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. 54, Issue 4
July-August

2019 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

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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)

SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA
[AGRICULTURAL BIOLOGY], 2019, Vol. 54, № 4

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 631-641
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Reviews, challenges

УДК 636.2:636.082.12

doi: 10.15389/agrobiology.2019.4.631eng
doi: 10.15389/agrobiology.2019.4.631rus

ANIMAL GENETIC RESOURCES: DEVELOPING THE RESEARCH OF ALLELE POOL OF RUSSIAN CATTLE BREEDS — MINIREVIEW

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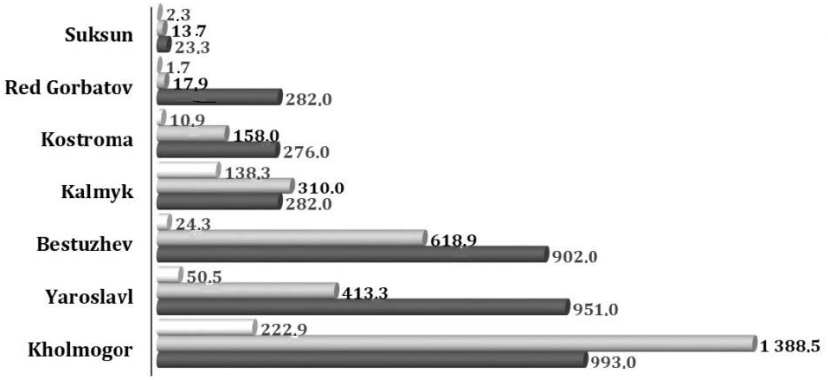
The authors declare no conflict of interests
Acknowledgements:
Supported financially by the Russian Science Foundation within Project No. 19-76-20012
Received March 7, 2019

Abstract

In modern biological science, the study and conservation of biodiversity is considered an important field of research (L.F. Groeneveld et al., 2010). In the twentieth century, a limited number of breeds were used in animal husbandry worldwide, leading to a significant decrease in the number of local breeds, which until recently, had been actively involved in agricultural production (B. Rischkowsky et al., 2007). This review describes the current state of knowledge of research on the cattle gene pool, with special attention paid to Russian genetic resources. The evolution of methods used for studying genetic diversity is briefly described. The results of studies of the allele pool of cattle breeds based on polymorphisms of mitochondrial DNA and microsatellites are summarized (M.-H. Li et al., 2009; J. Kantanen et al., 2009; P. V. Gorelov et al., 2011; T. Yu. Kiseleva et al., 2014; A. A. Traspov et al., 2011; R. Sharma 2015). The advantages of using single nucleotides polymorphisms (SNP) at the genome-wide level to study the population structure and genetic relationships between breeds are discussed (R. Fries, G. Durstewitz, 2001; R. Martinez-Arias et al., 2001; C. Xing et al., 2005). Data on the divergence of breeds based on whole-genome SNP analysis are presented (J. E. Decker et al., 2009; L. A. Kuehn et al., 2011; E. J. Mctavish et al., 2013; J. E. Decker et al., 2014; J. E. Decker et al., 2016; T. Iso-Touru et al., 2016). The allele pool of modern populations of Russian cattle breeds is characterized (N. A. Zinovieva et al., 2016; A. Yurchenko et al., 2018; A. A. Sermyagin et al., 2018). In comparative studies of Eurasian taurine breeds, high genetic divergence of Yakut cattle was detected. This review describes the maintenance of significant parts of authentic genetic components in several Russian breeds (Kholmogor, Yaroslavl, Red Gorbatov), bolstering their status as valuable national genetic resources and confirming the need for more in-depth studies and the preservation of these breeds. Notably, the use of powerful tools such as multiple SNP analysis does not always allow unambiguous interpretation of results from the point of view of the demographic history of Russian breeds due to the significant changes in the allele pool of modern populations of both Russian breeds and their presumed ancestor breeds. The informative power of molecular genetic analysis of breed evolution can be substantially enhanced by studying historical DNA samples, such as bone material from cranial and osteological collections (O. I. Boronetska et al., 2017). To date, methods have been developed to obtain DNA suitable for a wide range of molecular genetic studies of both mitochondrial and nuclear DNA, including individual gene and complete genome analysis (D. E. McHugh et al., 2000; A. Beja-Pereira et al., 2006; M. Gargani et al., 2015). Studying historical samples will provide new data on the allele pool evolution in Russian breeds and clarify the origin of modern populations. The results of such studies will be used to develop programs for breed conservation, as well as to establish organic production systems based on local genetic resources.

Keywords: biodiversity, Russian cattle breeds, DNA markers, historical DNA samples

The study and conservation of biodiversity are increasingly important parts of modern biological science [1]. Agricultural genetic resources are the biological capital essential to develop production systems in the future [2]. In the XX century, animal husbandry development around the world used a limited number of breeds, leading to a significant decrease in the number of local breeds, which until recently, were actively involved in agricultural production [3]. Thus, over the past half-century, the total number of six Russian local dairy cattle breeds (Kholmogor, Yaroslavl, Bestuzhev, Kostromsky, Red Gorbatov, and Suksun) has decreased by more than 10-fold—from 3.4 million heads in 1960 to 312.6 thousand heads in 2015; the number of Yaroslavl, Kostroma and Bestuzhev animals decreased by 19-37-fold. The current population of Red Gorbatov cattle is approximately 0.6% of that in the middle of the last century (Fig.).



Changes in the number of the Russian local cattle breeds (thousand heads): □ — year 2015, ■ — year 1991, ■ — year 1960 [4-6].

With the increasing demand for livestock products throughout the world as well as in Russia, we expect production systems to continue to use a few major breeds, which will result in further reductions in biodiversity.

Along with the decrease in the number of local breeds, crossbreeding within the small amount of purebred livestock puts local breeds at risk of extinction [3]. From a genetic point of view, crossbreeding leads to the disappearance of a number of unique alleles, especially rare ones, which can result in the loss of valuable traits and properties of breeds, including the composition of raw materials, disease resistance, the ability to adapt to the local and climatic conditions of specific regions, etc. In this regard, it is important to assess the current state of the allele pool of breeds and to identify populations and individuals that carry authentic breed-specific alleles and unique allelic combinations to develop programs to maintain the genetic authenticity of breeds.

This review describes the current state of knowledge in cattle gene pool research, with special attention paid to Russian local genetic resources. The possibilities of using historical samples to study the evolution of breeds are discussed.

Diverse types of DNA markers have been used to reconstruct the demographic history of domestic animal breeds [7]. In the 1960s, the main tools for characterizing genetic diversity, population structure and genealogical relationships between breeds were blood groups and milk protein polymorphisms [8, 9]. The detection of mtDNA polymorphisms and highly polymorphic microsatellites has since presented new opportunities for more powerful genetic studies [10]. Studies of polymorphisms of D-loop mtDNA have shown the presence of two

independent centres of the domestication of taurine and indicine cattle [11-13], which were subsequently confirmed by microsatellite analysis [14, 15]. Microsatellites were used to verify the hybrid origin of Middle Eastern cattle breeds [15, 16], demonstrate the various historical origins of Mediterranean and North European cattle populations [17], elucidate the classification of Eurasian cattle breeds [18], and determine the origin of some local cattle breeds [19, 20]. Mitochondrial DNA polymorphisms and microsatellites have been successfully used to study diversity, establish genetic relationships, and characterize the population structure of a number of Russian cattle breeds at the global [21] and regional levels [22-24]. The results obtained by M.-H. Li and J. Kantanen [18] confirmed the expansion of highly productive breeds, such as Danish Red, Angeln, Holstein-Friesian and Ayrshire, to Northern and Eastern Europe, including Russia. The authors pointed to the composite origin of the Yaroslavl, Istoben and Kholmogor cattle breeds, which, in their opinion, was a consequence of cross-breeding and may reflect the presence of multiple migration events from neighbouring regions of Europe, Asia and the Middle East. In all of the above-mentioned breeds, a significant component of European Black Pied lowland cattle was found. However, it should be noted that this conclusion contradicts the opinion of a number of authors who claim that Kholmogor, Yaroslavl and Istoben cattle were developed with a slight influence of Holstein-Friesian cattle [25, 26]. In comparative studies of paternal (Y-chromosomal microsatellite haplotypes) and material (mtDNA haplotypes) variability, J. Kantanen et al. [21] showed the accelerated loss of Y-chromosomal variation in locally derived cattle breeds, including Russian breeds (Kholmogor, Yaroslavl, Istoben, Suksun, and Red Gorbatov), due to the intensive culling of breeding bulls and the paternal forms of cross-breeding. Based on mtDNA analysis, the similarity of Finnish and Northern Russian cattle breeds was shown. The allele pool diversity and the genetic structure of Sychev and Simmental breeds were characterized [22, 24] with linkage disequilibrium data of 29 microsatellite loci in six Russian cattle populations [23].

Another method for simultaneously analysing polymorphisms in many loci, which has been widely used in research of agricultural animal gene pools, is the analysis of polymorphisms in DNA fragments flanked by inverted repeats of microsatellite loci, or Inter Simple Sequence Repeats (ISSR) markers. ISSR markers enable the evaluation of the similarities and differences in the gene pools of species and breeds (intra-breed groups) and the assessment of genetic patterns and purebred and genealogical relationships [27, 28]. Studies of Russian and commercial cattle breeds have identified a large number of ISSR loci—which can be used to distinguish *Bos taurus* and *Bos indicus*—that describe the "standard" of breeds, their genetic profile, and breed-specific patterns [29, 30]. Polymorphism analysis of mtDNA, microsatellites and ISSR markers has expanded our understanding of the origin and evolution of breeds; however, due to the insufficient power of these types of DNA markers, the results have not always been clearly interpreted.

The development of new high-throughput genotyping technologies has led to the widespread use of DNA markers based on single nucleotide polymorphisms (SNPs) in the study of animal genomes. Currently, SNPs are the preferred markers for genomic evaluation, establishing the relationship between individuals, determining the degree of inbreeding and hybridization, high-resolution genetic mapping and more complete characterization of genetic resources [31, 32]. The main advantages of SNPs compared to microsatellites are their wide distribution in the genome and clear mutation mechanism, with a low level of homoplasy and lower mutation degree. The technical advantages of SNP

analysis are the absence of special requirements for DNA quality (SNP analysis is mainly carried out by obtaining short amplicons of less than 100 bp), the lower degree of incorrect genotyping, the possibility of automating the process using high-throughput genotyping technologies, and the standardization of data [33-35]. SNPs provide wider genome coverage compared to STR and can be used to study both neutral and selection-affected genes [36, 37]. Despite the variety of SNP analysis methods [37], whole-genome analysis performed on the BeadArray™ platform (Illumina, Inc., San-Diego, CA, USA) is becoming increasingly widespread, allowing the simultaneous analysis of several tens to several hundred thousand SNPs [38]. The development and market introduction of commercial DNA chips that allow simultaneous analysis of tens and even hundreds of thousands of SNPs has led to the dominance of SNP markers in animal genome analysis [39].

SNP analysis at the whole-genome level performed using Bovine SNP50 BeadChip (Illumina, Inc., USA) has been successfully applied to elucidate the historical origin and characterize the current structure of cattle populations worldwide [40-45]. In a large-scale study by Decker J.E. et al. [40], a phylogenetic network of 48 cattle breeds (n = 372) was constructed, which provided an accurate description of the genetic relationships between breeds and improved our understanding of the history of domestication and development of breeds. McTavish E.J. et al. [38] showed that North American breeds, as well as many related breeds in southern Europe, are of hybrid origin, exhibiting both taurine and indicine genomic components. Using a wide range of analytical methods, Decker J.E. et al. [43] were able to clearly distribute 134 breeds of domestic cattle (n = 1543) among three groups: Asian indicine, Eurasian taurine, and African taurine. The African taurine group contains a large proportion of the African tour ancestors, leading to its divergence from the Eurasian taurine group. The influence of species other than *Bos taurus taurus* and *Bos taurus indicus* on the development of Asian breeds of cattle was shown. A significant contribution of European Shorthorns in the origin of European breeds was found. The introgression of African taurine in Iberian and Italian cattle breeds was revealed [43].

Studies of Russian local cattle breeds at the whole-genome level have been less extensive. A total of 35874 polymorphic SNPs were used to estimate the genetic diversity and population structure of modern populations of five cattle breeds, including Bestuzhev, Kholmogor, Kostromsky, Red Gorbatov and Yaroslavl [46]. Analysis of the obtained SNP genotypes differentiated the Russian breeds from each other and from the Holstein breed, which was used for comparison. All the studied Russian breeds had a complex origin, and the analysis revealed the presence of genomic components from the other Russian breeds and, in several cases, the admixture of Holsteins. The study of 274 individuals from 18 domestic breeds in comparison with 135 world breeds [47] showed their distribution into four clusters, reflecting their ancestral relationships with other breeds. It was shown that some breeds (for example, Kholmogor, Yakut, and Yaroslavl) have specific genomic components, which makes them a priority target for further in-depth research. A detailed study of the population structure and relationships of nine Russian cattle breeds (Bestuzhev, Black-and-White, Kalmyk, Kholmogor, Kostromsky, Red Gorbatov, Suksun, Yakut, and Yaroslavl) with 36 other Eurasian *taurine* breeds was carried out by Sermyagin A.A. et al. [48]. Multi-Dimension Scaling (MDS)-analysis confirmed the taurine origin of all the studied local breeds. According to the neighbour-net and admixture results, all the breeds could be divided into three groups. The Yakut and Kalmyk cattle formed a separate group due to their Turan-Mongolian origin. The Black-and-White, Kostroma and Suksun breeds had a significant proportion of ances-

tral components of transboundary European breeds, Holsteins, Brown Swiss and Danish Red, respectively. The Kholmogor, Yaroslavl, Red Gorbatov and Bestuzhev breeds were characterized by the lowest share of introgression of transboundary breeds, which makes them the most important national genetic resource.

On the other hand, it should be noted that the study of modern populations of local Russian cattle breeds — even with a powerful tool such as whole-genome SNP-analysis — does not always allow an unambiguous interpretation of the demographic history of breeds. This is because the allele pool of modern populations of both Russian breeds and their presumed ancestors has undergone significant changes during centuries-old development. On the one hand, this is due to the influence of the changing environmental conditions, and on the other hand, is due to the alteration in breeding goals. Intensive breeding for a limited number of traits, which has been practiced in recent decades, had undoubtedly led to the population-wide distribution of alleles positively associated with selected traits and the elimination of other alleles that are neutral or negatively associated with the selected traits. Another difficulty in the identification of authentic genetic components in modern populations of domestic livestock is the active use of crossbreeding with highly productive transboundary breeds, which has been practiced in recent decades. Thus, intensive use of Red Holstein cattle to improve the Bestuzhev cattle breed [49], Black-and-White Holstein cattle to improve the Kholmogor [54, 55] and Tagil breeds [56, 57], Brown Swiss to improve the Kostromsky breed [54-56], and others, has been observed. The Russian Black-and-White breed has been almost completely replaced by Holsteins; pedigree analysis of more than 500 AI bulls in Russia [57] showed the presence of only a few animals with 50% or more blood from the Black-and-White breed. Another difficulty in interpreting molecular genetic results is that some of the initial breeds involved in the origin of Russian cattle breeds have ceased to exist. This extinction happened, for example, with Tyrolean cattle, which were widespread in Europe in the XIX century [58]. It is assumed that Tyrolean cattle, whose massive export to Russia occurred in 1848 [59], significantly impacted the formation of the Bestuzhev and Red Gorbatov allele pools [60].

Results from molecular genetic analyses of the demographic origins of breeds can be improved by including historical DNA samples from animals that existed at least 100 years ago. DNA for this analysis can be derived from skull samples, which are stored in craniological collections [61]. Craniological methods were the main method for studying the origins of domestic animals before the discovery of biological markers. Craniological methods were first used in 1865 by the Swiss scientist and professor of comparative anatomy L. Rutimeyer (1825-1898) [cited according 62]. L. Rutimeyer's development of this method led to a series of similar kinds of work in different cattle breeds. In Russia, the comparative anatomical method has been used to examine cattle by K. Linderman (1873 and 1874), A. von-Middendorf (1884), P. Kuleshov (1888), E. Liskun (1910), Filipchenko (1915 and 1916), V. Ustyantsev (1915), and A. Browner (1919) [cited according 62]. The craniological method was most developed by the academician E.F. Liskun. He developed a detailed method of craniological research [63] that was widely used for more than 50 years to study farm animals from different breeds. E.F. Liskun started the unique craniological and osteological collection of domestic animals from different parts of Europe and Asia, which has no counterpart in our country or abroad. In 1947, the collection was donated to the Timiryazev Moscow Agricultural Academy. The collection contains more than 700 skulls of animals, including 350 skulls of 41 cattle breeds [64]. Considering that the exhibits of the collection are dated from the end of

XIX and beginning of the XX century, they may be used as reference profiles for cattle breeds in molecular genetic analyses and to trace the evolution of more than 100 years of history.

To date, methods have been developed to obtain DNA (both mitochondrial and nuclear DNA) that is suitable for a wide range of molecular genetic studies, including individual gene and complete genome analyses [65-67]. The first molecular genetic study using 10 microsatellite loci (TGLA227, BM2113, ETH10, SPS115, TGLA122, INRA23, TGLA126, BM1818, ETH225, and BM1824) revealed noticeable genetic differences between modern and historical samples of the Yaroslavl and Kholmogor cattle breeds. The development of research involving an additional number of historical samples and expanding the range of DNA markers used will enable the collection of new data on the allele pool evolution of Russian local cattle breeds over the last century.

Thus, the results of research conducted on the gene pools of Russian cattle breeds show the maintenance of the authentic genomic components in most breeds, which makes them the most important national genetic resources and a reserve of variability, which is necessary to sustain agricultural production systems in the future. Comparing modern and historical samples at the genomic level using a set of DNA markers will be useful for developing conservation programs for breeds, as well as for creating organic production systems based on the use of local genetic resources.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 642-654
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 573.6.086.83:577.21]:615.371

doi: 10.15389/agrobiology.2019.4.642eng
doi: 10.15389/agrobiology.2019.4.642rus

RESULTS OF Fc-PROTEIN FUSION TECHNOLOGY APPLICATION FOR VACCINE DESIGN AGAINST INFECTIOUS DISEASES OF ANIMALS AND HUMAN

(review)

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Foundation for Basic Research, grant mol_a No. 18-316-00092
Received November 27, 2018

Abstract

The main criteria for current vaccines design are effectiveness, efficaciousness and safety. Increasing requirements for vaccine safety and purity push forward not only classical vaccine development, but also new generation vaccine technology, including sub-unit, recombinant, anti-idiotypic, DNA vaccines etc. This recombinant technology has already demonstrated its advantage, efficaciousness and safety in a large field of therapeutic and curative drug development for animal and human (**S.** Khan et al., 2016). In 2011, six novel drugs were created based on the new Fc-fusion protein technology. Most of the newly developed drugs affect receptor-ligand interactions, acting as antagonists by blocking direct receptor binding, i.e. Enbrel® (etanercept; Amgen, USA), Zaltrap® (aflibercept; Sanofi, France), Arcalyst® (rilonacept; Regeneron, USA), or as agonists for direct stimulation of receptor function which augment immune response as Amevive® (alefacept, Astellas, USA) does, or decrease immune response as Nplate® (romiplostim; Amgen, USA) does. In this review, we pay attention to the most relevant results from the last few years for virus and bacterial vaccine designed based on Fc-fusion technology. The Fc-chimeras are hybrid sequences in which Fc-fragment of IgG (Fc-IgG) and targeted therapeutic protein are fused in an entire protein molecule (V. Pechtner et al., 2017). In this fusion, the hinge region of Fc-IgG is a flexible spacer between therapeutic protein and conservative part of IgG. It helps to minimize potential negative effect of two functional domains to each other. Therapeutic drugs based on Fc-fusion proteins are divided in three types, the receptor-Fc, peptide-Fc, and monomer-Fc. The Fc-fused proteins have tremendous therapeutic potential, since Fc domain in this molecules helps to specifically augment the pharmacodynamics values. Presence of Fc-domain in hybrid molecules prolongs half elimination of protein from plasma, which extends drug therapeutic activity and slows down kidney clearance for large molecules. Here, we summarize the most significant experimental data of Fc-fusion technology application against such pathogens as human immunodeficiency virus (D. Capon et al., 1989), Ebola virus (K. Konduru et al., 2011), Dengue virus (M.Y. Kim et al., 2018), influenza virus (L. Du et al., 2011), *Mycobacterium tuberculosis* (**S.** Soleimanpour et al., 2015), classical swine fever virus (Z. Liu et al., 2017). We also discuss the critical aspects of mechanism of action, drug design and Fc-fused protein production. Targeted activation of effector systems boosts protective potential of immunogenic molecules and broadens its application. The interest of this review is focused on an application of Fc-fused proteins as potential vaccines against infectious human and animal diseases. We also briefly discuss the perspectives of Fc-fused antigens for novel effective medicine developments using African swine fever virus as an example.

Keywords: Fc fragment, human immunodeficiency virus, Ebola virus, influenza virus, tuberculosis, classical swine fever virus, African swine fever virus, vaccination

Vaccination (immunization) is one of the most effective methods to manage infectious diseases of animals and humans. The number of developed

vaccines has increased significantly in recent years [1]. In 2017, the Pharmaceutical Research and Manufacturers of America (PhRMA) has published a list of 144 infectious disease vaccines under development [2]. The main requirements for modern vaccines are their efficiency, reliability, and absence of side effects [3]. Increasing requirements for vaccine safety and purity push forward not only classical vaccine development, but also new generation vaccine technologies, i.e. sub-unit vaccines, recombinant vaccines, anti-idiotypic vaccines, DNA vaccines, etc. New vaccines must also be targeted to successfully resist highly contagious infections that could not be treated before [4].

Emergent animal infections deserve special attention because of the need to prevent epidemics and restrict ability of pathogens to pass the interspecific barrier [5]. The creation of an effective vaccine against African swine fever virus (ASFV) remains one of the most acute and important problems over the past few years in domestic and world agriculture. Numerous studies are conducted by experts in many countries (Russia, USA, UK, Germany) to develop new and safe vaccines to cope with this virus [6].

The obtained results of the use of prototype vaccines against ASF virus demonstrate the protective effect of the homologous virus but do not provide protection against ASF viruses of heterologous origin. The problems are due to the biological characteristics of ASF virus and the lack of ability of antibodies to neutralize the virus [7]. Another distinctive feature of this virus is its extreme antigenic variability and heterogeneity. The data on the protective antigens of the virus and their role in the pathogenesis of the disease are also absent. The structure of the viral envelope, which includes a large number of glycated proteins, allows the virus to "mask" antigenic determinants and evade host's immune surveillance. One of the approaches to develop the means for treatment and prevention of African swine fever was the study of the role of virus surface antigens (CD2v protein, C-lectin-like protein, P54 virus membrane protein) [6, 8].

The technology of recombinant proteins has proved its advantage, effectiveness, and safety in a wide range of therapeutic and curative drugs against infectious diseases of humans and animals [9]. In 2015, the U.S. Food and Drug Administration (FDA) has approved more than 180 therapeutic fused proteins and peptides [10]. However, fused proteins have some disadvantages. Due to their small size and/or hydrolysis, these proteins are usually eliminated from the body rapidly. The short half-life of therapeutic proteins requires more frequent administration of the drug to maintain their effective concentration [11].

Two main strategies for improving the pharmacokinetics of the peptide or protein of interest exist. The first involves the formation of a repository or the introduction of an implant, which ensures the distribution of the drug from the site of introduction into the circulatory system using polymer and lipid microparticles [12]. The second is to reduce the rate of nephritic elimination of the target molecule by increasing its size [13]. It is achieved by increasing the hydrodynamic radius of the therapeutic protein by chemical conjugation with a large polymer such as polyethylene glycol (PEG) or by recombinant methods [14], as well as increasing the molecular weight of the protein to the threshold of passing through nephritic filtration (about 60-70 kDa) due to either noncovalent fusion of the therapeutic peptide with a larger carrier protein, or covalent fusion of the therapeutic peptide with the carrier protein using genetic recombination [15].

Quite often, an immunoglobulin fragment (IgG-Fc) is used as a fusion partner. A therapeutic protein fused with an IgG-Fc fragment may further provide a therapeutic effect that varies depending on the pathogenesis of the disease. Fc-fused proteins proved themselves well as therapeutic and prophylactic agents

[16]. In 2011, six drugs were created based on the Fc-fusion protein technology. Most of the Fc-fused proteins affect receptor-ligand interactions as antagonists either blocking direct receptor binding, i.e. Enbrel® (etanercept; Amgen, USA), Zaltrap® (aflibercept; Sanofi, France), Arcalyst® (rilonacept; Regeneron, USA), or as agonists for direct stimulation of receptor function which augment immune response as Amevive® (alefacept, Astellas, USA) does, or decrease immune response as Nplate® (romiplostim; Amgen, USA) does [17, 18]. The advantages of Fc-fusion drugs over other biopharmaceuticals are discussed in a number of papers [18-21]. The proteins obtained with this technology have greater therapeutic potential, as they are associated with the Fc-domain, which provides a targeted increase in the pharmacokinetics of the hybrid protein. It is proved that the presence of Fc-domain lengthens significantly the half-life of the proteins in the blood before their elimination, which prolongs therapeutic activity, and also leads to slower nephritic clearance for larger molecules [21].

This review has a particular emphasis on the most relevant recent findings in the application of Fc-fusion technology to create vaccines against viral and bacterial agents, with special attention on the prospects of this method for the development of drugs to prevent ASF.

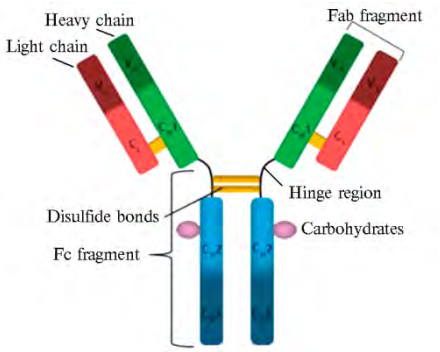

Fig. 1. Structure of IgG antibody [10].

The functions of the Fc-fragment. Immunoglobulins G (IgG) are antibodies involved in the neutralization of bacterial and viral toxins, stimulation of phagocytosis, and complement fixation. IgG trigger the effector mechanism of the immune response by interaction with the surface of leukocytes via Fc-receptor (FcR) [22, 23]. This is the manifestation of antibody-dependent cell cytotoxicity which leads to the lysis of pathogen-infected cells by cytotoxic T-cells (cytotoxic T-cell, T-killer, CD8+) [24]. About 85% of all serum immunoglobulins (A, D, E, G, M types) are IgG (Fig. 1) [13]. As well as albumin, IgG has the longest half-life compared to other plasma proteins [25]. Due to the small molecular weight (about 150 kDa), IgG molecules diffuse freely from the vascular bed into the extracellular space where they perform a protective function. IgG can penetrate the placental barrier from the mother's blood into the fetal blood [26].

Design and structure of Fc-fused proteins. IgG is a class of antibodies that are most commonly used to treat infectious diseases. Various ligands, extracellular domains of the soluble receptor, viral antigen, etc. can act as the target peptide or protein [27]. Due to the increased size and natural IgG conversion in the body, proteins fused to the Fc-fragment are protected from degradation because of their recycling which involves the neonatal FcRn receptor.

Fc-chimeric sequences are hybrid molecules in which the Fc-fragment of IgG (Fc-IgG) and the target therapeutic protein are a single protein product derived from a gene construct. In this fusion, the FC-IgG hinge site is a flexible spacer between the therapeutic protein and the constant part of the immunoglobulin, preventing the possible negative impact of the two functional domains on each other [15, 27].

The most common types of chimeric proteins are receptor-Fc, peptide-Fc, and monomer-Fc (Fig. 2).

One of the modern approaches in the creation of vaccines leading to the in-

duction of T-lymphocytes was implemented to produce a chimeric protein consisting of an antigen and an adjuvant based on the IgG Fc-fragment [27, 28]. Such vaccines may be used as a subunit vaccine (purified protein) or as a vector carrying the gene of this chimeric protein [29].

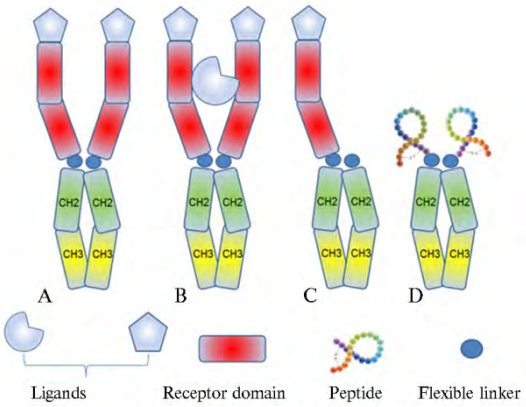

Fig. 2. Protein-protein fusion of IgG Fc-fragment with target fragments: dimeric fusion with receptor proteins (A, B; two versions of fusion with ligands), monomeric fusion with a target peptide (C), dimeric fusion with a target peptide (D).

The Fc-fusion technology has been successfully used to design vaccines against infectious diseases of humans and animals.

Fc-fused proteins as antiviral vaccines. *Fc-fused proteins as vaccines against human immunodeficiency virus.* The pioneering work by Capon et al. [30] on the use of Fc-fusion to develop remedies for acquired immunodeficiency syndrome (AIDS) has revealed the enormous potential of chimeric Fc-proteins for the treatment of a wide range of diseases. Capon and colleagues showed that a hybrid protein based on the extracellular part of the CD4+ receptor and Fc fragment of IgG, created for the treatment of HIV-1 (human immunodeficiency virus 1) infection, prevents the virus entering cells. Antibodies to CD4+ blocked the penetration of HIV-1 virus into T-cells, and the human cells transfected with complementary DNA (cDNA) of CD4+ became insensitive to the infection.

Soluble CD4+ (rCD4) receptor, devoid of transmembrane and cytoplasmic CD4+ sequences, can block the penetration of the HIV-1 virus into the cell, but this allows only temporary immunity to be formed [31, 32].

Fc-fused proteins as vaccines against the Ebola virus. The Ebola virus, belonging to the *Filoviridae* family, causes hemorrhagic fever in humans, which is characterized by high morbidity and mortality [33]. Filoviruses are classified as category A bioterrorism agents. The rVSV-ZEBOV vaccine is not commercially licensed but has been used under “extended access” conditions during Ebola outbreaks in North Kivu [34].

Currently, several types of recombinant vaccines based on different vectors are being developed, including adenovirus, parainfluenza virus, Venezuelan encephalitis virus, vesicular stomatitis virus, virus-like particles carrying viral glycoprotein [35]. Glycoprotein (Gp) of filovirus is the main protective antigen due to which protection against infection is provided.

In 2017, Konduru et al. [36] have reported the use of a chimeric protein of viral glycoprotein fused to the Fc-domain of immunoglobulin as a vaccine. The extracellular domain of the Zaire Ebola virus glycoprotein (ZEBOV) fused to the Fc-fragment of human immunoglobulin IgG1 (ZEBOVGP-Fc) was expressed in mammalian cells for this purpose. The results of the studies showed that the viral glycoprotein undergoes cleavage by furin [37]. Immunization of mice with the recombinant chimeric protein ZEBOVGP-Fc has activated T-cell immunity against ZEBOV virus and produced neutralizing antibodies against recombinant vesicular stomatitis virus (rVSV-GP). Mice vaccinated with the chimeric protein ZEBOVGP-Fc were protected from infection with a lethal dose of ZEBOV virus [38]. These results suggest that vaccination with only the chimeric protein ZEBOVGP-Fc may be sufficient to produce protective immunity

against the ZEBOV virus in mice.

High protection against a virulent ZEBOV virus induced by the ZEBOVGP-Fc hybrid protein indicates that a subunit vaccine based on hybrid proteins (Filovirus GP-Fc) can protect against viral infection [39]. Filo-virus GP-Fc, containing a glycoprotein bound to the Fc-domain, can be used as a standalone vaccine or in combination with other drugs such as DNA vaccines, virus-like particles or viral vector vaccines that are currently being developed. Production of the subunit vaccine based on Filovirus GP-Fc hybrid proteins is commercially quite simple, and in the case of its application the impact of side effects reduces. However, to confirm the safety of the vaccine being developed based on filovirus GP-Fc hybrid proteins, additional experiments on Guinea pigs and monkeys are necessary [40].

Fc-fused proteins as vaccines against the influenza virus. Hemagglutinins (HAs) of human influenza viruses (H1 and H3 subtypes) and avian influenza virus (H5 subtype) were obtained as recombinant proteins fused with the Fc-domain of a human immunoglobulin. Insect cells infected with baculovirus secreted recombinant HA-HuFc proteins (human influenza virus hemagglutinin fused with human Fc-domain of human immunoglobulin) as glycosylated oligomeric hemagglutinins. When mice were immunized with purified recombinant HA-HuFc protein in the absence of adjuvant, the obtained serum samples caused hemagglutination suppression, demonstrated epitope specificity and neutralized the virus. Based on the obtained results, the authors concluded that human influenza virus hemagglutinins fused with the Fc-domain of immunoglobulin can be candidate influenza vaccines [41-43].

Fc-fused proteins as vaccines against human papillomavirus. Human papillomavirus (HPV) is a huge problem in modern health care. There are 15 types of genital HPV, causing about 5% of carcinomas, primarily cervical, anogenital, and oropharyngeal transmitted sexually. All types of HPV affecting human skin tend to cause a benign form of cancer [44]. Licensed HPV vaccines based on virus-like particles carrying the main capsid antigen L1 are effective against widespread types of virus, but do not protect against other types that cause skin lesions, and are not therapeutic. Vaccines with enhanced adjuvant properties, including small capsid antigen L2, which use capsid display and fusion with early HPV antigens or Toll-like receptor antagonists, are under development.

According to Chen et al. [45], recombinant Fc-fused antigens of various viruses increase immunogenicity and induce synthesis of viral neutralizing antibodies against HPV, provide protective immunity against virulent herpes virus type II, influenza, and Ebola viruses. Chen et al. [45] showed for the first time that the fusion of HPV epitope 16 L2 (positions 17-36 bp) with a recombinant ligand for the FcRs receptor can significantly increase the immunogenicity of the L2 peptide and induce the production of cross-neutralizing antibodies and protective immunity against a number of phylogenetically distant types of human papillomavirus.

The modified Fc-fragment of human IgG1 can be used as a basis for the presentation of the L2 antigen to induce cross-neutralizing antibodies and protective immunity to different types of human papillomavirus. This type of recombinant fused protein is expressed in large quantities and can be easy purified. Therefore, the presentation of the L2 antigen together with the modified Fc-fragment provides new opportunities for the development of a vaccine against human papillomavirus [46, 47].

Fc-fused proteins as vaccines against tuberculosis. Tuberculosis caused by *Mycobacterium tuberculosis* (Mtb) ranks second in morbidity and mortality among infectious diseases worldwide [48]. As per WHO estimates, about 1.6 mil-

lion people died of tuberculosis in 2017, including 300,000 those HIV-infected [49]. An important feature of Mtb as a pathogen is its ability to survive for a long time inside the cell in a latent form that can later lead to an active tuberculous. The TB vaccine (Bacille Calmette-Guérin, BCG) is the only available licensed TB vaccine. It provides a sufficiently high protection against pulmonary tuberculosis, from 60% to 80%, but does not protect against hidden infections [50]. Consequently, the necessity for a new, safe and effective TB vaccine and an innovative vaccination strategy that could prevent all forms of TB, especially latent TB is obvious. Most new TB vaccines are currently in different stages of clinical trials or preclinical studies [51].

Simultaneous vaccination with multiple Mtb antigens can improve the protective effect against various forms of tuberculosis. Multistage fused proteins have been developed using ESAT-6 as an acute-phase antigen, with HspX protein as a latent antigen, with mouse Fcp2a fragment.

ESAT-6 antigen target is one of the most immunodominant and Mtb-specific target antigens containing multiple immunogenic T-cell epitopes capable of enhancing the cellular immune response. Mtb ESAT-6 is an important candidate antigen for the TB vaccine. In mice, Guinea pigs, and primates, TB vaccine containing ESAT-6 provides a higher protection than BCG [52].

HspX is a 16 kDa protein also known as α-crystallin Mtb that accumulates in dormant mycobacteria predominantly. It is highly immunogenic and can cause a strong cellular immune response in patients exposed to Mtb. Secreted proteins, the Mtb10.4 (Rv0288), Mtb8.4 (Rv1174c), ESAT-6 (Rv3875) and Ag85B (Rv1886c) antigens, are also highly immunogenic and can provide strong protective immunity against infection with *M. tuberculosis*, which suggests that these are promising candidate antigens [53, 54].

Profiling of immunogens to Fc-receptors (FcRs), as well as antigen-presenting cells (APCs) such as myeloid and plasmacytoid dendritic cells (DCs), monocytes, and macrophages can enhance the immune response in vitro and in vivo. This method is effective as it increases the half-life of the antigen and facilitates its uptake by APCs via FcRs and therefore increases cross-presentation efficiency for a powerful Th1 immune response. FcRI mediates selective uptake of antigens by dendritic cells, which leads to their delivery to the cytoplasm, where epitopes are recognized by the main class I histocompatibility complexes and presented to CD8+ cells. Cytotoxic T-lymphocytes (CTL) serve as an effective factor of cellular immunity for the destruction of intracellular pathogens. CTL activation via FcRs destroys an infected Mtb cell to form γ-interferon (IFN -γ), which activates infected macrophages to kill intracellular bacteria [55].

Fc-fused proteins as candidates to create a vaccine against Dengue fever. Dengue fever is an acute vector-borne viral disease that occurs with fever, intoxication, myalgia, arthralgia, rash, and lymphoid nodes enlargement. In some cases, Dengue fever develops the hemorrhagic syndrome, mainly in children under 15 years [56]. Dengue fever occurs mainly in South and South-East Asia, Africa, Oceania, and the Caribbean. The annual incidence is about 50 million people [57]. The causative agent of Dengue fever belongs to arboviruses of the *Flaviviridae* family of the *Flavivirus* genus (arboviruses of antigenic group B) [58].

The licensed Dengvaxia® vaccine (Sanofi Pasteur, France) does not protect children under the age of 9; therefore, additional vaccination strategies are necessary to stop this growing global epidemic. To obtain humanized and highly immunogenic polymeric immunoglobulin G scaffold (PIGS) fused with domain III of Dengue virus glycoprotein E (D-PIGS), the plant cell expression system was used [59]. The immunogenicity of this IgG-Fc receptor-targeted candidate vaccine has been demonstrated in transgenic mice expressing human

FcRI/CD64 [60]. In addition, recombinant molecules stimulated antigen-specific proliferation of CD4+ and CD8+ T-cells, as well as the production of neutralizing antibodies to IFN-γ. The purified D-PIGS fraction induced stronger immune activation than the monomeric form, indicating effective interaction with low-affinity Fcγ receptors on antigen-presenting cells. These results show that D-PIGS expressed in plants has the potential to be used as a single-component vaccine against Dengue fever of serotype 2 [60, 61].

A new approach to vaccination against Dengue fever is based on the application of Fc-fusion technology [62]. A design consisting of D-PIGS fused with Fc of mouse IgG2a showed high immunogenicity. To implement this approach, a version of the D-PIGS design using human immunoglobulin was created to obtain a candidate vaccine against Dengue fever. D-PIGS has shown high immunogenicity in transgenic mice expressing the human IgG receptor and, more importantly, in human tonsil cell culture. Thus, D-PIGS induced memory cell responses, IFN-γ production, and neutralizing antibodies against all four Dengue virus serotypes. This Dengue vaccine, based on human domain III and IgG1 polymer scaffold, has a potential advantage over other vaccines. It is easy to manufacture and scale, the risk of infection with animal pathogens is minimal and, most importantly, no antigenic interference, usually associated with the use of a tetravalent vaccine, is observed. The latter advantage is provided by the use of the EDIII domain III sequences of surface glycoprotein fused with human IgG1-Fc [63].

Fc-fused proteins as a vaccine against classical swine fever virus (CSFV). In recent years, a combinatorial approach based on the baculovirus vector has been widely used to create candidate vaccines against CSF virus [64]. Screening of a number of vectors based on baculovirus revealed that the baculovirus vector expressing the Fc domain of swine IgG1 has the greatest antagonism to complement (75.6%). Flow cytometry of transduced cells showed that in using this baculovirus vector the Fc-domain significantly increases the efficiency of transduction and transgenic expression of the reporter genes [65].

The E2 protein of the CSF virus was fused with the Fc-domain of swine IgG1 and translation enhancers *Syn21* and *P10UTR* were additionally linked to enhance antigen expression. The E2 protein of the CSF virus has been shown to be effectively expressed in both insect and mammalian cells. In pigs immunized with recombinant baculovirus, specific antibodies against the E2 protein responsible for the neutralization of the CSF virus, activation of the cellular immune response, and secretion of IFN-γ were synthesized with high titers. These results indicate the potential for widespread use of the Fc-domain of swine IgG1 and the surface antigen of the CSF virus [66, 67].

Thus, Fc-fusion technology has been successfully applied to the development of ways to fight many infectious diseases of viral and bacterial etiology. Structural, capsid proteins, and surface glycoproteins act as protective antigens. Both purified recombinant proteins and viral vectors (baculoviruses, adenoviruses, etc.) can provide antigen delivery [66, 68]. Regardless of the antigen delivery method, Fc-fused molecules induce strong cellular and humoral immune responses. When using recombinant Fc-fusion protein in the baculovirus system as a candidate vaccine against classical swine fever virus, intramuscular, intraperitoneal or intranasal vaccination with such constructs has been shown to induce a persistent humoral and cellular immune response. High titers of CSF-specific and neutralizing antibodies, as well as increased secretion of IFN-γ, indicate that baculovirus effectively delivers exogenous antigen to pig cells [65].

In other cases of Fc-technology application considered by the authors (Ebola, Dengue, human papillomavirus, tuberculosis causative agent), immun-

ization of animals with purified recombinant proteins was used to create antigenic constructions and effective activation of cellular and humoral immunity was also noted. In this regard, the technology of Fc-fused viral antigens as an approach to the creation of candidate vaccines looks promising in the case of ASF virus, in particular when using the viral protein CD2v, responsible for serospecificity.

So, the presented review demonstrates particular examples of the application of the proteins Fc-fusion strategy for the development of candidate vaccines against dangerous animal and human infections. Targeted activation of effectors increases the protective potential of immunogenic molecules and expands the scope of their application. Fc-fusion technology of recombinant antigens is effective to create therapeutic drugs. This approach can be promising in the development of candidate vaccines against African swine fever based on the CD2v antigen of the African swine fever virus.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 655-666
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.033/.034:636.082/.083:575.1

doi: 10.15389/agrobiology.2019.4.655eng
doi: 10.15389/agrobiology.2019.4.655rus

PRODUCTIVE LONGEVITY OF ANIMALS, METHODS OF ITS PREDICTION AND EXTENSION

(review)

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Received July 23, 2018

Abstract

Lengthening the terms of the productive use of animals is the most important problem in the cultivation of dairy and dairy and beef cattle, pig breeding and other branches of animal husbandry. The aim of this work was to review the influence of various genotypic and paratypic factors on life expectancy, productive longevity of farm animals, as well as analysis of studies to find modern ways of predicting and prolonging them. It was shown that with an increase in milk yield for lactation from 2500-3000 kg to 10000 kg of milk, the duration of productive use of cows decreases from 7-9 to 2-3 lactations, which increases the cost of milk production (I.I. Klimenok et al., 2001; J.R. Wright et al., 2016 et al.). An increase in milk productivity is accompanied by a decrease in reproductive function: the service period is prolonged, animal fertility decreases due to stress resulting from activation of the lactational dominant (A.I. Abilov et al., 2013; Y.S. Schuermann et al., 2016, etc.) To improve reproductive functions, duration of use, it is recommended to use special mineral-vitamin supplements (L.V. Romanenko et al., 2014; B. Close, 2007). Animal welfare is considered as an indicator of the stability of the system and is considered economically profitable (P.A. Oltenacu et al., 2010; L.V. Efimova et al., 2017). The duration of the productive use of sows, depending on the number of farrowing during use, fertility, survival of piglets and other factors is 3-4 farrowing instead of 4.5 in accordance with the accepted norm, which also affects economic indicators (M.D. Hoge et al., 2011). The indicators of heritability of signs of longevity in cattle and pigs are given (L. Canario et al., 2006), various feeding methods, breeding techniques, including the use of genetic markers to lengthen the economic use of animals. (C.N. Lopes et al., 2011; A.I. Sironen et al., 2010 et al.). Molecular markers related to the reproductive characteristics and duration of use of animals are given, which should also be used in genomic selection (N.S. Yudin et al., 2015; Q. Zhang et al., 2017). The theoretical provisions on the causes of aging, the influence of various stressors arising as a result of peroxide and antioxidant processes in the body are considered (E.S. Bauer, 1935; V.L. Voeikov, 2002).The role of reactive oxygen species, free radicals, and antioxidants of different nature on the reproductive function and viability of animals under stressful effects of different strengths is discussed (D.D. Boler et al., 2012; M. Sajeda Eidan, 2016). Thus, to increase the duration of the use and longevity of farm animals, combined with high productivity and adaptability to various, including adverse environmental factors, methods should be used that add up to several positions. It is necessary to develop and use proper feeding techniques that optimize the energy balance during all periods of the reproductive cycle, create favorable conditions for keeping animals, providing for exposure to certain stimulating factors that increase the biophysical potential of the body, affecting the functioning of biochemical systems. One should use the latest methods for predicting the level of free-radical oxidation of animal tissue lipids, which affect the manifestation of oestrus, oocyte and sperm viability, and the use of antioxidants with feed additives to balance oxidative and antioxidative processes. One more approach is to create herds (breeds, types) of animals with a high genetic potential for productivity and stress resistance using the most effective selection methods, genetic markers, genetic and mathematical models, and genetic engineering methods.

Keywords: productive longevity, milk yield, stress, reproductive function, heritability, genetic markers, free radicals

With the intensification of animal husbandry, especially commercial

farming technologies, animals do not lack close interaction with the natural environment. They do not receive microelements directly from the soil, insolation from the sun, and are subjected to the additional stress because of overcrowding and machineries. As a result, there is a reduction in life expectancy and economic use of livestock. Lengthening the productive life of animals is becoming a major problem in dairy and meat cattle breeding, pig breeding and other livestock industries.

The purpose of this review was to describe the influence of various genotypic and paratypic factors on life expectancy and productive longevity, as well as to carry out an analysis of studies to predict and search for modern ways to prolong them in farm animals.

In the 1990s, the productive longevity of the Siberian breed of black-and-white cows (3,736-4,122 kg milk yield per lactation, 15,500-17,600 kg life-time milk yield) when mating with Holstein bulls was 4.1-4.5 calvings [1]. With the increase in the milk yield of the first-calf cows, the time of their use in dairy herds even increased from 81.6 months for cows with milk yield of 2,501-3,000 kg to 105.6 months for cows with milk yield of 4,501-5,000 kg. The best in the duration of economic use in the Kholmogory breed were first-calf cows with milk productivity of at least 3,501-4,500 kg. They were used in dairy herds for at least 94.8-104.4 months [2].

In recent years, with the increase in animal productivity in many farms of the Russian Federation, the duration of the use of cows has begun to decrease to 2-3 lactations [3]. According to Strizhakov [4], the period of use is up to three calvings for cows with the productivity of 5,000-7,000 kg, and two calvings for cows with milk yield from 9,000 to 10,000 kg. As per Wright et al. [5], the use of cows in the USA equals 2.8 lactations. According to Hare et al. [6], this indicator is 2.8 for Holstein cows, 2.9 for Ayrshires, 2.4 for Guernsey cows, and 3.2 for Jersey cows. The average survival of cows to the 2nd calving is 73%, to the 3rd calving — 50%, to the 4th calving — 32%, from the 5th to the 8th calving — 19%, 10%, 5%, and 2%, respectively [6]. From Holstein-Friesian replacement heifers selected at 1 month of age at English dairy farms, 11% do not live up to the 1st calving. Of calving animals, 19% are rejected during the 1st lactation period, and 24% during the 2nd lactation period. Only 55% of replacement heifers successfully complete the 3rd lactation [7].

Fedoseeva et al. [8] believe that the main task of breeding highly productive animals should be not so much an increase in the milk yield as the creation of optimal conditions to realize existing genetic potential of milk productivity and especially the increase in productive longevity. The analysis of the productivity of Holsteinized cows of the Kholmogory breed has shown that productive longevity is only 3.3-3.8 calvings at the average annual milk yield of 7,400-7,800 kg. According to the authors, this indicator is influenced by many factors, the most important of which are stress, physical inactivity, nutritional imbalance, decreased adaptive abilities of the body, leading to impaired reproductive function and the birth of weak offspring.

In the last century, Shteiman [9], the outstanding breeder and the author of the Kostroma cattle breed paid special attention to the lengthening of use of highly productive cows. He believed that this provides an increase in the number of outstanding offspring and significantly reduce the cost of all products. At the Karavaevo breeding farm, some cows lived until they were 19-22 years old, their life-time milk yield was 98-103 tons. The Poslushnitsa II cow gave 14,115 kg of milk for 300 days of the 6th lactation with a fat content of 3.92%. Its daily ration with the maximum daily milk yield (61 kg of milk) consisted of concentrated (58%), succulent (35%) and rough (7%) feeds. Opytnitsa, the record-holder

cow, lived 19 years. Shteiman believed that the maximum milk yield for lactation could be reached if the cow was well-prepared during the dry period due to accumulation of necessary nutrients [9, p. 98]. This contributes to a more uniform coverage of the costs of milk production and allows avoiding excessive stress of cows throughout lactation.

The main causes of cow culling at present are infertility, abortions, mastitis, difficult calving, and the birth of small or dead calves [10-13]. Infertility is especially common among animals that produced more than 11,000 kg of milk during the first calving [14]. If the living conditions at the first calving do not correspond to high milk production, then the productive life is reduced, and the number of calvings decreases. As per Jaśkowski et al. [15], the high genetic potential of milk production in cows affects their fertility. They have a longer service period, a shorter estrus cycle and fewer chances of ovulation after calving. Oocytes of cows with a high genetic potential form a smaller number of blastocysts than in cows with an average potential. This leads to infertility and postpartum disorders.

Calving, in which animals need help or surgery, increases the risk of culling by 18% compared to that without assistance. The increased complexity of calving has a greater effect on culling in the first lactation than in subsequent ones. Difficult calving, mainly the first one, increases the cost of depreciation of the herd by 10% compared to the easy one [10]. There is a statistically significant relationship between the temperament and functional longevity of cows [16]. The very nervous (stress-sensitive) Holstein, Ayrshire and Jersey Canadian cows were 26%, 23% and 46% more prone to culling than very calm (stress-resistant) cows. The probability of culling very hard-milking cows of these breeds was 36%, 33% and 28% higher than average-hard-milking ones.

Recently, in order to increase the longevity of animals, special attention has been paid to some exterior parameters. Positive effects of posterior limb positioning, hoof angle and movement on life expectancy were found. Cows with a better structure of legs and hoofs are more likely to have long productive uses. Groups of cows with the highest and lowest scores for pelvic limbs differed in the duration of economic use by 931 days [17]. In the work of Zavadilová et al. [18], cows with crescent legs had lower longevity than cows with more straight legs.

Selection for plentiful milk production and elongation of lactation, causing activation of the lactational dominant, leads to stress in animals [19]. With an increase in milk productivity, the sexual dominant temporarily fades away, the duration of the service period increases, the effectiveness of artificial insemination decreases, and the duration of productive use decreases [3]. So, according to Sharkaeva et al. [20], in imported selection cows with a milk yield of 8,271 kg for the 1st lactation, the service period was 194.6 days compared to 114.9 days in Black-and-White cows with a milk yield of 5,688 kg. Moreover, the survival rate of imported cows before the 3rd lactation was only 38.6% versus 54.5% of cows of local selection. Schuermann et al. [21] consider longevity to be a key component of sustainable dairy farming. Highly productive dairy cows often suffer from ovarian dysfunction and infertility, resulting in reduced reproductive and productive lifespan. The authors attribute sterility to metabolic stress during the transition period (from the 3rd to the 12th week after calving), when there is an increase in cholesterol, triglycerides, total bile acids and a decrease in the concentration of glucose and glutathione compared to the period before calving.

With an increase in milk yield, animal welfare as a whole and the fertility of offspring decreases, problems with legs and metabolism appear, and life expectancy decreases. Many scientists consider the well-being of animals to be an economically advantageous condition, which is an indicator of the sustainable

husbandry with the high quality of products [22]. In the investigation of Efimova et al. [23] conducted on a large population the correlation coefficients between milk yield and reproductive ability in highly productive Holstein cows with loose housing on a deep non-replaceable bedding (milk yield 7,081 kg) were negative (r from –0.39 to –0.69 in daughters of different bulls; the level of statistical significance from $p < 0.05$ to $p < 0.001$). For cows that were kept loose in boxes, with a milk yield of 6,762 kg, these coefficients turned out to be positive, although insignificant (r from 0.01 to 0.25). That is, the content in the boxes, apparently, provided the animals with better well-being due to less exposure to stress and other environmental factors [23]. Studies conducted by Koketsu [24] indicate that achieving longevity and high reproductive ability of animals in breeding herds do not contradict each other. These indicators can be improved simultaneously.

Romanenko et al. [25] and Volgina et al. [26] proved that in order to realize the genetic potential of the milk productivity of cows with a milk yield of about 9,000 kg and above, it is necessary to optimize energy, protein, carbohydrate, and mineral-vitamin nutrition. To optimize energy supply during the stall period, they recommend increasing the amount of high-energy feed in the first phase of lactation. This contributes to a better realization of the genetic potential of cows for milk production in the following months.

During the early postpartum period, tissues of highly productive dairy cows undergo extensive catabolism due to the negative balance of nutrients. During this period, nutrients are distributed both in favor of lactation and to ensure the viability of the animal. However, metabolic disorders often lead to diseases that dramatically decrease the productive, reproductive abilities and the immune status [27]. The inadequate intake of nutrients and changes in feeding increase the risk of inflammation of the uterus. The strategy of manipulating health in the prenatal and postpartum periods should be aimed at minimizing the negative balance of nutrients, improving homeostasis and immunity through an appropriate diet. Supplements of unsaturated fatty acids of the n-3 and n-6 families usually improve fertility if they do not interfere with the metabolism of rumen microorganisms.

The use of mineral feeds that regulate the acid-base ratio, especially calcium, has a significant effect on the state of health, reproductive function, cow productivity and milk quality [28]. In the first weeks after calving, when the feed intake of the main feeds is reduced, the amount of calcium consumed is not enough for the cow. Feeding dry cows with special additives that create acidic conditions solves the problems of improving reproduction, increasing productivity during the lactation period, and reducing the cost of veterinary drugs. Vitamin E plays a vital role in calcium metabolism, bone formation and the incorporation of this element into the skeleton. Calcium salts of polyunsaturated fatty acids are used in beef cattle breeding to increase the reproductive ability of cows [29].

Negative consequences for the reproductive function and the duration of the productive use of animals are also observed in pig breeding with an increase in the milk production of sows due to the large size of the litter, which leads to excessive mobilization of body reserves during lactation. Life expectancy and productive use of sows are also becoming an economic problem, especially with high productivity. These indicators depend on the age at the first farrowing, the number of farrowing during the period of use, the length of the period between weaning and hunting, multiple fertility, the number of stillborn piglets and piglets born during the sow's life, growth rate and survival of suckling pigs. The higher the fertility, the lower the number of stillborn piglets and the higher the nest weight at 21 days of age, the less likely they are to be culled [30].

In pig breeding, there is a special attitude toward the preparation of dams in the dry period and during gestation, so that the sow has enough milk to feed the piglets while maintaining its continued use. In the conditions of pig farms, it is customary to receive 4.5 farrowing from the dam during the period of use. However, this is not always achieved, although 11-12 farrowings are obtained from some dams. The most common causes of culling sows, as in dairy cattle breeding, are reproductive problems (40-51%), leg problems (23-29%) and mortality (15-19%) [31]. Experiments have shown that the selection of pigs from highly productive and adapted dams, who survived 5 or more farrowing, and boars kept without a walk for at least 2 years, can be effective only if the animals for several months before insemination had motions [32]. The feeding and keeping conditions affect the fatness of pigs during the first farrowing, which, in turn, affects the condition of the body, leg strength and longevity. These indicators are improved when pigs are grown on floor coverings and bedding, which contribute to the development of healthy legs, and diets that limit the loss of live weight during lactation [33].

The main limiting factor in sow multiple pregnancy is embryonic loss during the first 2-3 weeks of gestation [34]. The reproductive quality of sows is largely dependent on the protein and lysine content of the diet. Low consumption of these substances weakens the development of follicles, reduces their ability to support oocyte maturation, becomes the main reason for sows to drop out due to agalactia, and leads to a decrease in milk production due to insufficient accumulation of nutrients in the body. During pregnancy, sows need significant amounts of minerals (Ca, P, Fe, Zn, Mn, Se, etc.) that are involved in the construction of embryo bones and milk synthesis. Close [35] believes that the content of these minerals in the diet should be increased at each subsequent gestation by 5%. To improve the economic performance of pig farming, it is necessary to reduce the time between weaning and hunting. This period, and at the same time, the duration of the entire productive use of sows is influenced by such factors as the timing and duration of ovulation, the correct determination of hunting and insemination techniques, seed quality, embryo mortality, fatness, and the immune status [36]. The period from weaning to insemination determines the multi-fetal pregnancy of sows. It was revealed that the multiplicity and weight of the nest of piglets during weaning, as well as the interval between weaning and hunting are higher in sows with later puberty (223-226 versus 185 days).

The selection of animals using statistical methods is not effective enough due to low heritability, relatively late manifestation of a trait or its manifestation only under the influence of certain factors, the presence of hidden carriers of undesirable traits, diseases. The heritability rate of survival in Holstein-Friesian heifers and cows in the UK, according to Pritchard et al. [37], amounted to 0.01-0.06. The heritability of the duration of the economic use of beef cows is small ($h^2 = 0.14$); therefore, it is believed that genetic improvement in longevity is difficult [38]. The coefficient of inheritance of life expectancy and reproductive traits in pigs varies from 0.14 to 0.17 [39]. For the heritability of the interval between weaning and the first hunt in sows after farrowing, this indicator is 0.17-0.18. The inheritance coefficient of the total number of piglets in the nest, live-born piglets, and the number of stillborn piglets is 0.10, 0.08 and 0.19, respectively [40)]

The modern science allows breeding not only through the estimation of the breeding value by statistical methods, but also at the genomic level. Animal genetics and breeding are developed on the basis of population genetics, from estimation of selection indices and the creation of complex genetic prediction systems to the development of accurate genome management systems. Based on

the information received, it is possible to conduct genetic selection and control specific genotypes at an early stage of the animal's life [41]. From determining the Estimated Breeding Value using the best linear unbiased prediction method (BLUP), based on the interaction of the genotype and the environment (heritability estimate), one can pass to marker-assisted selection (MAS) for certain genes, controlling economically useful traits, regardless of the degree of their inheritance.

Thus, the *DGAT1* gene located on the chromosome 14 (BTA14) [42] was identified as a genetic marker of the duration of the productive life of cattle, fat content, and milk productivity. Crossbred cows have single nucleotide polymorphisms (SNPs), associated with reproductive longevity, on chromosomes 4, 5, 15, and 19, which can be used to increase the life span of animals [43]. When identifying genomic regions in North American dairy cattle, the results of previous studies were confirmed and new sites were discovered that were associated with longevity, lactation resistance, reproductive function, and profit [44]. Zhang et al. [45] identified two important genomic regions located on chromosomes 6 (META-CHR6-88MB) and 18 (META-CHR18-58MB) which are associated with longevity. The *NPFFR2* was previously identified as a candidate gene for resistance to mastitis, the META-CHR18-58MB marker is associated with calving difficulties. The relationship of polymorphic DNA variants with milk productivity in cattle is discussed in detail by Yudin et al. [46].

About 30,000 genes were found for pigs [47] that can be used in genomic selection. It is particularly effective for signs that appear in the late stages of ontogenesis (life expectancy) or have low inheritance (reproductive capacity), as well as for resistance to disease and stress [41, 48]. At present, candidate genes associated with the reproductive characteristics of pigs (*ESR* and *PRLR*) [49], characteristics of the structure of legs, and life length of the sow [50-52] are of particular importance. Genetic markers have been found to improve milk production indicators that are associated with the reproductive qualities and life length of sows [53].

It was found that the duration of use of sows largely depends on several genes that affect these processes. Thus, the genetic markers of carnitine O-palmitoyltransferase (*CPT1A*) and C-C chemokine receptor (*CCR7*) were significantly ($p < 0.05$) associated with at least one reproductive trait. These results indicate that molecular markers should be considered for use in breeding in order to improve the duration of use of sows [54, 55].

It can be assumed that genomic selection for the life span and economic use of animals, as well as for productivity, will be more effective. In this case, the genome is tested using chips (matrices) for a large number of single nucleotide markers, the SNPs, covering the entire genome and associated with a quantitative trait locus (QTL), which makes it possible to determine the genotypes with the desired manifestation of productive traits and evaluate the breeding characteristics of the animal. Genomic selection allows decoding the genotype already at birth and selecting the best animals for breeding, which increases the accuracy and reliability of the evaluation of breeding pigs, significantly accelerates the selection process.

The search and use in the selection of markers of nuclear and mitochondrial DNA are becoming revolutionary. However, it should be borne in mind that genetic markers can indicate the location of certain genetic factors associated with longevity or productivity in animal chromosomes, be a means of early prediction of productivity and accelerated selection, but do not reveal the biochemical and biophysical processes that occur in the body during their phenotypic manifestation or interaction with each other. To a large extent, their mani-

festation depends on environmental factors, including various stressful irritations.

Theories of aging of organisms are divided into two types, i.e. genetic and free radical [56]. The aging process is a variable, stochastic and pleiotropic phenomenon which is regulated by various environmental and genetic factors [57]. The activity of the telomerase enzyme decreases in aging cells, which causes the shortening of the telomere end sites of chromosomes and does not ensure the preservation of DNA properties in successive generations of cell divisions [58]. Telomere shortening occurs in all somatic cells of the body in many diseases [59]. According to the free-radical theory of aging, the so-called free radicals appear in the process of life activity in the cytoplasm; they play a key role in the reproductive function of mammals, in the development of follicles, in the process of maturation of oocytes and sperm cells, their capacitation. The appearance of radicals depends on many factors, including the composition of the feed [60], the housing conditions, and the genetic characteristics of the animals [61]. In experiments on mice, it was shown that the oxidative process, which is observed in the blood serum, ovaries, and eggs and is caused by animal stresses, significantly affects the development of oocytes [62]. Spermatozoa, characterized by an extremely high rate of metabolism, produce particularly large amounts of free radicals [63, 64]. There are specific enzyme absorbers of reactive oxygen species, the glutathione peroxidase, catalase, superoxide dismutase (65], which convert active oxygen species into harmless molecules and play a huge role in the development of follicles and the maturation of oocytes [61, 65, 66]. Catalase has been tested by scientists to improve the quality of semen during freezing [66, 67]. A leading role in the molecular mechanisms of antioxidant protection and bringing the concentration of free radicals to the physiological norm belongs to thiol compounds that have SH-groups, showing high reactivity [68, 69]. Certain relationships between catalase and SH-groups in the blood lead to an improvement in sperm capacitation and thereby to better fertilization of the eggs [70, 71].

The latest ideas about the peculiarities of the processes involving free-radicals and the generation of electron-excited states in ontogenesis are based on the principles of theoretical biology formulated in the 1930s by Bauer [72]. According to them, the living system extracts substances and energy from the environment due to the higher thermodynamic (biophysical) potential. This requires a certain external impulse (signal), i.e. a stress stimulus within the physiological norm [59]. The animals, especially from reproductive groups, should not only be in favorable conditions, but also have systematic optimal loads to mobilize neuroendocrine systems. Experiments and practice have shown a positive effect of forced movement on the health and sperm production of producers, on the development of young animals, the productivity and viability of breeding stock.

Thus, to increase the longevity of farm animal use combined with high productivity and adaptability to various, including adverse, factors, a set of techniques should be applied. First, favorable feeding and housing conditions are mandatory. Nevertheless, certain stress factors at physiologically acceptable levels are also necessary to stimulate biochemical functions and production of reactive oxygen species, which causes electronically excited states in cells and thus increases the biophysical potential. Second, it is necessary to create herds (breeds, types) of animals with high genetic productivity potential using the most effective breeding methods, genetic markers and engineering, genetic and mathematical models.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 667-680
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.5:636.082:591.3

doi: 10.15389/agrobiology.2019.4.667eng
doi: 10.15389/agrobiology.2019.4.667rus

REPRODUCTIVE FUNCTION IN HYBRID POULTRY. V. THE EFFECT OF EGG STORAGE PRIOR TO INCUBATION

(review)

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Received May 14, 2019

Abstract

Storage of eggs causes the death of the blastoderm cells, including necrosis and apoptosis (S. Bloom et al., 1998). Depending on the storage conditions (duration, temperature, and air humidity), the physicochemical parameters of egg ingredients vary. Water and CO_2 move through the shell, leading to an increase in pH of the albumen and yolk, which changes the activity of enzymes, reduces bactericidal properties of albumen and yolk. Violation of amino acid composition and ratio is characteristic of the yolk, and lipid peroxidation is developing (M.N. Argunov et al., 2015). Destruction and increased permeability of the vitelline membrane and the internal eggshell membranes occur. The secondary sexual ratio shifts (M. McDonald, 1960; M. Tagirov, 2010; M. Boerjan, 2016). The efficiency of egg incubation decreases as well as chick yield (G. Fasenko, 1992; V. Christensen et al., 2001; K. Tona et al., 2003; P. Hristakieva, 2011; D. Terčič et al., 2016). Immunocompetence of chickens hatched from stored eggs lowers (M. Goliomytis et al., 2015). These hatchlings need elevated temperature of environment during the first weeks of life (S. Yalcin et al., 2014). Haugh units, albumen and yolk indexes, albumen pH, etc., being indicators of the stored egg quality (P. Tsarenko, 2015), vary depending on genotype, age and feeding, the stress of the parents, and the season. Each next day of storage following 2 days post egg laying needs an additional one-hour incubation period for compensation. The degree of embryo development in the freshly laid eggs is not the same in different bird species. The stage of embryogenesis depends on age, mother's type of use (for eggs, for meat, or for dual use), and the order of the egg in the cycle of egg laying. Eggs with embryos which reach the gastrula stage are less sensitive to storage (I. Reijrink et al., 2008). Reduced hatching can be partially leveled by different methods, including heating during storage which provides development to the specified stage of embryogenesis (I. Kosin, 1956; M. Petek et al, 2004; Y. Piestun et al, 2013; D. Nicholson et al., 2013; L. Dyadichkina et al., 2016). Their positive effect is limited by the egg quality, modes of storage and heating (temperature, frequency and duration of exposure), as well as incubation.

Keywords: poultry, egg hatching, storage, ontogenesis, quality assessment methods, preventive methods

The pre-incubation storage of eggs, which worsens not only the efficiency of their incubation, but also the quality of young stock [1-3], is one of the most significant factors limiting the efficiency of poultry reproduction. However, this technology is widely used at poultry enterprises providing the opportunity to limit the number of parental stock and thereby increase the profitability of production. In addition, it allows incubating large batches of eggs and, accordingly, obtaining chickens of the same age in quantities sufficient to implement the basic principle of commercial livestock: "All in —all out".

This paper describes the characteristics of the ontogeny of young stock of the main poultry species caused by pre-incubation storage during pre- and postnatal periods, as well as the prevention of negative consequences caused by pre-incubation storage.

The state of embryos in eggs laid by the hens. For ducks, tur-

keys and guinea fowls, embryos in laid eggs are mainly at development stages (DSs) 7-8 according to the classification of Eyal-Giladi et al. [4], for hens at the stage 10, for quails and geese at the stage 11 [5, 6]. The selection of poultry based on productivity characteristics caused the heterogeneity of ontogeny in genotypes, and in particular, early embryogenesis. In turkey eggs, which were selected to increase live weight gain, embryos are more likely to be at the early gastrula stage than their brothers from the divergent line [7]. In 3-4 days after egg laying by broiler hens of lines A and B, which differ in fertility and hatchability, the DS of the embryos was 10.3 and 10.7 [8], respectively, and after 7 days of incubation, it was 28.7 and 29.5 according to the classification of Hamburger et al. [9]. The area pellucida and area opaca have already formed in chicken embryos at the 10th DS, only the first signs of a hypoblast were distinguishable [5].

The condition of the embryos is also determined by the age of the mothers and the order of the egg in the oviposition cycle. The area of the blastoderm in fresh unincubated eggs expands in proportion to the aging of hens [10]. In the majority of eggs of 32- and 63-week-old broiler hens, DSs 11 and 12 are observed in embryos, respectively [11]. In the eggs laid in the first days of the cycle, the embryos are at the 10.36 DS, while in the eggs laid in the next days – at the 10.05 DS, respectively [12].

The conditions of hatching eggs with embryos at different stages of development are not the same. The hatchability of hen eggs with embryos that have not reached gastrula (<10 DS) at the time of laying is less than 55%, but if this stage has already formed (DS 12-13), then the number of chickens obtained is significantly increased [13, 14]. Based on this pattern, Fasenko et al. [15] formulated the hypothesis that eggs with embryos at the DS 12-13 have better keeping capacity in comparison with eggs with less developed embryos. Based on this hypothesis, a method has been developed to prevent the decrease in the value of stored eggs by heating them in an incubator (egg heating, EH), which will be considered below.

The laid eggs tend to be in an environment with a temperature lower than in the female body. Under the influence of hypothermia, embryogenesis ceases and the facultative diapause begins [16]. In hen eggs stored for 0, 4, and 21 days, such diapause was recorded at 14 °C, and embryos were assigned to the DSs 9.9, 10.0, and 9.9, respectively [17]. However, at ambient temperatures above the diapause threshold, genesis continues. The embryos in the hen eggs that were present in the nests for 1.5 hours (28.1 °C) were at the 10.4 DS, and within 6.5 hours (30.4 °C) they reached the 11.7 DS [18]. The presence of eggs from 59-week-old hens in the nest at 30 °C or 20 °C caused a 2.4% decrease in hatchability in the first compared to the second. The same patterns for eggs from 37-week-old hens were not found.

The blastoderm in laid hen eggs consists of 40-60 thousand cells, some of which die over time [4]. For example, in turkey eggs stored for 0 and 14 days (18 °C), the number of such cells decreased due to necrosis and apoptosis from 32 to 21 thousand, or by 34.4% [19]. The latter was found in 3.1% of blastoderm cells of eggs in newly laid hen eggs, and after 14 days of storage (12 °C) their share was already 13.9% [20]. In eggs stored for 14 days, the expression of a number of proapoptotic genes is enhanced compared with that in eggs stored for 4 days [21].

Thus, it can be stated that in the main species of poultry, the stage of embryogenesis at the time of egg-laying is different. The incubation properties of eggs with embryos at different stages of development are not the same. It is advisable to cool the laid eggs to ensure the stop of embryogenesis.

The effect of storage on the properties of hatching eggs. The

eggs are compressed during cooling, and water, carbon dioxide and air are transported through the shell to the outside. As a result, duck eggs stored for 8 and 15 days lose 0.53% and 0.78% of the weight, respectively [22], turkey eggs stored for 10 and 14 days lose 1.0% and 1.5% [23], hen and quail eggs stored for 10 and 20 days lose 1.8% and 3.5%; 1.9% and 4.7%, respectively [24, 25], while the size of the air chamber increases.

A decrease in the CO_2 content causes a rise in the protein pH from 7.6 in freshly laid eggs to 9.0 in eggs stored for 4 days, but afterward this indicator remains practically unchanged [14]. Along with alkalization, the viscosity of a dense protein decreases, which is due to the state of the ovomucin-lysozyme system formed by electrostatic bonds between its constituent molecules. The system has maximum stability at pH 7.0, and its disintegration occurs in the range of 9.0-9.5, probably due to alkaline hydrolysis [26]. The bactericidal activity of lysozyme in the protein of stored eggs decreases [27], the activity of other enzymes also changes. Due to the difference in osmotic pressure, protein water diffuses into the yolk, shifting the active reaction from pH 6.0-6.3 to pH 6.5-6.8 [14]. In proportion to the eggs aging, the protein content in the vitellin membrane changes [28], and its strength weakens [29]. Lower temperature slows down these processes, restricting the penetration of Salmonella enteritidis into the yolk [30].

The numerical values of a number of quality markers of stored eggs, including protein and yolk indices, as well as the number of Haugh Units (HU), are decreasing. So, in hen eggs that were stored for 14 days, a decrease was observed from 8.2% to 5.3%, from 45.5% to 42.8%, and from 79.7% to 62.2%, respectively [3]. The same occurs in pheasant eggs (a decrease from 2.4% to 1.8%; from 43.9% to 40.1%; from 83.0% to 76.5%), which correlates with a deviation in the young stock hatching (decrease from 66.7% to 41.6%) [31].

The higher the ambient temperature, the greater are abnormalities. In fresh hen eggs, HU reached 91.4, and in eggs stored for 10 days (5, 21 or 29 °C), this value decreased to 76.3, 53.7 or 40.6, respectively [32]. To a lesser extent, the egg condition is reduced due to storage at a 75-90% humidity [33].

During long-term storage in the deutoplasm, lipid peroxidation is recorded. In hens, after 21 days after laying the eggs, the amount of malondialdehyde in the yolk rises from 0.13 to 0.17 μmol/l [34]. At the same time, the antioxidant system is activated, which is expressed by an increase in the activity of catalase and glutathione peroxidase.

Thus, in the deutoplasm of stored eggs, the content of water and carbon dioxide decreases, protein and yolk are alkalized. The disintegration of the ovomucin-lysozyme system leads to the liquefaction of the dense protein. The ability of lysozyme to dissolve the wall of bacterial cells decreases, the activity of other enzymes changes. The lipid peroxidation is developing, the antioxidant defense system is activated. The properties of the vitellin membrane change. A change in the values of such quality markers of hatching eggs as the height of the air chamber, protein and yolk indices, HU and, ultimately, hatchability indicates a decrease in the value of eggs due to storage.

Embryogenesis in eggs incubated after storage. Embryogenesis after storage is slower than in freshly laid eggs. After the 42-hour incubation of eggs stored for 7 and 14 days, the lag was 5 and 12 hours, respectively, compared to freshly laid eggs [35]. Incubation until hatching 50% of the chickens was 16 hours longer in eggs stored for 18 days than in eggs stored for 3 days (502 and 486 hours, respectively) [2]. The prolongation was due to a delay in the beginning of stage I of the perinatal period and its lengthening, but not of stage II. Apparently, this is due to a delay in the increase in the blood concentration

of corticosterone which is necessary to increase the ratio of triiodothyronine and thyroxine involved in the regulation of hatching.

In general, the result of egg aging is retardation of the development of not only embryos, but also of hatched young stock. In chickens from eggs that were stored for 14 days, compared to those hatched from eggs that were stored for 3 days, the length of the jejunum, as well as the width and area of the villi in it, reduced [36]. During the first 7 days of growth, the bodyweight of chickens increased by 76% in case of 3-day pre-incubation storage of the eggs and by 64% in the case of 18-day storage [37].

The dynamics of the performance of eggs incubation varies depending on the duration of their storage. Thus, the young quail hatching averaged 86%, 88%, 84% and 82%, respectively, for the eggs after 1-, 3-, 5- and 7-day storage [38], and chicken hatching averaged 88%, 89%, 92% and 82% after 1-, 3-, 6- and 15-day storage [39]. The data presented indicate that as a result of short-term storage of eggs of both species (3 days for quails and 6 days for hens), the desired indicator increases. With an increase in the storage periods of eggs from 5 to 30 days, embryonic mortality during incubation increases in hens and ducks from 2.0% and 14.3% to 100%, respectively [16]. The presented data confirm the objectivity of the conclusion of a number of researchers [33] that the placement of eggs for incubation a few days after laying has a beneficial effect on embryogenesis and incubation performance.

The phenomenon of a shift in the secondary sexual ratio due to egg storage has been established, the manifestation of which is determined by a number of conditions. McDonald [41] found the predominance of females (54.6%) in the White Leghorn hens obtained from the eggs that were stored for 7 days at 4.4 °C, whereas 15.5 °C or 26.6 °C did not cause such an effect. In Rhode Island Red chickens hatched from eggs that were stored for 15 and 21 days at 11.5 °C, the sex ratio was 1.85♂:1♀, while in eggs hatched after storage for 3 days — 1♂:1♀ [42]. Among the chickens of the Lohmann cross (Lohmann Tierzucht, Germany) hatched from eggs that were stored for 11 days and to which EH was applied, females dominated [43]. At the same time, in the chickens of the Slobozhansky cross (National Academy of Agricultural Sciences, Ukraine) [42], as well as in the chickens of the Prelux-G cross (University of Ljubljana, Department of Animal Science, Slovenia), hatched from eggs laid by 24- and 65-week-old hens, stored from 3 to 15 days at 15 °C and then heated (EH), a similar pattern was not observed.

Consequently, the storage of hatching eggs provokes changes in the pattern of ontogenesis in both embryonic and postembryonic periods.

The influence of the genotype, age and physiological state of parents on the resistance of embryos developing after pre-incubation storage of eggs. Published data indicate a variation in the resistance of hatching eggs of modern highly productive poultry genotypes to storage. In ISA-White cross (Institut de Sélection Animale, France) eggs stored for 10 days, the protein level decreased from 9.7 to 4.7 mm, in ISA-Brown cross eggs from 8.3 to 4.1 mm, while the egg protein pH values in these genotypes did not differ (7.4-9.3) [44]. The duration of incubation of Peking duck eggs after storage for 3 and 14 days was 27.9 and 28.2 days, respectively, of Muscovy duck eggs — 33.7 and 34.6 days [45], that is, this period increased by 1.07% and 2.67%, respectively. In meat and egg quails, the hatchability of eggs stored for 10 days reached 83% and 85%, respectively, whereas after 14-day storage this indicator reached 78% and 83% [46]. It is conspicuous that in the egg laying genotype, hatchability decreases less significantly with an increase in the storage period.

In the poultry genotypes with different egg resistance to storage, the me-

tabolism of the embryos and chickens varies. So, the glycogen concentration in the liver of 17-day-old embryos from the eggs pre-stored for 14 days and 1 day were 19.7 and 30.0 mg/g in the broiler hen line L^+ with higher egg resistance to storage vs. 23.5 and 24.8 mg/g in the line L^- [47]. The hatchability of eggs after 14-day storage compared to 1-day storage decreased by 8% in L^+ and by 15% in L^-.

Poultry breeding has been proven to improve the resistance of stored eggs to bacterial contamination. Jones et al. [48] used eggs from hens of freely mating lines 5, 7, and 10 the selection of which was stopped in 1950, 1959, and 1970, respectively, as well as from hens of a commercial herd genetically associated with these lines. The authors found that the surface shell contamination in fresh eggs from hens of all genotypes was the same, but, for example, eggs from the herd stock after inoculation with *Salmonella enteritidis* (SE) and *Pseudomonas fluorescens* (PF) were more contaminated on day 7 of storage, while the eggs from line 10 were less contaminated. In this case, salmonella prevailed on the shell (the SE and PF average counts were 2.7 and 0.8 log CFU/ml, respectively). Egg components, on the other hand, were more infected with pseudomonas. The SE and PF concentrations in the air chamber, shell, and deutoplasm of eggs from laying hens of older age groups exceeded this indicator in eggs from young hens. In general, eggs from line 10 were less contaminated.

Egg quality parameters change and storage resistance is getting worse in proportion to the age of the laying hens. In eggs laid by 32- and 59-week-old hens, the pH of the protein increased (8.1 and 8.3, respectively), and its height, on the contrary, decreased (7.7 and 6.3 mm) [49]. After 7 days of storage of eggs laid by 35- and 45-week-old hens, the HU decreased from 77% to 69%, hatchability from 88% to 85%, and the quality of chickens from 97% to 79% [50]. The physiological state of chickens hatched from eggs which were pre-stored for 10 days differed between 27- and 61-week-old laying hens fed a phosphorus-deficient diet [51]. In the experiment, young stock obtained from an older age group had lower ash content in bones, and rickets were more often recorded than in herd mates hatched from young laying hens.

Chickens from eggs incubated after 3 and 14 days of storage were raised under brooders at the optimum temperature (OT, 32-28 °C), at elevated temperature (ET, 34-30 °C) or low temperature (LT, 30-27 °C) [52]. On day 2 in the chickens hatched from eggs pre-stored for 3 days, the body temperature did not depend on the ambient temperature, but it turned out to be the lowest in those chickens that were obtained from eggs pre-stored for 14 days and kept at LT. For the first 7 days of growing, a greater increase in bodyweight was recorded at OT for young stock from the eggs after 3-day pre-storage and at ET after 14-day pre-storage.

Artificial molting of hens did not adversely affect eggs [53]. The hatchability and bodyweight of 7-day-old chickens from eggs that were stored for 8-18 days before incubation and were obtained from molted hens were higher than those for a batch of eggs from non-molting hens. However, it should be noted that the effect of storage on the quality of hatching eggs from hens subjected to artificial molting has not been studied enough.

Prevention of a decrease in incubation parameters of eggs during storage. There are several methods to prevent the effects of egg aging, which differ in the mechanism, for example, by the use of a controlled gas environment [54, 55] or by placing eggs with the sharp end up [56, 57] and applying a layer of propolis to the shell [58], etc. One of these methods, the EH, is considered the most effective. The experiments carried out on the main species of poultry prove its effectiveness (Table).

Hatchability at single heating of eggs (EH) before storage

Poultry species	Age of laying hens, weeks	Conditions		Result	References
		storage	EH		
Broiler hens	32	11.5 °C; 60 %	37.50 °C; 6, 12 or 18 h	At St4, there is no effect; at St14 (for 6 h EH), hatchability is 79.0% (control — 70.5%)	[15]
	44	12.06 °C; 76 %	36.92 °C; 6 or 12 h	At St4 and St9 (for 6 h EH), hatchability is higher; at St14 (for 6 or 12 h), hatchability is lower	[66]
Turkeys	39-40	17.4 °C; 66 %	37.50 °C; 6 or 12 h	At St4, there is no effect; at St14 (for 12 h EH), hatchability is 75.3% (control — 70.6%)	[61]
Quail	20, 37	15 °C; 65 %	37.50 °C; 8 h	Hatchability in the experiment 82.6%, in the control — 70.6%; from 20-week-old laying hens — 84.7%, from 37-week-old laying hens — 77.6%. The differences between the variants St5 and St15 are minor	[62]
Guinea fowl	–	18 °C	37.50 °C; 3, 6 or 9 h	At St14 (for 6 h EH), hatchability is higher compared to the control	[67]

Note. For storage conditions, temperature and humidity are shown. St stands for pre-incubation storage of eggs (the number indicates the duration in days). Dash means that the age is not indicated [67].

The heating of freshly hatched eggs at 37.5-37.8 °C minimizes their quality deviation [59, 60]. It was established that the incubation indices correlate with storage and heating parameters. In particular, in chickens and turkeys, when the eggs were stored for 14 days before incubation, EH increased hatchability, which was not observed under 4-day storage [61]. A 6-hour heating was more favorable for the first group of eggs, while 12-hour heating for the second group.

The effectiveness of the EH depends on the age of the laying hens. In fresh quail eggs, which were processed once (8 h, 37.5 °C) and turned twice daily during storage [62], after 5 and 15 days of storage the hatchability due to EH improved by 4.4% for a batch of 20-week-old laying hens and only by 1.4% for 37-week-old laying hens, while the storage period did not affect the results of incubation. According to Gucbilmez et al. [63], the best result in batches of eggs from younger laying hens (27 and 29 weeks of age) is due to the fact that most embryos have not yet developed a hypoblast, and the EH ensures its formation, causing development to a stage more resistant to long-term aging [15].

EH effectiveness has a seasonality. Piestun et al. [64] in both the winter and summer periods used EH (at 30.2 °C for 12 hours) before 4- and 9-day storage of eggs. The hatchability of eggs in the control and experimental batches differed by 10% (84% and 94%, respectively) in the first case and only by 2% (85% and 87%) in the second one. In all cases, the bodyweight of 35-day-old young stock of both sexes increased, including due to the pectoral muscles.

In other experiment [65], eggs from 61- and 28-week-old hens were stored at 28 °C (mode 1) or 18 °C (mode 2). On the day of collection, EH was performed (37.8 °C for 6.0 and 4.5 hours for mode 1 and mode 2, respectively). As a result, variations were noted between the modes from 11.7 DS to 13.3 DS and from 9.0 DS to 12.6 DS, respectively. The hatching of chickens from eggs stored for 12 days for the mode 1 decreased compared to the control (74% and 80%, respectively), while in batches of eggs stored for 3, 5, and 8 days, the negative effect of EH on the analyzed parameter was not found. For eggs that were stored for 11 days, a positive result was observed for mode 2: hatchability in the experiment and control was 86% and 81%, respectively.

The effect of EH at the beginning of storage can change from positive to negative depending on the duration of the temperature exposure. Thus, in broiler hens, EH (6 h, 36.9 °C) of eggs of 4- and 9-day storage caused an increase in intestinal weight in 1-day-old chickens and did not affect the morphology of the mucous membrane of the duodenum [66]. However, a longer treatment (12 h) had a depressing effect on both the development of the mucosa and the live weight of the chickens. In guinea fowl, investigation of the effect of a single EH

at 37.5 °C for 3, 6, or 9 hours before storage revealed the advantage of a 6-hour exposure, namely an increase in the hatchability of eggs stored for 14 days [67].

Dymond et al. [68] performed 4-fold EH of hens, 4 h each at 37.5 °C, followed by storage for 4 and 21 days (the control was an intact batch stored for 21 days). EH caused an increase in the number of viable blastoderm cells. Hatchability was 92% and 84%, respectively vs. 71% in the control, and incubation time was 511 and 504 hours compared to 519 h in the control [68]. In turkey, EH at 37.8-38.0 °C for 5 hours on the 3rd and 5th day at a total storage period of 10 days, under the temperature and humidity regime differentiated with regard to requirements of each incubation period, reduced embryonic mortality, increased hatchability, shortened embryogenesis, and improved offspring rearing [69].

Thus, the development of embryos at egg-laying is not the same both in poultry of different species and within the same species [4-6], as well as in lines resulted from directional selection [7, 8]. The same was observed in embryos of the same genotype at different ages of mothers [10, 11] or depending on the order in the oviposition cycle [12]. Eggs with embryos at DS 12-13 were the best in storage parameters [15].

At ambient temperatures below physiological zero, embryogenesis does not occur in laid eggs. There is no consensus on the temperature range at which development ceases [10]. According to the reports, this range is 20-21 °C, 25-27 °C or 28-29 °C [70]. Apparently, such significant fluctuations are caused not only by the factors mentioned above, but also by differences in the thermal tolerance of genotypes, even with the same direction of use [71]. Eggs should be cooled after laying. Otherwise, the division of blastoderm cells will continue, but with an increase in the frequency of apoptosis, which will negatively affect the storage resistance. It is advisable to change the storage conditions depending on the intended duration, Thus, 19 °C and 70% humidity are optimal to store broiler hen eggs for 1-2 days, 12.5 °C and 90% are optimal for 13-16 days [72]; 15-18 °C are optimal for duck eggs under 1-3-day storage, 12-15 °C for 1-8-day storage and 8-12 °C for more than 8-day storage under 78-80% humidity [73].

As already noted, depending on the storage mode, the water reserves in the egg contents decrease due to evaporation through the shell, and the air chamber becomes more voluminous [23-25, 32], the protein and yolk are alkalinized, the ovomucin-lysozyme system disintegrates, the lytic activity of lysozyme decreases, the activity of other enzymes, the concentration and ratio of amino acids, including essential amino acids, change [74], the properties of under-shell membranes and the vitelline membrane are violated [28, 29], deuteroplasm is contaminated with microflora [30, 75], yolk lipids undergo peroxidation [34]. The main markers of the incubation value of such eggs are protein pH, protein and yolk indices, HU, and the air chamber height [3, 24].

During the incubation of old eggs, the embryogenesis and growth of hatched chickens lag compared to fresh eggs. Under incubation of turkey eggs pre-stored for 5, 10 and 11-15 days, 8-day-old embryos reached developmental stages 28.6, 27.7 and 27.0 [9] with a weight of 505, 437 and 406 mg, respectively [76]. One of the reasons causing a decrease in the embryo weight may be the activation of blastoderm cell death due to apoptosis during storage [21]. On average, during each next day from the laying to the beginning of incubation, its time becomes 1 hour longer, and hatchability decreases by 1% [77]. The physiological state of individuals at different hatching periods is not the same [78]. In the eggs subjected to long-term storage, a lower cardiovascular function occurs during the final period of embryogenesis, which is caused by the depletion of the energy reserves of the body, as evidenced by the activation of gluconeogenesis [47]. These circumstances must be taken into account, especially when breeding

highly productive broiler hens predisposed to the development of ascites syndrome due to their inherent chronic heart failure which is formed in embryogenesis [80].

Chickens hatched from stored eggs require elevated ambient temperature during the first week of active life [52]. Such young animals have reduced immunocompetence [81]. Considering the well-known role of lysine in the formation of immunodeficiency [82], it can be assumed that at least one of the reasons for the latter may be a decrease in the content of this essential amino acid in the protein of stored eggs [74].

The phenomenon of better incubation of eggs after short-term storage (3-6 days) compared to fresh eggs and eggs stored for a longer time is conspicuous [33, 38, 39]. It is likely that the increased hatching from the first ones is due to the fact that in them, unlike the second ones, the processes occurred before incubators are identical to those in the eggs during the nest formation in natural conditions. A characteristic feature of breeding individuals of this group, which includes most poultry species, is that during this period parents spend most of their time outside the nest. For example, ducks visit the nest only 1-2 times a day [16]. During this period, a pH gradient forms between the dorsal and basal sides of the blastoderm [40], the activity of enzymes changes, the viscosity of the protein decreases, and prerequisites are created for the formation of the embryonic fluid. During artificial incubation of fresh eggs, such a lag period is absent and the corresponding physicochemical processes ensuring the preparation of eggs for embryogenesis do not have time to occur. However, this period is short in terms of changes in the deuteroplasm that can occur under prolonged storage thus reducing quality of the hatching eggs.

With increasing age of the laying hens, the egg resistance to storage decreases [50, 51]. According to Damaziak et al. [83], the hatchability of stored eggs from 70- and 73-week-old broiler hens, as well as the quality of the obtained chickens are worse than for 49- and 52-week-old hens. Eggs from young hens can withstand longer storage at high temperature and low humidity than eggs of laying hens at the end of the productive period [84]. Embryos in stored eggs laid by hens of different ages differ in sensitivity to fluctuations in ambient temperature. There is a significant change in the embryogenesis in eggs from aging hens due to fluctuations in air temperature [85]. The authors simulated opening a warehouse door, where eggs were stored for 7 days (in poultry farms this usually happens at 09^{00}, 13^{00} and 17^{00} for the scheduled movement of batches of eggs), causing temperature fluctuations in the range of 18-21 °C for 40 minutes. As a result, there was a decrease in early embryonic mortality in the offspring of 27-week-old hens and, conversely, its increase in the offspring of 50-week-old laying hens compared to eggs stored at a constant temperature.

The growth rate of modern poultry genotypes derived through directional selection based on productivity traits varies, including at the stage of early embryogenesis. L^+ line turkeys, which were selected to increase bodyweight gain, lay eggs with less developed embryos than females of the L^- [7]. It is known that embryos that have not reached the gastrula stage are less resistant to storage [14]. This proves the need for individualization of both storage and EH modes for almost every cross. In particular, when studying the effects of the genotype, maternal age, and duration of storage on the egg condition [86], a higher HU value was established in stored eggs from Hy-Line Brown cross hens (Hy-Line International, USA) with brown shells compared to this in eggs from the Bovan Brown cross (Hendrix Genetics, Netherlands) with the same shell color and the crosses DeKalb White (Decalb Poultry Research, USA) and Hy-Line W36 with white egg shells. The dry matter content in the deutoplasm, the elasticity of the

vitellin membrane and its resistance to deformation also differed.

Considering the different needs of industrial poultry farming in males and females, the data on the displacement of the secondary sexual ratio compared to the standard (1:1) for different egg storage conditions is worth noticing [42, 43], especially since a shift in this ratio caused by the action of a certain temperature regime during egg incubation, fixed at the genetic level and transmitted to the next generation, was established [87].

The influence of EH as one of the most effective ways of preventing the decline in the egg quality during storage [61-69] has been tested on the main species of poultry. EH at a temperature of 37.5 °C should be carried out several days after laying, with repeating the procedure at 6-7-day intervals, while the total time of EH should not exceed 15 hours at a temperature above 32 °C [88]. Note that thermal effects on the egg occurs not only during pre-incubation storage (in the form of EH), but also during incubation through periodic changes in the temperature regime in order to train the hypothalamic-pituitary-cortico-adrenal system of embryos and to stimulate their adaptive capabilities [89-93]. These two methods are based on the use of thermal contrasts, which are similar to temperature fluctuations in natural conditions, during egg laying and hatching. EH during storage, as well as thermal training during artificial incubation, can optimize reproduction due to simulation of the processes that occur during the reproduction of birds in the natural habitat. At the same time, it is necessary to consider the unequal requirements of modern highly productive bird genotypes to environmental conditions. For example, developing embryos of the Ross 708 broiler cross (Aviagen, UK) are more sensitive to temperature conditions than the embryos of another Ross 308 broiler cross [94].

So, during the storage of eggs, changes in the physicochemical properties of the deutoplasm, vitellin membrane and shell membranes occur, the effectiveness of the protection against microflora and incubation efficiency become worse, and the conditions of the obtained young birds deteriorate. The development of embryos and the growth of chickens hatched from such eggs are slowed down. Methods have been developed to control the quality of stored eggs. In order to compensate for the decrease in the quality of hatching eggs, it is advisable to perform their heating during storage.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 681-692
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.084.524:637.4.04:615.322

doi: 10.15389/agrobiology.2019.4.681eng
doi: 10.15389/agrobiology.2019.4.681rus

FUNCTIONAL EGG PRODUCTION. III. THE ROLE OF THE CAROTENOIDS

(review)

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The authors declare no conflict of interests
Functional egg production. I. The role of ω-3 polyunsaturated fatty acids (review). Agricultural Biology, 2017, vol. 52, No 2: 349-366 (doi: 10.15389/agrobiology.2017.2.349rus, doi: 10.15389/agrobiology.2017.2.349eng).
Functional egg production. II. The roles of selenium, zinc, and iodine (review). Agricultural Biology, 2017, vol. 52, No 4: 700-715 (doi: 10.15389/agrobiology.2017.4.700rus, doi: 10.15389/agrobiology.2017.4.700eng).
Acknowledgements:
Supported financially by Russian Science Foundation, grant No 16-16-04047
Received June 6, 2019

Abstract

Recent growth of public awareness on the role of carotenoids (especially xanthophylls) in the prophylaxis and dietotherapy of certain oncologic, cardiovascular, and ocular diseases in human related to antioxidative and immunomodulating properties (E. Bakan et al., 2014) made these substances used earlier for the improvement of egg yolk color (and, as a consequence, attractibility of the eggs for consumers) valuable target substances in the production of dietetic, designer, and functional eggs (V.P. Singh et al., 2012). High bioavailability of carotenoids from table eggs due to the solubilization in yolk lipids makes the eggs a suitable source of carotenoids for the enrichment of human diet (H.-Y. Chung et al., 2004). In the study presented different aspects of practical production of functional eggs enriched with carotenoids are reviewed: the sources of carotenoids in the diets for laying hens and their comparative efficacy; metabolism of carotenoids in hens and its relation to the metabolism of lipids; the effects of different xanthophyll sources on health and productivity in hens, egg quality, the intensity of yolk coloration, concentrations of xanthophylls in yolk. The main advantage of synthetic preparations of carotenoids is their high availability for the layers (M. Marounek et al., 2016); however, due to their expensiveness these sources can be economically unprofitable in the production of enriched eggs. Natural sources of basic yolk carotenoids, lutein and zeaxanthin (S.M. Vostrikova et al., 2011), e.g. marigold (*Tagetes* spp.), frequently contain substantial part of these xanthophylls as ethers with fatty acids; the availability of these forms for layers is significantly lower in compare to the saponified forms (K. Lokaewmanee et al., 2011). These sources can be preliminary saponified to release the etherified xanthophylls: it will improve the availability of the latter for layers by 40-60 %, the transfer of these substances to egg yolks (H. Hencken, 1992), and prevent the resulting fatty acid profiles of eggs from the shift to higher percentage of saturated fatty acids (A. Altunta et al., 2014). The disadvantage of this approach is related to low stability of saponified preparations during the storage; researchers recommend these preparations to be stored frozen and to be used as soon as possible after the unpacking. Different effects of the sources of xanthophylls on the productive performance in layers (primarily the intensity of lay, egg weight and morphology) were reported: certain authors reported the significant improvements of these productivity parameters while other authors found these parameters to remain at the level of control treatments or be slightly lower. The majority of studied sources providing xanthophylls in the forms available for layers substantially and significantly improves the parameters of yolk coloration intensity (decreases index of lightness L* and increases indices of yellowness b* and redness a*) and concentrations of lutein and zeaxanthin in yolk. Reasonable choice of sources and doses can result in eggs enriched with xanthophylls to the extent where the eggs could be considered functional carotenoid sources for human (A. Sahoo et al., 2014), though no normal consumption rates for the xanthophylls irrespective to specific diseases were so far developed (E. Toti et al., 2018).

Keywords: functional eggs, carotenoids, lutein, zeaxanthin, layer diet, dietary carotenoid

In previous reports, the authors discussed the issues related to the production of ω-3 polyunsaturated fatty acid [1], selenium, zinc and iodine [2] functional hen eggs, as well as the enrichment (biofortification) of eggs with vitamins and carotenoids [3]. In this part of the reports series, special attention is paid to the production of carotenoid-functional eggs, as information is currently being accumulated on these substances as a means of preventing a number of human diseases.

Chemically, carotenoids are tetraterpene pigments with a color ranging from pale yellow to dark red [4], which are synthesized by many microorganisms and plants, as well as some algae and fungi. The animal organism is not capable of synthesizing de novo carotenoids [5]. More than 750 of these compounds are already known, and this list is updated every year [6]. They can form esters with fatty acids and complexes with sugars and proteins [7]. Some of them, for example, β- and γ-carotenes, cryptoxanthins, but not all of them, exhibit A-provitamin activity [8]. About 50 carotenoids with A-provitamin activity are known [9]. Carotenoid color is due to chains of conjugated double bonds acting as a chromophore group [10]. The same structures are responsible for the antioxidant properties of carotenoids: the more conjugated double bonds in the carotenoid molecule, the better its ability to bind the formed active oxygen species or to prevent their development [11]. The antioxidant properties of carotenoids in vivo are due to their interactions with other antioxidants, such as vitamins E and C [12].

The most important carotenoids for humans, based on the consumption with various products and plasma blood concentration, include α- and β-carotenes, lycopene (hydrocarbon carotenoids, or carotenes), lutein, zeaxanthin and β-cryptoxanthin (oxycarotenoids, or xanthophylls) [13]. In total, up to 40 different carotenoids can be present in the human diet [14]. It is known that carotenoids reduce the risk of certain types of malignant neoplasms (breast, lung, ovarian, prostate cancer), cardiovascular diseases, eye diseases (cataracts, age-related macular degeneration). The anti-cancer activity of α- and β-carotenes, lycopene, canthaxanthin and a number of other xanthophylls is associated with several possible mechanisms. Carotenoids with A-provitamin activity can affect the differentiation and division of cancer cells; antioxidant activity protects the DNA of healthy cells from damage by reactive oxygen species; the immunomodulatory effect enhances the control of carcinogenesis by the immune system; carotenoid enhancement of the intercellular signaling system inhibits the expansion of initiated cancer cells [15]. The role of carotenoids in the prevention of cardiovascular diseases is associated with their antioxidant activity: they protect low-density lipoproteins from oxidation, which contributes to the development of atherosclerosis, including coronary atherosclerosis [16]. The relationship between carotenoid intake with food and the frequency of cardiovascular diseases was confirmed by large-scale examinations of patients in Italy [17], Japan [18], EU countries [19], and Costa Rica [20].

The protective effect in eye diseases is carried out primarily by lutein and zeaxanthin, which are the only carotenoids present in the retina and lens of the eye [21]. They are part of the pigment of the so-called macula [22]. Two mechanisms have been proposed to explain the protective effect of carotenoids against age-related macular degeneration. The first is due to the fact that the macular pigment protects the photoreceptors and retinal epithelium from harmful blue light, which is well absorbed by both lutein and zeaxanthin [23]. The second mechanism is associated with the fact that the specified carotenoids reduce oxidative stress caused by light and photo-induced changes in the metabolism of

this tissue [24]. The antioxidant effect also explains the decrease in the frequency of cataracts due to these carotenoids (especially lutein). Thus, an in vitro experiment showed that lutein could inhibit lipid peroxidation induced by ultraviolet light in a culture of human retinal epithelial cells [25].

The main source of carotenoids in the human and animal diet is fruits and leaves of plants. The content of carotenoids in them and the bioavailability of these substances depend on the type of plant, its maturity, time of harvest, growing and storage conditions [26]. The bioavailability of carotenoids from concentrated preparations can also vary greatly [27]. For example, about 5% of carotenoids are absorbed from whole raw vegetables in the intestine, while up to 50% are absorbed from micellar solution, therefore the physical form in which carotenoids enter the intestinal mucosa cells is extremely important [28]. The chemical form of carotenoids is no less important. It is known that in hens free lutein is absorbed much more efficiently than its mono- or diethyl ethers, in the form of which this carotenoid can be found, for example, in marigold flowers, and that preliminary hydrolysis of these esters increases the absorption of lutein by 40-60%. The same applies to esters of zeaxanthin and capsanthin, the red pepper carotenoid [29]. It has been established that in the yolk and other tissues of hens' body, lutein is deposited mainly in its free form (80%) and to a lesser extent in the form of metabolites, mono- and diesters, and 3′-oxolutein [30]. On the other hand, it should be noted that both mono- and diesters of carotenoids are also effective antioxidants, since esterification does not affect or screen their polyisoprenoid chains [31].

Since most carotenoids are fat-soluble, their absorption and metabolism are associated with the absorption of fats. After absorption in the intestine and metabolization in the liver, carotenoids are transported into the blood by lipoproteins with low (non-polar carotenoids, i.e. carotenes) or high density (xanthophylls such as lutein or zeaxanthin). Then, these complexes with lipoproteins are decomposed by lipoprotein lipases with the release of free carotenoids [32].

The effectiveness of the absorption of carotenoids is affected by the composition of the diet. Thus, some plants, in addition to carotenoids, contain inhibitors of their absorption [33]. It has also been reported that carotenoid absorption worsens when there is a significant amount of coarse fiber in the diet [34]. Another factor is the interaction between individual carotenoids: it is assumed that the introduction of a large amount of a carotenoid into the diet affects the metabolism of other carotenoids, and can both reduce and increase their absorption. It has been shown that when mixtures of xanthophylls and β-carotene are added to the diet in approximately equal proportions, the latter inhibits the absorption of canthaxanthin [35] and lutein [36], as judged by the decrease in their concentration in blood plasma.

The bioavailability of carotenoids from food eggs is higher than from plant components of the human diet [37] due to solubilization in egg yolk lipids. So, one egg enriched with carotenoids can provide 5-10% of the daily human need [38]. According to Sahoo et al. [39], the total content of lutein and zeaxanthin in high-quality food eggs is 0.4-0.5 mg, and eggs with a content of about 2.2 mg/egg are already considered to be designer, that is, a functional food product. Russian researchers reported that the concentration of carotenoids in eggs from industrial laying hens receiving standard diets was 20 μg/g yolk [40].

Since the absorption of carotenoids in hens is also associated with the metabolism of feed fats, age and fat content in the diet affect their accessibility to poultry. It is known that at an early age, the efficiency of fat digestion in hens is low; therefore, the absorption of carotenoids during this period of ontogenesis is also small, but increases with age [41]. It was reported that the introduction of

an additional 6% of fat in the diet of chickens enriched with lutein leads to a 3-fold increase in its deposition in body tissues [42]. The same paper notes that some mycotoxins (aflatoxins, ochratoxin), as well as diseases (coccidiosis, Newcastle disease), reduce the efficiency of absorption and deposition of carotenoids in body tissue, especially in young animals. It should be noted that the introduction of carotene preparations into the diets of laying hens against the background of chronic mixed mycotoxicoses increased the survival rates of birds and reduced the intensity of the clinical manifestations of toxicosis [43].

The carotenoid profile of eggs is largely determined by the diet of laying hens, by varying which the carotenoid picture of eggs can also be changed directionally [44-46]. Carotenoids (e.g. canthaxanthin) are used as feed additives in industrial laying hens' diets to improve the color of the yolk; however, the amount and type of such additives may vary in accordance with the country's standards for the use of such additives and local prices [47]. It is known that xanthophylls are much more efficiently deposited in the yolk compared to carotenes [48] and to a much greater extent affect the color intensity of the yolk [49]. A low degree of transfer of carotenoids with A-provitamin activity to the yolk is due to the fact that they are mainly used by the body of the layer hen itself [50]. The most common xanthophylls in the yolk of eggs of various bird species are lutein and zeaxanthin [51, 52].

The efficiency of egg yolk pigmentation with carotenoids depends on their absorption, transport, excretion, deposition in tissue and bioconversion in laying hens, and all these factors are significantly influenced by carotenoid sources. In the study using radioactive labels, the degree of deposition of feed carotenoids into the yolk varied from 14% for astaxanthin to 30-40% for canthaxanthin [29]. The deposition of feed carotenoids into the yolk occurs within 48 hours, although the uniformity of staining is achieved later, after about 7-10 days [53].

As natural sources of carotenoids in chickens' rations, corn and its derivatives (gluten, alcohol bard) are used, including corn modified to increase the content of various carotenoids [54], tomatoes, carrots with different carotenoid picture, red pepper, flour from alfalfa, marigold flowers, marigold, carotenoid-rich algae, etc. [55]. Moreover, tomatoes, carrots and alfalfa contain more carotenes (β-carotene, lycopene) than xanthophylls [56, 57]. Synthetic carotenoids are also used, for which preparations of various compositions have been developed in recent decades [58, 59]. It should be borne in mind that not all of these sources of carotenoids are suitable for producing functional and/or dietary eggs. For example, specific red pepper carotenoids (capsanthin, capsorubin), in addition to antioxidant activity, can exhibit allergenic or toxic properties [60]. It is undesirable to use such sources (even with their low bioavailability for laying hens) when producing eggs with desired therapeutic properties.

In an experiment to investigate the effectiveness of the deposition of fodder lutein in the yolk [61], hens received lutein in doses of 0, 125, 250, 375, 500, 625, 750 and 1000 g/t with a basic corn-soy diet. With an increase in the feed lutein dose from 0 to 375 g/t, its content in eggs on the 7th day of the experiment significantly increased from 0.3 to 1.5 mg per 1 egg weighing 60 g ($p < 0.01$). The yolk coloring intensity score according to the Roche color scale increased from 6-7 to 13-14. However, with a further increase in the dose of lutein in the diet, a significant increase in its content in the eggs was not detected. It should be noted that in this experiment, the same diet supplemented with corn gluten and alfalfa flour increased the lutein content in eggs (up to 2.2 mg/egg at a lutein dose of 500 g/t), while the addition of flaxseed rather reduced this indicator. It can be assumed that the plateau of yolk saturation with lutein occurred as

a result of the limited ability of the liver to metabolize feed lutein and transfer it to the yolk during vitellogenesis, and not due to the limited ability of lutein to solubilize in yolk lipids. The source of lutein in this experiment was a 5% premix based on marigold flowers, that is, fodder lutein probably contained a significant amount of lutein mono- and diesters, which were significantly less absorbed. In a later experiment [62], different doses of flour from the flowers of marigolds and a hydrolyzed extract of this flour, where the lutein esters were saponified, were compared. Each source was fed at doses of 10, 20, 30 and 40 g/t per lutein. Neither the type nor the dose of the lutein preparation had a significant effect on the main indicators of egg quality and laying productivity. An improvement in the yolk coloring indicators was noted, and it was reliable only at a dose of 40 g/t for unprocessed flour and at doses of 20 to 40 g/t for hydrolyzed extract. The deposition in the yolk of common xanthophylls (lutein + zeaxanthin) in the groups receiving the flour, and not its hydrolyzed extract, increased from 2.0 (control) to 4.3 mg/100 g (dose 40 g/t), and in the groups receiving the extract up to 5.3 mg/100 g with the same dose of feed lutein. This increase was mainly due to increased lutein deposition, while zeaxanthin deposition increased slightly.

In a similar experiment with different doses of canthaxanthin (from 0.5 to 64 g/t of feed), its relative deposition in the yolk was 40%, regardless of the dose in the diet, although the absolute deposition increased a hundredfold, from 0.026 to 2.5 mg/1 egg [63]. A highly reliable ($p < 0.001$) decrease in the indices of lightness (L*) and yellowness (b*) of the yolk and an increase in the redness index (a*) were also noted. The color indices of the yolk were measured using Minolta colorimeters (Konika Minolta, Japan). These indices reflect lightness (L*: 0 = black, 100 = white), redness (a*: –100 = green, +100 = red) and yellowness (b*: –100 = blue, +100 = yellow) of the yolk. The egg-laying rate of the hens remained comparable to the control. Recently, it was reported that the introduction of canthaxanthin (3 and 6 g/t) into the diet of laying hens significantly increased not only their productive indicators (egg production rate, egg weight and feed conversion), but also the strength of the vitelline membrane in the yolks of freshly delivered eggs, which again decreased during storage [64]. Although the authors considered this indicator as a characteristic of the incubation qualities of an egg, it can have a certain value in terms of egg processing technology.

In the experiment of Czech authors [65], the effects of adding lutein (250 g/t), chlorella (12.5 kg/t) and a mixture of synthetic carotenoids Carophyll red and Carophyll yellow (DSM Nutritional Products, Switzerland; 20 and 15 g/t, respectively) to laying hens' diet on yolk coloration, the oxidative stability of yolk lipids, and hens' productivity were compared. All studied additives significantly increased the egg mass (Carophyll by 2.9%, lutein by 1.6%, chlorella by 2.0%), shell weight and thickness ($p < 0.001$), and also reduced the yolk/protein ratio. At the same time, lutein, unlike other additives, significantly increased the strength of the shell. With the same significance, synthetic carotenoids and chlorella increased the color intensity of the egg yolk. The yolk redness index (a*) was distributed among the experimental groups in the following order: control < chlorella < Carophyll < lutein; the yellowness index (b*) increased in the groups receiving chlorella and lutein. Lutein and chlorella significantly ($p < 0.001$) increased the concentration in the yolk of lutein (from 12.8 in the control to 133.9 and 49.0 mg/kg dry matter, respectively) and zeaxanthin (from 9.2 to 123.9 and 40.1 mg/kg dry matter). All three additives significantly increased the oxidative stability of yolk lipids, which was determined by the concentration of TBARS (thiobarbituric acid reactive substances) after egg storage for 28 days at 18 °C. Lutein additive also significantly increased the vitamin A content in the yolk and reduced the amount of vitamin E.

In a later experiment, the same authors [66] used marigold flower extract (0, 150, 250, and 350 g/t of feed) as a source of carotenoids, and compared the effectiveness of this extract (at a dose of 350 g/t) and a mixture of synthetic carotenoids (Carophyll red and yellow) in a production environment. An increase in the dose of marigolds led to a significant increase in the indices of redness and yellowness of the yolk ($p < 0.001$) and a decrease in the lightness index ($p < 0.05$). In this case, synthetic carotenoids in an industrial experiment reduced ($p < 0.001$) the yellowness index compared to the control without carotenoid additives. The deposition of xanthophylls in the yolk with an increase in the dose of marigold in the diet increased ($p < 0.001$): for lutein from 18.1 (control) to 29.8 mg/kg of yolk dry matter (dose of marigold 350 g/t), for zeaxanthin − from 12.3 to 19.2 mg/kg, respectively. It should be noted that in the industrial experiment, the introduction of synthetic carotenoids into the diet led to a decrease of almost 10% ($p < 0.001$) in the vitamin E content in the egg yolk (α-tocopherol), whereas in the group receiving marigolds, it did not have significant differences with the control. The authors were unable to identify a natural or synthetic carotenoid that affected the deposition of vitamins in the egg yolk. The authors consider the optimal dose of flour from the flowers of marigold to improve the color of the yolk is 250 g/t, and in terms of the economy of egg production — since at a dose of 250 g/t, the productivity indicators of the layers are slightly reduced. A series of experiments in which synthetic and natural sources of carotenoids were compared [59] led to the same conclusions.

In another experiment, Englmaierová et al. [67] introduced marigold extract with a lutein content of 21.26 and zeaxanthin 9.65 mg/kg in a corn-wheat diet at doses of 0, 150, 350, 550, 750 and 950 g/t of feed. An increase in the laying egg rate of laying hens in the 550 and 950 g/t variants and an increase in the weigh of eggs in the groups receiving the extract in doses of 550 and 750 g/t were revealed. The indices of redness and yellowness of the yolk increased with increasing the dose of the extract, and the index of lightness decreased. The content of lutein and zeaxanthin in egg yolks increased from 12.34 and 5.92 mg/kg of dry matter (control), respectively, to 36.33 and 25.59 mg/kg (extract dose 950 g/t). The authors believe that the extract at a dose of 550 g/t can be used as a replacement for synthetic xanthophylls.

The experiment of Spada et al. [68] compared the effects of natural (South American annatto shrubs) and synthetic (a mixture of red and yellow Carophyll) carotenoid sources in laying hens' diets on egg yolk color, the organoleptic properties of fresh and stored eggs (boiled and fried), the TBARS concentration in eggs and the emulsion properties of the yolk. It was established that the score for the color of the yolk of freshly boiled eggs from a bird receiving both sources of carotenoids was significantly higher than in the control group ($p < 0.05$); this difference gradually leveled during the storage of eggs. No significant differences in scores for the smell and texture of the yolk were observed. The control received higher scores for the color of the yolk of fried eggs, which was associated with the excessive, according to tasters, redness of the yolk from hens that received carotenoids. Lipid peroxidation (which was evaluated via the concentration of TBARS) during egg storage occurred in all groups, but in the eggs from hens that received carotenoids, it progressed somewhat more slowly. The emulsion properties of the yolk, which were evaluated by the electrical conductivity of yolk emulsions of different concentrations, did not undergo significant changes when both sources of carotenoids were introduced into hens' rations. The authors conclude that a natural source can be used as a rather effective replacement for synthetic carotenoids; however, to achieve the necessary organoleptic and technological properties of the yolk, it is necessary to more

carefully select the dosage of preparations, especially synthetic ones.

The presence of carotenoid esters in natural feed preparations for laying hens not only impairs the absorption and transport of carotenoids into the yolk, but can also affect the fatty acid picture of the yolk lipids. The experiment of Altunta et al. [69] considers the effect of feeding hens with flour from *Tagetes* flowers (10 or 20 g/kg feed) for 42 days on the fatty acid picture of yolk lipids of eggs laid in the last week of the experiment. In contrast to the study described above [67], here the egg weight at a dose of marigold 20 g/kg was lower than in the control. The addition of marigold increased the concentration in the yolk of the sum of saturated fatty acids and reduced the content of the sum of monounsaturated fatty acids. The authors attribute this to the fact that in marigolds, a certain part of lutein is in the form of esters with saturated palmitic and myristic acids. At the same time, such esters previously showed a very insignificant degree of deposition in the yolk, i.e. about 5% of the dose in the diet [53].

Enrichment of the diet with xanthophylls has a positive effect on the state of hens' bodies [70]. Gao et al. [71] studied the effect of introducing a mixture of xanthophylls (40% lutein and 60% zeaxanthin; 20 or 40 g/t) into the diet of laying hens at the age from the 34th week on liver and blood condition indicators characterizing the activity of the antioxidant system (activity of glutathione peroxidase, superoxide dismutase and catalase, total antioxidant capacity, the ratio of oxidized and reduced forms of glutathione, concentration of malondialdehyde). An increase in the activity of antioxidant enzymes and a decrease in the concentration of malondialdehyde have been established.

Since in eggs, especially dietary and/or functional eggs, an increase in the proportion of saturated fatty acids in yolk lipids is undesirable, the best sources of xanthophylls in laying hens' diets are synthetic products where they are not esterified with saturated fatty acids. It is also possible to use natural extracts of xanthophyll-rich plants previously hydrolyzed to saponify xanthophyll esters. Their use may be due to economic considerations, i.e. the availability and lower cost of natural sources compared to synthetic ones. At the same time, in the production of "organic", environmentally friendly eggs, it is quite possible to use intact natural sources of xanthophylls, which, if the composition and dose are selected correctly, can quite effectively replace synthetic carotenoids without significantly impairing the productivity of laying hens. A similar conclusion was made by the authors of many papers, where synthetic and natural carotenoids from terrestrial plants [59, 65-68] or seaweeds were compared [72].

When using natural sources of xanthophylls (especially saponified extracts), it is necessary to consider their instability in contact with atmospheric oxygen, as well as with certain nutrients and trace elements (for example, iron) that may be present in laying hens' diet. So, when storing an extract from marigold flowers, the content of carotenoids (in particular, β-carotene and lutein), total phenolic and total flavonoid compounds, as well as antioxidant activity, are gradually reduced, and the process speed depends on storage temperature: the most effective temperature was –20 °C, and its increase to +4 °C and further contributed to a faster decrease in indicators. The authors conclude that the best conditions for storing this product are air elimination with freezing up to –20 °C [73]. Therefore, after opening the factory packaging containing such preparation, it must be used as quickly as possible. Another method for stabilizing carotenoids, facilitating their transport through the digestive tract to the place of absorption (duodenum and jejunum) without associated oxidation, is solubilization in vegetable oils or absorption on lipid solids [74, 75].

Thus, correctly selected sources of carotenoids, primarily lutein and ze-

axanthin, in laying hens' diets allow obtaining eggs with the content of these and other carotenoids 2-3 mg/egg or more. Since the requirements for consumption of carotenoids (especially those that do not have A-provitamin activity) by humans are almost absent, it is difficult to say that eggs enriched to such an extent satisfy the quantitative criterion of functionality, according to which the product in an amount corresponding to its required daily intake should provide at least 30% of the recommended daily intake of the target substance. However, this percentage of the daily requirement may be significantly higher than 5-10%, i.e. the threshold specified by earlier studies on egg enrichment with carotenoids, which many authors of the papers of the last two decades agree with.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 693-704
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 637:577.161.2:[636.083+637.02

doi: 10.15389/agrobiology.2019.4.693eng
doi: 10.15389/agrobiology.2019.4.693rus

ULTRAVIOLET IRRADIATION TO ENRICH FOODS WITH VITAMIN D
(review)

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation, grant No. 16-16-04047
Received January 30, 2019

Abstract

Vitamin D deficiency found in 50-90 % of the adult and children's population in Russia (I.N. Zakharova et al., 2015; V.M. Kodentsova et al., 2017, 2018) and caused by inadequate intake and reduced endogenous synthesis in the skin due to insufficient solar irradiation, is associated with many chronic diseases and makes an important problem (A. Hossein-nezhad et al., 2013). One of the options for biofortification, called "bio-addition", is based on the ability of living organisms to form vitamin D from endogenous ergosterol by UV irradiation. Ultraviolet irradiation of animals allows minimizing seasonal variations in the concentration of vitamin D in cow's milk (R.R. Weir et al., 2017). A one-hour exposure of animals for 14-day to insolation at summer noon increased the vitamin D_3 content in pork ($p < 0.001$) to 0.716±0.097 μg/100 g (28.6±3.9 IU/100 g) which significantly exceeded the same indicator in the control animals (0.218±0.024 μg/100 g, or 8.7±1.0 IU per 100 g) (D.E. Larson-Meyer et al., 2017). UV irradiation effectively increased vitamin D level in chicken, from 0.16 to 0.96 μg per 100 g, even at 3000 IU/kg of dietary vitamin D_3 (A. Schutkowski et al., 2013).The amount of vitamin D_2 in shiitake mushrooms (*Lentinula edodes*) can achieve, under optimal conditions of UV irradiation, 29.87±1.38 μg per g dry weight. In the USA, Ireland, the Netherlands and Australia, fresh mushrooms are exposed to UV irradiation, which leads to an increase in the vitamin D_2 content to 10 μg/100 g wet weight (O. Taofiq et al., 2017; G. Cardwell et al., 2018). This is 50-100 % of the recommended daily consumption of the vitamin. The processing of baking yeast *Saccharomyces cerevisiae* by ultraviolet irradiation induces the conversion of ergosterol into vitamin D_2. The average content of vitamin D_2 is 3,065,417 IU/100 g (2,560,000-3,750,000 IU/100 g) or 770 μg/g (640-940 μg/g), which increases its initial concentration (less than 20 IU of vitamin D_2/100 g) almost 30-50-fold (EFSA, 2014). The vitamin D_2-enriched UV-treated yeast is allowed by The European Food Safety Authority (EFSA) for fortification of yeast-leavened bread, rolls and fine pastry at maximum D_2 dose of 5 μg per 100 g of the products. The concentration of vitamins D_2 and D_3 after UV irradiating of the wheat germ oil (1.6 mm oil layer) was 1035 and 37 ng/g, respectively (A.C. Baur et al., 2016). Similarly, there is an increase of the vitamin D content in eggs after exposure of chickens to UV irradiation or natural sunlight (A. Schutkowski et al., 2013; J. Kühn et al., 2014, 2015). In the conditions of the complete absence of the commercial production of vitamins in our country, bio-addition with vitamin D of chicken meat, eggs and dairy products by UV irradiation of animals, mushrooms, yeast, vegetable oils takes on particular significance.

Keywords: vitamin D, biofortification, bio-addition, poultry, eggs, cows' milk, mushrooms, vitamin D-enriched UV-treated baker's yeast, ultraviolet light irradiation, wheat germ oil

Functional products for healthy lifestyle and prevention of diseases caused by an inadequate and unbalanced diet are one of the main challenges and current global trends. Comparing the people's actual diet and the state of health indicates that the diet adequate for energy expenditure does not meet the needs in macro-

and micronutrients and minor biologically active substances. The quality of a functional food product (FFP) and the effects of its systematic consumption crucially depend on the food raw materials used. Vitamin value is the most important indicator to characterize the usefulness of food raw materials and the resulting FFPs, which determines the relevance of research aimed at increasing the concentration of vitamins in farm products. To improve vitamin availability, vitamins are added directly to foods or to raw foodstuffs [1]. However, as food fortification is still a voluntary initiative of producers, the portion of such products in the retail network is low. In addition, there is unfortunately a misconception among the public and sometimes in scientific literature that synthetic vitamins are poorly assimilated by the body.

Biofortification is a modern intensively developing technology to improve composition of biologically significant nutrients in foods. In animal and poultry production, this is achieved by adding vitamins, minerals, polyunsaturated fatty acids to feed [2], and in crop production by traditional and marker-assisted selection, biotechnology and agrotechnology [3]. The promising version of biofortification is based on the ability of living organisms to form vitamin D from endogenous ergosterol or 7-dehydrocholesterol under UV radiation. Vitamin D, which is synthesized in animals, mushrooms or yeast, undergoes the stages of biotransformation and is consumed by humans in its natural form.

This overview summarizes the opportunities for increasing the vitamin D content of agricultural products through ultraviolet irradiation (UV irradiation).

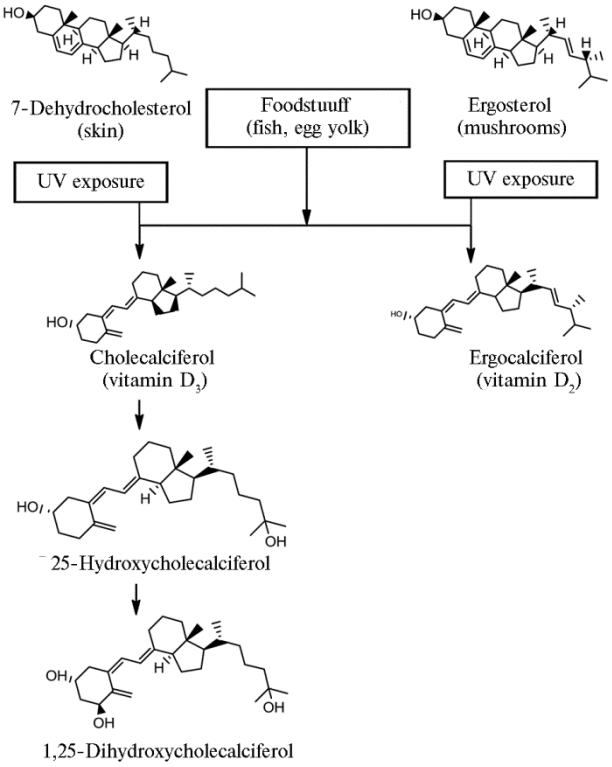
The essentiality of vitamin D (as well as other vitamins) for the human body is beyond doubt. Vitamins D, or calciferols, are represented by two compounds, the cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2). The difference between vitamin D and other vitamins is that it not only enters the body with food but can also be formed in the skin due to ultraviolet radiation (Fig.) [4, 5].

The main sources of vitamin D_3 in the human diet (in decreasing order of content) are cod liver, fish, eggs, liver, and butter [6]. In addition to cholecalciferol, 25-hydroxy-cholecalciferol ($25OHD_3$) contributes significantly to the vitamin value of meat and dairy products. In food of plant origin (algae, leaves and fruits of some plants), the vitamin D amounts are extremely low (from 0.03 to 0.67 μg/100 g dry matter) [7]. Vitamin D_2 is present in mushrooms. Vitamin D vitamers have different biological activity for humans. Vitamin D_3 is more effective than D_2 [8, 9]. It was found that in tackling inadequate vitamin D status 10 μg of vitamin D_3 is equivalent to 23 μg of vitamin D_2 or 6.8 μg of $25OHD_3$ [10, 11].

Calciferols are formed due to photo-isomerization of provitamins under UV radiation (see Fig.). Terrestrial animals are able to synthesize 7-dehydrocholesterol, the provitamin of cholecalciferol, from cholesterol; mushrooms and yeasts contain ergosterol, the provitamin of ergocalciferol [5]. Cholecalciferol is formed from 7-dehydrocholesterol when irradiated with sunlight or artificial ultraviolet light (λ = 280-320 nm) as a result of photochemical modification of 7-dehydrocholesterol followed by non-enzymatic isomerization.

It is believed that up to 80% of vitamin D can be synthesized in human skin under the influence of B ultraviolet radiation (λ = 290-315 nm) [12]. However, in Russia, due to insufficient sun exposure, endogenous vitamin D synthesis in the skin does not meet the body's need for this vitamin.

After entering the body, vitamin D is hydroxylated (see Fig.), turning first into 25-hydroxyvitamin D (25OHD) circulating in the blood (blood 25OHD is measured to assess individual vitamin D status) and then into its metabolically active form, the 1,25-dihydroxyvitamin D, possessing hormonal function [12].



UV dependent endogenous vitamin D synthesis and metabolism as adapted from [39].

Vitamin D deficiency in the population, caused by inadequate food intake and/or reduced endogenous synthesis in the skin due to insolation deficiency, has highly undesirable health effects [1, 13, 14], associated not only with skeletal but also with non-skeletal functions [15]. Vitamin D deficiency correlates with many chronic age-borne diseases [16], cardiovascular disorders [17], myocardial infarction, type 2 diabetes, tuberculosis, bronchial asthma, atopic dermatitis, urticaria, cancer of the prostate, breast, intestines, autoimmune diseases [17], and is accompanied by neurocognitive disorders, depressive states, reproductive dysfunction [12, 18, 19].

According to the results of studies of vitamin supply in the Russian population, vitamin D deficiency or insufficient intake occur in 50-90% of the adults and children [4, 14, 20, 21]. This problem is solved in some countries (USA, UK, Germany, Italy, Belgium, Finland) by legally regulated technological food fortification of mass consumption products [22]. As a result, technologically enriched liquid dairy products contribute 28-63% of vitamin D [23] to the total consumption. The effectiveness of such enrichment has been proven. In particular, there are reports on a decrease in the osteoporotic fractures [24, 25] and economic benefits [26, 27]. An alternative way to increase the vitamin D content of agricultural products is to irradiate them with ultraviolet light.

Cow's milk. Vitamin D content in milk depends on the intake of ergocalciferol with mushrooms and cholecalciferol from vitamin supplements, as well as endogenous synthesis in the skin [28]. Experiments using blankets and udder covers have shown that, despite the wool cover of the skin, vitamin D synthesis in cows is carried out evenly over the entire body surface [29]. Seasonal variations in the vitamin D content in cow's milk are well known, which are minimal in winter (0-0.04 μg/g fat) and maximum in summer (up to 0.014 μg/g fat) during intensive insolation at pasture [30]. Natural insolation is more effective in improving the vitamin D status in animals than the addition of vitamin D_3 or D_2 to feed [29, 31]. Artificial UV irradiation of cows for 24 days, imitating 1, 2, 3 or 4 hours of sun exposure in summer at 56° N, resulted in an increase in vitamin D_3 and $25OHD_3$ content in milk [30, 32]. Such irradiation minimizes seasonal variations in vitamin D concentration in cow's milk.

Animal meat. These products have traditionally not been considered an important source of vitamin D for humans, but in recent years there have been reports of the possibility of meat intravital modification.

Wavelength $\lambda = 296$ nm was optimal for the endogenous synthesis of vitamin D_3 in the pig skin. The maximum dose of 20 kJ/m^2 provides 3.5-4.0 μg of vitamin D_3/cm^2 of skin [33]. Exposure to sunlight in pigs has been found to increase the vitamin D content in the sirloin as well [34]. Exposure to sunlight increased ($p = 0.003$) the content of 25OHD, the form more effective for human, in muscle tissue and subcutaneous fat, but did not affect the amount of vitamin D_3 ($p = 0.56$). In pigs, after 14-day stay in the sun for 1 hour in summer afternoon, the content of vitamin D_3 in meat increased ($p < 0.001$) to 0.716±0.097 μg/100 g (28.6±3.9 MU/100 g), significantly exceeding the same indicator in the control group (0.218±0.024 μg/100 g or 8.7±1.0 MU/100 g) [34]. The amount of 25OHD$_3$ reached 0.281±0.014 μg/100 g vs. 0.130±0.016 μg/100 g. The total vitamin D content (D_3 + 25OHD$_3$) in the sirloin of pigs exposed to UV radiation increased 2.9-fold (0.997±0.094 μg/100 g vs. 0.348±0.027 μg/100 g, $p = 0.001$). The fact that the vitamin D_3 content in subcutaneous fat tissue did not change under sun exposure and was the same in pigs exposed to sunlight and in animals in the control group (with a 2-fold increase in the amount of 25OHD$_3$), has not been explained [34]. Sun exposure has led to better vitamin D availability in pigs, as per blood 25OHD$_3$ level, even with sufficient vitamin D content in the diet [35]. Daily UV irradiation of mini pigs (corresponding to sun exposure at noon for 10-20 minutes) stimulated the skin synthesis of vitamin D_3 and resulted in an increase in the amount of vitamin D_3 and 25OHD$_3$ in blood and the carcass. The vitamin D_3 concentration in adipose tissue of mini pigs was 150-260 ng/g if the animals were exposed to UV light, and 90-150 ng/g after D_3 oral administration at a dose of up to 60 μg per day, or 3.7-4.4 μg/kg bodyweight [36]. That is, UV irradiation was more effective. Based on the data obtained, it was concluded that changing the conditions for raising pigs when animals are allowed to be in the sun provides an effective natural increase in the vitamin D content of pork products.

Similar data were obtained for other animal species. Insolation improves the vitamin D supply in cattle. Thus, in late summer and autumn, the blood concentration of 25OHD in calf was significantly higher than in early June (55.2-63.8 vs. 26.3 ng/ml) [37]. Exposure to UV B increased the amount of vitamin D in the muscular tissue of chickens 4 times more effectively than feeding them with a diet with maximum permissible vitamin D_3 level. UV exposure increased the amount of vitamin D in chicken meat from 0.16 to 0.96 μg/100 g, even under 3,000 MU of vitamin D_3/kg feed [38].

Mushrooms. Many mushroom species contain high concentrations of ergosterol, which is transformed into vitamin D_2 under the UV radiation. Vitamin D_2 formation is influenced by temperature, humidity, UV spectrum (B or C), duration and dose of exposure [39-41]. The table shows the vitamin D_2 content of UV-irradiated freshly picked and freeze-dried mushrooms.

Vitamin D_2 content (μg/g of dry weight) **after UV irradiation of fresh and freeze-dried mushrooms** (*M*±SEM) [42-44]

Mushrooms	Fresh	Freeze-dried and crushed
Shiitake (*Lentinus edodes*)	29.46±2.21	60
Champignon (*Agaricus bisporus*)	3.55±0.11	119
Oyster mushroom (*Pleurotus ostreatus*)	58.96±1.15	34.6

The amount of vitamin D_2 under optimal conditions of UV irradiation of shiitake mushrooms (*Lentinula edodes*) was 29.87±1.38 μg/g dry weight [45]. Drying Shiitake mushrooms in the sun increased their vitamin D_2 content 16 times, from 100 to 1,600 MU per 100 g [15]. The original method of obtaining a functional food component was to obtain a shiitake mushroom extract with an ergoste-

rol content of 15%, and then convert it to vitamin D_2 under the influence of UV radiation at λ= 254 nm [6]. Freeze-dried shiitake extract after UV irradiation contained about 37 μg/g of vitamin D_2, which was more than 6 times its original amount. The extract can be used as a dietary supplement to food, 0.4 g of which fully meets the daily requirement for vitamin D [46]. The vitamin D_2 content in the fruitbody of six fungal genera (*Agaricus*, *Agrocybe*, *Auricularia*, *Hypsizygus*, *Lentinula* and *Pholiota*) and five species of *Pleurotus* irradiated with ultraviolet light for 2 hours increased significantly from 0-3.93 to 15.06-208.65 μg/g [47]. The maximum content (204.7 μg/g) was in oyster mushrooms. Drying of pre-cut mushrooms significantly increased the amount of vitamin D_2 produced during subsequent UV treatment, i.e. up to 406 vs. 45 μg/g of whole mushrooms [48]. Irradiation of dry white mushroom powder at room temperature for about 10 minutes resulted in an increase in the vitamin D_2 content to 741.50±23.75 μg/g [41].

Fresh mushrooms are currently exposed to UV radiation in the United States, Ireland, the Netherlands, and Australia, which leads to an increase in the vitamin D_2 content of up to 10 μg/100 g of raw weight [43, 49]. As a result, a portion of mushrooms (100 g) provides 50-100% of the recommended vitamin norm. The vitamin D content of the mushrooms treated in this way is similar to that of fatty fish [50]. This is essential for vegetarians typically lacking in vitamin D due to refusal of animal food [51-53]. The vitamin D_2 content in mushrooms with different methods of preparation is 62-88% of the original ($p \leq 0.05$) [54].

In rats with experimental vitamin D deficiency it was shown that vitamin D_2 from UV irradiated fungi is digested by these animals: the blood amounts of 25OHD and calcium increased and the mineral density of bone tissue was significantly higher ($p < 0.01$) than in the control rats receiving conventional mushrooms [55].

As per estimates of the vitamin D bioavailability from mushrooms, its absorption is sufficiently high. It was shown that consumption of mushrooms after their UV irradiation led to an increase in the blood concentration of $25OHD_2$ in human up to 24.2 nmol/l, especially in case of initial lack of this vitamin. However, there was a 12.6 nmol/l decrease in $25OHD_3$ [44]. Vitamin D_2 is well absorbed from UV irradiated shredded chanterelles and porcini mushrooms, helps to increase the $25OHD_2$ concentration and reduce the amount of $25OHD_3$ in blood [56]. It has been found that 2,000 MU of vitamin D_2 contained in mushrooms is as effective in increasing and maintaining the required amount of 25OHD in human blood as 2,000 MU of vitamin D_2 or D_3 [57].

A 30-day feeding calves with vitamin D2-enriched mushrooms prior to slaughter resulted in an increase in the vitamin D content of the meat, although less pronounced than that of the diet with vitamin D_3 [58].

Bakery yeast. The UV irradiation on *Saccharomyces cerevisiae* bakery yeast has induced the conversion of the ergosterol they contain into vitamin D_2 (see Fig.). The amount of vitamin D_2 was almost 30-50 times higher than its original concentration (less than 20 MU/100 g) and averaged 3,065 thousand MU/100 g (fluctuations within the range of 2,560-3,750 thousand MU/100 g), or 770 μg/g (640-940 μg/g) [59]. In 2012, the European Food Safety Authority (EFSA, Italy) approved UV-treated yeast enriched with vitamin D_2 as a new food ingredient in the production of yeast bread, rolls, flour confectionery products, the maximum dose is 5 μg/100 g. Such yeast is also permitted as a component of dietary supplements.

Consumption of bread made with D_2-rich yeast had a comparable effect with pure vitamin D_2 on the 25OHD concentration in women blood plasma [59]. The vitamin D_2 from UV-irradiated yeast used in bread baking was shown to be digestible and improve bone health in rats with an initial vitamin D defi-

ciency [60]. According to the effect on the content of 25OHD in experiments on growing rats, the effectiveness of the used form of vitamin D decreased in the series vitamin D_3 > vitamin D_2 > UV-treated yeast cells or their individual fractions [61].

In Canada, vitamin D_2 containing yeast are allowed as an ingredient of bread products in quantities up to 90 MU (2.25 μg) per 100 g, which is 23% of the D_2 recommended consumption rate [62]. In the United States, legislation was amended in 2012 to ensure the safe use of vitamin D_2 from baking yeast in baking products: it is permissible to add no more than 400 MU of vitamin D_2 per 100 g of finished products, or 50% of the recommended intake [63]. Food yeast remains a potential source of biologically active substances [64].

Vegetable oils. Vegetable oils can also be a potential source of vitamin D. They contain significant amounts of not only ergosterol but also 7-dehydrocholesterol (7-DHC) [65]. The ergosterol concentration is 22.1-34.2 μg/g in wheat germ oil, 4.2-23.4 μg/g in avocado oil, 7.9-17.4 μg/g in sunflower oil, 4.1-9.5 μg/g in rapeseed, soybean, flax oil, and < 4.5 μg/g in olive oil. UV-exposure resulted in the partial conversion of ergosterol and 7-DHC to vitamins D_2 and D_3 in these oils [65]. After 1-minute UV irradiation of 1.6 mm layer of wheat germ oil, the concentration of vitamins D_2 and D_3 was 1035 and 37 ng/g, respectively [65]. At the same time, such influence practically did not reduce the content of tocopherols and did not intensify peroxidation [65]. Assessment of the bioavailability of vitamin D showed that mice with an initial deficit of vitamin D improved, which was confirmed by an increase in the blood 25OHD concentration and accumulation in the liver compared to mice that received conventional wheat germ oil. However, the plasma content of 25OHD in mice fed UV-treated oil did not reach the values observed in the group that consumed oil with pure vitamin D added [65].

Chicken egg. Enrichment of eggs with vitamin D instead of adding it to the feed of chickens can be achieved by artificially irradiating birds with ultraviolet (bio-addition), or through free-range poultry.

In eggs from laying hens irradiated with ultraviolet for 3 hours daily for 4 weeks vs. those fed diet with the adequate vitamin D_3 content (3,000 MU/kg feed), the amount of vitamin D (cholecalciferol and 25OHD) usually reaches 2.5 μg. It is almost 5 times higher than in eggs of hens on the same diet but not UV irradiated [38]. Curiously, the endogenous formation of the vitamin occurs mainly in the legs of chickens, where the plumage is the smallest. The dependence of the increase in the vitamin D content in the egg yolk on the time of daily UV radiation is non-linear. With daily exposure to UV light for 300 minutes, the vitamin D_3 content increased to 28.6 μg/100 g of egg yolk dry weight, but did not reach the plateau, while the amount of 25OHD was already maximum with exposure for 60 min [66].

A similar increase in the vitamin D content is due to natural insolation. The amount of vitamin D_3 in the egg yolk in birds exposed to sunlight (free and closed/free keeping) was 3-4 times higher ($p < 0.001$) than its accumulation in the egg yolk of chickens in closed range [67]. The concentration of vitamin D_3 in the egg yolk of birds in free range was 14.3 μg/100 g vs. 3.8 μg/100 g of dry weight. The vitamin D content in the egg yolk of chicken eggs under the mixed keeping mode is in an intermediate position. The amount of $25OHD_3$ in the egg yolk also depended on sunlight, although it was lower than the concentration of vitamin D_3 ($p < 0.05$).

It should be noted that in recent years there has been a kind of rehabilitation of the chicken egg. Consumption of 6 to 12 eggs per week in balanced diets has no negative effect on major risk factors for cardiovascular disease and

type 2 diabetes [68]. A nutritional analysis of 7,216 participants aged 55-80 found that moderate egg intake was not associated with increased risk of cardiovascular disease in both diabetic and non-diabetic patients [69]. In addition, the simultaneous consumption of whole eggs cooked with fresh vegetable salad is an effective way to increase the absorption of α-tocopherol and γ-tocopherol, as well as carotenoids from plant foods [70, 71].

An adequate supply of vitamin D_3 is essential to maintain public health. In recent years, many countries have applied technological fortification of food products (yoghurt, bread, etc.) [27, 72, 73]. As to the role of biofortification, it should be noted that there is no production of vitamin substances in Russia. Food, medical and agricultural requirements for vitamin substances are met only through imports [72].

Thus, the use of alternative ways of enriching food products with vitamin D is vital, to some extent contributing to the solution of the problem of import substitution. Biofortification of chicken, pork, eggs and dairy products with vitamin D by UV irradiation of animals is perspective. So far, plant sources of vitamin D have not been given due importance, but the possibility of increasing the vitamin D content of mushrooms and plant oils through UV irradiation, makes it advisable to obtain these products that are important for vegetarians.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 705-712
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Functional structure of genome

UDC 636.4.033:636.082.12:575.113

doi: 10.15389/agrobiology.2019.4.705eng
doi: 10.15389/agrobiology.2019.4.705rus

STUDY OF GENETIC ARCHITECTURE OF FEED CONVERSION RATE IN DUROC YOUNG BOARS (*Sus scrofa*) BASED ON THE GENOME-WIDE SNP ANALYSIS

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The authors declare no conflict of interests
Acknowledgements:
The equipment of the Center for Biological Resources and Bioengineering of Farm Animals (Ernst Federal Science Center for Animal Husbandry) was used for the study.
Supported financially by the Ministry of Education and Science of the Russian Federation, a unique project number RFMEFI60417X0182
Received February 18, 2019

Abstract

Feed conversion (feed conversion ratio — FCR, kg/kg), calculated as the ratio of the amount of feed intake to the body weight gain, is the most important trait that determines the economic efficiency of pork production. The development of automated feeding stations allows researchers to carry out an accurate individual measurements of feed intake in the group-housed pigs, which became the basis for the integration of the FCR in the breeding programs. The development of high-throughput genotyping methods for tens of thousands of single-nucleotide polymorphisms (SNPs) made it possible to identify genetic factors associated with economically important animal traits at genome-wide level. Previous studies, performed in different pig breeds have shown the presence in the genome of the pig of multiple QTLs for FCR, while the regions of the genome identified in different studies were only partially overlapped. In this report, we present the genome-wide association studies results in one of the Russian Duroc boar population, which revealed the presence of 30 SNPs that were significantly associated with the feed conversion rate, as well as positional and functional candidate genes whose products are involved in the regulation of proliferation and differentiation various types of cells in lipid hematopoiesis and metabolism. The aim of the present work was to study the genetic factors affecting the feed efficiency in Duroc young boars, phenotyped individually for feed conversion rates and genotyped by ~ 70 thousand single-nucleotide polymorphisms at the genome-wide level. The study was performed on 715 young Duroc boars marked with electronic chips. Individual values of feed intake were recorded using automatic feeding stations MLP-RAP («Schauer Agrotronic AG», Switzerland) and GENSTAR («Cooperl Arc Atlantique», France). Genotyping was performed using a high-density DNA chip GGP Porcine HD (GeneSeek Genomic Profiler platform, Neogene, USA) containing of ~ 70 thousand SNPs. After quality control, 44810 SNPs were selected for genome-wide association studies (GWAS). Average daily gain (ADG) in the studied pigs amounted to 962.04±5.06 g/day, and feed conversion (FCR) was 2.53±0.2 kg/kg. Based on the GWAS analysis, 30 significant ($p < 0.00001$) SNPs localized at SSC2, SSC3, SSC4, SSC6, SSC7, SSC12 and SSC15 were identified, including three genome-wide significant SNPs, the H3GA0010441 ($p < 4.14\times10^{-7}$), ALGA0119936 ($p < 1.03\times10^{-6}$) on SSC3, and ASGA0028727 ($p < 1.17\times10^{-6}$) on SSC6. At SSC2, SSC6 and SSC15, the SNPs' blocks, consisting 10 (in the region of 29.0-30.9 cM, Sscrofa genome assembly 10.2), 7 (79.1-80.3 cM) and 3 SNPs (69.3-70.7 cM), respectively, were identified. Annotation of candidate genes localized in close proximity to significant SNPs revealed genes whose products are involved in heterogeneous biological processes, such as regulation of proliferation and differentiation of different cell types, hematopoiesis, lipid

metabolism. The additional studies aimed at validation of detected associations in other populations of pigs are necessary. Identification of novel QTLs for feed conversion rate will enhance our understanding of the genomic architecture of this important breeding trait.

Keywords: genome-wide association studies, feed conversion rate, average daily gain, back fat, Duroc boars.

Increasing productivity while reducing feed costs is one of the main goals in farm animal breeding [1, 2]. The feed conversion ratio (FCR), calculated as the ratio of the amount of eaten feed to the increase in live bodyweight, is the most important indicator that determines the economic efficiency of pork production, since the costs of feed reach 70% of the prime cost of pork [3, 4]. The development of automated feed stations made it possible to conduct accurate individual measurements of feed intake in group-housed pigs, which became the basis for integrating the FCR indicator into the breeding programs [5-7]. A detailed analysis of the heritability of feed conversion, conducted on three breeds of pigs [8], revealed a moderate genetic variation between individual breeds (h^2 = 0.30-0.54). The individual differences in feed conversion, based on genetic factors, allow identification of DNA markers of the trait in order to predict this indicator and to use it in pig breeding [9-13].

Based on the analysis of the genetic linkage of microsatellite markers, a number of QTLs were identified for the feed conversion indicator [14]. Thus, in the crossbred population (Meishan × Large White), QTLs for FCR were detected on SSC11 and SSC14 [15]. In the F_2 resource population (White Duroc × Erhual), using 183 microsatellites, 3 QTLs for FCR were found on SSC2, SSC7 and SSC9 [16]. The study of the Large White × Pietrain resource population for 118 microsatellite markers revealed the QTLs for FCR on SSC7 [17]. Mapping with 88 informative microsatellites performed in a crossbred pig population revealed the presence of QTLs for FCR on SSC2, SSC6 and SSC7 (for a feeding period from 90 to 120 kg) and on SSC2, SSC4 and SSC14 (for a feeding period from 60 to 140 kg) [18]. However, most QTLs, identified based on linkage analysis, were characterized by relatively low localization accuracy (in the range of more than 20 cM).

Improving the accuracy of QTL mapping and identification of the corresponding DNA markers became possible due to a DNA chip that allows the simultaneous analysis of tens of thousands of SNPs (Porcine60K BeadChip, Illumina, Inc., USA). In Duroc pigs, the presence of regions reliably associated with FCR was found on SSC4, SSC7, SSC8 and SSC14 [19], on SSC4 and SSC15 [20], and on SSC12 [21]. A genome-wide association study (GWAS) of the F_2 resource population showed the presence of QTL for FCR on SSC7 [22]. Maxgro terminal boars have 12 1-Mb regions on SSC6, SSC7, SSC9, SSC11, SSC14 and SSC15, responsible for more than 0.5% of the genetic variation of the trait [23]. Thus, the previous works showed the presence of multiple QTLs in the pig genome associated with feed conversion, while the parts of the genome, identified in different works, overlapped only partially. The involvement of new pig populations in the research expands the understanding of the genomic architecture of this important breeding trait.

This paper is the first to report data on the genetic conditionality of the inheritance of a complex and economically significant breeding indicator, the feed conversion, obtained with the use of microarray technology on a pig population of Russian reproduction. In this report, we present the results of genome-wide association analysis in one of the Russian populations of Duroc boars, phenotyped individually for feed conversion and genotyped for ~ 70 thousand single nucleotide polymorphisms (SNPs) at the genome-wide level. As a result, 30

SNPs were identified that were reliably associated with the feed conversion indicator, as well as positional and functional candidate genes the products of which are involved in the regulation of proliferation and differentiation of various types of cells, in hematopoiesis and lipid metabolism.

The subjective of the work was to study the genetic factors affecting the efficiency of feed use in Duroc boars.

Techniques. The studies were conducted in OOO "SGTs" (Voronezh Province, Verkhnyaya Khava settlement) from October 2017 to November 2018 on 715 Duroc boars labeled with electronic chips. The tissue samples (ear pluck) were collected from all animals, preserved with 96% alcohol and stored at –20 °C. The average age of the animals at the beginning and the end of the experiment was 77.6±0.3 and 156.2±0.4 days, respectively. The boars were kept at the quarters with slotted floors in groups of 15 animals each (floor area 1.30 m^2/animal) at 18 °C. The animals had unlimited access to food and water. Feed consumption was individually measured using automatic feed stations MLPII-RAP (Schauer Agrotronic AG, Switzerland) and GENSTAR (Cooperl Arc Atlantique, France).

The FCR value was calculated for each animal as the ratio of the eaten feed to the increase in live bodyweight over the entire growing period. The values for the parameters of the initial and final weight of the boars, daily average weight gain and duration of testing at the stations were checked for compliance with the normal distribution ($M \pm 3\sigma$). Given the differences in the duration of the growing period between the groups, the heterogeneity of the formation of groups of animals by live weight (both when setting and removing from fattening), as well as the rate of growth, to obtain comparable feed conversion values, was assessed using the multiple linear regression equation of the FCR indicator, calculated with STATISTICA 10 (StatSoft, Inc., USA):

$$FCR_{(r)} = 4.6738 - 0.0158x_1 - 0.0170x_2 + 0.0183x_3 - 0.0024x_4,$$

where $FCR_{(r)}$ is the regression value of feed conversion; x_1 is the duration of the feeding period at the station; x_2 is live weight at the beginning of fattening; x_3 is live weight at the end of fattening; x_4 is the average daily gain in live weight over a period; 4.6738 is a free member of the equation, a constant value.

To isolate DNA from the tissue samples, the DNA Extran 2 kit (OOO NPF Syntol, Russia) was used in accordance with the manufacturer's recommendations. The concentration of double-stranded DNA was determined using a Qubit 2.0 fluorimeter (Invitrogen/Life Technologies, USA). OD_{260}/OD_{280} ratio was measured (NanoDrop8000 spectrophotometer, Thermo Fisher Scientific, USA) to determine the DNA quality. DNA with OD_{260}/OD_{280} = 1.6-1.8 was used in the analysis. In addition, the DNA quality was evaluated by gel electrophoresis on a 1% agarose gel.

Genome-wide genotyping was performed with a high-density DNA chip Porcine GGP HD (GeneSeek Genomic Profiler platform, Neogene, USA) containing ~ 70 thousand SNPs. Quality control and filtering of genotyping data for each SNP and each sample was performed with the PLINK 1.9 software package (http://zzz.bwh.har-vard.edu/plink/) applying the following filters (the corresponding commands in the PLINK program are given in brackets): the quality of genotyping for all studied SNPs for an individual is not lower than 90% (--mind); the quality of genotyping for each of the studied SNPs in all individuals is not less than 90 % (--geno); the frequency of occurrence of minor alleles (MAF) is 0.03 (--maf); deviation of SNP genotypes from the Hardy-Weinberg distribution in the aggregate of tested samples with a p-value < 10^{-6} (--hwe). After the quality control, 44,810 SNPs were selected for GWAS analysis.

Regression analysis in the PLINK 1.90 (--assoc --adjust --qt-means) was used to identify associations of SNP markers with the FCR indicator. To

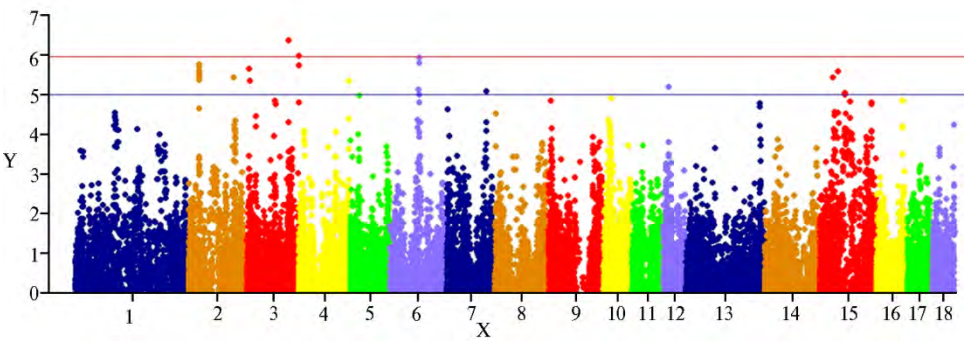
confirm the reliable effect of SNP and to determine significant regions in the pig genome, we tested the Bonferroni null hypotheses at a threshold value of $p < 1.12\times10^{-6}$, $^{0.05}/_{44,810}$. The data was visualized in the qqman package using the programming language R [24]. To search for candidate genes localized in the region identified by SNP, the genomic resource Sscrofa10.2 was used (https://www.ncbi.nlm.nih.gov/assembly/GCF_000003025.5/, reference date of April 2, 2019). Functional gene annotations were performed using the GeneCards database (http://www.genecards.org/, reference date of April 2, 2019). The arithmetic mean (*M*), the standard error of the mean (±SEM), and the coefficient of variation (*Cv*, %) were calculated with MS Excel 2013.

Results. The initial and final live weight of the boars used in the studies was 35.3±0.2 and 110.5±0.5 kg, respectively, with daily average gain over the control period of 962.0±5.1 g and the average feed conversion of 2.53±0.02 kg/kg (Table 1).

1. Phenotypic indicators in the studied sample of Duroc boars (*Sus scrofa*) (*n* = 715, OOO SGTs, Voronezh Province, Verkhnyaya Khava settlement, October 2017-November 2018)

Indicator	*M*±SEM	Min	Max	C_V, %
Initial live weight, kg	35.3±0.2	19.9	53.0	15.7
Final live weight, kg	107.3±0.5	75.0	157.0	10.9
ADG, g/day	962.0±5.1	547.5	1507.7	14.0
ADFI, g/day	2411±15	1318	3762	16.4
FCR, kg/kg	2.53±0.02	1.33	4.03	16.2

N o t e. ADG, g/day — daily average gain; ADFI, g/day — average daily feed intake; FCR, kg/kg — feed conversion; *M* — mean value; SEM — error of the mean; Min — minimum value, Max — maximum value; C_V, % — coefficient of variation (the ratio of the standard deviation σ of a random variable to its expected value).



GWAS analysis for the feed conversion indicator in the studied sample of Duroc boars (*Sus scrofa*): X-axis — the chromosome number; Y-axis — the inverse decimal logarithm of the confidence level $-\log_{10}(p)$; upper horizontal line — confidence threshold for genome-wide associations $-\log_{10}(p) = 1.2\times10^{-6}$; lower horizontal line — confidence threshold for suggestive associations $-\log_{10}(p) = 1.02\times10^{-5}$ (*n* = 715, OOO SGTs, Voronezh Province, Verkhnyaya Khava settlement, October 2017-November 2018).

Using GWAS analysis, we identified 30 SNPs on SSC2, SSC3, SSC4, SSC6, SSC7, SSC12 and SSC15, which were reliably associated with the FCR indicator ($p < 0.00001$), while blocks from 3-10 SNPs were found on SSC2, SSC6 and SSC15. For three SNPs (H3GA0010441, ALGA0119936 on SSC3 and ASGA0028727 on SSC6), the confidence level exceeded the threshold for genome-wide studies ($p < 1.2\times10^{-6}$) (Fig., Table 2).

The most interesting positional and functional candidate genes identified in close proximity to significant SNPs include the *ABTB2, CAPRIN1* (located on SSC3 inside a block of 10 SNP candidates in the 29.0-30.9 cM region), *RBAK* (located on SSC3 in the immediate vicinity, –10825, from the genome-wide SNP ALGA0119936), *PTPRU, MECR, MED18, PHACTR4* and *RCC1* (located on SSC6 inside a block of 7 SNP candidates in the 79.1-80.3 cM region).

2. Reliably significant ($p < 0.00001$) SNPs associated with feed conversion in Duroc boars (*Sus scrofa*), and candidate genes (n = 715, OOO SGTs, Voronezh Province, Verkhnyaya Khava settlement, October 2017-November 2018)

SSC	SNP number	SNP	Position	p	Candidate gene (position)
2	10	INRA0059415	28977392	2.57×10^{-6}	*ABTB2* 28,946,563 ... 29,149,547; *NAT10*
		ASGA0082316	29159639	2.84×10^{-6}	29,153,541 ... 29,194,433; *CAPRIN1* 29,199,142 ...
		ALGA0012786	29501376	3.92×10^{-6}	29,242,779; *LMO2* 29,401,270 ... 29,422,801;
		WU_10.2_2_30191053	30191053	2.00×10^{-6}	*KIAA1549L* 29,617,451 ... 29,746,696; *CD59*
		ASGA0097788	30337163	4.32×10^{-6}	29,777,121 ... 29,797,735; *FBXO3* 29,800,868 ...
		WU_10.2_2_30595322	30595322	3.19×10^{-6}	29,812,278; *HIPK3* 30,028,373 ... 30,057,948;
		WU_10.2_2_30650235	30650235	3.19×10^{-6}	*DEPDC7* 30,336,221 ... 30,344,776; *TCP11L1*
		ALGA0012822	30781606	2.01×10^{-6}	30,353,850 ... 30,399,032; *CSTF3* 30,402,895 ...
		ASGA0009813	30860286	1.72×10^{-6}	30,418,796; *QSER1* 30,443,575 ... 30,528,992;
		ALGA0012826	30894959	3.28×10^{-6}	*PRRG4* 30,549,353 ... 30,569,161; *CCDC73* 30,598,386 ... 30,748,173; *EIF3M* 30,748,559 ... 30,772,863; *WT1* 30,902,160 ... 30,946,266
	1	ALGA0015465	124437922	3.73×10^{-6}	*CCDC112* 124,337,488 ... 124,341,509; *TICAM2* 124,610,898 ... 124,613,028; *TMED7* 124,682,078 ... 124,691,898
3	1	H3GA0008543	7379529	2.24×10^{-6}	*LAMTOR4* 7,350,308 ... 7,402,741
	1	WU_10.2_3_8562889	8562889	4.56×10^{-6}	*COL26A1* 8,559,750 ... 8,703,022; *MYL10* 8,726,761 ... 8,738,595
	1	H3GA0010441[a]	116920902	4.14×10^{-7}	*CLIP4* 116,931,705 ... 117,021,159
	2	WU_10.2_3_144118805	144118805	1.83×10^{-6}	*RBAK* 144,137,154 ... 144,154,400
		ALGA0119936[a]	144165225	1.03×10^{-6}	
4	1	WU_10.2_4_140678150	140678150	4.40×10^{-6}	*LMO4-201* 141,341,709...141,354,291
6	7	WU_10.2_6_79124865	79124865	7.22×10^{-6}	*MED18* 79,123,55...79,156,756; *PHACTR4*
		WU_10.2_6_79310063	79310063	7.22×10^{-6}	79,256,482...79,343,715; *RCC1*
		ASGA0087502	80148516	9.87×10^{-6}	79,351,068...79,378,340; *TRNAU1AP*
		ASGA0028727[a]	80164977	1.17×10^{-6}	79,381,689...79,406,923; *RAB42*
		ALGA0035788	80216205	1.57×10^{-6}	79,417,316...79,419,319; *TAF12*
		ALGA0114520	80246537	1.57×10^{-6}	79,432,412...79,441,136; *YTHDF2*
		WU_10.2_6_80303210	80303210	1.57×10^{-6}	79,553,445...79,583,689; *OPRD1* 79,611,950...79,662,042; *EPB41* 79,809,404...79,916,611; *TMEM200B* 79,916,247...79,920,674; *SRSF4* 79,952,072...79,979,921; *MECR* 79,986,968...80,018,140; *PTPRU* 80,024,234...80,106,252
7	1	H3GA0022804	109049333	7.98×10^{-6}	*DIO2* 109,299,323...109,308,576
12	1	WU_10.2_12_15902684	15902684	6.47×10^{-6}	*MARCH10* 15,861,982...15,944,352
15	1	MARC0002947	35674169	3.69×10^{-6}	*PTPN18* 35,772,241...35,792,658
	1	DRGA0015116	50414290	2.54×10^{-6}	*TENM3* 50,292,659...50,995,047
	3	H3GA0044471	69276645	1.02×10^{-5}	*KCNJ3* 69,329,216...69,481,280; *NR4A2* 70,671,034 ... 70,689,181; *GPD2* 70,841,147...70,946,575
		H3GA0044472	69300270	9.18×10^{-6}	
		ALGA0085736	70725225	9.18×10^{-6}	

N o t e. [a] is SNPs with a confidence level that exceeds the threshold for genome-wide associations. The positions in the assembly of the Sscrofa10.2 genome are indicated (https://www.ncbi.nlm.nih.gov/assembly/GCF_000003025.5/).

The product of *ABTB2* has activity of protein heterodimerization, the product of *CAPRIN1* is involved in the regulation of proliferation and migration of various cell types. The protein encoded by *LMO2* plays a central role in the development of hematopoiesis, the protein encoded by *RBAK* interacts with the androgen receptor which affects cell proliferation and differentiation, as well as with the transcription factor E2F which plays a crucial role in the control of the cell cycle. PTPRU is a signaling molecule that regulates a wide range of cellular processes, including growth, differentiation, and the mitotic cycle. Among the known functions of *MECR* is participation in the metabolism and biosynthesis of fatty acids. The *MED18* product, a component of the mediator complex, is involved in the regulation of transcription, and its participation in lipid metabolism has also been established. The functions of the protein encoded by *PHACTR4* include participation in the proliferation of nervous cells, as well as interaction with the α-actin of the skeletal muscles, which are involved in motility of different cell types. In humans, a relationship is established between polymorphisms in the *CAPRIN1*, *LMO2*, *MECR*, *PTPRU*, *PHACTR4* and *RCC1* genes with sys-

tolic pressure, in *LMO2*, *PTPRU* and *ABTB2* — with the blood protein concentration, in *CAPRIN1*, *LMO2*, *MED18*, *PHACTR4* and *RBAK* — with parameters of erythrocytes. It is known that the level of systolic pressure is associated with the growth rate in the juvenile period [25]. In turn, a relationship was established between the hematological parameters and the growth rate of pigs [26, 27], which, as is known, correlates with feed conversion [5]. The relationship is shown between the protein (glycoprotein and haptoglobin) content in the blood of pigs and the growth rate and the efficiency of feed use [28].

Thus, the conducted genome-wide studies of the Duroc boar population revealed 30 SNPs reliably associated with the feed conversion index and located on SSC2, SSC3, SSC4, SSC6, SSC7, SSC12 and SSC15 ($p < 0.00001$), with blocks of 3-10 SNPs found on SSC2, SSC6, and SSC15. The analysis of genomic regions with reliable SNPs shows several positional and functional candidate genes, the products of which are involved in the regulation of proliferation and differentiation of various cell types, hematopoiesis, and lipid metabolism. Further studies are required to validate the associations found in other pig populations. Identification of QTLs based on feed conversion expands understanding of the genomic architecture of this crucial breeding trait.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 713-722
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.033:636.4:636.082.12:577.2

doi: 10.15389/agrobiology.2019.4.713eng
doi: 10.15389/agrobiology.2019.4.713rus

STUDY OF WUR10000125 POLYMORPHISM ASSOCIATION WITH MEAT, FATTENING AND REPRODUCTIVE TRAITS OF LANDRACE AND LARGE WHITE PIG BREEDS

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The authors declare no conflict of interests
Acknowledgements:
The equipment of the Center for Biological Resources and Bioengineering of Farm Animals (Ernst Federal Science Center for Animal Husbandry) was used for the study.
Supported financially by the Ministry of Education and Science of the Russian Federation, a unique project number RFMEFI60417X0182
Received February 18, 2019

Abstract

Integration of DNA markers associated with disease resistance into breeding programs is one of the most promising approaches to control infections of livestock. The identification and implementation of such a marker for the porcine reproductive and respiratory syndrome is particularly topical. The disease causes significant economic losses in the industry, and the proposed vaccines against PRRS are ineffective and associated with a risk of developing viremia after immunization. A promising DNA marker of resistance to this disease is the single nucleotide polymorphism WUR10000125 (*WUR*) localized in the *GBP1* gene. The aim of the study was to assess the reproductive, fattening and meat qualities of Large White and Landrace pigs bred in PRRS-free nucleus farms, considering the genetic variant of the WUR gene. Studies were conducted in 2018-2019 on pigs of Large White and Landrace pigs reared in Selection and Hybrid Center LLC (Voronezh region). Genotypes of 206 sows of Large White and 112 sows of Landrace pig breeds were determined by PCR with using the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, USA). The reproductive qualities of sows (number of piglets born alive per litter; total litter weight at birth; number of stillborn pigs per litter; number of mummified pigs per litter; total number born per litter) were estimated based on the first three litters: for Large White pig breed in the period from 2008 to 2018 and for Landrace pig breed in the period from 2010 to 2018. Characteristics of meat and fattening qualities, including the age of 100 kg of body weight, the back-fat thickness, measured in three points, muscle depth (lifetime measurements), were evaluated. To assess the effect of genotype on *WUR* on the productivity traits the model equations for multivariate analysis of variance were used. The results of animal genotyping showed that the studied pigs were characterized by relatively low frequencies of the "desirable" allele *G* responsible of resistance to PRRS (2.9 and 13.4 %) and *GG* genotype (0.49 and 4.46 %) in pigs of Large White and Landrace breeds, respectively. The analysis of values of estimates of the *WUR* genotypes obtained by the least square means (LSM) method showed a statistically significant superiority of carriers of the *AA* genotype over animals with *AG* variant by the total number born per litter, prolificacy and total litter weight at birth in pigs of Large White breed, but the similar tendency in Landrace pigs breed was not found. We noted some superiority of the *AA* genotype carriers over the *AG* genotype carriers among sows of Large White breed by EBV of total litter weight at birth. Comparison of meat and fattening parameters did not reveal significant differences either by direct phenotypic estimates or by EBV values. Thus, assessment of the productive traits of Large White and Landrace pigs from PRRS-free nucleus farms did not show a significant effect of the *WUR* genotypes on the meat and fattening parameters, as well as on the reproductive qualities of Landrace pigs. The increasing of the *G* allele and *GG* genotype frequencies under nucleus conditions will lead to an increase in the number of animals with preferable character-

istics under PRRS conditions.

Keywords: *Sus scrofa*, pigs, large white breed, landrace, WUR10000125, reproductive-respiratory syndrome, linear regression, productivity, evaluation of breeding value, DNA marker.

The integration of DNA markers associated with disease resistance into breeding programs is a promising approach to control infections of livestock [1]. Porcine reproductive and respiratory syndrome (PRRS), a highly contagious viral disease caused by a small virus with single-stranded non-segmented RNA (PRRSV), is a widespread disease that causes significant economic damage to pig breeding [2]. PRRSV disrupts the cellular immune response and damages mucosal surfaces. Clinical signs of the reproductive and respiratory syndrome are infertility, agalactia, lower conception frequency, a significant increase in the number of abortions in the late stages and the presence of stillborn, mummified or weakened piglets [3-5]. The economic loss is caused by the death of sows and young animals, early forced slaughter during fattening, reduced meat and fattening productivity, and lower sanitary quality of meat.

The use of vaccines against PRRS is not effective enough, which, most likely, is associated with different virulence and degree of antigenic relationship of vaccine and field viruses [6, 7]. Vaccination with a live modified vaccine provides effective protection against genetically homologous wild-type PRRSV strains, but only partially protects or completely does not protect against heterologous strains [8, 9]. Another disadvantage of vaccination is the possibility of isolating persistent virus strains from vaccinated animals. It was shown that viremia may develop in pigs vaccinated with live modified vaccine within 4 weeks after immunization, which leads to the spread of the vaccine virus among uninfected animals [8, 10]. There is also evidence of recombination between a live modified vaccine strain and wild-type strains [10, 11].

The selection of pigs that are genetically more resistant to PRRS is attractive to improve herd health [12]. As a result of genome-wide association studies (GWAS), single nucleotide polymorphism (SNP) WUR10000125 (*WUR*) was identified which is located next to the putative polyadenylation site in the 3′-untranslated region of the *GBP1* gene (interferon-inducible guanylate-binding protein-1-encoding gene). *WUR* may affect the stability of the transcript with consequences for protein synthesis and expression [13]. It was shown that pigs with a susceptible genotype (allele A of WUR10000125) expressed less GBP5 than pigs with a resistant genotype, and truncated protein was produced as a result of alternative splicing [14]. This polymorphism was responsible for 13.2% of the viremia variability and 9.1% of the variability in the average daily gain of pigs under viral load [15]. The discovered effect of *WUR* was successfully confirmed in pig populations of various genetic origins [16, 17]. GWAS studies conducted before and after the outbreak of PRRS revealed a close relationship between *WUR* polymorphism and the number of stillborn and non-viable piglets, as well as the presence of antibodies to PRRSV [18]. Different ($p < 0.05$) expression of *GBP5* gene, a member of the family of interferon-activated guanylate binding protein (*GBP*) genes located next to the *WUR* [14], in pigs with different genotypes on *WUR* confirms that the *WUR* is a DNA marker. The *WUR* effect was validated in pigs of different breeds vaccinated against PRRSV, infected with various PRRSV isolates, and also coinfected with PRRSV and pig type 2b circovirus (PCV2b) [19-21].

WUR polymorphism is due to the A→G nucleotide substitution at the position 139666819 SSC4 (rs80800372, Sscrofa10.2). Dominant G allele is desirable under the viral load due to both infection and vaccination. A study of *WUR* polymorphism showed a relatively low frequency of the desired G allele in pigs of the Large White (0.08), Landrace (0.02-0.22) and Duroc (0.08-0.12) breeds of

foreign breeding [17]. In pigs of Russian selection, the frequency of the G allele was also relatively low, 0.03 in animals of the Large White breed, 0.18 in Landrases, and 0.07 in Duroc pigs [22]. Considering that pure-bred pigs are used in an industrial cross-breeding (hybridization) system, and the young animals obtained are raised in commercial herds, with a significantly higher pathogenic load and vaccination against PRRS, selection of carriers of the *G* allele of *WUR* is relevant. The use of *WUR* as a DNA marker in pig breeding should be preceded by an assessment of the *WUR* effects on the most important economically useful traits. Based on the relatively low frequency of the *G* allele in various pig breeds, a negative relationship was suggested between the *G* allele and the most important economically significant traits and, as a result, selection against this allele was characteristic of breeding herds with a high health status [23]. Thus, in the absence of the virus, a higher fattening rate was established in pigs with the *WUR AA* genotype [24]. However, another investigation of cross-breed pigs (Yorkshire × Landrace) in the presence of pathogenic microflora revealed a significant superiority in the growth rate before weaning in piglets with the *G* allele.

Comprehensive studies of the reproductive, fattening and meat qualities of pigs in relation to the *WUR* genotype have not yet been conducted.

In this work we have found for the first time that the *WUR* genotype is not associated with productivity traits in Landrace pigs. The Large White pigs showed some superiority of the *AA* genotype carriers over heterozygous individuals in larger litter size and in the number of piglets born per farrow. It was found that the *WUR* DNA marker can be used to obtain fattened pigs on pedigree farms free from PRRS infection.

Our purpose was to study the influence of the *WUR* genotype on the reproductive, fattening, and meat qualities of the Large White and Landrace pigs under the conditions of nucleus farms free from pig reproductive and respiratory syndrome (PRRS).

Techniques. The investigations were carried out on Large White and Landrace sows (*Sus scrofa*) (Selective Hybrid Center LLC, Voronezh Region, 2018-2019).

Genomic DNA was isolated from tissue samples (ear pluck) using the DNA Extran-2 Reagent Kit (Syntol LLC, Russia). DNA quality and concentration were determined on a Qubit 2.0 fluorimeter (Invitrogen/Life Technologies, USA) and a NanoDrop8000 spectrophotometer (Thermo Fisher Scientific, USA).

WUR genotypes (A→G at the position 139666819 SSC4, rs80800372, Sscrofa10.2) (https://www.ncbi.nlm.nih.gov/assembly/GCF_000003025.5/) were determined by the real-time PCR method (PCRq) (a QuantStudio 5, Thermo Fisher Scientific, USA) with a test system based on the use of two specific primers WUR-SN-F and WUR-SN-R and two allele-specific fluorescently labeled probes, a probe for identifying *G* allele associated with pig resistance to PRRS was labeled with FAM, and a probe for allele *A* with CY5.

The reproductive traits of Large White sows (n = 206) and Landrace sows (n = 112) were evaluated for the first three farrowing: for the Large White sows from 2008 to 2018, and for the Landrace sows from 2010 to 2018. The meat and fattening qualities of the Large White (n = 200) and Landrace (n = 108) pigs, including early maturity (age of 100 kg body weight), fat thickness at three points, and muscle depth (intravital measurements) were also measured.

Descriptive statistical parameters were determined to characterize the studied productivity indicators, i.e. M — arithmetic mean for the trait in the sample, ±SEM — standard error of the mean; the standard deviation for the trait in the sample (σ) was used in the calculations.

In assessing the breeding value of animals for reproduction traits, the equation of the BLUP-AM model was used:

$$y = YM + b_1Par + animal + pe + e,$$

where y is the productivity indicator for the traits: the number of live piglets born per farrow (TBA), the weight of the nest at birth (BW), the number of stillborn piglets per farrow (SB), the number of mummified piglets per farrow (MUM), the total number of all piglets born per farrow (TNB); YM — "year-month of farrowing" factor; b_1Par — regression effect of "farrowing number" and regression coefficient; animal — a randomized effect of an animal; pe — permanent environmental effects; e — residual effects not included in the model.

The following models of assessing the breeding value of sows according to their own indicators of meat and fattening qualities were applied:

$$y = YM + b_1W + animal + e,$$

where y is the weighing age for calculating precocity estimates; b_1W — regression effect of "live weight during weighing" and regression coefficient;

$$y = YM + b_1Age + animal + e,$$

where y is the phenotypic indicator of the traits: the thickness of the fat at the first measurement point (the 6th-7th rib, mm) (BF1), the thickness of the fat at the second measurement point (the 1st rib, mm) (BF2), the thickness of the fat at the third measurement point (the 14th rib, mm) (BF3), muscle depth (LD); b_1Age — regression effect of "age at weighing" and regression coefficient.

When assessing the effect of the *WUR* genotype on reproductive traits, the model equation was used for multivariate analysis of variance without interaction:

$$y = YM + b_1Par + G + e,$$

where y is the evaluated trait; *G* is the effect due to the influence of the *WUR* factor. The effect of the *WUR* genotype on meat and fattening qualities was evaluated using the model equation for multivariate analysis of variance without interaction:

$$y = YM + A + G + e,$$

where y is the evaluated parameter; A — age at weighing (for traits BF1, BF2, BF3, LD) and live weight at weighing (for Age100, the precocity trait).

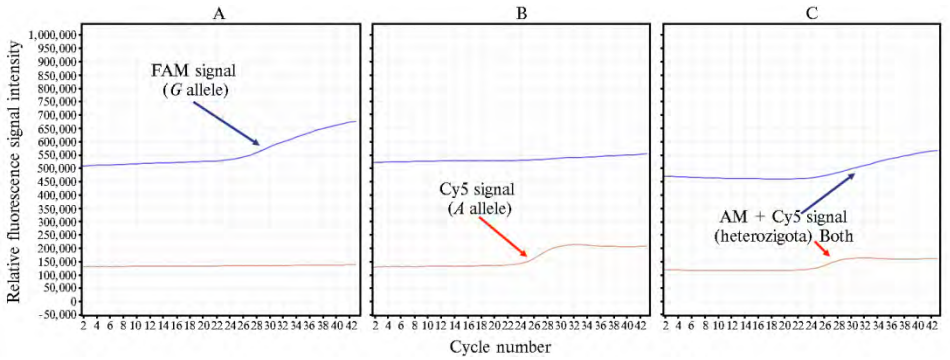
To assess the statistical significance of the influence of the factors taken into account, we used the Fisher test (the ratio of the variance of the factor taken into account to the residual variance) for the corresponding number of degrees of freedom (df). The significance of differences in the average values of traits in the compared groups of genotypes was determined using Student's *t*-test for the corresponding number of degrees of freedom and levels of confidence probability $P > 0.95$; $P > 0.99$; $P > 0.999$. Calculations for analysis of variance and the LSM method (Least Square Means) were performed using the STATISTICA 10 software (StatSoft, Inc., USA). The assessment of the breeding value of animals and the analysis of variants were carried out using the BLUPF90 family programs [26].

Results. Using the developed test system, genetic variants of Large White pigs and Landrace pigs by the WUR marker were identified (Fig.).

The studied sample of pigs had relatively low frequencies of the allele desirable for resistance to PRRS (2.9% and 13.4% in Large White and Landrace pigs, respectively) and the *GG* genotype (0.49% and 4.46%) (Table 1).

The analysis of the weighted values of the *WUR* genotype estimates obtained by the least squares (LSM) method for sow fertility revealed a statistically significant superiority of carriers of the *AA* genotype over animals with *AG* in

terms of the total number of piglets per farrow and multiple pregnancy in Large White pigs (Table 2). Comparison with productivity indices for carriers of the *GG* genotype was not possible as there was only one Large White sow with the *GG* genotype in the studied sample. In Landrace pigs, there were no statistically significant differences between groups with different *WUR* genotypes.



The results of genotyping Large White and Landrace sows (*Sus scrofa*) for *WUR* using real-time PCR (QuantStudio 5, Thermo Fisher Scientific, USA): A — *GG*, B — *AG*, C — *AA* (Selective Hybrid Center LLC, Voronezhskaya Province, 2018-2019).

1. **Frequencies of *WUR* genotypes and alleles in the studied samples of Large White and Landrace sows (*Sus scrofa*)** (Selective Hybrid Center LLC, Voronezhskaya Province, 2018-2019)

Breed	Frequency of genotypes			Frequency of alleles	
	AA	*AG*	*GG*	*A*	*G*
Large White	94.66	4.85	0.49	0.971	0.029
Landrace	77.68	17.86	4.46	0.866	0.134

2. **Weighted values of the *WUR* genotype estimates obtained by the least squares** (LSM) **for fertility traits of Large White and Landrace sows (*Sus scrofa*)** (*M*±SEM, Selective Hybrid Center LLC, Voronezhskaya Province, 2018-2019)

Genotype	TBA	BW	SB	MUM	TNB
		Large White (n = 206)			
AA	14.18±0.40[a]	19.29±0.51	1.82±0.15	0.17±0.07	16.17±0.44[a]
AG	12.30±0.66[a]	17.55±0.85	1.58±0.25	0.19±0.11	14.07±0.73[a]
GG	14.47±1.71	18.88±2.18	1.41±0.66	0.01±0.30	15.89±1.88
F-test	5.96*	3.09*	0.83	0.18	6.05*
		Landrace (n = 112)			
AA	12.41±0.48	17.90±0.62	1.41±0.27	0.20±0.09	14.02±0.53
AG	12.90±0.56	18.72±0.73	1.57±0.31	0.14±0.10	14.61±0.62
GG	12.02±0.82	17.25±1.06	1.50±0.46	0.12±0.15	13.63±0.90
F-test	1.13	1.89	0.35	0.55	1.30

Note. TBA — the number of live piglets born per farrow, BW — the weight of the nest at birth, SB — the number of stillborn piglets per farrow, MUM — the number of mummified piglets per farrow, TNB — the total number of all piglets born per farrow; [a] — differences between the marked genotypes are statistically significant at $p \le 0.05$.
* The value of the Fisher test is statistically significant at $p \le 0.05$.

A study of the breeding value of sows depending on the WUR genotype showed a statistically significant effect of variant *AA* on the estimation of breeding value (EBV) of nest weight at birth ($F = 3.33$) in Large White pigs. No significant differences in other reproductive traits were found in sows with unequal *WUR* genotypes (Table 3).

The analysis of variance did not reveal a statistically significant effect of the *WUR* genotype on meat and fattening productivity traits both in Large White and in Landrace pigs (Table 4). A study of the breeding value of sows with different *WUR* genotypes did not show a statistically significant effect of *WUR* genotypes on EBV for meat and fattening productivity (Table 5).

3. Association of breeding value estimates for fertility in Large White and Landrace sows (*Sus scrofa*) depending on the *WUR* genotype (*M*±SEM, Selective Hybrid Center LLC, Voronezhskaya Province, 2018-2019)

Genotype	TBA	BW	SB	MUM	TNB
		Large White (*n* = 206)			
AA	0.003±0.06	0.02±0.10	–0.01±0.02	0.003±0.003	0.003±0.07[a]
AG	–0.59±0.26	–0.52±0.43	–0.23±0.11	0.02±0.01	–0.87±0.33[a]
GG	–0.00±0.83	–0.27±1.37	–0.11±0.34	–0.02±0.04	–0.13±1.04
F-test	2.42	0.75	1.98	0.67	3.33*
		Landrace (*n* = 112)			
AA	–0.02±0.03	–0.00±0.00	0.07±0.05	–	0.05±0.10
AG	0.01±0.06	0.01±0.01	0.21±0.10	–	0.34±0.22
GG	–0.04±0.12	–0.01±0.01	–0.00±0.20	–	–0.08±0.44
F-test	0.10	1.36	0.87		0.81

Note. TBA — the number of live piglets born per farrow, BW — the weight of the nest at birth, SB — the number of stillborn piglets per farrow, MUM — the number of mummified piglets per farrow, TNB — the total number of all piglets born per farrow; [a] — differences between the marked genotypes are statistically significant at $p \leq 0.05$. Dashes mean that in the studied sample the variability is completely due to the individual characteristics of individuals, that is, non-additive genetic effects.
* The value of the Fisher test is statistically significant at $p \leq 0.05$.

4. Weighted values of the *WUR* genotype estimates obtained by the least squares (LSM) **for meat and fattening productivity of Large White and Landrace sows (*Sus scrofa*)** (*M*±SEM, Selective Hybrid Center LLC, Voronezhskaya Province, 2018-2019)

Genotype	$Age100_{corr}$	BF1	BF2	BF3	LD
		Large White (*n* = 200)			
AA	152.98±0.68	14.83±0.37	11.93±0.29	11.89±0.27	56.76±0.58
AG	152.44±2.57	15.10±1.20	12.17±0.93	11.85±0.88	56.15±1.68
GG	148.00±7.46	17.83±3.57	12.30±2.76	14.11±2.61	55.50±5.54
F-test	0.24	0.38	0.04	0.37	0.08
		Landrace (*n* = 108)			
AA	155.50±0.94	14.52±0.63	12.59±0.50	11.61±0.46	55.16±0.94
AG	158.01±2.02	13.82±1.27	10.30±1.00	10.60±0.93	50.49±1.88
GG	152.34±3.51	11.75±2.04	10.34±1.60	11.26±1.49	58.54±3.02
F-test	1.24	0.94	2.76	0.50	3.89

Note. $Age100_{corr}$ — precocity, adjusted for a mass of 100 kg, BF1 — the thickness of the fat at the first measurement point (in the region of the 6th-7th rib, mm), BF2 — the thickness of the fat at the second measurement point (in the region of the 10th rib, mm), BF3 — the thickness of the fat at the third measurement point (in the region of the 14th rib, mm) (BF3), LD — muscle depth.

5. Association of breeding value estimates for meat and fattening productivity in Large White and Landrace sows (*Sus scrofa*) depending on the *WUR* genotype (*M*±SEM, Selective Hybrid Center LLC, Voronezhskaya Province, 2018-2019)

Genotype	$Age100_{corr}$	BF1	BF2	BF3	LD
		Large White (*n* = 200)			
AA	0.08±0.13	–0.00±0.07	0.00±0.02	–0.03±0.04	–0.09±0.03
AG	–0.00±0.58	–0.31±0.29	–0.01±0.07	–0.04±0.19	–0.11±0.13
GG	–1.61±1.82	1.04±0.93	0.04±0.22	0.29±0.59	–0.03±0.41
F-test	0.44	1.15	0.02	0.15	0.03
		Landrace (*n* = 108)			
AA	0.00±0.00	0.10±0.07	0.05±0.04	0.06±0.04	0.07±0.05
AG	0.01±0.01	0.23±0.16	0.00±0.09	0.18±0.09	0.03±0.11
GG	–0.01±0.01	–0.03±0.30	–0.08±0.17	0.04±0.18	0.23±0.21
F-test	1.02	0.40	0.33	0.72	0.37

Note. $Age100_{corr}$ — precocity, adjusted for a mass of 100 kg, BF1 — the thickness of the fat at the first measurement point (in the region of the 6th-7th rib, mm), BF2 — the thickness of the fat at the second measurement point (in the region of the 10th rib, mm), BF3 — the thickness of the fat at the third measurement point (in the region of the 14th rib, mm) (BF3), LD — muscle depth.

Pig reproductive and respiratory syndrome, which has a significant negative impact on the economic efficiency of the industry, causes a significant increase in mortality rates (up to 30-50% of suckling pigs and 4-20% of piglets after weaning), and also leads to the manifestation of clinical signs (shortness of breath, anorexia, lethargy, skin hyperemia, weight loss) in animals after weaning and growing [2]. It also causes changes in the reproductive system of young animals associated with the chronic PRRS, which, in turn, reduce fertility [5].

The use of a DNA marker in breeding, which is associated with leveling the negative effects of vaccination or increasing the ability of an animal to be less affected by viruses, is of great practical importance. However, a limitation for the large-scale introduction of this marker may be its connection with the fertility traits, meat and fattening productivity. Our studies of the Large White and Landrace sows did not reveal such a relationship. However, these findings are consistent with the studies of Dunkelberger et al. [23] who did not note a significant effect of *WUR* genotypes on the reproductive and fattening traits of Landrace and Large White pigs. The authors showed the effect of this marker ($p < 0.001$) on the survival of Pietrain piglets. In terminal pigs, a correlation was found between the *G* allele, which is desirable for resistance to PRRSV, with significantly lower feed intake ($p = 0.004$) and, consequently, a decrease in daily gain during life ($p = 0.001$) and daily gain during testing ($p = 0.002$). An opposite relationship was found for the Pietrain pig line, where the *G* allele was associated with significantly higher feed intake ($p < 0.001$) and a tendency to increase average daily growth during testing ($p = 0.09$). The influence of *WUR* on the values of the breeding index for all indicators was not significant for any of the studied breeds ($p \geq 0.15$) [23]. At the same time, another study of contact of cross-bred pigs (Yorkshire × Landrace) with pathogenic microflora establishes significant superiority in the growth rate before weaning in piglets carrying the *G* allele: the average daily increase in *AA* piglets was 339 g versus 365 g in *AG* piglets ($p = 0.013$) [25].

Thus, the investigations of the productive indicators in Large White and Landrace pigs under the conditions of nucleus farms free of pig reproductive and respiratory syndrome (PRRS) did not show a significant effect of the *WUR* genotype on meat and fattening traits, as well as the reproductive qualities of Landrace pigs. The obtained relationships between *WUR* genetic variants and reproductive qualities of Large White pigs should be clarified on a large number of carriers of the *GG* genotype. Selection for the *G* allele is expected to lead to an increase in the number of livestock that has more preferable parameters under PRRS infection, and is not inferior in productivity to other genetic variants for *WUR* in conditions free of viral load. It will also contribute to an increase in the frequency of the desired genotype in herds that are most vulnerable and susceptible to diseases, since it is in them that the degree of pathogenic load is significantly higher.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 723-731
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Functional morphology of tissues

UDC 636.5:591.463.12:591.8.086.2

doi: 10.15389/agrobiology.2019.4.723eng
doi: 10.15389/agrobiology.2019.4.723rus

TESTIS HISTOSTRUCTRURE DYNAMICS DURING QUAIL (*Coturnix coturnix*) SPERMATOGENESIS

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation, grant No. 16-16-04104
Received December 5, 2018

Abstract

Male sex cells are unique objects for scientific research in the field of genetics and physiology and in the study of the development biological basis in animal husbandry. Maturation and differentiation processes in male animals and birds germ cells are of great interest for comparative embryology, developmental biology, medicine and biotechnology. Quails characterized by early puberty and a short generation period are perspective for these experimental works. The greatest interest is the use of spermatogonia, the testes stem cells which are currently being actively studied as promising targets for the introduction of recombinant DNA in obtaining transgenic individuals. However, the morphology of germ cells from male poultry in their formation process is not fully covered. For the first time, we describe in detail the histological features of spermatogenical quail epithelium tissue at different stages of spermatogenesis and the dynamics of spermatogonial testis cells populations in this study. The aim of the study was to identify age-related features of spermatogenesis associated with the dynamics of the different cell type development in the epithelial spermatogenous layer of the seminiferous tubules in quail. For this, we examined the histological structure of the testes in quail (*Coturnix coturnix*) of the Estonian breed at the age of 1, 2, 3, 4, 5, 6, 12, and 24 weeks. In each age group, there were 10 males. Testis tissue was fixed in Bouins's solution, dehydrated in alcohols of increasing concentration and embedded in paraffin. Five to six micron histological sections were stained with hematoxylin-eosin. The composition of spermatogenic cells and their ratio in the seminiferous tubules was investigated. At least 30 seminiferous tubules were examined from each male. The diameter of the seminiferous tubules in the quail testes changed during ontogenesis and at the age of 1, 2, 3, 4, 5, 6, 12 and 24 weeks reaching 42±1, 71±2, 91±2, 117±2, 237±4, 278±5, 28 ±7 and 291±6 μm, respectively. Sertoli cells and generative cells were parts of cell population of the quail seminiferous tubules at different stages of differentiation, i.e. spermatogonia, spermatocytes, spermatids and sperm cell maturation. The number of spermatogenic cells inside the seminiferous tubules increased with age ($p < 0.01$) and was 18±1, 24±1, 58±4, 80±6, 249±16, 587±34, 658±24 and 540±41 in quails aged 1, 2, 3, 4, 5, 6, 12 and 24 weeks, respectively. In 1-week aged quails, Sertoli cells dominate in seminiferous tubules (12±1 per seminiferous tubule) while spermatogonia are few, 1 to 4 cells per tubule. The number of spermatogonia increases with age. The percentage of spermatogonia is maximum in 3-week aged birds, 76±2 % of the total number of spermatogenic cells. In 4-week aged quails, primary and secondary spermatocytes are visualized in the seminiferous tubules, and from week 5 spermatids are found. At the age of 5 weeks, we detected single spermatozoa, the number of which increased in the quail semen tubules by the 6-week age. Thus, the quails' age from 1 to 3 weeks is optimal for manipulating spermatogonia as targets for introducing recombinant DNA in order to obtain transgenic offspring or biological material to preserve the genetic resources of farm birds in cryobanks.

Keywords: quail, testes, Sertoli cells, spermatogenesis, spermatogonia

The study of maturation and differentiation of male germ cells of ani-

mals and birds is of great interest for comparative embryology, developmental biology, medicine and biotechnology in general. This is especially true for spermatogonia as the precursors of sperm, the mature germ cells. Spermatogonia are promising targets to produce transgenic individuals via the introduction of recombinant DNA [1]. Such technology involves the isolation and in vitro transformation of spermatogonia with their subsequent transplantation into the testes of male recipients in which their own spermatogenesis is previously blocked [2, 3]. Transplanted spermatogonia subsequently differentiate into sperm cells, which are used to produce transgenic offspring. Such a possibility of obtaining chimeric and genetically modified individuals was shown in a number of works on laboratory animals [4, 5], pigs [6, 7], sheep [8], as well as on roosters [9, 10]. Male gonad cells are also used as valuable genetic material for creating cryobanks as part of the conservation and maintenance of the gene pool of valuable breeds of animals and birds [11, 12].

Unlike mammals, in birds, including quails, testes remain in the abdominal cavity (in the place of their development) throughout life [13, 14]. Features of the genital organs of chickens, turkeys, ducks, and quails are rather deeply described in terms of anatomy, while the morphology of the cells of male poultry is described incompletely [15].

In birds, as in mammals, spermatogenesis is a long process of the gradual transformation of germ cells into spermatozoa within the boundaries of the seminiferous tubules of the testis and includes three successive stages, the spermatocytogenesis, spermatidogenesis, and spermiogenesis. The physiological and anatomical characteristics associated with spermatogenesis in birds are the subject of extensive research [16, 17]. During spermatogenesis, cell proliferation occurs with repeated mitotic divisions, duplication of chromosomes, meiotic cell division, etc. for the further development of haploid spermatids and their subsequent differentiation into mature sperm [18].

In the present paper, the histological features of the tissue of the spermatogenic quail epithelium at various stages of spermatogenesis were investigated for the first time in detail with characterization of the dynamics of the spermatogenic testis cell populations.

The purpose was to reveal the age-related features of spermatogenesis in quail in connection with the dynamics of the development of different types of cells of the epithelial spermatogenic layer of the seminiferous tubules.

Tehcniques. In the experiments, Estonian male quail (*Coturnix coturnix*) at the age of 1, 2, 3, 4, 5, 6, 12, and 24 weeks were used (groups of eight age categories, 10 animals per group). The biomaterial was the testes collected during slaughter.

The selected testis tissues were fixed for 48 h in a Bouin solution consisting of picric, acetic acids and formalin (15:1:5), then the samples were embedded in paraffin to prepare histological sections of a 5-6 μm thickness [12, 13]. The preparations were stained with hematoxylin and eosin (BioVitrum, Russia).

During histological analysis, the seminiferous tubules having a rounded shape and a lumen (transverse section) were examined. Cell types of spermatogenic epithelium were identified morphologically [14, 15]. Histological preparations were examined using light microscopy (Ni-U, Nikon, Japan; the microscope is equipped with a software for image processing and analysis NIS-Elements, Nikon, Japan). The diameter of the seminiferous tubules, the number and types of spermatogenic cells located in them were evaluated.

Statistical processing was performed using the MS Excel 2016 data analysis package (t-test). The tables present arithmetic means (*M*) and standard errors of

means (±SEM). Differences were considered statistically significant at $p < 0.01$.

Results. The histological structure of the testes in quail was similar to that in mammals. The parenchymal tissue of the organ was formed by a system of convoluted seminiferous tubules containing various types of cells (Sertoli cells, spermatogonia, spermatocytes, spermatids, and sperm cells).

The size of the seminiferous tubules in quail varied during ontogenesis (Table 1). This indicator slightly increased with age during the early postnatal development. The diameter of the seminiferous tubules increased by 69% ($p < 0.01$) from week 1 to week 2, by 28% ($p < 0.01$) from week 2 to week 3, and by 29% ($p < 0.01$) from week 3 to week 4. From week 4 to week 5, an increase in the size of the seminiferous tubules was the most apparent. The diameter and area of the seminiferous tubules in 5-week-old quail were 2 times greater than that in individuals aged 4 weeks. From week 6 to week 24, the sizes of the seminiferous tubules almost did not change.

1. Age-related dynamics of morphological parameters of the testis histostructure in Estonian quail (*Coturnix coturnix*) ($n = 80$, M±SEM)

Age, weeks	Diameter of seminiferous tubules, microns	Area of seminiferous tubules, μm^2	Number of spermatogenic cells per seminiferous tubule
1	42±1	1329±55	18±1
2	71±2^{ab}	4117±222^{ab}	24±1^{ab}
3	91±2^{ab}	6172±177^{ab}	58±4^{ab}
4	117±2^{ab}	9932±310^{ab}	80±6^{ab}
5	237±4^{ab}	40688±993^{ab}	249±16^{ab}
6	278±5^{ab}	56232±868^{ab}	587±34^{ab}
12	282±7^{ac}	57023±766^{ac}	598±23^{ac}
24	291±6 ^{ac}	55985±812^{ac}	570±41^{ac}

$^{a, b}$ Differences with the previous age group are statistically significant at $p < 0.01$.

$^{a, c}$ Differences with the same indicator at the age of 1 week are statistically significant at $p < 0.01$.

An increase in the diameter and area of the seminiferous tubules in quail with aging was due to the growth and differentiation of spermatogenic cells (Table 1). In 1-week-old males, the number of spermatogenic cells in one seminiferous tubule ranged from 11 to 26 and averaged 18±1. In 2-week-old males, this indicator was 33% ($p < 0.01$) higher compared to 1-week-old animals, and it was 142% ($p < 0.01$) higher from week 2 to week 3, and 86% ($p < 0.01$) higher from week 3 to week 4. Significant growth and differentiation of spermatogenic cells were recorded from week 4 to week 6. In the seminiferous tubules of 5-week-old males, as compared to 4-week-old males, the number of spermatogenic cells increased 3.1-fold, from week 5 to week 6 2-fold. After reaching maturity (6 weeks), the growth and development of males practically were not accompanied by changes in the number of spermatogenic cells in the seminiferous tubules of the testes. The differences in this indicator, established in males aged 6 weeks and at a later age (3 and 6 months), did not exceed 1.8%.

The presence, number, and ratio of spermatogenic cells inside the seminiferous tubules of the testes varied depending on the age of the quail (Table 2). In 1-week-old quail, the basal membrane of the seminiferous tubules of the testes was lined with Sertoli cells and single spermatogonia (Fig. 1, A), while the number of Sertoli cells prevailed with a percentage of 71% vs. 29%, respectively. Sertoli cells had a dark-colored nucleus of a pyramidal shape located on the basal membrane. Spermatogonia were located along the seminiferous tubule but not on the basal membrane; they were mainly represented by type A (testis stem cells). Cells of this type were large and were characterized by the presence of a nucleus of an elliptical or round shape, located usually on the basal membrane of the tubule. Nuclear chromatin in the core was concentrated in one area.

2. Characterization of the spermatogenic epithelial cells population in the seminiferous tubules of the testes of Estonian quail (*Coturnix coturnix*) of different ages (n = 80, M±SEM)

Age, weeks	Cell type				
	Sertoli cells	spermatogonia	1st-order spermatocytes	2nd-order spermatocytes	spermatids
1	12±1	5±1	0	0	0
2	13±1	10±1[ab]	0	0	0
3	15±1	44±4[ab]	0	–	0
4	14±1	49±3	11±1	5±1	0
5	16±1	92±5[ab]	21±3[ab]	25±3[ab]	96±13
6	16±1	112±4[ab]	61±5[ab]	84±2[ab]	192±3[ab]
3	18±3	113±9	65±5	95±8	194±5
6	18±2	119±6	63±4	93±4	192±3
12	12±1	5±1	0	0	0
24	13±1	10±1[ab]	0	0	0

[a, b] Differences with the previous age group are statistically significant at $p < 0.01$.

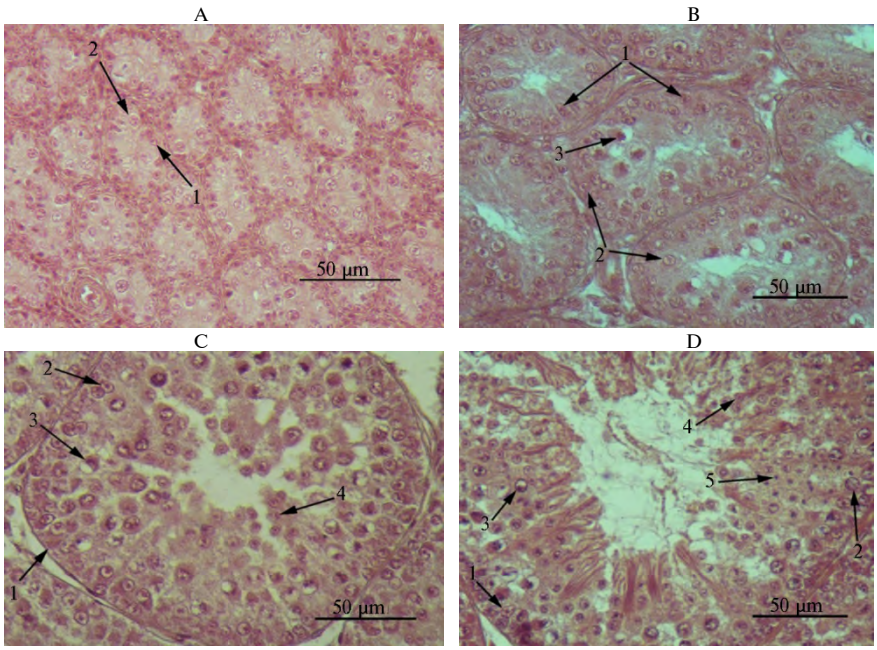

Fig. 1. Histological structure of the seminiferous tubules of the testes of Estonian quail (*Coturnix coturnix*) at the age of 1 week (A)**, 3 weeks** (B)**, 4 weeks** (C) **and 6 weeks** (D)**:** 1 — Sertoli cells, 2 — spermatogonia, 3 — 1st-order spermatocytes, 4 — 2nd-order spermatocytes, 5 — spermatids. Hematoxylin and eosin staining, light microscopy (Ni-U, Nikon, Japan), magnification ×400.

In 2-week-old quail, spermatogenic epithelial cells of the seminiferous tubules were also represented by two types — Sertoli cells and spermatogonia. The number of spermatogonia of various types in the seminiferous tubule increased to 10±1 ($p < 0.01$). At the same time, the number of Sertoli cells changed insignificantly (see Table 2). Spermatogonia were found both on the periphery and inside the seminiferous tubule. Along with type A spermatogonia, we detected spermatogonia of the intermediate type and type B. Spermatogonia of the intermediate type were slightly smaller than type A spermatogonia, the chromatin in their nucleus merged into one or two nucleoli and had a darker color. Type B spermatogonia were characterized by the presence of a large round or elliptical nucleus; chromatin flakes in the nucleus were distributed throughout the endoplasm. At this stage of development, the lumen in the seminiferous tubule was absent.

Development in the seminiferous tubules of the lumen necessary for the

release of sperm in adults was recorded at the age of 3 weeks (see Fig. 1, B). Spermatogonia were located on the basal membrane. Their number compared to the previous period increased 4.4 times ($p < 0.01$).

At the age of 4 weeks, small developing lumens were present in all seminiferous tubules. In some of them, spermatogenic epithelial cells were lined up in 5-6 rows. Along with Sertoli cells and spermatogonia, there were spermatocytes of the 1st and 2nd order, located closer to the center of the tubule. First-order spermatocytes were large cells with a large oval nucleus. Second-order spermatocytes were smaller in size (see Fig. 1, B). The average number of 1st- and 2nd-order spermatocytes in the seminiferous tubule did not exceed 11±1 and 5±1, respectively. The number of Sertoli cells and spermatogonia at this age did not noticeably change compared to the previous period: differences for indicators did not exceed 6 and 13%, respectively.

In 5-week-old animals, the population of spermatogenic cells in the seminiferous tubules was represented by Sertoli cells, spermatogonia located on the basal membrane, and spermatocytes of the 1st and 2nd order (see Table 2). Near the lumen, the presence of spermatids in the form of immature small formations of a rounded shape with a clearly visible nucleus was recorded. Individual spermatozoa occurred in the lumen of some seminiferous tubules.

At the age of 6 weeks, elongated spermatids with tails occurred near the lumen of the seminiferous tubule; their number increased 2.0-fold compared to the previous period ($p < 0.01$, see Table 2, Fig. 1, D). The presence of spermatids at different stages of spermiogenesis was recorded, which led to their unequal shape.

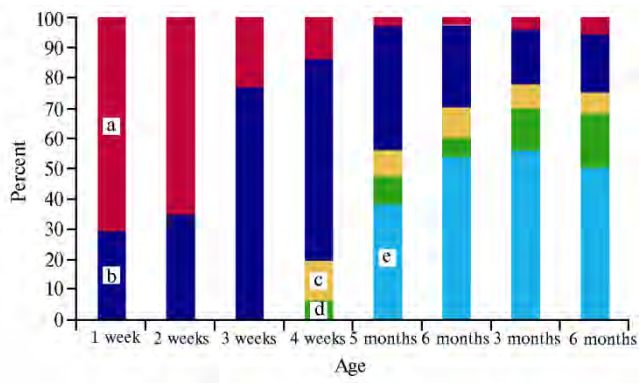

Fig. 2. The ratio of spermatogenic epithelial cells of the seminiferous tubules in the testes of Estonian quail (*Coturnix coturnix*) of different ages: a — Sertoli cells, b — spermatogonia, c — 1st-order spermatocytes, d — 2nd-order spermatocytes, e — spermatids.

So, with approaching the lumen of the seminiferous tubules, spermatids decreased in size and were cells with an oval elongated head of dark color and long tails. In individual seminiferous tubules, spermatids in the form of bundles were found. Developed spermatozoa were in the lumens of most seminiferous tubules. Sertoli cells often had an elongated shape (in the form of strands located almost from the basal membrane to the lumen of the tubule). The total number of cells in the seminiferous tubules compared to the 5 weeks of age was higher 2.4 times mainly due to an increase in the number of spermatocytes, spermatids, and spermatozoa ($p < 0.01$).

In adult males aged 3 and 6 months, all spermatogenic epithelial cells were present in the seminiferous tubules; the average values of these indicators were almost the same. Differences in the content in the seminiferous tubules of spermatogenic cells of different types, found in 6-week-old and 3- and 6 month-old quail, did not exceed 11%.

Thus, the studies showed that in quail of different ages, the number of different types of spermatogenic cells and their ratio in the seminiferous tubules of the testes vary. The share of Sertoli cells in the total number of spermatogenic cells in the seminiferous tubule decreased with age due to spermatocytes and

spermatids (Fig. 2). During the early period of postnatal development of males, the proportion of spermatogonia increased as the animal matured, reaching a maximum value (up to 76.6%) by the age of 3 weeks. In the subsequent period, this indicator decreased to 13% at the age of 6 months.

The results of this study are consistent with the data that the authors obtained earlier in studying the age-related characteristics of spermatogenesis in rabbits [19], roosters [20], and guinea fowls [21]. In males of these species, the composition of spermatogenic cells in the seminiferous tubules of the testes is shown to change during ontogenesis. During the postnatal period, an uneven increase in the size of the seminiferous tubules and differences in the presence, number and ratio of spermatogenic cells in them occur. During the early ontogenesis, the size of the seminiferous tubules and the number of spermatogenic cells in them constantly and significantly increase. Intensive growth and differentiation of spermatogenic cells and, as a result, an increase in the size of the seminiferous tubules are detected in the period preceding the maturity: in male rabbits between the ages of 5 and 6 months, in roosters and guinea fowls from 4 to 5 months. It should be noted that similar studies performed by other authors were not found.

Among the available information sources, we also did not find papers on age-related changes in the morphometric characteristics of the seminiferous tubules of the testes in quail and the quantitative composition of spermatogenic cells. Studies on the birds, including quail, were mainly associated with the morphometric indicators (diameter, area, etc.) of spermatogenic cells and their structural units in the course of differentiation [22, 23]. There are a number of papers of other authors on the study of the anatomical structure and morphometric data of the genitals in male quail [24-27]. So, the morphometric parameters of the testes were assessed for 60-day-old quail, in particular, the sizes of the testes, their anatomical structure and histological structure [24]. The results of histological studies presented in this paper are consistent with those we obtained. Kannan et al. [25] studied the anatomical and morphological features of quail testes in age dynamics, in young quail, upon reaching maturity and in adult males. An increase in the size of the testes to the age of 22 weeks has been shown. Similar data were obtained by Bausova [26]. A number of studies have noted the effect of various feeds, hormones, herbicides and other substances [28-30], as well as natural and artificial lighting, on quail spermatogenesis [31].

So, in quail (*Coturnix coturnix*), the size of the seminiferous tubules, the number and composition of spermatogenic cells in them varies depending on age. At the age of 1-3 weeks, the population of cells of the epithelial spermatogenic layer of the seminiferous tubules is represented by two types — Sertoli cells and spermatogonia. In 3-week-old animals, the seminal lumen begins to develop, the histological sections of the testes show spermatocytes of the 1st and 2nd order from week 4, and spermatids from week 5. In the seminiferous tubules of the 6-week quail, all types of cells of the epithelial spermatogenic layer are present, i.e. Sertoli cells, spermatogonia of various types, spermatocytes of the 1st and 2nd order, spermatids, and sperm cells. These data expand the understanding of the morphology of developing germ cells in male poultry and suggest that quail age from 1 to 3 weeks is the optimal period for manipulating spermatogonia when used as biological material to preserve the genetic resources of poultry in cryobanks, as well as targets for the introduction of gene constructs to obtain transgenic birds.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 732-743
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.2/.3:636.018:591.469:591.8

doi: 10.15389/agrobiology.2019.4.732eng
doi: 10.15389/agrobiology.2019.4.732rus

AGE VARIABILITY OF MAMMARY GLAND HISTOSTRUCTURE IN DAIRY AND NON-DAIRY ANIMALS OF DIFFERENT BREEDS AND ORIGIN

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The authors declare no conflict of interests
Received September 25, 2018

Abstract

Increase in milk production and improvement of milk quality must be addressed, along with of paratypic factors, through further researches of breast morphology and physiology. These studies are targeted not only at its morphological and functional characterization, but also at establishing basis for changing the body's activities and improvement of dairy animals. For most dairy breeds in the process of breeding almost reached the biological threshold of productivity. In addition, with the development of highly mechanized and automated technologies for milk production, the requirements for the selection of animals for adaptation to such conditions, in particular for the state of the udder, have increased. Therefore, an additional reserve can be the improvement of dairy cattle and improve the quality of milk on the basis of in-depth studies of the morphological, functional characteristics and physiology of the breast. The most informative data are presented on the structure and function of the udder in species with high initial milk yield and high quality indicators in comparison to the most common breeds of cattle. In the scientific literature there are no comparative data on histological mammary gland of cows and female-yaks. The results presented by us in this study fill this gap. Investigations of histostructure of udder in cows were carried out on black-motley and Brown Swiss breeds and their crosses with Holsteins (F_1 and F_2, Lenin APS Farm), and on the local population of Brown Swiss cows and female-yaks imported from Tuva and Kyrgyzstan (LLS "Elbrus Agroinvest" farm). The microstructure was investigated using 2×3 cm^2 specimens taken between dairy base and the base of the tank throughout the udder fraction along a line passing through the center of the teat canal and lactic sinus in the right front of teat. In the mammary gland of $^1/_2$ Holstein-Swiss crossbred cows the diameter of alveoli were the largest and reached 119 μm in the cows of the third lactation, which is 6,2-3,7 % higher compared to peers of other groups ($P > 0.999$). The smallest diameter of the fat cells are in the black-motley cows (68.3 μm) and the largest ones were in Holstein-Swiss crossbred hybrids of the third lactation, the rest of cows take an intermediate position. The greatest increase in the diameter of the fat cells with age was in Holstein × black-motley hybrids F_2 in which the figure ranges from 68.6 μm in heifers to 77.3 μm in cows of the third calving. Consequently, according to the development and size of structure elements most important for milk production (the size of the alveoli, and the diameter of the fat globules), the animals improved by Holsteins are superior to the peers of pure Brown Swiss and black-motley cows. It was established that in local Brown Swiss cows the alveoli diameter is higher than that of female-yaks, regardless of their origin, and averages 9.1-11.3 μm. By the fat cell diameter, the female-yaks were superior to the Brown Swiss peers by 4.5-5.4 μm on average. Thus, mammary glands of Brown Swiss cows and female-yaks imported from Tuva and Kyrgyzstan have characteristics features, namely Brown Swiss cows of local population have larger quantity of glandular epithelial cells, the diameter of alveoli, and the thickness of the main strands, whereas female-yaks have a higher concentration of adipose tissue, a smaller diameter of fat cells and the thickness of interlobular strands. For comparison, we compared the mammary gland in another animal species, the goats of different origin. It was shown that the Zaanen breed has better developed mammary gland with higher biomechanical properties as compared to the local goats' population.

Keywords: cows, yaks, goats, genotype, mammary gland, histological structure, alveoli, adi-

pose cells, connective-woven strands

A deeper understanding of cow mammary gland physiology is a way to increase milk production [1]. In addition, such investigations improve the fundamental understanding of the biology of this organ determining the development of mammals [2, 3]. In Russia, such research was initiated by Liskun who in 1912 described significant differences in the histogram of the udder in dairy (Red Steppe and Yaroslavl breeds) and draft (Gray Steppe) cattle [4].

The shape, size, and condition of the udder determine the resistance to mastitis, milk yield and the productive period longevity [5, 6]. In the Netherlands, for example, the damage from a single case of clinical mastitis ranged from 164 to 235 euros, depending on the month of lactation, and could reach 100 million euros in total [7]. Mammary gland morbidity in the U.S. dairy industry during the year reaches 51%, and the cost of treatment is estimated at an average of $2 billion. The frequency of clinical mastitis in the UK is slightly lower, 32-38%, while in Germany it is up to 60% [8, 9]. The incidence of mastitis in Russian farms ranges from 10-20% to 70-80%. The economic damage caused by this disease to dairy cattle breeding is 150-200 billion rubles per year [10, 11]. A certain relationship between the development of mammary gland pathology in cows and the shape of the nipple and udder was pointed out by Mitev et al. [12] and Asadpour et al. [13]. In recent decades, high milk yielding breeds of dairy cattle have prevailed in livestock, which is accompanied by an increase in the frequency of various pathologies, including mastitis. Counting the number of somatic cells in milk and ultrasonic scanning made it possible to reduce the incidence of mastitis, increase productivity and extend the period of cows' commercial use [14].

Endocrine changes associated with pregnancy stimulate extensive cell proliferation in the mammary gland, which continues in the early stages of lactation. The cell population then decreases markedly until involution is complete [15]. The study of SP (side population) cells of the mammary gland revealed that they represented an undifferentiated subfraction of epithelial cells. SP cells retain the potential for differentiation in vitro into clones typical of the mammary gland and normal lobular and duct structures in vivo. Mammary gland stem cells [16, 17] were studied and identified with their preliminary labeling and mammary gland transplantation for subsequent localization analysis [18].

The mammary gland is one of the most labile and easily changing organs in animals. The reasons for these variations are very diverse and depend on the breed [19, 20], age, the month of lactation [19], pregnancy, feeding and housing conditions, cow milking technology [21-24]. The central nervous system, digestive organs, respiratory organs, internal secretion and, to a large extent, blood circulation intensity have a significant influence on the amount of secreted milk [25-27].

To improve the productive and technological qualities of domestic livestock, along with the intra-breed selection, the gene pool of the best foreign breeds is often used for crossing with domestic livestock. Histological studies of the mammary gland in Ayrshire cattle have shown that the daughters of bulls of the Finnish origin have the most developed glandular udder tissue (the number of alveoli per 1 mm^2 was 27.3), while in daughters of bulls of the Canadian origin it was 25.1, with a diameter of 125.1 and 123.5 μm, respectively [28]. Differences between udder quarters in morphology of glandular and connective tissues (different types of duct system branching) were revealed in native Kostroma cows aged 16 months. The best ratio of glandular and connective tissues (38.1% and 61.9%, respectively) was established in quarters with the trunk type of duct

system [29].

Similar features are also typical of non-dairy cattle. In young ewes at 6 months of age, the size of the mammary gland tissue anlage was 39.5 μm [30]. In newborn young ewes, glandular tissue is localized in the center of the fatty lobes and has the form of epithelial anlages in the form of islets [31]. In the mammary gland of newborn young ewes, connective tissue prevails over glandular tissue (the average ratio is 0.7:1.0). Adult sheep show a similar trend, with the portion of connective tissue in the mammary gland increasing to 65% vs. to a 35% increases in glandular tissue. During pregnancy, the development of glandular tissue in the mammary gland of ewes intensifies.

In the mammary gland of the Saanen goats the adipose tissue surrounded by the bands of stromal connective tissue prevails during the neonatal period. Parenchyma is comprised of lobules separated by zones of connective tissue. Adipose tissue with a small amount of glandular tissue in the form of epithelial anlages is localized directly in the lobule. One-year-old Saanen goats have 33.5% of adipose tissue, 41.5% of glandular tissue and 25.0% of connective tissue. Goats are thought to have a fully developed mammary gland at this age [32].

We have not found any reports on the morphology and histological structure of the mammary gland in female yaks.

Diseases caused by metabolic disorders and reduced resistance to environmental factors are known to occur when the genetic potential of animals is fully realized [33, 34]. In addition, modern machinery and automated technologies in dairy farming necessitate breeding animals for adaptation to such conditions, in particular, for udder health [35, 36].

In the Kabardino-Balkarian Republic, the improvement of Brown Swiss and Black-and-White cattle is carried out with the use of Holstein livestock gene pool of Black-and-White cattle. However, there is no data on the effect of crossbreeding with Holstein cows on the structure and function of the mammary gland. There is also no information on the histological structure and biomechanical properties of the mammary gland of female yak and goats, the breeding of which is expedient seeing the environmental and climatic factors of the region.

In this paper we present for the first time comparative data on the mammary gland histogram of Brown Swiss cows, their crossbreeds with Holstein cows (different generations), as well as the yaks imported from Tuva and Kyrgyzstan, and goats bred in the Republic. The results obtained complement the existing understanding of the structural and functional organization of this mammalian organ.

The aim of the work was histological investigation of the peculiarities of the mammary gland structure in animals of different species and breeds depending on the origin and type of use.

Techniques. The investigations were conducted from 2012 to 2017.

In Lenin Agricultural Production Cooperative (Urvansky District, Kabardino-Balkarian Republic), six groups of cows were formed, $n = 5$ each. Group I comprised Swiss animals, Group II crossbreed cows (Holstein × Swiss) F_1, Group III their peers F_2, Group IV Black-and-White cows, Group V half-bred cows (Holstein × Black-and-White cattle) F_1, Group VI their peers (blood $^1/_4$ Black-and-White cattle + $^3/_4$ Holstein) F_2. In Elbrus Agroinvest LLC (Bezengi village, Chereksky District, Kabardino-Balkarian Republic) female yaks of populations imported from Tuva and Kyrgyzstan and Brown Swiss cows of the local population (5 animals per each group) were selected. Saanen goats (3 ewes) were from the Sarsky farm (Maysky District, Kabardino-Balkarian Republic), and local goats (3 ewes) from private farms (Arik village, Tersky District, Kabardino-Balkarian Republic). All farms were free from infectious and invasive diseases.

For histological examination, 2×3 cm tissue samples were taken between the base of the milk tank and the entire base of the udder quarter along the line through the middle of the teat canal and the breast sinus of the right front teat. The samples were preserved in a 10% formalin solution for 24 hours and put to 5% solution for permanent storage. The 5-10 μm-thick sections were prepared on a sled section cutter MPS-2 (Russia), 10-15-μm-thick sections on a freezing section cutter MZ-2 (Russia). Preparations were stained with hematoxylin and eosin to examine the overall histological structure (a microscope MBS-10, LZOS JSC, Russia, magnification of ×4, ×10, ×40, ×100 for and ×10 for eyepiece). The quantitative tissue ratio (glandular, adipose and connective) was determined by the triangle method. With the help of a drawing machine, the diameter of milk alveoli and the thickness of connective-tissue bands were measured, and the number of glandular epithelium cells was calculated per 1 mm^2. To assess structures, the tissue samples were embedded into paraffin using the standard method; paraffin sections were prepared on a MPS-2 section cutter. The histological study was carried out at the Kabardino-Balkarian Pathological Anatomical Bureau of the Ministry of Health of the KBR. A total sample comprise 5 histological sections from each group of cows and female yaks from Lenin Agricultural Production Cooperative and Elbrus Agroinvest LLC, 3 histological sections from Saanen goats from Sarsky farm and 3 histological sections from local goats from Arik village.

Biomechanical properties (elasticity, flexibility, and strength) were assessed in udder tissue sampled from the central part of the suspensory ligament. To test 6 samples from two groups of goats, i.e. the local and Saanen populations, a universal bursting machine REM-50-A (Metrotest LLC, Russia) was used.

Biometric data processing was performed as per the description [37]. The arithmetic mean (*M*), standard errors of the mean (±SEM), and the reliability of differences between mean values were estimated by Student's *t*-test at three confidence levels ($P > 0.95$; $P > 0.99$ and $P > 0.999$).

Results. The monitoring of the mammary gland microstructure in the examined animals showed a different ratio of tissue types depending on the breed (genotype) of individuals (Table 1).

1. Udder tissue ratio in the 2nd calving cows depending on the origin (*M*±SEM, Lenin Agricultural Production Cooperative, Urvansky District, Kabardino-Balkarian Republic, 2012-2015)

Group (n = 5)	Tissue		
	glandular	connective	fatty
I	66.7±1.20	23.2±1.13	10.1±0.67
II	71.2±0.93*	19.2±1.21*	9.6±0.80
III	74.6±1.10**	16.7±1.68*	8.7±1.04
IV	68.4±1.32	21.9±0.98	9.7±0.59
V	73.6±0.85*	17.2±1.75*	9.2±0.73
VI	76.7±1.04**	15.3±1.87*	8.0±0.92

N o t e. I – Swiss; II – Holstein × Swiss, F_1; III – Holstein × Swiss, F_2; IV – Black-and-White; V – Holstein × Black-and-White, F_1; VI – Holstein × Black-and-White, F_2.

*, ** Differences are statistically significant at $P > 0.95$ and $P > 0.99$, respectively (for Group II and Group III as compared to Group I, for Group V and Group VI as compared to Group IV).

The greater share of glandular tissue was found in the F_2 Holstein and Black-and-White crossbreeds with the advantage of 3.1% ($P > 0.95$) over peers in F_1 and 8.3% ($P > 0.99$) over purebred Black-and-White cows. When comparing the Swiss and Holstein-Swiss cattle, the amount of glandular tissue was higher in crossbreeds (by 4.5-7.9%, $P > 0.95$-0.99). In F_2 cows, the share of connective and fatty tissues decreased due to the increase in the amount of glandular tissue. Thus, the amount of connective tissue in the Swiss cows was 4.0-6.5% higher ($P > 0.95$) than that of the crossbreed Holstein-Swiss peers in

F_1 and F_2, and the fat tissue was 0.5-1.4% higher. The differences between Black-and-White cows and Holstein and Black-and-White crossbreeds in distribution of these tissues were similar. In the Swiss cows, a higher proportion of fatty tissue in the udder is likely to be associated with a better predisposition to milk fat synthesis.

The histological data we obtained indicate distinctive features in the structure of the mammary gland of the Black-and-White and Brown Swiss cows, as well as their crossbreeds with Holstein cattle at all ages (Table 2).

2. Histostructure of the mammary gland depending of breed and age of cows (*M*±SEM, Lenin Agricultural Production Cooperative, Urvansky District, Kabardino-Balkarian Republic, 2012-2015)

Group (n = 5)	Calving	Diameter, μm		Thickness of connective-tissue bands, μm			Number of epithelial cells per 1 mm^2
		alveoli	adipocytes	main	interlobular	intralobular	
I	1st	73.5±1.32	70.4±0.68	471.3±6.09	84.3±1.47	30.3±1.08	3.56±0.11
	2nd	80.0±0.41	72.6±2.10	480.3±4.26	88.6±1.77	31.0±1.41	3.76±0.10
	3rd	89.0±1.41	78.3±1.47	492.3±6.17	91.0±0.41	34.0±0.70	4.06±0.14
II	1st	90.3±1.77***	72.3±1.47	530.0±6.28***	70.6±1.77**	29.0±1.41	3.80±0.14
	2nd	97.3±1.78***	78.0±2.54	534.3±5.75***	74.0±1.41***	31.0±0.41	3.93±0.08
	3rd	112.0±2.54***	80.6±1.78	550.0±6.36***	77.3±1.47***	33.0±0.07	4.03±0.14
III	1st	101.3±4.60***	74.3±2.85	539.0±8.33***	68.0±4.24***	29.3±2.48	3.90±0.21
	2nd	116.0±4.24***	76.0±3.94	555.3±11.66***	70.0±3.24***	30.7±2.48	4.10±0.20
	3rd	119.0±4.95***	80.4±6.30	570.0±8.86***	77.0±4.41**	34.3±2.58	4.23±0.20
IV	1st	74.0±1.41	68.3±1.47	467.0±4.30	79.0±1.41	32.0±0.71	3.50±0.12
	2nd	79.0±1.40	70.0±1.22	480.0±3.53	80.3±1.47	33.0±0.70	3.60±0.12
	3rd	90.0±1.87	73.0±1.41	491.0±1.13	88.0±0.41	35.0±0.70	3.80±0.13
V	1st	90.3±2.16***	68.6±1.77	495.0±6.81**	70.0±1.87**	31.0±1.41	3.66±0.10
	2nd	99.6±2.48***	73.3±2.16	502.0±7.64*	72.0±2.12*	31.3±1.47	3.80±0.12
	3rd	110.6±2.48***	77.3±2.16	515.0±5.33**	76.3±1.77***	34.0±1.41	4.00±0.12
VI	1st	111.0±3.53***	70.3±2.48	520.0±7.07***	65.0±2.12***	30.0±1.87	3.90±0.14
	2nd	115.0±3.93***	75.0±2.82	540.0±7.87***	69.0±2.82**	32.0±2.12	3.90±0.18
	3rd	118.0±3.24***	77.3±2.16	556.0±8.83***	75.0±2.54**	33.0±1.41	4.10±0.14

Note. For a description of the groups, see Technique section.
*, **, *** Differences are statistically significant at P > 0,95; P > 0,99 and P > 0,999, respectively (for Group II and Group III as compared to Group I, for Group V and Group VI as compared to Group IV).

Regardless of the breed and genotype of cows, all analyzed parameters increased with age. Other things being equal, they were higher in crossbreds with Holstein cattle. A larger diameter of alveoli was characteristic of the F_2 Holstein crossbreeds with Swiss and Black-and-White cattle, at a difference of 27.8 μm (P > 0.999) and 37.0 μm (P > 0.999), respectively, for individuals of the 1st calving, of 36.0 μm each (P > 0.999) for the 2nd calving, and 30.0 μm (P > 0.999) and 28.0 μm (P > 0.999), respectively, for the 3rd calving. Crossbreeds F_1, regardless of the parent breed, occupied an intermediate position relative to the extreme values. In F_2 (Swiss × Holstein) of the 3rd calving the diameters of alveoli were largest, being 119 μm on average which is 6.2-3.7% higher than the same indicator in female peers from Groups I, II, IV, and V (P > 0.999). Differences in this indicator between F_2 animals of Groups III and VI (0.8%) were unreliable. It should be noted that the maximum increase in the alveoli diameter from the 1st to the 3rd calving was characteristic of half-bred crossbreeds of Holstein × Swiss (+21.7 μm, P > 0.999) and Holstein × Black-and-White cattle (+20.3 μm, P > 0.999).

The smallest diameter of fat cells in the 1st lactation was characteristic of Black-and-White cows (68.3 μm), while the largest one was in $^1/_2$- and $^3/_4$-bred crossbreeds Holstein × Swiss (F_1 and F_2) (72.3 and 74.3 μm, respectively); all other groups occupied an intermediate position. The tendencies were similar at the age of the 2nd and 3rd lactations. A greater age-related increase in the diameter of fat cells was characteristic of the crossbreeds Holstein × Black-and-White cattle in F_2 with a variation from 70.3 μm in first calves to 77.3 μm in animals of the 3rd calving.

These data on the differences in the mammary gland histogram between domestic breeds and their crossbreeds with the improved dairy cattle breeds are consistent with the results of earlier reports [20, 24].

Alveoli and excretory ducts are surrounded by connective tissue. Penetrating between the lobes, lobules, and alveoli, connective tissue forms a mesh network consisting of bands and interlayers [1]. The data we obtained lead to the conclusion that with age the thickness of connective tissue bands increases in all groups of dairy cattle. To the greatest extent it was characteristic of the main bands, in the least — of intralobular bands. When comparing the indices of Black-and-White cows and crossbreeds of Holstein with Black-and-White cattle, it turned out that thicker main bands (regardless of the number of lactation) are characteristic of cows in F_2, whose superiority over purebred peers was 53 μm ($P > 0.999$), 60 μm ($P > 0.999$), and 65 μm ($P > 0.999$). In crossbred Holstein and Swiss (F_2) cows, these bands were thicker in the 3rd lactation (2.5-16.0% higher than the other groups).

A slightly different trend was observed in the thickness of the interlobular bands: they have increased with age, and the increase in the Holstein thorough-bredness has led to a decrease in this figure. For example, in F_1 and F_2, the thickness of interlobular bands increased from 68.0 to 77.3 μm for the Holstein × Brown Swiss cows, and from 65.0 to 76.3 μm for the Holstein × Black-and-White cows. It should be noted that regardless of lactation, the thickness of the interlobular connective tissue bands was higher in purebred Brown Swiss and Black-and-White individuals. The corresponding superiority of Swiss cows over Holstein-Swiss cows was 13.7-16.3 μm ($P > 0.99$-0.999) for the 1st calving, 14.6-18.6 μm ($P > 0.999$) for the 2nd calving, and 13.7-14.0 μm ($P > 0.99$-0.999) for the 3rd calving. Similar differences occurred between groups of Black-and-White and crossbreed Holstein-Black-and-White cattle with the superiority of Black-and-White cows.

The variation in the diameter of the intralobular bands was slightly lower than that of the main and interlobular ones: in cows of all studied genotypes, irrespective of the lactation, this index ranged within 29.0-35.0 μm and changed insignificantly with higher Holstein thorough-bredness.

The number of epithelial cells per 1 mm^2 of the alveoli area was the smallest in the Black-and-White first calving heifers, and the largest in F_2 animals, regardless of the used mother breed. Thus, the differences in this indicator were 0.24-0.34 cells per mm^2 between Swiss and Holstein-Swiss first calving heifers, in favor of crossbreeds, and 0.16-0.4 cells per mm^2 between Black-and-White and Holstein-Black-and-White cows. The superiority of crossbreeds (of different genotype) over purebred peers in the number of epithelial cells per 1 mm^2 area of the alveoli was also noted in the 2nd and 3rd calving.

Thus, the study of mammary gland tissue specimens of cows obtained from crossing of Brown Swiss and Black-and-White breeds with bulls of the Holstein breed of black-and-white color indicates that the development of structural elements of the mammary gland, which are the most important for milk secretion, in improved animals and original breeds differs. The size of the milk alveoli and the diameter of the fat globules in the crossbreeds were larger than those of the Brown Swiss and Black-and-White peers, and these figures, with an increase in the Holstein breed, increased with a slight age-related decrease in the thickness of the interlobular bands.

A comparison of the mammary gland tissue types of the Swiss cows of the local population and the yak females imported from Tuva and Kyrgyzstan showed (Table 3) that the share of connective tissue in yaks of different popula-

tions was almost the same, the 30.8-30.9% which was 5.7-5.8% higher than in the peers of the Brown Swiss breed (P > 0.999). However, the yaks imported to the region were inferior to the local population as to the relative amount of glandular tissue (this indicator is usually used to judge the milk yielding). Differences in the glandular tissue fraction between Swiss cows and yaks varied in the range of 7.5-9.7% (P > 0.999). As to fatty tissue proportion in the mammary gland, yaks from Kyrgyzstan were superior with a 2.1% excess (P > 0.999) compared to peers from Tuva and 3.9% excess (P > 0.999) compared to Brown Swiss cows of the local population, which explains the higher fat concentration in yak milk.

3. Tissues ratio (%) in the mammary gland in the local population of Brown Swiss cows and female yaks imported from Tuva and Kyrgyzstan (n = 5 in each group, M±SEM, Elbrus Agroinvest LLC, Bezengi village, Chereksky District, Kabardino-Balkarian Republic, 2015-2016)

Tissue	Local Brown Swiss cows	Imported yak females	
		from Tuva	from Kyrgyzstan
Glandular	64.3±1.30	56.8±0.90*	54.6±0.70*
Connective	25.1±0.50	30.8±0.60*	30.9±0.60*
Fatty	10.6±0.20	12.4±0.10*	14.5±0.20*

N o t e. Animals of the 3rd calving were examined.
* Differences with indicators in Brown Swiss cows are statistically significant at P > 0.999.

A comparison of the udder histogram at the age of the 3rd calving in the Swiss cows of the local population and female yaks showed (Table 4) that the alveolar diameter in the local Swiss cows is larger than in the yaks, regardless of the origin of the latter, with the average excess by 9.1-11.3 μm, P > 0.999, which indicates insignificant differences in this parameter between yaks of different origins and the advantage of double use cows. The diameter of fat cells in yaks was 4.5-5.4 μm (P > 0.99) larger than in Swiss peers. More developed fat cells of the mammary gland contribute to higher milk quality in yaks.

4. Histostructure of the mammary gland in local Brown Swiss cows and female yaks imported from Tuva and Kyrgyzstan (n = 5 in each group, M±SEM, Elbrus Agroinvest LLC, Bezengi village, Chereksky District, Kabardino-Balkarian Republic, 2015-2016)

Indicator	Local Brown Swiss cows	Imported yak females	
		from Tuva	from Kyrgyzstan
Diameter, μm:			
alveoli	90.6±1.45	79.3±1.22***	81.5±1.06***
adipocytes	77.1±0.88	82.5±1.03**	81.6±0.90**
Thickness of bands, μm:			
main	501.4±5.13	472.5±3.96***	476.3±4.78**
interlobular	75.4±1.98	93.0±2.46***	90.4±2.31**
intralobular	33.4±1.36	34.5±1.45	34.1±1.60
Number of epithelial cells per 1 mm^2 alveoli surface	4.20±0.08	3.84±0.06**	3.91±0.07*

N o t e. Animals of the 3rd calving were examined.
*, **, *** Differences with indicators in Brown Swiss cows are statistically significant at P > 0.95; P > 0.99 and P > 0,999, respectively.

The thickness of the connective tissue bands varied depending on the origin of the animals. Analysis of connective tissue parameters in yak females imported from Tuva and Kyrgyzstan indicates the absence of significant differences. At the same time, the thickness of the main bands was higher in the local Swiss population, by 25.1-28.9 μm on average, of interlobular bands — in yaks, by 15.0-17.6 μm on average (P > 0.99-0.999). We did not find any significant differences in the thickness of intralobular bands between animals of different origins in these groups. The number of glandular epithelium cells per 1 mm^2 alveoli area was higher in the Brown Swiss cows of the local population than in yaks of

different origins, by an average of 0.29-0.36 cells per mm^2 ($P > 0.95$-0.99).

As in dairy cattle [20, 24, 28], the productive qualities of dairy goats [27, 32, 38] in many respects are due to the development (histogenesis) of a mammary gland and the prevalence of different tissues. So we compared histogram and biomechanical parameters of mammary gland tissue in goats of different origins (Table 5).

5. Tissues ratio, histogram and biomechanical properties of the mammary gland in goats of different origins (n = 3 in each group, M±SEM, Kabardino-Balkarian Republic, 2016-2017)

Indicator	Breed, origin		Saanen goats compared to the local goats
	Saanen	local population	
Tissue ratio, %:			
glandular	65.3±1.70	48.6±1.50	+16.7***
connecting	20.6±0.40	18.7±0.50	+1.9*
fatty	14.1±0.30	32.7±0.90	–18.6***
Diameter, microns:			
alveoli	94.5±1.50	78.6±1.30	+15.9**
adipocytes	78.0±1.50	75.3±1.20	+2.7
Thickness of bands in the connective tissue stroma:			
main	63.4±1.00	49.5±0.80	+13.9***
interlobular	42.6±0.70	36.0±0.50	+6.6**
intralobular	18.3±0.30	17.4±0.30	+0.9
Biomechanical properties of glandular tissues:			
tensile strength, MPa	10.3±0.20	8.9±0.10	+1.4**
flexibility, MPa	5113±36.70	4562±28.40	+551***
elasticity, %	19.3±0.40	17.4±0.30	+1.9*

N o t e. Saanen goats were from Sarsky farm (Maysky District), local population goats — from Arik village (Tersky District), animals were examined in the 3rd lactation.

*, **, *** Differences with goats of the local population are statistically significant at $P > 0.95$; $P > 0.99$ and $P > 0.999$, respectively.

The analysis of goat mammary gland tissue specimens revealed a different ratio of tissue types. For example, Saanen goats had the largest volume of glandular tissue, 65.3% which was 16.7% higher than that of local goats ($P > 0.999$). Differences in the connective tissue content between goat groups varied by 1.9% ($P > 0.95$), with the best ratio of parenchyma and stroma, 1.88:1, in the mammary gland of the Saanen goats as compared to local goat population with 0.94:1. At the same time, fat tissue was less developed in the mammary gland of Saanen goats (by 18.6% on average, $P > 0.999$). Large diameters of alveoli (by 15.9 μm, $P > 0.99$) were characteristic of Saanen goats, while no significant intergroup differences in the area of fat cells were found. There were differences in the thickness of connective tissue bands of different origins, especially between the main and interlobular bands, with an excess of 13.9 μm ($P > 0.999$) and 6.6 μm ($P > 0.99$), respectively, in the Saanen animals.

The maximum values of strength, flexibility, and elasticity of mammary gland tissues in the central region of the suspensory ligament were characteristic of Saanen goats. Thus, their advantage was 1.4 MPa ($P > 0.99$) in terms of strength, 551 MPa ($P > 0.999$) in terms of elastic modulus, and 1.9% in terms of maximum elastic deformation ($P > 0.95$). The higher biomechanical properties of the mammary gland in Saanen goats compared to local individuals are likely to be due to the selection of breeds for both milk productivity and udder morphology.

Thus, the results of the analysis of the histological structure and properties of the mammary gland of cattle, yaks, and goats allow drawing the following conclusions. Animals improved by holsteinization exceed the peers of the Brown Swiss and Black-and-White breeds both in proportion of glandular tissue in the

mammary gland and in the development and size of structural elements the most important for the milk secretion (milk alveoli, fat globules). A comparison of the mammary gland of the Brown Swiss cattle and yaks imported from Tuva and Kyrgyzstan revealed the following peculiarities. In Swiss cows of local population, there are more glandular epithelial cells, a larger alveoli diameter and thickness of main bands. In yak females of different populations, fatty tissue proportion and the thickness of interlobular bands are increased and the diameter of fat cells is decreased. Among goats of different origins, a more developed mammary gland with high biomechanical properties is characteristic of the Saanen breed. Regardless of species, more yielding dairy animals differed from the less productive livestock by the predominance of glandular tissue, epithelial cells, thicker main bands and improved biomechanical properties of the mammary gland tissues.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 744-753
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Veterinary microbiology

UDC 636.294:579.62:577.2

doi: 10.15389/agrobiology.2019.4.744eng
doi: 10.15389/agrobiology.2019.4.744rus

THE RUMEN MICROBIOTA OF REINDEER (*Rangifer tarandus*) WITH CLINICAL MANIFESTATIONS OF NECROBACTERIOSIS

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation for project No. 17-76-20026 "Rumen microbiocenosis in Rangifer tarandus of the Russian Arctic as a fundamentals for promising animal biotechnologies"
Received April 28, 2019

Abstract

Necrobacteriosis is an infectious disease that affects many species of domestic and wild mammals, birds, and humans. The main clinical manifestations of the disease are associated with the development of purulent-necrotic lesions of the skin, mucous membranes, internal organs, and extremities as a result of infection of the host organism by strictly anaerobic pathogenic fuzobacteria, the *Fusobacterium necrophorum*. For reindeer herding, the problem of necrobacteriosis among other diseases of reindeer is one of the most significant, since it brings the most significant damage to the economic activity of the population of the Russian Arctic. This paper for the first time shows the obtained results on rumen microbiota composition differences between the clinically healthy *Rangifer tarandus* reindeer of the Russian Arctic and reindeer with necrobacteriosis. The purpose of the study was to characterize the distinctive features of rumen microbiota in the reindeer with the clinical manifestations of necrobacteriosis. The study was carried out on calves (4-6 months) and adults (3-6 years) animals, including clinically healthy individuals and those with necrobacteriosis. Samples of the rumen content were collected during the summer-autumn period in 2017 ($n = 3$ from each age group) in the Yamalo-Nenets Autonomous District. The total number of bacteria and fungi of the class *Neocallimastigales* was analyzed by quantitative PCR, the composition of the bacterial community was analyzed by T-RFLP (terminal restriction fragment length polymorphism) method. In individuals with clinical manifestations of necrobacteriosis, a significantly higher content of fusobacteria was detected, 1.79-fold in adults ($p < 0.05$), and 2.65-fold in calves ($p < 0.05$). In sick animals of both age groups, there was a significantly higher presence of bacteria of the genus *Staphylococus* ($p < 0.05$) and the family *Pseudomonadaceae* ($p < 0.05$), some species of which may cause purulent-necrotic lesions of animals. The 4-6 month old calves showed a significant increase ($p < 0.05$) in the content of family *Campylobacteriaceae* and family *Enterobacteriaceae* compared to clinically healthy animals. At the same time healthy individuals showed a greater number of cellulolytic and acid-utilizing bacteria. In general, it was noted that the rumen microbiome of calves with clinical signs of necrobacteriosis is characterized by large changes compared to the adult animals. In particular, in young reindeer with necrobacteriosis, there was a significant increase in the Shannon's diversity index of the rumen microbial community ($p < 0.05$), which indicates a greater heterogeneity of the bacterial community compared to healthy individuals. In addition, a significant ($p < 0.05$) decrease of cellulolytic chytridiomycetes of the class *Neocallimastigales* was detected in the rumen of this animal group. In this regard, the identified patterns may be due to the physiological features of this

stage of animal development in *Rangifer tarandus*. The obtained results can be a basis for recommendations to improve anti-necrobacteriosis measures in reindeer and to reduce mortality during summer and autumn period.

Keywords: *Rangifer tarandus*, reindeer, necrobacteriosis, laminitis, Fusobacteria, T-RFLP analysis, quantitative PCR, rumen microbiome, Russian Arctic

Infectious and invasive diseases are constantly recorded in the natural area of reindeer (*Rangifer tarandus*) [1]. They cause significant damage to reindeer husbandry which is strategically important for the population of the Far North. Parasitic diseases transmitted by blood-sucking insects, water infestations (edemagenosis, cephenomyiosis) and necrobacteriosis, an infectious bacterial disease, are of particular threat. The proportion of animals with necrobacteriosis averages from 7% to 33%. Necrobacteriosis is most pronounced in summer, when the number of sick animals can reach 65-70% [2].

Necrobacteriosis affects many species of domestic and wild animals, birds and humans. The main clinical manifestations of the disease are purulent necrosis of the skin, mucous membranes, and internal organs as a result of infection by strictly anaerobic pathogenic fusobacteria, mainly *Fusobacterium necrophorum* [3, 4]. The limbs are affected in ruminants. In reindeer, purulent lesions of hooves are noted only in the summer-autumn period [2, 5], mainly in July, August, with the onset of heat. In September, the number of diseases decreases sharply, and in October new cases are no longer recorded. This is due to the creation of optimal seasonal conditions for the development of infections, i.e. positive air temperatures, animal exhaustion, and insect activity. Outbreaks of necrobacteriosis occur both in the Russian Arctic [5] and in other areas of reindeer habitat [6].

Necrobacillosis is well-studied in cattle [6, 7], while studies on reindeer are much fewer. Recently, the possibility of interrelations between the causative agent of necrobacteriosis and the microflora of other ecotopes of the animal organism, in particular, the rumen, a being most actively discussed [8, 9].

Many authors believe that the health of ruminants is highly dependent on rumen digestion and is therefore ensured by the presence of certain microorganisms in the rumen [10]. According to modern concepts, *F. necroforum* belongs to the normal flora of the digestive tract of ruminants, especially the rumen, and can spread in the environment through excrement [6, 11]. It is known that fusobacteria are able to secrete a number of toxins that lead to tissue necrosis and the occurrence of secondary infections caused by actinobacteria. It has been reported that fusobacteria are able to penetrate only into damaged tissues, for example, with necrotic lesions of the extremities [12]. It has also been established that fusobacteria enter the animal through hemorrhagic lesions in the digestive tract [7].

The reasons of an increased reindeer susceptibility to infection with fusobacteria have not been studied much. Apparently, stress, high or low temperature, overpopulation, and poor nutrition can provoke it [12, 13]. Thus, Norwegian researchers, having studied the outbreak of necrobacteriosis in reindeer living in the north of Norway (2007-2008), confirmed that the *F. necroforum* was the causative agent of the infection, and concluded that the disease was provoked by the higher (compared to medium) values of temperature and humidity [6].

Biodiversity of pathogenic and conditionally pathogenic bacteria in the reindeer rumen under necrobacteriosis is practically not described.

This paper is the first report on the differences between the composition of rumen microbiota in clinically healthy reindeer in the Arctic zone of the Russian Federation and animals with manifestations of necrobacteriosis. Individuals with symptoms of necrobacteriosis have fewer cellulose-decomposing and acid-

reclaiming bacteria and more abundant fusobacteria and other pathogens (staphylococcus, pseudomonads, etc.), some of which may cause purulent necrotic lesions in animals. The most pronounced changes in the composition of the rumen microbiome as a result of necrobacteriosis were detected in 4-6-month-old calves in which the rumen bacterial community was more heterogeneous and characterized by increased counts of campylobacteria, enterobacteria, and decreased representation of *Chytridiomycetes* fungi.

The aim of the work was molecular analysis of the microbiota composition in the rumen of reindeer with clinical manifestations of necrobacteriosis.

Techniques. Samples of the rumen content were collected via a probe from 4-6-month-old calves and 3-6-year-old adult reindeer (*Rangifer tarandus*) of the Nenets breed (clinically healthy animals and individuals with manifestations of necrobacteriosis; $n = 3$ from each age group) in the summer-autumn period in 2017 in the Yamalo-Nenets Autonomous Area (Kharp urban settlement, forest-tundra natural climatic zone). Samples were frozen at –20 °C until analysis.

Total DNA from the samples was isolated using Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) in accordance with the manufacturer's recommendations. The final concentration of the isolated total DNA in the solution was determined on a Qubit fluorimeter (Invitrogen, Inc., USA) with Qubit dsDNA BR Assay Kit (Invitrogen, Inc., USA) as per the manufacturer's instructions.

The total number of bacteria and *Chytridiomycetes* fungi of the *Neocallimastigales* class was assessed by quantitative PCR (DT Lite-4 amplifier; NPO DNA-Technology LLC, Russia) with a set of reagents for qPCR in the presence of EVA Green intercalating dye (Syntol CJSC, Russia). The following primers were used: F — 5′-ACTCCTAC-GGGAGGCAGCAG-3′, R — 5′-ATTACC-GCGGCTGCTGG-3′ (bacteria); F — 5′-GCACTTCATTGTGTGTACTG-3′, R — 5′-GGATGAAACTCGTTG-ACTTC-3′ (fungi). Amplification mode: 3 min at 95 °C (1 cycle); 13 s at 95 °C, 13 s at 57 °C, 30 s at 72 °C (40 cycles).

The composition of the bacterial community of the reindeer rumen was analyzed by T-RFLP (terminal restriction fragment length polymorphism) [14]. PCR was performed on a Verity DNA amplifier (Life Technologies, Inc., USA) with eubacterial primers 63F (5′-CAGGCCTAACACATGCAAGTC-3′) labeled at the 5′-end (WellRed D4 fluorophore, Beckman Coulter, USA) and 1492R (5′-TACGGHTACCTTGTTACGACTT-3′), which allow amplification of a 16S rRNA gene fragment (from position 63 to 1492; numbering is indicated for *Escherichia coli* 16S rRNA gene), in the following mode: 3 min at 95 °C (1 cycle); 30 s at 95 °C, 40 s at 55 °C, 60 s at 72 °C (35 cycles); 5 min at 72 °C.

The fluorescently labeled amplicons of the 16S rRNA gene fragment were purified by the standard method [15]; 30-50 ng DNA was treated with restriction enzymes HaeIII, HhaI and MspI (Fermentas, Lithuania) for 2 h at 37 °C. Restriction products were precipitated with ethanol, then 0.2 μl of Size Standart-600 molecular weight marker (Beckman Coulter, USA) and 10 μl of Sample Loading Solution formamide (Beckman Coulter, USA) were added. The analysis was performed on CEQ 8000 (Beckman Coulter, USA) according to the manufacturer's recommendations (instrument error not more than 5%). The data obtained were processed with Fragment Analysis software (Beckman Coulter, USA). The height of the peaks and their area were calculated, based on which subtypes (phylotypes) were identified with an error of 1 nucleotide accepted in the study, and their proportion in the microbial community was estimated.

The taxonomic affiliation of bacteria was determined using the database (http://mica.ibest.uidaho.edu/trflp.php).

Statistical processing of the results was carried out by the dispersion analy-

sis method using the Microsoft Excel 2010 software. The mean (*M*) and the standard error of the mean (±SEM) were calculated, the significance of differences between the groups was evaluated by Student's *t*-test. The Past program calculated the Shannon and Simpson biodiversity indices (http://folk.uio.no/ohammer/past/).

Resultes. According to the assessment of the bacterial community by the T-RFLP method, most of the phylotypes belonged to the *Firmicutes* phylum (their total number reached 61% in adult reindeer). To a lesser extent, bacteria of the phyla *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* were found in the rumen microbiome of clinically healthy animals and individuals with symptoms of necrobacteriosis. Members of the *Fusobacteria*, *Acidobacteria*, and *Cyanobacteria* phyla comprised a minor amount. Also, there was significant proportion of phylotypes that could not be identified from the databases. The amount of unidentified taxa was the largest (up to 22.18%) in adults.

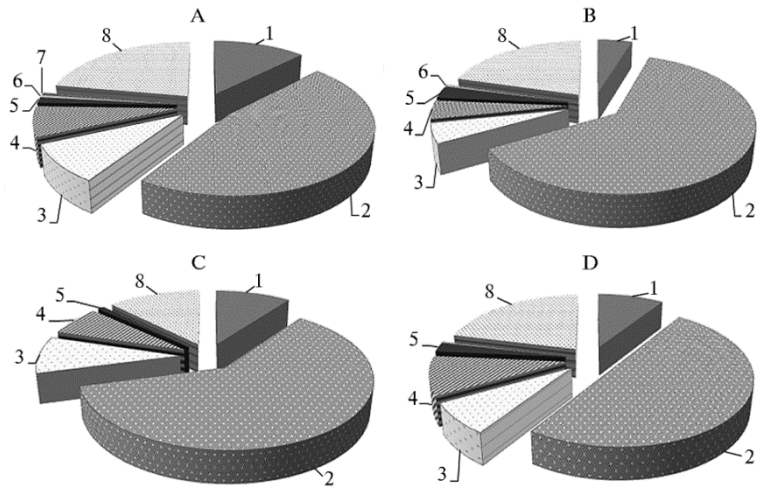

Fig. 1. Bacterial phyla in the reindeer (*Rangifer tarandus*) rumen community: A — adult individuals, B — adult individuals with clinical signs of necrobacteriosis, C — calves, D — calves with clinical signs of necrobacteriosis; 1 — phylum *Bacteroidetes*, 2 — phylum *Firmicutes*, 3 — phylum *Actinobacteria*, 4 — phylum *Proteobacteria*, 5 — phylum *Fusobacteria*, 6 — phylum *Cyanobacteria*, 7 — phylum *Acidobacteria*, 8 — unclassified nucleotide sequences (Yamal-Nenets Autonomous Area, urban settlement Kharp, 2017).

The rumen microbiome of the animals with clinical manifestations of necrobacteriosis differed notably from that of healthy individuals. First, there should be noted a significantly higher presence of the *Fusobacteria* phylum in reindeer with symptoms of necrobacteriosis (Fig. 1), 1.79-fodl ($p < 0.05$) in adults and 2.65-fold ($p < 0.05$) in calves. The results obtained once again confirm the role of bacteria of the *Fusobacterium* genus (including *F. necrophorum*) in the etiology of necrobacteriosis.

Nevertheless, there are reports whose authors expressed doubts about this or provided evidence of the main role of other types of microorganisms in the etiology of necrobacteriosis in ruminants [16]. In this connection, the fact of joint presence and growth of a number of pathogens, including pathogens of purulent-necrotic infections, in the rumen of the individuals with symptoms of necrobacteriosis is of interest. Thus, in both age groups of reindeer with necrobacteriotic manifestations, the proportion of bacteria of the genus *Staphylococcus* ($p < 0.05$) and the family of *Pseudomonadaceae* ($p < 0.05$) significantly increased, some of which can cause purulent-necrotic infections of animals. Moreover, we revealed a 1.96-fold ($p < 0.05$) and 2.38-fold ($p < 0.05$) increase in campylobacteria (*Campylobacteriaceae* family) and enterobacteria (*Enterobacteriaceae*

family) populations, respectively, in 4-6-month-old calves as compared to clinically healthy animals.

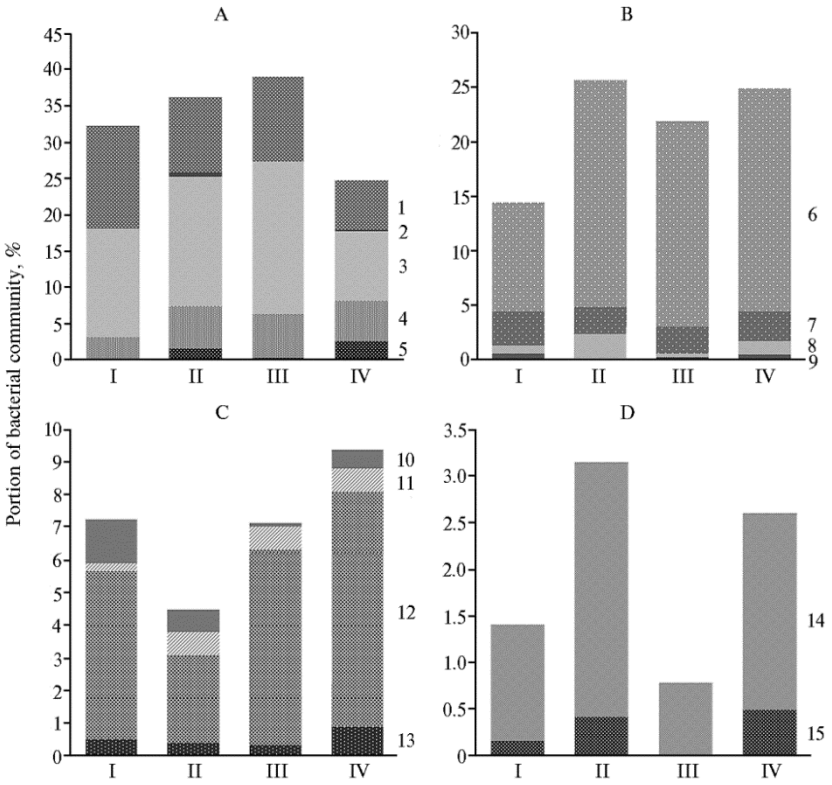

Fig. 2. Representation of bacterial taxa in rumen community of reindeer (*Rangifer tarandus*): I — adult individuals, II — adult individuals with clinical signs of necrobacteriosis, III — calves, IV — calves with clinical signs of necrobacteriosis (Yamal-Nenets Autonomous Area, urban settlement Kharp, 2017).

A — class *Clostridia* of phylum *Firmicutes*: 1 — family *Thermoanaerobacteraceae*, 2 — family *Lachnospiraceae*, 3 — family *Eubacteriaceae*, 4 — family *Ruminococcaceae*, 5 — family *Clostridiaceae*.

B — other *Clostridia* members of phylum *Firmicutes*: 6 — order *Negativicutes*, 7 — genus *Bacillus*, 8 — genus *Lactobacillus,* 9 — genus *Peptococcus*.

C — phylum *Proteobacteria*: 10 — family *Burkholderiaceae*; 11 — family *Pseudomonadaceae*, 12 — family *Campylobacteriacea*e, 13 — family *Enterobacteriaceae*.

D — others: 14 — phylum *Fusobacteria*, 15 — genus *Staphylococcus*.

The obtained results confirm reports on the polyetiological nature of necrobacteriosis. Smith et al. [17, 18] showed the presence of microorganisms concomitant to fusobacteria, including enterobacteria and actinobacteria, which complicate the course of necrobacteriosis. The same opinion was held by Petrov and Tashev (quoted in [3]), who during the examination of 134 reindeer with purulent limb lesions revealed a blue pus bacillus (*Pseudomonas aeruginosa*), streptococcus (*Streptococcus* genus), staphylococcus (*Staphylococcus* genus) and their associations. Laishev et al. [5] report the isolation of micrococci, *E. coli*, staphylococcus, Proteus together with fusobacteria in experimental infection of reindeer with a pure culture of necrobacteriosis pathogen. According to Nocek [7], proliferation of pathogens living on the extremities of cattle (fusobacteria, staphylococcus, streptococcus, enterobacteria), along with an increase in blood levels of endotoxins and histamine (products of scarlet microbial lysis) and damage to blood vessels causes inflammation and necrosis of the dermal layers of hooves. Ostrovsky et al. [19] explained the mass spread of limb diseases in reindeer by the violation of the management technology, leading to injuries and

maceration of the skin, weakening its protective properties, followed by the introduction of microflora in the tissue.

In our work, the bacterial community of the rumen of clinically healthy animals differed from that of individuals with symptoms of necrobacteriosis by a high percentage of microorganisms involved in the fermentation of carbohydrates in plant feeds. For example, in calves with symptoms of necrobacteriosis, the proportion of *Clostridia* class bacteria (including those of the *Lachnospiraceae*, *Eubacteriaceae* and *Clostridiaceae* families), potentially capable of fermentation of plant feed polysaccharides with the formation of volatile fatty acids, was 1.32 times lower ($p < 0.05$). In addition, the number of *Bacteroidetes* phylum bacteria (including *Bacteroides*, *Prevotella*), which ferment starch, fiber, a number of other carbohydrates, proteins, and deaminate acids, was significantly lower ($p < 0.05$) in adults and young animals compared to healthy ones. Less calves had bacteria of the *Negativicutes* order (*Megasphaera*, *Selenomonas* genera), which prevent the pH reduction and lactate acidosis due to the utilization of organic acids (propionic, acetic, oil, lactic, etc.) formed during fermentation of plant tissue [7, 20, 21].

The calculation of environmental indices also demonstrated a general decrease in biodiversity in the rumen of animals with clinical manifestations of necrobacteriosis (Fig. 3). In particular, the Shannon index increases ($p < 0.05$) in young animals with necrobacteriosis, which indicates a greater heterogeneity of the bacterial community of their rumens compared to healthy individuals.

It should be noted that the total number of bacteria in healthy and diseased individuals did not differ significantly (Fig. 4). However, a significant decrease ($p < 0.05$) in the number of chytridiomycetes, which are known to produce a wide range of multifunctional polysaccharide enzymes, was revealed in the rumen of the young reindeer [22].

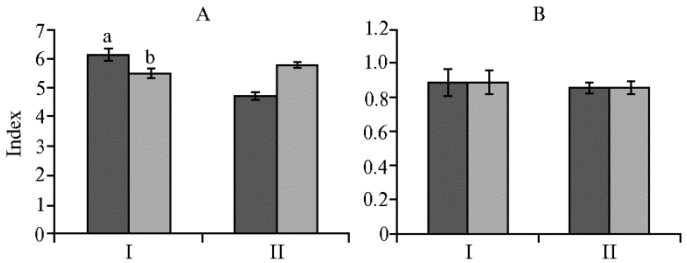

Fig. 3. Biodiversity of the rumen bacterial community in adult reindeers (*Rangifer tarandus*) (I) and calves (II): A — Shannon index, B — Simpson index; a — clinically healthy animals, b — animals with clinical signs of necrobacteriosis (*M*±SEM, (Yamal-Nenets Autonomous Area, urban settlement Kharp, 2017).

Previously, a microbiotic imbalance in the digestive tract under necrobacteriosis infection was reported to lead to a faster proliferation of the pathogenic strain of fusobacteria [23, 24]. In addition, infection of cattle with fusobacteria may occur as a result of the lactate acidosis syndrome, which is characterized by abnormal rumen microbiota composition [4, 7]. At the same time, the number of cellulose-decomposing and acid-recycling bacteria decreases in the cattle rumen; the number of *Streptococcus* and *Lactobacillus* genera synthesizing lactates increases, which reduces the rumen pH [25]. Lactate acidosis leads to damage to the rumen epithelium and penetration of pathogenic fusobacteria through the mucous membrane into the bloodstream. In turn, this leads to further infection of the body, in which, depending on the biotype of the pathogen (A, B or AB), a corresponding clinical picture is observed (lesions of hooves, mucous membranes or skin, liver abscesses or other internal organs). Simultaneous isolation of fusobacteria from other organs (liver, kidneys) and the rumen

has been repeatedly shown for the cattle [7, 8]. Interestingly, there was no increase in the representation of lactobacillus in the rumen of the reindeer we surveyed, which indicates the need to continue studying the relationship between the rumen microbiome and the development of necrobacteriosis. For example, the causes of necrobacteriosis in reindeer may be contaminated feed or the penetration of microorganisms through the respiratory tract. The latter was shown in the United States during outbreak of necrobacteriosis caused by lesions of the respiratory tract of white-tailed deer (*Odocoileus virginianus*) by fusobacteria *F. necrophorum* and *F. varium* [8, 26].

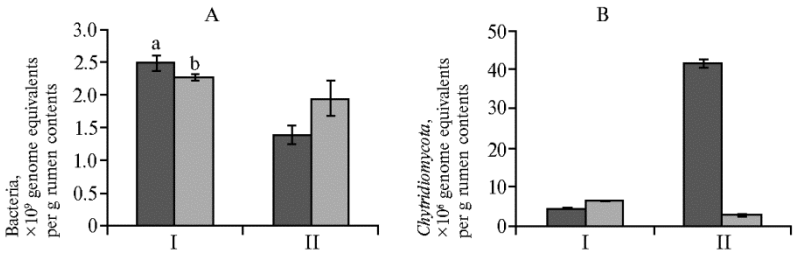

Fig. 4. The counts of microorganisms in rumen of adult reindeers (*Rangifer tarandus*) (I) and calves (II): A — bacteria, B — chytridiomycetes; a — clinically healthy animals, b — animals with clinical signs of necrobacteriosis (*M*±SEM, (Yamal-Nenets Autonomous Area, urban settlement Kharp, 2017).

Thus, in reindeer with manifestations of necrobacteriosis the composition of rumen microbiome is significantly disturbed compared to clinically healthy animals. The latter had more cellulose-decomposing and acid-utilizing bacteria and less fusobacteria and other pathogens (*Staphylococcus*, *Pseudomonas*, etc.). The rumen microbiome in calves with clinical signs of necrobacteriosis is characterized by more significant changes than in sick adults. These are expressed in greater heterogeneity of the bacterial community, increased representation of campylobacteria, enterobacteria, as well as a decrease in the number of chytridiomycetes. Therefore, the revealed regularities could be conditioned by physiological peculiarities of this stage of development in reindeer. The obtained results can be a base for recommendations on effective protection against reindeer necrobacteriosis and reducing mortality.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 754-766
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.52/.58:579.62

doi: 10.15389/agrobiology.2019.4.754eng
doi: 10.15389/agrobiology.2019.4.754rus

DIVERSITY AND ANTIBIOTIC RESISTANCE OF ENTEROBACTERIA ISOLATED FROM BROILERS IN A POULTRY FARM OF PERM KRAI: A 14-YEAR STUDY

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by the Government of Perm Krai (grant No. C-26/792)
Received January 31, 2019

Abstract

Sanitary and anti-epizootic measures in poultry industry should restrict spread and circulation of antibiotic-resistant bacteria, including pathogens which are the causative agents of epidemic diseases. This paper is our first report on prevalence and antibiotic resistance of enterobacteria in a commercial poultry flock population during a 14-year period with an assessment of the effectiveness of the most common fluoroquinolone- and colistin-containing veterinary medicines. Our goal was to compare species diversity and the antibiotic resistance of enterobacterial strains isolated from cross Ross 308 broiler chickens (*Gallus gallus*) in a large poultry complex (JSC PRODO Perm Poultry Farm, Perm Krai). Dead embryos and trachea, lungs, heart, liver, spleen, femur and tibia bones collected from slaughtered broilers were the pathomaterial (995 samples in 2004-2009, and 991 samples in 2010-2017). The organs of healthy broilers served as a control. In special experiments, we assessed the effectiveness of veterinary drugs used against certain pathogens. It was found that the frequency of *Enterobacteriaceae* family members remained stably high during the whole observation and significantly exceeded that of gram-positive microorganisms ($p < 0.00001$). In 2010-2017, bacterial contamination was higher in heart ($p < 0.05$) and spleen ($p < 0.01$), while the rate of infected dead embryos averaged 25 % and was lower ($p < 0.05$) compared to 2004-2009. *Escherichia coli* dominated, and *Proteus mirabilis* was a subdominant species. The prevalence of avian pathogenic *E. coli* (APEC) in the microbial community did not change, *Proteus* isolates ($p < 0.0001$) increased, and salmonella decreased ($p < 0.05$), the prevalence of other enterobacteria did not differ significantly. The prevalence of antibiotic-resistant *E. coli* was multidirectional: the ciprofloxacin-resistant strains increased from 47.4 to 75.9 % ($p < 0.0005$), whereas the amikacin-resistant strains decreased from 32.8 to 16.4 % ($p < 0.0001$). It was shown that Coliflox (NEC Agrovetszashchita SP, LLC, Russia) which contains maximum dosage of colistin and enrofloxacin is the most active among four studied colistin-containing medicines. Further down medicines are Vitrocil (Interchemie werken De Adelaar BV, Netherlands), which contains 10 times less colistin and 2 times less enrofloxacin, Pulmosol® (VIK — Animal Health LLC, Belarus) and Aquaprim (SP Veterinaria, SA, Spain) with 1.1 million and 400,000 IU colistin, respectively. Pulmosol® and Aquaprim, lacking of fluoroquinolones, showed the least activity (37.5 and 35.7 % of resistant *E. coli*, respectively, and 50.0 and 37.5 % of resistant *Proteus* spp.). Thus, combined veterinary medicines containing enrofloxacin had the highest activity against enterobacteria, but the effectiveness of these drugs has decreased in recent years. The long-term use of fluoroquinolones as dietary additives to prevent infections among poultry seems to be a risk factor leading to the selection of resistant strains.

Keywords: poultry farms, monitoring, enterobacteria, *Escherichia coli*, antibiotic resistance

Bacterial infections are second only to viral infections and cause significant damage to poultry production worldwide, as they can cause up to 35% mortality [1]. This problem is particularly acute due to the increase in food-borne diseases [2, 3]. Ensuring effective protection of poultry from infectious pathology has

been and remains one of the main tasks of veterinary medicine [4, 5].

The conditions of large poultry farms with high planting densities cause circulation among birds and people of different microorganisms, creating the risk of disease outbreaks. Animals can be a source of pathogenic and conditionally pathogenic bacteria — *Salmonella enterica*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* [6-9]. As a rule, gram-negative bacteria cause an acute course of diseases. The same chemicals are used for their treatment and prevention for a long time. This leads to the spread of antibiotic-resistant strains of microorganisms, primarily enterobacteria, which become the main depot of antibiotic-resistance genes. *E. coli* is already almost completely insensitive to tetracyclines and nalidixic acid [10]. Koga et al. [11] revealed a significant number of *E. coli* isolates producing extended-spectrum β-lactamases (*ESBL*). In salmonella isolated from birds, multiple resistances to antibacterial drugs is often found [8, 12, 13]. The fluoroquinolone-resistant species of enterobacteria circulating in poultry enterprises have been described [14, 15].

It is known that bacteria infectivity, species and antibiotic resistance of the livestock vary considerably from farm to farm. Control of bacterial pathogens is necessary to assess the epizootic situation in poultry farms and to select active antibacterial agents. In addition, since most of the antibiotics used in the poultry industry are also used in medicine, it is of interest to analyze the prevalence and resistance profiles of enterobacteria found in poultry farms.

This study presents for the first time in the comparative aspect the data of the long-term monitoring of the prevalence and antibiotic resistance of enterobacteria with the complex evaluation of the effectiveness of the main veterinary drugs containing fluoroquinolones and colistins. It has been established that the isolation rate of *Enterobacteriaceae* bacteria from bird organs was much higher than that of gram-positive microorganisms, and the number of generalized forms of colibacillosis increased. Enrofloxacin-containing preparations were the most active against enterobacteria, but as a result of long-term use for antibacterial therapy in industrial poultry production facilities, their effectiveness was reduced.

The aim of the work was to study species diversity and carry out a comparative assessment of antibiotic resistance of enterobacterial strains isolated from broiler chickens in a large poultry complex.

Techniques. Pathological material (trachea, lungs, heart, liver, spleen, bone tissue − femoral and tibial bones) were collected from broiler chickens (*Gallus gallus*) (Ross 308 cross; PRODO Perm Poultry Farm JSC, Perm Region) after slaughter and from died (not hatched) chickens. A total of 995 samples were tested in 2004-2009 [16] and 991 samples in 2010-2017. The control was the organs of healthy birds. The biomaterial was selected monthly. The age of the birds varied from 1 to 34 days and averaged 19.7±4.4 days. In addition, water samples from the drinking system were analyzed (as a likely source of birds' infection).

In bacteriological analysis, the biomaterial collected aseptically was used for plating by Gold's method on MacConkey medium (Sigma, USA) and blood agar. The isolated pure cultures were identified to a species using the diagnostic systems Entero-Test16 and NefermTest24 (Lachema, Czech Republic). The sensitivity of isolated strains to antibacterial drugs was determined by the disc-diffusion method according to MG (methodological guidelines) 4.2.1890-04 using Muller-Hinton agar (Merk, USA) and commercial discs manufactured by NITsF LLC (St. Petersburg, Russia) or HiMedia Laboratories Pvt. Ltd. (India) containing the following antibiotics (μg): ampicillin — 10, amoxicillin-clavulanate — 20/10, cefotaxime — 5, meropenem — 10, gentamicin — 10, amika-

cin — 30, ciprofloxacin — 5, levofloxacin — 5, chloramphenicol — 30, tetracycline — 30, nalidixic acid — 30 and furazolidone — 50. The *Escherichia coli* reference strain ATCC 25922 served as an internal control.

In a series of special experiments, the effectiveness of veterinary drugs used in the specified farm was determined in relation to some pathogens of bacterial diseases of birds. The following drugs were used: Aquaprim (SP Veterinaria, S.A., Spain), Vitrocil (Interchemie werken De Adelaar B.V., Netherlands), Koliflox (LLC NEC Agrovetzashchita S-P., Russia), Pulmosol® (LLC VIC − Animal Health, Belarus), Triflon (LLC Vitavet, Russia), Ciprovet (LLC NEC Agrovetzashchita S-P., Russia), Enroflon (LLC VIC — Animal Health, Belarus). All preparations are included in the Register of Medicines and Feed Additives for Animals approved for use in Russia, belong to hazard class III or IV according to GOST 12.1.007-76 (except for Triflon, hazard class II) and are assigned to broiler chickens with therapeutic and preventive purposes. Their effectiveness was evaluated by serial dilutions with regard of the working dose of the drug as per the manufacturer's instructions.

Statistical processing of the obtained data was carried out using Microsoft Excel 2016 and STATISTICA 10 software (StatSoft, Inc., USA). The indicators are presented as the arithmetic mean and its error ($M \pm SEM$). To identify statistically significant differences between the two independent samples, χ^2 or χ^2 with the correction of Yates (Frank Yates) was determined.

Results. During our observation, the isolation of gram-negative microorganisms from the organs of broiler chickens after forced slaughter remained high (Fig. 1). In 2012-2014, there was a slight decrease in the isolation rate, but since 2015 it again exceeded 50% of all the samples studied. Despite the fact that in recent years there has also been an increase in poultry infection with gram-positive microorganisms, in 2010-2017 the samples with gram-negative bacteria (59.0%) significantly exceeded the same indicator for gram-positive bacteria (14.4%) ($p < 0.00001$). In general, in 2004-2009 and 2010-2017, the average infection rates of poultry with bacterial microflora did not differ significantly (56.9±2.2% and 59.0±13.3%, respectively). The infection of addled eggs at the second stage of the study was 25% and was lower than that found in 2004-2009 ($p = 0.0462$).

In water from the drinking system, enterobacteria were isolated in more than 80% of cases in both observation periods.

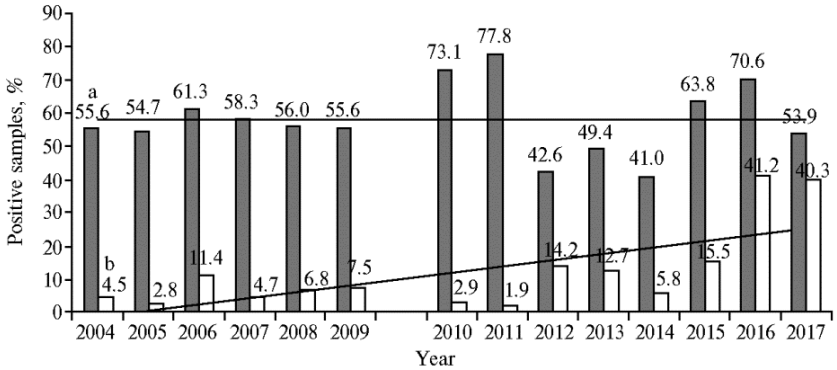

Fig. 1. Microbial contamination of the internal organs of broiler chickens (*Gallus gallus*) of the Ross 308 cross with gram-negative (a) **and gram-positive** (b) **bacteria in 2004-2009 [16] and in 2010-2017** (PRODO Perm Poultry Farm JSC, Perm Region). The straight lines reflect trends in representation for each group of microorganisms.

Infection with gram-negative bacteria of trachea, lungs, heart, liver,

spleen, and broiler bone reached, 31.2%; 73.1%; 64.3%; 62.5%; 65.7% and 32.3%, respectively (Table 1). Bacteria were isolated less often from bone tissue than from parenchymal organs and lungs ($p < 0.0001$). There were no statistically significant differences between infection of the heart, liver, and spleen. The trachea was infected less than the lungs ($p < 0.0005$), which turned out to be the most bacterized of all the studied material. Compared to 2004-2009, bacterial contamination of the heart ($p < 0.05$) and spleen ($p < 0.01$) were significantly higher.

1. Contamination by gram-negative bacteria of the internal organs of cross Ross 308 dead embryos, broiler chickens (*Gallus gallus*) and water from the drinking system in different years (PRODO Perm Poultry Farm JSC, Perm Region)

Source	No.	Organ, biotope	2004-2009 [16]		2010-2017	
			n	positive, *n* (%)	*n*	positive, *n* (%)
Broilers	1	Trachea	–	–	16	5 (31.2 %)
	2	Lungs	6	5 (83.3 %)	104	76 (73.1 %) $p_{1-2} < 0.0005$
	3	Heart	264	143 (54.2 %)	244	157 (64.3 %)
	4	Liver	276	186 (67.4 %) $p_{3-4} < 0.01$	307	192 (62.5 %)
	5	Spleen	180	90 (50 %) $p_{4-5} < 0.01$	134	88 (65.7 %)
	6	Bone	–	–	155	50 (32.3 %) $p_{2-6} < 0.0001$ $p_{3-6} < 0.0001$ $p_{4-6} < 0.0001$ $p_{5-6} < 0.0001$
	7	Kidneys	–	–	12	0
	8	Ovary	110	64 (58.2 %)	–	–
	9	Other organs	10	6 (60 %)	3	2 (66.7 %)
	10	All organs	846	494 (58.4 %)	975	570 (58.5 %)
Embryos	11		149	75 (50 %)	16	4 (25 %) $p_{10-11} < 0.05$
Drink system	12	Water	142	124 (87.3 %)	15	13 (86.6 %)

N o t e. Dashes indicate a lack of data for the specified period. The subscripts at p are the numbers of the compared samples.

As expected, the infection of the birds by *E. coli* prevailed throughout the investigation, averaging 43.4±7.7% and 53.8±12.4%, respectively, for 2004-2009 and 2010-2017 (see Fig. 2, A). Among enterobacteria, *Escherichia* comprised 63.3% in 2004-2009, 69.6% in the subsequent period, that is, did not significantly differ (see Fig. 2, B). *Proteus* spp. (*Proteus mirabilis*, *P. vulgaris*, etc.) varied from 7.4% in 2006 to 45.7% in 2016 and averaged 22.8% over the past 8 years, which was almost 2 times more than in 2004-2009 (12.2%, $p < 0.0001$). The Salmonella infection rate was high (26.6%) in 2009 with a significant proportion of isolates of embryonic origin. In 2010-2017, they were significantly less than in the previous period. In general, for 2010-2017, the species diversity of enterobacteria isolated from poultry after forced slaughter did not change significantly compared to 2004-2009, while we found a significant increase in the counts of the *Proteus* genus representatives, a tendency to an increase in *Escherichia* infection and, which is essential, a decrease in the frequency of salmonella, including in embryonic material.

Since *E. coli* and *Proteus* spp. were the dominant bacteria throughout the observation period, we assessed the antibiotic sensitivity of these microorganisms using the disk diffusion method. A total of 511 strains of *Escherichia* and 172 strains of *Proteus* were analyzed. When choosing the tested antibacterial drugs, both the duration of their use in veterinary practice and the species characteristics of the isolates were taken into account. The strains of *E. coli* resistant or conditionally resistant to ampicillin and cefotaxime among the strains isolated in 2010-2017 comprised 74.1% and 17.8%, respectively (Table 2). In the first stage of the study (in 2004-2009), all bacteria were sensitive to meropenem. Amikacin

resistance ranged from 5.9% to 34.9% and averaged 16.4% over 8 years, which is statistically significantly lower than in the previous period ($p < 0.0001$). Similar trends were found for gentamicin: in 2010, the gentamicin-resistant *E. coli* strains reached almost 60.0%, and in 2017 only 6.2%. Bacterial resistance to ciprofloxacin, on the contrary, statistically significantly increased to 75.9% in the second period of the study ($p < 0.0005$). The number of strains of *E. coli* resistant to levofloxacin was lower, but they still accounted for about half of the cultures. Similar trends were observed for *Proteus*: a significant portion of strains resistant to ampicillin (42.5%) and fluoroquinolones, the ciprofloxacin (36.8%), and norfloxacin (33.4%), were detected, while resistance to gentamicin and amikacin declined (20.2% and 2.1%, respectively). Almost all cultures of the studied enterobacteria were insensitive to furazolidone, tetracycline, and nalidixic acid.

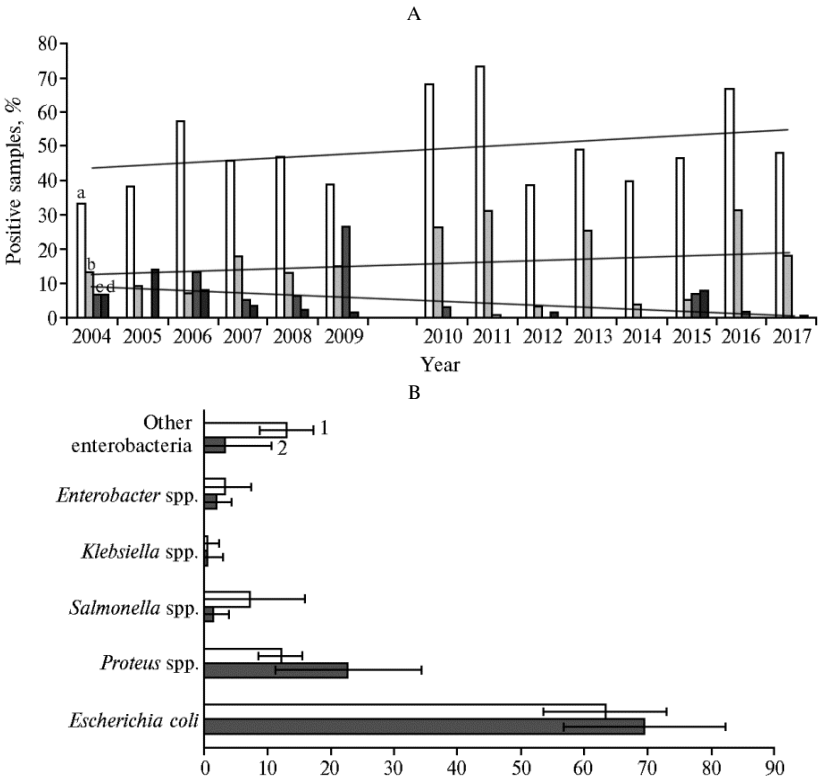

Fig. 2. Contamination by *Enterobacteriaceae* of Ross 308 (A) **cross broiler chickens *(Gallus gallus)* and specific weight** (%) of ***Enterobacteriaceae*** (B) **in different years:** a — *Escherichia coli*, b — *Proteus* spp., c — *Salmonella* spp., d — другие энтеробактерии; 1 — 2004-2009 [16], 2 — 2010-2017. *Proteus* spp. comprised *P. mirabilis*, *P. vulgaris*; *Salmonella* spp.comprised *Salmonella* serovar Enteritidis, *S. tiphimurium*, *S. arizonae* etc.; *Klebsiella* spp. comprised *K. pneumoniae*, *K. oxytoca*; *Enterobacter* spp. comprised *E. sakazakii*, *E. aerogenes*, *E. cloacae*; enterobacteria comprised *Escherichia vulneris*, *Citrobacter freundii*, *Morganella morganii* (PRODO Perm Poultry Farm JSC, Perm Region). The straight lines reflect trends in representation for each group of microorganisms.

2. Antibiotic resistance of *Escherichia coli* isolates (absolute number of resistant isolates/%) **from the internal organs of Ross 308 cross broiler chickens (*Gallus gallus*) in different years (**PRODO Perm Poultry Farm JSC, Perm Region)

Ampicillin	2004-2009 (n = 390) [16]	2010-2017 годы (n = 511)
Amoksiklav	321/82.3	379/74.1
Cefotaxime	181/46.4	–
Meropenem	87/22.3	91/17.8
Gentamicin	0	0
Amikacin	212/48.9	54/10.6*
Ciprofloxacin	128/32.8	84/16.4*

Table 2 continued

Levofloxacin	185/47.4	388/75.9*
Chloramphenicol	–	221/43.2
Tetracycline	208/53.3	–
Nalidixic acid	385/98.7	443/86.7
Furazolidone	–	459/89.8
Ampicillin	359/2.1	476/93.1

N o t e. Dashes indicate a lack of data for the specified period.
* Differences with the previous period are statistically significant at $p < 0.05$.

3. Resistance of *Escherichia coli* and *Proteus* spp. isolates from the internal organs of Ross 308 cross broiler chickens (*Gallus gallus*) to some drugs (PRODO Perm Poultry Farm JSC, Perm Region)

Drug (working dose), active substance	Amount of active substance	Strians, *n*/%					
		E. coli		*Proteus* spp.		enterobacteria	
		total	resistant	total	resistant	total	resistant
Aquaprim (1 ml/l):							
lincomycin	50 mg						
sulfamethoxol	200 mg						
trimethoprim	20 mg						
colistin	400,000 IU	16	6/37.5	6	3/50.0	22	9/40.9
Vitrocil (0.5 ml/l):							
enrofloxacin	50 mg						
colistin	600,000 IU	24	8/33.3	11	2/18.2	35	10/28.6
Coliflox (1 ml/l):							
enrofloxacin	100 mg						
colistin	7 million IU	12	1/8.3	5	1/20.0	17	2/11.8
Pulmosol® (150 g/l):							
kitasamycin	52.5 mg						
colistin	1.1 million IU	42	15/35.7	16	6/37.5	58	21/36.2
Triflon (1 ml/l):							
enrofloxacin	100 mg						
trimethoprim	50 mg	5	0	2	1/50.0	7	1/14.3
Ciprovet (1 g/l):							
ciprofloxacin	100 mg	39	6/15.3	18	4/22.2	57	10/17.5
Enroflon (1 g/l):							
enrofloxacin	100 mg	53	8/15.1	25	3/12.0	78	11/14.1

N o t e. Dilutions (working doses) is given according to the manufacturer's instructions. Data for *Salmonella* of Enteritidis serovar are not presented.

In a special series of experiments, we assessed the effectiveness of seven veterinary drugs against the main pathogens of bacterial infection of birds, *E. coli*, *Proteus* spp. and *Salmonella* of Enteritidis serovar. Of the four studied colistin-containing drugs, Coliflox, represented by a combination of colistin at the maximum dose and enrofloxacin, had the highest activity (Table 3). Vitrocil which contained 10 times less colistin and 2 times enrofloxacin, Pulmosol® and Aquaprim (the dose of colistin 1.1 million IU and 400,000 IU respectively), followed in descending order. The last two preparations that do not have fluoroquinolones in their composition showed the lowest activity: 37.5% and 35.7% of *E. coli* cultures, 50% and 37.5% of *Proteus* spp. cultures were resistant to them.

Mono-preparations containing fluoroquinolones suppressed the growth of more than 80% of all tested enterobacteria. Enroflon was not statistically significant, but more effective than Ciprovet, primarily due to the effect on *Proteus* strains. At the same time, Triflon, in which trimethoprim is also present, was less active against *Proteus* than Enroflon, but this may be due to a smaller sample of cultures. All cultures of *Salmonella* of Enteritidis serovar ($n = 3$) were sensitive to the studied combined veterinary drugs in a working dose. It should be noted that in most cases, bacterial growth was inhibited when diluting drugs in a dose less than 2 times lower than the working one, for example, when testing Coliflox (data not shown). In this case, the growth of both cultures resistant to Coliflox in the working dose was inhibited by the drug added in a double dose. Similar data were obtained for the remaining tested drugs. Enterobacteria were

also resistant to increased concentration of the drug, and their number increased in dynamics: for Aquaprim there were 7 of 9 such cultures (77.7%; 1 culture in 2015 and 6 in 2017), for Vitrocil — 6 out of 10 (60%; all cultures were identified in 2017), for Pulmosol® — 11 out of 21 (52.4%; 3 cultures in 2015 and 8 cultures in 2017), for Ciprovet — 5 out of 11 resistant cultures (45.4%; all cultures highlighted in 2017).

As per annual medical statistics, bacteria of the genera *Salmonella*, *Escherichia*, and *Campylobacter* are the most common causative agents of anthropozoonoses that are associated with poultry products. The industrial maintenance of the poultry serves as the powerful stress by itself modulating reactions of the congenital and induced immunity, reducing the resistance of birds even to own microbiota that creates preconditions for the influence of bacteria on an organism and fast distribution of pathogens of diseases. In recent years, the circulation of opportunistic and pathogenic microorganisms that can cause human food poisoning has increased in poultry farms [17-19]. The need to study the dynamics of the spread of antibiotic resistance in representatives of the *Enterobacteriaceae* family is not in doubt.

It is known that in specialized poultry farms of Russia, the death of birds from coli infection remains high for many years [20]. Thus, according to the All-Russian Scientific Research Veterinary Institute of Poultry (St. Petersburg), the specific rate of *E. coli*, isolated in recent years from various species of birds in several regions of Russia, is at least 40% [21]. Microbiological monitoring at 11 poultry farms in Western Siberia established the dominance of *E. coli* among gram-negative microorganisms (48%). Representatives of the *Citrobacter* and *Proteus* genera were isolated only in 13% and 6% of cases [22]. In several poultry farms in Ukraine, *Escherichia* amounted to 30.8% in the microbiota of dead birds [23].

The most important problem for all commercial poultry is systemic coli infection, as well as respiratory diseases such as aerosacculitis [20, 24]. In a multicenter monitoring study conducted by Kalinin et al. [25], it was shown that in bird respiratory syndrome, a gram-negative flora was isolated in most cases (*E. coli* — 36.5%, *P. vulgaris* — 11.7%, *P. aeruginosa* — 5.8%, *Salmonella* of Enteritidis serovar — 3.9%). In this study we revealed the *E. coli* infection to prevail, and the rate of *Escherichia* among enterobacteria was stably higher than 60%. A predominant proportion of *E. coli* was observed in all organs and tissues. *E. coli* was isolated from the heart in 80.3% of cases, which coincides with the fact that fibrinous pericarditis is the most typical sign of colibacteriosis [21].

During the incubation and rearing of birds, the counts of microorganisms and diversity of microflora increases, reaching a maximum during hatching [22]. Indeed, we found the embryos to be less infected than broiler chickens. Earlier, during a 6-year observation of the development of birds in dynamics from days 1 to days 43-45, we showed that infection is determined by the age of the bird [16]. If in 1-day-old chickens the internal organs were practically sterile, then by day 40 their bacterial contamination reached 78.3%.

The problem of antimicrobial resistance over the past 20 years has gained particular importance in connection with the intensification of agriculture. The 68th World Health Assembly adopted the Global Plan of Action to Reduce Antimicrobial Resistance (resolution WHA68.7 dated May 26, 2015). A comprehensive and intersectoral approach, which provides for a system to control the circulation of antibiotics, is proposed for industries where they are actively used, including for agriculture.

Our results indicate that over the years, the antibiotic resistance of

E. coli strains at the Perm Territory's poultry farm to some drugs has varied widely, while to other drugs has remained relatively stable. It should be noted that since 2011, antibiotics have not been added to feed. Thus, the resistance of the strains to ampicillin, tetracycline, furazolidone and nalidixic acid remained almost unchanged, and resistance to these drugs can be considered as a fixed sign, which, apparently, is associated with the prolonged use of these chemotherapeutic agents in the poultry farm. Nevertheless, it was possible to identify the dynamics of changes in the resistance of strains to aminoglycosides and fluoroquinolones. In the first case, the number of resistant strains isolated from birds decreased statistically significantly, which is probably due to the complete cessation of the use of these drugs since 2013, which contributes to the elimination of such strains from the ecosystem. The results obtained indicate that antibiotic rotation can be effective in controlling strain resistance.

Resistance to fluoroquinolones, on the contrary, has increased significantly, which may be associated with prolonged use of monocomponent and complex fluoroquinolone-containing preparations (Enroksil, Ciprovet, Enroflon, Hydroquinocol, Coliflox, Quinoprim, Triflon). The active substance of most of them is enrofloxacin, which is partially metabolized in the liver to ciprofloxacin and inhibits bacterial DNA gyrase and topoisomerase IV. At the same time, fluoroquinolone-containing preparations showed the greatest antimicrobial activity (as assessed by the serial dilution method), although their effectiveness began to gradually decrease.

Our data on the effectiveness of a number of drugs are consistent with studies by other authors. In the article of Miles et al. [10], it was found that 82.4% strains of *E. coli* isolated from chickens were resistant to tetracycline, 85.3% to nalidixic acid, and only 8.8% to ciprofloxacin. Moreover, almost 30% strains were stable and about 40% were moderately resistant to enrofloxacin. In another work, the sensitivity tests of enterobacteria to 15 drugs showed that aminoglycosides, neomycin, and gentamicin were effective, and most of the *Escherichia* were resistant to other drugs, including the fluoroquinolone series (enroflox, enroflon) [19]. Thorsteinsdottir et al. [26] found that enrofloxacin-resistant *E. coli* strains were found in 33.6% of cases when screening for isolates from broiler chickens and in 52% when screening for food products (broiler meat). In Iran, among 318 APEC strains (avian pathogenic *E. coli*) isolated from broiler chickens with generalized coli infection, 37.7% were resistant to enrofloxacin, but only 7.5% were resistant to ciprofloxacin and 5.7% to gentamicin [27]. According to the verification of veterinary drugs in the framework of microbiological monitoring at poultry farms in Ukraine, a high bactericidal activity of fluoroquinolones, the enroxil and sarafloxacin, was revealed [22]. An observation at federal poultry processing plants in Canada showed that in chicken herds all *E. coli* strains were sensitive to amicacin, ciprofloxacin and enrofloxacin, but it should be noted that these data were obtained in 2003-2004 [28].

Consequently, the trend towards an increase in the number of antibiotic-resistant strains of bacteria is characteristic of different countries. A connection has been shown between the use of specific drugs in poultry enterprises and the resistance of circulating bacteria to them [28]. Most reports indicate almost complete resistance of APEC to tetracycline, mediated by the *tetB* and *tetD* efflux-genes which are often associated with conjugative plasmids [10]. At the same time, sensitivity to aminoglycosides remains rather high. The data regarding quinolones and fluoroquinolones are contradictory: in a large part of the studies, high resistance to nalidixic acid is described, but the rate of strains resistant to ciprofloxacin and enrofloxacin can vary significantly. It has been proven that isolates resistant to fluoroquinolones can circulate even in the absence of

further drug exposure, and therefore some countries have abandoned the use of fluoroquinolones in poultry farming [29]. The issue of the prospect of using colistin (the polymyxin group) is being actively discussed: first, because of the increasing bacterial resistance due to the spread of the *mcr-1* gene the treatment of colibacteriosis and salmonellosis in animals may be ineffective, and second, in medicine it is necessary to maintain the effectiveness of the drugs used for combating pathogens with multidrug resistance [30]. Providing systematic monitoring of the spread of antimicrobial resistance has become part of the program "The Strategy for Preventing the Distribution of Antimicrobial Resistance in the Russian Federation until 2030" (Decree of the Government of the Russian Federation No. 2045-r dated October 25, 2017).

Thus, this paper represents results of monitoring enterobacteria in the microbiota of broiler chickens in a large poultry farm in the Perm Territory. It was found that the rate of enterobacteria isolated from poultry organs during all the observation periods remained stably high and significantly exceeded the corresponding indicator for gram-positive microorganisms. In 2004-2009 and 2010-2017, the average contamination of poultry organs with gram-negative bacteria did not statistically significantly differ (56.9±2.2% and 59.0 ± 13.3%, respectively). Lungs, heart, liver, broiler spleen were contaminated in more than 50% of cases, trachea and bone tissue in every third bird. Apparently, the bacterial contamination of various organs of industrial poultry is associated with the generalization of the infectious process typical of broilers, which is due to reduced immunological resistance because of the peculiarities of housing conditions. The proportion of APEC strains (avian pathogenic *Escherichia coli*) in the total microbial composition did not change, while the *Proteus* strains increased and salmonella decreased, the frequency of other representatives did not differ significantly. The revealed trends in the prevalence of antibiotic-resistant *E. coli* were multidirectional. The number of ciprofloxacin-resistant strains increased, and the rate of amikacin-resistant decreased. Antibiotic resistance of bacteria (bacterial factor) and immunological suppression (macroorganism factor) contributed to the generalization of coli infection: indicators of bacterial contamination of the heart and spleen were significantly higher compared to the previous period. Of the studied veterinary antibacterial drugs, Coliflox, the combination of colistin and enrofloxacin, has the greatest activity against enterobacteria circulating in the enterprise. Despite the fact that the effectiveness of fluoroquinolone-containing preparations has been decreasing in recent years, the rejection of their use and replacement with others is apparently inadvisable today. The data obtained confirm the need for systematic monitoring bacterial pathogens in poultry enterprises and their antibiotic resistance, which will allow adequate and effective use of antimicrobials in veterinary practice.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 767-776
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Physiology of stress

UDC 636.92:619:591.1:616-092.9

doi: 10.15389/agrobiology.2019.4.767eng
doi: 10.15389/agrobiology.2019.4.767rus

DYNAMICS OF OXIDATIVE STATE INDICATORS IN RABBITS (*Oryctolagus cuniculus* L.) UNDER SIMULATED TECHNOLOGICAL STRESS AND ITS PHARMACOLOGICAL CORRECTION

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The authors declare no conflict of interests
Received August 6, 2018

Abstract

Stress is the most important livestock problem, causing great damage to the industry. The emergence of technological stress contributes to a large number of factors, from transportation to conditions of keeping and feeding. The development of pathological processes under stress intensifies free radical processes in the body, with the excessive formation of free radicals. Therefore, there is a need in drugs based on substances with high antioxidant activity to pharmacologically correct technological stress in farm animals. In our experiment, we simulated conditions of technological stress in Soviet chinchilla rabbits aged 6-7 months by immobilization. Antioxidant and anti-stress drugs developed at Stavropol State Agrarian University were used as agents. Group 1 of animals was control. Rabbits of group 2 received Drug to correct stress in farm animals (Patent RU 2428992 of 09.20.11), group 3 received Mebisel (Patent RU 2418579 of 05.20.11), these drugs have a pronounced anti-stress effect; group 4 received Antioxidant preparation for animals (Patent RU 2435572 of 12.10.11) and group 5 received Polyoxidol (Patent RU 2538666 of 01.10.15), the antioxidants. Blood levels of cortisol, thyroxine, lipid peroxidation and antioxidant protection were assessed. It was shown that immobilization of experimental animals provokes a significant production of cortisol (5,8 times higher) and a decrease in the thyroxine level up to 60,9 % ($p \le 0,01$), the blood concentration of diene conjugates increases 2.6 times ($p \le 0,01$), malondialdehyde by 55,8 % ($p \le 0,01$) and fluorescent Schiff bases 2,2 times ($p \le 0,01$). The restricted mobility adversely affectes the activity of antioxidative defence enzymes, with a significant decrease in glutathione peroxidase activity (by 35.2 %), superoxide dismutase (by 36.4 %), catalase (by 40.7 %) ($p \le 0.01$) and the content of reduced glutathione (by 33.3 %, $p \le 0.01$). Administration of antioxidant and antistress preparations contributes to the normalization of the studied parameters in experimental animals, the values of which during the experiment were statistically significantly different from the data recorded in the control group. In the dynamics of activity of antioxidant enzymes and products of lipid peroxidation, there were significant differences between the indices of animals from the control group and rabbits which received preventive agents. The animals of the control group showed a progressive increase in the concentration of lipoperoxides and a decrease in the activity of glutathione peroxidase, superoxide dismutase, catalase, and reduced glutathione. The use of antioxidant and antistress drugs three days before immobilization contributed to the optimization of these indicators. The applied prevention regimens allowed reduction of negative impact of stress, which resulted in statistically significant differences in the numerical values of the results of the laboratory blood test of animals from the groups 2, 3, 4 and 5 conoared to the control. At the end of the experiment glutathione peroxidase was 48.2-107.4 % higher ($p \le 0.01$), superoxide dismutase 31.1-85.9 % higher ($p \le 0.01$), catalase 12.9-40.1 % higher ($p \le 0.05$ in groups III, IV and V), while glutathione was 34.8-60.8 % lower ($p \le 0.01$), thyroxine 27.2-82.7 % lower ($p \le 0.05$). The cortisol level declined by 83.5-207.0 % ($p \le 0.01$), diene conjugates by 37.2-84.3 % ($p \le 0.01$), malondialdehyde by 26.1-46.9 % ($p \le 0.05$), and fluorescent Schiff bases by 22.03-118.1 % ($p \le 0.05$). The use of drugs accelerates post-stress adaptation, which was expressed in an increase in the average daily weight gain of rabbits from experimental groups, i.e. 28 g for group 2, 34 g for group 3, 36 g for group 4, and 38 g for group 5 compared to 24 g for the control group. Our results on the stress-born hormone dynamics are indicative of significant changes in the antioxidant defense system functioning and lipid peroxidation. These data allow us to recom-

mend the developed tranquilizers and antioxidants for physiological correction of technological stresses in animals.

Keywords: *Oryctolagus cuniculus* L., rabbits, technological stress, immobilization, antistress agent, antioxidant preparation, antioxidant system, lipid peroxidation, hormones, enzymes

Stress is one of the most important factors disturbing homeostasis in animals and humans. Biologically active substances balance changes under the influence of stress reaction leading to the development of pathologies. Many researchers agree that the imbalance of antioxidant and pro-oxidant processes is one of the first manifestations of metabolic disorders under stress [1-4]. The stress problem in animal husbandry is extremely acute. Stress leads to a decrease in productivity and in the quality of products, an increase in animal morbidity, a decrease in the rate of reproduction and, as a consequence, the profitability of animal husbandry [5-7]. Animal welfare is important not only in the context of humanizing human activities but also in terms of economic benefits and therefore attracts the attention of researchers and practitioners.

Unfortunately, it has to be stated that in animal husbandry technological stress accompanies almost all production process. It occurs during animals' transportation, during feeding and care operations, can be observed at sharp changes in diets and maintenance conditions, its development can be affected by changes in the microclimate and many other factors [8-10]. In addition to technological stress, animals experience physiological stress due to the most intense periods of exploitation, such as pregnancy and parturition [11, 12].

Normally, free radical oxidation ensures normal cell functioning and metabolic processes in the cell [13, 14]. Pathological changes in these processes trigger the mechanism of chain lesions of cells and tissues due to the ability of free radicals to disrupt the structure and integrity of biological membranes [15-17]. The intensification of free-radical reactions out of control of the antioxidant protection system is the most probable mechanism of organism damage under stressful load [18, 19].

Currently, animal husbandry is focused on improving the welfare of livestock. However, due to the need to increase production at minimal cost, it is not always possible to modify technologies to reduce the number of stress factors and their impact [20]. Consequently, the pharmacological prevention of stress-related disorders remains appropriate. The mode of action of modern means and methods of correction of the changes caused by the negative influence of stress factors on the body must be effective and safe [21, 22].

In the present study, based on the assessment of the state of the antioxidant protection system, cortisol and thyroxine status in the simulation of technological stress, we have shown for the first time the anti-stress effect of the developed antioxidant and anti-stress drugs.

The aim was to investigate the influence of experimental stress on the dynamics of free-radical oxidation and antioxidant protection in rabbits under correction by complex antioxidant and anti-stress drugs.

Techniques. According to the principle of analogs, 6 groups of Soviet Chinchilla rabbits (*Oryctolagus cuniculus* L.) aged 6-7 months (20 animals per group) were formed. Technological stress was simulated by placing the test animals in specially manufactured modules of 0.12 m^2 for 5 days (immobilization with space limitation). Group II rabbits were intramuscularly injected with a drug for correction of stress states in farm animals (anti-stress drug) [23] at a dose of 3.9 mg/kg of live weight (by active substance) 3 days and 1 hour before immobilization, Group III used Mebisel [24] at a dose of 6.0 mg/kg, Group IV

used antioxidant agent for animals [25] at a dose of 5.4 mg/kg, and Group V used Polyoxidol at a dose of 5.0 mg/kg [26]. No drugs were used in Group I (control group). All used drugs are developed at the Department of Therapy and Pharmacology of Stavropol State University. The test drugs in Group II and Group III contain active substances with an anti-stress effect, in Group IV and Group V with an antioxidant effect.

Blood was taken from the auricular vein before drug administration, immediately before immobilization, in 1 day and 5 days after the beginning of stress simulation and in 5 days after the end of restriction of animal mobility, at the same time the animals were weighed. Blood cortisol and thyroxine concentrations, lipid peroxidation and antioxidant protection parameters were determined. Cortisol was measured on a Chem Well Combi automatic enzyme-linked analyzer (Awareness Technology, USA) with reagent kits (Hema LLC, Russia). The antioxidant protection parameters were determined as per the description [27]. A UNICO 2800 UV/VIS spectrophotometer (United Products & Instruments, Inc., USA) was used to measure the activity of catalase, superoxide dismutase, glutathione peroxidase, reduced glutathione content, and lipid peroxidation product concentrations.

The mean (*M*) and standard error of mean (±SEM) were calculated during data processing. The reliability of the differences was assessed by Student's *t*-test. Differences were considered statistically significant at $p \leq 0.05$.

Results. It is known that space-constrained immobilization is one of the strongest stressors [28-31]. The analysis of the data obtained in the laboratory blood test indicates that the immobilization stress simulated in rabbits leads to a multiple increase in the amount of cortisol and a decrease in the thyroxine production (Table 1). It was found that in animals not subjected to prophylactic treatment, the concentration of blood cortisol for 1 day in a limited space increased 5.8 times and remained high during the whole experiment. It should be noted that in the blood of animals injected with anti-stress and antioxidant drugs before the provocation of the stress response, cortisol also increased significantly, and the peak of such an increase was on day 1 of stressing. Even at its peak, it was less than in the control group, by 65.2% in Group II, by 44.7% in Group III, by 22.9% in Group IV, and by 29.5% in Group V; the difference between the groups in this and subsequent stages of the experiment was statistically significant ($p \leq 0.05$). After the cessation of the stress factor, the tendency to normalize the amount of cortisol in the blood was more pronounced in animals undergoing pharmacological preparation. In 5 days after the end of immobilization, rabbits in Group I had the highest values for this parameter (they were 1.8 times or higher than in other groups).

The use of anti-stress and antioxidant drugs contributed to an increase in the amount of blood thyroxine in rabbits (see Table 1). Three days after the drug introduction, the concentration of this hormone in animals of Group II increased by 42.3%, in Group III by 48.6%, in Groups IV and V by 9.1 and 31.1%, respectively. After the rabbits were moved to limited space, the amount of thyroxine in all groups decreased, in most of them by more than 50%. In blood samples taken 5 days after the end of immobilization, the hormone concentration increased in all groups. This indicator was statistically significantly lower in the control group, by 64.6% ($p \leq 0.01$) compared to Group II, by 82.7% ($p \leq 0.01$) compared to Group III, by 37.7 ($p \leq 0.01$) and 27.2% ($p \leq 0.02$), respectively, compared to Group IV and Group V. The dynamics of thyroxine indicates that stress response has a pronounced inhibitory effect on its synthesis, and the drugs used have a significant preventive effect.

1. Blood concentration of stress hormones and lipid peroxidation products in Soviet Chinchilla rabbits (*Oryctolagus cuniculus* L.) under simulation of immobilization stress (M±SEM, n = 20)

Group	Cortisol, nmol/l	Thyroxin, nmol/l	Diene conjugates, OD/mg lipids	Malone dialdehyde, μmol/l	Schiff bases, rel. units/ml
Before drug administration					
I	38.26±2.69	27.18±1.94	0.33±0.03	1.29±0.09	0.30±0.02
II	34.67±2.12	29.43±2.15	0.29±0.02	1.17±0.08	0.27±0.02
III	37.19±2.74	26.83±1.71	0.34±0.03	1.31±0.09	0.30±0.03
IV	35.72±2.44	33.62±1.98	0.31±0.02	1.24±0.08	0.31±0.02
V	39.12±2.81	28.52±2.03	0.34±0.03	1.32±0.09	0.28±0.02
Before immobilization					
I	40.73±2.56	25.44±1.58	0.34±0.03	1.32±0.09	0.31±0.02
II	22.21±1.70[a]	42.27±2.99[a]	0.27±0.02	1.19±0.08	0.27±0.02
III	24.96±1.94[a]	39.87±3.11[a]	0.30±0.02	1.28±0.09	0.31±0.02
IV	36.09±2.48[e]	36.70±2.78[a]	0.24±0.02[c]	1.20±0.09	0.29±0.02
V	38.47±2.61[e]	37.39±2.63[a]	0.21±0.02[d]	1.23±0.08	0.26±0.02
1 day after the beginning of immobilization					
I	238.23±16.89	13.47±1.00	0.62±0.05	1.57±0.11	0.35±0.03
II	144.19±11.17[a]	20.53±1.46[a]	0.46±0.04[a]	1.48±0.10	0.31±0.02
III	131.74±9.75[a]	23.66±1.59[a]	0.42±0.03[a]	1.43±0.09	0.33±0.03
IV	183.51±13.90[d]	17.32±1.34[c]	0.37±0.03[a]	1.25±0.08[a]	0.27±0.02[a]
V	167.91±12.07[c]	19.48±1.48[a]	0.33±0.02[d]	1.22±0.08[b]	0.26±0.02[a]
5 days after the beginning of immobilization					
I	181.14±12.93	9.93±0.72	0.87±0.07	2.01±0.15	0.67±0.05
II	74.60±5.21[a]	16.58±1.26[a]	0.62±0.05[a]	1.73±0.13	0.56±0.04
III	86.89±6.34[a]	18.21±1.33[a]	0.58±0.04[a]	1.59±0.11[a]	0.52±0.04[a]
IV	111.24±7.72[d]	12.97±0.93[d]	0.41±0.03[d]	1.41±0.10[a]	0.48±0.04[a]
V	100.76±7.14[b]	13.22±1.18[c]	0.45±0.03[d]	1.36±0.10[b]	0.41±0.03[b]
5 days after the immobilization completed					
I	149.12±11.65	11.84±0.89	0.59±0.04	1.88±0.14	0.72±0.05
II	48.57±3.65[a]	19.49±1.39[a]	0.43±0.03[a]	1.49±0.10[a]	0.59±0.04[a]
III	60.08±4.62[a]	21.63±1.70[a]	0.39±0.03[a]	1.42±0.10[a]	0.46±0.03[b]
IV	81.24±5.76[d]	15.06±1.12[d]	0.36±0.03[a]	1.31±0.09[a]	0.39±0.03[b]
V	76.43±5.53[d]	16.31±1.21[c]	0.32±0.02[b]	1.28±0.09[a]	0.33±0.03[d]

Note. [a] – between this group and Group I the difference is statistically significant; [b] – between this group, Group I and Group II the difference is statistically significant; [c] – between this group, Group I and Group III the difference is statistically significant; [d] – between this group, Group I, Group II and Group III the difference is statistically significant; [e] – between this group, Group II and Group III, the difference is statistically significant ($p \leq 0.05$).

Our findings indicate that the effect of the stress factor is accompanied by the intensification of lipid peroxidation, as evidenced by the change in the concentration of peroxidation products. The concentration of diene conjugates in the control group for the 5 days of immobilization increased 2.5 times. Under the conditions of 5-day immobilization, the index increased by 82.3% in animals from Group II that received the anti-stress drug, by 93.3% in rabbits that received Mebisel, by 70.8% in Group IV where another antioxidant drug was used, and more than 2-fold in Group V where Polyoxidol was administered. After immobilization, the concentration of diene conjugates decreased in all five groups, but in the control group, it was significantly higher ($p \leq 0.05$) than in the test groups.

Comparing the values for malondialdehyde (MDA) between the groups, it is worth noting that the highest content of this peroxidation product was in the control group throughout the experiment. In blood samples obtained 5 days after immobilization, the MDA concentration in Group I was 20.7% ($p = 0.16$) higher than in Group II, 24.5% ($p \leq 0.05$) higher than in Group III, 30.3% ($p \leq 0.05$) and 31.9% ($p \leq 0.05$) higher than in Group IV and Group V, respectively (see Table 1).

The dynamics of accumulation of fluorescent Schiff bases in the blood of animals from all groups practically did not change after 3 days from the drug administration. Differences began to appear in 1 day after the beginning of simulation of stress exposure. During the 5 days of immobilization, the concentration of

Schiff bases in Group I was 16.4% higher than in Group II ((p = 0.09), 22.4% higher than in Group III (p = 0.02), 28.4% higher than in Group IV (p ≤ 0.01), and 38.8% higher than in Group V (p ≤ 0.01) (see Table 1).

Assessing the dynamics of the enzymatic link of the antioxidant protection system, it should be noted that the use of antioxidant and anti-stress drugs has led to an increase in the activity of enzymes (Table 2). At the end of the experiment, the activity of glutathione peroxidase in Group I was 48.2% lower than in Group II (p ≤ 0.01), 76.8% lower (p ≤ 0.01) than in Group III, 2.0 times and 2.1 times lower than in Group IV and Group V, respectively. This difference can be explained by the fact that the active substances of all used drugs include a selenium-containing compound.

2. Antioxidant blood protection parameters and dynamics of bodyweight in Soviet Chinchilla rabbits (*Oryctolagus cuniculus* L.) under simulation of immobilization stress (*M*±SEM, *n* = 20)

Group	GPx, μmol G-SH/(l · min · 10^3)	SOD, units/mg hemoglobin	Catalase, μmol H_2O_2/(l · min · 10^3)	Reduced glutathione, mmol/l	Bodyweight, kg
Before drug administration					
I	7.39±0.53	4.72±0.36	24.13±1.78	0.31±0.02	3.58±0.26
II	8.43±0.64	5.11±0.42	23.27±1.51	0.35±0.03	3.44±0.23
III	7.87±0.57	4.93±0.39	23.79±1.82	0.33±0.03	3.61±0.29
IV	8.18±0.69	5.02±0.46	24.42±1.96	0.34±0.03	3.49±0.24
V	7.34±0.56	4.81±0.34	22.94±1.35	0.29±0.02	3.70±0.31
Before immobilization					
I	7.22±0.36	4.64±0.39	24.05±1.66	0.30±0.02	3.64±0.30
II	10.31±0.76[a]	5.63±0.58	23.89±1.47	0.35±0.03	3.42±0.28
III	11.13±0.69[a]	5.49±0.43	24.01±1.59	0.35±0.03	3.65±0.32
IV	12.41±0.88[a]	6.18±0.53[a]	26.95±1.91	0.37±0.03	3.57±0.29
V	12.93±0.82[b]	5.92±0.55	27.11±2.13	0.32±0.03	3.72±0.34
1 day after the beginning of immobilization					
I	5.07±0.42	3.22±0.25	19.47±1.28	0.26±0.02	3.37±0.21
II	10.56±0.71[a]	5.41±0.44[a]	21.60±1.43	0.30±0.02	3.31±0.29
III	13.22±0.94[b]	5.78±0.50[a]	22.11±1.52	0.32±0.02[a]	3.48±0.27
IV	14.49±1.09[b]	5.89±0.52[a]	24.76±1.73[a]	0.35±0.03[a]	3.34±0.31
V	15.01±1.03[b]	6.14±0.57[a]	25.31±1.80[a]	0.30±0.02	3.51±0.33
5 days after the beginning of immobilization					
I	4.68±0.34	2.95±0.23	14.53±1.12	0.20±0.02	3.09±0.24
II	9.69±0.67[a]	4.26±0.29[a]	16.82±1.20	0.27±0.02[a]	3.24±0.27
III	10.92±0.83[a]	4.71±0.33[a]	18.63±1.36[a]	0.30±0.02[a]	3.51±0.32
IV	12.45±0.88[b]	5.04±0.40[a]	21.26±1.49[b]	0.32±0.02[a]	3.22±0.25
V	12.95±0.96[b]	5.63±0.44[b]	23.15±1.42[c]	0.34±0.03[a]	3.45±0.29
5 days after the immobilization completed					
I	6.33±0.51	3.41±0.27	18.49±1.54	0.23±0.02	3.21±0.28
II	9.38±0.59[a]	4.47±0.32[a]	20.88±1.41	0.31±0.02[a]	3.38±0.31
III	11.19±0.82[a]	5.23±0.41[a]	24.31±1.89[a]	0.34±0.03[a]	3.68±0.32
IV	12.86±0.89[b]	5.79±0.38[b]	26.52±2.33[b]	0.36±0.03[a]	3.40±0.26
V	13.13±0.97[b]	6.34±0.52[b]	25.91±2.07[a]	0.37±0.03[a]	3.64±0.32

Note. GPx — glutathione peroxidase, SOD —superoxide dismutase.
[a] — the difference between this group and Group I is statistically significant; [b] — the difference between this group, Group I and Group II is statistically significant; [c] — the difference between this group, Group II and Group III is statistically significant (p ≤ 0.05).

The activity of blood superoxide dismutase significantly increased in animals that received antioxidant and anti-stress drugs, with a decrease in the control. The simulated technological stress led to a marked decrease in the activity of blood superoxide dismutase in rabbits from Group I (control). During 5 days of immobilization, the statistically significant differences were 44.4% (p ≤ 0.01) between Group I and Group II, 59.6% between Group I and Group III, 70.8% (p ≤ 0.01) between Group I and Group IV, and 90.8% (p ≤ 0.01) between Group I and Group V.

After the rabbits were returned to their usual conditions, their catalase activity normalized, but in the control group this indicator was much lower than in other groups, the difference with Group II was 12.9% (p = 0.25), with Group

III was 31.5% ($p \leq 0.02$), with Group IV was 43.4% ($p \leq 0.01$), and with Group V was 40.1% ($p \leq 0.01$) (see Table 2).

Glutathione is one of the most important factors of antioxidant protection [32, 33] and critical markers of its functioning [34, 35]. After 5-day immobilization of rabbits, the concentration of reduced glutathione in Group I was 35% lower than in Group II ($p \leq 0.02$), 50% lower ($p \leq 0.01$) compared to Group III, 60% ($p \leq 0.01$) and 70% ($p \leq 0.01$) lower compared to Group IV and Group V, respectively (see Table 2).

The simulated technological stress had a negative impact on the dynamics of the rabbits' bodyweight. The use of antioxidant and anti-stress drugs accelerated post-stress adaptation, resulting in an increase in the average daily bodyweight gain of animals. Over 5 days since the cessation of restriction of mobility, the average daily gain of rabbits was 24.2 g in Group I, 28.4 g in Group II, 34.1 g in Group III, 35.6 g in Group IV, and 38.3 g in Group V.

So the developing stress response appears as a sharp increase in the blood cortisol level, up to 238.23±16.89 nmol/l, with the maximum values on day 1 of stressing. Based on this, it can be assumed that day 1 is the most critical for the course of the pathological process during immobilization stress in animals. Also, at technological stress during the whole period of its influence, there is a decrease in the concentration of thyroxine from 27.18±1.94 to 9.93±0.72 nmol/l. The simulated stress affected the antioxidant protection function, as it was shown by a decrease in the activity of glutathione peroxidase enzymes by 36.7%, superoxide dismutase by 37.9% and catalase by 39.8%, as well as a decrease in the concentration of reduced glutathione by 35.9%. The depressive state of the antioxidant system is accompanied by a significant accumulation of lipid peroxidation products in the blood (diene conjugates, malondialdehyde and fluorescent Schiff bases). A decrease in bodyweight by 490 g in 5 days of immobilization was also a result of the negative impact of stress on rabbits.

The use of four developed anti-stress drugs to prevent the negative effects of technological stress ensures stabilization of the level of cortisol, thyroxine, the activity of antioxidant enzymes and the concentration of lipid peroxidation products. At the same time, tranquilizers to a greater extent reduce the concentration of cortisol and normalize the amount of thyroxine, which contributes to improving free radical oxidation and antioxidant protection. Antioxidants activate the function of the enzymatic link of antioxidant protection and reduce the accumulation of lipid peroxidation products in rabbits, resulting in a decrease in the amount of thyroxine and cortisol. This allows the developed means to be suggested for pharmacological prevention of technological stress.

Thus, it has been established that under the experimental stress (immobilization), rabbits develop oxidative stress, which is expressed in an increase in the blood concentration of lipid peroxidation products, i.e. diene conjugates, malondialdehyde and fluorescent Schiff bases, as well as a reduced activity of glutathione peroxidase, superoxide dismutase and catalase in the presence of increased concentration of cortisol and decreased thyroxine. The use of new medicines for animals (i.e. a drug for stress correction in farm animals, an antioxidant drug for animals, Mebisel and Polyoxidol) effectively prevents technological stress and can be used in practical veterinary medicine. Normalization of the antioxidant status in rabbits leads to a decrease in the blood level of cortisol and an increase in the concentration of thyroxine. These results allow us to recommend the drugs with antioxidant activity in the scheme of technological stress prevention in animals.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 777-786
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 619:616-099:582.28

doi: 10.15389/agrobiology.2019.4.777eng
doi: 10.15389/agrobiology.2019.4.777rus

MANIFESTATIONS OF CHRONIC FEED MYCOTOXICOSIS IN LABORATORY RATS UNDER EXPERIMENTAL CONDITIONS

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The authors declare no conflict of interests
Received April 14, 2019

Abstract

Mycotoxicoses are specific human and animal diseases caused by the certain types of microspores fungi that during their life form toxic substances, the mycotoxins. Toxic effects of these diseases are diverse and depend on the dose of toxin, exposure, animal species, age and sex. Lack of data on physiological mechanism of the pathological influence under combined mycotoxicoses significantly reduces the development of new drugs and methods for treatment of animal mycotoxicoses. This paper is our first report on the features of the clinical, biochemical, and pathomorphological signs of associative mycotoxicoses under experimental exposure of laboratory animals to the most common mycotoxins. The aim of the research was studying clinical signs of chronic combined mycotoxicosis and pathoanatomical changes in organs and tissues under experimental mycotoxins in laboratory rats, as well as the characteristics of intoxication symptoms, the effect of mycotoxins on the reproductive function of rats and their progeny, and morphobiochemical blood parameters. Experiments were carried out on 66 white non-linear rats of both sexes with a body weight of 80-120 g (Krasnodar Research Veterinary Institute vivarium, 2018). After 14-day acclimatization (quarantine) the rats matching experimental conditions were divided into two groups, 33 animals each, according to paired analogue criterium. The experimental rats were fed for 21 days with the feed naturally contaminated by mycotoxins. The control rats ate toxin-free feed. The spore counts in the contaminated feed was 5.7×10^4 for *Fusarium* sp., 1.2×10^4 for *Mucor* sp., and 2.0×10^4 for *Penicillum* sp. Mycotoxin concentration in the samples exceeded the maximum permissible level (3.6 mg/kg for zearalenone, 0.2 mg/kg for ochratoxin A, 6.2 mg/kg for fumonisin B_1), which was confirmed by bioassay on laboratory animals (mice). During the experimental period, all animals were clinically monitored for general status, feed consumption, behavior, response to external stimuli, motor activity, skin and fur condition, tactile sensitivity, functions of the digestive and urinary organs, corneal and dermal reflexes, and dynamics of weight gain. Hematologic blood tests were also performed. At the end of the study, three rats were euthanized in the experimental group and three rats in control group to identify pathologic and anatomical changes. It was determined that toxic feed leads to a 21 % decrease in body weight compared to the control, and also negatively impacts upon ontogenesis and reproduction causing a higher number of stillborn offspring and lower body weight and vitality of the newborn rats. In the blood of experimental animals the number of erythrocytes decreases by 17.0 % ($p \le 0.05$), hemoglobin by 13.0 % ($p \le 0.05$), total protein by 23.7 % ($p \le 0.01$), glucose by 22.7 %, cholesterol by 28.9 %, and triglycerides by 22.7 % ($p \le 0.05$). Reactive leukocytosis developed as a response of leukopoiesis to intoxication and a possible allergic process. Activity of blood alanine and aspartate aminotransferases was 40.5 and 61.3 % ($p \le 0.01$) higher, respectively, compared to the control rats. We also revealed exudative hemorrhagic inflammation of the mucous membrane of the stomach, thin and thick intestine, swelling and plethora of the lungs, enlarged liver, kidneys and heart. Histological examination of liver tissues showed a decrease in the amount of glycogen in hepatocytes, as well as the areas with granular and fatty dystrophy, vacuolization of hepatocytes, proliferation of the bile ducts, which may indicate severe destructive and necrotic processes. In the kidneys, a granular and fatty dystrophy of the convoluted tubule epithelium, desquamation of epithelial cells and proliferation occur, in the heart there are changes in the transverse striations of fibers, problems with blood circulation in the heart muscle and endocardial thickening. A depletion of lymphoid elements and a decrease in the lymphatic follicles (malpighian bodies) are characteristic

of spleen. Proliferation and mitosis of muscle cells are found in the uterus. Thus, the combined mycotoxicosis deeply violates the homeostasis of laboratory animals and leads to multiple pathological changes in the organs and systems of the body.

Keywords: mycotoxins, biochemical indicators of blood, laboratory rats, pathological anatomical studies, organs

The productive and physiological health of farm animals largely depends on the composition and quality of the consumed feed rations, which is also determined by the content of mold fungi and mycotoxins [1-4].

Mold fungi use for growth most of the constituent elements of grain, which leads to significant losses of nutrient and biologically active substances. The color, smell, and taste of the grain changes. However, the accumulation of highly toxic metabolites of microsporic fungi, the mycotoxins, is even more dangerous, of which trichothecene mycotoxins (T-2 toxin, deoxynivalenol – DON, zearalenone), aflatoxin, ochratoxin, and sterigmatocystin are the most common [5, 6]. The list of mycotoxins continues to expand; to date, about 350 species of toxin-forming fungi (14 genera) and more than 520 mycotoxins that are dangerous to humans and animals have been identified [7-9].

It has been reliably established that the consumption of feed containing mycotoxins causes a decrease in productivity (and, as a result, a decrease in live weight gain of young animals), overspending of feed per unit of production, and deterioration in product quality [10-12]. Moreover, mycotoxins are detected not only in feed for farm animals and poultry, but can also get into food products that have undergone technological processing, which may lead to the development of a number of human diseases, including oncological ones. This has attracted attention to obtaining biologically complete and harmless livestock products from many transnational communities, i.e. the World Health Organization, Food and Agriculture Organization, United Nations Environment Programme, and International Agency for Research on Cancer [13-15]. With the simultaneous intake of two or more mycotoxins, or their combinations with toxic pollutants (pesticides, dioxins, heavy metals), the mycotoxicological danger increases by many times, which can not only significantly increase the toxicity of the metabolic byproducts of microsporic fungi, but also has a significant negative effect on the health of animals [16, 17].

The action of mycotoxins on the body and the severity of the pathological process depend on many factors, which include doses, the duration of toxins in the body, the animal species, gender and age. However, in all cases, damage to vital organs and body systems occurs [18, 19]. At the same time, the pathological effect of combined mycotoxicoses on physiological systems and organs, as well as the mechanism of such an effect, is still not well understood, which reduces the possibility of developing drugs and methods of treating animals. The increase in export and import of grain between countries and the gradual climate change in the world contributes to a significant increase in the widespread prevalence of feed crops with various mycotoxins, which can lead to uncontrolled contamination of feeds with toxic metabolites of fungi [20-22].

In the case of the combined effect of mycotoxins, in which their combined effect on the organism increases dramatically, it is quite difficult to assess the severity of the pathological development of mycotoxicosis. It depends not only on the association of individual mycotoxins, but also on their concentrations, which raises questions of monitoring the clinical picture of mycotoxicoses in animals at one of the first places in terms of the relevance of research programs on mycotoxicology [23-25].

In this work, for the first time, the clinical, biochemical and pathomorphological manifestations of associative mycotoxicoses were revealed during ex-

perimental exposure laboratory animals to the most common mycotoxins.

The goal was to study the clinical picture of chronic combined mycotoxicosis and pathoanatomical changes in organs and tissues, damaged by mycotoxins, as well as the peculiarities of intoxication symptoms in laboratory rats, the effect of mycotoxins on the reproductive function of animals, offspring obtained from them, as well as morpho-biochemical parameters of the blood.

Techniques. An experimental chronic associative mycotoxicosis was simulated on 66 white non-linear rats of both sexes with a body weight of 80-120 g, divided into two groups of 33 animals each (15 females and 18 males) (stationary conditions of the Krasnodar Research Veterinary Institute vivarium, 2018). For the experiment, clinically healthy animals were selected that had a smooth, shiny fur, pale pink color in visible mucous membranes, and a good appetite. The duration of the quarantine (acclimatization period) was 14 days. They were fed at a fixed time with a full standard diet in accordance with established standards. Access to water was not limited.

During 21 days, the experimental group of rats received feed, naturally contaminated with mycotoxins, the control group received high-quality feed. In both groups, water was given ad libitum. From the date of replanting males into groups when pregnancy was detected, the effect of toxic feed on embryonic development and generative function of animals was determined.

In the process of mycological, toxico-biological and enzyme-linked immunosorbent assay of samples taken in accordance with the regulations for the selection and transportation of feed for sanitary-hygienic and chemical-toxicological studies (as per GOST 13586.3-83), the content of fungi spores was determined. During the experimental period, all animals underwent clinical control according to the following criteria: general condition, feed intake, behavior, reaction to external stimuli, nature of motor activity, condition of the skin and fur, tactile sensitivity, functions of the digestive and urinary organs, corneal and dermal reflexes, and dynamics of bodyweight gain.

Blood for research was taken from five rats from each group at the end of the experimental period directly from the heart under ether anesthesia. Hematological blood tests were performed on an automatic hematological analyzer for in vitro diagnostics Mythic18 (C2 DIAGNOSTICS S.A., Switzerland/France), biochemical tests were performed on an automatic biochemical analyzer Vitalab Flexor Junior (Vital Scientific N.V., Netherlands) using kits of the company ELITech Clinical Systems (France).

At the end of the study, three rats were killed in the experimental and control groups using ether anesthesia (following the principles of bioethics) to identify pathoanatomical changes. The effect of mycotoxins on the macro- and microstructure of the internal organs of white rats was evaluated by post-mortem examination of the animals with complete removal of the internal organs. The material was fixed in 10% neutral formalin; the diagnosis was conducted by methods generally accepted in pathomorphology [26]. The samples were stained with hematoxylin and eosin. For microphotography, an MS-300 microscope (Micros, Austria) and a digital 10-megapixel camera Digital IXUS 970 IS (Canon, Inc., Japan) were used; magnification ×150 (ocular ×15, lens ×10); ×300 (ocular ×15, × lens 20) and ×600 (ocular ×15, lens ×40).

The results were processed using the software package Statistica 6.0 (StatSoft, Inc., USA). The data were presented as mean (M) and standard error of the mean (±SEM). The significance of differences between the series was determined using Student's t-test.

Results. The contamination with fungal spores of the feed, which was given to the animals of the experimental group, exceeded the maximum permis-

sible level (MPL) (5.7×104 per 1 g feed), that is, the feed was found to be toxic. The number of spores was 2.5×10^4 for *Fusarium* sp., 1.2×10^4 for *Mucor* sp., and 2.0×10^4 for *Penicillum* sp. The mycotoxins in the sample (zearalenone — 3.6 mg/kg, ochratoxin A — 0.2 mg/kg, fumonisin B_1 — 6.2 mg/kg) also exceeded the MPLs, which was confirmed by bioassay in laboratory animals (mice).

The first signs of intoxication with mycotoxins in rats from the experimental group were already recorded on day 5 to day 7 of the experiment. This was manifested by excessive timidity with a simultaneous increase in excitability against together with a decrease in spontaneous motor activity and the development of adynamia. The fur was ruffled, not shiny, with areas of loss and contamination of wool and alopecia. There was an increase in thirst with a decrease in appetite, which led to a noticeable growth lag by the end of the first stage of the experiment (Table 1).

1. Live weight of non-linear white rats fed with feed contaminated with spores and metabolites of microscopic fungi (M±SEM, n = 33)

Group	Body weight, g		Average daily gain, g	To the control, %
	initial	final		
Control	106.1±2.38	131.7±3.12	1.22±0.03	100
Test	103.8±2.33	124.2±2.94	0.97±0.04*	79.5

N o t e. For a description of the groups, see the Techniques section.П р и м е ч а н и е. Описание групп см. в разделе «Методика».

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

The average daily weight gain in rats of the experimental group was lower if compared to the control. The excess of this indicator in control analogs was 21% ($p \le 0.05$). In absolute units, the average body weight of the control animals exceeded that of rats from the experimental group by 5.2 g.

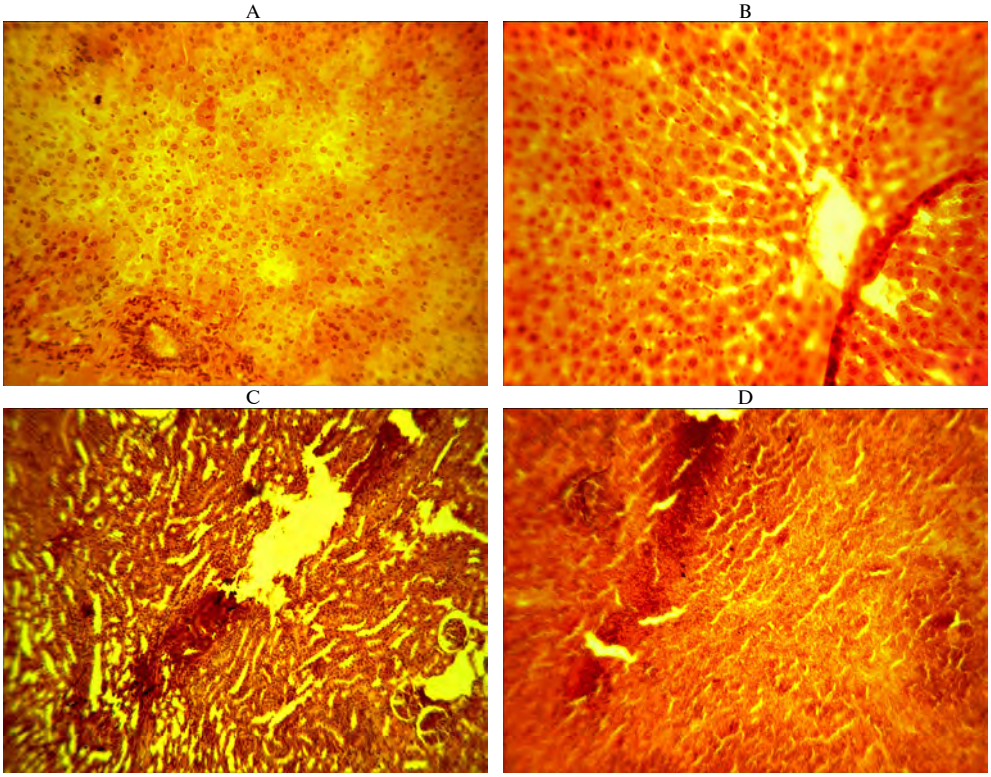
Long-term consumption of toxic feed had a negative effect on gynogenesis and animal development. By the end of the studies, 6 pregnant females were detected in the experimental group, and 9 ones in the control group. Although in rats receiving feed affected by mycotoxins, the pregnancy ended in 23-24 days with natural birth and did not differ in duration from pregnancy in control females, the number of newborn rats in such animals was insignificant (1-2 rats per a female). At the same time, out of the total number of young rats that were born to females of the experimental group (10 animals), two were stillborn, three more were not viable and died during the first 2 days. However, deformities and anomalies in their development were not noted. The average weight of young rats in the experimental group was 2.9±0.2 g. In the control group, 29 rats were born with an average weight of 3.5±0.3 g, of which two were non-viable.

2. Morphological and biochemical blood parameters in non-linear white rats fed with feed contaminated with spores and metabolites of microscopic fungi (M±SEM, n = 5)

Indicator	Group	
	test	control
Erythrocytes, $\times10^{12}$/l	5.4±0.23***	6.3±0.31
Leukocytes, $\times10^9$/l	9.8±0.57**	8.07±0.29
Hemoglobin, g/l	113±3.74***	127.3±4.25
Total protein, g/l	64.6±4.33**	79.9±2.94
Aspartate aminotransferase, units/l	150.7±6.41*	93.4±3.06
Alanine aminotransferase, units/l	97.8±3.47*	69.6±6.13
Alkaline phosphatase, units/l	621.5±13.07	547.3±21.84
Glucose, mmol/l	6.6±0.42	8.1±0.63
Urea, mmol/l	8.4±0.31	7.5±0.47
Creatinine, μmol/l	28.3±1.15	25.3±1.1
Cholesterol, mmol/l	1.76±0.05*	2.27±0.14
Triglycerides, mmol/l	0.44±0.03***	0.54±0.03

*, ** and *** Differences with control are statistically significant at $p \le 0.001$; $p \le 0.01$ and $p \le 0.05$.

The intoxication was assessed by the morpho-biochemical profile of the blood of experimental animals (Table 2). In rats from the experimental group, a decrease in hematological parameters was found. By day 21, there was a decrease in the number of erythrocytes by 17.0% ($p \leq 0.05$), in hemoglobin by 13.0% ($p \leq 0.05$) and an increase in leukocytes by 21.4% ($p \leq 0.05$) compared to the control. Therefore, it may indicate the inhibition of erythro- and hematopoiesis as a result of long intoxication. The presence of reactive leukocytosis in the blood, which occurs as a response of leukopoiesis to intoxication and a possible allergic process, confirms this assumption.



Granular dystrophy (A) **and areas of fatty degeneration of the liver** (B)**, proliferation in the kidneys** (C) **and spleen** (D) **in non-linear white rats with the consumption of feed contaminated with spores and metabolites of microscopic fungi.** Light microscopy (MS-300, Micros, Austria), staining with hematoxylin and eosin, magnification ×150 (A), ×300 (B, D) and ×600 (C).

At the end of the study, during the external examination in rats of the experimental group, we noted cyanosis of visible mucous membranes, rumpledness and dullness of the fur. A visual examination of the internal organs of rats from the experimental and control groups did not reveal any abnormalities in their location and structure. However, pathological changes were revealed in rats of the experimental group as hemorrhagic inflammation of the gastric mucosa, thin and thick intestines, swelling and pulmonary congestion. The liver was dark red, flabby, enlarged, in some places there were grayish areas of necrosis, the gall bladder was full. The heart was enlarged, with foci of micronecrosis, the heart muscle was flabby. The kidneys were enlarged, pink-gray, the vulva of the females was swollen, the testes of the males were enlarged.

During histological examination (Fig.), the most characteristic changes were found in liver tissues: the amount of glycogen in hepatocytes decreased, there were areas with granular and fatty degeneration in the cytoplasm, hepatocyte vacuolization and proliferation in the bile ducts were visible. In the hepatic

lobules, the radial arrangement of the blocks was disturbed due to the rounding of hepatocytes. All this indicated the presence of severe destructive and necrotic processes in the liver. Granular and fatty degeneration of the convoluted tubule epithelium, desquamation of epithelial cells, and proliferation were observed in the kidneys, in the heart there were changes in the transverse striation of the fibers, impaired blood circulation in the heart muscle, and thickening of the endocardium. In the spleen, depletion of lymphoid elements and a decrease in the Malpighian layer occurred. Proliferation and mitosis of muscle cells were noted in the uterus.

In the study, we found high contamination of the feed used in the experiment with spores of fungi producing the main types of mycotoxins — zearalenone, ochratoxin A, fumonisin B_1, which are classified as potent and highly toxic compounds. Such a combination leads to an increase in the synergistic effect of mycotoxins on the organism, as a result of which the clinical signs of toxicosis were noted in rats already during week 1 of the experiment. Further consumption of toxic feed increased the negative effect of mycotoxins and led to serious physiological changes and pathological processes in the reproductive organs. In our opinion, such manifestations are associated with zearalenone which is 1.8 times higher than the maximum permissible level. It was established that this mycotoxin can cause infertility, abortion and cyst formation in animals. The negative effect of the association of zearalenone, ochratoxin A, and fumonisin B_1 on the conception in rats and the embryonic development of the offspring, which was found experimentally, is consistent with the established properties of T-2 toxin and DON to induce apoptosis in animals' embryos, including poultry [27].

The presence of high doses of ochratoxin A (4 times higher than the maximum permissible level. MPL) in the feed leads to inhibition of hemo- and leukopoiesis, synthesis of protein and a number of enzymes, damage to the liver and kidneys, a decrease in live weight and growth retardation, and a combination of several mycotoxins enhances their joint pathological effect on the organism [28]. It was reported that experimental mycotoxicosis in laboratory mice, caused by compound feed contaminated with ochratoxin and T-2 toxin, was manifested by the following clinical signs: hyperemia of the visible mucous membranes, disturbance of the nervous system, impaired feed intake, decreased live weight, gastrointestinal tract damage, a change in the biochemical parameters of blood, i.e. a decrease in the content of amylase and cholesterol while increasing the amount of urea and creatinine [29].

A number of researchers note that when consuming feed contaminated with mycotoxins in vivo, the toxic effect is more pronounced than when an equivalent amount of pure mycotoxin was received in the experiment. In respect of T-2 toxin and aflatoxin, trichothecenes and fusaric acid, zearalenone and deoxynivalenol, there are data confirming the effects of their synergism [30-34].

When discussing the problem of chronic mycotoxicoses, it should be emphasized that the increased lipid peroxidation, observed during mycotoxicoses, leads to damage to the membranes of hepatocytes, inhibition of various liver functions, and ultimately to the development of hepatopathies [35-38]. Our research indicates that under combined natural contamination of feed with zearalenone, ochratoxin A and fumonisin B_1, pathological processes in the liver developed in accordance with fatty degeneration type.

Thus, in non-linear white rats, experimental combined mycotoxicosis was characterized by multiple toxic manifestations, i.e. excessive timidity, rumpledness and dullness of the fur, alopecia, increased thirst and decreased appe-

tite, lag in growth and development, negative impact on fertility of females and fetal development (a small number of newborn rats, high level of stillbirth and mortality, low body weight at birth). Morpho-biochemical blood parameters of rats with associative mycotoxicosis were lower in erythrocytes and hemoglobin levels (by 17.0 and 13.0% at $p \leq 0.05$ compared to animals from the control group) together with the development of reactive leukocytosis, hypoproteinemia and hypoglycemia, lipid metabolism disorders show a simultaneous increase in activity of liver transaminases (by 40.5% for alanine aminotransferase and by 61.3% for aspartate aminotransferase at $p \leq 0.01$). Histological examination of the liver of test animals revealed areas of granular and fatty degeneration, vacuolization and a decrease in the amount of glycogen in hepatocytes, as well as proliferation in the bile ducts. Granular and fatty dystrophy of the convoluted tubule epithelium, desquamation of epithelial cells, as well as proliferation occur in the kidneys, in the heart there are changes in the transverse striation of the fibers, impaired blood circulation in the heart muscle and the thickening of the endocardium. In the spleen, there is a depletion of lymphoid elements and a decrease in lymphatic follicles (Malpighian bodies), in the uterus the proliferation and karyokinesis of muscle cells occurs. The results of the research indicate deep violations of homeostasis in laboratory animals, as well as multiple pathological changes in organs and systems of the body caused by combined mycotoxicosis.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 787-797
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Basic animal nutrition and feeding

UDC 636.2:591.132:636.084.41

doi: 10.15389/agrobiology.2019.4.787eng
doi: 10.15389/agrobiology.2019.4.787rus

PHYSIOLOGICAL ASPECTS OF COMPLETE MIXED DIET DIGESTION IN COMPLEX STOMACH OF RUMINANTS ON THE EXAMPLE OF CATTLE (*Bos taurus taurus*)

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The authors declare no conflict of interests
Received March 22, 2019

Abstract

Modern systems of normalized cattle feeding are based on the position that different feeding technologies do not affect the processes of digestion and absorption of nutrients of the same diet. In our studies, data were obtained for the first time, indicating changes in the parameters of the ration digestion process in the complex stomach of cattle, depending on the method of feeding: separate delivery of separate feeds and simultaneous delivery by the form of the Total Mixed Ration (TMR). To determine mechanisms of increasing feed dry matter intake in cattle when they were switched to Total Mixed Ration feeding, daily flux rates of chyme, its chemical composition and digestibility of main nutrient groups in the compound stomach of young cattle were studied. A Latin square design experiment was carried out using two diets differing in the forage fodder to concentrated fodder ratio and two methods of feeding (separate distribution of feed and in the form of TMR) by the method of periods with two Ukrainian red-motley bulls (*Bos taurus taurus*) of 300 kg live weight, with a sluice T-shaped duodenal canula. As a result of switching to the TMR feeding, both the nature of chyme influx to the duodenum and nutrient digestibility in the complex stomach were found to change. For 1 hour, the volume of chyme during TMR feeding increased by 417±71 ml/h as compared to separate high concentrate diet feeding, while compared with low concentrate feeding, it increased by 221±81 ml/h, or by 14.42 % and 8.36 %, respectively ($p < 0.001$). TMR increased Ca, P and total mineral residue influx to the duodenum (by 5.9 %, 10.1 and 8.5 %, respectively). At that, the increase in Ca with the first diet and in general ash with the second one was established at a level of trend, whereas for all the others the increase was significant ($p < 0.05$). This fact as well as the observed tendency to a change in the Ca and P concentrations in the duodenal chyme composition suggest an increase in the outflow of chyme from the rumen caused by both increased salivation and consumption of water by the animals. The feeding of animals with the TMR was shown to cause multidirectional changes in digestibility of all nutrients: digestibility of raw fat and crude protein decreased in both diets (by 39.6 % and 27.5 %, respectively), while digestibility of crude fiber increased (by 6.9 %); digestibility of nitrogen-free extractives did not change significantly in the first diet, whereas it tended to decrease in the second one so that the total digestibility of the dry and organic matter of the diet changed insignificantly. Raw fiber should be considered as an integral marker for assessing the TMR effect for rumen's digestion, since it is only digested in the rumen and is not synthesized by the microflora. Due to this, the increase in the chyme flow that we established, when we changed feeding method in the same type of diet, can reduce fiber digestibility due to accelerated outflow of rumen's content with small particles, whereas with the other type of diet, on the contrary, can increase digestion due to improvement of fermentation conditions.

Keywords: Ukrainian red-motley cattle, digestion, chyme volume, fiber, total mixed ration

Digestion of feed nutrients in ruminants can be divided into two processes. On the one hand, it is transformation of nutrients in the forestomachs where both the digestion of feed particles due to the enzymes produced by symbiotic microflora of the rumen and the synthesis of nutrients, constituting the microbial body itself, take place, on the other hand – digestion in the guts with own enzymes and the absorption of nutrients in the internal environment of the

animal body. The second stage of digestion begins with the true stomach, which is the analog of non-ruminants' (monnogastric) stomach. The division into microbial digestion and own digestion is quite conditional: some products enter the internal environment of the macroorganism directly from the forestomachs, and part of the food is digested by the microflora of the small and large bowels [1, 2]. The main retention of feed masses occurs in the rumen since their promotion to the following bowel segments is possible only after grinding to a particle size of about 1 mm [3-5].

Many studies show [6-8] that changing to cattle feeding with Total Mixed Ration (TMR) increases the total intake of dry matter as compared to separate delivery of components of the same ration. The lack of selective feeding and reduced competitive relationships between animals are other positive effects [9-11]. We have previously revealed the effect of ration feeding technology on nutrient digestibility [12].

In this paper, we first obtained data on changes in the digestive process in young cattle depending on the mode of feeding the same ration. For example, the digestibility of raw fat and crude protein decreased in both high-concentrate and low-concentrate rations (by 39.6% and 27.5%, respectively), while the digestibility of crude fiber increased (by 6.9%); the digestibility of nitrogen-free extractives did not change significantly in the high-concentrate ration, whereas it tended to decrease in the low-concentrate ration.

The work objective was to study the dynamics of the daily flow of chyme, its chemical composition and digestibility of the main groups of nutrients in young cattle during switching to the Total Mixed Ration of feeding.

Techniques. The experiments (the physiological yard of Institute of Animal Science of the National Academy of Agrarian Sciences of Ukraine, 2015-2016) were carried out on castrated Ukrainian Red-and-White bulls (*Bos taurus taurus*), 300 kg bodyweight, with duodenal cannulas set at the beginning of the duodenum. The design of cannulas ensured the collection of chyme coming from the rennet into the duodenum, its assessment and return to the digestive system [13]. Animal were fed twice a day (at 8 a.m. and 5 p.m.) in equal portions. The chyme flow was measured for 9 hours after morning feeding. The obtained values of the chyme volume for 9 h were extrapolated to the daily interval, which made it possible to calculate the digestibility of nutrients in the complex stomach after the chemical analysis of selected samples of chyme and feed ration.

The effect of the Total Mixed Ration on the volume of duodenal chyme and the digestibility of nutrients in the complex stomach was studied according to the scheme of the Latin square with two rations differing in the ratio of voluminous and concentrate feeds in two periods on one group (2 animals). The volume of incoming chyme was measured in each animal in 3-fold repetition.

The available energy of rations was calculated by the formula:

$$AEC = 14.46 - 0.0007 \times CP + 0.0168 \times CF - 0.0192 \times CFb - 0.00028 \times NFE,$$

where CP, CF, CFb, NFE are the concentrations of crude protein, crude fat, crude fiber and nitrogen-free extractives in the ration, respectively, g/kg dry matter (DM), AEc is the available energy concentration, mJ/kg DM [14].

In the first period, hay and silage were given first, and dry concentrate feed was on top of the silage. Adaptation of young cattle digestion to the used ration occurred during 14 days. Then, the influx of chyme in the duodenum was measured during 14 days for a 9-hour interval after morning feeding ($n = 6$). Breaks for 1-2 days for the rest of animals were made between measurements. In the second period (28 days), pre-chopped hay, silage and mash were weighed and mixed (for each animal separately) with a drum mixer (manufactured in the

laboratory of mechanization of livestock processes of the Institute of Animal Science of the NAASU). This technique ensured the delivery of all ration components in the form of TMR to each animal with the same accuracy as with separate feeding.

The chyme influx in the duodenum was measured as described above. Chemical analysis of the main nutrients of rations, chymus, and feces was carried as per standard techniques [15]. The animals were weighed before and after the experiment.

The Office Standard 2010 32-bit Russian software (license GGWA-A) (https://www.microsoft.com/ru-ru/downlo-ad/office.aspx) with dispersion analysis techniques was used for statistical calculations and drawing charts and diagrams. The arithmetic mean values (*M*), standard errors of means (±SEM), and mean square deviation (±σ) were calculated. The significance of differences was evaluated by paired Student's *t*-test (for allied series), which allowed elimination of the systemic error resulting from to individual peculiarities of animal digestion.

Results. Rations for test animals were made in such a way that their characteristics differed as much as possible (Table 1). This was made to assess the changes in the physiological parameters of digestion under the influence of TMR feeding vs. diametrically opposite conditions that occur in the rumen under low- and high-concentrate rations. Differences in energy and protein availability between rations were more than 20%.

1. High- and low-concentrate rations in experiments on young Ukrainian Red-and-White cattle (*Bos taurus taurus*) (Institute of Animal Science of the National Academy of Agrarian Sciences of Ukraine, 2015-2016)

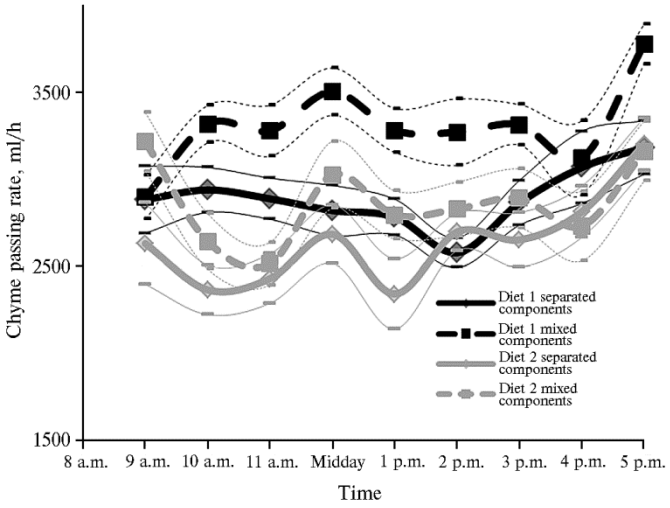
Ingredients, characterization	Ration		Comparison of rations (ration No. 2 to ration No. 1, %)
	high-concentrate (No. 1)	low-concentrate (No. 2)	
Corn silage, kg	11.00	16.80	
Alfalfa hay, kg	1.00	2.08	
Wheat groats, kg	0.30	–	
Corn groats, kg	0.30	–	
Sunflower cake, kg	3.30	–	
Barley groats, kg	–	0.72	
Mineral additive, kg	0.03	0.03	
Dry matter, g	7008	6355	90.68
Organic matter, g	6615	5996	90.65
Ash, g	393	359	91.28
Crude protein, g	1413	501	35.48
Crude fat, g	563	112	19.80
Crude fiber, g	1955	2170	111.01
Nitrogen-free extractives, g	2684	3213	119.74
Calcium, g	42.55	30.74	72.24
Phosphorus, g	25.62	21.18	82.67
Available energy, mJ	71.52	50.86	71.10
Available energy content, mJ/kg	10.21	8.00	78.41
Energy-protein ratio, g/mJ	19.76	9.86	49.89
Crude protein content, %	20.17	7.89	39.12
Basal metabolism provision, mJ/kg	1.03	0.63	61.56

N o t e. The basal metabolism provision is given per 1 kg of metabolic body weight (body weight to the power of 0.75, $W^{0.75}$). The dash indicates the absence of a component in the ration.

The average daily growth in body weight during the investigation was 1.12 and 0.26 kg with a swing of 0.95-1.29 and 0.21-0.32 kg, respectively, on rations No. 1 and No. 2, which is consistent with the literature data [16, 17].

The chyme influx from the complex stomach to the duodenum was uneven. At eating, the volume of chyme per time decreased compared to the period of animals' rest. Based on previous measurements, it was found that the volume of chyme for 9 hours after morning feeding was 37.47±0.97% with a coefficient of variation of 3.73% relative to the daily volume, which almost coincides with the time interval selected in the experiment: 9:24 × 100% = 37.5%. The chosen

period facilitated the investigation greatly and, most importantly, reduced the stress impact on the operated animals.



Dynamics of chyme influx in the duodenum of young Ukrainian Red-and-White cattle (*Bos taurus taurus*) under separate feeding and feeding with Total Mixed Ration. For a description of the rations and conditions of the experiments, see the Techniques section (Institute of Animal Science of the National Academy of Agrarian Sciences of Ukraine, 2015-2016).

In switching to the TMR, the chyme influx in the duodenum and the digestibility of nutrients in the complex stomach changed. During the whole experiment, the rate of chyme influx from the rennet into the duodenum was higher when using the TMR (Fig.). At average, for 1 h, the chyme volume in feeding with the TMR vs. separate feeding increased by 417±71 ml (ration No. 1) and by 221±81 ml (ration No. 2), or by 14.42 and 8.36%, respectively ($p < 0.001$). With the TMR, not only significant changes in the volume of the chyme but also an increase in the flow rate of minerals from the complex stomach to the duodenum occurred in all cases.

Feeding the TMR increased the intake of calcium, phosphorus, and total mineral residue in the duodenum (Table 2). At the same time, an increase in the amount of Ca for ration No. 1 and total ash for ration No. 2 was found to be insignificant, at the trend level ($0.1 > p > 0.05$), for all other indicators, the increase was significant ($p < 0.05$). The concentration of minerals in the incoming chyme, except of Ca, decreased during the switching to TMR No. 2 while overall mineralization of the duodenal chymus markedly increased. Hence, it can be concluded that feed in the TMR form to a greater extent stimulates the flow of water into the complex stomach.

2. Mineral substances in the chyme in the initial section of the duodenum of young Ukrainian Red-and-White cattle (*Bos taurus taurus*) under separate and Total Mixed Ration of feeding (Institute of Animal Science of the National Academy of Agrarian Sciences of Ukraine, 2015-2016)

Ration	Separate feeding			Total mixed ration		
	Ca	P	ash	Ca	P	ash
	Came to the duodenum, g/day					
No. 1	60.71	48.99	602.5	64.27*	53.94**	653.5**
No. 2	60.35	38.70	615.3	71.75***	42.16****	669.5
	Minerals concentration in the chyme, mg%					
No. 1	87.68	70.51	868.0	81.75**	68.57	832.4****
No. 2	96.88	61.94	982.1	104.51***	61.03*	971.0

Note. For a description of the rations and conditions of the experiments, see the Techniques section.
* Differences for the Total Mixed Ration with indicators at separate feeding are statistically insignificant ($0.1 > p > 0.05$).
, *, **** Differences for the Total Mixed Ration with indicators at separate feeding are statistically significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Variance analysis (Table 3) revealed a noticeable effect (more than 50% on the Chaddock scale) of the feeding mode on the intake of P (both rations) and Ca (ration No. 2) in the duodenum. At the same time, the studied factors

(the composition of the ration and the form of feeding) influenced the total content of the mineral residue in the duodenum chyme.

3. Variance analysis of the influence of the feeding mode and the composition of rations on the intake of minerals in the duodenal chyme of young Ukrainian Red-and-White bulls (*Bos taurus taurus*) (Institute of Animal Science of the National Academy of Agrarian Sciences of Ukraine, 2015-2016)

Factor	Influence, %		
	Ca	P	ash
Ration composition			
Ration No. 1	10.5	57.8	12.3
Ration No. 2	59.2	52.8	13.7
Feeding mode			
Separate feeding	23.3	48.8	12.8
Total mixed ration	46.4	61.8	13.2

Note. For a description of the rations and conditions of the experiments, see the Techniques section.

According to the data on the salivary secretions in cows [18-20], Na prevails among cations, comprising 126 mEq/l (up to 85%), while K concentration is only 6 mEq/l (4%). The intake of Ca and Mg with saliva is less important for ionic equilibrium. Concentration of Ca ions is of 0.2 to 0.5 mEq/l. Among anions, bicarbonates (103-125 mEq/l) and phosphates (25-64 mEq/l) are of particular importance, determining the alkaline properties of saliva [18, 21]. High alkalinity, due to the content of bicarbonates and phosphates, is necessary to neutralize the acids formed in the pancreas as a result of fermentation. The composition of saliva is characterized by relative constancy [22-24], and changes in the intake of its components are associated with changes in the total amount of saliva. Apparently, it happens due to the fact that saliva has a micellar structure. The micelle consists of a nucleus, which is based on calcium phosphate. The nucleus is surrounded by hydrogen phosphate ions and an outer diffuse shell based on calcium ions [25].

Based on a significant increase in the intake of P in the duodenum for both rations, Ca for ration No. 2 and total ash for ration No. 1 (see Table 2), it can be assumed that the increase in the chyme outflow from the rumen found in our experiments is associated with increased salivation. At the same time, the data obtained on a certain decrease in the concentration of the studied minerals indicate an increase in the intake of water into the rumen in addition to its intake in the composition of saliva. Due to the lack of data on the dynamics of water consumption by animals, it is not possible to draw a conclusion about the origin of its additional amount. The increase in the consumption and flow of water directly through the rumen wall is highly likely. The very fact of dilution of the rumen fluid indicates rather an increase in water consumption since its flow from the intercellular fluid is likely to be accompanied by an increase in the number of electrolytes in the rumen content without the effect of dilution.

Some authors observed the effect of increased outflow rate associated with increased water consumption when using granular feed [26]. Given the fact that a significant amount of fluid is absorbed during the passage of the ruminal digesta through the omasum, and the flow of electrolytes from the gastric secretion into the rennet to some extent should smooth the effect of diluting the ruminal digesta with water drunk, then the increase in the flow of water into the rumen in the case of TMR feeding can be much higher than we have revealed. It is also known about the function of the omasum in regulating water-salt balance of the fluid coming from the rumen. Consequently, the fact of dilution of the ruminal fluid in the duodenal indicates a significant influence of the TMR on the water-salt balance in the rumen. The probable cause of the effect of dilu-

tion of Ca and P in the duodenal chyme we observed could be that the regulation of the body water balance is carried out by Na-dependent receptors [27, 28], and the maintenance of the water-salt balance in the digestive tract is mainly due to Na ions. An indirect confirmation can be the absence of any noticeable influence of both the ration and the feeding method on the amount of total ash in the duodenal contents (see Table 3).

As a result of switching from separate feeding to TMR, multidirectional changes in the digestibility of all nutrients in the complex stomach occurred but due to mutual compensation, the total digestibility of dry and organic matter of the ration changed slightly (Table 4).

4. Digestibility (%) of the main groups of nutrients in the complex stomach of young Ukrainian Red-and-White bulls (*Bos taurus taurus*) (n = 6, M±SEM, Institute of Animal Science of the National Academy of Agrarian Sciences of Ukraine, 2015-2016)

Ration	Indicator					
	DM	OM	CF	CP	CFb	NFE
Separate feeding						
No. 1	50.09±1.19	56.23±1.90	18.40±18.38	44.62±3.77	70.50±2.35	61.07±2.60
No. 2	52.44±1.76	60.98±1.43	–64.04±27.87	–15.28±32.60	76.26±2.79	66.64±3.48
Total mixed ration						
No. 1	48.75±3.16**	55.32±2.91	11.11±5.03*	32.34±7.06**	75.40±4.40**	62.07±3.75
No. 2	50.37±1.40*	60.06±1.13*	–44.98±8.18	–36.19±7.42**	78.67±3.03	64.19±2.66

Note. DM — dry matter, OM — organic matter, CP — crude protein, — crude fat, CFb — crude fiber, NFE — nitrogen-free extractives.
* Differences for the Total Mixed Ration with indicators at separate feeding are statistically insignificant ($0.1 > p > 0.05$).
** Differences for the Total Mixed Ration with indicators for separate feeding are statistically significant at $p < 0.05$.

As a result of changes in digestive processes under the TMR treatment, the digestibility of crude fat (CF) and crude protein (CP) decreased for both rations, and the digestibility of crude fiber (CF) increased. The digestibility of nitrogen-free extractives (NFE) for ration No. 1 did not change significantly, for ration No. 2 had a tendency to decrease. In our opinion, higher outflow rate of the liquid fraction from the rumen had a positive effect on the rumen microflora, especially cellulolytic. It is evidenced by significant differences in CP and CFb between separate feeding and TMR in the case of ration No. 1 and differences at the trend level ($0.1 > p > 0.05$) for ration No. 2. The active growth of the microbial populations of the rumen led to an increase in the amount of newly synthesized microbial CF and CP in the chyme coming from forestomachs. This had an impact on the decrease in the apparent digestibility of these groups of nutrients under TMR feeding for CF at the trend level, and for CP significantly ($p < 0.05$) (see Table 4). When using ration No. 2 with a low level of concentrates and a significant proportion of bulky feeds, the synthesis of microbial CF and CP exceeded the consumption of these nutrients with feeds. In this case, it is more correct to assess not the digestibility, but the availability of nutrients for digestion in the small bowel, i.e., the amount of nutrients received in the duodenum, in relation to those consumed with food. CF availability for ration No. 2 was 156% at separate feeding, 147% at TMR; CP availability was 120% and 138%, respectively.

The increased amount of CF coming from the complex stomach to the duodenum, compared to the content in the consumed feed, was associated with the transformation of some substances due to activity of the microbial population of the rumen. In this case, more fats, newly synthesized from carbohydrates and carbohydrate skeletons after deamination of amino acids, were produced compared to the content in the consumed feed. The increased content of nitrogenous substances in the chyme coming to the duodenum was also due to micro-

bial synthesis, but the origin of additional nitrogen compared to the incoming feed is explained by its recycling inside the body.

As a result of deamination of amino acids in the process of basic metabolism, ammonia, toxic to the cell, is formed in the body tissues. For its neutralization, an energy-consuming mechanism of urea synthesis was developed based on three free amino groups [29]. It should be noted that the synthesis of urea occurs not only in the liver of animals, but also in the rumen wall directly [30-32]. The urea synthesized in this way enters the blood and forms a so-called pool of urea, from which it is partially directed to the rumen, and partially excreted in the urine. The main mechanism for guiding the urea in the rumen is its excretion with saliva. When it enters the rumen, urea is destroyed by ureases of the microflora to ammonia, which is used in microbial synthesis for the formation of amino acids and the subsequent synthesis of microbial and protozoal protein. The excretion of urea is most effective in the parotid gland (20-30 mg%), the blood concentration of this compound is 10-15 mg%, the concentration in the secret of the submandibular and sublingual glands is 10-15 and 8-10 mg%, respectively [33]. Depending on the intensity and uniformity of urea formation during the day, the ratio of its recycling and excretion may vary significantly. A significant role in this is played by the energy of the ration available for the microflora, especially digestible carbohydrates [34].

Based on our findings, both the feed factor and the intensity of urea entering the rumen as part of saliva affect the synthesis of microbial CP. Thus, in the case of ration No. 1 after the transition to TMR, the digestibility of CP in the rumen significantly increased, which indicates a decrease in the involvement of urea in nitrogen recycling and its more active excretion from the body. In other words, the amount of energy was not enough for microbial crude protein synthesis from nitrogen available in the rumen. When using ration No. 2, the potential supply of available energy to the microflora was higher, as a consequence, endogenous nitrogen was also involved in the microbial synthesis. Increased salivation caused by the TMR and the associated increased urea intake into the rumen contributed to the additional synthesis of microbial CP, which resulted in an increase in CP intake into the duodenum.

It has already been noted before that the increased outflow of fluid from the complex stomach during switching to the TMR positively affected the microbial population of the rumen. This was observed both on the high-concentrate ration, when the increased acidity due to the synthesis of a significant amount of propionic acid was noted, and on the low-concentrate ration with a predominance of the acetic-acid type of fermentation, typical for the digestion of fibrous foods rich in fiber. However, this mechanism had some differences depending on the composition of the ration. Increased fluid outflow from the rumen regulated microbial synthesis by reducing the concentration of microflora-born products in the first case, and by increasing the amount of the substrates used in the second case.

It should be taken into account that with the increased fluid outflow from the rumen, the removal of finely divided insoluble fraction of feed particles is accelerated. It can be assumed that increasing this fractional outflow would reduce the time of microbial fermentation of feed particles, which would inevitably lead to a decrease in the apparent digestibility of nutrients. In our experiments, we revealed the increase in the digestibility of NFE with a decrease in the digestibility of CF for ration No. 1 and a reduced digestibility of NFE with an increase in digestibility of CF for ration No. 2. Apparently, the increase in the intensity of microbial fermentation in one case was quite high and prevailed

over the decrease in digestibility due to an increased outflow rate, and in the other, on the contrary, the microbial synthesis did not compensate a decrease in digestibility because of an increase in the fractional outflow rate, which affected the increase in the overall apparent digestibility.

Another mechanism of the influence of increased salivation on microbial fermentation in the rumen which is difficult to take into account is the fact that in ruminants, saliva has a relatively low surface tension (almost 1.5 times lower than in water). This property of saliva prevents the formation of a foamy mass in the rumen and mesh. The low surface tension of saliva has a detrimental effect on particular species of bacteria; therefore, it can participate in the regulation of the species composition of microorganisms inhabiting the forestomachs. This fact, in turn, affects the intensity of cleavage of certain components of plant food cells and the accumulation of newly synthesized microbial fat and protein in the rumen fluid.

The adequate interpretation of the obtained data is impossible without taking into account the fact that nutrients in the rumen are not only destroyed, but also, being synthesized again, accumulated in the composition of microbial biomass. To assess the interaction of the accelerated fractional outflow of small particles of feed and improved fermentation conditions due to the accelerated removal of microbial fermentation products, crude fiber should be considered as a marker since it is only digested in the rumen and not synthesized by microflora. In this regard, the increase in the chyme flow, revealed when changing the mode of feeding the same type of ration, may decrease the digestibility of fiber due to the accelerated outflow of small particles of the rumen content, whereas with another type of ration, on the contrary, its digestion may increase due to improved fermentation conditions (pH shift to the alkaline side).

Thus, in bulls of the Ukrainian Red-and-White breed, as a result of the switching to the Total Mixed Ration (TMR), the chyme influx into the duodenum and the digestibility of nutrients in the complex stomach changed. The chyme volume for TMR with high energy content increased by 417±71 ml/h, with low energy content by 221±81 ml/h. The TMR treatment increased the intake of calcium, phosphorus, and total mineral residue in the duodenum. The established increase in the intake of mineral components in the duodenal chyme and the change in their concentration indicate that switching to the TMR increases the outflow of fluid from the complex stomach both due to increased salivation and due to other mechanisms of increasing water intake into the complex stomach. The digestibility of all nutrients in the complex stomach when feeding the TMR varies in different directions. For crude fat and crude protein the digestibility decreases at both rations and for crude fiber it increases; no significant change occurs for nitrogen-free extractives at the high concentrate ration and a trend to the decrease appears at the low concentrate ration. As a result, total digestibility of dry matter and organic matter of the ration show a slight change. It is possible to use the index of digestibility of crude fiber as an integral marker for assessing the impact of the TMR on rumen digestion.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 798-809
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.52/.58:636.084.416:579.6

doi: 10.15389/agrobiology.2019.4.798eng
doi: 10.15389/agrobiology.2019.4.798rus

POULTRY DIETS WITHOUT ANTIBIOTICS. II. INTESTINAL MICROBIOTA AND PERFORMANCE OF BROILER (*Gallus gallus* L.) BREEDERS FED DIETS WITH A PHYTOBIOTIC

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The authors declare no conflict of interests

Acknowledgements:

Supported financially by Russian Science Foundation, grant No. 16-16-04089-P, for the study of the physiological and microbiological characteristics of the embryonic and postembryonic digestion in meat chicken to develop feeding programs ensuring complete use of genotype potential"

Received April 30, 2019

Abstract

The worldwide experience is explicitly evidencing that genetically conditioned productivity potential in poultry can be realized only in healthy birds. Since the implementation of the antibiotic bans in EC countries a constant search for the effective alternatives to in-feed antibiotic growth promoters (AGP) is still in progress. The additives of different types (probiotics, prebiotics, synbiotics, symbiotics, acidifiers, phytobiotics) with growth-stimulating efficiency close to that in antibiotics and inducing no harmful effects become increasingly popular in practical poultry nutrition. The efficiency of phytobiotic Intebio based on the essential oils in diets for growing chicken of preparental lines B5 and B9 (selected by Smena Center for Genetic Selection) was studied. The parameters of growth efficiency, duodenal and circulatory activity of the digestive enzymes in fistulated birds, the results of molecular genetic analysis of the composition of duodenal and cecal microbiota are presented. It was found that live bodyweight in males and females in both lines at 21 weeks of age was similar in control treatments fed diets supplemented with AGP and experimental treatments fed diets supplemented with Intebio (3172 g in males and 2318 in females vs. 3169 and 2316 g, respectively, in control in B5 line; 2590 and 1917 g vs. 2589 and 1920 g in males and females, respectively, in B9 line). Reproductive organs (testicles in males and ovaries and oviducts in females) were normally developed in all lines and treatments. Supplementation of diets with the phytobiotic significantly increased lipase activity in the duodenal digesta in B5 line (by 30.9 %, $p \le 0.05$) and B9 line (by 98.3 %, $p \le 0.01$), and protease activity in B5 line (by 36.4 %, $p \le 0.05$). The activity of lipase in B9 line was significantly ($p \le 0.001$) lower in compare to B5 line in the duodenal digesta (by 59.9 %) and in blood serum (by 48.3 %). Digestibility of dietary dry matter in males and females of B5 line was higher by 3.11 % in compare to B9 line, digestibility of fat higher by 2.95 %, nitrogen retention higher by 2.12 %. The taxonomic composition of duodenal microbiota in both lines is found to be affected by the dietary phytobiotic. In phytobiotic-treated birds the significant increases were found in the duodenal populations of certain polysaccharide-fermenting species (phylum *Bacteroides*, class *Clostridiales*) and species with high antagonistic activity against avian pathogens (*Bifidobacterium spp., Bacillus* spp.).

Keywords: growing chicken, preparental lines, live bodyweight, digestive enzymes, phytobiotic, intestinal microbiota

Meat poultry may achieve high productive and reproductive qualities on-

ly under full valuable feeding which largely influences the effectiveness of geneticists and breeders work. The nutritional value of feeds, their quantity and quality must provide the planned selected indicators according to weeks of life since the 7-day age of poultry. It is necessary to feed compound feeds balanced in terms of available energy, nutritious, mineral, and biologically active substances taking into account their availability at all stages of breeding young stock of the original lines. They must comply with existing veterinary, sanitary, and hygienic requirements and be non-toxic [1].

Currently, most European countries have implemented a ban on the inclusion of feed antibiotics in poultry feeds. The focus is on feed additives that might replace feed antibiotics without significant changes in feed recipes [2-4].

The widespread use of antibiotics and chemical antibacterial agents often leads to the deterioration of poultry health associated with the development of uncontrolled secondary infections: salmonelloses, campylobacterioses, staphylococcoses, clostridioses, as well as polymicrobial diseases [5, 6]. Pathogenic microorganisms cause a violation of the intestinal microbiota composition, lead to changes in the thickness, appearance, muscle tone, strength, and increased paracellular permeability of the intestinal walls for toxic metabolites, which negatively affects the health and productivity of poultry ultimately. Contamination of poultry products by various causative agents of human infectious diseases also remains relevant [7, 8].

The study of the properties of plant extracts and essential oils is considered one of the most promising approaches to the creation of preparations for preventing diseases and increasing poultry productivity. Unlike antibiotics and drugs obtained through chemical synthesis, substances extracted from plants are less toxic, do not accumulate in the body and may become an ideal raw material for the creation of drugs [9]. In addition, plants are an unlimited renewable source of biologically active substances, including up to 12 thousand compounds, among which about 2 thousand are described [10]. Interest in them is due to their antibacterial effect and the possibility of using instead of therapeutic and feed antibiotics, as well as other properties that affect the metabolism and productivity of animals [11-15]. However, data on many aspects of the effect of essential oils are still contradictory, which may be explained by the difference in the nutritional diet, maintenance conditions, and poultry breed characteristics.

The need for breeding stock for broiler farming in the country is mainly met by foreign poultry crosses. However, in recent years, the Smena Center for Genetic Selection in collaboration with scientists of All-Russian Research and Technological Poultry Institute RAS has been working to create a new Russian cross of meat chickens [16, 17]. Its main advantages over foreign analogs include high viability and genetic potential of productivity, as well as adaptation to the local conditions of feeding and maintenance.

The study of the influence of phytobiotics on the microbiome of poultry bowels of different genetic lines is of substantial interest. A decrease in the risk of infectious pathologies is associated with the formation of healthy microbiota of the digestive tract, which is able to provide high resistance to colonization of the bowel by pathogens [18, 19] due to the synthesis of volatile fatty acids (VFAs), bacteriocins and other compounds that inhibit the growth and development of pathogenic species [20, 21]. It is known that microorganisms, interacting with each other, as well as with the host organism, are able to have a profound impact on immunity, nonspecific resistance to infections, and general processes of poultry life [22]. In addition, the active participation of the microbial community of poultry bowels in digestive processes, in particular, in the cleavage of complex polysaccharides and proteins [23, 24], in the use and for-

mation of nutrients, the synthesis of vitamins [25], the development of intestinal villi, increasing the absorbable surface [26, 27], was shown.

The most promising modern approaches in microbiology avoiding microorganisms' culture are based on molecular genetic methods, the NGS-sequencing (next-generation sequencing) and T-RFLP-analysis (terminal restriction fragment length polymorphism) [27-30].

The data on the activity of digestive enzymes in fistulated birds that received the phytobiotic Intebio (developed and produced by OOO BIOTROF, St. Petersburg) which confirm that this preparation may serve as a replacement to fodder antibiotics are given in the presented paper for the first time.

The work objective was to study the effect of phytobiotics based on essential oils on the growth and sexual development of young meat chickens (*Gallus gallus domesticus*), the activity of digestive enzymes and the state of intestinal microbiocenosis as compared to feed antibiotic.

Techniques. Zootechnical and physiological experiments were carried out on the original lines of poultry B5 (paternal line of the paternal parental breed of the Cornish form) and B9 (maternal line of the maternal parental breed of the Plymouth Rock form) in the genetic and selection center Zagorskoe Experimental Breeding Farm (EBF) (Sergiev Posad, Moscow Province) in 2017. From 1-day to 21-week age, birds were kept in cages (50 birds in a group). Humidity, temperature, and light regimes, feeding and watering were consistent with the recommendations of ARRTPI [31]. The viability and live weight of poultry, as well as the weight of reproductive organs (testicles and ovaries with oviducts), were estimated.

In the 1st week, the young stock received feed free, without limitation in the quantity. Then the quantity of feed was fixed weekly, thus normalizing the feeding. The control group received mashes of plant type, balanced in all nutrients according to age periods, with the addition of the Bacitracin-30 feed antibiotic (42 U/mg) in an amount of 100 g/t during the entire experimental period. Poultry of the experimental group received feed additive Intebio (OOO BIOTROF, St. Petersburg) at 1000 g/t feed. Intebio is a phytobiotic (TU 9362-011-50932298-2011) consisting of a carrier (wheat bran, GOST 7169-66) and a mixture of essential oils (garlic, lemon, thyme, and eucalyptus). The poultry of the original lines was fed with crumbled mashes of the following nutritional value: 1st-21st day — 280 kcal/100 g of metabolic energy, 20% of crude protein, 1.0% calcium, 0.7% of phosphorus, 1.15% of total lysine, 0.95% of available lysine, 0.45% of total methionine, 0.39% of available methionine; 22nd-35th day — 275 kcal/100 g and 18%; 1.0%; 0.7%; 0.9%; 0.76%; 0.38%; 0.32%, respectively; 36th-105th day — 265 kcal/100 g and 14%; 1.0%; 0.65%; 0.65%; 0.58%; 0.30%; 0.26%, respectively; 106th-147th day — 270 kcal/100 g and 15%; 1.5%; 0.7%; 0.64%; 0.57%; 0.30%; 0.26%, respectively.

To obtain the duodenum digesta, the young stock was operated at the age of 6 weeks to implant a T-shaped cannula of 1 cm from the confluence of three pancreatic and two bile ducts into the bowel. In 5 days after surgery, when the bird recovered, it was used in experiments. Five birds were selected for experiments from the control and experimental groups; the test period lasted 10 days. In the morning after 14-hour starvation, the birds received 30 g of feed, the duodenal chyme (5.0 ml) was sampled in 1 h after feeding, centrifuged at 5,000 rpm for 3 minutes (DM0412, Dragonlab, PRC), the supernatant was diluted with Ringer's solution 10 times and the activity of digestive enzymes was determined. Amylase activity was evaluated by Smith-Roy-Ugolev [32], with colorimetry (a KFK-3, OAO Zagorsk Optical and Mechanical Plant, Russia) at $\lambda = 670$ nm and expressed as the amount of disorganized starch (mg) per 1 ml

of chyme for 1 min ($mg \cdot ml^{-1} \cdot min^{-1}$). The activity of proteolytic enzymes was determined photometrically by the amount of cleaved casein ($mg \cdot ml^{-1} \cdot min^{-1}$) (KFK-3, ZOMP, $\lambda = 450$ nm) [33], lipase activity was evaluated on a semi-automatic biochemical analyzer BS3000P (SINNOWA Medical Science & Technology Co., Ltd., China) with a kit for lipase determination (DIACON-VET, Russia). Biochemical analysis of blood collected on an empty stomach from the axillary vein were performed on an automatic biochemical analyzer Chem Well 2900 (T) (Awareness Technology, USA) with the appropriate reagent kits (Human GmbH, Germany), and on a semi-automatic biochemical analyzer BS3000P (SINNOWA Medical Science & Technology Co., Ltd., China).

Samples of the duodenum digesta for the analysis of microflora in females of both lines of the experimental and control groups ($n = 3$) were collected at the end of the experiment (three repetitions from each group) with strict observance of sterility [33] and frozen immediately.

The composition of microflora was investigated by the T-RFLP method. Total DNA from the samples was isolated using the DNA Purification Kit (Fermentas, Inc., Lithuania), following the manufacturer's recommendations. PCR was performed with a Verity DNA amplifier (Life Technologies, Inc., USA) using eubacterial primers 63F (5′-CAGGCCTAACACATGCAAGTC-3′) labeled at the 5′-end (fluorophore D4 WellRed) and 1492R (5′-TACGGHTACCTTG-TTACGACTT-3′), which allow amplification of the 16S rRNA gene fragment from the positions 63 to 1492 (enumeration is specified for the 16S rRNA gene of *Escherichia coli*), in the following mode: 3 min at 95 °C (1 cycle); 30 s at 95 °C, 40 s at 55 °C, 60 s at 72 °C (35 cycles); 5 min at 72 °C. Fluorescently labeled 16S rRNA gene amplicons were purified with standard methods [35]. The concentration of purified DNA fragments of the 16S rRNA gene was determined (a Qubit 2.0 fluorimeter, Invitrogen, Germany). 30-50 ng amplicons of 16S rRNA were treated with HaeIII, HhaI and MspI restrictases (Fermentas, Lithuania). Restriction products were analyzed (a CEQ 8000 sequencer, Beckman Coulter, USA) according to the manufacturer's recommendations.

Bacteria were attributed to a certain taxon with Fragment Sorter software and the database (http://www.oardc.ohiostate.edu/trflpfragsort/index.php).

Statistical processing of the results was performed with Microsoft Excel, by determining the mean values (M) and standard errors of the means (±SEM). The significance of differences was assessed according to Student's t-test. The differences were statistically significant at $p < 0.05$. The Past program calculated the Shannon (H) and Simpson (D) biodiversity indices (http://folk.uio.no/ohammer/past/).

Results. Our findings have shown that both test and control groups had 100% viability. The live weight of the young stock of the lines in the control and test groups was almost identical, which indicates a positive effect of the phytobiotic on the growth of poultry when compared to a feed antibiotic (Table 1). For example, at the end of growing, males of the B5 line in the control group weighted 3,169 g, in the experimental group 3,172 g. Females at the age of 21 weeks showed the same trend. The males' bodyweight in both groups of the B9 line also did not differ, although in absolute values it was lower than in the B5 line, which is due to the breeding direction. Similar results were obtained for females. The feed consumption per 1 bird for the entire period was 11,305 kg in the B5 line and 10,934 kg in the B9 line. Feed conversion in the experimental groups had no significant differences with the control of both studied lines.

The dietary Intebio contributed to an increase in lipase activity compared to the control (by 30.9% at $p \leq 0.05$ in B5 line chickens, by 98.3% at $p \leq 0.001$ in B9 line chickens) and proteases (by 36.4% at $p \leq 0.05$ in B5 line chickens) in the duodenal digesta (Table 2). It can be assumed that essential oils have a stimulating

effect on the production of gastric juice of poultry, which increases the activity of pancreatic enzymes. Lipolytic activity in the intestinal digesta and blood in B9 line was lower than in B5 line by 59.9% and 48.3%, respectively.

1. Age-depended live weight (g) **of young meat chickens (*Gallus gallus domesticus*) of two lines and feed consumption** (g/bird per day) **under the use of dietary antibiotic Bacitracin-30 or essential oils-based phytobiotic Intebio** (*M*±SEM, GSC Zagorskoe EBF, Sergiev Posad, Moscow Region, 2017)

Age, weeks	Line									
	B5					B9				
	♂		♀		FC	♂		♀		FC
	C	T	C	T		C	T	C	T	
1	229±5.4	219±6.2	220±4.8	215±4.3	266	209±3.8	201±5.0	213±2.9	207±4.5	259
2	340±6.3	335±6.5	332±5.9	327±5.8	294	187±6.0	180±6.5	270±5.6	260±4.8	280
3	630±8.6	629±8.8	605±7.8	600±6.9	350	527±7.5	521±6.8	492±6.6	489±7.2	336
4	780±16.3	775±15.8	700±15.2	689±16.0	434	679±12.5	667±13.4	605±10.6	600±11.3	420
5	938±20.3	940±21.8	807±18.8	800±18.1	469	849±18.6	835±20.1	718±17.7	710±16.5	455
6	1110±21.6	1115±20.8	980±17.6	982±18.0	476	992±19.3	990±18.4	850±17.0	842±16.8	462
7	1260±22.3	1262±222.1	1005±118.4	1001±18.6	504	1222±20.3	1215±18.6	1039±16.6	1030±16.8	490
8	1450±23.6	1440±24.0	1170±20.7	1162±20.1	511	1390±21.4	1382±19.9	1127±18.8	1122±19.2	497
9	1595±25.2	1589±24.8	1245±23.3	1240±22.9	518	1450±22.6	1455±23.1	1200±20.4	1207±21.1	504
10	1790±28.3	1794±26.9	1440±27.9	1437±26.8	525	1590±27.6	1587±28.0	1295±25.2	1290±26.4	511
11	1900±30.2	1910±31.0	1550±28.3	1555±27.8	546	1605±28.8	1600±30.3	1375±25.5	1367±24.8	532
12	2020±32.3	2015±30.8	1700±26.6	1692±27.0	553	1810±25.6	1812±26.1	1460±24.4	1462±23.7	539
13	2110±30.6	2117±31.2	1740±27.6	1747±26.5	560	1890±28.0	1884±27.4	1530±22.6	1525±23.1	546
14	2275±31.2	2269±29.9	1875±28.3	1880±27.6	574	1940±25.6	1932±24.8	1600±20.7	1592±21.4	560
15	2495±28.8	2490±28.0	1910±26.4	1905±25.9	602	1995±27.7	1990±26.3	1687±24.4	1680±22.6	588
16	2530±32.4	2537±29.9	1947±27.7	1951±26.8	623	2140±28.5	2147±29.6	1710±26.6	1712±25.7	609
17	2650±31.7	2649±28.8	2005±30.3	2001±30.0	630	2267±30.1	2260±31.2	1775±28.0	1771±27.8	616
18	2795±32.5	2790±28.4	2190±29.6	2185±27.7	658	2368±32.5	2351±33.0	1804±26.5	1801±24.7	644
10	2940±30.8	2947±31.1	2210±29.6	2215±29.1	714	2478±33.4	2480±34.6	1843±30.2	1845±28.8	672
20	3075±32.6	3077±33.2	2235±28.8	2240±29.0	728	2505±36.2	2500±35.8	1885±27.7	1890±27.0	728
21	3169±35.0	3172±35.7	2316±30.9	2318±30.7	770	2589±38.8	2590±39.2	1920±29.8	1917±28.7	756

N o t e. C and T — control and test, respectively, FC — feed consumption for 1 week. For a description of the groups, see the Techniques section.

2. Enzymatic activity of duodenal chyme and activity of blood pancreatic enzymes in meat chickens (*Gallus gallus domesticus*) of two lines under the use of dietary antibiotic Bacitracin-30 or essential oils-based phytobiotic Intebio (*M*±SEM, GSC Zagorskoe EBF, Sergiev Posad, Moscow Region, 2017)

Indicator	Group		To control, %
	control (n = 5)	test (n = 5)	
	L i n e B5		
	Enzymatic activity of the chyme		
Amylase, mg · ml^{-1} · min^{-1}	219±21.1	231±25.5	105.5
Lipase, U/l	750±54.7	982±76.5*	130.9
Proteases, mg · ml^{-1} · min^{-1}	22±1.8	30±1.7*	136.4
	Enzymatic activity of the blood		
Amylase, mg · ml^{-1} · min^{-1}	395±43.5	322±20.5	81.5
Lipase, U/l	29±2.2	28±2.1	96.5
Trypsin, U/l	35±5.4	34±3.5	97.1
	L i n e B9		
	Enzymatic activity of the chyme		
Amylase, mg · ml^{-1} · min^{-1}	266±31.0	305±41.0	114.7
Lipase, U/l	301±37.5	597±50.3**	198.3
Proteases, mg · ml^{-1} · min^{-1}	36±0.8	36±1.0	100.0
	Enzymatic activity of the blood		
Amylase, mg · ml^{-1} · min^{-1}	290±25.1	263±6.5	90.7
Lipase, U/l	15±0.9	19±0.6*	126.7
Trypsin, U/l	29±0.5	30±0.9	103.4

N o t e. For a description of the groups, see the Techniques section.
* and ** Differences with control are statistically significant at $p \le 0.05$ and $p \le 0.01$, respectively.

The live weight of poultry under the use of feed antibiotic and phytobiotic indicates almost the same their effects, which was confirmed by the digestibility and use of feed nutrients. Indicators of the digestibility of dry matter, fat, and nitrogen between the test and control males and females had no significant

differences. It was noted only that the males and females of the B5 line digested the dry matter better (by 3.11%), digested fat better (by 2.95%) and used nitrogen better (by 2.12%).

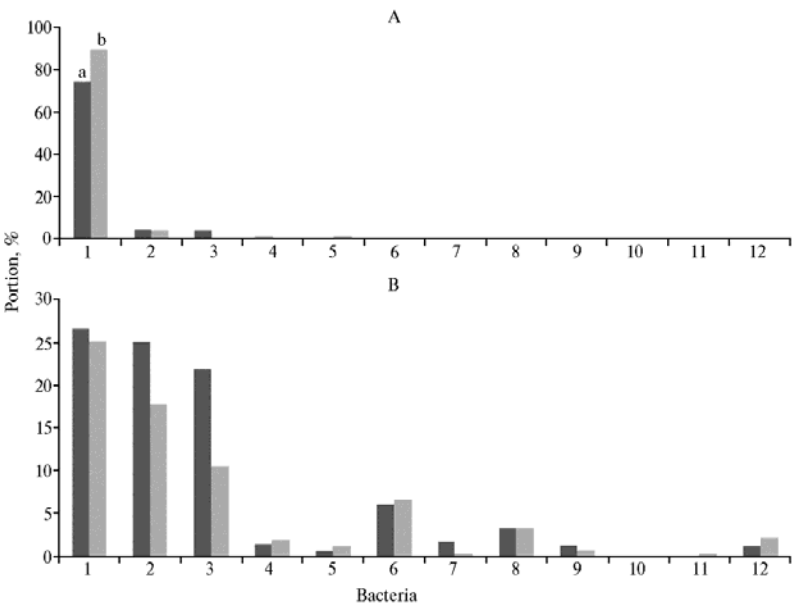
The rearing flocks should have not only the live weight corresponding to standards but also well-developed reproductive organs (testicles, ovaries, and oviducts). The weight of testicles in 21-week-old males of the B5 line in the control group was 7.5-9.1 g, in the experimental group 7.7-9.3 g, i.e., the differences were insignificant. In the B9 line in the control group, the indicators were 7.2-8.7 g, in the experimental group 7.4-9.0 g, which is almost equivalent. A similar trend occurred in reproductive organs of females. Thus, the weight of ovaries and oviducts in the control group of the B5 line was 1.75-1.86 and 5.52-5.61 g, in the test group 1.74-1.91 and 5.64-5.70 g; for the B9 line — 1.73-1.95 and 5.61-5.64 g, respectively (control group), 1.77-1.95 and 5.67-5.72 g (test group).

T-RFLP-analysis of the bacterial community of the bowel revealed a significant number of phylotypes of microorganisms, the total number of which was from 125.65±3.12 to 170.36±6.09 depending on the poultry origin and the use of phytobiotics (Table 3). Part of the phylotypes, ranging from 3.98±0.42% to 24.88±1.61%, depending on the group, could not be identified to phylum, which indicates the presence of absolutely unknown microorganisms in the poultry bowel digesta, whose nucleotide sequences have no analogs with the described taxa. Unidentified sequences have also been identified at lower taxonomic levels.

3. Biodiversity of the bacterial community in the duodenum digesta in meat chickens (*Gallus gallus domesticus*) of two lines under the use of dietary antibiotic Bacitracin-30 or essential oils-based phytobiotic Intebio (*M*±SEM, GSC Zagorskoe EBF, Sergiev Posad, Moscow Region, 2017)

Parameter	Line B5		Line B9	
	control (n = 3)	test (n = 3)	control (n = 3)	test (n = 3)
Shannon biodiversity index (H)	2.53±0.11	2.43±0.09	3.39±0.15	1.67±0.07
Simpson biodiversity index (D)	0.82±0.04	0.82±0.06	0.91±0.02	0.68±0.08
The number of phylotypes	149.05±5.23	130.82±3.68	125.65±3.12	170.36±6.09

N o t e. For a description of the groups, see the Techniques section.



Bacteria of the duodenum digesta in parent lines of broiler chickens (*Gallus gallus domesticus*) B9 (a) **and B5** (b) **in the control** (A) **and test** (B) **groups:** 1 — order *Lactobacillales*, 2 — unidentifiable bacteria, 3 — genus *Bacillus*, 4 — phylum *Actinobacteria*, 5 — femily *Campylobacteriaceae*, 6 — phylum *Bacteroidetes*, 7 — class *Clostridiales*, 8 — order *Selenomonadales*, 9 — genus *Bifidobacterum*, 10 —

phylum *Fusobacteria*, 11 — genus *Staphylococcus*, 12 — order *Pseudomonadales*. For a description of the groups, see the Techniques section.

According to the taxonomic affiliation, the majority of the identified phylotypes were attributed to three phyla, the *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, comprising in total not less than 77.37±4.29% and reaching a maximum of 95.69±6.15% (Fig.). To a lesser extent, the bacteria of the phylum *Actinobacteria* were represented; members of the phyla *Tenericutes* and *Fusobacteria* were the minority.

A significant number of opportunistic and pathogenic microorganisms were detected in the bacterial community of gut, the dominant among which were members of the family *Campylobacteriaceae*. This fact arouses interest since the presence and distribution of infectious agents in the chyme of the duodenum is not well-studied.

It should be noted that the obtained data, in general, correspond to modern ideas about poultry gut the microbiota [24, 36-39]. Thus, the representatives of 13 bacterial phyla, with more than 90% of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, was found in the chicken and turkey gut during the taxonomic analysis of about 5,000 sequences from GenBank (https://www.ncbi.nlm.nih.gov/genbank/), Ribosomal Database Project (https://rdp.cme.msu.edu/), and Silva (https://www.arb-silva.de/) databases [24].

In general, the comparison of ecological indices between poultry revealed lower biodiversity for the Plymouth Rock breed of chickens, which indicates a lower entropy accumulation and a higher organization and uniformity of the bacterial community compared to that of the Cornish breed.

Comparative analysis of the bacterial community of the duodenum digesta allowed us to determine statistically significant differences in the composition of the microbiota associated with the use of dietary phytobiotic. Some differences in the structure of microbiocenosis of the digestive tract depending on the breed of poultry were noted. As per Shannon biodiversity and Simpson dominance indices, in Cornish birds, phytobiotic contributed to a significant ($p \leq 0.05$) decrease in the heterogeneity of the intestinal microbiota. According to the results of the taxonomic assessment, significant changes in response to phytobiotic occurred in the bacterial community of the duodenum of birds in both lines. First, the significant increase in the representation of the members of the *Bacteroidetes* phylum, as well as the *Clostridiales* class, including representatives of the families *Eubacteriaceae*, *Clostridiaceae*, and *Lachnospiraceae* was observed ($p \leq 0.05$), which indicates an increase in potential of fermentation of plant polysaccharides by the microbial community, since these microorganisms have the ability to metabolize starch, fiber, and some other carbohydrates, proteins and deaminate amino acids.

The results we obtained on the intestinal microbiota composition are quite expected and have a clear relationship with the physiological state of birds. For example, the increase in the microbiota having enzymatic activity is consistent with the above data on the increase in the activity of pancreatic enzymes in the duodenal digesta. Our data support reports that some obligate inhabitants of the birds' gut are able to directly impact on birds' productivity. For example, Torok et al. [37] in a series of experiments revealed a significant correlation between the composition of microorganisms in the caecum and the efficiency of feed digestion. The presence of a relationship for bacteria of the *Firmicutes* phylum was noted [40], including representatives of the genera *Eubacterium* (*Eubacteriaceae* family), *Roseburia* (*Lachnospiraceae* family), *Faecalibacterium* (*Ruminococcaceae* family) [41]. Metabolism of these microorganisms is associated with the synthesis of various volatile fatty acids (butyric, acetic, etc.), which are

necessary for poultry as a source of energy. Some acids (e.g., butyric acid) increase the size of the intestinal epithelium [42, 43], thus providing a barrier to toxic agents [44].

It should be noted that the increase in the counts of VFA-synthesizing microorganisms in our experiments with dietary phytobiotics had a positive impact on the representation in the intestine of *Selenomonadales* bacteria, which can transform organic acids to various useful compounds thus participating actively in metabolism.

In addition, interesting changes related to the phytobiotic were observed in respect of the obligate representatives of the poultry gut, the lactictobacteria of *Lactobacillus*, *Enterococcus*, *Bacillus* and bifidobacteria of the genus *Bifidobacterium*, which, due to the synthesis of different organic acids and bacteriocins, are capable of antagonistic displacement of intestinal pathogens such as salmonellae, protea, staphylococci, *E. coli*, pseudomonades, and streptococci [21, 41]. It was found that in response to the use of dietary phytobiotics in the of birds of both breeds, the representation of bacteria of the genera *Bacillus* and B*ifidobacterium* increased significantly ($p \leq 0.05$), along with a decrease in the number of other microorganisms having similar properties.

Among the bacteria that cause infectious diseases, we have found pathogens of campylobacteriosis (family *Campylobacteraceae* – *Arcobacter*, *Campylobacter*), pasteurellosis (family *Pasteurellaceae* – *Pasteurella, Haemophilus*), mycoplasmosis (phylum *Tenericutes* – *Mycoplasma*), necrotic enteritidis (phylum *Fusobacteria*), purulonecrotic infections (genus *Staphylococcus*), *Clostridia* (species *Clostridium novyi* and *C. perfringens*) in birds. Most of these microorganisms were minor is gut community, with the exception of *Campylobacteria*.

The maximum number of opportunistic bacteria was found in Cornish birds, which also indicates some imbalance in their gut microbial community (see Fig.). The presence of *Campylobacter*, Fusobacterium, as well as *C. perfringens* and *Clostridium novyi* species was lower in birds of the maternal line. Any regularity characterizing similar changes in the number of pathogens in the duodenal digesta of the poultry of maternal and paternal lines in response to the use of phytobiotics was not found. This fact probably is connected with the genetic differences of the bird and requires additional elucidation.

The obtained results also indicate that the Cornish birds had a tendency to decrease the number of pathogenic fusobacteria, the causative agents of necrotic enteritidis leading to lesions of internal organs and joints, in the duodenal content.

Thus, our data draw to a conclusion that replacement of a fodder antibiotic by essential oils of plant origin in mashes for young meat chickens (original lines B5 and B9) makes it possible to reach an almost identical live weight of poultry. The development of reproductive organs of males (testicles) and females (ovaries and oviducts) in both groups remained normal without significant differences. The revealed increase in lipase activity in the duodenal chyme by 30.9% in the B5 line and by 98.3% in the B9 line, as well as proteases in the B5 line by 36.4% is consistent with data on the digestibility of fat and nitrogen in these groups. The results of this investigation indicate the noticeable changes in the bacterial community of duodenal digesta in both breeds, associated primarily with an increase in the number of microorganisms with enzymatic activity towards complex polysaccharides (class *Clostridia*, phylum *Bacteroidetes*), as well as bacteria with high antagonistic properties (*Bifidobacterium*, *Bacillus*, etc.).

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 810-819
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Ultradisperse dietary additives

UDC 636.52/.58:591.1:636.085.12:546.763

doi: 10.15389/agrobiology.2019.4.810eng
doi: 10.15389/agrobiology.2019.4.810rus

THE EFFECTS OF CHROMIUM MICROADDITIVE IN DIFFERENT DIETS FOR LAYING HENS (*Gallus gallus* L.) ON THE INTESTINAL DIGESTION AND CERTAIN BIOCHEMICAL BLOOD PARAMETERS

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by the Program on study of the mechanisms of digestive adaptation of mammals and poultry to different diets (Resolution of the Presidium RAS No. 132, dated 05.07.2017).
Received April 4, 2019

Abstract

Cromium (Cr) is a biogenic element necessary for normal growth and development in animals and poultry. Cr regulates the synthesis of fats, carbohydrate exchange, and circulatory glucose concentration. Cr additives in the diets were reported to affect growth efficiency positively in broiler chicks (I.Z. Gubaydulina et al., 2018); supplementation of diets with Cr nanoparticles (100-200 ppb) stimulated mineral exchange in broilers. With the lack of the research related to the effects of Cr microadditives on the digestion in animals and the absence of the studies on animals with chronic intestinal fistulae, the aim of our study was to investigate the effects of Cr microadditive (100 ppb) as nanoparticles in different wheat-based diets for laying hens (with soybean cake or sunflower cake as main protein sources) on the intestinal digestion and certain biochemical blood parameters. The trial was performed on Hisex White chicken (*Gallus gallus* L.) (10-12 months of age, 5 birds per treatment, diet shifts in 7-10 day periods) with chronic duodenal fistulae. Cr (III) oxide (99.8 %) nanoparticles (d = 91 nm, specific surface area 9 m^2/g, Z-potential 93±0.52 mV) (Platina LLC, Moscow, Russia) were produced by plasmochemical synthesis. The activities of amylase, lipase and total proteases were determined in the duodenal digesta sampled in 1 hour after the feeding. The blood was sampled from the axillary vein in the morning from starved birds and centrifuged with sodium citrate to obtain serum. The activities of trypsin and alkaline phosphatase, concentrations of glucose, total protein, triglycerides, uric acid, alanine and aspartic acid transaminases in serum were determined using semiautomatic flow biochemical analyzer BS-3000P (SINNOWA Medical Science & Technology Co., Ltd, China). The activities of amylase and lipase in serum were determined using analyzer Chem Well 2900 (T) (Awareness Technology, USA) and reagent kits (Human GmbH, Germany). Cr microadditive was found to produce different effects within different diets. The digestibility of protein and fiber from the diets with sunflower cake tended to be higher in compare to soybean cake. When the birds were fed Cr-supplemented diets the serum amylase activity increased by 37.8-50.2 % irrespective of the diet, with simultaneous reduction in serum glucose concentration by 26.6-17.5 % evidencing the improvement in glucose assimilation. The latter is in agreement with the negative correlation between the activity of amylase in the duodenal digesta and serum glucose concentration (r = -0.72 and -0.45 for different diets, $p < 0.05$). The supplementation of soybean-based diet with Cr decreased the duodenal activity of lipase with simultaneous reduction in certain biochemical blood parameters by 22-40 %; however, these parameters remained within the physiologically normal ranges. The supplementation of sunflower-containing diet with Cr increased duodenal activities of amylase, lipase, and total proteases; it is probably related to the presence of antinutritive factors in sunflower cake. These results lead to the conclusion that the effects of Cr oxide nanoparticles depend on the composition of basic diet; this fact one should take into account in practice of poultry nutrition.

Keywords: *Gallus gallus* L., laying hens, duodenal digesta, activities of digestive enzymes, digestive enzymes in blood serum, chromium (III) oxide

Chromium refers to the biogenic elements that are found in the tissues of plants and animals and are necessary for the healthy growth and functioning of the body. Its most important biological role is to regulate fats synthesis, carbohydrate metabolism, and blood glucose. Chromium is part of the low molecular weight organic complex — glucose tolerance factor, which ensures its normal content in the blood. It acts as a regulator of the amount of sugar in the blood [1, 2], providing the normal activity of insulin. Chromium is involved in the metabolic control of cholesterol (part of trypsin) and serves as an activator of some enzymes, by participating in maintaining the normal functioning of the cardiac muscle and the functioning of blood vessels. Chromium also contributes to the excretion of toxins, salts of heavy metals, radionuclides [3].

Chromium biological activity is mainly due to the ability of Cr^{3+} ions to form complex compounds. Cr^{3+} ions are involved in stabilizing the structure of nucleic acids. Chromium affects the hemopoiesis and has the ability to activate trypsin since it is part of the crystalline trypsin in the form of a labile compound capable of splitting off chromium ions [4]. Chromium penetrates through the intestinal wall, and the rate of its absorption increases depending on the concentration, with a decrease in particle size and the presence of digestive agents (vitamins, phytates, amino acids) [5, 6].

It is known about the positive effect of chromium supplements in feeding young chickens [7, 8]. It is shown [9, 10] that the introduction of chromium nanoparticles into the diet of broiler chickens in the doses of 100-200 ppb stimulates the exchange of chemical elements. The contrary opinion [11-14] that chromium can have a negative effect on the body exists as well. Since the response to a certain amount of heavy metal is different, when chromium is introduced into the diet in the doses of 100 ppb, both stimulation and inhibition of some processes occur, which directly depend on its amount in the feed.

In the present paper on laying hens with duodenal fistula, it was shown for the first time that when a chromium microadditive is introduced into the diet, the activity of enzymes in the digestive tract of chickens which react differently to the presence of heavy metal in different feed ingredients, changes. The blood biochemical parameters (the activity of amylase, trypsin, glucose, triglycerides, total protein, uric acid, alkaline phosphatase) change as well.

The work objective was to study the effect of Cr_2O_3 additive on digestion and blood biochemistry of laying hens when the additive is introduced in microdoses (100 ppb of feed) into feed of different composition.

Techniques. The experiments were performed with Hisex White laying hens (*Gallus gallus* L.) aged 10-12 months (Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, 2018). All manipulations were carried out in accordance with the requirements of the European Convention for the protection of vertebrates used for experimental and other scientific purposes (ETS No. 123, Strasbourg, 1986; https://www.msu.ru/bioetika/doc/konv.doc). To collect the duodenum digesta, the poultry was operated by implanting a cannula in the duodenum, opposite to the confluence of the pancreatic and bile ducts. Surgical operations were performed with the use of sedatives and painkillers. The chicken was fixed in the left lateral position in a special machine.

The incision was made on the right side of the last rib on the edge of the lateral process of the keel bone at 4-5 cm. The duodenum was extracted, the place of ducts confluence in the duodenum was found and a purse suture of 0.5-0.6 cm was placed opposite to it. The incision was made inside the purse suture, a cannula was inserted and a purse suture was tightened. The area around the implanted cannula was carefully treated, additional purse suture was put, if necessary. The bowel was immersed deep into the thoracoabdominal cavity and the

surgical wound was sutured with knotted sutures, capturing all the layers. After the operation, the poultry had access to water for 16-18 hours but did not receive the feed. After 5-7 days after surgery, when the poultry health was fully restored, physiological experiments have been started.

The physiological experiment was performed by the method of group-periods (5 animal units in each group), formed based on the principle of analogues. The experiment included two periods (7-10 days each): during the control period, the poultry did not receive an additive, during the experimental period, Cr_2O_3 ultrafine preparation (LLC Platinum, Russia; produced by plasma chemical synthesis, d = 91 nm, specific surface area 9 m^2/g, Z-potential 93±0.52 mV, Cr_2O_3 content 99.8%) at a dose of 100 ppb was added to the main diet. In the first series of experiments, soy cake was included in the main diet, in the second series this was sunflower cake. To obtain significant results, at least three digestion experiments were performed on each chicken in each test period.

The feed was prepared in accordance with zootechnical norms. The poultry received 30 g per bird in the morning on an empty stomach, the rest was fed during the day. After 1 h after feeding, duodenal chyme (5 ml) was sampled, the samples were immediately centrifuged (5 min at 5000 rpm) and diluted with a cooled Ringer solution (1:10).

Amylase activity in chyme was determined by starch hydrolysis [15] using a photometer KFK-3 (Zagorsk Optical and Mechanical Plant, Russia) at λ = 670 nm and expressed in milligrams of cleaved starch per 1 ml of chyme for 1 min. Lipolytic activity was measured on a semi-automatic flow biochemical analyzer BS-3000P (SINNOWA Medical Science & Technology Co., Ltd., China) using a kit of reagents for lipase (OOO DIACON-VET, Russia). Protease activity was determined with casein as a substrate by Hammerstein (EMD Millipore Corp., Billerica, USA) with colorimetry (KFK-3 at λ = 450 nm) [16].

The blood was sampled from the axillary vein in the morning from starved birds and centrifuged with sodium citrate for 3 min at 5000 rpm. The activities of amylase and lipase were determined using an analyzer Chem Well 2900 (T) (Awareness Technology, USA) and reagent kits (Human GmbH, Germany). The activity of trypsin [17] was determined using a semiautomatic flow biochemical analyzer BS-3000P (SINNOWA Medical Science & Technology Co., Ltd., China).

Biochemical blood tests were performed on a semi-automatic flow biochemical analyzer BS-3000P (SINNOWA Medical Science & Technology Co., Ltd., China) with a reagent kit (OOO DIACON-VET, Russia).

The conditions of keeping and feeding poultry during the period of the experiments conform to the standards of ARRTPI (Guide for Optimizing Mash Recipes for Poultry. Sergiev Posad, 2014). Feeds were prepared on the basis of wheat and barley. Feed No. 1 contained 19.4% of soybean cake, feed No. 2 21.4% of sunflower cake. The amount of available energy in the feeds was the same (265 kcal/100 g). The sunflower cake feed contained 2.11% more crude fiber and 1.07% more crude fat.

JMP Trial 14.1.0 (SAS, USA) software was used for statistical processing of (https://www.jmp.com/en_nl/of-fers/free-trial.html), by which the mean value (*M*), the standard error of the mean (±SEM) and the correlation (*r*) were calculated, the significance of the differences was assessed by the Student *t*-test. The differences were considered statistically significant at $p < 0.05$.

Results. It is known that pancreatic enzymes adapt to the quality of food entering the body [18]. In our experiments, replacing soy cake with sunflower cake in the diet of laying hens, led to a change in the activity of digestive enzymes in the duodenal chyme (Table 1).

1. Activity of digestive enzymes in the duodenal digesta of Hisex White cross laying hens (*Gallus gallus* L.) fed with dietary chromium oxide Cr_2O_3 nanoparticles as influenced by the feed ingredient composition (M±SEM, $n = 5$)

Indicator	Feed No. 1		Feed No. 2	
	control	test	control	test
Amylase, $mg \cdot ml^{-1} \cdot min^{-1}$	1426±144.8	1086±70.0	666±147.6	1747±187.3*
Lipase, U/l	3231±484.0	1762±187.6*	2669±144.5	4573±644.5*
Proteases, $mg \cdot ml^{-1} \cdot min^{-1}$	66±6.0	65±3.6	65±4.3	80±8.0*

N o t e. For a description of groups and feeds, see the Techniques section.
* Differences with the corresponding control are statistically significant at $p < 0.05$.

Amylase activity decreased by 53.3% ($p < 0.05$) when replacing soybean cake with sunflower cake in the diet. The activity of lipase and total proteases remained unchanged. Chromium microadditive, introduced into the diet on the background of soybean cake, reduced the activity of amylase by 24.0%, lipase by 45.5% ($p < 0.05$) compared to the control (feed No. 1). The addition of chromium to the feed with the sunflower cake (feed No. 2) led to an increase in the activity of amylase by 162.3% ($p < 0.05$), lipase by 71.4% ($p < 0.05$), protease by 23.1% ($p < 0.05$). Consequently, the chromium microadditive acted as a stimulant of the secretory function of the pancreas at low enzymatic activity in the gut, and inhibited lipase activity at high enzymatic activity.

Digestibility of nutrients reflected the adaptation of digestive enzymes to feed ingredients and microelements (Table 2).

2. Digestibility of nutrients in Hisex White cross laying hens (*Gallus gallus* L.) fed with dietary chromium oxide Cr_2O_3 nanoparticles as influenced by the feed ingredient composition (M±SEM, $n = 5$)

Digestibility, %	Feed No. 1		Feed No. 2	
	control	test	control	test
Protein	91.5±0.25	92.1±0.46	89.9±0.33*	90.7±0.25
Fiber	33.3±2.40	31.6±2.04	20.9±2.10*	23.1±1.78
Fat	91.2±0.38	90.4±0.34	93.4±3.10	91.3±0.47

N o t e. For a description of groups and feeds, see the Techniques section.
* Differences between controls are statistically significant at $p < 0.05$.

Protein digestibility when replacing soybean cake with sunflower cake (control periods) decreased by 1.5% ($p < 0.05$). The amount of lysine excreted with poultry manure in the control period (feed No. 2) exceeded the same indicator for feed No. 1 by 25.0% ($p < 0.05$), the content of methionine in the control periods did not change significantly and amounted to 0.001±0.0002 g. The crude fiber in the feed containing soybean cake was digested by 12.4% better ($p < 0.05$) compared to sunflower cake. Consequently, against the background of higher activity of digestive enzymes, the digestibility of protein and fiber for soybean cake was higher than for sunflower cake. The introduction of chromium nanoparticles into the diet did not significantly affect the digestibility of nutrients when using different protein ingredients in the feed.

The data we obtained earlier indicate a change in the activity of digestive enzymes in blood of meat chickens when various biopreparations are used in the diet [19]. In these experiments, biochemical parameters of blood in poultry did not have significant differences when using different ingredient composition of feed. The exception was triglycerides, the amount of which was 2 times higher with feed No. 2 compared to feed No. 1 (Table 3), indicating better fat absorption and the same lipase activity in the duodenal digesta. When chromium was added together with soy protein, an increase in amylase activity in blood comprised 37.8% ($p < 0.05$), the remaining indicators tended to decrease compared to the control: for trypsin — by 23.8% ($p < 0.05$), for glucose — by 26.3% ($p < 0.05$), for alkaline phosphatase — by 26.1% ($p < 0.05$), for total protein — by 21.4%

($p < 0.05$), for triglycerides — by 22.2% ($p < 0.05$), for uric acid — by 25.7% ($p < 0.05$), for alanine aminotransferase (AlAT) — by 29.9% ($p < 0.05$), for aspartate aminotransferase (AsAT) — by 40.1% ($p < 0.05$). When using chromium microadditives with sunflower cake, the activity of amylase increased by 50.1% while reducing glucose by 17.2% ($p < 0.05$).

3. Blood biochemical parameters in Hisex White cross laying hens (*Gallus gallus* L.) fed with dietary chromium oxide Cr_2O_3 nanoparticles as influenced by the feed ingredient composition ($M \pm SEM$, $n = 5$)

Parameter	Feed No. 1		Feed No. 2	
	control	test	control	test
Amylase, U/l	288±18.8	397±32.7*	271±25.0	407±35.2*
Lipase, U/l	58±2.8	62±7.5	61±3.0	63±0.2
Trypsin, U/l	202±43.7	154±16.8	95±37.9	135±53.6
Glucose, mmol/l	11.3±0.40	8.3±0.51*	12.0±0.33	9.9±0.42*
Alkaline phosphatase, u/l	1155±161.3	853±97.3	1293±68.8	1635±185.6
Total protein, g/l	41.9±1.91	33.0±1.12*	42.4±1.71	38.7±2.30
Triglycerides, mmol/l	1.8±0.30	1.4±0.21	3.6±0.40	4.3±0.40
Uric acid, mmol/l	245±34.7	182±19.1	233±21.1	180±16.2
Alanine aminotransferase, U/l	7.7±0.91	5.4±0.53*	7.9±0.54	7.5±0.91
Aspartate aminotransferase, U/l	197.2±8.81	118.3±16.63*	211.2±6.65	190.2±11.21

N o t e. For a description of groups and feeds, see the Techniques section.
* Differences with the corresponding control are statistically significant at $p < 0.05$.

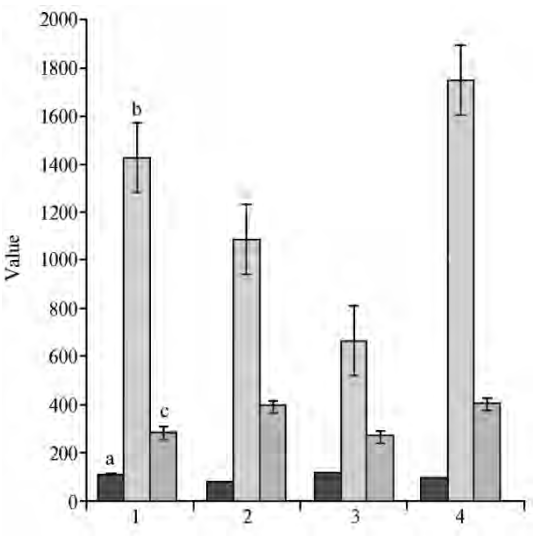

Fig. 1. Blood glucose concentration (mmol/l) (a) **and amylase activity** (U/l) (b)**, and amylase activity in duodenal digesta** ($mg \cdot ml^{-1} \cdot min^{-1}$) (b) **in Hisex White cross laying hens (*Gallus gallus* L.) fed with dietary chromium oxide Cr_2O_3 nanoparticles as influenced by the feed ingredient composition:** 1 — control (feed No. 1), 2 — test (feed No. 1), 3 — control (feed No. 2), 4 — test (feed No. 2). For a description of groups and feeds, see the Techniques section. Indicators for glucose are increased by 10 times.

A relationship has been established between amylase activity in the duodenal chyme and the glucose content: the higher the activity of the enzyme, the less glucose contained in the blood (Fig. 1). If the correlation in the control was not significant, then in the experiment the correlation coefficient between the activity of amylase in the duodenal chyme and glucose in blood remained steadily negative: for feed No. 1 $r = -0.72$ ($p < 0.05$), for feed No. 2 — $r = -0.45$. This fact allows us to suggest that chromium significantly affects carbohydrate metabolism due to the better use of glucose by the body. Chromium is known to regulate glucose homeostasis by activating insulin receptors, thereby enhancing signal transduction and increasing insulin sensitivity [20-22].

Triglycerides are esters that serve as the main constituents of fat in all living organisms. Getting into the gut together with food, triglycerides are turned to glycerin and fatty acids by lipase of gastric juice and pancreas [23], but the issues of fat metabolism in the poultry body are not fully studied [24]. According to our data, the increase in lipase activity in the chyme increased the number of triglycerides in blood (Fig. 2).

The value of the lipase-fat ratio in blood, when used feed with soy additive in the diet of laying hens, is almost 2 times higher than the control indicators with the addition of sunflower cake. A direct relationship was observed between lipase

activity in the duodenal chyme and triglycerides, i.e., the better the hydrolysis of fats in the bowel was, the more lipids entered the blood (correlation coefficient r ranged from 0.42 to 0.63; $p < 0.05$). Microadditive chromium enhanced these processes, improving the absorption of fats.

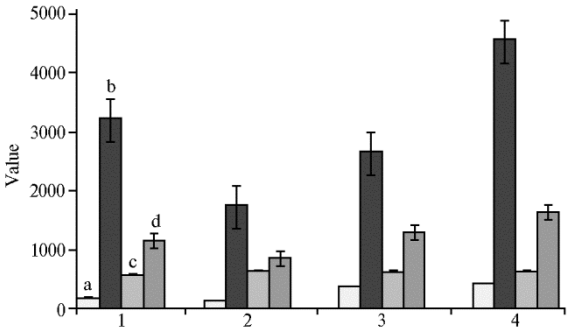

Fig. 2. Blood concentration of triglycerides (mmol/l) (a)**, activity of lipase** (U/l) (b) **and alkaline phosphatase** (U/l) (c)**, lipase activity in duodenal digest** (U/l) (d) **in Hisex White cross laying hens (*Gallus gallus* L.) fed with dietary chromium oxide Cr_2O_3 nanoparticles as influenced by the feed ingredient composition:** 1 — control (feed No. 1), 2 — test (feed No. 1), 3 — control (feed No. 2), 4 — test (feed No. 2). For a description of groups and feeds, see the Techniques section. Indicators for triglycerides are increased 100-fold, for blood lipase 10-fold.

The activity of alkaline phosphatase, which is secreted in the liver, depends on the lipase content in the duodenal chyme [25]. A relationship between lipase activity in duodenal digesta and alkaline phosphatase activity in blood was found (see Fig. 2). The correlation between these indicators was strong in some cases ($r = –0.90$, $p < 0.05$).

Total blood protein is a relatively stable indicator in healthy animals; its fluctuations can be associated with low protein content in the feed or with a disease. Blood proteins are mainly represented by albumins, which provide maintenance of oncotic blood pressure. In our experiment, the decrease in the total protein content within the physiological norm was associated with the introduction of chromium microadditives into the feed (Fig. 3). At the same time, the amount of uric acid in the blood and AlAT activity changed, indicating a normalization of the liver and pancreatic function [26].

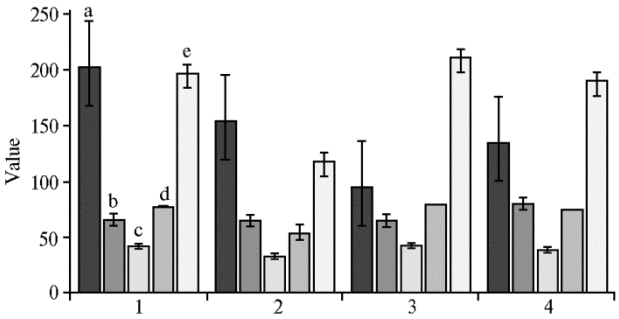

Fig. 3. Blood trypsin activity (U/l) (a)**, total proteins** (g/l) (b)**, alanine aminotransferase activity** (U/l) (c)**, aspartate aminotransferase activity** (U/l) (d)**, and duodenal protease activity** ($mg \cdot ml^{-1} \cdot min^{-1}$) (e) **in Hisex White cross laying hens (*Gallus gallus* L.) fed with dietary chromium oxide Cr_2O_3 nanoparticles as influenced by the feed ingredient composition:** 1 — control (feed No. 1), 2 — test (feed No. 1), 3 — control (feed No. 2), 4 — test (feed No. 2). For a description of groups and feeds, see the Techniques section. Indicators for alanine aminotransferase are increased 10-fold.

The analysis of the enzyme content in the blood is widely used in medicine to assess the state of the heart and liver. Fernando De Ritis found in 1957 that special significance has not only the AlAT and AsAT activity in blood but also their ratio (de Ritis ratio). Fluctuations in this indicator in humans are in the range from 1 to 2. In the test chickens, its value was extremely high, 21.8-26.7. Probably, the calculation of the de Ritis ratio for chickens should be refused, but it is important to note that the correlation analysis we performed showed a direct and fairly stable relationship between AsAT and AlAT, for which the value of r reached 0.98 ($p < 0.05$). With the addition of chromium oxide nanoparticles, the de Ritis ratio decreased slightly in the test group poultry compared to the control. Unlike tissue enzymes, pancreatic enzymes hydrolyze proteins, fats, and carbohydrates in the

gastrointestinal tract to monomers, and then enter the blood, where they participate in the regulation of metabolism. As known [27], there is a direct relationship between the activity of trypsin in the blood and the amount of glucose: the more trypsin in the blood, the higher the glucose content. This correlation was confirmed in studies on poultry in the postprandial period [28].

Our experimental data are also consistent with the results of studies [1, 2], which show that chromium regulates blood glucose homeostasis by activating insulin receptors and thus, as already noted, modulates signal transduction and increases insulin sensitivity [29, 30]. As for lipid metabolism, unlike Oganyan et al. [3], no significant effect of chromium microadditive on blood triglyceride concentration was found in this investigation. However, it was found that chromium microadditive acts as a stimulator of duodenal enzymes activity at low enzymatic activity in the gut, and at high enzyme activity it inhibits lipase activity. Consequently, it is reasonable to suppose that at low enzymatic activity, chromium supplementation has a positive effect on lipid metabolism. In protein metabolism when Cr_2O_3 was added to the feed, there were some changes: total protein decreased within the physiological norm (by 24.1%) when the chickens received the diet with the soybean cake, and protease activity increased by 23.1% when sunflower cake was used, but the digestibility of crude protein did not change significantly.

In experiments on rats [31], a comparative analysis of different chromium forms in the diet showed that the activity of amylase in the pancreas increases at a dose of chromium oxide nanoparticles 300 ppb. The use of chromium picolinate (CrPic) in a similar dose in feed for rats stimulates the activity of lipase and protease. The increased dose (500 ppb) of $CrCl_3$ and CrPic reduces lipase activity in the duodenum and reduces the activity of amylase and lipase in blood, which indicates the depressing effect of high doses of chromium on enteropancreatic circulation of digestive enzymes and metabolic imbalance of Mg and Fe in the blood. The amount of triglycerides decreases at the maximum doses of chromium in the form of chloride and picolinate, which confirms their participation in lipid metabolism. In addition, rats showed a significant increase in the frequency of DNA damages in peripheral blood and liver leucocytes after exposure to chromium oxide at doses of 300 and 1000 mg/kg [32].

The results we obtained on chickens are consistent with the action of chromium oxide nanoparticles on rats. The advantage of the our experiments is that this is the first to study the effect of chromium oxide in vivo on cannulated chickens under the influence of different protein sources.

Thus, dietary chromium microadditive to different protein components of the ration has an ambiguous effect in laying hens of the Hisex White cross. In the experimental period, regardless of the diet, with the introduction of chromium oxide supplements, the activity of blood amylase increases by 37.8-50.2% ($p < 0.05$) while the glucose content in the basal period reduces by 26.6-17.5% ($p < 0.05$), which indicates an improvement in glucose absorption. The activity of amylase in the duodenal digesta and the amount of glucose in the blood correlate, the correlation coefficients in the test period for different diets comprised –0.72 ($p < 0.05$) and –0.45. For a wheat diet with the addition of soy cake, the chromium oxide reduce the activity of lipase in the bowel digesta while the blood biochemical parameters of laying hens reduced by 22-40% ($p < 0.05$) within the physiological norm. The use of chromium oxide together with sunflower cake increases the activity of amylase, lipase and protease in the duodenum, which is associated, apparently, with the anti-nutrients in sunflower cake. This indicates the role of the basic diet in the action of chromium oxide on the bird body.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 820-831
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.52/.58:591.1:636.085.12:546.763

doi: 10.15389/agrobiology.2019.4.820eng
doi: 10.15389/agrobiology.2019.4.820rus

EFFECTS CAUSED BY DIFFERENT DOSES OF DIETARY CHROMIUM NANOPARTICLES FED TO BROILER CHICKENS

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by the Ministry of Science and Education of the Russian Federation for basic research according to the program of RAS Presidium (Agreement No. 075-02-2019-1847)
Received January 26, 2019

Abstract

Chromium is important chemical element for humans and animals which essentiality is manifested in reducing the amount of glucose and cholesterol in blood, reducing body fat. Factors which influence the absorption of chromium are source, size and composition of diet. Reducing size of Cr particles allows one to increase absorption. Replacement of traditional sources of microelements for organic and ultrafine metal forms is prospective due to their surface area, higher reactivity and bioavailability. In this paper, we show for the first time that Cr_2O_3 nanoparticles (NPs) at doses of 50 to 100 μg/kg of feed have no toxic effect, improve productive performance through stimulation of digestive enzymes and have positive effect on accumulation of the element in broiler chicken carcass. Our goal was to estimate effects of various doses of dietary chromium nanoparticles on the activity of digestive enzymes, biochemical blood parameters and gut microbiota in Arbor Aikres broiler chickens (*Gallus gallus*) (OAO Orenburg Poultry Farm, 2018). Five groups of chickens were formed, control and test groups 1, 2, 3, and 4 (n = 30 each) with live weight from 160 to 180 g. The control birds during experiment (0-14-21-42 days) received the basic diet, the birds of groups 1, 2, 3, and 4 additionally received 50, 100, 200 and 400 μg/kg feed of dietary Cr_2O_3 NPs (d = 91 nm; Platina LLC, Moscow, Russia). Addition of 200-400 μg/kg Cr_2O_3 NPs increased body weight and improved feed conversion by 3.1-3.9 and 7-11 %, respectively ($p \leq 0.05$), compared to control. Cr incorporation into carcass was 28.2 and 25.6 % higher when broilers were fed with NPs at 200 and 400 μg/kg, respectively, while this index in droppings was 15 % lower. Increased $Cr_{feed}/Cr_{droppings}$ (1.5-2.5) and $Cr_{feed}/Cr_{carcass}$ (4.6-6.4) values in the test groups indicate better absorption of chromium in the gastrointestinal tract. Cr_2O_3 NPs caused higher activity of endogenous transferases, the alanine aminotransferase and aspartate aminotransferase. Catalase and superoxide dismutase activity remained unchanged as well as concentration of malonic dialdehyde. That is, chromium acts as antioxidant, with up to 18 % increase ($p \leq 0.05$) in blood NO-metabolites. Cr_2O_3 NPs stimulate activity of blood enzymes: by 29.5 % (group IV, $p \leq 0.05$) on day 21 for amylase, by 19-30 % (group III and IV, $p \leq 0.05$) on days 21 and 42 for lipase, followed by a decrease in lipolytic activity by the end of the experiment in the test groups compared to day. NPs of 50 and 400 μg/kg suppressed amylase and activated lipase and protease in the duodenal chymeduction, with an increase in pH of the intestinal contents from 4.62 to 9.34 in all test groups. In droppings, digestive enzymes showed a reverse trend. Dietary Cr_2O_3 NPs at 50 μg/kg decreased the number of bifidobacteria, staphylococci and Salmonella in droppings, at 100 μg/kg increased the counts of enterobacteria, and at 400 μg/kg, on the contrary, reduced enterobacteria by 20 %, with simultaneous restriction Salmonella abundance in the cecum. Dietary Cr_2O_3 NPs decreased bifidobacteria. Thus, the dietary Cr_2O_3 NPs at 50-100 μg/kg has more pronounced positive effect and can be used as a chromium additive for poultry (for example, in premixes or vitamin-mineral complexes).

Keywords: chicken-broilers, antioxidant enzymes, Cr, productivity, concentration of Cr,

Chromium is an important chemical element for humans and animals, the essentiality of which, according to standards [1], is manifested in reducing the amount of glucose and cholesterol in the blood, reducing fat deposits, stimulation of muscular tissue formation [2]. Insufficient intake of chromium in the body, associated with the type and quality of nutrition, is accompanied by a slowdown in growth and deterioration of glucose tolerance [3]. Cr stimulates the insulin function by enhancing the function of the receptors of insulin-sensitive cells [4-6].

An important tool in regulating the metabolism of chromium received in the body is its ability to penetrate through the intestinal wall. This process is accelerated by reducing the size of chromium particles and the presence of digestive agents (vitamins, phytates, amino acids). Chromium has an extremely low digestibility, is poorly absorbed (25% for organic forms, 3% for inorganic), while the absorption of Cr^{3+} occurs mainly through the kidneys (80-95%) [7] with losses when deposited in the hair, excretion through the sebaceous glands and bile (45%) [8], indicating rapid absorption and recreation of Cr. Regardless of the increase in the dose of chromium in the diet (40-240 rg/day), the degree of its uptake remains constant – 0.4-2.0% [9]. Organic Cr has a more beneficial effect on poultry compared to inorganic forms due to increased absorption and bioavailability [10]. Consequently, the factors that determine the absorption of chromium are its source, particle size, and composition of the diet, while reducing the size of Cr particles allows increasing the rate of chromium absorption in the body.

The prospect of replacing traditional sources of microelements with organic and ultrafine forms of metals is determined by the high specific surface area of the latter, greater reactivity, and bioavailability. By taking into account small sizes and high penetrating power of nanoparticles (NPs), it is necessary to remember that each part of the gastrointestinal tract has a unique medium with a specific set of enzymes and pH [11]. Nanoparticles must be able to overcome these obstacles to exhibit biological activity in the small bowel [11].

The biological effect of chromium nanoparticles is associated with the participation in the metabolism of nucleic acids, an increase in the muscle area, the accumulation of chromium in tissues, and a decrease in the amount of fat [12, 13]. In poultry experiments, the addition of chromium to the diet increased the amount of protein in the chest and thigh muscles and reduced the cholesterol content in the muscles [14].

It should be noted that reports on the influence of various chromium sources on the activity of digestive enzymes in animals are rare, although the study of the possibility to use NPs as modulators of digestive enzymes activity seems promising. It is known that several regulatory centers specialized in relation to different effectors exist in enzyme molecules [15]. According to some researchers, rapid conformational transitions may occur as a result of the activity of modifiers [16]. Alternative forms of microelements can be a necessary tool in the management of digestive processes to improve nutrient conversion, the productivity and nutritional value of poultry products.

In this paper, we show for the first time that Cr_2O_3 nanoparticles (NPs) at doses of 50 to 100 μg/kg feed have no toxic effect, improve productive performance through stimulation of digestive enzymes and have a positive effect on the absorption of the element in broiler chickens' carcass.

The work objective was the biological certification of different doses of chromium nanoparticles in the diet of broiler chickens (*Gallus gallus*) on the activity of digestive enzymes, biochemical, and microbiological parameters.

Techniques. Investigations were carried out on broiler chickens of the Arbor Aikres cross (OAO Orenburg Poultry Farm, http://www.pfo56.ru, 2018). The experimental part of the work was carried out in accordance with the protocols of the Geneva Convention, the principles of good laboratory practice (National Standard of the Russian Federation GOST R 53434-2009, good laboratory practice for preclinical studies in the RF (GOST 3 5100.4-96) and The Guide for Care and Use of Laboratory Animals (National Academy Press Washington, D.C., 1996). The birds were kept in KUN-05 cages with an area of 4,050 cm^2 (90×45×45 cm) and marked with plastic foot tags. On the basis of daily weighing by the method of pairs-analogs, 5 groups were formed: one control and four experimental (n = 30, weight from 160 to 180 g). The birds were fed 2 times a day with a diet prepared by taking into account the recommendations [17] in accordance with the need for different age periods.

The composition of the main diet (MD) in the starting and growth period was as follows: wheat grain (respectively 27.1% and 41.2%), corn (16% and 22%), soybean cake (25% and 15%), sunflower cake (18% and 8%), sunflower oil (5% and 2.8%), lysine monohydrochloride, 98% (0.35% and 0.17%), DL-methionine (0.10% and 0.13%), L-threonine (0.03% and 0.54%), kitchen salt (0.28% and 0.3%), monocalcium phosphate (0.7% and 0.7%), fodder chalk (0.5% and 0.4%), limestone meal (1.0% and 0.7%), premix (2%) (OOO Koudijs MKorma, Russia). Drinking was free. Weighing was carried out every week. The control birds received the main diet throughout the whole experiment. NPs of Cr_2O_3 (d = 91 nm, specific surface area 9 m^2/g, Z-potential 93±0.52 mV, Cr content 99.8%, produced by plasma chemical synthesis; Platinum, LLC, Moscow, Russia) were additionally introduced in the main diet of birds of the test groups during the experiment (14-42 days) in the following doses: Group I — 50, II — 100, III — 200 and IV — 400 rg/kg. Feed dosages were chosen by taking into account the previously obtained positive effect of chromium on the growth and biochemical parameters of broiler chickens [18, 19]. Mash was prepared with the step mixing method; NPs were introduced after dispersion in a saline solution (UZDN-2T, NPP Akadempribor, Russia; 35 kHz, 300 W, 10 μA, 30 min).

Blood for analysis was taken before slaughter at 21- and 42-day age in the morning on an empty stomach from the axillary vein. Blood serum biochemical parameters were assessed (an automatic analyzer CS-T240, DIRUI Industrial Co., Ltd, China) using commercial veterinary kits DiaVetTest (OOO Diacon-Vet, Russia) and Randox Laboratories Limited (Randox Laboratories, Ltd., Great Britain).

The biomaterial was obtained after decapitation of broilers under Nembutal anesthesia on the 21st and 42nd days. Post-slaughter anatomical dressing of carcasses was carried out; the absolute and relative weight of internal organs was measured, followed by grinding and ashing (Multiwave 3000, Anton Paar, Austria). Microelement analysis was carried by the atomic emission spectrometry (Optima 2000 V, Perkin Elmer, USA) and mass spectrometry (Elan 9000, Perkin Elmer, USA) methods according to the manufacturer's recommendations.

To assess the activity of digestive enzymes, the bowel was extracted immediately after surgical autopsy, the pancreas and duodenum were sampled in sterile tubes. The activity of pancreatic enzymes was measured (an automatic biochemical analyzer CS-T240, Dirui Industrial Co., Ltd, China) using commercial biochemical kits for veterinary DiaVetTest (OOO Diacon-Vet, Russia), protease activity by hydrolysis of casein [20].

The qualitative and quantitative composition of broilers' gut microbiocenosis was determined by the standard method [21]. Endo-agar (OOO SRCP, Russia) was used for enterobacteria with normal enzymatic activity and oppor-

tunistic lactose-negative enterobacteria, meat-and-peptone agar (MPA) (OOO SRCP, Russia) for aerobic flora, Rogosa agar (Himedia, India) for lactobacilli, Bifido agar (Himedia, India) for bifidobacteria, yolk-salt agar (YSA) (OOO SRCP, Russia) for counting staphylococci, BSA (Himedia, India) for pathogenic salmonella. The inoculations were incubated for 24-72 hours at 37 °C. The number of microorganisms of each group in 1 g of intestinal digesta (M) was calculated by the formula $M = N \times 10n$, where N is the number of colonies, n is the dilution. The final result per 1 g of the caecum digesta was expressed as CFU/g.

Statistical analysis was performed by the ANOVA method (Statistica 10.0 software, StatSoft, Inc., USA) and in Microsoft Excel. The statistical significance of differences between the compared indicators was estimated according to Student's *t*-test. The values at $p \leq 0.05$ were considered significant. The data are presented as mean values (*M*) and standard errors of means (±SEM).

Results. During the experiment, the feed consumption per 1 kg of live weight gain in the control was 1.85 kg, depending on the dose of Cr_2O_3 NPs, the difference with the control was from 8% to 16%. The poultry in the experimental Group III and Group IV was characterized by the highest growth rates (Table 1). A similar growth-stimulating effect was obtained by other authors when Fe, Cu, Zn NPs were tested as an additive [22, 23]. The data on the negative effect of the chromium preparation at a dose of 400 g/t on the productivity and safety of broilers [24] were obtained, while doses up to 1200 μg/kg had a positive effect on the live weight and efficiency of feed consumption [18, 19, 25].

1. Growth rates and chromium concentration in biosubstrates and carcasses of Arbor Aikres cross broilers depending on the dose of Cr_2O_3 nanoparticles in the diet (*M*±SEM, *n* = 30, vivarium conditions, day 42)

Indicator	Group				
	control	I	II	III	IV
Initial weight, g	224±2.4	230±4.3	234±3.0	224±3.2	232±23.1
Final weight, g	2266±20.3	2366±40.1	2431±36.4	2533±59.8	2536±78.2
Feed consumption, g:					
total	3774.30	3649.80	3739.53	3855.70	3853.97
per 1 kg gain	1.85	1.71	1.70	1.67	1.55
Cr content, rg/kg:					
in the diet	540±1.5	587±1.5	637±2.5*	736±2.7*	937±2.4**
in droppings	433±3.5	373±4.6	466±6.1	363±4.2	366±3.8
in carcass	108±0.2	125±2.4**	129±4.1**	132±3.2*	145±2.8**
Cr ratio:					
feed/droppings	1.2	1.5	1.3	2.0	2.5
feed/carcass	5.0	4.6	4.9	5.5	6.4

N o t e. See the description of groups in the Techniques section.
*, ** Differences with the control are statistically significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

It is known that the biological role of minerals in the body depends on their biochemical availability. In the authors' experiment, the Cr content in the broiler carcass was by 28.2% and 25.6% higher than in the control at maximum doses of 200 and 400 μg/kg, with a 15% decrease in droppings. The coefficient $Cr_{feed}/Cr_{droppings}$ was the highest in groups with high chromium content in the diet, which indicates its best use. At the same time, the digestion from feed, expressed by the coefficient $Cr_{feed}/Cr_{carcass}$, was in the range of 4.6-6.4, which indicates the absence of agglomerations (formation of larger secondary particles) typical for nanoparticles entering the body in high doses, as well as the existence of a regulatory mechanism in chromium metabolism [23]. By taking into account that the absorption efficiency of 100-nm nanoparticles in bowel tissue cells is 15-250 times higher than that of larger microparticles [26], chromium deposition due to penetration into the cytoplasm [27] may be increased due to the deficiency of the transport protein transferrin and the formation of stable hard-to-adsorb hydrates in the duodenum, caecum, and colon [28].

The introduction of Cr_2O_3 NPs in the diet of broiler chickens was accompanied by the absence of oxidative stress, as indicated by the activity of catalase (CAT), superoxide dismutase (SOD) and the concentration of malondialdehyde (MDA) (Table 2) in blood. In particular, significant differences ($p \leq 0.05$) with the control were typical for Group II (51.3%), the CAT content was consistently low and decreased in response to an increase in the dose of Cr_2O_3 NPs in the diet, with a decrease in SOD in all experimental groups. The absence of toxicity and the growth-stimulating effect of Cr_2O_3 NPs were determined by an increase in the amount of NO-metabolites in the experimental Group II and Group III by 18.8% and 9.2%, respectively, compared to control ($p \leq 0.05$). The differences compared to the control did not exceed 5% in other groups.

2. Activity of catalase (CAT)**, superoxide dismutase** (SOD)**, the concentration of malondialdehyde** (MDA) **and NO-metabolites in blood of Arbor Aikres cross broilers depending on the dose of Cr_2O_3 nanoparticles in the diet** (M±SEM, n = 30, vivarium conditions, day 42)

Indicator	Group				
	control	I	II	III	IV
SOD, % of epinephrine autooxidation inhibition	568±68.6	405±57.0	277±18.6*	509±28.6	435±48.0
CAT, μmol $H_2O_2 \cdot l^{-1} \cdot min^{-1}$	2363±54.9	1050±82.8*	1871±51.38*	1510±47.4*	1116±64.1*
MDA, nmol/ml	0.65±0.260	0.36±0.006	0.41±0.120	0.29±0.090	0.18±0.040
NO-metabolites, μmol/l	59.8±2.53	61.4±3.42	73.6±3.62*	65.8±1.51*	62.9±1.83

N o t e. See the description of groups in the Techniques section.
* Differences with the control are statistically significant at $p \leq 0.05$.

Differences in the mechanism of chromium NPs action in different dosages led to unequal changes in blood biochemical parameters (Table 3). The effect of Cr_2O_3 NPs was expressed in the stimulation of the activity of alanine-aminotransferase (AlAT) and aspartate aminotransferase (AsAT) on the 14th day. Thus, the AlAT activity in Group II and Group IV was almost 2 times higher than in the control ($p \leq 0.05$). Significant differences were typical for all experimental groups for AsAT on the 21st day (see Table 3).

3. Biochemical blood parameters in broiler chickens of the Arbor Aikres cross on the 21st and 42nd days depending on the dose of Cr_2O_3 nanoparticles in the diet (M±SEM, n = 30, vivarium conditions)

Indicator	Group				
	control	I	II	III	IV
			Dat 21		
AlAT, U/l	7.3±0.50	8.3±2.40	14.2±1.00***	7.1±3.20	13.2±1.70**
AsAT, U/l	52.5±33.00	80.2±50.10*	190.4±76.90**	188.7±33.20**	106.4±20.00**
Glucose, mmol/l	14.0±0.40	14.8±0.80	14.4±1.60	15.3±0.50	14.8±0.50
Cholesterol, mmol/l	3.0±0.10	3.4±0.20	2.9±0.60	2.7±0.30	3.2±0.30
Triglycerides, mmol/l	0.6±0.20	0.6±0.20	1.6±0.20*	0.6±0.30	1.2±0.10*
Amylase, U/l	436±19.1	422±195.1	303±64.1	403±25.7	617±42.9*
Lipase, U/l	6.4±0.30	7.2±0.30	6.8±0.30	7.9±0.80	8.3±2.40
			Day 42		
AlAT, U/l	23.0±2.10	17.1±1.00	26.3±1.10	24.0±2.40	15.4±1.10
AsAT, U/l	70.5±6.10	152.0±8.50***	63.8±7.80	69.4±13.70	116.6±20.90**
Glucose, mmol/l	15.4±0.40	14.3±0.60	13.4±1.10	14.0±0.50	14.2±0.20
Cholesterol, mmol/l	3.8±0.30	3.3±0.10	3.0±0.30	3.1±0.30	3.9±0.40
Triglycerides, mmol/l	0.6±0.10	0.4±0.20	0.3±0.10	1.1±0.30*	2.1±0.70*
Amylase, U/l	173±5.6	180±5.6	157±10.4	166±1.2	186±1.2
Lipase, U/l	2.7±0.80	2.3±0.80	1.4±0.70	2.8±0.70	5.4±4.50**

N o t e. See the description of groups in the Techniques section. AlAT stands for alanine aminotransferase, AsAT stands for aspartate aminotransferase.
*, **, *** Differences with the control are statistically significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

By the end of the experiment, the blood AlAT activity (with a statistically insignificant difference with the control) decreased in the groups with the minimum and maximum loads of Cr_2O_3 NPs, while AsAT, on the contrary, increased by 53.7% and 39.6% ($p \leq 0.05$). The high activity of endogenous trans-

ferases may be a sign of liver, kidney and pancreatic dysfunctions, but by taking into account the absence of inflammatory markers (SOD and CAT indicators), it is reasonable to assume that the chromium metabolic function is associated with fine mechanisms involved in stimulating the production of chromodulin (LMWCCr) [29]. Chromodulin accepts chromium molecules bound by biological molecules, including transferrin, and stimulates hepatoprotective activity [30]. Changes in the glucose and cholesterol indicators in the case of chromium introduction in the diet were not noted.

The concentration of triglycerides in the blood is a marker of energy and lipid metabolism. In broilers, receiving Cr_2O_3 NPs in the diet at a dose of 100 and 400 μg/kg, this indicator on the 21st day was higher by 61.6% and 48.5% respectively, compared to the control. The effect was prolonged by the end of the accounting period, which does not confirm the results of studies [31], where high cholesterol and triglycerides content was typical for chrome deficiency states. This ambiguous reaction of the organism may be related to both the high bioavailability of chromium nanoparticles and the restructuring of the enzymatic system [32].

The activity of amylolytic enzymes in the blood on the 21st day was the highest in Group IV, the difference with the control values was 29.5% ($p \leq 0.05$). In other groups, no significant deviations were found. Similar dynamics were typical for lipase. In the groups that received the highest doses of Cr_2O_3 NPs (200 and 400 μg/kg), the activity of this enzyme on the 21st and 42nd days was 19-30% higher than in the control. An increase in the blood enzyme activity may result from the synthesis or resynthesis of micronutrients, increased permeability of cell membranes, and translocation of digestive enzymes into the bloodstream [33].

A number of authors [33, 34] postulate that enzymes circulating with the bloodstream represent a repository for subsequent pancreatic recreation. The second mechanism of formation of pancreatic hydrolases in the blood is their resorption from the excretory ducts of the gland (the so-called escape of enzymes), the third mechanism is the resorption of enzymes from the small bowel [35].

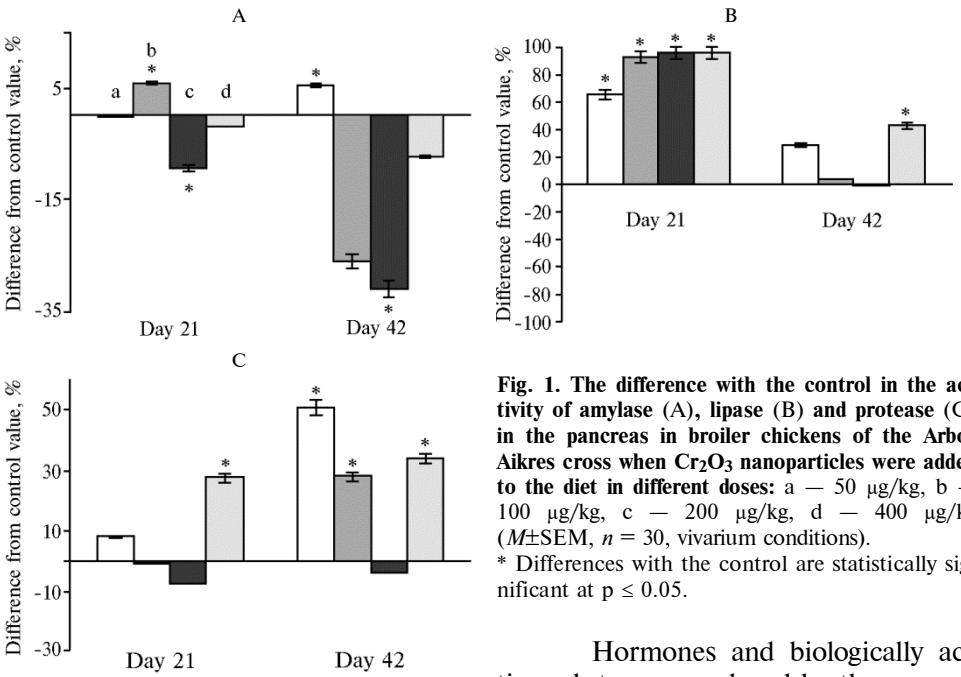

Fig. 1. The difference with the control in the activity of amylase (A)**, lipase** (B) **and protease** (C) **in the pancreas in broiler chickens of the Arbor Aikres cross when Cr_2O_3 nanoparticles were added to the diet in different doses:** a — 50 μg/kg, b — 100 μg/kg, c — 200 μg/kg, d — 400 μg/kg ($M \pm SEM$, $n = 30$, vivarium conditions).
* Differences with the control are statistically significant at $p \leq 0.05$.

Hormones and biologically active substances produced by the pancreas are necessary for the effective absorption of nutrients through the bowel mucosa. Pancreatic enzymes were character-

ized by an increase in lipase activity on the 21st day (up to 96% in all experimental groups). The peak activity of amylase and protease corresponded to Cr NPs doses of 100 and 400 μg/kg ($p \leq 0.05$). The dose-dependent difference on the 42nd day of the experiment was expressed in amylase inhibition in the dose range from 100 to 400 μg/kg with an increase in lipase and protease activity at low and high concentrations of Cr NPs (Fig. 1).

In this age period (on the 42nd day of the experiment), functional stress of enzyme incretion under the influence of Cr NPs is due to a decrease in the activity of amylase and lipase in the pancreas against the background of increased proteolytic activity. The readsorption of enzymes into the blood and then into the lumen of the small bowel is considered as a possible adaptation of enteropancreatic regulation in different age periods [36]. This mechanism is probably related to the observed change in enzyme activity in the duodenum to values diametrically opposite to the corresponding indicators in the pancreas.

Thus, it has been shown that broiler chickens that consumed Cr NPs in the diet have dose-dependent multidirectional changes in the activity of digestive enzymes in the pancreas at different age periods. Our data on the modification of enzymatic activity in the presence of nanoparticles is consistent with the results obtained by other authors [37-39]. In their experiments, the formation of chymotrypsin complex with selenium NPs contributed to the pH shift of hydrolytic activity to the alkaline side with a simultaneous increase in the maximum enzymatic activity in comparison with the free enzyme.

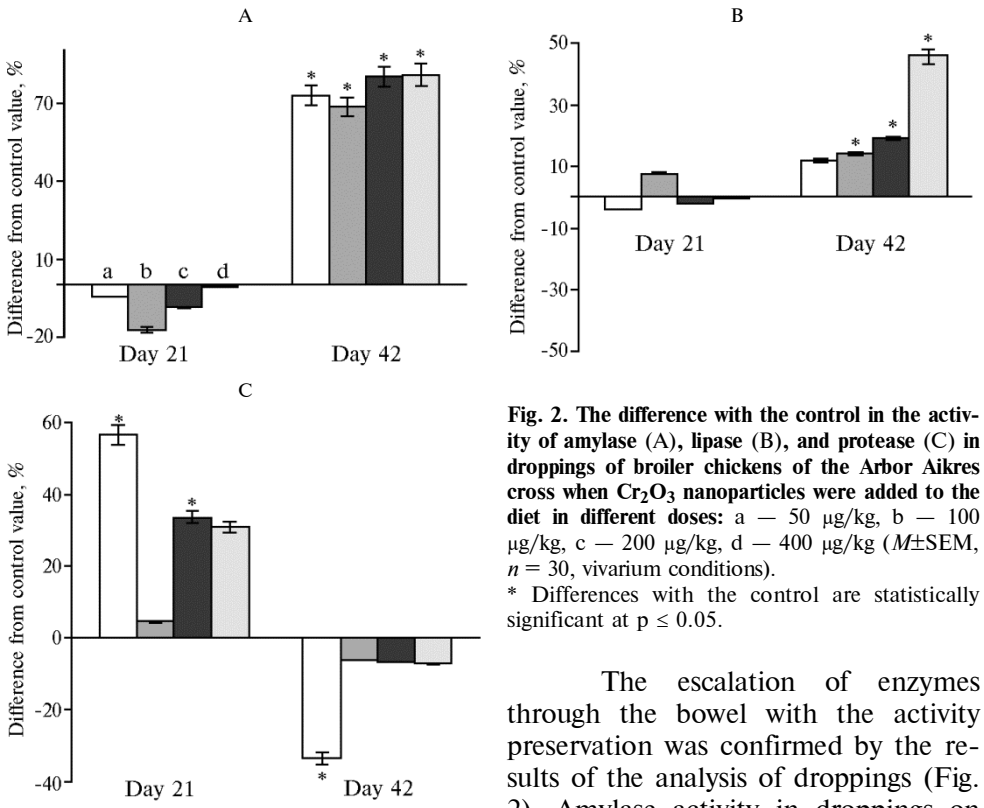

Fig. 2. The difference with the control in the activity of amylase (A)**, lipase** (B)**, and protease** (C) **in droppings of broiler chickens of the Arbor Aikres cross when Cr_2O_3 nanoparticles were added to the diet in different doses:** a — 50 μg/kg, b — 100 μg/kg, c — 200 μg/kg, d — 400 μg/kg (M±SEM, $n = 30$, vivarium conditions).
* Differences with the control are statistically significant at $p \leq 0.05$.

The escalation of enzymes through the bowel with the activity preservation was confirmed by the results of the analysis of droppings (Fig. 2). Amylase activity in droppings on the 21st day decreased, but increased by the end of the accounting period. Lipase activity did not change in the first period and increased by 70-75% on the 42nd day ($p \leq 0.05$). Protease activity, on the contrary, was higher than control on the 21st day, followed by a decrease by the end of the experiment. On the 21st day, the control group had the lowest pH

value of intestinal digesta, while in other groups, this value varied within the pH range of 4.62-7.53. Only in the group received 400 μg/kg of Cr_2O_3 NPs the acidity index on the 42nd day was pH 9.34 and differed from the control. Preserving and increasing the activity of amylase and lipase at this pH may be the example of adaptation of enzymes, as well as their stabilization by nanoparticles. The decrease in amylase activity in Group IV probably indicates the sensitivity of this enzyme to changes in the acid-base balance of the medium.

One of the reasons for the decrease in the intestinal digestion function in birds is the excessive growth of microbial flora in the intestinal lumen, which leads to a decrease in the promotion of chyme and premature deconjugation of primary bile acids [40]. Excessive microbial flora may cause damage to the small bowel epithelium since the metabolites of some microorganisms are cytotoxic. Determining the number of microorganisms in the caecum of broilers is an important step in monitoring the viability of the organism [41]. There was a decrease in total number of microorganisms (by 88.4%) ($p \leq 0.05$), enterobacteria and bifidobacteria (by 28.0 and 65.4%, respectively) ($p \leq 0.05$) in the caecum of broilers on the 21st day in the group that received 400 μg/kg of Cr in feed, and the counts of salmonella increased (by 21.7%, $p \leq 0.05$) (Table 4). At a dose of 50 μg/kg, the number of bifidobacteria and lactobacilli decreased (by 35.6 and 53.8% respectively) ($p \leq 0.05$), a dose of 100 μg/kg increased the number of enterobacteria, but reduced the number of salmonellae, 200 μg/kg contributed to the growth of the number of staphylococci, enterobacteria, salmonella, while reducing the number of bifidobacteria and lactobacilli. The number of bifidobacteria in Group I continued to decline with a simultaneous reduction in the number of staphylococci and salmonella on the 42nd day. The number of enterobacteria increased and bifidobacteria decreased in Group II. The representation of bifidobacteria decreased in Group III, the representation of enterobacteria, bifidobacteria, and salmonella decreased in Group IV on the 42nd day (as well as on the 21st day).

4. The number of different groups of microorganisms in the caecum of broiler chickens of the Arbor Aikres cross on the 21st and 42nd days depending on the dose of Cr_2O_3 nanoparticles in the diet ($M \pm$SEM, $n = 30$, vivarium conditions)

Group	Total microbial number	Staphylococci	Enterobacteria	Salmonellae	Bifidobacteria	Lactobacteria	Cellulose-fermenting bacteria
				Day 21			
Control	37.6±3.20	0.5±0.10	8.9±0.60	15.0±0.70	6.2±0.40	1.4±0.20	0.9±0.20
I	40.0±5.10	0.9±0.10	11.7±1.20	16.5±0.80	4.0±0.30*	0.6±0.09*	1.1±0.40
II	44.8±2.30	1.3±0.20	12.8±1.20*	10.3±0.30*	6.6±0.50	0.9±0.20	0.8±0.20
III	42.6±3.10	2.3±0.20*	15.1±2.10*	20.2±1.40*	3.0±0.20*	0.2±0.01*	1.1±0.30
IV	4.1±0.60*	0.9±0.10	6.4±0.30*	19.2±1.00*	2.1±0.20*	1.7±0.40	0.5±0.10
				Day 42			
Control	48.6±4.10	3.1±0.60	15.1±0.60	2.5±0.30	31.0±2.60	67.0±5.90	2.1±0.30
I	56.1±4.90	1.2±0.10*	18.6±1.30	0.9±0.10*	20.0±2.90*	52.3±4.80	1.8±0.40
II	46.6±3.80	2.2±0.20	19.2±1.10*	0	20.7±2.50*	56.3±5.50	1.9±0.20
III	60.6±5.80	2.0±0.10	17.4±1.90	3.5±0.60	18.7±2.70*	52.3±5.30	2.3±0.30
IV	62.2±5.20	1.8±0.10	12.3±0.80*	0.7±0.20*	17.7±3.10*	62.2±6.80	2.2±0.40

Note. See the description of groups in the Techniques section.
* Differences with the control are statistically significant at $p \leq 0.05$.

In this context, the observed "enzymatic release" indicates violations in the microbial ecology of the bowel, which happens due to a number of reasons: hyperperistalsis, the altered composition of chyme entering the large bowel, hyperproduction of alkaline secretions in the case of pathologies [39]. It is known that under the influence of microbial proteolytic enzymes, the droppings pH shifts towards alkaline values, contributing to the preservation of enzyme activity in the bowel [38, 42, 43].

The multiple physiological effects of Cr NPs observed earlier in rats [44] and, in the present study, in broiler chickens are related to the physicochemical properties of nanoparticles facilitating their interaction with biological objects [45].

So, Cr_2O_3 nanoparticles in the diet are not toxic, as indicated by the absence of changes in the activity of catalase, superoxide dismutase and in the accumulation of malondialdehyde. In this case, chromium acts as an antioxidant, increasing the concentration of NO-metabolites in the blood. The biological role of chromium nanoparticles when introduced in the diet of broiler chickens at doses of 50 and 100 μg/kg is manifested in stimulating growth, production of digestive enzymes and reducing feed costs. At high concentrations of NPs in different age periods (Day 21 and Day 42), the partial suppression of digestive enzymes absorption into the blood and their activity in the bowel (amylase) occurs while maintaining activity in droppings (amylase, lipase) due to the weakening growth of microorganisms (bifidobacteria, staphylococci, and salmonella) and the shift of pH to the alkaline side.

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AGRICULTURAL BIOLOGY,
2019, V. 54, № 4, pp. 832-841
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Radioecology of fodder plants

UDC 633.2:581.1:54.027:631.4

doi: 10.15389/agrobiology.2019.4.832eng
doi: 10.15389/agrobiology.2019.4.832rus

^{137}Cs REMOVAL FROM CONTAMINATED SOIL BY PERENNIAL BLUEGRASS HERBS DEPENDING ON MINERAL NUTRITION AND SOIL WATER AVAILABILITY

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The authors declare no conflict of interests
Received July 15, 2018

Abstract

The expansion of zones of anthropogenically affected agricultural lands and soil pollution pose a serious environmental threat. Radionuclides with long half-lives of fallout from the anthropogenic nuclear disasters are among the most dangerous pollutants. At present, the world scientific literature has accumulated extensive data on the effect of ameliorants, organic and mineral fertilizers on the yield and biological removal of ^{137}Cs from the soil by crops. This paper is our first report on the influence of natural and anthropogenic factors on ^{137}Cs migration from the contaminated soil to bluegrass forage plants many years following the Chernobyl accident (South-West of the Bryansk region, 2009-2011). Our subjective was to study ^{137}Cs removal from the soil depending on plant species and doses of full fertilizers. The soil of the site was alluvial meadow sandy, with pH_{KCl} = 5.2-5.6, 3.08-3.33 % humus, 620-840 mg/kg mobile phosphorus, 133-180 mg/kg exchangeable potassium, and ^{137}Cs contamination of 493-872 kBq/m^2. Effects of $N_{90}P_{60}K_{90}$, $N_{90}P_{60}K_{120}$, $N_{90}P_{60}K_{150}$, $N_{120}P_{60}K_{120}$, $N_{120}P_{60}K_{150}$, and $N_{120}P_{60}K_{180}$ used as ammonium nitrate, granulated superphosphate, and potassium chloride on monospecies crops of perennial bluegrasses *Dactylis glomerata* L., *Festuca pratensis* Huds., and *Phalaroides arundinacea* L. were compared. Fertilizers were used annually, with N and K applied in equal amounts at the first and second mowing and P full dose applied at the first mowing and P full dose applied at the first mowing. The period of vegetation in 2010 was characterized by increased radiation balance. In 2011, bioclimatic conditions were optimal for plant growth and development. The period between the first and the second mowing differed from that before the first mowing in the increased radiation balance and evaporability. The deficiency of soil moisture during the time from beginning of plant growth to the first mowing did not affect water supply of plants because of close groundwater after periodic flooding of the plain. We determined transpiration, transpiration coefficient, a relative transpiration, the rate of decrease in 137Cs specific activity of the biomass, intensity of ^{137}Cs removal from the soil to justify an inverse relationship of ^{137}Cs specific activity in the biomass from a dose of full mineral fertilizer. It has been shown that the intensity of ^{137}Cs bio-removal depends on a dose of full mineral fertilizer. The intensity of ^{137}Cs removal is the smallest in *Dactylis glomerata* and the greatest in *Phalaroides arundinacea*. The main mechanism of biological removing ^{137}Cs from the soil is relative transpiration which determines the availability of soil moisture for plant roots and Pe value reflecting the ratio of diffusion and convection in the moisture flow. The relationship between Pe and relative transpiration in the three studied bluegrass species is high (r = 0.8-0.9). We propose the equation of ^{137}Cs bio-removal by perennial bluegrass with the use of mineral fertilizers, which expresses essential pattern of ^{137}Cs activity in the biomass as influenced by the fertilizers and is fair for the studied species of bluegrass herbs.

Keywords: alluvial meadow sandy soil, ^{137}Cs, removal rate, perennial bluegrass herbs, tran-

Natural forage lands, which are one of the main sources of coarse and succulent feed for livestock breeding, also play a multifunctional role in the formation of a sustainable agricultural landscape. Therefore, the study of mechanisms to increase their productivity and recovery after anthropogenic damage is of great interest [1-5]. In the conditions of radioactive contamination, along with increasing the productivity of hayfields and pastures, it becomes important to obtain the feed that meets the standard for the permissible content of radionuclides [6-9].

A large amount of data has been accumulated on the effect of ameliorants, organic and mineral fertilizers on the yield and biological removal of ^{137}Cs from the soil by agricultural crops [10-13]. It has been experimentally proved that potassium fertilizers reduce the specific activity of ^{137}Cs of plant products [14-18]. The introduction of potassium fertilizers has become the main agrochemical method in the conditions of radioactive soil contamination. When studying different doses of N, P, and K as part of a complete mineral fertilizer, it was found that the transition of ^{137}Cs from the soil to plant production depends not only on the dose of potassium, but also on the ratio of the doses of potassium and nitrogen, as well as the amount of nitrogen in the composition of the complete mineral fertilizer [19-21].

For the first time in the distant period after the Chernobyl accident, we investigated the role of natural and anthropogenic factors in the process of ^{137}Cs migration in the soil-plant system (by the example of the use of different types of *Poaceae* as feed) and proposed a mechanism that regulates the biological removal of ^{137}Cs from the soil through relative transpiration, which determines the availability of soil moisture for plants.

The aim of the work was to study the effect of different doses of full mineral fertilizer on the availability of soil moisture for the root system and the intensity of the process of biological removal of ^{137}Cs from the soil by monospecies crops of perennial bluegrass.

Techniques. The studies were conducted in the southwestern part of the Bryansk Region on the meadow section of the central floodplain of the Iput River. The soil of the experimental plot is alluvial meadow shallow medium-humic, sandy on sandy loam alluvium with the following division of the profile into genetic horizons: A_d (0-4 cm), A_1 (4-18 cm); B_1 (18-40 cm); B_g (40-60 cm), Cg (60-90 cm). The agrochemical characteristics of the soil are as follows: pH_{KCl} 5.2-5.6, hydrolytic acidity 2.6-2.8 mEq/100 g of soil, the amount of absorbed bases 11.3-13.1 mEq/100 g of soil, cation exchange capacity 12.9-15.9 mEq/100 g of soil, base saturation of 81-82%; humus content 3.08-3.33% (according to Tyurin), mobile phosphorus 620-840 mg/kg, exchange potassium 133-180 mg/kg (according to Kirsanov). The contamination density of the ^{137}Cs experimental site during the period of the work was 493-872 kBq/m^2.

The intensity of ^{137}Cs bio-removal from the soil at different doses of full mineral fertilizer was studied on monospecies crops of perennial bluegrass plants. Against the background of two-tier plowing, cocksfoot grass (*Dactylis glomerata* L., cultivar VIC 61), meadow fescue grass (*Festuca pratensis* Huds., cultivar Dedinovska) and reed canary grass (*Phalaroides arundinacea* L., cultivar Pripyatsky) were sown; the seeding rates of all seeds were 15 kg/ha. The scheme of the experiment: I — the control without fertilizers, II — $N_{90}P_{60}K_{90}$, III — $N_{90}P_{60}K_{120}$, IV — $N_{90}P_{60}K_{150}$, V — $N_{120}P_{60}K_{120}$, VI – $N_{120}P_{60}K_{150}$, VII – $N_{120}P_{60}K_{180}$. Ammonium nitrate, simple granulated superphosphate, and potas-

sium chloride were used. Fertilizers were introduced annually: nitrogen and potassium ones in two doses (half of the calculated dose for the first mowing, the second half – for the second mowing), phosphate – one full dose for the first mowing. The sown area was 63 m^2, the harvesting area was 24 m^2, and the experiment was conducted with threefold repetition.

The yield of grass was calculated by the method of total sampling for weighting and the selection of the sample bundle. Two cuts were carried out per year (the first mowing – from June 1 to 10, the second mowing – from August 23 to September 1).

The transpiration value was determined by the formula of Penman [22], evaporation – by Budyko [23]. The transpiration coefficient was calculated as $C_t = \Sigma_g E_t / Y$ (1), where $\Sigma_g E_t$ is the total transpiration during the growing season, mm; Y is yield of air-dry phytomass of grass, t/ha. Relative transpiration was calculated using the formula: $\alpha = \Sigma_g E_t / \Sigma_g E_0$ (2), where $\Sigma_g E_t$ and $\Sigma_g E_0$ are the total transpiration and evaporation, respectively, during the growing season.

The specific activity of ^{137}Cs in the studied plant samples was determined using the Gamma Plus universal spectrometric complex (Scientific Production Enterprise Doza, Russia), the established measurement error of not more than 10%.

The daily average values of the short-wave part of the radiation balance were conducted according to urgent observations using the trapezoidal method [24]. To calculate the average daily values of the total radiation balance, empirical coefficients were used [25, 26]. The daily average values of photosynthetically active radiation (PAR) were calculated in accordance with the description [27].

The obtained data were subjected to the analysis of variance using the Excel 7.0 and Statistic 7.0 software (StatSoft, Inc., USA). The mean values are presented (*M*). The significance of differences with the control and between the variants was evaluated by the least significant difference (LSD_{05}). The differences were considered statistically significant when going beyond the borders of the LSD.

Results. Table 1 shows the phytoclimatic and meteorological indicators for the duration of the experiments. The vegetation season of 2010, in comparison with other years, was characterized by an increased radiation balance. Phytoclimatic conditions, optimal for the growth and development of crops from the first to second mowing developed in 2011. The period from the first mowing to the second mowing differed from that before the first mowing by a higher radiation balance and, correspondingly, greater evaporation.

1. Phytoclimatic conditions of spring-summer vegetation of bluegrass crops by mowings and years of observation (Bryansk Region)

Indicator	2009		2010		2011	
	1	2	1	2	1	2
The sum of the average daily values of the radiation balance during the growing season, MJ/m^2	394	569	439	688	432	485
The sum of average daily values of photosynthetically active radiation during the growing season, MJ/m^2	266	460	268	426	256	336
Air temperature, °C	13.9	19.4	16.2	25.0	16.3	21.1
Specific heat of evaporation, MJ/kg	2.47	2.46	2.47	2.45	2.47	2.45
Evaporation during the growing season, mm	160	231	178	281	175	198
Amount of precipitation during the growing season, mm	75.8	155.3	86.7	200.7	84.1	169.1
Moisture deficit during the growing season, mm	–84	–76	–91	–80	–91	–29
Humidity coefficient	0.47	0.67	0.49	0.71	0.48	0.85

N o t e. 1 — before the first mowing; 2 — from the first to the second mowing.

The deficit of soil moisture during the period from the resumption of the growing season to the first mowing did not affect the water regime of grass crops due to the close standing of groundwater after periodic flooding of the floodplain.

In the period from the first to the second mowing, a particularly large moisture deficit was in 2010, the minimum in 2011. The decrease in groundwater level and soil moisture deficit affected the water regime of crops and, as it appeared, the productivity of bluegrass plants in the period before the second mowing.

The transpiration coefficient is equal to the transpiration moisture consumption for the formation of 1 ton of hay of bluegrass plants. As it follows from Table 2, prior to the first mowing, when the soil moisture is sufficient, the values of C_t were determined by the phytoclimatic conditions of cultivation and did not depend on the species characteristics of the crops. Species features were manifested given the lack of soil moisture in 2010, caused by a decrease in groundwater level, an increase in radiation balance, evaporation and air temperature during the growing season from the first to second mowing. Under these conditions, only the reed canary grass reduced PAR absorption and water consumption for yield formation.

2. Transpiration coefficient C_t for perennial bluegrass plants by year and vegetation period (Bryansk Region)

Plants	2009	2010	2011
From the resumption of vegetation to the first mowing			
Cocksfoot grass	452	500	515
Meadow fescue grass	450	500	510
Reed canary grass	450	500	510
From the first to the second mowing			
Cocksfoot grass	472	490	444
Meadow fescue grass	472	497	439
Reed canary grass	472	465	442

In the experimental plot, the initial non-uniformity of ^{137}Cs contamination was observed. In the plots occupied by cocksfoot grass, meadow fescue grass, and reed canary grass, the density of ^{137}Cs contamination of the arable horizon of soil varied within the limits of 725-837, 615-671 and 493-631 kBq/m^2, respectively. Such values of the density of surface contamination ^{137}Cs are characterized as high [28].

3. Contamination density ^{137}Cs (kBq/m^2) in the arable horizon of soil under the studied crops according to the variants of the experiment and years of research (Bryansk Region)

Variant	Cocksfoot grass			Meadow fescue grass			Reed canary grass		
	2009	2010	2011	2009	2010	2011	2009	2010	2011
Control	726	726	725	668	669	667	493	493	493
$N_{90}P_{60}K_{90}$	842	839	841	650	649	651	629	631	629
$N_{90}P_{60}K_{120}$	790	785	785	637	634	633	541	541	539
$N_{90}P_{60}K_{150}$	812	809	809	667	668	671	524	526	522
$N_{120}P_{60}K_{120}$	837	840	834	670	670	667	546	542	545
$N_{120}P_{60}K_{150}$	800	794	803	678	679	669	498	496	496
$N_{120}P_{60}K_{180}$	764	766	761	615	609	622	510	502	537

The highest yields of bluegrass plants in all experimental variants in the first mowing were obtained in 2010 with a PAR value of 268 MJ/m^2 and C_t = 500. In the period from the first to second mowing, the highest crop yields in all variants were noted in 2011 with a PAR value of 336 MJ/m^2 and C_t = 442. In all cases in the first and second mowing, hay yield was higher given an increase in the dose of mineral fertilizer from 150 to 210 kg of active matter per 1 ha. Table 5 shows the specific activity of ^{137}Cs obtained in the experiment of air-dry phytomass of bluegrass plants. As follows from the data in Tables 4 and 5, an inverse proportion was revealed between the yield and specific activity of ^{137}Cs of hay: an increase in crop yield with increasing doses of mineral fertilizers led to a de-

crease in the specific activity of ^{137}Cs in the phytomass.

4. Yield (t/ha) **of the air-dry mass of perennial bluegrass plants according to the variants of the experiment and years of research** (Bryansk Region)

Variant	Cocksfoot grass				Meadow fescue grass				Reed canary grass			
	2009	2010	2011	on average	2009	2010	2011	on average	2009	2010	2011	on average
The first mowing												
Control	1.12	2.43	1.75	1.77	1.17	2.34	1.9	1.80	1.23	2.48	1.86	1.86
$N_{45}P_{60}K_{45}$	3.96	8.94	7.8	6.90	4.25	8.91	8.51	7.22	4.37	9.32	8.78	7.49
$N_{45}P_{60}K_{60}$	3.98	9.48	7.87	7.11	4.44	8.42	8.6	7.15	4.51	9.55	8.86	7.64
$N_{45}P_{60}K_{75}$	4.28	9.62	8.06	7.32	4.43	9.72	9.27	7.81	4.69	9.77	9.31	7.92
$N_{60}P_{60}K_{60}$	4.55	9.33	8.59	7.49	4.87	9.38	8.9	7.72	4.89	9.41	9.14	7.81
$N_{60}P_{60}K_{75}$	5.02	5.58	8.61	6.40	5.22	9.67	9.16	8.02	5.59	9.72	9.26	8.19
$N_{60}P_{60}K_{90}$	5.06	9,82	9,31	8,06	5,47	9,96	9,33	8,25	6,12	10,23	9,45	8,60
LSD_{05}	4.26	4,35	4,04	4,18	3,98	3,84	4,44	4,12	3,89	4,08	4,26	4,16
The second mowing												
Контроль	0.61	1.14	0.96	0.90	0.64	1.21	0.93	0.93	0.67	1.28	1.02	0.99
$N_{45}K_{45}$	1.97	3.09	3.7	2.92	2.04	3.15	3.67	2.95	2.10	3.20	4.05	3.12
$N_{45}K_{60}$	2.06	3.35	3.85	3.09	2.09	3.37	3.77	3.08	2.18	3.40	4.18	3.25
$N_{45}K_{75}$	2.16	3.49	3.94	3.20	2.33	3.46	3.89	3.23	2.23	3.51	4.25	3.33
$N_{60}K_{60}$	2.23	3.42	4.22	3.29	2.38	3.54	4.25	3.39	2.45	3.62	4.51	3.53
$N_{60}K_{75}$	2.59	3.57	4.37	3.51	2.54	3.61	4.59	3.58	2.54	3.69	4.75	3.66
$N_{60}K_{90}$	2.62	3.76	4.44	3.61	2.59	3.78	4.67	3.68	3.86	3.81	5.21	4.29
LSD_{05}	1.29	0.98	1.24	1.47	1.26	1.48	1.67	1.51	1.58	1.36	1.72	1.56

5. Specific activity of ^{137}Cs (Bq/kg) **of the air-dry mass of perennial bluegrass plants according to the variants of the experiment and years of research** (Bryansk Region)

Variant	Cocksfoot grass				Meadow fescue grass				Reed canary grass			
	2009	2010	2011	on average	2009	2010	2011	on average	2009	2010	2011	on average
The first mowing												
Control	2990	2866	2952	2936	2880	2796	2877	2851	2325	2296	2378	2333
$N_{45}P_{60}K_{45}$	1322	1318	1338	1326	1215	1208	1231	1218	1208	1186	1227	1207
$N_{45}P_{60}K_{60}$	845	809	848	834	834	811	842	829	736	698	744	726
$N_{45}P_{60}K_{75}$	479	437	461	459	440	421	456	439	398	363	418	393
$N_{60}P_{60}K_{60}$	469	479	492	480	476	422	467	455	426	412	458	432
$N_{60}P_{60}K_{75}$	280	286	313	293	312	285	321	306	259	238	268	255
$N_{60}P_{60}K_{90}$	275	268	297	280	289	256	286	277	255	231	258	248
LSD_{05}	56	68	55	50	42	56	55	45	66	59	75	45
The second mowing												
Control	2862	2788	2966	2872	2910	2655	2793	2786	2264	2196	2308	2256
$N_{45}K_{45}$	1308	1285	1301	1298	1290	1213	1262	1255	1309	1195	1210	1238
$N_{45}K_{60}$	802	776	798	792	754	708	743	735	717	637	698	684
$N_{45}K_{75}$	347	345	352	348	363	312	342	339	330	308	337	325
$N_{60}K_{60}$	434	437	452	441	446	409	453	436	436	411	437	428
$N_{60}K_{75}$	376	381	368	375	352	388	361	367	359	318	385	354
$N_{60}K_{90}$	305	333	346	328	315	302	352	323	310	296	342	316
LSD_{05}	84	76	68	63	105	98	87	95	63	57	66	69

6. Coefficients of ^{137}Cs accumulation in the air-dry mass of perennial bluegrass plants according to the variants of the experiment and years of research (Bryansk Region)

Variant	Cocksfoot grass			Meadow fescue grass			Reed canary grass		
	2009	2010	2011	2009	2010	2011	2009	2010	2011
The first mowing									
Control	1.03	0.99	1.02	1.08	1.04	1.08	1.18	1.16	1.20
$N_{45}P_{60}K_{45}$	0.39	0.39	0.40	0.47	0.47	0.47	0.48	0.47	0.49
$N_{45}P_{60}K_{60}$	0.27	0.26	0.27	0.33	0.32	0.33	0.34	0.32	0.34
$N_{45}P_{60}K_{75}$	0.15	0.14	0.14	0.17	0.16	0.15	0.19	0.17	0.20
$N_{60}P_{60}K_{60}$	0.14	0.14	0.15	0.18	0.16	0.18	0.20	0.19	0.21
$N_{60}P_{60}K_{75}$	0.09	0.09	0.10	0.12	0.11	0.12	0.13	0.12	0.14
$N_{60}P_{60}K_{90}$	0.09	0.09	0.10	0.12	0.11	0.12	0.13	0.12	0.12
The second mowing									
Control	0.87	0.96	1.02	1.09	0.99	1.05	1.15	1.11	1.17
$N_{45}K_{45}$	0.39	0.38	0.39	0.50	0.47	0.49	0.52	0.48	0.48
$N_{45}K_{60}$	0.26	0.25	0.25	0.30	0.28	0.29	0.33	0.30	0.32
$N_{45}K_{75}$	0.11	0.10	0.11	0.14	0.12	0.13	0.16	0.15	0.16
$N_{60}K_{60}$	0.13	0.13	0.14	0.17	0.16	0.17	0.20	0.19	0.20
$N_{60}K_{75}$	0.12	0.12	0.12	0.13	0.15	0.14	0.18	0.16	0.19
$N_{60}K_{90}$	0.10	0.11	0.11	0.13	0.12	0.14	0.18	0.17	0.19

The calculation of ^{137}Cs accumulation coefficients in the air-dry mass of bluegrass plants according to the experimental variants (Table 6) made it possible to establish an inversely proportional relationship between these coefficients and the yield, as well as the doses of mineral fertilizers. To reveal the mechanism of such a dependence, we calculated the values of relative transpiration ($\Sigma_g E_t/\Sigma_g E_0$) according to the experimental variants in the first and second mowing in different years. Relative transpiration is used to determine the degree of water supply for crops, as well as the period of irrigation of crops. This value characterizes the availability of soil moisture to the root system of the plant. Mineral fertilizers increase the transpiration of crops [29, 30] and, correspondingly, the value of $\Sigma_g E_t/\Sigma_g E_0$. Under optimal conditions of the water regime, the relative transpiration of crops is 0.70-0.85 [31].

7. Relative transpiration of crops of perennial bluegrass plants according to the variants of the experiment and years of research (Bryansk Region)

Variant	2009			2010			2011 год		
	1	2	3	1	2	3	1	2	3
From the resumption of vegetation to the first mowing									
$N_{45}P_{60}K_{45}$	1.12	1.21	1.24	2.51	2.51	2.62	2.29	2.50	2.58
$N_{45}P_{60}K_{60}$	1.12	1.26	1.27	2.66	2.36	2.68	2.31	2.50	2.61
$N_{45}P_{60}K_{75}$	1.21	1.26	1.32	2.7	2.73	2.74	2.37	2.73	2.74
$N_{60}P_{60}K_{60}$	1.29	1.50	1.38	2.62	2.63	2.65	2.53	2.62	2.69
$N_{60}P_{60}K_{75}$	1.42	1.47	1.58	1.57	2.71	2.73	2.53	2.68	2.73
$N_{60}P_{60}K_{90}$	1.43	1.55	1.73	2.76	2.8	2.88	2.74	2.74	2.78
From the first to the second mowing									
$N_{45}K_{45}$	0.40	0.42	0.43	0.55	0.55	0.56	0.83	0.55	0.91
$N_{45}K_{60}$	0.42	0.43	0.45	0.59	0.59	0.6	0.86	0.59	0.93
$N_{45}K_{75}$	0.44	0.48	0.45	0.62	0.61	0.62	0.88	0.61	0.93
$N_{60}K_{60}$	0.45	0.48	0.50	0.60	0.62	0.64	0.94	0.62	1.01
$N_{60}K_{75}$	0.53	0.52	0.52	0.63	0.64	0.65	0.98	0.64	1.07
$N_{60}K_{90}$	0.54	0.53	0.79	0.66	0.67	0.67	1.00	0.67	1.17

Note. 1 — cocksfoot grass, 2 — meadow fescue grass, 3 — reed canary grass.

During the growing season before the first mowing, the value of relative transpiration in all variants exceeded 1.0 (Table 7). These data indicate that the crops experienced an excess of moisture caused by a close occurrence of groundwater after flooding in the floodplain in April. From the first to second mowing, this figure exceeded 1.0 only in 2011. In 2009 and 2010, with a large moisture deficit of 76-80 mm, relative transpiration did not reach the optimum value in any variant of crops. The meteorological and phytoclimatic conditions were optimal for the growth and development of crops in 2011, when the seeds developed in the conditions of abundance of soil moisture.

The calculation results (see Table 7) show that with an increase in the dose of complete mineral fertilizer, the relative transpiration value increases with a lack and excess of soil moisture. Electrolytes that are part of mineral fertilizers, causing compression of double electric layers on the walls of soil capillaries, reduce the proportion of adsorbed ions ^{137}Cs in the solution flow to the plant root system [24]. An increase in the dose of mineral fertilizer increases the availability of soil moisture for plant roots, reduces the Pe value, and leads to a decrease in the specific activity of ^{137}Cs in the phytomass of grasses. Relative transpiration, which determines the bioavailability of soil moisture and the Pe number at different levels of mineral nutrition, is one of the main reasons for the inverse proportion between the specific activity of ^{137}Cs and the phytomass yield in bluegrass plants.

The data on the specific activity of ^{137}Cs in the phytomass of bluegrass plants (see Table 5) reflect the relationship between the specific activity in the control and the use of different doses of mineral fertilizers. This relationship is described by the following formula: $A_i = A_k \times \exp(-\lambda \Sigma b E_T)$ (3), where A_i and

A_k are the specific activity of ^{137}Cs in the phytomass, respectively, in the variant *i* and in the control, Bq/kg; ΣbE_T is transpiration during the growing season, mm; λ is the relative intensity of bio-removal (a constant value for certain conditions; the larger the value of λ, the lower the specific phytomass activity in the variant *i* is, compared to the control). With an increase in the dose of full mineral fertilizers above 195 kg of active matter per 1 ha, the difference in the values of λ is 0 or very small. These data confirm the exponential dependence of the specific activity of the phytomass of herbs in the variant *i* on transpiration and the adequacy of formula (3) to the real process of bio-removal.

It should be noted that under equal conditions of cultivation in each of the six experimental variants in the first and second mowing, changes in the intensity of ^{137}Cs bio-removal by cultures depending on the species were noted, as reported by other authors [5, 32-35]. In our experiment, cocksfoot grass had the lowest intensity of ^{137}Cs biological removal, and reed canary grass the highest one.

8. Relative intensity (λ) of ^{137}Cs bio-removal from the soil by the phytomass of perennial bluegrass plants according to the experimental variants and years of research (Bryansk Region)

Variant	2009 год			2010 год			2011 год		
	1	2	3	1	2	3	1	2	3
From the resumption of vegetation to the first mowing									
$N_{45}P_{60}K_{45}$	5.7	5.0	4.7	2.3	2.3	1.8	2.4	2.1	1.8
$N_{45}P_{60}K_{60}$	8.3	7.4	6.6	3.2	3.4	3.0	3.6	3.1	3.0
$N_{45}P_{60}K_{75}$	10.8	10.8	9.7	4.4	4.3	3.9	4.7	4.2	3.7
$N_{60}P_{60}K_{60}$	9.7	8.5	9.0	4.5	4.4	4.1	4.5	4.2	3.8
$N_{60}P_{60}K_{75}$	11.4	10.5	9.4	9.3	5.2	4.7	5.6	5.1	4.3
$N_{60}P_{60}K_{90}$	11.4	10.3	10.0	5.3	5.2	4.8	5.2	5.2	4.5
From the first to the second mowing									
$N_{45}K_{45}$	12.8	12.0	9.8	7.7	7.7	6.8	7.5	7.1	5.4
$N_{45}K_{60}$	14.9	14.7	12.9	8.4	8.4	7.7	7.8	7.9	6.7
$N_{45}K_{75}$	20.3	18.9	21.3	11.6	11.6	12.3	11.6	11.0	10.3
$N_{60}K_{60}$	18.8	15.9	14.8	11.3	11.2	10.0	9.6	9.4	7.8
$N_{60}K_{75}$	21.6	21.1	22.0	13.5	13.8	12.2	11.3	10.7	9.0
$N_{60}K_{90}$	21.8	21.9	15.3	13.3	13.9	12.3	12.2	10.6	8.7

Note. 1 — cocksfoot grass, 2 — meadow fescue grass, 3 — reed canary grass.

Therefore, the formula (3) can be used for a comparative assessment of ^{137}Cs bio-removal from the soil by different species of bluegrass plants when applying mineral fertilizers.

Thus, an analysis of the results of field experiments conducted on monospecies crops of bluegrass forage plants revealed the following features of the process of ^{137}Cs bio-removal from the soil. The transpiration coefficient of the studied grass species with sufficient moisture supply does not depend on the dose and type of fertilizer, but with a lack of moisture, it depends on the radiation balance and the type of plant. One of the main reasons for the inverse proportion between the specific activity of ^{137}Cs and the yield of grass phytomass is an increase in relative transpiration with increasing doses of complete mineral fertilizer and a decrease in the proportion of adsorbed ions ^{137}Cs in the soil moisture flow to the root system caused by the compression of double electric layers on the walls pores and a decrease in the Pe value with increasing convection in the flow. We suggest the equation of ^{137}Cs bio-removal from the soil by the phytomass of perennial bluegrass plants using mineral fertilizers, which expresses the pattern of change in the specific activity of ^{137}Cs phytomass under the influence of introduced fertilizers and is valid for the studied species of bluegrass plants. It was revealed that the intensity of ^{137}Cs bio-removal from the soil to the first mowing differs from that in the period from the first to the second mowing, which is characterized by a significantly greater decrease in ^{137}Cs bio-removal by sowing of grass due to low relative transpiration and low bioavailability of mois-

ture, which is accompanied by a decrease in the intensity of convective flow of moisture to the root system.

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