 μm and 201/mm^2) than in the control (1.9 μm and 177/mm^2), that is, the test group had a denser arrangement of fibers and a longer sarcomere length (Fig. 5, 6).

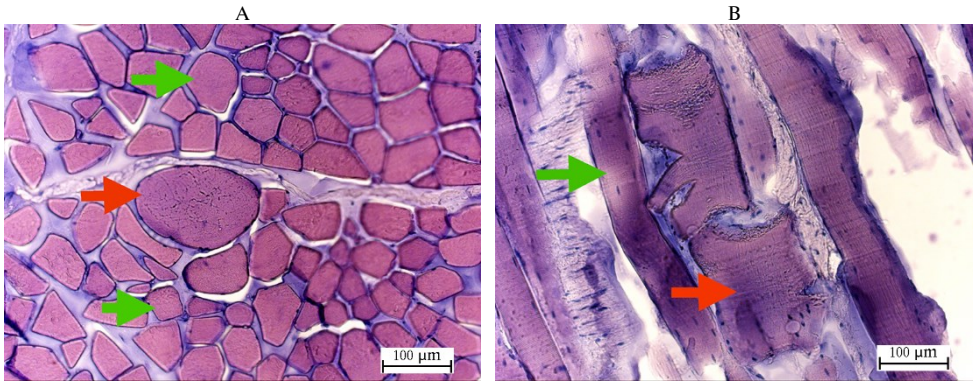


Fig. 4. Giant (red arrow) **and normal** (green arrow) **fibers in transverse** (A) **and longitudinal** (B) **sections of *musculus longissimus dorsi* of crossbred piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc].** Hematoxylin and eosin staining, objective magnification ×20, eyepiece ×10, microscope AxioImaiger A1, Carl Zeiss, Germany; Gorbatov Federal Research Center for Food Systems RAS, 2022).

3. Morphometric tissue parameters of musculus longissimus dorsi in crossbred piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Gorbatov Federal Research Center for Food Systems RAS, 2022)

Parameter		Group	
		comtrol	trst
Muscle fiber diameter, µm	k	1000	1000
	M±SD	56.8±16.7**	55.4±15.6**
	min-max	17.5-121.6	16.8-107.1
	Me	55.1	53.7
	SEM	0.5	0.5
	95 % CI	55.7-57.8	54.4-56.3
Sarcomere length, µm	k	100	100
	M±SD	1.9±0.2*	2.1±0.3*
	min-max	1.5-2.2	1.6-2.7
	Me	1.9	2.1
	SEM	0.02	0.03
	95 % CI	1.9-1.9	2.1-2.2
Muscle fiber density, number per unit cross section area (mm^2)	k	30	30
	M±SD	182±39*	206±40*
	min-max	124-265	146-286
	Me	177	201
	SEM	7	7
	95 % CI	169-198	189-218
Diameter of giant fibers, µm	k	213	116
	M±SD	123.7±14.7	121.9±15.6
	min-max	91.7-169.1	91.6-177.6
	Me	122.5	119.5
	SEM	1.0	1.5
	95 % CI	120.6-124.5	118.8-124.5
Area of giant fibers in cross section, μm^2	k	213	116
	M±SD	11999±2892	11850±3129
	min-max	6597-22460	6588-24774
	Me	11784	11206
	SEM	198	291
	95 % CI	11572-12337	11222-12358

N o t e. k — number of observations, *M* — mean, SD — standard deviation, min-max — minimum and maximum values, *Me* — median, SEM — standard error of the mean, CI — confidence interval. For a description of groups and options, see the Materials and methods section.
* and ** Differences from control are statistically significant at $p < 0,05$ and $p = 0,05$, respectively.

When examining histological sections in samples from the control and experimental groups, 213 and 116 giant fibers were identified, respectively, but no significant differences were established between the control and experimental groups in either the average diameter or the average area of giant fibers.

Average values for groups observed in biological experiments are usually not indicative of varying parameters. Even though group means are the same, traits may differ significantly in the degree and pattern of variation in specific samples within each group [53]). In our experiment, the most significant variation was

observed in the average diameter and density of muscle fibers, as well as the average area and number of giant fibers (Table 4).

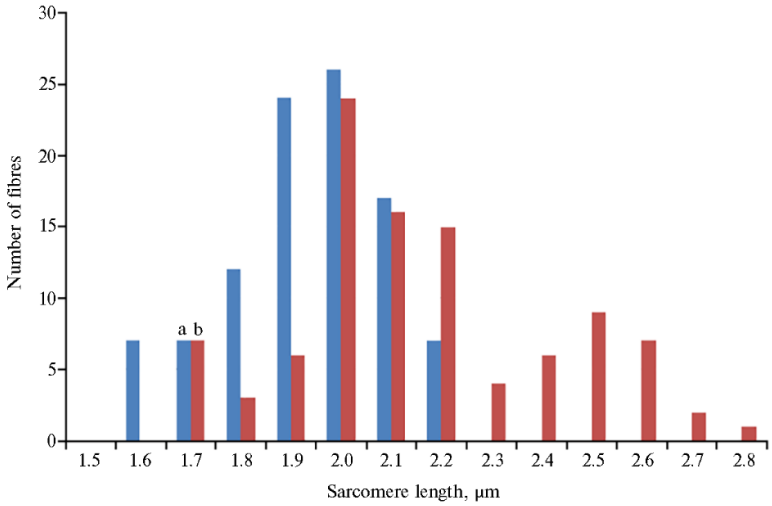


Fig. 5. Distribution of fibers by the length of sarcomeres in *musculus longissimus dorsi* of crossbred piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] in control (a) **and when fed a complex of adaptogens dihydroquercetin and vitamins E, C** (b) (Gorbatov Federal Research Center for Food Systems RAS, 2022)**.**

4. Coefficient of variation (%) **of *musculus longissimus dorsi* morphometric parameters in crossbred piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] fed a complex of adaptogens dihydroquercetin and vitamins E, C** (n = 10; Gorbatov Federal Research Center for Food Systems RAS, 2022)

Parameter	Group	
	control	test
Average muscle fiber diameter	29.39	31.12
Average sarcomere length	8.51	12.32
Muscle fiber density, number per unit cross section area	21.42	19.42
Average diameter of giant fibers	12.00	12.81
Average area of giant fibers on a cross section	24.11	26.40
Giant fibers density, number per unit cross section area	70.83	100.00

N o t e. For a description of groups and options, see the Materials and methods section.

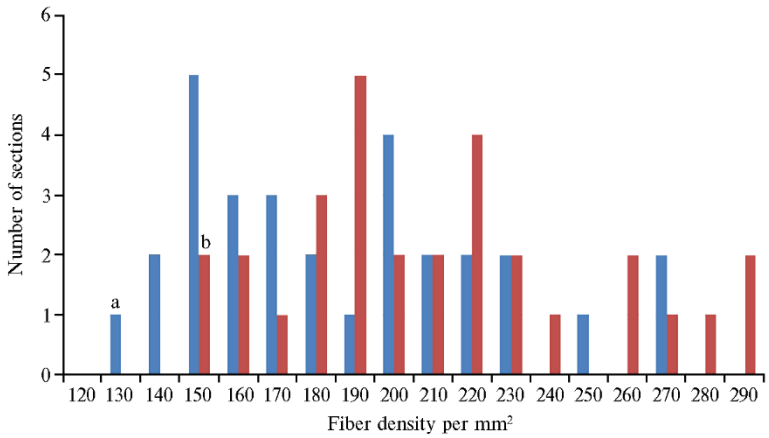


Fig. 6. Distribution of sections by the density of muscle fibers in *musculus longissimus dorsi* of crossbred piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] in control (a) **and when fed a complex of adaptogens dihydroquercetin and vitamins E, C** (b) (Gorbatov Federal Research Center for Food Systems RAS, 2022)**.**

There was also a tendency towards significant differences between the control

and experimental groups in the average diameter of muscle fibers (p = 0.05), which varied respectively within the range of 55.7-57.8 and 54.4-56.3 μm. Moreover, the median in the experimental group was less than in the control group (53.7 vs. 55.1 μm), which indicated a finer-fiber structure of the samples from the experimental group. However, the nature of the distribution of the values of this indicator for the samples of the control and experimental groups was similar (Fig. 7).

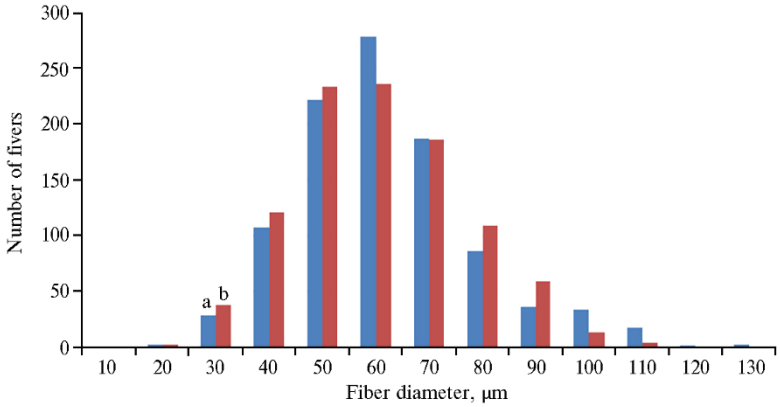
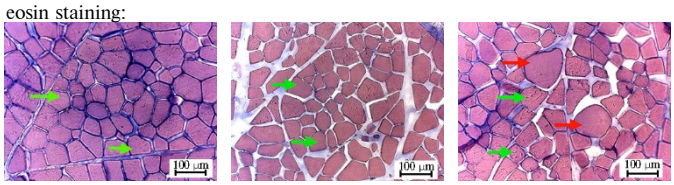


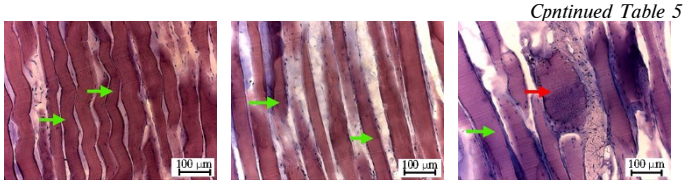
Fig. 7. Distribution of muscle fibers by diameter in *musculus longissimus dorsi* of crossbred piglets (*Sus scrofa domesticus*) F2 [(Large White × Landrace) × Duroc] in control (a) **and when fed a complex of adaptogens dihydroquercetin and vitamins E, C** (b) (Gorbatov Federal Research Center for Food Systems RAS, 2022)**.**

Given a significant variability of morphometric parameters, as well as the advisability of using, along with numerical data, a number of descriptive histological characteristics, we adapted and applied a semi-quantitative method based on scoring, developed jointly by the Gorbatov Federal Scientific Center for Food Systems RAS and Federal Research Center for Livestock Husbandry — Ernst VIZh [52] (Table 5).

5. Scheme for scoring the myopathy expression based on microstructural images of *musculus longissimus dorsi* of crossbred piglets (*Sus scrofa domesticus*) F2 [(Large White × Landrace) × Duroc]

Parameter	Description (value) of the indicator/points assigned		
	no myopathy signs	moderate myopathy	severe myopathy
Shape of muscle fibers	Slightly wavy, tightly spaced/1	Mostly straight, tightly spaced/2	Straight, lie freely in relation to each other/3
State of transverse striation	Clearly expressed/1	Small, close together, smoothed, uneven/2	Small, close together, smoothed, uneven/2
Average sarcomere length, rm	From 2.0 (inclusive) and more/1	1.6-1.9 (inclusive)/2	Less than 1.6/3
The presence of destructive changes in the sarcolemma		There are single ruptures of the sarcolemma/2	There are multiple breaks in the sarcolemma/3
Presence of giant fibers (contraction nodes), pcs/cm^2	Not detected/1	From 10 (inclusive) to 30/2	From 30 (inclusive) and more/3
Average area of giant fibers on a cross section, rm^2	Not detected and/or single detected (up to 10)/1	From 10,000 (inclusive) to 15,000/2	From 15,000 (inclusive) and more/3
Density of muscle fibers (number per unit area of cross section), pcs/mm^2	Up to 10,000/1	From 150 (inclusive) to 250/2	Up to 150/3
The proportion of muscle fibers whose diameter is less than or greater than $^1/_3$ of the average fiber diameter, %	From 250 (inclusive) and more/1	From 8 (inclusive) to 30/2	From 30 (inclusive) and more/3
Microstructure by hematoxylin and eosin staining: cross section			

longitudinal section



N o t e. Muscle tissue without signs of myopathy, with destructive changes corresponding to the normal development of autolytic processes, included samples that did not have a score of 3 points for any indicator and scored no more than 12 points inclusive. Muscle tissue with moderately expressed myopathy included samples that scored from 13 to 16 points inclusive; samples with moderate myopathy could not contain or contain individual giant fibers in the muscle tissue. Photos of the microstructure of such muscle tissue without giant fibers are shown above. Samples scoring over 16 points were classified as muscle tissue with pronounced signs of myopathy. In the photographs, green arrows indicate normal fibers, red arrows indicate giant fibers (staining with hematoxylin and eosin, objective magnification ×20, eyepiece ×10, AxioImaiger A1 microscope, Carl Zeiss, Germany).

According to the results of the scoring of the microstructure of muscle tissue (Table 6), in the control group only 1 sample was found that had no signs of myopathy, 4 samples had signs of moderate myopathy and 5 samples had signs of severe myopathy. In the test group, there were 4 samples without signs of myopathy, and 6 samples with signs of moderate myopathy. In the experimental group, not a single sample with severe myopathy was identified.

6. Individual scoring of *musculus longissimus dorsi* microstructure in crossbred piglets (*Sus scrofa domesticus*) F2 [(Large White × Landrace) × Duroc] fed a complex of adaptogens dihydroquercetin and vitamins E, C (*n* = 10; Gorbatov Federal Research Center for Food Systems RAS, 2022)

Parameter	Group	Scores									
		1	2	3	4	5	6	7	8	9	10
Shape of muscle fibers	C	1	1	2	2	2	3	3	2	2	2
	T	2	2	2	1	1	3	1	1	3	3
State of transverse striation	C	1	1	1	1	1	1	1	2	2	2
	T	1	1	1	1	1	1	1	1	1	1
Average sarcomere length	C	2	2	2	2	2	1	2	2	2	3
	T	1	1	1	2	2	1	1	2	1	1
Destructive changes in the sarcolemma	C	3	1	3	1	2	1	1	3	3	3
	T	1	3	1	1	2	1	1	1	1	1
Presence of giant fibers (contraction nodes)	C	3	1	3	1	2	1	1	3	3	3
	T	1	3	2	1	1	2	2	1	1	1
Average area of giant fibers on a cross section	C	2	1	2	3	1	3	3	1	2	2
	T	1	2	2	2	2	1	2	2	1	1
Density of giant fibers (number per unit cross section area)	C	3	1	2	3	2	2	3	2	2	2
	T	1	2	2	2	2	2	3	2	1	2
Proportion of muscle fibers whose diameter is less than or greater than 1/3 of the average fiber diameter	C	2	2	2	2	2	2	2	2	2	3
	T	2	2	2	2	2	2	2	2	2	1
Total points	C	17	10	17	15	14	14	16	17	18	20
	T	10	16	13	12	13	13	13	12	11	11
Conclusion about the signs of myopathy	C	S	N	S	M	M	M	M	S	S	S
	T	N	M	M	N	M	M	M	N	N	N

N o t e. C — control group, T — test group; N — no myopathy signs, M — moderate myopathy, S — severe myopathy. For a description of groups and options, see the Materials and methods section.

Statistical processing of the results of scoring the severity of signs of myopathy in muscle tissue samples (Table 7) showed that carcasses from the control and experimental groups had significant differences in the average length of sarcomeres ($p = 0.006$) and the presence of destructive changes ($p = 0.04$). According to the average number of points (see Table 7), reflecting the overall severity of myopathy, both groups differed significantly ($p = 0.004$). Nevertheless, based on the average number of points, both the control and experimental groups should be classified as samples of muscle tissue with moderately severe myopathy. However, the difference was obvious: the control group approached the state of severe myopathy (over 16 points), and the experimental group approached the state without

signs of myopathy (up to 12 points inclusive).

7. Average score (in points) **of the myopathy severity in crossbred piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] fed a complex of adaptogens dihydroquercetin and vitamins E, C** (n = 10, M±SEM; Gorbatov Federal Research Center for Food Systems RAS, 2022)

Parameter	Group	
	control	test
Shape of muscle fibers	2.0±0.2	1.9±0.3
State of transverse striation	1.3±0.2	1.0±0.0
Average sarcomere length	2.0±0.3*	1.3±0.2*
The presence of destructive changes in the sarcolemma	2.1±0.3*	1.3±0.2*
Presence of giant fibers (contraction nodes)	2.1±0.3	1.5±0.2
Average area of giant fibers on a cross section	2.0±0.2	1.6±0.2
Muscle fiber density (number per unit cross section area)	2.2±0.2	1.9±0.2
Proportion of muscle fibers whose diameter is less than or greater than $^1/_3$ of the average fiber diameter	2.1±0.1	1.9±0.1
Average points	15.8±0.9*	12.4±0.5*

N o t e. For a description of groups and options, see the Materials and methods section.
* Differences from control are statistically significant at $p < 0.05$.

An increasing number of myopathies have been consistently reported in animals raised intensively for meat. At the same time, there is a decline in the consumer quality of meat products [53, 54], which leads to huge economic losses for the industry. In our study, only one carcass was identified in the control group, where we did not find signs of myopathy in the muscle tissue, while half of the examined samples were classified as muscle tissue with severe myopathy. The high incidence of myopathic changes in the control group indicates the presence of a problem and confirms the concern of many researchers about the decline in the consumer characteristics of meat.

Recently, the use of adaptogens to reduce the effects of oxidative stress caused by factors associated with animal husbandry and slaughter has become increasingly popular in livestock and poultry farming [55, 56]. Polyphenols of plant origin, fat- and water-soluble vitamins are considered as adaptogens-antioxidants [57, 58]. Their effectiveness in in vivo experiments is judged mainly by blood parameters characterizing the antioxidant status of individuals receiving the adaptogen, as well as by some organoleptic qualities of meat that are important from the consumer's point of view, for example, the color of muscle tissue [59]. However, very few studies have been devoted to the study of the microstructure of muscle tissue in meat depending on the antioxidant status of animals. In this regard, our study is of interest in both theoretical and practical aspects.

To increase the antioxidant status of crossbred piglets, we used a complex of adaptogens - dihydroquercetin, vitamins E and C, which was selected based on an analysis of literature data and our own research. In the experimental group, we observed an improvement in the microstructure of muscle tissue. The muscle fibers were located more densely, the transverse striations were more pronounced, the length of the sarcomeres increased, and fewer destructive changes in the sarcolemma of the muscle fibers were observed. Muscle fibers are the key element of muscle tissue. The connection between these changes in microstructure and improved meat quality was noted in the works of other authors [60, 61]. The predominance of fibers of small and medium diameter without destructive features helps to improve the quality of meat [62]. Changes in the number and diameter of muscle fibers entail changes in meat quality [63].

The characteristics of muscle fibers depend on genetic factors [64]. Intensively growing animals obtained through two- and three-breed crossings are inferior to slow-growing purebred individuals in terms of muscle fiber density [60, 61, 63]. A positive effect of increased values of this indicator on the quality of meat

is noted [62]. In our experiment, due to the nutritional factor in animals of the same genetic origin, the F_2 [(Large White × Landrace) × Duroc] the density of muscle fibers was increased by 14% which can be considered a significant effect.

Giant fibers should be viewed as pathological consequences of past stress rather than as an additional type of normal muscle cell [23]. The presence of a large number of giant fibers in muscle tissue is also more typical for hybrid animals, suggesting the development of the PSE defect and low consumer characteristics of meat [62]. In samples of muscle tissue from piglets from the experimental group, we detected 46% fewer giant fibers.

Thus, when feeding crossbred piglets with a complex of adaptogens dihydroquercetin and vitamins E, C (DEC), significant differences were revealed between samples of the control and experimental groups in the average length of sarcomeres, the density of muscle fibers, the presence of destructive changes in the sarcolemma and the overall score of the severity of myopathic changes. The nature of the identified differences indicated an improvement in the condition of muscle tissue in animals receiving the adaptogen complex. It should be noted the effect of DEC on reducing the number of giant fibers which indicates positive changes in muscle metabolism before and after slaughter. In our experiment, factors such as genetic, breed, conditions of keeping and handling of animals were excluded. That is, the reduction in the risk of obtaining low-quality meat in the experimental group was achieved only through the use of DEC in vivo, which allows us to conclude that the adaptogen complex influences the stress resistance of animals and glycolysis in meat. The decrease in the number of giant fibers in the experimental group by almost 2 times (11.6 vs. 21.3/cm^2 in the control) indicated a significant improvement in their microstructure. The less pronounced nature of destructive changes in the sarcolemma in the experimental group also indicated an increase in the stress resistance of animals. Our studies of the muscle microstructure give reason to believe that the problem of reducing pork quality in fast-growing hybrids can be solved by coorrectly added small doses of dietary adaptogens.

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AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 2, pp. 373-385
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.52/.58:636.085.8

doi: 10.15389/agrobiology.2023.2.373eng
doi: 10.15389/agrobiology.2023.2.373rus

THE EFFECTIVENESS OF VARIOUS FORMS OF Zn AS STIMULATORS OF THE IMMUNE RESPONSE IN BROILER CHICKENS (*Gallus gallus* L.)

E.A. SIZOVA[1] ✉, S.A. MIROSHNIKOV[2], K.S. NECHITAILO[1]

[1]*Federal Research Centre of Biological Systems and Agrotechnologies RAS,* 29, ul. 9 Yanvarya, Orenburg, 460000 Russia, e-mail Sizova.L78@yandex.ru (✉ corresponding author), k.nechit@mail.ru;
[2]*Orenburg State University*, 13, prosp. Pobedy, Orenburg, 460018 Russia, e-mail sergey_ru01@mail.ru
ORCID:
Sizova E.A. orcid.org/0000-0002-5125-5981 Nechitailo K.S. orcid.org/0000-0002-8755-414X
Miroshnikov S.A. orcid.org/0000-0003-1173-1952
The authors declare no conflict of interests
Acknowledgements:
Supported financially from the Russian Science Foundation (project No. 20-16-00078)
Final revision received June 28, 2022
Accepted August 12, 2022

Abstract

A significant problem of modern poultry farming is insufficient viability of broiler chickens due to various reasons, including immunosuppression. Various biologically active substances and trace elements are successfully used to increase the adaptive capacity and immunoreactivity of birds. The use of zinc (Zn) in feed additives which has immunotropic properties, stimulates immune and antioxidant systems, increases the productivity and safety of animals is of undoubted interest. Here, for the first time the influence of zinc from different sources on natural resistance and morphofunctional reorganization of immunocompetent organs of Smena 7 cross broiler chickens (*Gallus gallus* L.) was shown. These data also point to the advantage of organic and ultrafine dietary forms of zinc for chick feeding compared to the inorganic form. Our goal was to compare the effectiveness of using different forms of zinc in the diet as modulators of the immune system based on biochemical parameters and characterization of the microstructure of immunocompetent organs. Studies were performed on broiler chickens of Smena 7 cross (three groups, 24 animals in each group) in the vivarium of the Federal Research Center of Biological Systems and Agrotechnologies RAS (Orenburg). Sources of trace elements were asparaginate Zn (organic form, OF; LLC V-Min+, Sergiev Posad, Russia), mineral salts $ZnSO_4 \cdot 7H_2O$ (inorganic form, IF; Lenreaktiv, St. Petersburg, Russia) and powder of ultradispersed Zn particles (UDP Zn; LLC Advanced Powder Technologies, Tomsk, Russia). Chickens in the control group received a basic diet throughout the experiment, in which Zn was normalized by the introduction of $ZnSO_4 \cdot 7H_2O$. In the test groups from day 14 to day 42 Zn sulphate was replaced with UDP Zn at a dose of 49 mg/kg feed (group I) or with asparaginate Zn at the same dose (group II). Samples for analysis were collected after poultry slaughtering at 3, 4, 5, and 6 weeks of age. Biochemical studies of blood serum were performed on an automatic analyzer CS-T240 (DIRUI Industrial Co., Ltd., China) using commercial kits for veterinary research DiaVetTest (JSC Diakon-DS, Russia). Morphological composition was determined using an automatic hematological analyzer URIT-2900 Vet Plus (URIT Medial Electronic Co., Ltd., China). Iindices of natural resistance, i.e., the bactericidal activity of blood serum (BABS), lysozyme activity (AL), β-lysine activity (Aβ-L), and immunological indices, the phagocyte number (PN) and phagocytic index (PI) were evaluated. Morphological characteristics of cloacal bursa (CB), thymus and spleen were determined on 5-6 microns thick histological sections stained with hematoxylin and eosin. General structural changes were assessed on paraffin sections stained with hematoxylin and eosin using a light-optical microscope with MT 5300L software (Meiji Techno Co., Ltd., Japan). The area of the follicle and medullary region, the width of the cortical zone were determined in the CB sections; the area of red and white pulp, cell density of red and white pulp in the spleen; the area of cortical and brain substance, their ratio (cortical index), cell density of red and white pulp in the thymus. The area of structures was determined on 125,000 μm^2, density on 1 mm^2. At 3 weeks of age, chickens of group II showed a 37.5 % ($p \leq 0.05$) increase in leukocytes compared to control. By 4 weeks of age, the index also increased by 40.7% ($p \leq 0.05$) when UDP Zn was fed. The increase was due to lymphocytes and monocytes. In the test groups, by the end of the experiment, the number of leukocytes was lower than the control. All indicators of white blood cells were within the normal range. Introduction of UDP Zn contributed to an increase in BABS in the range from

5.8 % to 16.7 % and AL by 8.2 % in the later stages of the experiment compared to control. The tendency to an increase in Aβ-L was recorded at 5 weeks of age with a subsequent decrease by the end of the experiment. Application of OF Zn led to a statistically significant ($p \leq 0.01$) increase in the activity of BABS by 13.4 % at 3-weeks of age and AL at the end of the experiment (by 8.8 %). The OF Zn provided a smooth progressive increase in Aβ-L throughout the experiment. Histological evaluation revealed that dietary OF Zn increased CB activity with an increase in lymphoid follicle area by 64.5 % ($p \leq 0.01$) due to expansion of medulla zones, as well as increased cellular density of the cortical zone with a large number of macrophages and mitoses. In the thymus, together with a 20.9 % expansion of the medullary zone, there was a greater number of Hassall's corpuscles, thickening of cells in the cortical layer and proliferative activity of lymphocytes with activation of macrophages. In the spleen after the introduction of UDP Zn, enlargement of follicles by increasing the area of white pulp 2.2-fold ($p \leq 0.05$) was noted. Thus, Smena 7 broiler chickens fed organic and ultradisperse Zn additives exhibit higher blood bactericidal activity and activity of lysozyme and β-lysines, which indicates the stimulation of natural resistance. The response of the immune system to Zn was also more pronounced for dietary UDP Zn and OF Zn compared to inorganic form.

Keywords: ultrafine particles, chickens, feeding, zinc, immune system, thymus, spleen, cloacal bursa, microstructure

In modern poultry farming, mortality among broiler chickens remains a problem which is due, in particular, to immunosuppression. The reasons may be imbalances in diets, physical inactivity, overcrowding, technological stress, as well as high antigen load during immunization. To increase adaptive capacity and immunoreactivity, in recent years biologically active substances have been successfully used, namely, vitamins [1], amino acids and trace elements [2]. Of undoubted interest is zinc as feed additive, which has immunotropic properties, stimulates the activity of the immune and antioxidant systems, and increases the productivity and safety of animals [3].

Feed additives contain Zn of different digestibility, i.e., inorganic, organic and chelated Zn sourses [4). Because elements in inorganic compounds can form insoluble forms that are not absorbed [5], the bioavailability of Zn is key to meeting the body's requirement for this element. Due to its low bioavailability, the inorganic form (IF) of Zn can cause a deficiency of the element in the body, which leads to a decrease in the number of T-cells, the production of interleukins and, as a consequence, inhibition of cellular and humoral immunity [6].

A promising approach may be the use of nanoforms of microelements [7, 8] which have unique properties that allow them to be competitive sources of chemical elements for mineral additives [9, 10]. A number of studies confirmes the high efficiency of metal nanoforms compared to inorganic salts and other sources [11-13].

The feasibility of using Zn in potentiating cell-mediated immune responces [14] as an inducer of phagocytosis and stimulator of a humoral response [15) has been shown. Zn is critical for the development and maintenance of immune function. The amount of Zn is a critical factor that influences antiviral immunity [16]. Therefore, Zn may be useful in immunotherapy as an additional immunoadjuvant. A search query using the keywords "zinc, immunity" in PubMed (https://pubmed.ncbi.nlm.nih.gov/) over the past 20 years yields more than 4900 references, which clearly demonstrates the diverse role of Zn in the functioning of the immune system. However, the effectiveness of Zn depends on many factors, including the form of the substance.

Here, we demonstrate for the first time the influence of different zinc sources on natural resistance and morphofunctional reorganization of immunocompetent organs of broiler chickens (*Gallus gallus* L.) Smena 7 cross. The sourses of organic and ultrafine zinc are superiior to the inorganic zinc.

This work aimes to compare the blood serum biochemical parameters and the microstructure of immunocompetent organs of Smena 7 broiler chickens in

order to assess the effectiveness of various dietary zinc supplements as immunomodulators.

Materials and methods. Experiments were carried out in accordance with the recommendations of the Guide for the care and use of laboratory animals of National Research Council (US) Institute for Laboratory Animal Research (Guide for the care and use of laboratory animals. National Academies Press, Washington DC, 1996). All methods were approved by the Bioethics Commission of the Federal Scientific Center for Biological Systems and Agricultural Technologies RAS (protocol No. 3 of March 21, 2018).

The sources of microelements were Zn aspartate (organic form, OF; V-Min+ LLC, Sergiev Posad, Russia), mineral salts $ZnSO_4 \cdot 7H_2O$ (inorganic form, IF; Lenreaktiv, St. Petersburg, Russia) and powder ultrafine Zn particles (UFF Zn; OOO Advanced Powder Technologies, Tomsk, Russia). Zn UFFs were produced by electric explosion of a wire in an argon atmosphere. Certification of materials included electron scanning and transillumination microscopy using JSM 7401F and JEM-2000FX instruments (JEOL, Japan). X-ray phase analysis was performed on a DRON-7 diffractometer (NPO Burevestnik, Russia). According to the certification results, the particles had a hydrodynamic radius of 164±31.2 nm, Z-potential was 25±0.5 mV, specific surface area 5±1.6 m^2/g. To obtain lyosols, aqueous suspensions of Zn UFF were treated in an ultrasonic dispersant UZDN-2T (NPP Academpribor, Russia) at 35 kHz, 300/450 W, 10 μA for 30 min. The resulting lyosols were stepwise-mixed with the feed.

Broiler chickens of the Smena 7 cross (n = 72) were kept in the vivarium of the Federal Scientific Center for Biological Systems and Agricultural Technologies RAS (Orenburg). At the age of 2 weeks, birds analogues by weight were assigned to three treatments (one group for control feeding and two groups for test feeding, n = 24 each). Feeding was carried out according to recommendations for age periods [17].

Chickens of the control group throughout the experiment received a basal diet in which Zn was normalized by introducing $ZnSO_4 \cdot 7H_2O$. In the test groups, from day 14 to day 42, Zn sulfate was replaced with Zn UDP at 49 mg/kg of feed (group I) or with Zn aspartate at the same dosage (group II). The dosage was based on the recommendations [17], however, given the bioavailability of elements from organic and ultrafine sourses, the values were reduced by 30%.

At the age of 3, 4, 5 and 6 weeks, 6 chickens from each group were killed. Blood was sampled from the axillary vein. Biochemical studies of blood serum were comducted in accordance with the manufacturers' protocols (a CS-T240 automatic analyzer, DIRUI Industrial Co., Ltd, China; commercial kits for veterinary research DiaVetTest, AO Diakon-DS, Russia). The blood morphology was assessed using an automatic hematology analyzer URIT-2900 Vet Plus (URIT Medial Electronic Co., Ltd, China). Serum bactericidal activity (BABS, bactericidal activity of blood serum), lysozyme activity (AL), activity of β-lysines (Lβ-A), and immunological parameters, the phagocyte number (PN) and phagocytic index (PI) were measured as described [18].

After slaughter at the age of 42 days, tissue samples for morphological analysis of the cloacal bursa (CB), thymus, and spleen were obtained. The samples were fixed in 10% neutral formalin and embedded in the HistoMix paraffin mixture (BioVitrum, Russia). Histological sections 5-6 μm thick were prepared on a semi-automatic microtome MVP 01 (KB Technom, Russia) and stained with hematoxylin and eosin [19]. General structural changes were examined by light optical microscopy with MT 5300L software (Meiji Techno Co., Ltd, Japan).

On the CB sections, the follicle and medullary zone areas and the cortical zone width were measured, in the spleen, the ares of the red and white pulp and

the density of red and white pulp cells were measured; in the thymus, the area of the cortex and medulla, their ratio (cortical index), and the density of red and white pulp cells were measured. The areas of the structures were determined using a 125,000 μm^2 counting grid, the density was assessed for a 1 mm^2 area. Laboratory tests were conducted at the Center for Collective Use of Biological Systems and Agricultural Technologies RAS (CCU BST RAS).

Statistica 10.0 software package (StatSoft, Inc., USA) and Microsoft Excel were used for statistical data processing. The mean values of the parameters (*M*) and standard errors of the means (±SEM) were calculated. Differences between groups were assessed by Student's *t*-test. If the distribution differed from normal, the Mann-Whitney U test was applied. Differences were considered significant at $p \leq 0.05$.

Results. At 3 weeks of age, when Zn was added (group II), chickens showed an increase in the number of blood leukocytes by 37.5% ($p \leq 0.05$) compared to the control. By 4 weeks of age, the number of leukocytes also increased by 40.7% ($p \leq 0.05$) in poultry fed UDP Zn. The increase occurred due to lymphocytes and monocytes. Thus, at 3 weeks of age, the difference in the number of lymphocytes was 10.3% for group I and 8.7% for group II. The counts of monocytes differed significantly ($p \leq 0.05$) only in favor of group II at 4 weeks of age (Table 1). A similar increase in the number of blood lymphocytes is observed with increased immunoreactivity of the birds, since lymphocytes serve as the main executive link in the cellular and humoral defense of the body. An increase in the number of monocytes when using OF Zn may indicate activation of the monocyte-phagocytic system and, consequently, an increase in the protective properties of broiler chickens [20]. By the end of the experiment, the counts of leukocytes in the test groups were lower than the control. All white blood cell parameters were within the normative values.

1. Counts of the blood white cells in broiler chicken (*Gallus gallus* L.) cross Smena 7 of different ages when fed various forms of Zn (n = 6, M±SEM, the vivarium of the Federal Scientific Center for Biological Systems and Agrotechnologies RAS, Orenburg, 2020)

Group	Age, weeks			
	3	4	5	6
Leukocytes, $\times 10^9$/l (norm 20.0-40.0)				
Control (IF)	18.45±1.850	24.30±1.921	26.33±2.998	35.73±1.307
I test (UDP)	22.30±1.188	34.20±2.112*	25.83±0.581	27.27±1.946*
II test (OF)	25.37±1.159*	27.33±1.700	28.60±1.517	30.90±1.361
Lymphocytes, % (norm 52-70)				
Control (IF)	79.55±4.950	85.70±1.343	89.17±1.281	88.70±1.102
I test (UDP)	87.80±1.686*	87.93±2.331	87.37±1.235	89.47±1.674
II test (OF)	86.53±1.317*	90.23±1.441*	88.27±1.906	89.07±1.667
Monocytes, % (norm 4,0-10,0)				
Control (IF)	5.30±0.900	6.67±0.176	6.20±0.529	6.33±0.491
I test (UDP)	6.80±0.436	6.63±0.996	6.70±0.436	6.03±0.203
II test (OF)	6.20±0.721	7.60±0.173*	6.33±0.393	6.23±0.120

Note. IF — inorganic form, UDP — ultra-dispedsed partiles, OF — organic form. For a description of the groups, see the Materials and methods section.
* Differences from control are statistically significant at $p \leq 0.05$.

Administration of the organic Zn (group II) led to a statistically significant ($p \leq 0.05$) increase in BABS at 3 weeks of age by 13.4% and at the end of the experiment by 10.1% compared to the control. The introduction of UFF Zn (group I) contributed to an increase in BABS in the later stages of the experiment: at 4 weeks of age by 16.7% ($p \leq 0.01$), at 5 weeks of age by 14.6% ($p \leq 0.01$), by the end of the experiment the difference was 5.8%.

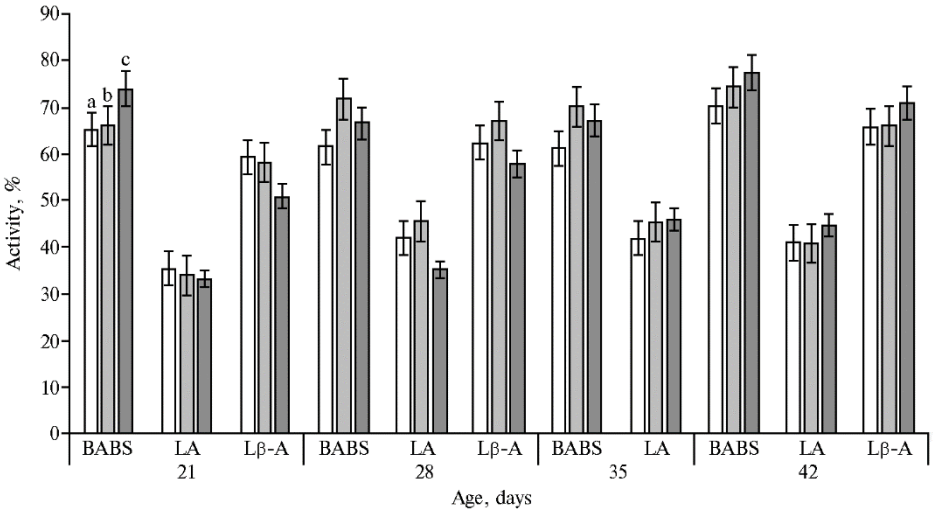

Fig. 1. Age-dependent parameters of nonspecific resistance of broiler chickens (*Gallus gallus* L.) cross Smena 7 fed different forms of Zn: BABS — bactericidal activity of blood serum, LA — lysozyme activity, Lβ-A — activity of β-lysines; a — control, b — test group I, c — test group II (n = 6, M±SEM, the vivarium of the Federal Scientific Center for Biological Systems and Agrotechnologies RAS, Orenburg, 2020). For a description of the groups, see the Materials and methods section.
*, ** Differences from control are statistically significant at $p \leq 0.05$ and $p \leq 0.01$.

Some white blood cells, in particular monocytes, serve as producers of nonspecific body defense factors, including lysozyme [21]. In chickens fed UDP Zn, serum AL increased at 4 weeks of age by 8.5% ($p \leq 0.05$), at 5 weeks of age by 8.2% ($p \leq 0.05$) compared to the control. Organic Zn added to the diet increased AL by 10.0% ($p \leq 0.05$) and 8.8% ($p \leq 0.05$) at 5 weeks and 6 weeks of age, respectively (Fig. 1). An increased blood lysozyme activity in birds of different ages contributes to an increase in their nonspecific resistance.

Various forms of Zn also influenced the activity of β-lysines. In birds fed UDP Zn, this parameter increased by 16.1% ($p \leq 0.05$) at 5 weeks of age followed by a decrease towards the end of the experiment. OF Zn provided a smooth increase in activity throughout the experiment (see Fig. 1). Being an important serum bactericidal systems, β-lysines are thermostable and selective against gram-positive bacteria. β-Lysine is a cationic protein of platelet origin [22]. An increased activity of β-lysines in the test groups may indicate a higer bird's resistance to gram-positive microorganisms [23].

The effect of Zn on the defense response was realized through an increase in AL, activity of β-lysines and, therefore, in bactericidal activity of blood serum as an integral indicator of humoral resistance which indicates the ability of the blood to self-purify. A decrease in serum bactericidal activity is more often than its increase, which is typical mainly for various stressful situations, violations of feeding and housing conditions, and in diseases [24]. Organic and UDP Zn stimulated serum bactericidal activity to a greater extent than inorganic Zn.

Zn contributed to an increase in phagocytic activity, expressed as PN. In group I, PN increased by 10.4 ($p \leq 0.05$) and 19.5% ($p \leq 0.05$) vs. control at 3 and 4 weeks of age, respectively. In group II, PN exceeded control by 8.6% ($p \leq 0.05$) at 4 and 5 weeks of age (Fig. 2).

The intensity of phagocytosis (PI) in test group I exceeded control values at 4 weeks of age by 19.1% ($p \leq 0.05$), and decreased by the end of the experiment. In group II, the differences vs. control were 16.6% at 3 weeks of age ($p \leq 0.05$), and by the end of the experiment, PI decreased by 12.2% vs. control (see Fig. 2).

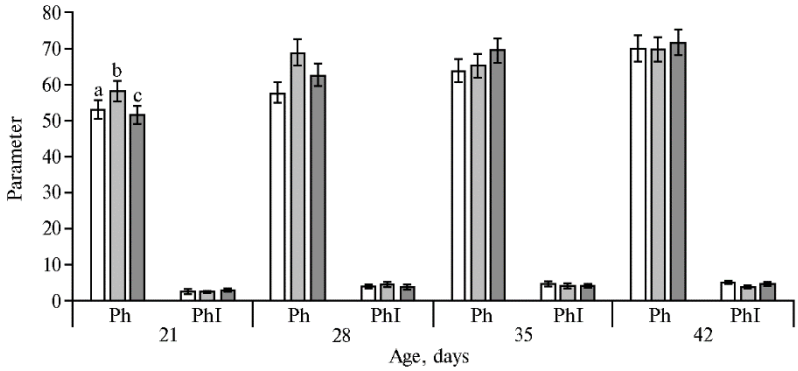

Fig. 2. Age-dependent blood phagocytic activity in broiler chickens (*Gallus gallus* L.) cross Smena 7 fed different forms of Zn: Ph — the number of phagocytes (PN) involved into phagocytosis, %, PhI — phagocytic index (PI); a — control, b — test group I, c — test group II ($n = 6$, $M \pm$SEM, the vivarium of the Federal Scientific Center for Biological Systems and Agrotechnologies RAS, Orenburg, 2020). For a description of the groups, see the Materials and methods section.
* Differences from control are statistically significant at $p \leq 0.05$.

Along with factors of nonspecific resistance, the histostructure of immunocompetent organs is informative to evaluate cause-and-effect relationships and the functioning of the immune system. Since CB plays a key role in the formation of poultry immunity, the study of its morphofunctional characteristics in connection with the search for effective methods to optimize mineral nutrition is among the most important tasks.

In chickens of group I, the microstructure of the CB (Fig. 3, A) differed from the control (see Fig. 3, B) in the moderate enlargement of follicles by 15.4% ($p \leq 0.05$) (Table 2) with an increase in the cortical zone width. There were numerous mitoses in the lymphoid cells of the cortical zone. In the cells of the glandular epithelium, small vacuoles were visible, elongated nuclei were located basally, and moderate focal proliferation occurred. Small cysts were detected.

The general histological structure of CB in experimental group II differed from the control by an increase in the area of the follicles by 64.5% ($p \leq 0.01$), mainly due to an increase in the area of their medullary zone (up to 82.7%) (see Table 2), as well as a more dense population of cells in both zones. Small cysts were found in individual follicles. In the narrow cortical zone, a large number of eosinophilic granulocytes were noted (see Fig. 3, B). Among the lymphoid cells, many macrophages and mitotically active cells were identified. The medullary zone was wide. An increase in the area of the follicular apparatus and a decrease in the connective tissue of the CB stroma indicated the activation of the reaction and, as a consequence, an increase in the adaptive reserve. It is known that Zn can improve the productivity and histomorphology of immune organs [25].

The spleen of mammals is primarily considered as a blood depot, while the spleen of birds serves exclusively as an immunocompetent organ [26]. In spleen of chickens from group I, we revealed functional activation in the white pulp microstructure, namely, the enlargement of follicles, an increase in their number, and a 2.3 times more dense arrangement of cells vs. control ($p \leq 0.001$) with a predominance of mature lymphocytes, lymphoblasts, plasma and macrophage cells. The area of the white pulp increased by 2.2 times ($p \leq 0.05$) (see Table 2). On the contrary, the activity of the red pulp decreased, as indicated by the presence of zones with depletion of cellular contents and exposure of the organ stroma (see Fig. 3, D). Macrophages were identified in large numbers among the predominant lymphoid cells of the pulp. The content of lymphoblasts and plasma cells decreased markedly.

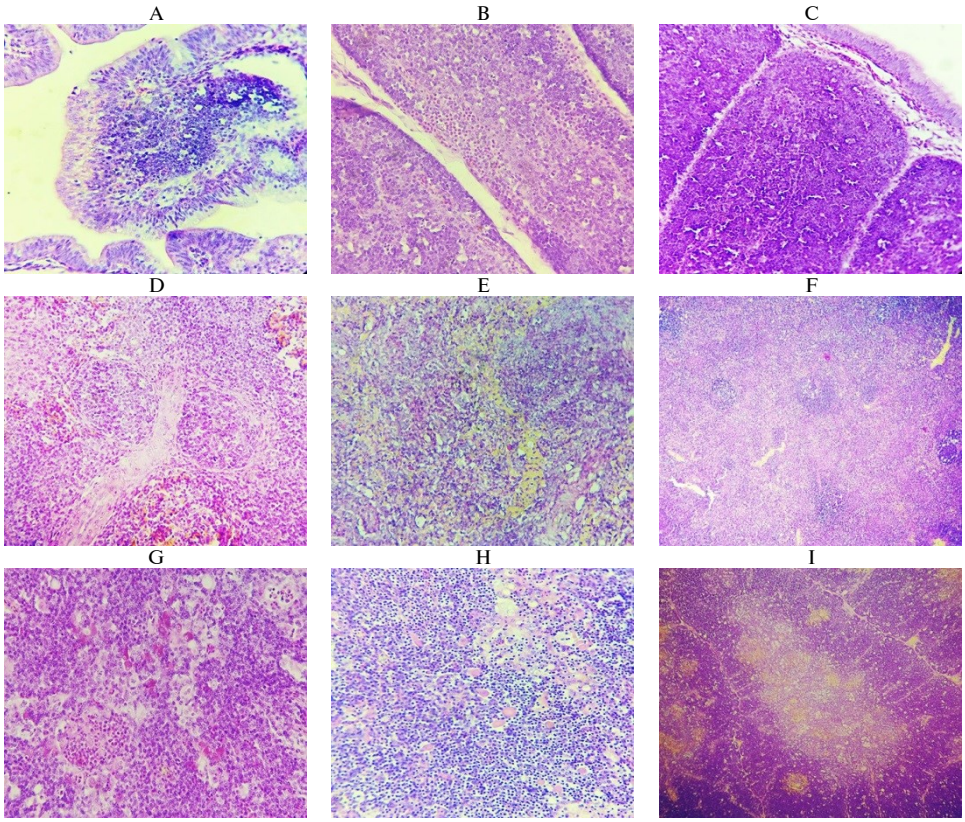

Fig. 3. Microstructure of the cloacal bursa (A, B, C)**, spleen** (D, E, F) **and thymus** (G, H, I) **in 42-day-old broiler chickens (*Gallus gallus* L.) cross Smena 7 fed different forms of Zn** (the end of the experiment)**:** A, D, G — test group I (A — increased epithelial proliferation, ×100; D — clear boundaries of the follicles, plethora of the red pulp with areas of cellular sparseness, ×400; G — cortical layer with accumulation of macrophages, eosinophilic granulocytes, ×400); B, E, H — test group II (B — wide medullary zone, a large number of eosinophilic granulocytes in a narrow cortical zone, ×400; E — hyperplasia of lymphoid tissue without clear boundaries with red pulp, ×400; H — a large number of lymphoid cells and Hassall bodies, ×400); C, F, I — control group (magnification ×100) (the vivarium of the Federal Scientific Center for Biological Systems and Agrotechnologies RAS, Orenburg, 2020). Hematoxylin and eosin staining, a MT 5300L microscope (Meiji Techno Co., Ltd, Japan).

The morphofunctional state of the spleen in chickens from test group II (see Fig. 3, E) indicated a higher activity of both zones of the parenchyma compared to the control (see Fig. 3, F). The area of the white pulp increased 1.7 times ($p \leq 0.05$). The density of follicle cells increased by 70.5% ($p \leq 0.001$) compared to the control; macrophage cells and immature forms of lymphocytes were present among them (see Table 2). In the red pulp, general plethora and increased cellular density were noted. Given active participation of the spleen in immune responses, reactive structural changes in this organ are natural [25]. In our experiment, similar changes occurred to a greater extent when chickens were fed organic Zn.

In the thymus histostructure of chickens from group I (see Fig. 3, G) vs. control (see Fig. 3, I), the area of the medullary zone was 20.9% larger ($p \leq 0.05$) and the cell density was higher in both the cortex and medulla by 31.4 ($p \leq 0.001$) and 37.5% ($p \leq 0.01$), respectively. In the cortical layer, there was a significant number of elements of the macrophage system with visible minimal degenerative changes, forming small clusters. Signs of proliferative processes in the lymphatic cells of the cortical zone were noticeable. The medullary zone was characterized by a small number of small-sized Hassall bodies.

2. Morphometric parameters of the cloacal bursa, spleen and thymus microstructures in 42-day-old broiler chickens (*Gallus gallus* L.) cross Smena 7 fed different forms of Zn (n = 6, M±SEM, the vivarium of the Federal Scientific Center for Biological Systems and Agrotechnologies RAS, Orenburg, 2020)

Parameter	Group		
	control (IF)	test I (UDP)	test II (OF)
Cloacal bursa			
Follicle area, mm^2	0,039±0,0010	0,045±0,0026	0,063±0,0018**
Area of the medullary zone of the follicle, mm^2	0,029±0,0008	0,032±0,0015*	0,053±0,0019**
Proportion of the medullary zone in the follicle, %	74,7±1,32	70,9±0,99	83,6±1,69
Width of the cortical zone of the follicle, μm	34,16±1,081	57,00±0,950***	32,50±0,510
Spleen			
Red pulp area, mm2	0,175±0,0121	0,089±0,0061*	0,118±0,0081
White pulp area, mm2	0,075±0,0081	0,162±0,0080*	0,133±0,0082*
Red pulp cell density	2051,2±56,10	1856,0±49,23	2329,6±62,83
White pulp cell density	1865,6±52,33	4201,6±87,21***	3180,8±54,63***
Thymus			
Area of the cortical zone, mm^2	0,665±0,0281	0,545±0,0113*	0,626±0,0126
Brain zone area, mm^2	0,583±0,0184	0,705±0,0158*	0,624±0,0191
Cortical index	1,14±0,093	0,77±0,019	1,00±0,030
Cortical cell density	2808,0±59,21	3690,7±51,73***	4805,3±65,01***
Medulla cell density	1749,3±44,91	2405,3±50,13**	2728,0±60,63**

Note. IF — inorganic form, UDP — ultra-disperded particles, OF — organic form. For a description of the groups, see the Materials and methods section.
*, **, *** Differences from control are statistically significant at $p \leq 0,05$, $p \leq 0.01$, and $p \leq 0.001$

The histostructure of the thymus in group II (see Fig. 3, H) turned out to be generally similar to that for group I. There was a higher immunological activity of the thymus, expressed in a larger medullary zone, a higher cell density in the cortical and medullary zones, by 71.9 ($p \leq 0.001$) and 55.9% ($p \leq 0.001$) (see Table 2), respectively, and the number of mitotic cells was higher compared to the control.

The described effects could be the result of good absorption of Zn from OF and UDP in the intestines of broiler chickens. In addition, apparently, OF Zn better overcomes antagonistic and other inhibitory factors of the digestive system, therefore the boundaries of the absorption fund of the element expand [27].

The works of other authors have shown that dietary correction with Zn [28, 29] and other substances, pre- and probiotics [30], butyric acid [31], sodium butyrate [32] leads to changes in the microarchitecture of lymphoid organs and the intestinal wall: the length increases and the surface area of the villi, the number of goblet cells.

Since the ingredient composition of the diet in our experiment was the same in all groups, except for different Zn sourses, we assume that the immunostimulation in the test groups compared to the control resulted from structural changes in the organs of the immune system, the spleen, thymus and CB.

Dietary Zn shows good absorption in the intestines and, influencing the immune system, activates a number of mechanisms. In general, these mechanisms are similar for many trace elements and involve interactions with specific receptors, the influence on phagocytosis, apoptosis, chemotaxis, adhesion, on the activity of enzymes, hormones, on carrier proteins, and on enhanced production of immunoglobulins. Zn^{2+} cations can stimulate the proliferation of cells of immunocompetent organs, in particular the spleen [15, 33]. Zn potentiates the body's cell-mediated defense responses to bacteria and viruses [14].

Thus, dietary organic (OF) and ultradispersed (UDP) Zn contribute to higher bactericidal activity of blood serum, activity of lysozyme and β-lysines, thereby stimulating the natural resistance of the Smena 7 broiler chickens. We did not reveal pathological changes of white blood. The tendency to increased numbers of leukocytes, lymphocytes and monocytes at the age of 3-4 weeks was noted to a greater extent in broilers fed dietary organic Zn. However, UDP Zn contributed

to an increase in the bactericidal activity of blood serum in the range from 5.8 to 16.7% and AL by 8.2% at later stages of the experiment compared to the control. A trend towards an increase in β-lysine activity was recorded at 5 weeks of age, followed by a decrease towards the end of the experiment. The use of the organic Zn led to a statistically significant ($p \leq 0.01$) increase in the bactericidal activity of blood serum by 13.4% at 3 weeks of age and of lysozyme by 8.8% at the end of the experiment. Organic Zn provided a smooth progressive increase in activity of β-lysine throughout the experiment. Histological evaluation revelaed that the organic Zn provided functional activation of the cloacal bursa with an increase in the area of lymphoid follicles by 64.5% ($p \leq 0.01$) due to the expansion of the medullary zones and to an increase in the cellular density of the cortical zone with a large number of macrophages and mitoses. In the thymus, along with an expansion of the medullary zone by 20.9%, there were a greater number of Hassall bodies, compaction of cells of the cortical layer and proliferative activity of lymphocytes with activation of macrophages. In the spleen, upon administration of UDP Zn, we noted enlargement of follicles due to an increase in the area of the white pulp by 2.2 times ($p \leq 0.05$). In general, it can be argued that the immune response to various sourses of dietary zinc was more pronounced for UDP Zn and organic Zn compared to inorganic Zn.

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AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 2, pp. 386-398
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.52/.58.084:636.085.12

doi: 10.15389/agrobiology.2023.2.386eng
doi: 10.15389/agrobiology.2023.2.386rus

AGE-DEPENDENT ACCUMULATION OF ESSENTIAL AND TOXIC CHEMICAL ELEMENTS IN FEATHER OF ARBOR ACRES BROILERS (*Gallus gallus* L.) REARED IN THE SOUTH URAL BIOGEOCHEMICAL PROVINCE OF RUSSIA

S.V. LEBEDEV, O.A. ZAV'YALOV ✉, A.N. FROLOV, M.YA. KURILKINA, V.V. GRECHKINA

Federal Research Centre of Biological Systems and Agrotechnologies RAS, 29, ul. 9 Yanvarya, Orenburg, 460000 Russia, e-mail lsv74@list.ru, Oleg-Zavyalov83@mail.ru (✉ corresponding author), forleh@mail.ru, k_marina4@mail.ru, viktoria1985too@mail.ru
ORCID:
Lebedev S.V. orcid.org/0000-0001-9485-7010
Zav'yalov O.A. orcid.org/0000-0003-2033-3956
Frolov A.N. orcid.org/0000-0003-4525-2554
Kurilkina M.Ya. orcid.org/0000-0003-0253-7867
Grechkina V.V. orcid.org/0000-0002-1159-0531
The authors declare no conflict of interests
Acknowledgements:
Supported financially from the Russian Science Foundation, project No. 21-16-00009
Final revision received June 20, 2022
Accepted July 22, 2022

Abstract

Microelement imbalances are a serious threat to the health and productive traits of farm animals and poultry, so it is important to control and optimize input of chemical elements with regard to the conditions of a biogeochemical province and anthropogenic pollution. Currently, blood is used as a biosubstrate to determine the elemental status in birds. However, preference is given to feather when it comes to assessing the elemental status over a relatively long period, also because obtaining this biosubstrate is minimally invasive. The amount of available information on macro- and microelements content in the feather of poultry breeds and crosses is limited (especially in terms of age), which makes it difficult to objectively interpret laboratory data. In the presented work, we confirmed the possibility of using feathers as a biomaterial for determining the essential and toxic chemical elements status in Arbor Acres cross broiler chickens in commercial breeding in the conditions of South Ural biogeochemical province with areas of technogenic pollution. In addition, the age-related changes in elements content in chickens' feathers under the specified conditions were characterized for the first time, and the percentile ranking of the obtained indicators was performed according to the method recommended for determining the physiological norm in the sample. The aim of the study was to determine the essential and toxic elements content in Arbor Acres broiler chickens of different ages under the conditions of South Ural biogeochemical province and to rank the obtained indicators by percentiles to estimate the limits of the age norm for the sample. The research was carried out on the territory of South Ural biogeochemical province of Russia on clinically healthy Arbor Acres broiler chickens at the age of 7 days (n = 120), 21 days (n = 120) and 35 days (n = 120). The content of nutrients, macro- and microelements in the diet of the examined birds was within the limits of the requirements for broiler chickens during the corresponding growing periods. Blood and feathers (the flight feathers were plucked out) were used as compared biosubstrates. The proximal part of the feather weighing at least 0.4 g was selected for research. The elemental composition of feathers and blood serum was determined by inductively coupled plasma atomic emission and mass spectrometry (ICP-DRC-MS). The results of the quantitative determination of each element in feather were ranked in ascending gradation in the form of an ordered series according to the recommendations of the American Society for Veterinary Clinical Pathology (2.5 and 97.5 percentile). It was found that the content of P, Cr, I, Se, Li, Si, As, Hg, Sn, Sr in feather of broiler chickens varied significantly depending on age. In this regard, the age factor must be taken into account when developing reference intervals for specific regions. In this study, percentile ranking (2.5 and 97.7 percentiles with a confidence interval CI = 90 %) of data on 25 chemical elements (Al, As, B, Ca, Cd, Co, Cr, Cu, Fe, I, K , Li, Mg, Mn, Na, Ni, P, Pb, Se, Si, Sn, Hg, Sr, V, Zn) in feather of physiologically healthy Arbor Acres cross broiler chickens at different ages (7; 21; 35 days). The obtained data can be useful in detecting elementoses in Arbor Acres broiler chickens.

Keywords: broiler chickens, Arbor Acres, blood, feather, essential elements, toxic elements, age, percentile ranking

Imbalance of minerals poses a serious threat to the health and productivity of farm animals and poultry [1, 2], and it is important to take into account the intake of both vital and toxic chemical elements into the body [3]. Excess of toxic metals can weaken the immune system by causing oxidative stress [4] which negatively affects biochemical parameters, reproductive function and productivity, increases the incidence of heart disease and leads to various mutations and neoplasms [5].

The South Ural territory is a biogeochemical mosaic with a deficiency of iodine, fluorine, selenium, and the presence of local areas with high levels of zinc, copper, strontium and nickel. Along with natural biogeochemical provinces, technogenic provinces of nickel, manganese, fluorine, and lead are being formed in local territories [6, 7]. Modern poultry farming uses water treatment systems, mixed feed, feed additives and rationing for nutrients and minerals with regard to the age and physiological needs of crosses developed for diferent purposes. This sufficiently standardizes feeding regimes and avoids effects of local conditions on the growth and productivity performance of poultry. However, when developing and adapting such regulations, local biogeochemical factors must be taken into account, including the nature and level of technogenic pollution [3-5].

It is obvious that when monitoring and optimizing the intake of mineral substances, non-invasive methods for assessing metabolic status, including determining the elemental composition in biosubstrates, are most acceptable. Such work was carried out on beef [8] and dairy [9] cattle, in sports horse breeding [10], and in commercial poultry farming [11]. Currently, blood mostly serves as a biosubstrate to analyze the elemental status of poultry [12], but in long and low-sress monitoring feathers are preferable [13]. In addition, it has been proven that the concentration of heavy metals in bird feathers is an informative indicator when assessing the contamination of territories with heavy metals [14]. It has also been found out that the amount of trace elements in feathers can generally characterize the metabolic pool of the main essential and toxic elements in broiler chickens and laying hens [15].

However, the available information on the accumulation of macro-, micro- and toxic elements in poultry (in particular, broiler chickens) during different periods of ontogenesis is limited. Moreover, for most breeds and crosses there are no reference intervals calculated by the recommendations of the American Society of Veterinary Clinical Pathology [16], including those for local breeding conditions. This hinders to objectively interpret and compare results when analyzing the bioelemental status of poultry.

Arbor Acres is a large meat cross with strong bones, high meat yield from the carcass (up to 72%), developed muscles, which reduces the fat content of the product, and shortened fattening periods [17]. According to data on 2016, in Africa, over 10 years, the cross has occupied up to 20% of the market; the cross is popular in Algeria, Egypt, Tunisia, Mozambique, and is raised for meat in the USA and China [18].

Here, we confirm that feathers are suitable to evaluate the essential and toxic elements of Arbor Acres cross broilers in the South Ural biogeochemical province with areas of technogenic pollution. For the first time, we revealed age-related changes in the accumulation of chemical elements in feathers under these conditions. The experimental data was processed according to the unified methodology of the American Society of Veterinary Clinical Pathology that is recommended for assessment of the physiological norm in the sample. The obtained percentile ranking can serve for detection of elementosis in Arbor Acres broilers during growth and for

metabolic correction of timely detected disorders using mineral complexes.

Our goal was to determine the concentration of essential and toxic elements in the feathers of Arbor Acres cross broiler chickens of different ages under the conditions of the South Ural biogeochemical province and to rank the obtained indicators by percentile to assess the boundaries of the age norm for the sample.

Materials and methods. Experiments were consistent with the instructions, recommendations, and protocols of the Geneva Convention and the principles of good laboratory practice (Order of the USSR Ministry of Health No. 755 of 08/12/1977 “On measures to further improve work using experimental animals”, “National Standard of the Russian Federation GOST R 53434-20092"). All manipulations were performed in accordance with the rules of the Animal Ethics Committee of BST RAS. All efforts were made to minimize animal suffering and reduce the number of samples analyzed.

The poultry was raised at three poultry farms (ZAO Orenburg Poultry Farm, Individual Peasant Farmer T.P. Tuzikov, Peasant Farmer V.A. Malyshev) located in the South Ural biogeochemical province (Orenburg Province) [6, 7].

Clinically healthy Arbor Acres broilers aged 7 days (n = 120), 21 days (n = 120), and 35 days (n = 120) were used. Feeding and housing conditions for all chickens were approximately identical and corresponded to recommendarions for the cross. The birds were kept in metal cages (145×70×250 cm width×depth×height) with mesh floors. Nutrients of the diets corresponded to the requirements of broiler chickens during each growing period [19]. Complete feeds (Russia) PK-0 (0-10 days of life), PK-5 (11-24 days), PK-6 (25 days and older) were added with the premix Koudais MKorma (Russia) (25 kg/t) as a mineral component. The premix comtains mineral components (69.10%), limestone (21.6%), and bran (9.3%).

Feather and blood for analysis were collected from three individuals aged 7, 21 and 35 days in each of 40 randomly selected cages from different sections of workshops. Five fight feathers were plucked from the wing; the proximal part of the feather weighing at least 0.4 g was selected for analysis. Blood (at least 2 ml) from the axillary vein of birds aged 21 and 35 days was sampled in the morning into 7 ml vacuum tubes with a blood clotting activator (Hebei Xing Sky & Co., China). Blood serum was separated by centrifugation for 10 minutes at 1000 rpm. The tubes were cooled to –8 °C and stored until elemental analysis.

The feather was ground to a fraction of no more than 2 mm. The resulting feather samples were washed in acetone (Sigma-Aldrich, Co., USA) to remove external contaminants, then three times in deionized water (18 MOM/cm) and dried at 60 °C to constant weight. Feather samples, 50 mg each, were placed in Teflon tubes with 5 ml of concentrated nitric acid (Sigma-Aldrich, Co., USA) and processed in a microwave system (Bergh of Products + Instruments GmbH, Germany). The resulting solutions were poured into 15 ml polypropylene tubes, diluted with deionized water to a final volume of 15 ml and mixed thoroughly by shaking.

Concentration of essential, conditionally essential (Ca, K, Mg, Na, P, Co, Cr, Cu, Fe, I, Li, Mn, Se, Si, Sr, V, Zn), toxic and potentially toxic (As, B, Cd, Hg, Ni, Pb and Sn) elements were measured (a NexION 300D spectrometer, Perkin Elmer, USA, with an ESI SC-2 DX4 autosampler, Scientific, Inc., USA). For calibration, standard solutions with different concentrations of microelements were used, prepared from Universal Data Acquisition Standards Kits (Perkin Elmer, Inc., USA). Single element purity standard (10 g/) for yttrium and rhodium (Perkin Elmer, Inc., USA) were used for internal online standardization. For laboratory quality control, continuous analysis of a certified reference substrate (the hair GBW09101, Shanghai Institute of Nuclear Research, China) was used. The analysis was carried out before and after each stage of the survey. The degree of extraction of all analyzed trace elements was no less than 88%.

The results of the quantification of each element were reranked in ascending gradation as an ordered series according to the recommendations of the American Society for Veterinary Clinical Pathology [16] with concentration intervals of chemical elements by percentiles, 2.5 and 97.5. The reliability of differences was assessed by Mann-Whitney U-test. The significance level (p) was taken to be less than or equal to 0.05. To calculate the correlation between the assessed parameters, the Spearman rank correlation method was used. The tables show the mean values (*M*) and their standard deviations (±SD). Data processing used the Statistica 10.0 application package (StatSoft, Inc., USA).

Results. In Russia, a significant part of the Arbor Acres cross population is bred in the conditions of the South Ural biogeochemical province. According to data from open sources, at the ZAO Orenburg Poultry Farm, the annual turnover of this cross is 1.8 million chickens, which indicates its prospects. AO Bashkir Broiler (Republic of Bashkortostan) is a second-order reproducer for the production of Arbor Acres hatching eggs for the Republic of Bashkortostan and the Orenburg Province.

The territory of the South Ural biochemical province, along with a deficiency of some elements, is characterized by an uneven distribution of various sources of pollution, e.g., enterprises of the chemical, petrochemical, fuel, mining industries (iron, copper, nickel ores, asbestos, oil) and ferrous and non-ferrous metallurgy (production of nickel, cobalt, copper) [6, 7].

Table 1 submits the concrnetration of chemical elements in the daily diet of broiler chickens at different age periods.

1. Content of chemical elements in the daily diet of the Arbor Acres cross broiler chickens by age periods of reared (Orenburg Province, South Ural biogeochemical province, 2022)

Element	Age, days		
	0-14	15-28	29-42
Macronutrients			
Ca	287.2	821.5	1533.6
K	251.7	339.6	452.4
Mg	60.8	87.9	127.1
Na	41.2	57.1	66.5
P	203.6	589.7	1102.3
Essential elements			
Co	0.007	0.010	0.011
Cr	0.304	1.883	4.526
Cu	1.25	1.55	2.00
Fe	5.69	10.36	15.98
Mn	3.51	6.55	9.38
I	0.027	0.044	0.034
Se	0.006	0.015	0.025
Zn	4.42	8.25	11.55
Conditionally essential elements			
B	0.504	0.695	0.860
Li	0.0021	0.0025	0.0030
Si	1.07	2.54	5.86
Ni	0.042	0.095	0.174
V	0.023	0.167	0.473
Toxic elements			
Al	0.337	0.524	0.800
As	0.001	0.028	0.043
Cd	0.001	0.002	0.003
Hg	0.000	0.000	0.000
Pb	0.002	0.015	0.022
Sn	0.000	0.000	0.000
Sr	0.400	0.754	1.258

In all farms and at all age periods, the chickens were physiologically healthy, developed normally and had relatively high dynamics of live weight and its growth (Table 2).

2. Bodyweight dynamics in the Arbor Acres cross broiler chickens at poultry breeding enterprises (n = 120, M±SD, Orenburg Province, South Ural biogeochemical province, 2022)

Parameter	Enterprise		
	ZAO Orenburg Poultry Farm	Individual Peasant Farmer T.P. Tuzikov	Peasant Farmer V.A. Malyshev
Bodyweight, г:			
7 days	183.2±19.34	179.4±14.48	180.2±18.61
21 days	653.2±84.34	634.3±76.61	652.1±87.64
35 days	1862.4±201.09	1811.6±187.69	1830.3±202.11
Average daily weight gain, g/(head · day)	60.0±21.17	58.3±16.34	58.9±18.64
Absolute weight gain, kg/(head · test)	1.68±0.49	1.63±0.38	16.65±0.43

3. Content (mg/kg) **of macro-, essential and toxic elements in the feathers of Arbor Acres cross broiler chickens of different ages at poultry farms** (n = 120, M±SD, Orenburg Province, South Ural biogeochemical province, 2022)

Element	Age, days		
	7	21	35
Macronutrients			
Ca	1496±124.9	1214±144.4	928.1±118.1
K	2429±622.0	2510±155.7	2283±199.3
Mg	314.0±64.41	435.2±51.31	419.3±83.13
Na	1346±311.4	1665±98.15	1801±105.6
P	1060±428.0	1382±102.5	1072±86.28[b]
Essential elements			
Co	0.0741±0.0151	0.0588±0.0075	0.0618±0.0120
Cr	3.02±0.264	4.63±0.421	3.03±0.261[b]
Cu	13.32±2.22	9.60±1.55	10.64±1.78
Fe	89.74±14.38	81.93±16.35	57.23±6.20
I	8.54±2.03	0.838±0.0828[a]	0.477±0.0562[ab]
Mn	10.27±1.86	16.38±3.25	15.66±4.46
Se	1.020±0.1417	0.597±0.0204[a]	0.568±0.0184[a]
Zn	176.0±13.48	314.0±50.54	268.2±42.99
Conditionally essential elements			
B	1.84±0.359	2.65±0.261	2.43±0.354
Li	0.106±0.0236	0.0464±0.0071[a]	0.0467±0.0047[a]
Ni	0.879±0.125	1.38±0.266	1.36±0.342
Si	103.0±12.58	80.55±5.02	39.52±5.52[ab]
V	0.225±0.0247	0.473±0.0604	0.254±0.0370[b]
Toxic elements			
Al	21.74±4.20	13.09±2.09	13.44±2.43
As	0.0672±0.0065	0.0617±0.0047	0.0496±0.0032[b]
Cd	0.0310±0.0091	0.0318±0.0147	0.0142±0.0035
Hg	0.0357±0.0127	0.0094±0.0012[a]	0.0130±0.0016[a]
Pb	1.78±0.335	1.84±0.384	2.44±0.700
Sn	0.234±0.117	1.50±1.34	0.0715±0.0102[a]
Sr	4.57±0.790	2.45±0.348[a]	2.14±0.352[a]

Note. Data are provided for three enterprises (CJSC Orenburg Poultry Farm, Individual Peasant Farmer T.P. Tuzikov, Peasant Farmer V.A. Malyshev).

[a] Dfferences for the values on days 21 and 35 compared to day 7 are statistically significant at $p \leq 0.05$.

[b] Dfferences for the values on day 35 compared to day 28 are statistically significant at $p \leq 0,05$.

A comparative analysis revealed a significant difference in the content of the main essential and toxic elements in the re-examined birds at different periods of growth and development (Table 3).

In the feather of 7-day-old broiler chickens, we found the highest content of P, I, Se, Li, Si, As, Hg, Sn and Sr. As they grew older, in the period from day 7 to days 21 and 35, the values significantly decreased (see Table 3). It has been previously reported that age may influence micronutrient metabolism in poultry [20]. A possible reason for the decrease in parameters we identified as the bird matures may be that elements such as Ca, P, Zn, I, Cu play an important role as cofactors of enzyme systems associated with bone mineralization, a process subject to age-related changes [21]. Thus, it has been established that during the formation of the organic matrix of bone tissue and its mineralization, two ages stand out as critical, 14 and 35 days, when the mineral saturation of the organic bone matrix decreases, which weakens the bones

of the supporting limbs [22]. It is noteworthy that the minimum content of almost all assessed chemical elements in our experiment was noted during the period of the beginning of molting at the age of 35 days. The molting period is usually accompanied by a natural decrease in feed intake and suppression of micronutrient metabolism [23]. When determining the age-related dynamics of the amount of toxic elements, we revealed a significant ($p \leq 0.05$) decrease in the accumulation of As, Hg, Sn, Sr in the feather of broiler chickens at the age of 21 and 35 days compared to 7 days of age. These differences may be associated with the metabolism of metallothionein, a protein that is synthesized in the body of animals in response to the intake of heavy metals from the external environment. Metallothionein levels, in turn, are regulated by sex hormones [24], the concentration of which depends on age [25]. It should be noted, however, that in our other study, toxic elements, on the contrary, accumulated with age in poultry (we believe, due to a longer intake of toxic metals with feed and drinking water) [11].

4. Concentration (mg/l) **of macro-, essential and toxic elements in the blood serum of Arbor Acres cross broiler chickens of different ages at poultry farms** (n = 120, M±SD, Orenburg Province, South Ural biogeochemical province, 2022)

Element	Age, days	
	21	35
Macronutrients		
Ca	0.159±0.0151	0.125±0.0132*
K	0.289±0.0447	0.282±0.0421
Mg	0.0372±0.0014	0.0322±0.0015
Na	3.01±0.482	3.22±0.484
P	0.263±0.0221	0.196±0.0225*
Essential elements		
Co	0.0021±0.0003	0.0036±0.0005**
Cr	0.0112±0.0021	0.0053±0.0022*
Cu	0.131±0.0154	0.178±0.0195*
Fe	1.98±0.182	1.32±0.144**
I	0.0621±0.0061	0.0452±0.0052**
Mn	0.0351±0.0133	0.0231±0.0034
Se	0.212±0.0423	0.154±0.0331
Zn	2.32±0.355	2.22±0.328
Conditionally essential elements		
B	0.714±0.145	0.537±0.115
Li	0.0188±0.0034	0.0247±0.0042
Ni	0.0093±0.0034	0.0082±0.0027
V	0.0052±0.0021	0.0074±0.0025
Toxic elements		
As	0.0032±0.0006	0.0051±0.0007*
Al	0.0633±0.0082	0.0854±0.0076*
Cd	0.0011±0.0002	0.0004±0.0002*
Hg	0.0002±0.0002	0.0007±0.0003
Pb	0.0003±0.0001	0.0003±0.0001
Sn	0.0009±0.0003	0.0007±0.0003
Sr	0.142±0.0264	0.135±0.0277

Note. Data are provided for three enterprises (CJSC Orenburg Poultry Farm, Individual Peasant Farmer T.P. Tuzikov, Peasant Farmer V.A. Malyshev).
*, ** Dfferences from the values on day 21 are statistically significant at $p \leq 0.05$ and $p \leq 0.01$.

To assess the possibility of using bird feathers as a biosubstrate for elemental analysis, we compared the data obtained for feather and for blood samples (Table 4). These data showed that the dynamics of element concentrations in the blood serum generally corresponded to those in feathers of the birds.

Correlation analysis (Table 5) revealed a significant ($p \leq 0.05$) positive relationship between the concentration of bioelements in feathers and blood serum in our study was established for Al, Ca, Cr, Cd, Fe, I, Mg, P, Pb, Se, Zn.

Thus, it can be stated that, in general, the elemental analysis of feathers reflects the concentration of chemical elements in the blood serum, which allows us to recommend flight feathers as a biosubstrate for non-invasive assessing the elemental status of broiler chickens. We note that for As and V there were significant

($p \leq 0.05$) but negative correlations. A possible reason for such differences is that the composition of blood is much more dynamic and reflects changes over a much shorter period of time, and feathers as a biosubstrate make it possible to characterize the elemental status over a long period [26]. In addition, the content of chemical elements in feathers is higher than in blood serum. This increases the analytical sensitivity of the determination and the information content of the assessment of changes in elemental status when using a pen.

5. Spearman correlation coefficients (*r*) between macro-, essential and toxic element content in the feathers and blood serum of Arbor Acres cross broiler chickens at poultry farms (n = 360, Orenburg Province, South Ural biogeochemical province, 2022)

Element	Al	As	B	Ca	Cd	Co	Cr	Cu	Fe	Hg	I	K	Li	Mg	Mn	Ni	P	Pb	Se	Sn	Sr	V	Zn
Al	0.8*	-0,4	0,0	-0,2	0,4	0,1	-0,4	0,4	-0,6	0,0	-0,3	-0,2	0,0	-0,3	0,2	0,4	-0,3	-0,2	-0,2	-0,7	-0,3	0,3	0,0
As	0.4	-0,8*	0,4	0,0	0,5	-0,2	-0,6	0,6	-0,5	0,2	-0,2	0,1	0,0	-0,5	0,5	0,5	-0,3	-0,1	-0,1	-0,8	-0,4	0,2	-0,3
B	0.4	-0,3	0,4	0,0	0,5	-0,2	-0,6	0,6	-0,5	0,2	-0,2	0,1	0,0	-0,2	0,5	0,5	-0,3	-0,1	-0,1	-0,8	-0,4	0,2	-0,3
Ca	0.4	-0,4	0,4	0,7*	0,5	-0,2	-0,6	0,6	-0,5	0,2	-0,2	0,1	0,0	-0,2	0,5	0,5	-0,3	-0,1	-0,1	-0,8	-0,4	0,2	-0,3
Cd	0.4	-0.4	0.2	-0.1	0.6*	-0.1	-0.6	0.6	-0.5	0.1	-0.4	0.0	0.1	-0.4	0.4	0.6	-0.2	-0.1	-0.4	-0.8	-0.4	0.3	-0.2
Co	0.4	-0.5	0.4	0.0	0.5	-0.2	-0.6	0.6	-0.5	0.2	-0.2	0.1	0.0	-0.5	0.5	0.5	-0.3	-0.1	-0.4	-0.8	-0.4	0.2	-0.3
Cr	0.5	-0.5	0.5	0.1	0.4	-0.3	-0.6	0.7	-0.4	0.3	-0.2	0.2	-0.1	-0.6	0.5	0.4	-0.3	0.0	-0.3	-0.7	-0.3	0.2	-0.5
Cu	0.2	-0.6	0.0	-0.2	0.4	0.1	-0.4	0.4	-0.6	0.0	-0.3	-0.2	0.0	-0.4	0.2	0.4	-0.3	-0.2	-0.2	-0.7	-0.3	0.3	0.0
Fe	0.4	-0.5	0.4	0.0	0.5	-0.2	-0.6	0.6	0.6*	0.2	-0.2	0.1	0.0	-0.5	0.5	0.5	-0.3	-0.1	-0.4	-0.8	-0.4	0.2	-0.3
Hg	0.0	-0.3	-0.2	-0.3	0.2	0.2	-0.2	0.2	-0.6	-0.2	-0.2	-0.6	0.1	-0.4	0.0	0.3	-0.2	-0.2	-0.2	-0.5	-0.1	0.3	0.2
I	0.5	-0.3	0.5	0.1	0.4	-0.3	-0.6	0.7	-0.4	0.3	0.8*	0.2	-0.1	-0.4	0.5	0.4	-0.3	0.0	-0.2	-0.7	-0.3	0.2	-0.3
K	0.4	-0.4	0.4	0.0	0.5	-0.2	-0.6	0.6	-0.5	0.2	-0.2	0.1	0.0	-0.3	0.5	0.5	-0.3	-0.1	-0.2	-0.8	-0.4	0.2	-0.3
Li	0.1	-0.2	0.0	-0.3	0.2	0.0	-0.3	0.5	-0.6	-0.1	-0.2	-0.3	0.1	-0.4	0.2	0.4	-0.2	-0.2	-0.4	-0.7	-0.3	0.3	0.1
Mg	0.4	-0.3	0.4	0.0	0.5	-0.2	-0.6	0.6	-0.5	0.2	-0.2	0.1	0.0	0.7*	0.5	0.5	-0.3	-0.1	-0.3	-0.8	-0.4	0.2	-0.3
Mn	0.4	-0.4	0.4	0.0	0.5	-0.2	-0.6	0.6	-0.5	0.2	-0.2	0.1	0.0	-0.3	0.5	0.5	-0.3	-0.1	-0.3	-0.8	-0.4	0.2	-0.3
Na	0.2	-0.2	0.0	-0.2	0.4	0.1	-0.4	0.4	-0.6	0.0	-0.3	-0.2	0.0	-0.3	0.2	0.4	-0.3	-0.2	-0.5	-0.7	-0.3	0.3	0.0
Ni	0.4	-0.4	0.4	0.0	0.5	-0.2	-0.6	0.6	-0.5	0.2	-0.2	0.1	0.0	-0.4	0.5	0.5	-0.3	-0.1	-0.3	-0.8	-0.4	0.2	-0.3
P	0.4	-0.4	0.3	0.2	0.3	-0.3	-0.5	0.7	-0.4	0.1	-0.1	0.1	-0.2	-0.4	0.4	0.3	0.8*	0.0	-0.4	-0.7	-0.3	0.2	-0.4
Pb	0.2	-0.2	-0.1	-0.2	0.3	0.2	-0.4	0.3	-0.5	0.0	-0.4	-0.3	0.0	-0.3	0.1	0.4	-0.3	0.7*	-0.4	-0.6	-0.3	0.4	0.1
Se	0.2	-0.3	0.2	-0.1	0.4	-0.1	-0.4	0.5	-0.6	0.1	-0.1	-0.2	0.0	-0.5	0.3	0.3	-0.4	-0.2	0.6*	-0.7	-0.3	0.2	-0.1
Si	0.7	-0.3	0.7	0.3	0.5	-0.4	-0.8	0.6	-0.2	0.4	-0.1	0.5	-0.1	-0.2	0.7	0.5	-0.5	0.2	-0.3	-0.7	-0.2	-0.1	-0.4
Sn	0.2	-0.3	0.0	-0.2	0.4	0.1	-0.4	0.4	-0.6	0.0	-0.3	-0.2	0.0	-0.4	0.2	0.4	-0.3	-0.2	-0.5	-0.7	-0.3	0.3	0.0
Sr	0.3	-0.2	0.3	0.0	0.3	-0.2	-0.5	0.6	-0.5	0.1	-0.2	0.0	0.0	-0.5	0.4	0.4	-0.3	-0.1	-0.6	-0.8	-0.4	0.2	-0.2
V	0.6	-0.4	0.6	0.2	0.4	-0.4	-0.7	0.7	-0.3	0.3	-0.2	0.4	-0.1	-0.2	0.6	0.6	-0.4	0.1	-0.8	-0.8	-0.3	-0.6*	-0.5
Zn	0.3	-0.5	0.2	-0.2	0.5	0.1	-0.5	0.4	-0.5	0.2	-0.4	-0.1	0.1	-0.3	0.3	0.5	-0.3	-0.2	-0.6	-0.7	-0.3	0.3	0.7*

N o t e. Data are provided for three enterprises (ZAO Orenburg Poultry Farm, Individual Peasant Farmer T.P. Tuzikov, Peasant Farmer V.A. Malyshev).

* Correlations are statistically significant at $p \leq 0.05$.

The essence of the percentile method of ranking measurement results [16] that we used is that the series covering the entire range of quantitative fluctuations of the attribute (100%) is divided into 100 intervals and percentile probabilities are established, the intervals between which constitute percentile intervals. In the calculations, we followed the international recommendations for veterinary laboratory standards [16] according to which, after excluding outliers (abnormally high and/or low values of the analyzed indicator), it is proposed to use the interval from 2.5 to 97.5 percentile as a physiological norm in a sample studied. A prerequisite for the use of this method is the calculation of 90% confidence intervals for the upper and lower limits, which make it possible with a known probability to estimate the mathematical expectation of the general population with further expansion of the experimental sample.

The content of chemical elements in the feather of broiler chickens at different periods of growth and development (7, 21, 35 days) differed significantly ($p \leq 0.05$), so we ranked the values of the indicators for each age separately (Table 6).

The largest intervals for almost all studied elements (with the exception of Na, P, Se, Li, Hg) occurred in broiler chickens at the age of 7 days. The widest ranges for K, Mg, Co, Cu, Mn, Se, Zn, B, Ni, Al, Pb, Sr were characteristic of young birds aged 35 days. In general, wider concentration ranges detected in birds at an early age indicate instability of metabolism for the noted elements, which may be associated with the individual characteristics of individuals.

6. Percentile rank intervals for macro-, essential and toxic element content (mg/kg) **in the feathers of Arbor Acres cross broiler chickens at poultry farms** (n = 120, Orenburg Province, South Ural biogeochemical province, 2022)

Element	2.5 (CI = 90 %)	97.5 (CI = 90 %)
7-day age		
Macronutrients		
Ca	1222 (794.3-1649)	1821 (1183-2458)
K	1053 (684.4-1421)	3972 (2581-5362)
Mg	177.0 (115.1-238.9)	475.0 (308.7-641.2)
Na	665.2 (432.3-897.7)	2094 (1361-2826)
P	302.3 (196.3-407.7)	2892 (1879-3904)
Essential elements		
Co	0.0343 (0.0223-0.0465)	0.104 (0.0676-0.140)
Cr	2.63 (1.71-3.55)	3.76 (2.44-5.07)
Cu	7.33 (4.77-9.89)	17.91 (11.64-24.17)
Fe	50.3 (32.7-67.91)	118.0 (76.7-159.3)
I	4.94 (3.21-6.67)	13.49 (8.76-18.21)
Mn	5.34 (3.47-7.21)	13.34 (8.67-18.01)
Se	0.637 (0.414-0.860)	1.29 (0.838-1.74)
Zn	144.2 (93.63-194.4)	210.0 (136.5-283.5)
Conditionally essential elements		
B	1.24 (0.806-1.67)	2.70 (1.75-3.64)
Li	0.0644 (0.0423-0.0873)	0.174 (0.113-0.234)
Ni	0.552 (0.359-0.745)	2.16 (1.40-2.91)
Si	76.67 (49.83-103.5)	126.0 (81.9-170.1)
V	0.170 (0.111-0.230)	0.290 (0.188-0.391)
Toxic elements		
Al	10.45 (6.79-14.12)	30.68 (19.94-41.41)
As	0.0481 (0.0313-0.0653)	0.0786 (0.0507-0.1050)
Cd	0.0082 (0.0054-0.0114)	0.0582 (0.0373-0.0776)
Hg	0.0154 (0.0102-0.0203)	0.112 (0.0728-0.151)
Pb	0.848 (0.551-1.14)	2.39 (1.55-3.22)
Sn	0.0974 (0.0634-0.131)	0.584 (0.379-0.788)
Sr	3.03 (1.97-4.09)	6.60 (4.29-8.91)
21-day age		
Macronutrients		
Ca	462.0 (300.3-623.7)	3592 (2334-4849)
K	1594 (1036-2151)	4740 (3081-6399)
Mg	209.0 (135.8-282.1)	1298 (843.7-1752)
Na	981.0 (637.6-1324)	3254 (2115-4392)
Essential elements		
Co	0.0135 (0.0087-0.0173)	0.158 (0.102-0.213)
Cr	1.66 (1.08-2.24)	9.98 (6.48-13.47)
Cu	3.34 (2.17-4.51)	43.04 (27.97-58.10)
Fe	29.13 (18.93-39.33)	427.0 (277.5-576.4)
I	0.230 (0.150-0.311)	1.73 (1.12-2.33)
Mn	1.39 (0.904-1.87)	69.89 (45.42-94.35)
Se	0.441 (0.287-0.595)	0.791 (0.514-1.060)
Zn	136.0 (88.40-183.6)	929.2 (603.8-1254)
Conditionally essential elements		
B	0.791 (0.514-1.07)	6.43 (4.17-8.68)
Li	0.0093 (0.0062-0.0134)	0.167 (0.108-0.225)
Ni	0.154 (0.100-0.208)	4.62 (3.01-6.23)
Si	42.45 (27.59-57.31)	143.0 (92.95-193.1)
V	0.112 (0.0732-0.151)	1.33 (0.86-1.79)
Toxic elements		
Al	2.79 (1.81-3.76)	44.12 (28.67-59.56)
As	0.0301 (0.0193-0.0407)	0.124 (0.0806-0.167)
Cd	0.0034 (0.0023-0.0045)	0.372 (0.241-0.502)
Hg	0.0022 (0.0014-0.0025)	0.0284 (0.0184-0.0383)
Pb	0.226 (0.147-0.305)	6.44 (4.18-8.69)
Sn	0.0194 (0.0125-0.0265)	33.60 (21.84-45.36)
Sr	0.595 (0.387-0.803)	7.31 (4.75-9.86)
35-day age		
Macronutrients		
Ca	349.0 (226.8-471.1)	3390 (2203-4576)
K	949.0 (616.8-1281)	7422 (4824-10019)
Mg	148.0 (96.20-199.8)	2736 (1778-3693)
Na	876.0 (569.4-1182)	2838 (1844-3831)
P	371.0 (241.1-500.8)	2750 (1787-3712)

Continued Table 5

	Essential elements	
Co	0.0065 (0.0043-0.0094)	0.277 (0.180-0.373)
Cr	1.47 (0.956-1.98)	7.12 (4.62-9.61)
Cu	4.08 (2.65-5.51)	59.12 (38.42-79.81)
Fe	22.99 (14.94-31.03)	191.0 (124.15-257.8)
I	0.158 (0.103-0.213)	1.84 (1.19-2.48)
Mn	0.894 (0.581-1.20)	133.0 (86.45-179.50)
Se	0.338 (0.220-0.456)	0.933 (0.606-1.250)
Zn	125.0 (81.25-168.7)	1362 (885.3-1838)
	Conditionally essential elements	
B	1.07 (0.696-1.44)	12.25 (7.96-16.53)
Li	0.0089 (0.0062-0.0124)	0.125 (0.0812-0.168)
Ni	0.0957 (0.0623-0.129)	9.52 (6.18-12.85)
Si	10.63 (6.91-14.35)	140.0 (91.21-189.5)
V	0.0473 (0.0315-0.0643)	1.06 (0.689-1.430)
	Toxic elements	
Al	1.87 (1.21-2.52)	75.90 (49.33-102.4)
As	0.0267 (0.0173-0.0365)	0.0934 (0.0607-0.126)
Cd	0.0013 (0.001-0.002)	0.107 (0.0695-0.144)
Hg	0.0018 (0.0012-0.0026)	0.0474 (0.0302-0.0629)
Pb	0.243 (0.158-0.328)	21.87 (14.21-29.52)
Sn	0.0078 (0.0053-0.0113)	0.238 (0.154-0.321)
Sr	0.365 (0.237-0.493)	9.66 (6.27-13.04)

N o t e. Data are provided for three enterprises (ZAO Orenburg Poultry Farm, Individual Peasant Farmer T.P. Tuzikov, Peasant Farmer V.A. Malyshev).

Feeds contain a wide range of microelements, some of which have nutritional value (Fe, Cu, Zn, Mn, Se, Co, Cr), while others have toxic properties (Pb, Cd, Ni) [27]. According to various studies, the content of bioelements is related to the geochemical conditions of the poultry breeding region [6]. Thus, in our experiment, the indicators for Zn were higher than those for chickens (*Gallus gallus domesticus*) bred in environmentally safe regions of Malaysia [28], but almost comparable to those obtained for this cross in Pakistan [29], for Cu and Mn, the values were comparable to the results for Korea [30] and significantly lower than in Belgium [31]. The content of Pb and Cd in the fether of broiler chickens in our study (South Ural biogeochemical province of Russia) was significantly lower than in chickens bred in regions with high technogenic load, in particular in South Korea (cross Ross 308, influencing factor is proximity to a large metropolis) [32], in China (cross is unknown, the influencing factor is mining metallurgy) [33], in the Republic of Kosovo (cross *G. gallus domesticus*, the influencing factor is proximity to a large metropolis) [34], but it differed little from indicators for poultry (*G. gallus domesticus*) raised near the capital of Oyo State in Nigeria [35]. The observed differences may be due to differences in regional and local background toxic metal pollution in the study areas [36]. Cross and species can also have a significant impact on the mineral composition of feathers. Thus, in a comparative assessment of the content of chemical elements in chickens of crosses *G. gallus domesticus* and *Coturnixcoturnix japonica*, bred in the same biochemical province, significant differences were revealed, in *C. japonica* the accumulation of Al, Mn, Co, Cu, Fe and Pb was higher than in the *G. gallus domesticus* cross [28]. In addition, the methodology for selecting, evaluating, and statistically analyzing the mineral composition of biosubstrates can have a significant impact on the results obtained, which makes it difficult to compare the results obtained in different studies [37, 38].

So, the accumulation of P, Cr, I, Se, Li, Si, As, Hg, Sn and Sr in the feather of Arbor Acres broilers raised in the South Ural biogeochemical province (Russia) varied significantly ($p \leq 0.05$) depending on age. In this regard, the age factor must be considered when developing reference intervals for the physiological norm of macro- and microelements and limit values of toxicants in poultry in specific regions. We quantified 25 chemical elements (Al, As, B, Ca, Cd, Co, Cr, Cu, Fe, I, K, Li, Mg, Mn, Na, Ni, P, Pb, Se, Si, Sn, Hg , Sr, V, Zn) in feathers of

physiologically healthy Arbor Acres broilers at different ages (7, 21 and 35 days). Following international veterinary standards for calculating percentile intervals to determine the boundaries of the physiological norm in the analyzed sample (2.5 and 97.5 percentiles at CI = 90%), we ranked the obtained data for each age of the bird. These results may serve for identifying elementosis in Arbor Acres cross chickens.

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